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Phylogeny and Ontogeny of Regeneration in Vertebrates

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ABSTRACT

This brief paper lays a conceptual link between ontogeny and phylogeny in vertebrate regeneration, inspired by the investigation in an anuran amphibian. Regenerative ability in *Xenopus* declines most rapidly after the climax of metamorphosis. In order to analyze the cause of this decrease during metamorphosis, we have made comparative experiments in larvae and adults after removing the anterior half of telencephalon. As a result, it was found that brain cells actively proliferated even in non-regenerating adults, just as in regenerating larvae, after partial removal of the telencephalon. Moreover, it was found that even adult-brain-derived dispersed cells reconstituted a subnormal structure of the lost part when transplanted to the partially truncated telencephalon. We consider that it is critical for the ependymal cells to cover the cerebral ventricle at an initial stage of wound healing, for massive organ regeneration, as is the case in larvae. On the other hand, in adults, these cells are strongly stuck in position and unable to move to seal off the exposed ventricle, which probably make the adult brain non-regenerative.

Keywords: Phylogeny, Ontogeny, Amphibians, *Xenopus laevis*, Metamorphosis

INTRODUCTION

Adult urodele and larval anuran amphibians regenerate not only limbs but also the tail, lens, jaw, liver, and even brain. Larval urodeles and fishes are also known to possess a similar regeneration ability. Needless to say, human beings cannot regenerate many of their organs. That means, in turn, that humans have lost their ability for regeneration during evolution. Our project intends to clarify the mechanism of the loss of regeneration ability during evolutionary process by studying the loss during ontogeny in an anuran amphibian. It would clearly be informative to compare a regenerative and non-regenerative phase in one organism and to understand how the mechanism is altered or disabled during ontogeny. It seems most promising to focus on a particular aspect of regeneration that can be analyzed at

a cellular and molecular level, and this is possible in the anuran *Xenopus*, where a lot of information, especially on developmental biology, has been accumulated during the last half-century.

In higher vertebrate animals than amphibians, the central nervous system (CNS), particularly the brain, is hard to regenerate. In mammals, including humans, it was recently observed that there are multipotent neural stem cells (NSCs) in CNS even in adults [1, 2]. In spite of this, when the CNS is lesioned, only the regrowth of axons is observed, while the massive brain regeneration prerequisite for neuron differentiation is hardly observed. In fishes and urodele amphibians on the other hand, the telencephalon or the optic tectum regenerates completely after partial removal [3, 4]. An anuran amphibian, the South African clawed frog (*Xenopus laevis*), can also regenerate the telencephalon, the mesencephala-

lon and the cerebellum during larval life and immediately after metamorphosis [5–8], but it is no longer possible in the adult [9].

Although many cells around the lateral ventricle are actively cycling in larvae, these cells are fewer in adults. But even in froglets, cell proliferation is promoted after ablation of the partial brain [10]. Because these cells are located in the surfaces of lateral ventricles where mammalian NSCs are located [1, 2], they may be genuine neural stem cells. The regeneration of the *Xenopus* telencephalon does not occur in adults [9]. Filoni and others [10] reported that the decline in the regenerative capacity of brain is related to a gradual loss of the undifferentiated cell populations as metamorphosis. We suspected that there might be other reasons for the decline than the loss of the undifferentiated cell populations, because the loss of regeneration capacity is not well correlated with the decrease in the number of cycling ependymal cells. Therefore, in order to know the possible difference between larvae and adults in the cellular environment related to regeneration, several experiments were conducted on larvae and froglets (the latter considered “adults” in terms of regenerative capability) after partial removal of the telencephalon.

MATERIALS AND METHODS

Animals

The animals used in the present study were from the J strain of the South African clawed frog, *Xenopus laevis*. All frogs were maintained in our laboratory. Larvae and metamorphosed animals were reared at 23°C in dechlorinated tap water. Developmental animals were staged according to the normal table of Nieuwkoop and Faber [11].

