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Functional Integration of Adult-Born Neurons

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Summary

Over the past decade, it has become clear that neural stem cells in the adult mammalian brain continuously generate new neurons, predominantly in the hippocampus and olfactory bulb [1]. However, the central issue of whether these new neurons participate in functional synaptic circuitry has yet to be resolved. Here, we use virus-based transsynaptic neuronal tracing and c-Fos mapping of odor-induced neuronal activity to demonstrate that neurons generated in the adult functionally integrate into the synaptic circuitry of the brain.

Results and Discussion

In the adult mammalian brain, neural stem cells residing in the ventricular zone of the lateral ventricle give rise to neuronal precursors. These precursors migrate along the rostral migratory stream to the olfactory bulb (OB), where they ultimately take up residence in the glomerular and granule cell layers and differentiate into interneurons [2]. To analyze whether these adult-born neurons integrate in synaptic circuitry, we created a green fluorescent protein (GFP)-expressing pseudorabies virus (PRV GS518) (Figure 1A). Directly injected into the brain, this neurotropic virus selectively infects and replicates in neurons. The virus is actively transported along axons and dendrites to synapses, where it passes on to infect higher order neurons within a circuit, in a manner strictly dependent on synaptic contact [3, 4].

To label dividing neuronal progenitor cells and their progeny, adult C57BL/6 mice received BrdU (1 mg/ml) in their drinking water for 4 weeks followed by a 3-week chase period to allow for cell migration, neuronal differentiation, and potential synaptic integration. This procedure results in extensive labeling of newborn granule cells and periglomerular neurons in the OB. At the end of the chase period, we injected 7.5×10^4 particles of PRV GS518 into the piriform cortex ($n = 4$). Four days later, we analyzed OB neurons for BrdU incorporation and viral infection (Figure 1B). Immunohistochemical analysis of periglomerular neurons revealed colocalization of virally encoded GFP with BrdU (Figure 1C), indicating that neurons labeled by the virus had been generated during the period of BrdU administration. The observed viral labeling of these neurons cannot be attributed to direct infection by PRV GS518 injected into the piriform cortex, as periglomerular neurons extend axons only locally within the glomerular layer of the OB and do not project to regions as distant as the piriform cortex [5]. They do, however, receive synaptic inputs from OB projection neurons that send axons to the piriform cortex [5] (see Figure 1B). The spread of PRV GS518 to a periglomerular neuron therefore requires transmission of virus across a synapse with a long-distance projection neuron. Furthermore, cholera toxin B (CTB), a retrograde neuronal tracer that is unable to cross synapses, was coinjected with PRV GS518 into the piriform cortex ($n = 2$). Thus, labeling of a neuron with PRV GS518 and CTB reveals a direct infection, whereas labeling of a neuron with PRV GS518 alone indicates that the virus has infected the neuron transsynaptically. Accordingly, BrdU-labeled periglomerular neurons that were infected with virus were never labeled with CTB (data not shown), further confirming the transsynaptic nature of viral infection. Additionally, BrdU-labeled neurons residing in the granule cell layer were also frequently double labeled with PRV GS518 (data not shown). The present findings demonstrate that neurons generated in the adult OB become synaptically integrated into the existing circuitry.

The hippocampus is a second site of neurogenesis in the adult brain. Previous studies have shown that newborn neurons in the adult dentate gyrus, upon maturation, extend appropriate axonal processes to the CA3 subregion of the hippocampus [6]. But whether these dentate gyrus neurons, or newborn neurons of other hippocampal regions, integrate into synaptic circuitry remains to be directly demonstrated. To address this question, we employed the same experimental paradigm as was used for the OB. As expected, BrdU administration resulted in the labeling of numerous newborn neurons in the dentate gyrus and, less frequently, in the hilus, CA1, and CA3 regions [7]. To assess whether these newborn hippocampal neurons are integrated into synaptic circuitry, we coinjected PRV GS518 and CTB into the piriform cortex and analyzed the ipsilateral hippocampus ($n = 4$), or we injected the CA1 subregion of the hippocampus and analyzed the contralateral hippocampus ($n = 2$). Viral labeling of hippocampal neurons was quite refined and resulted in extensive transsynaptic labeling of hilus and dentate gyrus neurons (in the case of CA1 injections) or of neurons in the pyramidal layers of CA3 (in the case of piriform cortex injections). Colocalization of viral infection and BrdU incorporation (in the absence of CTB labeling) successfully identified transsynaptically labeled newborn neurons throughout the granule cell layer of the dentate gyrus as well as in

