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Resolving vertebrate brain evolution through salamander brain development and regeneration

The emergence of the brain was a radical event in evolution. The global architecture of the brain is established during early embryonic development and is highly correlated with its function, with considerable morphological similarities across diverse vertebrates (Figure 1A). With evolution, however, neuronal diversity and spatial distribution of neuronal subtypes and circuits generally become more complex in the brain, endowing species with better functionality and adaptability to the environment. In addition, different species exhibit varied abilities to repair brain injury, which is clinically relevant. Therefore, comparing brains among different species can be used to analyze the evolutionary origin and diversification of this structure. To gain insight into the gradual increase in structural and functional complexity of the brain, culminating in humans, it is necessary to incorporate advanced technologies, such as single-cell, single-nucleus, and spatial transcriptomics, as well as cell labeling and tracing tools. These tools can help identify cell type composition, cell dynamics during development, and the molecular mechanisms underpinning cell differentiation in critical taxonomically related vertebrate species.

Previous anatomical, cytological, and molecular studies of the brain have been conducted in cyclostomes and amniotes, including jawless fish (lampreys) (Lamanna et al., 2022), reptiles (turtles and lizards) (Tosches et al., 2018), birds (Colquitt et al., 2021), and mammals (mice and humans) (Hodge et al., 2019). These studies revealed conserved and species-specific cell type components pertaining to their divergent structural and functional evolution. Within vertebrate evolution, amphibians are taxonomically close to amniotes. In addition, amphibian species, such as the axolotls (*Ambystoma mexicanum*) and newts (*Pleurodeles waltl*), exhibit a robust ability to regenerate damaged tissues, including the brain (Burr, 1916). This characteristic makes the salamander a useful model system for studying organ regeneration. However, little is known about the cell types in the amphibian brain during development and regeneration, and how they differ from amniote brains.

To fill these knowledge gaps, three papers recently published in *Science* explored cell-type heterogeneity in the axolotl (Lust et al., 2022; Wei et al., 2022) and newt telencephalon (Woych et al., 2022) using single-cell, single-nucleus, and spatial transcriptomics. The comprehensive single-cell atlas of the salamander brain showed regional distribution of neuronal and non-neuronal cells in the axolotl and newt telencephalon, as reported in earlier research (Brox et al., 2004), but previously underestimated complexity and diversity. Furthermore, glutamatergic neurons of the axolotl telencephalon also showed transcriptional similarities with the hippocampus, dorsal cortex, and olfactory cortex of reptiles and mammals. Another study reported in *Science* further identified a core set of neuronal cell types showing high transcriptomic similarity between the brains of lizards and mice (Hain et al., 2022). These findings provide evidence at the single-cell level that all tetrapods share complex cell identities of similar ancestral origin in an evolutionarily conserved brain architecture. The brains of reptiles and amphibians also show divergent neuronal cell types compared to mammals (Hain et al., 2022; Lust et al., 2022; Woych et al., 2022), suggesting functional divergence of the brain within each taxonomic species.

As a structurally complex and remarkable organ, the brain performs many central functions in vertebrates. Although mammals, especially humans, have evolved highly sophisticated brains, certain features, such as regenerative capability, have markedly declined with the increase in structural and functional complexity (Iismaa et al., 2018). Brain damage from physical injury or neurodegenerative disease is irreversible, leading to the loss of neuronal cells and glial scarring in mammals. Given their pivotal evolutionary position

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within vertebrates and robust regenerative capacity throughout life, salamanders are ideal species for investigating the mechanisms underlying tissue regeneration. Exploring the cell types involved in neuronal regeneration in salamanders, such as the axolotls, should provide insight into repairing of injured brains in higher vertebrates.