Partial Removal of the Telencephalon

Larvae (stage 53) were anesthetized in 1 : 2,000 MS222 (ethyl-*m*-aminobenzoate methane sulfonate, Nakarai Tesque) in Steinberg's solution. Froglets (10 days after metamorphosis) were anesthetized in 1 : 500 MS222 in De Boer's solution. Operations were done manually with a scalpel, fine forceps and iridectomy scissors under a binocular microscope.

Transplantation Experiment

After partial removal of the telencephalon of froglets, a brain cell suspension (containing ca. 20,000 viable cells obtained by mechanical and enzymatic treatment) was injected by micropipette into the space made by brain removal. After transplantation,

the skull and skin were returned to their normal position. Operated individuals were reared in De Boer's solution for 4 days without food, and then they were transferred to the usual rearing conditions.

BrdU Pulse Labeling

To label proliferating cells, larvae were injected with 5 µl of 0.25% BrdU (5-Bromo-2'-Deoxyuridine, Sigma) in Steinberg's solution into the abdominal cavity. Froglets were injected with 50 µl of 0.25% BrdU in De Boer's solution into the abdominal cavity. Sectioned fixed materials were examined immunohistochemically. The mean mitotic index, calculated as the percentage of BrdU incorporated cells of the total number of cells, was obtained by counting all cells of 3 randomly selected sections per each 3 animals at each point and examined statistically by Student's *t*-test.

Immunohistochemistry

For identification of the phenotypes of proliferating cells, a double-staining technique was applied using several different anti-BrdU antibodies and antibodies for cell markers, i.e., rat monoclonal anti-Musashi1 (a gift from Prof. H. Okano, Keio University), mouse monoclonal anti-GFAP, and mouse monoclonal anti-NeuN. After processing with Cy3- or FITC-conjugated secondary antibodies, nuclei were counterstained with Hoechst 33342.

Histology

For normal histological examination, animals were anesthetized with MS222, and fixed overnight in Bouin's solution, embedded in paraffin, sectioned serially at 5 µm, and stained with Delafield's hematoxylin and eosin.

RESULTS

Regeneration after Removal of the Anterior Half of the Telencephalon

In order to know the difference between the larval and adult brain after partial removal of the telencephalon, stage 53 larvae and froglets 10 days after metamorphosis were compared. Thirty days after the operation in larvae, the space made by partial removal is filled with the regenerated brain, and the brain was re-connected with the olfactory nerve extended from the anteriorly-located olfactory organs (Fig. 1 B-G). In contrast, in adults, the space made by partial removal remained as it was even after 30 days, and no connection with the olfactory nerve was seen (Fig. 2 B, C).

Histological examination of the regenerating larval brain showed that the cerebral lateral ventricles, which were opened by the operation, were sealed off by a cell mass by the 4th day. Many of the constituent cells were ependymal cells, which were apparently derived from the periphery (Fig. 1 I-K). The covered area then gradually increased in thickness with time, the regenerated brain was extended to the front, differentiation into gray and white matter was seen in the regenerated domain on the 30th day, and it appeared that the morphological regeneration had finished by this time (Fig. 1 L-N). In contrast, in adults, the lateral ventricle was not closed up, and there was no indication of regeneration even on the 30th day (Fig. 2 E, F).

