Adult neural stem cells: Long-term self-renewal, replenishment by the immune system, or both?

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The current model of adult neurogenesis in mammals suggests that adult-born neurons are generated by stem cells that undergo long-term self-renewal, and that a lifetime supply of stem cells resides in the brain. In contrast, it has recently been demonstrated that adult-born neurons in crayfish are generated by precursors originating in the immune system. This is particularly interesting because studies done many years ago suggest that a similar mechanism might exist in rodents and humans, with bone marrow providing stem cells that can generate neurons. However, the relevance of these findings for natural mechanisms underlying adult neurogenesis in mammals is not clear, because of uncertainties at many levels. We argue here that the recent findings in crayfish send a strong signal to re-examine existing data from rodents and humans, and to design new experiments that will directly test the contributions of the immune system to adult neurogenesis in mammals.

Keywords:
adult neurogenesis; blood; blood brain barrier; bone marrow; hemocyte; mesenchymal stem cell

Introduction

It has been nearly two decades since studies in mammals, including humans, suggested that cells from bone marrow are capable of migrating into the brain and generating glia and neurons [1–5]. The potential applications of these findings for regenerative medicine are indisputable. However, the possible significance of bone marrow as a natural source of neuronal precursors is less clear, for reasons summarized below. Data from a non-vertebrate model now point to precisely this mechanism – the immune system providing neuronal precursors responsible for adult neurogenesis – as a normal physiological process [6]. Might this mechanism be conserved in later branches of the metazoan phylogeny, as are so many aspects of neuronal development and function? This possibility and the existing literature on the relationship between bone marrow and brain in mammals will hopefully motivate new studies specifically testing the contribution of the immune system to adult neurogenesis. Such studies may be of vital importance in understanding neurological conditions and diseases that have been associated with adult neurogenesis [7]. If adult neurogenesis in mammals is dependent on the immune system in critical ways, as is apparently the case in some decapod crustaceans, our understanding of the etiology of these diseases will advance and thereby reveal new therapeutic opportunities via the immune system.

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Cells from the immune system generate adult-born neurons in a non-vertebrate model

Our research group has recently published findings demonstrating that the immune system is the source of neuronal precursors underlying adult neurogenesis in the crayfish *Procambarus clarkii* and *Pacifasticus leniusculus* [6]. As in other decapod crustaceans, neurogenesis persists in the adult brains of these organisms, and there are many structural and mechanistic parallels with adult neurogenesis in mammals [8]. The 1st-generation neuronal precursors, which are functionally analogous to neuronal stem cells in vertebrate species, reside in a vascularized neurogenic niche that has many of the same features as neurogenic niches in

