## Title

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# *Xenopus* leads the way: Frogs as a pioneering model to understand the human brain

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## Abstract

From its long history in the field of embryology to its recent advances in genetics, *Xenopus* has been an indispensable model for understanding the human brain. Foundational studies that gave us our first insights into major embryonic patterning events serve as a crucial backdrop for newer avenues of investigation into organogenesis and organ function. The vast array of tools available in *Xenopus laevis* and *tropicalis* allows interrogation of developmental phenomena at all levels, from the molecular to the behavioral, and the application of CRISPR technology has enabled the investigation of human disorder risk genes in a higher-throughput manner. As the only major tetrapod model in which all developmental stages are easily manipulated and observed, frogs provide the unique opportunity to study organ development from the earliest stages. All of these features make *Xenopus* a premier model for studying the development of the brain, a notoriously complex process that demands an understanding of all stages from fertilization to organogenesis and beyond. Importantly, core processes of brain development are conserved between *Xenopus* and human, underlining the advantages of this model. This review begins by summarizing discoveries made in amphibians that form the cornerstones of vertebrate neurodevelopmental biology and goes on to discuss recent advances that have catapulted our understanding of brain development in *Xenopus* and in relation to human development and disease. As we engage in a new era of patient-driven gene discovery, *Xenopus* offers exceptional potential to uncover conserved biology underlying human brain disorders and move towards rational drug design.

### Keywords

Amphibian; neural; organogenesis; genetics; birth defects

## Introduction

The development of the vertebrate central nervous system is a famously intricate and complicated process. Human brain development is particularly difficult to characterize, due both to its extraordinary complexity and to its inaccessibility. Nevertheless, because it is the primary organ that determines how we experience and interact with the world, the brain is

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the holy grail in terms of our desire to understand how we exist and function. A variety of model systems have been used to glean insight into how the brain forms and operates, and continue to contribute to our growing knowledge about the development of the nervous system and the disorders that perturb its functions.

A major goal of neurobiology has been to determine the etiology of disorders of the brain, which constitute a large fraction of medical diagnoses and often suffer from widespread stigmatization (Rössler 2016). Reaching this goal requires a thorough understanding of typical neurodevelopment, as well as the ability to identify and test a plethora of interacting genetic and environmental factors and correctly interpret their effects. Recently, impressive strides have been made in the discovery of disorder risk genes through massive patient sequencing studies at the exome or whole genome level, particularly in psychiatric and developmental disorders like autism (An et al. 2018; Grove et al. 2019; Satterstrom et al. 2020; Wang et al. 2020). The result of these efforts has been an ever-growing list of genetic loci, each predicted to contribute in some degree to the likelihood that a disorder will present in an individual who exhibits variation in them. The identification of risk genes lays a foundation for elaborating the molecular mechanisms underlying each disorder. However, these genetic risks are also affected by background genetic heterogeneity and environmental factors, which adds complexity to the analysis of their contribution. Further complicating the elucidation of disorder etiology, these genes likely have pleiotropic functions in different cell types during different developmental periods (Sestan and State 2018). Similarly, any potential treatment (pharmaceutical or otherwise) may affect the activity of more than one target, and testing both the efficacy and the possible off-target effects of these treatments is critical before they can be implemented.

This scenario demands a model in which multiple factors can be investigated in a high-throughput manner, to match the accelerating pace of gene discovery, the large number of genes that can carry risk for a single disorder, and the accompanying prospect of potential treatments (A. J. Willsey et al. 2018; Sestan and State 2018). Rodents and primates, although commonly employed in the investigation of brain disorders, are not appropriate for such large scale and exploratory work. An alternative model is required to begin to tackle the staggering scope of potential etiological factors and generate focused hypotheses that can then be tested in mammalian systems. Fortunately, mounting evidence suggests that the neurodevelopment of other tetrapods may be more similar to the human case than previously appreciated (Clinton et al. 2014; Fernandez et al. 1998; Martínez-Cerdeño et al. 2016; Medina and Abellán 2009; Norimoto et al. 2020; Tosches et al. 2018). Without a doubt, fundamental discoveries made in other models have already given invaluable insight into mechanisms of human brain development, in addition to guiding the establishment of new *in vitro* models, including human iPSCs and organoids (Andrews and Nowakowski 2019; Bhaduri et al. 2020; McCammon and Sive 2015; Munoz-Sanjuan and Brivanlou 2002; Pasca 2018; Pollen et al. 2019; Simunovic and Brivanlou 2017).

In this review, we summarize why frogs of the genus *Xenopus* are an ideal model at this juncture. As we describe below, the same features that have made *Xenopus laevis* a favored system for embryological studies and a fertile ground for the mechanistic characterization of neurodevelopmental processes remain key advantages in their continued use as models of

human disorders. Importantly, recent technological advances in gene editing have also flung open the doors for *Xenopus tropicalis* to emerge as a higher-throughput genetic model. The combination of these features, especially in light of the deeply conserved nature of tetrapod brain development, makes *Xenopus* a strong member of the model organism armamentarium in the pursuit of an understanding of neurodevelopmental disorder biology.

## Amphibian embryology sets the stage

Part of the reason that amphibians are a major model today is the richness of their history as a model system and the consequent deep understanding of their development that permits contextualization of ongoing discoveries. Over a century ago, experimentalists chose amphibian embryos, both anuran (frogs, including *Xenopus)* and urodele (newts and salamanders), as the ideal candidates to develop and apply the embryological techniques necessary to answer central questions about animal development (Gurdon and Hopwood 2000; Wlizla, McNamara, and Horb 2018). The unique convergence of several appealing features of amphibian development made them the obvious choice at the time and are major strengths to this day. Intuitive among these are amphibian embryos' rapid, external, and robust development and large size; the large number of embryos that can be obtained from a single mating pair; the ability to induce mating year-round in species including *Xenopus*; and the cost-effectiveness of animal care (Gurdon and Hopwood 2000; McNamara, Wlizla, and Horb 2018; Wlizla, McNamara, and Horb 2018). Additionally, embryos can be cultured easily through all developmental stages in simple saline solution and are remarkably amenable to explant, transplant, and ablation techniques (Schoenwolf 2001; Sive, Grainger, and Harland 2000). No other tetrapod model boasts this combination of traits. Aside from these inherent advantages, careful observation and lineage tracing using either endogenous pigments or applied dyes allowed the generation of reliable developmental time tables and fate maps, which have served as a crucial framework for future experiments (Dale and Slack 1987; Keller 1975, 1976; Moody 1987a,b). Further advantages relevant to molecular investigations are discussed below.

All of these features made amphibians a favored model, and can explain why foundational principles of embryonic (including neural) development were discovered in this system. These discoveries have been thoroughly reviewed by many others (De Robertis etal. 2000; Gurdon and Hopwood 2000; Harland and Grainger 2011; Kimelman 2006), but for the purpose of contextualizing the rest of this review, two examples will be briefly summarized here. Famously, Mangold and Spemann used transplantation and lineage tracing techniques to demonstrate the powerful inducing activities of the dorsal mesoderm, which came to be known as the Organizer (Spemann and Mangold 1924). This tissue, like its homologs in other vertebrates, is responsible for establishing the dorsal-ventral axis of the embryo, including the induction of neural identity in the dorsal ectoderm (De Robertis et al. 2000; Niehrs 2004). Another major discovery, made by Barth and similarly by Holtfreter, was that dissociated cells of the blastula stage embryo differentiate with neural identity (Barth 1941; Holtfreter 1944). This led to the idea that the "default" identity of cells is to assume a neural fate. Following from these and other experiments came the long-standing two-step "activation and transformation" model of neural specification, proposed by Nieuwkoop, wherein the neural territory is first induced in the dorsal ectoderm with anterior identity, and

posterior neural fates are subsequently induced in more caudal positions (Nieuwkoop 1952a,b; Nieuwkoop et al. 1952). This model has been deeply influential in ongoing efforts to characterize the mechanisms of neural induction and patterning across vertebrates (see below).

These discoveries exemplify some of the most essential contributions of amphibians as classical embryological models. As a prelude to the explosion of molecular discoveries that would describe a litany of mechanistic detail, amphibians were already a preeminent model in the investigation of vertebrate neurodevelopment. As the field of developmental biology turned towards a molecular approach, additional characteristics of amphibian embryos served to further strengthen their position, as discussed below.

## *Drilling down into mechanisms: Unique opportunities presented by the* Xenopus *toolkit*

The aforementioned embryological work demonstrated which embryonic tissues participate in neural induction, but the molecules responsible for this process remained a mystery for several decades, despite fervent efforts to identify them in various vertebrate models. In the 1990s, however, unique technical capabilities of the *Xenopus* embryo synergized with elegant and creative experimental design to identify these molecules for the first time (references to follow in this section). This foundational work unlocked a molecular treasure chest for the detailed characterization of vertebrate neurodevelopment by relying on a core set of tools offered only by the *Xenopus* system. A brief summary of this essential toolkit is given in this section and presented graphically in Figure 1, followed by a discussion in the next section of major molecular discoveries made possible by *Xenopus* research.

Along with the embryological advantages summarized above, several key features kept *Xenopus* in the spotlight, especially as more molecular tools became available. Chief among these is the ability to easily inject reagents of choice into individual blastomeres at early stages, and, with the use of lineage tracing dyes and detailed fate maps, observe their direct and indirect effects throughout subsequent developmental stages (Figure 1 B,E). Injectable reagents include plasmids and mRNA for overexpression experiments, morpholinos for knockdown of maternal or zygotic expression, and, recently, CRISPR/Cas9 for genome editing (Aslan et al. 2017; Bhattacharya etal. 2015; Blitz et al. 2013; Guo et al. 2014; Naert et al. 2020; Naert and Vleminckx 2018; Nakayama, Grainger, and Cha 2020; Tandon et al. 2017). Although all of these tools can be used in *Xenopus laevis* or *X. tropicalis*, CRISPR approaches have more commonly been deployed in *X. tropicalis* due to its diploid genome, whereas the pseudotetraploid *X. laevis* is often preferred for overexpression experiments and for embryological, cell biological, and biochemical approaches because of its larger size (Harland and Grainger 2011; Kakebeen and Wills 2019).