Proliferating Cells in the Brain after Partial Removal of the Telencephalon

In order to investigate whether the reason for the inability of adults to regenerate is that cell division does not become active after removal of the telencephalon, the uptake of BrdU by cells in the remaining telencephalon domain was compared between regenerating stage 53 larvae and non-regenerating froglets. Even in intact stage 53 larvae, a large number of cells lining the cerebral ventricle had taken up BrdU (Fig. 3A). By the 8th day after partial removal of the telencephalon, the number of BrdU-positive cells had increased even in the area apart from the amputated plane (Fig. 3B). The mitotic index (MI, indicating the rate of cell proliferation) was 1.5 times that of the intact individuals ($P <$

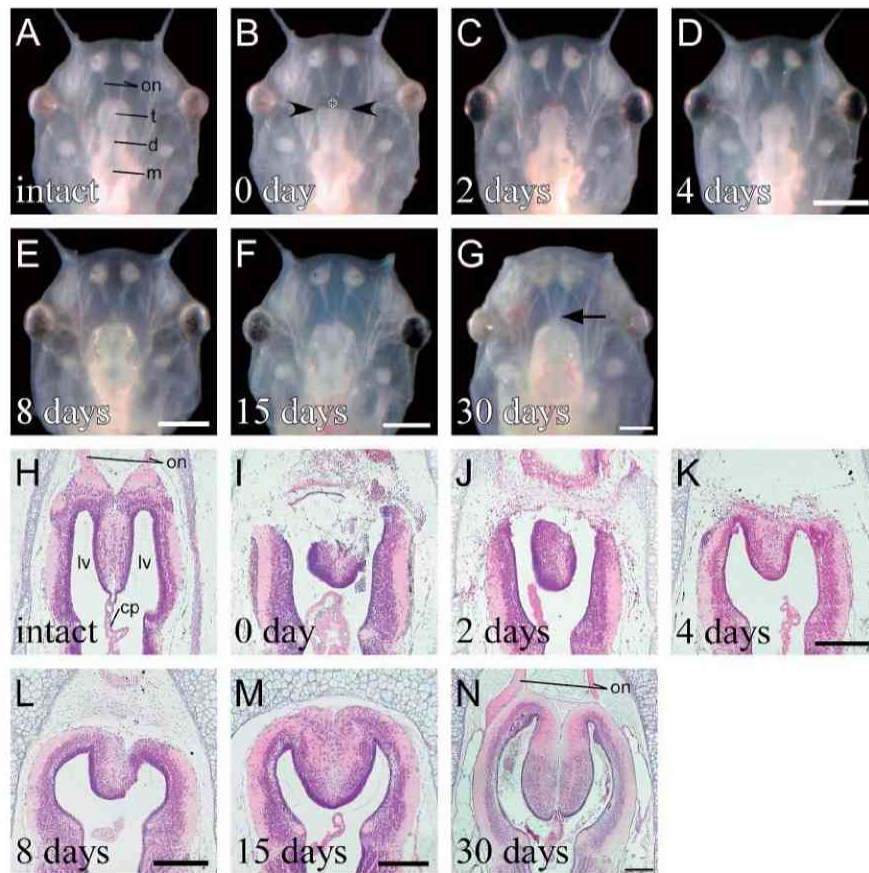


Fig. 1 Regeneration of larval telencephalon. Anterior half of the telencephalon was removed from stage 53 tadpoles (A and H), then observed macroscopically (A-G) and histologically on horizontally sectioned materials (H-N). Just after the operation, the ventricles were open at the anterior end (B and I). As early as 4 days after the operation, the ventricle was completely covered by the cells apparently gathered from neighborhood (D and K). In 30 days, the telencephalon returned to the normal structure with olfactory nerve bundles, which was connected to the olfactory organs. Arrowheads in B indicate the anterior end of the remaining telencephalon domain. Asterisk in B indicates the space made by the partial removal. Arrow in G indicates the connection between the olfactory nerve and the regenerated telencephalon. on, olfactory nerve. t, telencephalon. d, diencephalon. m, mesencephalon. lv, lateral ventricle. cp, choroid plexus. Scale bar for panels A-G = 2mm, Scale bar for panels H-N = 300 μ m.

0.003). The number of proliferating cells decreased after a peak observed on the 8th day until 30th day, when regeneration was complete.