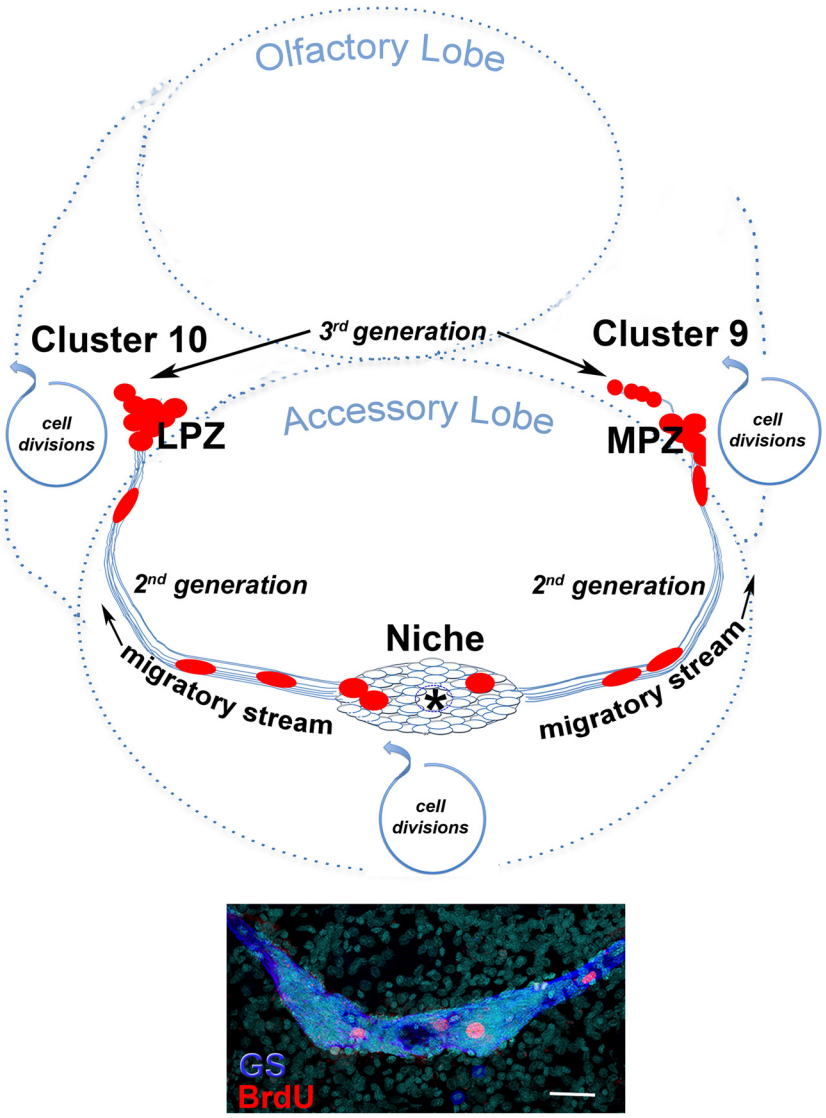

Figure 1. A model summarizing the events leading to the production of olfactory and accessory lobe interneurons in the adult crayfish brain. The cellular machinery consists of a neurogenic niche containing 1st-generation neural precursors, migratory streams containing 2nd-generation precursors, and two clusters of olfactory interneurons (clusters 9 and 10). The spatial relationships between these regions are indicated in the schematic drawing, supplemented with a representative image of BrdU-labeled (red) cells in the niche and proximal parts of the migratory streams, which are labeled with an antibody to glutamine synthetase (blue). First-generation precursors in a neurogenic niche divide symmetrically, both daughters migrating to proliferation zones (MPZ, LPZ) in clusters 9 and 10, where they divide at least once more before progeny differentiate into neurons. Solid black arrows next to the streams indicate the direction of migration; blue circular arrows indicate locations of cell divisions. The niche is connected to the blood system via a central ''vascular cavity'' in the niche, illustrated in the diagram (asterisk), and appearing as a black region in the center of the niche image. Hoechst (cyan) labeling of niche cell nuclei is also shown. Scale bar: niche image, 50 μm.

mammals [8, 9] (Fig. 1). Their daughters, the 2nd-generation precursors, migrate along processes of bipolar niche cells to arrive at two brain cell clusters that contain interneurons in the olfactory and accessory (multimodal) pathways [10]. These precursors then divide at least once more, finally differentiating into neurons that are indistinguishable from other mature neurons in these clusters in terms of their transmitter types and targets [11, 12]. The production of adult-born neurons in crustacean species is also sensitive to many of the same environmental and endogenous factors as in mammals, including circadian phenomena [13], seasonality [14], serotonin levels [15], nitric oxide [16], physical activity [17], and environmental enrichment [18, 19].

A critical distinction between adult neurogenesis in crustaceans and mammals is that the 1st-generation neuronal precursors in crayfish are not self-renewing [20, 21]. This has been unequivocally demonstrated using pulse-chase double nucleoside labeling (e.g. 5-bromo-2′-deoxyuridine [BrdU], followed after an interval by 5-ethynyl-2′[EdU]) [20]. This experiment has shown that the 1st-generation neuronal precursors in the niche do not retain BrdU as would be expected if these divisions were self-renewing. Further, both daughters of these divisions (the 2nd-generation precursors) migrate away from the niche towards the proliferation zones (see Fig. 1). Rapid cycling of the niche precursors (and hence dilution of the BrdU label) cannot explain these results because the cycle time of these cells is ≥48 hours [20]. However, in spite of the absence of self-renewal among these 1st-generation precursor cells, the neurogenic niche is never depleted, and neurons continue to be generated throughout the long lives of these animals (up to 20 years for some species). This suggests that the neurogenic niche in crayfish is not a closed system, and that 1st-generation neuronal precursors must be replenished from a source extrinsic to the niche.