In both species, targeted injection of single blastomeres has been utilized extensively to restrict the direct effects of such perturbations to tissues of choice, such as to the dorsal ectoderm that gives rise to the brain (Moody 1987a,b). One important and widely used version of this strategy is to inject only one cell at the 2-cell stage. Crucially, in *Xenopus,* unlike the other major vertebrate models, the progeny of each of these cells stay mostly

restricted to either the left or the right sides of the animal, without much mixing. Thus, any molecules injected into only one of these two cells will be inherited by one side of the animal, while the other side will be left as an internal control (Figure 1 B,E). This makes it possible to compare manipulated tissue to contralateral control tissue within an individual animal, enabling the detection of subtle phenotypes that are difficult or impossible to detect via comparisons between individuals (DeLay et al. 2018; H. R. Willsey et al. 2018; Willsey et al. 2020). To complement the spatial precision afforded by targeted injections, some of the available tools can also achieve temporal specificity under the experimenter's control (for example, drug- or heat-inducible constructs) (Chae, Zimmerman, and Grainger 2002; Horb et al. 2019; Roose et al. 2009; Wheeler, Hamilton, and Hoppler 2000).

Other common approaches similarly take advantage of the amphibian embryo's size and accessibility. Conveniently, the aquatic nature of *Xenopus* development facilitates straightforward treatment with pharmacological agents (Figure 1 C), either on their own or to induce the activity of injected molecules (Tomlinson, Hendry, and Wheeler 2012; Wheeler and Brandli 2009; Willsey et al. 2020). Many of these can subsequently be washed out by returning embryos to drug-free medium. Injected reagents and drug treatments can also be applied in combination with explant or transplant techniques (Figure 1 D) (Dingwell and Smith 2018; Lowery et al. 2012), a tactic that has been applied extensively to give molecular insight into major embryological discoveries like the ones discussed above.

The effects of these manipulations are also easily assessed in *Xenopus* (Figure 1 E–l). Observation of gross embryonic phenotypes alone can often give a quick and convenient readout. *In situ* RNA hybridization and immunostaining techniques allow simple visualization of transcripts and proteins or other molecules in whole mount embryos, explants, or sectioned tissue. Transgenic lines expressing fluorescent reporters that facilitate live imaging are increasing in their availability through the efforts of individual labs and resource centers (Horb et al. 2019; Nenni et al. 2019; Pearl et al. 2012; Tandon et al. 2017). Standard molecular biology techniques like RT-PCR, co-immunoprecipitation, and Western blot are commonly employed to gain additional information, and tools for studying physiological and behavioral metrics are also readily available (references below). More recently, omics techniques have been applied to characterize transcript and protein expression, chromatin accessibility, and metabolic features in different tissues over developmental time, with or without experimental manipulation and often at single-cell resolution (Aztekin et al. 2019; Briggs et al. 2018; Kakebeen et al. 2020; Lombard-Banek, Choi, and Nemes 2020; Lombard-Banek et al. 2017; Niu et al. 2020; Owens et al. 2016; Peshkin et al. 2015; Sun, Champion, et al. 2016; Sun, Dubiak, et al. 2016; Willsey et al. 2020). The result of all this experimental power has been a steadily growing body of knowledge about *Xenopus* development that supports ongoing work (Heasman 2006; Houston 2017). As described below, the application of this suite of techniques in *Xenopus* has facilitated the discovery of highly conserved molecular features fundamental to vertebrate neural development.

## *Understanding neural induction, neural patterning, and neurogenesis: Fundamental discoveries made in* Xenopus

Equipped by previous embryological discoveries with a knowledge of the tissue interactions important for neural induction and patterning, as discussed above, the field had turned an eager eye toward the identification of the molecular players responsible. Research (references to follow in this section) took advantage of the exceptional tractability of the *X. laevis* embryo to isolate and clone novel genes and to assess their functions through overexpression, knockdown, and rescue experiments. Several studies used an expression cloning approach to isolate factors whose inductive capabilities were then tested by injecting synthetic mRNA or morpholino oligonucleotides and observing the resulting embryonic phenotype, such as induction of ectopic dorsoanterior structures or reduction of head features. Much of the early work also made use of the animal cap, an explanted region of the embryonic ectoderm already known to be sensitive to inductive signals from other tissues, to assay the effects of candidate neural-inducing molecules; hypotheses generated by this approach were then tested in whole embryos to confirm their relevance. The identification of several molecular markers of neural identity, often of particular brain regions (see below), provided additional robust and definitive molecular readouts of the activity of newly identified factors. Once again, the remarkable amenability of the *Xenopus* embryo made it an efficient and productive system for making foundational discoveries about a growing list of molecular players, which would later be shown to have conserved roles in the neurodevelopment of other vertebrates, including humans.

During the 1990s, innovative experimentation took advantage of the unique combination of embryological and molecular tools available in *X. laevis* to identify the factors responsible for vertebrate neural induction for the first time. Pivotal experiments began with the isolation of mRNA from dorsal tissues and the construction of cDNA libraries, which were subjected to a variety of tests to home in on individual genes that function in dorsal induction. One particularly elegant approach was to inject pools of these cDNAs into embryos that had been ventralized by UV irradiation; single cDNA clones that could rescue the phenotype and induce dorsal (including neural) fates were subsequently isolated by iteratively fractionating and testing the cDNA pools (Smith and Harland 1991; Smith and Harland 1992; Smith et al. 1995). A parallel strategy was to demonstrate dorsal-inducing activity by injecting into the ventral side of un-irradiated embryos to identify clones able to induce a secondary axis (Cho et al. 1991; Sasai et al. 1994; Sokol et al. 1991). These experiments were only possible in *Xenopus,* due to the ability to easily generate embryos in large enough numbers and to screen plasmid libraries via injection with rapid phenotyping. Other tests, which required a similarly large number of embryos, involved *in situ* mRNA hybridization staining to demonstrate specific expression in endogenous or experimentally-induced dorsal tissues. These approaches identified many molecules expressed by the Organizer and anterior endoderm that are capable of inducing dorsal identity. Further experiments in *X. laevis* showed that several of these molecules are BMP antagonists, and their activity is required to prevent the neural ectoderm from being converted to epidermal fate by BMP signals produced on the ventral side (Fainsod et al. 1997; Khokha et al. 2005; Piccolo et al. 1996; Zimmerman, De Jesús-Escobar, and Harland 1996). Likewise, their ectopic overexpression

is sufficient to induce a secondary axis, which explains at the molecular level how transplantation of an Organizer into a new host has the same consequence. Three of these antagonists (Chordin, Noggin, and Xnr3) were originally discovered in *Xenopus* using these strategies (Sasai et al. 1994; Smith and Harland 1992; Smith et al. 1995), and their function in dorsal induction is conserved throughout vertebrates. Indeed, their activity is so central to neural induction that they are exploited by current protocols that generate neurons from human iPSCs in culture (Lee, Lee, and Moody 2014; Sasai et al. 2008).

This work constituted a major breakthrough, and served as a crucial turning point in neurodevelopmental biology for at least three reasons. First, it highlighted some previously unappreciated parallels between core principles of *Drosophila* and vertebrate neural development, namely that BMP antagonism is required for neural induction, even though many of the vertebrate genes involved were novel (i.e., had no known fly ortholog). Importantly, these vertebrate genes had been discovered not by intentionally testing invertebrate players, but by the independent approach of expression screening experiments described above. This discovery inspired the targeted investigation of other vertebrate homologs of genes known to regulate *Drosophila* neural development (see below). Second, although most publications focused only on one or two genes at a time, the expression cloning approach had generated vast libraries of plasmids encoding candidate regulators of neural specification, neural patterning, and neurogenesis. This trove of potential molecular regulators was the subject of systematic investigation over the coming years, and has continued to reliably identify new players to this day. Third, this work generated an invaluable experimental paradigm for the rapid characterization of new molecules, whether brought to attention due to homology with *Drosophila* genes or directly through empirical methods in vertebrates. The endogenous neural plate of *Xenopus* had become a simple, robust, and familiar ground for assaying neural identity. Furthermore, the identification of BMP antagonists as powerful neural inducers allowed investigators to reproducibly generate large quantities of neural tissue in the form of neuralized animal caps, which could then be subjected to further manipulations to characterize candidate regulators.

Indeed, many experiments capitalized on the accessibility of animal caps or explanted neural plates to test for novel functions in neural patterning. A common approach involved injection of mRNA encoding suspected anteriorizing or posteriorizing factors directly into the embryo; explants could then interrogated via *in situ* mRNA hybridization, Western blot, RT-PCR, or other assays to detect regional neural markers (e.g., *otx2* for the anterior neural plate, *egr2* (*krox20*) for rhombomeres 3/5 of the hindbrain, or *hoxb9* for the spinal cord (Nieto, Bradley, and Wilkinson 1991; Pannese et al. 1995; Wright et al. 1990). Neural identity could be similarly queried after coculture with other regions of the embryo, especially after overexpression or knockdown of candidate regulators in these regions. Explants thus provided a straightforward readout of the effects of such manipulations on neural fate. Meanwhile, the intact *Xenopus* embryo was readily available to test the resulting hypotheses in a more physiological context. Overexpression and depletion experiments in whole embryos were often assessed through phenotypic readouts or by *in situ* mRNA hybridization for regional neural markers. Importantly, these stains in whole embryos allowed for a spatially intact comparison between regional markers, to distinguish the effects of candidate regulators on different regions along the anteroposterior axis. For example, co-

staining for *otx2, krox20,* and *hoxb9* within the same embryo could show a loss of one domain, a shift in another, and an expansion in the third. Furthermore, unilateral perturbations through targeted blastomere injection further empowered these analyses by permitting comparison to the contralateral control. This retention of spatial information within embryos was critical for detecting the effects of signaling gradients and counter gradients of secreted antagonists (see below).