Although there were far fewer cells, BrdU-positive ependymal cells in the paraventricular regions were observed even in intact froglets (Fig. 3 D). After partial removal of the telencephalon, the MI increased to become 5 or more times that of an unhurt individual by the 4th day ($P < 0.001$), and it exceeded the MI of stage 53 larva (Fig. 3 E, G). The number of labeled cells then gradually decreased to normal levels by 30 days.

Antigenic Characteristics of Proliferating Cells

In order to determine whether the BrdU incorporating cells surrounding the cerebral ventricle are neural stem cells, the antigenic profiles of BrdU-positive cells were examined in intact stage 53 larvae and froglets. The BrdU-positive cells of larvae extended GFAP-positive projections to the periphery of the telencephalon (Fig. 4 A-C). Moreover, they were positive for Musashi1, the marker by which a neural stem cell is characterized (Fig. 4 D-F).

In adults, proliferating cells were negative for NeuN, a nerve cell marker (data not shown). They extended the GFAP-positive projections like the larvae (Fig. 4 G-I), and were also Musashi1-positive (Fig.

4 J-L). The BrdU label was seen in various cells (mitral and granular cells in the olfactory bulb, pyramidal and ependymal cells in the cerebrum) in the telencephalon when the brain of froglets was observed 30 days after injection of BrdU. Although the cells remaining near the lateral ventricle were NeuN-negative at this time, it turned out that the cells which were distributed distantly from the ventricle had differentiated into neurons, as shown by the NeuN positivity (Fig. 4 M-O).

Transplantation Experiment

In order to investigate whether the brain of an adult can be reconstituted by adult cells, dispersed cells obtained from larvae and adults were introduced to the space made in the adult brain by partially removing the telencephalon. When the larval cells were transplanted to an adult, not only were cells of donor origin observed in all individuals (4/4), but also it was observed that these cells existed in the reorganized brain structure. The structure formed was also connected to the host's olfactory nerve and cerebrum. Strikingly, even when adult cells were transplanted to an adult, the cells of donor origin were observed in many individuals (3/4), along with partially restored brain structure. Although the shape of the reconstructed brain and ventricle was usually deformed, the three-layer struc-

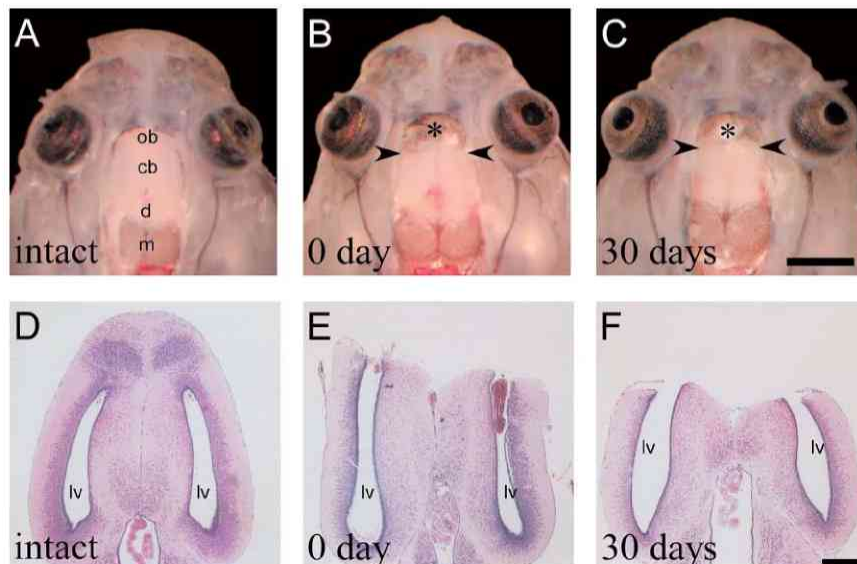


Fig. 2 No regeneration occurred in adult telencephalon. Anterior half of the telencephalon was removed from froglet 10 days after metamorphosis (A and D), observed macroscopically (A-C) and histologically on horizontal sections (D-F). Just after the operation, the ventricles were open at the anterior end (B and E). As late as 30 days after operation, the ventricles were still open at the anterior end (C and F). Arrowheads in B, C indicate the anterior end of the remaining telencephalon domain. Asterisk in B, C indicates the space made by partial removal. ob, olfactory bulb. cb, cerebrum. d, diencephalon. m, mesencephalon. lv, lateral ventricle. Scale bar for panels A-C = 2 mm, Scale bar for panels D-F = 300 μ m.