There are two major concerns in successful brain regeneration, i.e., restoration of damaged structures and recovery of original function. These complex processes depend on stem cells to replenish lost cells and re-establishment of appropriate neural connections. Previous studies in regenerative species have shown that ependymoglial cells (EGCs), equivalent to neural stem cells in mammals, contribute to neurogenesis during brain regeneration (Berg et al., 2010; Kirkham et al., 2014). However, the underlying cellular and molecular mechanisms of brain regeneration remain unknown. Based on single-cell RNA sequencing (scRNA-seq) and spatial transcriptomics, the above studies explored brain regeneration after mechanical injury and identified injury-induced cell types, their roles in regeneration, and neural circuit remodeling. Notably, a distinct sub-population of EGCs that specifically emerges during brain regeneration, but not development, is rapidly induced after injury, defined in two studies as injury-specific state EGCs and reactive EGCs (reaEGCs) (Lust et al., 2022; Wei et al., 2022). For nomenclature consistency, we rename this sub-population as regeneration-specific EGCs (hereafter RSEGCs). These RSEGCs are activated into an injury-induced regenerative state, with up-regulation of genes involved in wound healing and cell migration at the early stages. Genes involved in cell proliferation, such as cell cycle- and ribosomal-related genes, are also up-regulated in this cell population. Concomitant with regenerative neurogenesis, RSEGCs are diminished at the late stages of regeneration. Comparing the global gene expression signature of RSEGCs with all other EGCs that appear during development and in adulthood, although RSEGCs are clearly distinct from all other EGCs, they are rather similar to early developmental EGCs, but distinct from those in the adult brain. Trajectory analysis suggested that these RSEGCs originate from residential adult EGCs, adjacent to the wound area, and may be the source of cells that give rise to neurons in brain regeneration. Reprogramming adult EGCs to an early embryonic state, namely rejuvenation, may be a crucial step required for successful brain regeneration.

In addition to the EGC population, another group of cells, called wound-stimulated neurons (WSNs), exists transiently during the early regeneration stages (Figure 1B). Genes involved in neuronal maturation and axonal growth are up-regulated in WSNs, whereas genes enriched in mature neurons are down-regulated. The transcriptomic profile of WSNs is similar to that of immature neurons. These results suggest that WSNs may be reprogrammed to an immature state via a transcriptomic remodeling while responding to injury, further integrating with other immature neurons and re-establishing neural network connections for functional recovery. However, these studies have several limitations. For example, scRNA-seq can only reflect the transcriptional state of the regenerative process, and further functional profiling is needed to understand the recovery status of the regenerating brain.

Further comparison between regenerative and developmental neurogenesis revealed that neurogenesis after injury progresses is similar to developmental neurogenesis and results in the re-establishment of lost neurons and input projections. Specifically, after an initial expansion phase, RSEGCs undergo a series of transitions into intermediate progenitors, immature neurons, and eventually mature neurons (Figure 1B) to replenish lost neurons (Lust et al., 2022; Wei et al., 2022). These lineage patterns during regenerative neurogenesis closely resemble those in telencephalon development. Based on analysis of gene regulatory networks in telencephalon regeneration and development, similar gene sets are employed to control these two processes. This evidence suggests that brain regeneration partially recapitulates developmental processes. Furthermore, the discovery of hundreds of regeneration-specific genes compared to brain development provides a starting point for future studies on developmental and regenerative gene regulatory networks and understanding the nature of what governs organ regeneration.

Regeneration occurs widely in the animal kingdom, although regenerative capacity varies considerably. Many invertebrates can regenerate entirely, such as planarians (Van Wolfswinkel et al., 2014) and hydra (Wittlieb et al., 2006). In addition, phylogenetically primitive vertebrates, such as fish, frogs, and amphibians, can regenerate a broad array of tissues and organs. In contrast, however, mammals exhibit a very limited regenerative capacity. Severe damage to certain tissues and organs does not elicit a regenerative response, but rather simple healing with fibrotic scar formation. This seems to indicate that regenerative capacity generally reduces during evolution. Moreover, regenerative capacity tends to decline during ontogenic development. These phenomena raise several interesting questions. Notably, what is the relationship between evolution and regeneration? Is regenerative capacity acquired during evolution or simply lost in some species? Is the regenerative ability of primitive vertebrates related to their relatively simple tissue and organ structure and function? Answering these questions will require further exploration of cell type composition and transcriptional state at the evolutionary scale.

The above studies have shed light on important issues related to vertebrate brain evolution, development, and regeneration, especially in salamanders. As keystone species in vertebrate evolution, salamanders demonstrate an unparalleled ability to regenerate injured organs. The new studies published in *Science* highlight the importance of using salamanders for regeneration research. Notably, research on cellular diversity and dynamics in salamanders not only provides novel insight into the molecular mechanisms of brain development and regeneration, but also provides a fundamental resource to compare brains over a large evolutionary scale and to guide future efforts in understanding behavior and cognition. Furthermore, the rapid emergence of single-cell transcriptomic data from multiple species will provide unprecedented opportunities to study the evolution of a variety of organ systems. Likewise, combining scRNA-seq