Patterning of identities within the neural ectoderm was pioneered using these approaches in *Xenopus,* fueled by previous expression screens and with a growing focus on major developmental signaling pathways as likely regulators. Again, the demand for an efficient system in which many candidate molecules could be tested quickly, rigorously, and in a uniform manner made *Xenopus* a central model during this era. Discoveries in *Xenopus* showed that, as neural specification proceeds, Wnt, FGF, and retinoic acid (RA) signals produced in caudal regions of the embryo induce posterior neural identities in those positions (Cox and Hemmati-Brivanlou 1995; Durston et al. 1989; Holowacz and Sokol 1999; Kiecker and Niehrs 2001; Kolm and Sive 1997; Kolm, Apekin, and Sive 1997; McGrew, Hoppler, and Moon 1997; Pownall et al. 1996; Ribisi et al. 2000; Sharpe 1991). Echoing the role of BMP antagonism in establishing neural identity, and giving molecular character to the “default” model, inhibitors of these signaling pathways preserve the anterior character of the rostral neurectoderm (Bouwmeester et al. 1996; Glinka et al. 1998; Kazanskaya, Glinka, and Niehrs 2000; Leyns et al. 1997; Pera and De Robertis 2000; Piccolo et al. 1999; Shibata et al. 2000; Wang et al. 1997). Several of these inhibitors, including the Wnt antagonists Dickkopf-1 and Frzb and the trivalent BMP, Nodal, and Wnt inhibitor Cerberus, were identified as anterior neural inducers by work in *X. laevis* before being shown to have the same role in other vertebrates. Targets of these patterning pathways were also described in *Xenopus* and continue to be used as markers of different regions of the brain and spinal cord. The evidence supports the major tenets of the “activation and transformation” model (Carron and Shi 2016; De Robertis et al. 2000), although modifications to this model have been proposed, also based on work in *Xenopus* (Polevoy et al. 2019). These events and players are, as usual, highly conserved among vertebrates.

Another major area of contribution by work in *Xenopus* has been in the discovery and functional characterization of molecules that regulate neurogenesis. Much of this work has used primary neurogenesis as a model, due in large part to its early occurrence during embryogenesis and its easy visualization on the dorsal surface of the amphibian embryo (Chitnis et al. 1995; Hartenstein 1989, 1993). Mechanisms of neurogenesis discovered in *Drosophila* were frequently functionally tested in vertebrates for the first time using *Xenopus,* through gain-and loss-of-function schemes in whole embryos via the techniques described above. Once again, the visual and experimental accessibility of the amphibian embryo made it a powerful platform for the isolation and functional characterization of a host of new molecules. Now-famous proneural factors, including the Neurogenin, NeuroD, and AscI families, were functionally characterized for the first time in *X. laevis* in the 1990s (Ferreiro et al. 1993, 1994; Lee et al. 1995; Ma, Kintner, and Anderson 1996). Likewise, the function of the Notch/Delta pathway in lateral inhibition in the neurogenic ectoderm, as well as its interactions with proneural genes, was elucidated in *Xenopus* (Chitnis et al. 1995; Chitnis and Kintner 1996; Wettstein, Turner, and Kintner 1997), with some striking

similarities to the *Drosophila* system. Moreover, the regulation of neurogenesis by these factors has been shown to operate in many instances of neuron production, including in the frog brain at later stages (D'Amico, Boujard, and Coumailleau 2013; Thuret, Auger, and Papalopulu 2015; Wullimann et al. 2005) and also in the mammalian brain (del Corral and Storey 2001).

It is worth emphasizing how much of an impact work like this in *Xenopus* has had on the field of neurodevelopmental biology. First, it has crucially informed work in other models, which has confirmed that similar mechanisms of neural specification, neural patterning, and neuronal differentiation are at play across vertebrates (Ozair, Kintner, and Brivanlou 2013; Stern 2005, 2006). Second, work in other models has been reciprocally tested in *Xenopus,* with the same outcome. As the only anamniote tetrapods, amphibians play an essential role in elucidating how neural development has evolved in vertebrates; characterization of *Xenopus* as representative anuran amphibians is a critical part of this investigation. Third, information gained from *Xenopus* research has been a driving force in the establishment and advancement of additional models of neural development, including human neural stem cell and organoid culture. Gurdon's serial cloning experiments in frogs were seminal to the understanding of pluripotency and the invention of iPSCs (Gurdon, Elsdale, and Fischberg 1958). Furthermore, the identification of major mechanisms of neural development by research in *Xenopus* has directed strategies for generating neural precursors and differentiated neurons, often with particular regional identities, in cell culture using human cells (Lee, Lee, and Moody 2014; Sasai et al. 2008). For example, BMP antagonists are applied to induce neural identity in culture, Wnt antagonists are applied to induce anterior fates, and Neurogenin expression is used to drive neuronal differentiation (Busskamp et al. 2014; Khan et al. 2020; Sloan et al. 2018; Zhang et al. 2013); these decisions follow directly and explicitly from foundational discoveries made in *Xenopus.* The refinement of human cell culture strategies has been a major innovation whose impact and potential cannot be overstated: it allows the testing of hypotheses in human cells and tissues in a manner not previously possible, and even opens the door to testing cell lines derived from individual people (Busskamp et al. 2014; Sloan et al. 2018; Zhang et al. 2013). These models will be invaluable in the daunting effort to identify patient-specific genetic factors and treatment effects, especially against the backdrop of the heterogeneity and pleiotropy characteristic of neurodevelopmental disorders. Finally, as research in human systems creates an explosion of *in vitro* data, hypotheses generated by this work can be tested in a high-throughput manner *in vivo* using *Xenopus* (A. J. Willsey et al. 2018). These complementary approaches offer valuable opportunities for the coming decades.

## Deep conservation among tetrapod brains

The similarities between amphibian and human brains extend beyond basic mechanisms of early neural development. Counter to tempting notions regarding the uniqueness of the human brain, many features of later embryonic brain development seen in humans are also represented in other tetrapods, including frogs (Clinton et al. 2014; Fernandez et al. 1998; Martínez-Cerdeño et al. 2016; Norimoto et al. 2020; Tosches et al. 2018). In fact, early embryonic brains from mammals and frogs resemble each other to a surprising degree, and lineage tracing experiments have shown that homologous embryonic regions give rise to

homologous adult brain structures. A brief overview of several embryonic and larval stages of brain development in *Xenopus* is shown in Figures 2 and 3.

Particularly exciting comparisons can be drawn between conserved patterning mechanisms that regulate regionalization of the brain, and even about further subdivision and neurogenesis within these regions. A thorough analysis of marker gene expression has shown over the last three decades that the factors that characterize various brain regions in mammals are largely similar in frog, although there are some differences. Briefly, amphibian and amniote brains are patterned by conserved signals, including BMP and Wntfrom the roof plate, RA from the non-neural ectoderm, Shh from the floor plate, and FGF from the anterior neural ridge (ANR) (Echevarria et al. 2003; Stern 2006; Vieira et al. 2010). These induce conserved targets in the neural tube, and pattern the alar (dorsal) and basal (ventral) aspects of the resulting forebrain, midbrain, and hindbrain. Two additional signaling centers, the midbrain-hindbrain boundary (MHB) and the zona limitans intrathalamica (ZLI) are also induced, and signals secreted by these regions pattern the brain at finer scales (Echevarría et al. 2003; Vieira et al. 2010). The expression and activity of these major signaling pathways in brain patterning is highly conserved between frogs and mammals, although the particulars of their targets' expression varies in some cases, most often in the telencephalon (described further in the next section).

A summary of some commonly used markers is presented in Figure 4, and shows tetrapod-typical expression of these factors in the developing *Xenopus* brain. These include *foxg1*, expressed throughout the telencephalon (Bourguignon, Li, and Papalopulu 1998); *tcf4* (also known as fc/7/2), expressed in the alar diencephalon and midbrain (Bandin, Morona, and Gonzalez 2015); *otx2,* expressed throughout the forebrain and midbrain (Pannese et al. 1995); *gbx2,* expressed in the hindbrain in addition to alar prosomere 2 of the diencephalon (Morona et al. 2011; Tour et al. 2001; von Bubnoff, Schmidt, and Kimelman 1996); *foxa2,* expressed in the ventral midbrain and hindbrain (Lee et al. 1997; Ruiz i Altaba, Jessell, and Roelink 1995); and Hox family genes, expressed in the hindbrain (Frank and Sela-Donenfeld 2019; Pownall et al. 1996). Note also that FGF, Wnt, and *en2* are expressed at the MHB (Brivanlou and Harland 1989; Hemmati-Brivanlou et al. 1991; Isaacs, Tannahill, and Slack 1992; Wolda, Moody, and Moon 1993), while Shh is expressed in the floor plate and the ZLI (Domínguez, González, and Moreno 2010). These patterns hold for both *Xenopus laevis* and *X. tropicalis*, as for other vertebrates.

Excitingly, features of brain development are even more similar between frogs and humans than a simple check of regional markers indicates. Recent transcriptomic analysis of isolated *Xenopus tropicalis* brains has allowed a comparative mapping to human brain development over a range of stages, and the pair track with each other in a surprisingly similar manner over time (Willsey et al., under review). The results show that stages 40-47 of *Xenopus* brain development compare closely to human mid-fetal development, as summarized in Figure 5. This underlines the utility of frogs, already prized for their other advantages, as promising models for the investigation of neurodevelopmental processes relevant to humans.