The presence of an extrinsic source of neuronal precursors was confirmed with an experiment in which crayfish received a single injection of BrdU, and labeling in the niche was documented daily for 1 week, and at longer intervals until 21 days after injection (Fig. 2) [6]. On days 1–4 after injection, precursor cells in the niche were BrdU-positive, as previously shown (e.g. [8, 20, 22]). There was no labeling on days 5–7 because the BrdU clearing time (<2 days; [20]) was over, and the cells that were originally labeled by the BrdU pulse had divided and the daughters migrated away from the niche, towards the brain cell clusters. However, on days 8–14 following BrdU exposure, intensely labeled cells were again found in the niche. As BrdU was no longer available for renewed labeling of neuronal precursors in the niche at these time

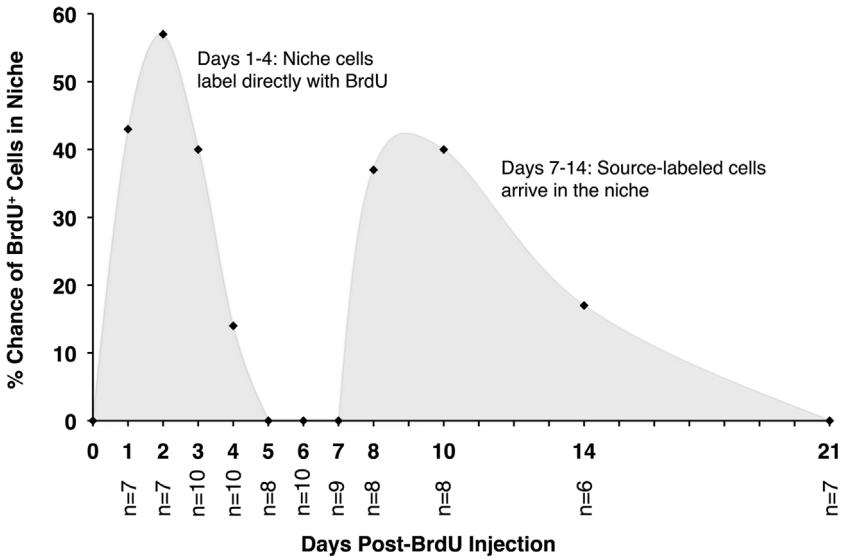

Figure 2. Actively proliferating (BrdU-labeled) cells in the neurogenic niche have a bimodal temporal distribution following a single injection of BrdU. BrdU-labeled cells were quantified in the niches of crayfish that were sacrificed daily for 1 week after injection, and at intervals thereafter for 21 days. The probability of observing BrdU-labeled cells in the niche was then plotted for each of the sampling days. BrdU-labeled cells are observed in the niche on days 1–4 following injection. On days 5–7, niches contain no BrdU-labeled cells. However, between days 8–14 after injection, BrdU-labeled cells are once again observed in the niche. (Figure adapted from [6]).

points, our interpretation is that these late-appearing BrdU-positive cells must have incorporated the nucleoside while still in the source tissue. Thus, the presence of the gap in labeling between days 5 and 7 confirms that the nucleoside has cleared and that no BrdU was retained in the niche precursor cells, while the second peak indicates the arrival of source-labeled cells in the niche [6].

The identity of the source tissue for neuronal precursors in the niche has been investigated both in vitro [20] and in vivo [6]. Hemocytes, but not other cell types, were attracted to the niche in short-term co-cultures [20]. Adoptive transfer experiments in which EdU-labeled hemocytes from a donor were transferred to recipient crayfish, demonstrated that donor-labeled hemocytes rapidly find their way to the recipient neurogenic niche – but not to other rapidly-proliferating tissues (e.g. hepatopancreas)[6]. Additional observations demonstrated that donor cells not only invade the niche, but also traverse the migratory streams and populate the two brain clusters where adult-born neurons are normally found. Further, after several weeks, these donor-labeled cells express neurotransmitters that are appropriate for the olfactory interneurons that occupy these cell clusters [6]. Finally, labeled hepatopancreas cells transferred from the donor to the recipient crayfish were not attracted to the niche, thus showing that the attraction of transferred hemocytes to the niche is a highly specific phenomenon that cannot be attributed either to a transfer of the label to recipient cells or to cell fusion events – two doubts that were raised about the prior studies in mammals (e.g. [23, 24]). These studies also ruled out persistent neuroblasts (i.e. traditional ectodermal precursors of neurons) as a source of adult-born neurons in crayfish [9, 20, 21], as was previously proposed [25].