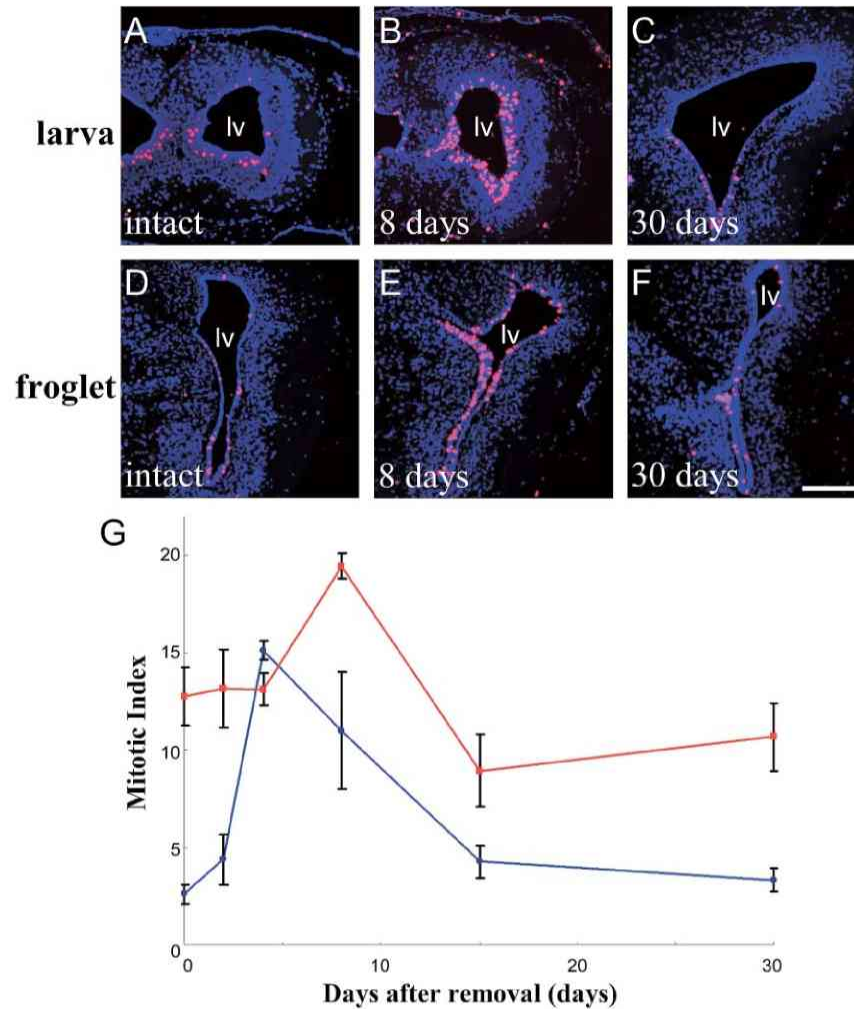


Fig. 3 Mitotic changes before and after telencephalon removal. After removing the anterior half of the telencephalon, mitotic figures significantly increased (B, E as compared with A, D) both in stage 53 larvae (A-C, red line in G) and 10 day froglets (D-F, blue line in G), as examined histochemically on cross sectioned materials (A-F). Mean mitotic indices in G were calculated on BrdU incorporated ependymal cells per all cells on 3 randomly selected sections in 1 animal. Three animals were used in each point. Vertical bars indicate SD of 9 samples (3 sections in each 3 animal). lv, lateral ventricle. Scale bar = 200 μ m.

ture composed of ependymal, pyramidal cell, and a fibrillous layers was recognized in many part of re-constituted part of the brain (data not shown).