## A focus on the forebrain

It is clear that early mechanisms and core features of neurodevelopment in frogs and other tetrapods, including humans, are deeply conserved (Binder, Hirokawa, and Windhorst 2008; Medina, 2009; Medina and Abelian 2009). However, the brains of adult humans are in many ways obviously different from adult frog brains, both in morphology and in the kinds of behaviors they support. Undoubtedly, differences in neurodevelopmental programs have driven the disproportionate expansion of parts of the forebrain (Kriegstein, Noctor, and Martinez-Cerdeno 2016), in particular the cerebral cortex, and future comparative, genomic, and molecular studies are urgently needed to understand the conservation of these mechanisms across species. Such studies will be vital for translating insights from frog and other animal models to human. In particular, with the advent of single cell genomics technologies, comparative single cell molecular studies herald a new era of translating insights across experimental models (Arendt et al. 2016).

Many “higher order” behaviors displayed by mammals, including primates, and humans specifically, have been proposed to be supported by uniquely evolved neurodevelopmental programs executed during forebrain development in these lineages (Kriegstein, Noctor, and Martínez-Cerdeño 2016; Namba and Huttner 2017; Namba, Vaid, and Huttner 2019; Nowakowski et al. 2016). This suggestion is intuitively appealing for humans, particularly when trying to frame knowledge about complex psychological processes (including multisensory, cognitive, and emotional processes) associated with human brain function and human behavior. This presumed uniqueness is even more tempting with respect to understanding psychiatric disorders, which are human-specific by definition, and in this context it is frequently assumed that human brains are in a class of their own. Certainly, there are differences between human brain development and brain development in other animals. However, compelling evidence suggests that many more features of forebrain development are conserved among tetrapods than previously appreciated (Clinton et al. 2014; Jímenez et al. 2020; Martínez-Cerdeño et al. 2016; Moreno and González 2017; Tosches and Laurent, 2019). This includes recent work using single cell RNA sequencing to assess the degree of conservation between progenitor and differentiated cell types in various regions of the forebrain, which has revealed striking similarities between reptiles and mammals and suggests that even mammalian-specific features appear to be elaborations of ancestral programs (Norimoto et al. 2020; Tosches et al. 2018). Such an analysis has not yet been undertaken in frogs, but other evidence in this vein comes from extensive work in *Xenopus* to catalog the expression patterns of known markers of forebrain regions and cell types.

Indeed, canonical forebrain markers exhibit conserved expression patterns in *Xenopus* and mammalian brains (Figure 4). As development and conservation of several regions of the diencephalon have been recently highlighted (Bandín, Morona, and González 2015; Domínguez et al. 2013; Domínguez, González, and Moreno 2014; Domínguez, González, and Moreno 2015; Moreno and González 2020; Moreno et al. 2017; Morona et al. 2020), we will restrict the present discussion to the telencephalon. Within the developing *foxg1+* telencephalon (Figure 2), the subpallium expresses *nkx2.1, isl1, ascl1, dlx2/5, gsx1/2* (Bachy, Berthon, and Rétaux 2002; Brox et al. 2002, 2003; Hollemann and Pieler 2000;

Illes, Winterbottom, and Isaacs 2009; Moreno et al. 2008; Papalopulu and Kintner 1993; Small et al. 2000; van den Akker et al. 2008; Winterbottom et al. 2010) while the pallium is marked by the expression of *pax6, emx1/2, ngn, neuroD,* and *tbr1/2* (Bachy, Berthon, and Rétaux 2002; Brox et al. 2004; D'Amico, Boujard, and Coumailleau 2013; Fernandez et al. 1998; Hirsch and Harris 1997; Pannese et al. 1998; Ryan et al. 1998; Wullimann et al. 2005). Overlapping domains of LIM-homeodomain gene expression mark various regions in a manner similar to that seen in mammals (Bachy, Berthon, and Retaux 2002; Bachy, Vernier, and Retaux 2001; Moreno et al. 2004). The developing subpallium can be subdivided into the lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE), which generate the striatum and the pallidum, respectively, as in mammals (Morona and Gonzalez 2013). A neurogenic territory caudal to the frog LGE and MGE has been proposed to be homologous to the caudal ganglionic eminence (CGE) of mammals (Moreno et al. 2008), but additional molecular, structural, and functional analysis must be carried out to determine the extent of this potential homology (Jiménez et al. 2020). The LGE expresses *gsx2, dlx2/5,* and *isl1,* while the MGE expresses *gsx1, dlx2/5, isl1,* and *nkx2.1* (Bachy, Berthon, and Retaux 2002; Illes, Winterbottom, and Isaacs 2009; Moreno et al. 2008). A subset of these, *nkx2.1, gsx1/2,* and *dlx2,* are expressed both within and outside the subpallial VZ, whereas *isl1* and *dlx5* are expressed only outside the VZ (Illes, Winterbottom, and Isaacs 2009; Moreno et al. 2008). Neurogenesis in the subpallium is driven by *ascii,* echoing this proneural gene's role in primary neurogenesis during earlier stages (Ferreiro et al. 1993, 1994). The major differentiated neuronal cell type produced by the subpallium at these stages is GABAergic interneurons, some of which migrate tangentially into the pallium (Moreno, González, and Rétaux 2008).

Likewise, the pallium can be further subdivided into the medial, dorsal, lateral, and ventral pallium; these give rise to the hippocampus, (neo)cortex, olfactory cortex and amygdala, and claustrum and amygdala, respectively (Morona and Gonzalez 2013). The ventral pallium is distinguished from the other regions in that it does not exhibit *emx1* expression in the ventricular zone (VZ), and shows a comparatively higher level of *pax6* and *tbr2* outside the VZ (discussed further below) (Bachy, Berthon, and Retaux 2002; Brox et al. 2004; Moreno, Rétaux, and González 2008). *emx2* and *lhx2* are expressed throughout the pallium; *pax6* is expressed throughout the pallial VZ; and *tbr1/2* are expressed throughout the pallium outside the VZ (Bachy, Berthon, and Retaux 2002; Brox et al. 2004; Moreno, Rétaux, and González 2008; Pannese et al. 1998). As is true for the subpallium, these features closely parallel the mammalian case. Neurogenesis in the pallium is driven by Neurogenin and NeuroD, as in primary neurogenesis (Lee et al. 1995; Ma, Kintner, and Anderson 1996; Wullimann et al. 2005). Thus, not only do the same embryonic regions give rise to homologous brain regions in frog and human adults; the molecular features of these regions and the cell types within them also appear to be conserved. Furthermore, functional tests have confirmed once again that these conserved molecules also enact conserved regulatory programs during telencephalon development. For example, Pax6 is required for normal expression of *neurogenin* and subsequent neurogenesis in the pallium (Nakayama et al. 2015), and *nkx2.1* is induced by Shh signaling to confer ventral identities in the subpallium (van den Akker et al. 2008).

Although most of these core features of forebrain gene expression appear to be conserved between *Xenopus* and mammals, there are a few aspects that appear to have diverged between these lineages. Most notably, the combinatorial expression of Lhx-family genes differs in frog and mouse; whereas the expression of this family shows concordance in the diencephalon and hypothalamus, *lhx2/9* and *lhx1/5* are reduced in the telencephalon of *Xenopus* compared to mouse (Bachy et al. 2001). However, while the complexities in the spatial overlap of these specific paralogs differs, telencephalon expression of the family overall is conserved. These data suggest that the family as a whole may execute conserved functions to regulate regionalization and connectivity in this domain, whereas the differences in unique paralog expression may underlie cortical differences between frogs and mammals (Bachy et al. 2001; Yang et al. 2020). Another striking difference is in the induction of *nkx2.1* by Shh signaling in the ventral forebrain. In *Xenopus, nkx2.1* is induced in the alar hypothalamus in addition to the basal hypothalamus, in contrast to its restriction to the basal hypothalamus in mouse (van den Akker et al. 2008). This divergence likely affects the patterning of the ventral forebrain and may explain some differences in pallial-hypothalamic connectivity between frogs and mammals (van den Akker et al. 2008), some of which are involved in social and maternal behaviors (Medina, 2009). A more subtle difference is observable in the expression of *gsx1/2,* which appear to overlap in the subpallium VZ in *Xenopus* but occupy more mutually exclusive domains in mouse and which may therefore regulate ventral forebrain neurogenesis trajectories differently between the two groups (Illes et al. 2009). The developmental mechanisms that drive these differences, their exact consequences on brain development, and their implications for brain evolution have yet to be fully explored.

Neurogenesis in the tetrapod pallium has been the subject of much investigation. In mammals, including humans, division of Pax6+ radial glia (RGs) in the VZ gives rise to Tbr2+ Ngn2+ intermediate progenitors (IPs) of the SVZ (Bayatti et al. 2008; Englund et al. 2005; Haubensak et al. 2004; Noctor et al. 2001; Noctor et al. 2004; Sessa et al. 2008). These divide and activate expression of NeuroD, which drives terminal differentiation into Tbr1+ postmitotic neurons, particularly glutamatergic neurons (Martynoga, Drechsel, and Guillemot 2012). As cells proceed down this differentiation pathway, they migrate away from the VZ, into the SVZ, and out into the mantle, following radial paths along continuous basal processes maintained by the RGs (Paridaen and Huttner 2014; Rakic 1971). This sequence of gene expression is thus correlated with spatial position, and it also reflects functional interactions between these several transcription factors (Elsen et al. 2018; Martynoga, Drechsel, and Guillemot 2012). Importantly, neurogenesis in the pallium of *Xenopus* also follows this sequence over developmental time and space and is driven by conserved functional interactions between the same factors (Brox et al. 2004; Wullimann et al. 2005). Indeed, some of these factors were discovered in *Xenopus,* including Neurogenin (discussed above) and Tbr2 (also known as *eomesodermin (eomes),* so named for its originally identified role in mesoderm development) (Ma, Kintner, and Anderson 1996; Ryan et al. 1996, 1998).