Fundamental neural mechanisms are conserved during evolution

Experiments in crayfish have demonstrated that cells from the innate immune system generate adult-born neurons [6]. These data challenge the canonical view that the ectodermal origins of embryonic neural tissues are the sole source of neurons in the adult brain. Indeed, platyhelminths and acoels provide the only other examples of a life-long non-ectodermal source of neurons; in these species, pluripotent stem cells (neoblasts) replenish all cell types in the organism, including neurons [26, 27].

Historically, invertebrate species have contributed in vital ways to our understanding of the nervous system, and evolution has often provided critical clues to neuronal function. Prime examples are the experiments using the squid giant axon that first elucidated the ionic currents underlying the action potential [28], and work in *Aplysia californica* demonstrating the synaptic basis of learning and memory [29]. Studies such as these underscore the often high degree of evolutionary conservation in fundamental neural mechanisms [30]. There is a long history of contributions from non-vertebrate organisms that have led the way towards new understanding in the mammalian brain. With this perspective in mind, it is therefore intriguing to entertain the possibility that the immune system in mammals, and perhaps even in humans, provides neural stem cells underlying adult neurogenesis. The many studies reviewed below suggest that cells from bone marrow, and specifically mesenchymal stem cells, do have the capacity to generate neurons. The ultimate question is whether this is a natural, ongoing mechanism for replenishment of neural stem cells in the mammalian brain, as proposed over a decade ago [1, 31].

Bone marrow and brain: Are neuronal stem cells replenished in mammals?

The proposal in crayfish that cells from the immune system play a central role in adult neurogenesis is not a novel idea. The existing literature includes many studies suggesting that the immune system in mammals may also be capable of providing neuronal precursors. In vitro work has shown that bone marrow cells can be induced by various means to express neuronal properties, including electrical responsiveness to depolarizing stimuli [32–35]. Further, a subset of

hematopoietic progenitor cells that were not pre-treated with neuralizing agents expressed specific neural or oligodendroglial genes [36, 37], and when CD34+ cells were transplanted into adult brain, cells containing neural and glial markers segregated into non-overlapping populations [36]. Thus, expression of neural proteins by a subset of bone marrow-derived cells requires no specific induction, and it is therefore concluded that some bone marrow cells naturally express neural or glial genes. Ex vivo studies have further shown that an unselected [3, 31, 38, 39] or selected [40] sub-population of cells from bone marrow, or cells derived from cultured bone marrow cells (e.g. [41, 42]), tend to migrate to the brain and express neuronal markers. Very few exceptions to this trend have been published (e.g. [43]; but see response in [5]).

The prevailing interpretation of these studies has been that while bone marrow cells can generate new neurons in the adult brain, this is not a normal physiological process. However, two retrospective studies in humans [1, 5] that used sex-mismatched female bone marrow transplantation patients perhaps provide the most compelling data related to the possibility of bone marrow as a source of neural stem cells. While the neuronal yield was relatively small in the human studies (2-5 Y-positive neurons per 10,000 [5], and 1% of hippocampal neurons [1]), particularly compared to adoptive transfers of bone marrow in mice, the presence of these transgender neurons in humans demonstrated that bone marrow cells can migrate to brain tissues; once there, these differentiate into cells that express neuronal markers and are morphologically indistinguishable from nearby resident neurons. Cell fusion and the possibility of microchimerism were ruled out either in the initial study [1] or in follow-up studies [44]. Another concern with these adoptive transfers was the possible influence of radiation on the permeability of the blood brain barrier (BBB) [45–47], and whether mesenchymal stem cells were able to cross into the brain only because the integrity of the barrier was compromised. Studies have demonstrated that the BBB also is compromised during brain inflammation or injury, and that trafficking of leukocytes across the BBB is upregulated during these periods [48]. Transmigration of leukocytes (diapedesis or extravasation) occurs by both paracellular (between endothelial cells of the BBB) and transcellular (through individual endothelial cells) routes [49, 50], and mechanisms of leukocyte transmigration have been described. However, it is not clear whether mesenchymal stem cells can use similar mechanisms to cross the BBB [51]. In mouse models of ischemic stroke and Alzheimer's disease, mesenchymal stem cells introduced intravenously were able to cross the BBB and migrate into the brain, but it is assumed the BBB is compromised in these situations and that this migratory ability may not represent an active mechanism in a healthy individual [52, 53]. Mesenchymal stem cells do express some chemokines and cell adhesion molecules that are associated with leukocyte homing behavior [54, 55], but experiments directly testing the interactions between mesenchymal stem cells and endothelial cells have yielded inconsistent results regarding the potential for transmigration of mesenchymal stem cells across the BBB under healthy circumstances [56–60]; reviewed in [51].