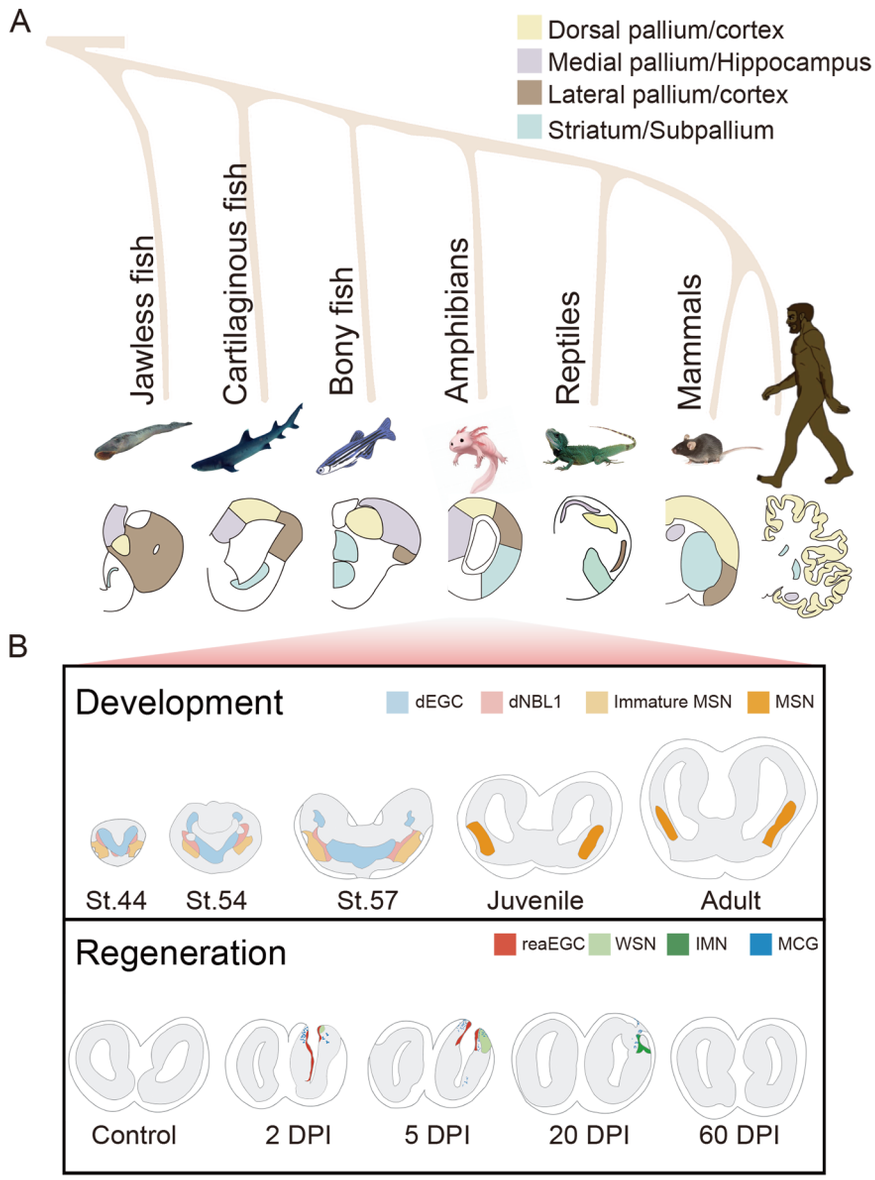

Figure 1 Salamander brain development and regeneration during vertebrate brain evolution

A: Phylogenetic tree displaying main vertebrate lineages and their approximate brain anatomies in cross-section. Colors show different regions of pallium/cortex and striatum/subpallium. B: Spatial visualization of selected cell types on Stereo-seq maps (Wei et al., 2022) during axolotl telencephalon development (stage 44 (St.44), stage 54 (St.54), stage 57 (St.57), juvenile, and adult), as well as regeneration (control, 2 days post injury (DPI), 5 DPI, 20 DPI, and 60 DPI). dEGC, developmental EGC; dNBL, developmental neuroblast; MSN, medium spiny neuron; reaEGC, reactive EGC; IMN, immature neuron; WSN, wound-stimulated neuron; MCG, microglial cell.

with epigenetic information, such as single-cell analysis of transposase-accessible chromatin using sequencing, will help elucidate gene regulatory networks and underlying epigenetic mechanisms. Such studies will bring us one step closer to understand the complexity of brain development and regeneration during evolution, which may provide potential clinical therapies for neurodegenerative diseases and brain injury in humans.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

X.Y.P., Y.M.L., and J.F.F. conceived and wrote the draft. Y.Y.Z. drew the figure. All authors read and approved the final version of the manuscript.

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