While many of these core features are clearly conserved, anuran amphibians do not exhibit a subventricular zone as distinct as the mammalian SVZ (Moreno and González 2017). Nevertheless, Tbr2 and Ngn2 are expressed in the pallium in embryonic and larval frogs

during neurogenesis (Figure 4), and their expression patterns are similar to those in mammals (Brox et al. 2004; Moreno et al. 2003; Nieber, Pieler, and Henningfeld 2009; Wullimann et al. 2005). While there is some debate about the proliferative state of Tbr2+ cells in *Xenopus* in stages beyond neurogenesis (Hevner 2019; Moreno and González 2017), birds and some reptiles, like mammals, do have Tbr2+ proliferative cells outside the VZ in the pallium (Cheung et al. 2007; Clinton et al. 2014; Martinez-Cerdeno et al. 2016; Montiel et al. 2016); consequently, amphibians are in the spotlight in terms of this cell population's evolution and development. It should also be noted that although a morphologically distinct SVZ may be a convenient feature for nomenclature purposes, it is not a prerequisite for the existence of proliferative IPs in amphibians, which may merely reside within the VZ along with RGs.

Another difference between frogs and humans is in the construction of the cortex. In mammals, neurogenesis in the dorsal pallium generates a six-layered neocortex in an "inside-out" fashion, as newly differentiating neurons migrate out past their predecessors to form new layers (Molnar et al. 2006; Paridaen and Huttner 2014). The *Xenopus* cortex is considerably simpler, even in comparison to the three-layered cortex found in some sauropsids, and neurons migrate to their final positions in an "outside-in" order (Cárdenas and Borrell 2020; Moreno and González 2017). In addition, human and some other mammalian cortices undergo extensive folding, which amphibian brains do not (Molnár et al. 2006; Rakic 2009). Some questions about corticogenesis are therefore likely to be more completely studied in mammalian models. However, although the spatial choreography is less complex in frogs, the molecular mechanisms that regulate neuronal differentiation and migration are conserved (Brox et al. 2004; Wullimann et al. 2005). As such, *Xenopus* retains its value as a model for core elements of corticogenesis in tetrapods, and the many aspects of neurodevelopment that evidently are conserved cement its position as a highly useful model of forebrain development.

## *Beyond fundamentals: modeling disorders of the brain in* Xenopus

The remarkable technical strengths of the *Xenopus* system have already made it a productive model for human disorders in recent years (Blum and Ott 2018; Getwan and Lienkamp 2017; Hwang, Marquez, and Khokha 2019; McCammon and Sive 2015; Nenni et al. 2019; Sater and Moody 2017; Walentek and Quigley 2017). Recent improvements to both the *X. laevis* and *X. tropicalis* genomes have confirmed that the majority of human disorder risk genes are conserved in terms of sequence and syntenny in the frog (Hellsten et al. 2010; Mitros et al. 2019; Session et al. 2016). With this in mind, the optimization of CRISPR-mediated genome editing in *Xenopus* (Aslan et al. 2017; Bhattacharya et al. 2015; Blitz et al. 2013; Guo et al. 2014; Naert and Vleminckx 2018; Naert et al. 2020; Nakayama, Grainger, and Cha 2020; Tandon et al. 2017) has allowed the targeted mutagenesis of disorder risk genes identified by patient sequencing efforts, with an ever-growing list to investigate. Many of these experiments take advantage of the unilateral mutagenesis approach in F0 animals to allow the identification of subtle phenotypes by comparison to the contralateral control. Importantly, mutant phenotypes may also be rescued by injection of the *Xenopus* or human homolog of the CRISPR-targeted gene, or by expression of suspected compensatory factors. Rescues can also be attempted through the use of pharmacological

agents, with the additional potential of large-scale drug screens on mutants after integration of CRISPR-induced mutations into the germline. This strategy provides an attractive opportunity in the search for potential drug treatments for disorders. Drugs can also be screened as potential causative agents that may affect the same cell populations or behaviors as genetic mutations. All of these approaches continue to build on the usual benefits of the *Xenopus* system, as described in previous sections.

In addition to these general advantages, several features of *Xenopus* make it a particularly powerful model for studying brain development, specifically. During early stages, the neural tissue exists on the surface of the embryo (Chitnis et al. 1995; Hartenstein 1989, 1993); at later stages, even once internalized through neurulation, the nervous system is still easily observed and manipulated due to its extreme dorsal position within the optically transparent and externally developing embryo. Conveniently, early embryonic effects of blastomere injection can be circumvented by electroporation techniques that target the brain directly (Bestman and Cline 2020). Several imaging tools, including injectable calcium dyes, *in vivo* lineage and axon tracing techniques, and transgenic animals (e.g., GcAMP6:GFP, brainbow, hsp70-CRE, and I-SceI lines), make the imaging of live developing brains simple compared to other vertebrate systems (Ablondi et al. 2020; Hiramoto and Cline 2009; Hiramoto and Cline 2020; Horb et al. 2019; Koser et al. 2016; Offner et al. 2020; Paudel et al. 2019; Qian et al. 2020; Tandon et al. 2017; Thompson et al. 2019). The *Xenopus* oocyte is a long-favored model for electrophysiology studies of channel and other protein function (Kusano, Miledi, and Stinnakre 1977; Limon, Reyes-Ruiz, and Miledi 2008; Miledi et al. 2004; Sigel and Minier 2005; Ullah et al. 2015; Vindas-Smith et al. 2016), and electrophysiology tools have been adapted for use in embryos, tadpoles, and adults (Barkan, Zornik, and Kelley 2017; Pratt and Khakhalin 2013). Behavioral assays in tadpoles give a window into the outputs and functions of the nervous system (Khakhalin 2020; Khakhalin et al., 2020), during typical development and after genetic, pharmacological, or surgical perturbation. Many of these techniques have also been used to study adults at the molecular, cellular, tissue, and behavioral level (Barkan, Zornik, and Kelley 2017; Kelley et al. 2017; Pratt and Khakhalin 2013).

*Xenopus* has already been established as a successful model for elucidating mechanisms of a wide range of disorders, some of which are commonly thought of as unique to human neurobiology. Frogs have been employed to make key discoveries regarding convergent mechanisms of complex and heterogeneous genetic disorders of the brain. One recent publication identifies a shared role of autism spectrum disorder risk genes in regulation of neural progenitor cell biology during forebrain neurogenesis (Willsey et al., under review), and two others suggest a specific role on microtubules (H. R. Willsey et al. 2018; Wilsey et al. 2020). These findings have provided long-sought hypotheses about the basis of this disorder, and have also given insight into the recognized comorbidity of autism with other congenital disorders. *Xenopus* has also been used to model disorders associated with dysregulated neuronal activity, particularly epilepsy (Sega et al. 2019), through developmental studies in embryos in addition to decades of electrophysiological studies using oocytes. Mechanisms of Fragile X syndrome have been dissected at the molecular, cellular, electrophysiological, and behavioral level using *Xenopus* (Faulkner et al. 2015; Truszkowski et al. 2016), which is particularly fascinating given that *Xenopus* does not have

an X chromosome, allowing the experimental isolation of the effects of single genes from the context of mammalian chromosomal sex determination. Recent work has also modeled elements of neurodegenerative disorders, such as Parkinson's and Alzheimer's (Horowitz et al. 2001; Liu et al. 2018; Paganelli et al. 2001); neuroinflammatory disorders, including Huntington's (Haremaki, Deglincerti, and Brivanlou 2015); and disorders of myelination, such as multiple sclerosis (Kaya et al. 2012; Mannioui et al. 2018). Insights into the mechanisms of neuroblastoma have also resulted from work in *Xenopus* (Wylie et al. 2015). The fact that research in *Xenopus* has shed light on such a variety of human disorders, the etiologies of which involve diverse brain regions, cell types, and mechanisms, is a testament to its extraordinary utility as a model. The emergence of *Xenopus tropicalis* as a model for genetic disorders of the brain parallels recent recognition of its value in the endeavor to understand disorders of other tissues and organs (Blum and Ott 2018; Hwang, Marquez, and Khokha 2019; Kakebeen and Wills 2019; Nenni et al. 2019; Sater and Moody 2017), including the heart (Deniz et al. 2018; Duncan and Khokha 2016; Hoppler and Conlon 2020; Kaltenbrun et al. 2011; Warkman and Krieg 2007), kidney (Blackburn and Miller 2019; Blackburn et al. 2019; Krneta-Stankic, DeLay, and Miller 2017; Lienkamp 2016; Marquez et al. 2020), neural tube (Wallingford 2005; Wallingford et al. 2013), airways (Tu et al. 2018; Walentek and Quigley 2017), esophagus (Nasr et al. 2019), and neural crest (Lasser et al. 2019; Mills et al. 2019).

## *Outlook on* Xenopus *as a model of brain development and disease*

*Xenopus* has been a prominent model of vertebrate neural development for the last century, with the promise of many decades to come. Its advantages include useful characteristics such as size and availability, which made it an early favorite for embryology; its amenability to molecular, genetic, and pharmacological experimentation, which has driven the discovery of many fundamental mechanisms of vertebrate neurodevelopment; and its deep conservation with humans and recent technical advances, which have already given key insights into human neurodevelopmental disorders. All of these have generated knowledge about embryonic brain development, and have also informed the establishment of new model systems, particularly human cell and organoid culture. *Xenopus tropicalis* is poised to contribute further to our understanding of human disorders as a higher-throughput genetic model that can be used to generate targeted hypotheses about disorder risk gene function.

Beyond the aspects of brain development discussed here, frogs have also been a valuable system for modeling other neurodevelopmental processes. The morphogenetic movements and regulation of convergent extension have been studied extensively in *Xenopus* (Keller et al. 2000; Shindo 2018), with several implications for our understanding of neural tube closure and associated congenital defects. Axon guidance (Erdogan, Ebbert, and Lowery 2016; Koser et al. 2016; Slater, Hayrapetian, and Lowery 2017; Thompson et al. 2019), synapse biology (Sakaki et al. 2020), circuit function (Barkan, Zornik, and Kelley 2017; Kelley et al. 2017), and behavior (Khakhalin 2020; Khakhalin et al., 2020) are all conveniently studied in frogs, and their evolutionary position affords them similar importance in answering questions about the evolution of these biological programs. Studies of the olfactory system and eye have shed light on how sensory organs develop and how they interface with different regions of the brain, in terms of both molecular interactions and

connectivity (Liu, Hamodi, and Pratt 2016). Frogs also have glia produced by conserved mechanisms (D'Amico, Boujard, and Coumailleau 2011; Goodbrand and Gaze 1991; Yoshida 2001), and as glia have been shown to be important in the development of a variety of disorders, *Xenopus* presents the opportunity to further investigate the roles of these cell types in the brain.