Conflicting explanations led to a stalemate and many unanswered questions

Why has the response to the bone marrow transplant data in mammals been so muted? First, several alternative interpretations have been proposed. Among these, it was shown that bone marrow cells transplanted to the hippocampus or striatum of the adult brain can be rejected by an inflammatory response and can transfer their donor label to neurons and glia in the host [24]. The use of BrdU or other nucleosides as the donor cell label was cited as particularly vulnerable in terms of transference to cells in the recipient, as label from dead cells or fibroblasts also found its way into neurons and glia; methods based on thymidine analogs were therefore thought to be especially unreliable [23]. Other studies found that adult hematopoietic stem cells show little plasticity [61], suggesting that cell fusion might account for the acquisition of broad properties by descendant lineages [62–64]. However, cell fusion-independent differentiation of neural stem cells into cells in the endothelial lineage has also been documented in vitro [64], suggesting that transdifferentiation can occur without cell fusion. Finally, there is strong evidence that mesenchymal, hematopoietic, and neural stem cells play important roles in regulating trophic factors and cytokines in vivo [66–68], suggesting that the introduction of these cells from donor to recipient may trigger responses that could (perhaps in combination with cell fusion) explain the apparent expansion of labeled donor cell populations in recipient tissues. In sum, these studies cited specific concerns related to the approach and technologies being used in adoptive transfer studies, which weakened the impact of findings in terms of the possible significance of mesenchymal stem cells as precursors of neurons by natural physiological mechanisms.

Do stem cells undergo long-term self-renewal?

The discussion surrounding mesenchymal stem cells as neuronal precursors also extends well beyond technical concerns related to specific experimental approaches. The first key issue is the presumed long-term self-renewal proposed for adult stem cells, an idea that is deeply rooted in stem cell biology. The National Institutes of Health (NIH) Stem Cell Basics site states, "The adult stem cell can renew itself and can differentiate to yield some or all of the major specialized cell types of the tissue or organ." (http://stemcells.nih.gov/info/basics/pages/basics4.aspx). While self-renewing divisions of adult neural stem cells have been documented in the nervous system [69], there is no evidence for long-term self-renewal of neural stem cells in vivo. Rather, this idea was adopted from work showing self-renewal and multi-potentiality of stem cells in culture [70]. Thus, self-renewal tends to dominate our thinking. As a result, experiments are not testing for potential sources of neural stem cells outside the nervous system, because prevailing thought suggests that the

stem cell population does not need to be replenished.

Is transdifferentiation a normal physiological process?

The idea that cells might naturally transdifferentiate from one germ cell lineage to produce cells in an organ generated by another germ layer also is hotly debated. It is known that cells can be "reprogrammed" in culture and induced to transdifferentiate (e.g. [65]), but whether this is also a natural physiological process is not yet clear. The 19th century biologists who studied the three germ layers and their lineages [71, 72] described relationships among different tissue types and their tendencies towards discrete and separate lineages within each germ layer. The perceived boundaries between these layers may be purely hypothetical, and transdetermination and/or transdifferentiation may indeed be natural processes. In vertebrates, the initial induction of neural ectoderm by mesodermal cells of the notochord suggests an early and intense relationship between these two tissues. In this light, the possibility that the immune system may provide neural precursors underlying adult neurogenesis could be viewed as an extension of this close relationship. The importance and complexities of interactions between the immune and nervous systems are underscored by the advent of "neuroimmunology," a rapidly expanding field that has spawned an international society and a number of professional journals.