DISCUSSION AND CONCLUSIONS

Regeneration Occurs Only in Larvae

As previously reported [5–9], it was reconfirmed that the larval telencephalon completely returned to the normal structure within 30 days. On the other hand, no regeneration at all was observed for at least 30 days in adults of only 10 days after metamorphosis. A major difference between larvae and adults was apparent as early as 4 days after the operation, when the ventricular openings made by the opera-

tion were already covered by the surrounding neural cells in larvae, but remained open after 30 days in froglets.

Mitotic Activity after Partial Removal of the Telencephalon

Proliferating cells existed not only in the larval brain but also in adults, although the ratio of proliferating cells among all ependymal cells was much lower in the adult brain. However, strikingly, the frequency of proliferating cells increased even in the non-regenerating adult brain after partial removal of telencephalon, as in the larval brain. Moreover, cell division became active not only in the telencephalon but also in the diencephalon and the mesencephalon

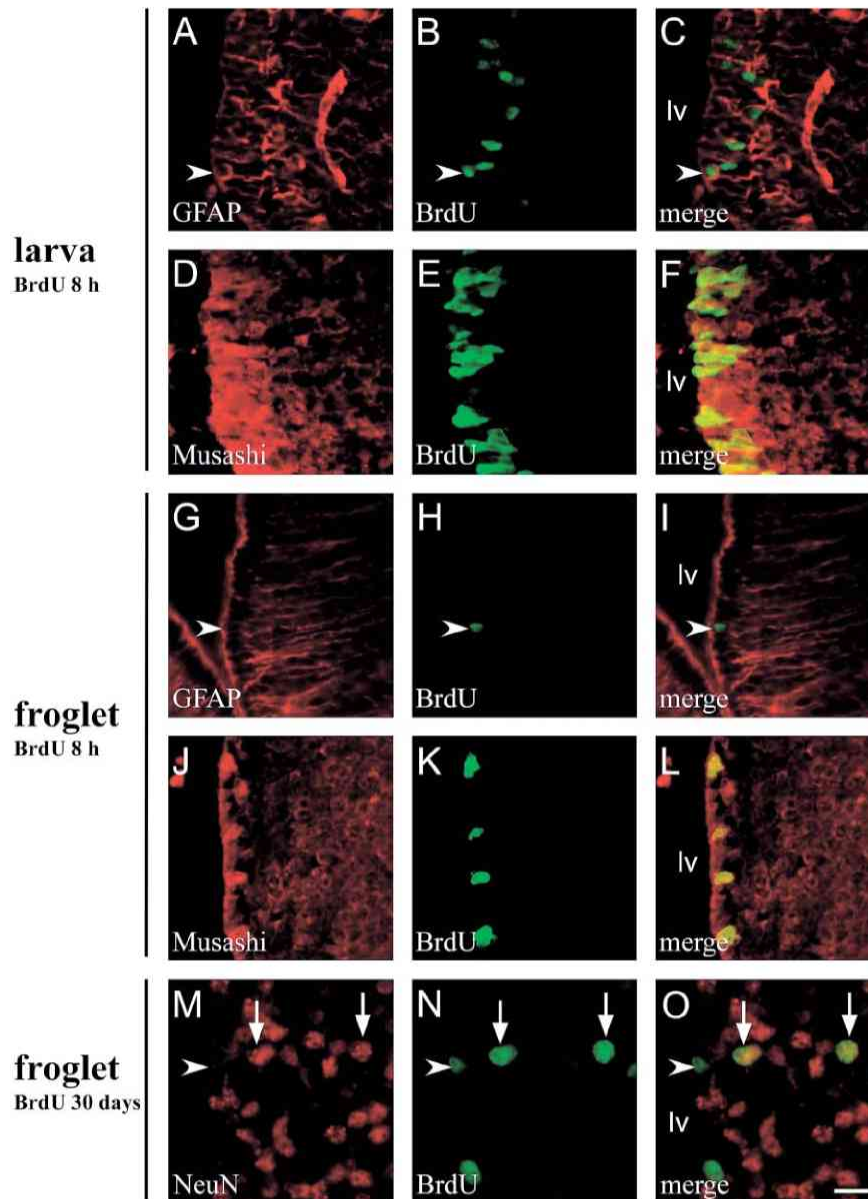


Fig. 4 Antigenic characterization of mitotically active ependymal cells surrounding the lateral ventricle. Intact larvae (A-F) and froglets (G-L) were injected with BrdU, and telencephalon was examined histochemically 8 hours later (A-L). Anti-BrdU antibody indicates replicating cells (green); anti-GFAP indicates radial projection of ependymal cells (red); anti-Musashi1 indicates undifferentiated neuroblasts (red). In M-O, froglets were injected with BrdU and the telencephalon was examined 30 days later in order to learn whether mitotically active ependymal cells gave rise to the differentiated neurons positive for NeuN antigens. The right-most panel shows merged figures indicating that yellow cells are double-positive for cell proliferation and characteristic molecules for each cell type. Arrowhead in A-C, G-I indicates the replicating cells with radial projection. Arrowhead in M-O indicates the replicated cells 30 days ago which did not differentiate into neuron. Arrows in M-O indicate the replicated cells 30 days ago differentiated into neuron. on, olfactory nerve. t, telencephalon. d, diencephalon. m, mesencephalon. lv, lateral ventricle. Scale bar = 200 μ m.

(data not shown) [10, 12–14]. This indicates, in turn, that cell proliferation might be mediated by the diffusible factor(s), released into the cerebrospinal fluid, which stimulate the division of broadly distributed neural stem cells [15].