Of course, there are also some differences between frog and human brain development. One striking example is that corticogenesis in humans produces a six-layered structure, derived from the dorsal pallium. The cortex forms by the migration of neurons out past their recently differentiated sisters in the mantle, in an "inside-out" fashion (Molnár et al. 2006; Paridaen and Huttner 2014). The frog brain contains no such layering, and radial migration builds the cortex in an "outside-in" fashion more typical of non-mammalian vertebrates (Moreno and González 2017). However, the mechanisms that regulate neural progenitor cell proliferation and neuronal migration and differentiation are largely conserved (Brox et al. 2004; Wullimann et al. 2005), so the frog case is useful as a simplified example of cortical neurogenesis. Another marked difference is the elaborate folding of the human brain, compared to the lissencephalicfrog brain (Molnár et al. 2006, 2019; Rakic 2009). Mechanisms that drive this folding appear to be restricted to a few mammalian lineages, although the functions of the associated regulators may still be investigated effectively in other tetrapods.

A recently popular idea has been that the emergence of complex behaviors in some tetrapods (for example, mammals in general and primates more specifically) was made possible by the evolution of novel mechanisms of brain development proposed to be unique to those lineages (Briscoe and Ragsdale 2018; Molnar et al. 2019). Some of these features, such as the remarkable folding that generates the brains of humans and other gyrencephalic mammals, do appear to be innovations specific to those lineages (Sun and Hevner 2014). However, recent evidence has shown that some features previously thought to be restricted to mammalian development, such as the existence of proliferative Tbr2+ intermediate progenitors in an SVZ-like structure, may actually be synapomorphies among tetrapods (Martinez-Cerdeno et al. 2016). Deeply conserved neurodevelopment mechanisms like these would mean that many more aspects of human brain development can be modeled effectively in other tetrapods than previously appreciated, and amphibians are a crucial piece of that puzzle.

Fortunately, several of the existing differences between frogs and humans may provide hidden opportunities rather than serving as deterrents to using frogs. In general, understanding any differences between frogs and humans will shed light on the evolution of both, which is useful in itself. More specifically, frogs exhibit a few biological processes that humans do not, but which may give insight into human biology nonetheless. Frogs undergo metamorphosis, a well-characterized and major developmental event driven by circulating hormones whose production is regulated by the brain (Furlow and Neff 2006); this provides a powerful model to understand interactions between the brain, gonads, hormones, and the rest of the body, which has already become the subject of enthusiastic investigation in frogs (Buchholz 2015, 2017). Frog embryos and larvae also exhibit high regenerative capacity (Kakebeen and Wills 2019; Kha et al. 2019; Lee-Liu et al. 2017; Slack, Lin, and Chen 2008;

Tseng and Levin 2008), including of neural tissues (for example, the spinal cord and elements of the limb), and this capacity decreases after metamorphosis (Slack et al. 2004; Slack, Lin, and Chen 2008). Both the ability to regenerate and the loss of this ability provide attractive opportunities to study regeneration and to contrast it with the case of humans, who exhibit little or no regeneration of most tissues across their lifetime. Recent time-course single cell RNA-seq analyses of regenerating *X. laevis* and *X. tropicalis* spinal cords provide invaluable tools for those interested in this avenue (Aztekin et al. 2019; Kakebeen et al. 2020).

Due to both its similarities and differences with human neurodevelopment, *Xenopus* provides some additional opportunities in areas of recent keen interest. Frogs develop a blood-brain barrier during embryonic stages (Andino et al. 2016; Lau, Li, and Cline 2017), opening the door to genetic and pharmacological studies to characterize its formation and function. Its aquatic development is a convenient feature for those interested in neuroendocrine biology (particularly given that frogs undergo metamorphosis) or the effects of environmental toxicants on brain development (Buchholz 2017). As in humans, the brains of *Xenopus* embryos are lateralized in their structure and function (Blackiston and Levin 2013; Pai et al. 2012), providing the opportunity to study left/right patterning of the brain. Frogs also provide a fascinating platform to investigate the effects of sex as a biological variable in brain development (Kelley 1986; Kelley et al. 2020; Zornik and Kelley 2011; Zornik and Kelley 2017; Zornik and Yamaguchi 2008). The experimental strengths of *Xenopus* make it an excellent platform for investigating the genetic and environmental factors that interface with sex and development, in the brain and other organs.

One crucial role for *Xenopus* in the coming years will be to serve as an *in vivo* model for the abundance of hypotheses that will inevitably be generated by work in human cell and organoid culture. It is already clear that many mechanisms are conserved between the two, as discoveries in *Xenopus* have directed the refinement of these human-derived models (Lee, Lee, and Moody 2014; Sasai et al. 2008). In turn, work *in vitro* is likely to have parallels in the embryonic brain, but these will need to be tested directly in a vertebrate model to ascertain their applicability outside of culture conditions (A. J. Willsey et al. 2018). Thus, we predict a close hand-in-hand relationship between *Xenopus* and cell culture *in vitro* systems, wherein iterative exchange and the complementary advantages of each model collaborate to drive rapid discovery.

Where *Xenopus* will truly shine over the next decades, though, is as a higher-throughput *in vivo* model for the growing number of disorder risk genes identified by patient sequencing efforts (A. J. Willsey et al. 2018). Psychiatric disorders, including autism spectrum disorder, schizophrenia, Tourette disorder, and obsessive compulsive disorder, have been a point of particularly intense focus and productivity (Cappi et al. 2020; Satterstrom et al. 2019; Satterstrom et al. 2020; Wang et al. 2018). For many disorders, these lists are already hundreds of genes long, and increasing rapidly. Some of these genes have never been characterized in terms of a functional role in development, and those that have been investigated have diverse cellular roles across developmental time and space, making it difficult to pinpoint which functions are relevant to disorder pathobiology (A. J. Willsey et al. 2018; Sestan and State 2018). For these reasons, investigating risk genes one by one is

both impractical and probably under-informative, and a model in which dozens or hundreds of genes can be studied in uniform and in parallel is therefore a necessity. Mammalian and other amniote models are not amenable to such large-scale approaches, and the tetraploid genome and more divergent brain development of teleost fish pose some technical and theoretical challenges to their use, although they can certainly contribute valuable insights nonetheless (Joo et al. 2020; Thyme et al. 2019). Due to its experimental capabilities and conservation of neurodevelopmental processes, *Xenopus* (more specifically, the diploid *X. tropicalis*) is an optimal model in which to undertake the higher-throughput genetic screens necessary to allow the generation of targeted hypotheses about neurodevelopmental disorder etiology. CRISPR-based approaches in *X. tropicalis* have already proven to be informative to human disorders in this regard, and ongoing omics approaches, including whole-organism single-cell analyses, will continue to support incisive discovery in this system. In addition, frogs can act as a much-needed higher-throughput platform for drug screening, especially as complementary work in other models generates further hypotheses for potential treatment opportunities.

## Summary

*Xenopus* has been a trailblazer in the discovery of major events and mechanisms of brain development that are deeply conserved among vertebrates, in uncovering the etiology of human neurodevelopmental disorders, and in guiding the implementation of new models of human brain development. Like every other available model, frogs cannot model every aspect of human brain development down to the last detail; however, the use of *Xenopus* to generate targeted hypotheses for further testing in other models, and vice versa, will be absolutely indispensable in the face of the ongoing avalanche of disorder risk gene discovery. In summary, *Xenopus* is an ideal choice as a well-established and high-throughput tetrapod model that the coming decades of patient sequencing and gene discovery will demand.

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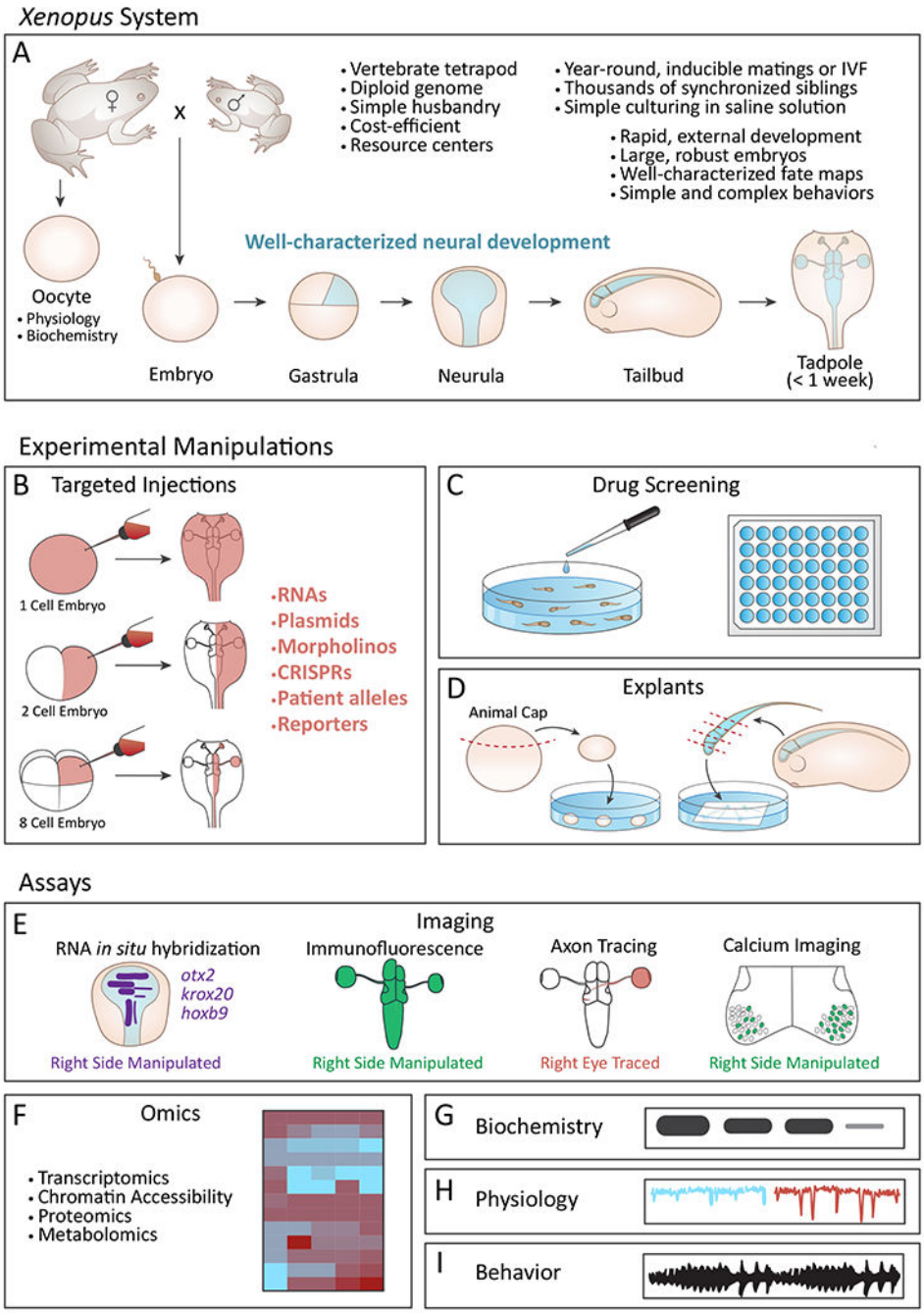