Adult neurogenesis and disease: Urgent questions and few answers

Studies using rodent disease models have shown that adult neurogenesis is altered in a number of psychiatric, cancerous and neurodegenerative diseases [70]. Three primary effects on adult neurogenesis have been associated with these disorders: a reduction in neurogenesis due to decreased neural stem cell activity or survival of newborn cells, over-production of neurons, or abnormal differentiation and faulty integration of the new neurons [7]. Although faulty neurogenesis in the adult brain has been implicated in several diseases, clinical depression and epilepsy have received the most attention. Theories about adult neurogenesis and depression date back to Jacobs et al. [73] who proposed that "...the waxing and waning of adult neurogenesis in the hippocampal formation are important causal factors, respectively, in the precipitation of, and recovery from, episodes of clinical depression." This theory was based on the finding that fluoxetine and other antidepressants enhance neurogenesis, and that stressors associated with the onset of clinical depression often reduce neurogenesis. In further support, it was proposed that the maturation time for new neurons might account for the delayed effectiveness of fluoxetine. However, it has now been shown that the delay in efficacy is most likely associated with gradual effects on serotonin transporter turnover in raphe neurons and the acquisition of serotonergic properties by neurons in the locus coeruleus [74]. Nevertheless, it is clear that the effectiveness of some antidepressants depends upon ongoing neurogenesis [75], although this is not universally true [76]. It has been proposed that hippocampal neurogenesis may be important as a buffer for the stress response, via hippocampal regulation of the hypothalamic-pituitary-adrenal pathway [77]. While it is unlikely that alterations in adult neurogenesis alone cause major depression, newborn neurons are nevertheless viewed as an important therapeutic target [78]. Equally compelling are the changes in hippocampal adult neurogenesis associated with epilepsy, in which an overproduction of neurons and faulty maturation and migration have been documented [79–85]. Genetic studies also suggest that changes in adult neurogenesis are sufficient to induce epileptic activity [86].

The dominant hypothesis concerning the genesis of primary brain cancers is also pertinent to this discussion, as a compelling amount of literature now suggests that neural stem cells are the founder cells that initiate tumor formation [87, 88]. Brain tumor-derived stem cells share several properties with neural stem cells, expressing markers typical of neural stem cells found in neurogenic regions [88], and also share cell surface markers with hematopoietic lineage cells [89]. Thus, stem cells involved in adult neurogenesis are implicated in tumorigenesis in the brain.

There are many unanswered questions about the role of stem cells and adult neurogenesis in disease mechanisms, as well as the relationship between bone marrow and brain. Nevertheless, many beneficial clinical therapies are based on an infusion of bone marrow, without a complete understanding of why these treatments are helpful. For example, following adoptive transfer of human mesenchymal stem cells, rats show remarkable recovery from stroke [67]. In addition, for boys with cerebral X-linked adrenoleukodystrophy (ALD), a bone marrow or cord blood transplant early in the course of the disease can stop the progression of the disease [90]. Likewise, bone marrow-derived mesenchymal stem cell therapies hold promise for treatment of Parkinson's disease and multiple system atrophy (MSA), both neurodegenerative disorders [91]. It is generally thought that the trophic influences of bone marrow cells account for many of the clinical benefits of these transplants [67, 68], but a direct contribution of stem cells derived from bone marrow has not been ruled out.

The critical questions moving forward will challenge our technical skills and our receptiveness to new ideas. Do adult neural stem cells undergo long-term self-renewal in vivo? If neural stem cells must be replenished, what is the source of new stem cells – an existing storage depot in the brain, or sources extrinsic to the brain? Can mesenchymal stem cells cross the blood brain barrier in healthy, untreated mammals? Alternatively, might the immune system in mammals replenish existing neural stem cells in the brain, during periods when the BBB is compromised by disease? Is transdifferentiation a natural process involved in adult neurogenesis in mammals? Do neural stem cell niches provide an environment for nurturing long-lived stem cells, as currently thought, or rather a place where pluripotent stem cells may transform into 1st-generation neuronal precursors?

Conclusions and outlook

Following the initial report of adult neurogenesis in rats [92], it took decades of debate and study before the general applicability of these findings was understood. Today, we know that lifelong neurogenesis in at least some brain regions is more the rule than the exception. Therapies and cures for many diseases depend upon our understanding of stem cells, their capacity for self-renewal in vivo, their potencies, and their ability to access the brain. Perhaps most important of all in terms of the clinical picture is to resolve whether the immune system can provide neural stem cells that generate adult-born neurons. Even if such a mechanism were just part of the story, perhaps active only during brain disease or injury when the BBB is compromised, the demonstration of a direct connection between these systems would provide a new avenue for therapeutic development, for understanding disease mechanisms, and for discovering cures. Considering the high degree of evolutionary conservation of neural mechanisms, the recent findings in crayfish send a strong message that existing data from rodents and humans should be reconsidered. Further, there is an urgent need for new experiments that directly test the contributions of the immune system to adult neurogenesis in mammals.

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