Neural Stem Cells

Mitotically active cells detected in both larval and adult brains were antigenically characterized by GFAP and Musashi1. GFAP staining indicates that the cells have radially extending glial fibers and that

the positive cells in the subventricular regions are ependymal cells. Musashi1 indicates that the cells are undifferentiated neuroblasts [16]. Mitotically active Musashi1-positive ependymal cells can be designated as neural stem cells by definition. Finally, the present study also showed that some of these cells did differentiate into NeuN positive neurons [17]. The overall results indicate without a doubt that neural stem cells in the brain exist not only in larvae but also in adults.

Transplantation Experiment

Larval and adult brain cells were examined for their ability to reconstruct the brain structure by introduction of dispersed brain cells into the space made by the partial removal of the telencephalon in froglets. The results showed that adult brain cells reconstitute a subnormal adult brain structure if they are dispersed and introduced in the damaged brain.

Why is the Adult Brain Non-regenerative?

It can now be concluded that the reason why adult frogs cannot regenerate the telencephalon is not because they lack proliferating neural stem cells, nor because these cells lose the ability to reorganize the brain structure. The most tenable hypothesis at this point is that in adults the brain tissues around the exposed ventricle fail to cover the exposed area. In turn, the inability of the adult brain to cover the ventricles might be because the migration of subventricular cells is prevented by the firmly constructed, complex web structure of the adult brain [18]. Quite recently, it was reported that the lack of neuronal turnover and/or replacement of injured neurons in the adult human brain is not due to the absence of potential neural stem cells [19]. Rather, it is more likely due to a remarkable resistance to accept such cells into the network structure of the mature brain. Our working hypothesis is that during ontogeny as well as phylogeny, the ability for active migration of neural stem cells is lost as a consequence of the acquisition of highly complex neural networks, which resulted in the loss of regeneration in highly organized brains.

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