**Figure 1: Summary of key techniques used in *Xenopus* to study brain development.**

Several important techniques core to the *Xenopus* neurodevelopmental biology toolkit are diagrammed here, although the authors note that this figure is not meant to be an exhaustive summary of available technologies, and that these methods are also applicable to the study of other developmental processes. Top panel (A): Summary of advantages of the *Xenopus* systems and brief overview of central nervous system development. Light blue indicates neural tissues at the embryonic and larval stages shown. Orientations: lateral view with animal pole to the top (oocyte, embryo), lateral view with dorsal to the right (gastrula),

dorsal view with anterior to the top (neurula and tadpole), lateral view with anterior to the left (tailbud). Middle panels (B-D): Common techniques used to manipulate *Xenopus* development, including targeted injection with any of several reagents (B), treatment with pharmacological agents (C), and two examples of explant techniques (D). Note that these methods can be used separately or in combination, as appropriate for the scientific questions of interest. Bottom panels (E-I): Common methods for characterizing typical development or assessing the consequences of experimental manipulations (see B-D) on development. Diagrams depict hypothetical example results based on data from several references; see text for citations. In (E), from left to right: mRNA *in situ* hybridization shows a reduction in *krox20* and *hoxb9* expression, a posterior shift in *krox20* expression, and no change in *otx2* expression on the injected side of a unilaterally manipulated embryo; staining with an antibody against a pan-neural protein shows reduced brain size on the injected side of a unilaterally manipulated embryo; tracing shows axon projections from the right eye to the left tectum; calcium imaging shows increased activity on the injected side of a unilaterally manipulated embryo. (F) shows a heatmap from an omics analysis. (G) shows Western blot results from a co-immunoprecipitation experiment. (H) shows results comparing excitatory post-synaptic current (EPSC) recordings from a control animal (blue) and a manipulated sibling (red). (I) shows sound pulses from an advertisement vocal call.

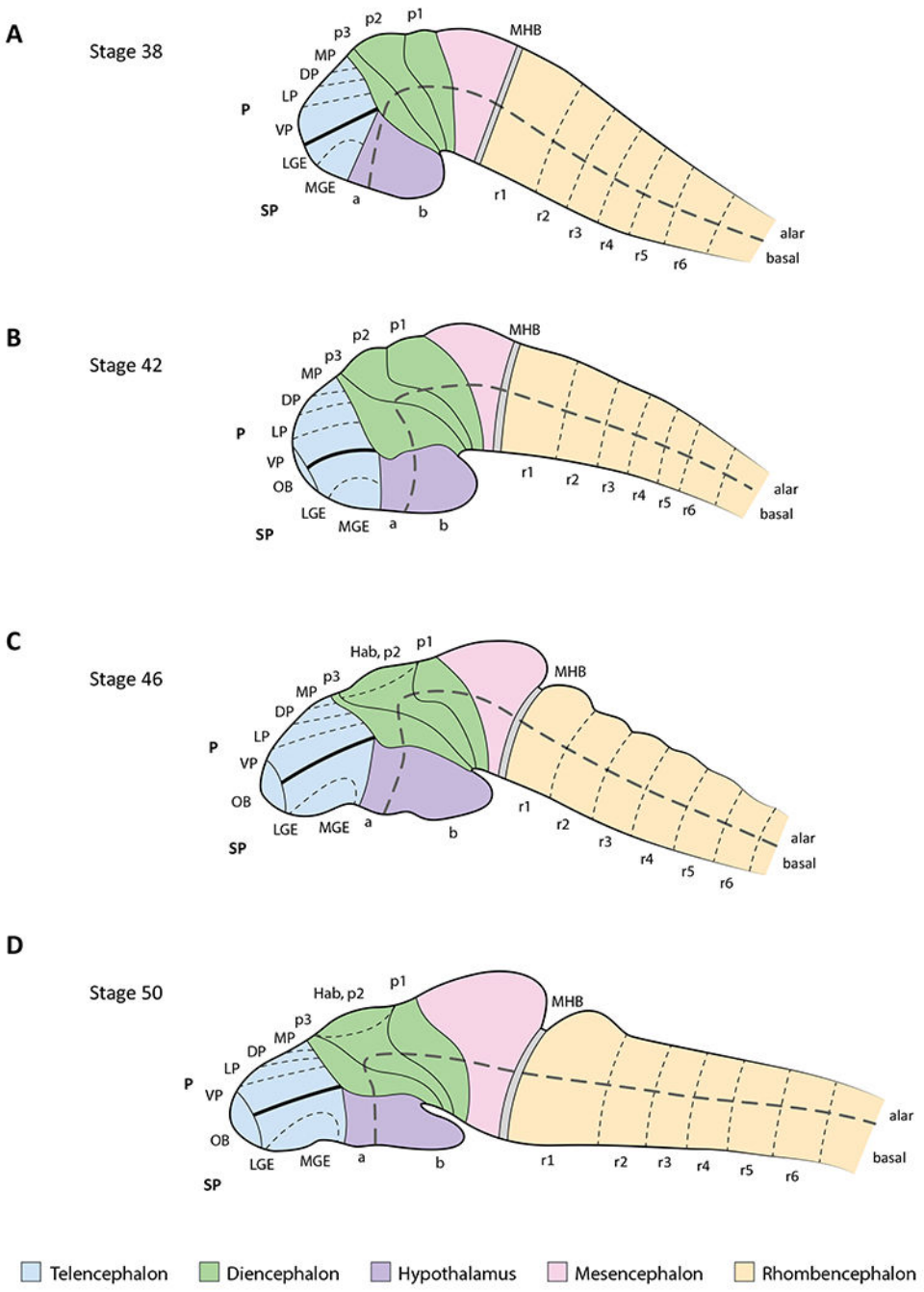


**Figure 2: Schematic representations of the developing *Xenopus* brain.**

Lateral views of the *Xenopus* brain (anterior to the left and dorsal at the top) at NF (Nieuwkoop & Faber) stages 38 (A), 42 (B), 46 (C), and 50 (D). Colors demarcate the developing telencephalon (blue), hypothalamus (purple), diencephalon (green), mesencephalon (pink), midbrain-hindbrain boundary (MHB, grey), and rhombencephalon (yellow). Images are representative of *X. laevis* and *X. tropicalis*. See text for anatomical references. *Xenopus* stages according to Nieuwkoop & Faber, 1994 (Nieuwkoop 1994). Abbreviations: P pallium; SP subpallium; MP medial pallium; DP dorsal pallium; LP lateral

pallium; VP ventral pallium; LGE lateral ganglionic eminence; MGE medial ganglionic eminence; a alar; b basal; p prosomere; r rhombomere; Hab habenula; MHB midbrain-hindbrain boundary; OB olfactory bulb.

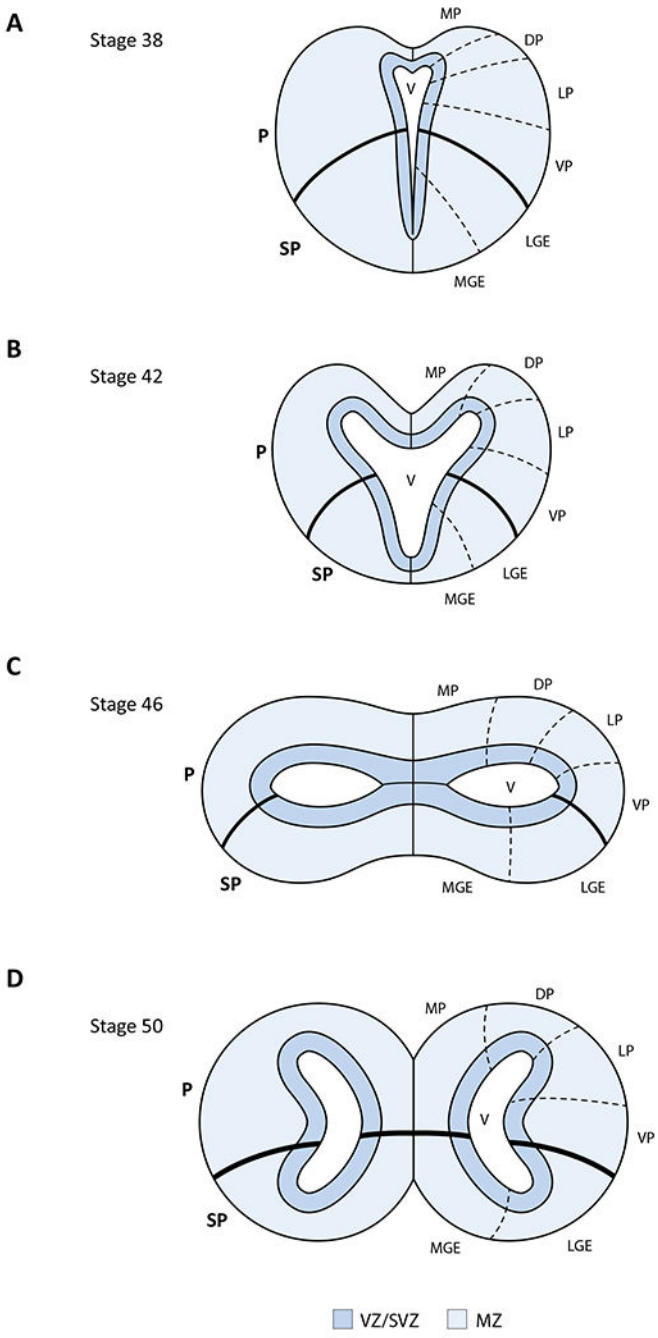


**Figure 3: Schematic representations of *Xenopus* forebrain sections during development**

Cross-sectional views of the *Xenopus* telencephalon (dorsal at the top) at NF stages 38 (A), 42 (B), 46 (C), and 50 (D). Images are representative of *X. laevis* and *X. tropicalis.* See text for anatomical references. *Xenopus* stages according to Nieuwkoop & Faber, 1994 (Nieuwkoop 1994). Abbreviations as in Figure 2, and: V ventricle; VZ ventricular zone; SVZ subventricular zone; MZ marginal zone.

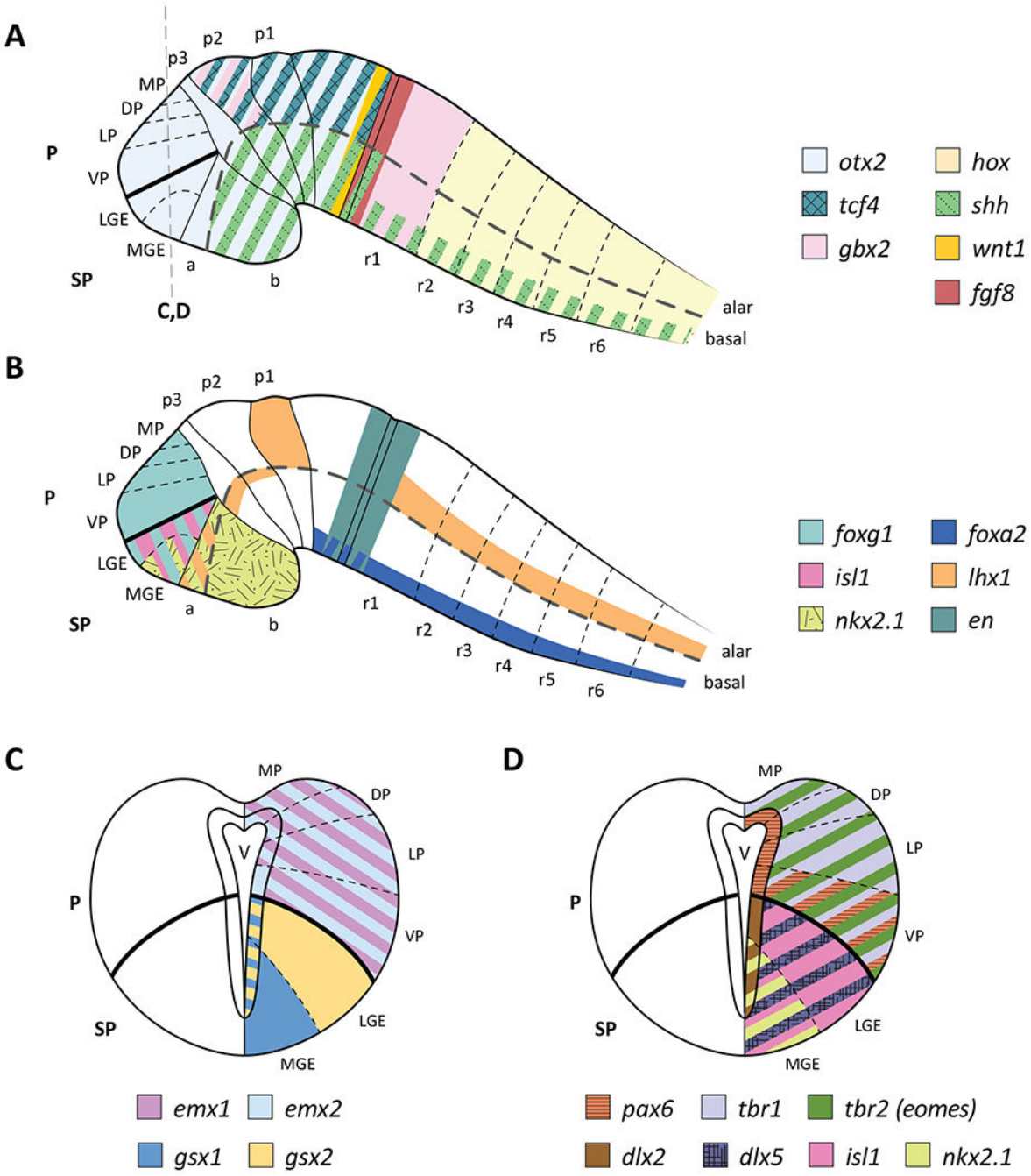


**Figure 4: Schematic representations of *Xenopus* stage 38 expression patterns.**

Lateral (A, B) and telencephalon cross-sectional (C, D) views showing expression domains of key patterning genes at NF stage 38. Stripes indicate co-expression of genes. See key in figure for color coding. Expression patterns are highly conserved between frogs and mammals (see text for references). *Xenopus* stage according to Nieuwkoop & Faber, 1994 (Nieuwkoop 1994). Dotted grey line in A indicates sectional plane shown in C and D. Abbreviations as in Figures 2 and 3.

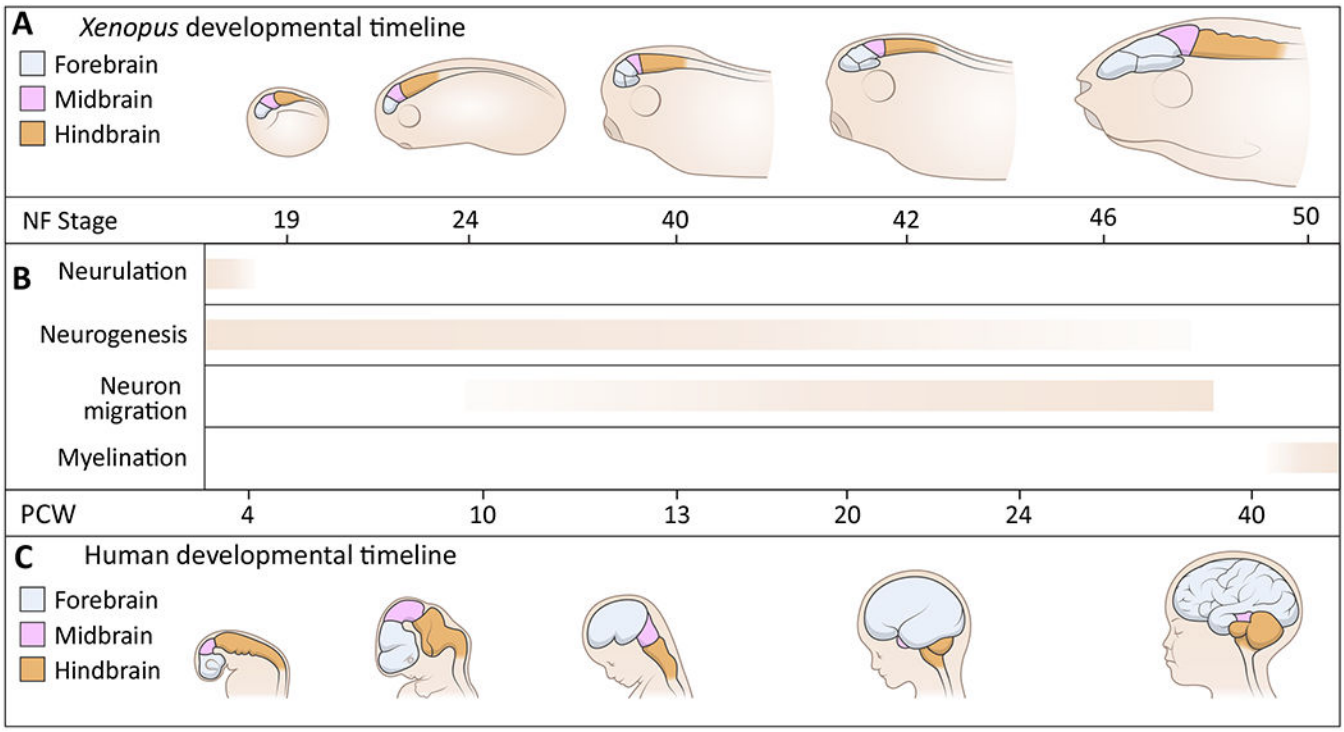


**Figure 5: Comparison of *Xenopus* and human brain development after neural tube closure.**

Summary of major events in dorsal pallium development over time (B), comparing *Xenopus* (A) and human (C) development. See key in figure for brain region color coding. *Xenopus* stages according to Nieuwkoop & Faber, 1994 (Nieuwkoop 1994). Human developmental epochs described as in Sestan & State, 2018 (Sestan and State 2018). Abbreviations: NF Nieuwkoop & Faber, PCW post-conception weeks.