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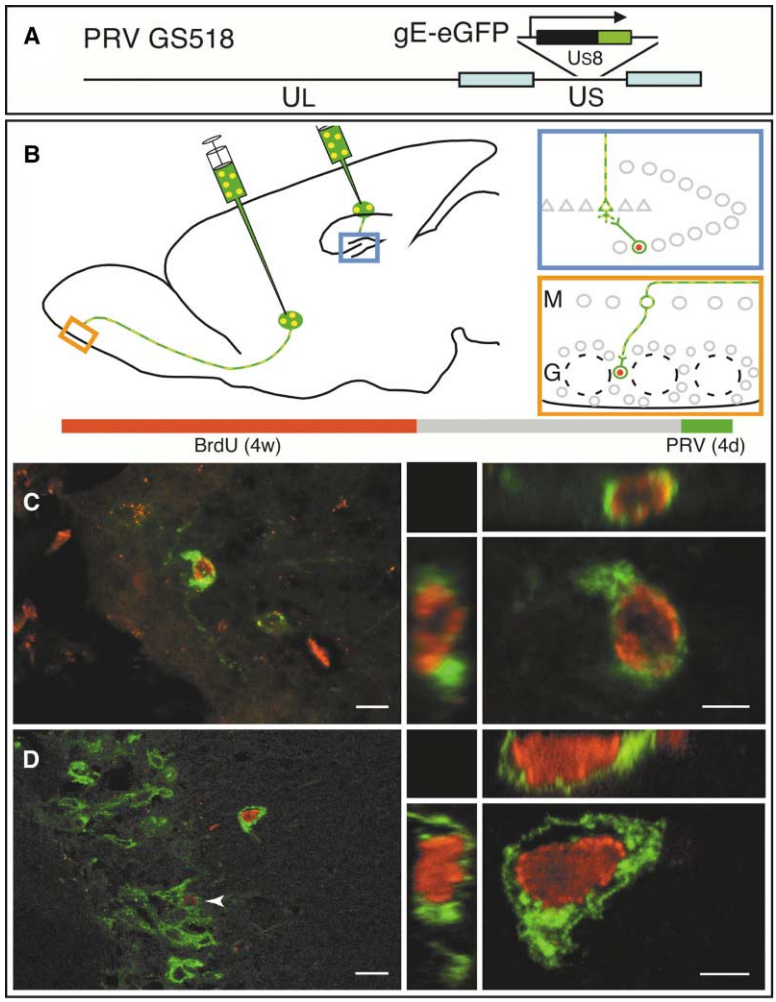

Figure 1. Functional Integration of Adult-Generated Neurons into Synaptic Circuitry

(A) Structure of the PRV GS518 virus genome. PRV GS518 was created by fusing the eGFP open reading frame (Clontech) at codon 457 of the PRV GS518 gE gene (Us8), resulting in a gE-eGFP fusion protein containing the entire ecto- and transmembrane domains of gE, and the first three amino acids of the cytosolic tail, fused to the eGFP protein. The fusion allele was recombined into the pBecker3 infectious clone in *Escherichia coli*.

(B) Transsynaptic neuronal labeling of adult-generated neurons in olfactory and hippocampal circuitry. Adult mice received a 4-week "pulse" of BrdU in their drinking water, followed by a 3-week "chase" period. We then injected PRV GS518 virus (shown in green) together with CTB (shown in yellow) into the piriform cortex or into the hippocampal CA1 region and examined OB and hippocampal neurons 4 days later. Top inset, injected virus and CTB are transported from CA1, the site of injection, to the CA3 region of the hippocampus. Virus, but not CTB, spreads to neurons of the dentate gyrus (DG) via synaptic connections. Bottom inset, OB mitral neurons that project to the site of injection are located in the mitral cell layer (M). A mitral neuron retrogradely transports virus and CTB to its cell body in the OB. Here, the virus, but not CTB, crosses at least one synapse to infect periglomerular interneurons located in the glomerular layer (G).

(C) Left: confocal image of an OB periglomerular neuron that has previously incorporated BrdU (red) into its DNA during mitosis and has subsequently been transsynaptically infected by PRV GS518 (green) injected into the piriform cortex (the scale bar represents 10 μm). Right: higher magnification image with *z* axis projections showing colocalization of viral infection (green) and BrdU incorporation (red) in all three planes in a single periglomerular neuron (the scale bar represents 5 μm).

(D) Left: confocal image of hippocampal neurons that are labeled by both viral infection (green) and BrdU incorporation (red), but not CTB (the scale bar represents 20 μm). Right: higher magnification image of a hilar neuron with the *z* axis projections showing colocalization of viral infection (green) and BrdU (red) in all three planes (the scale bar represents 4 μm). Confocal analysis of a dentate gyrus granular neuron (indicated by arrowhead at left) also confirmed colocalization of virus and BrdU in all three planes (data not shown). Viral tracing studies were performed as previously described [4].

the hilus and CA3 subregions of the hippocampus (Figure 1D and data not shown), indicating that the synaptic integration of adult-generated neurons is not specific to the olfactory system, but rather is a more general phenomenon of adult neurogenesis. Previous work has demonstrated that newborn neurons integrate into synaptic circuitry in the adult avian brain [8], and the present data extends this to the adult mammal.

Previous studies have proven the utility of using the induction of c-Fos, an immediate early gene product, as a marker for mapping the synaptic activation of neurons [9] and indeed for defining odor-responsive neurons in the rodent OB [10]. Therefore, to ask whether neurons generated in the adult brain can respond to a physiological stimulus, we exposed mice to a cocktail of odorants and examined the induction of neural activity as indicated by the upregulation of c-Fos expression (Figure 2A). Mice exposed to odorants ($n = 6$) displayed a marked increase in c-Fos induction in periglomerular neurons (Figure 2B) compared to controls exposed to a neutral odor ($n = 5$). The ability of odorants to activate adult-generated neurons was revealed by the colocalization of c-Fos induction with BrdU incorporation and expression of the neuronal marker NeuN within single periglomerular neurons of activated glomeruli (Figure 2B). Within odorant-activated glomeruli, approximately 17% $\pm$ 3% (mean $\pm$ SE) of c-Fos-expressing cells were also BrdU positive, indicating a substantial contribution of adult-born neurons to the olfactory response. The induction of neuronal activity by odor demonstrates that adult-generated neurons are not only synaptically integrated, but also functional, as they respond to a physiologically relevant stimulus.

A recent study correlated the ablation of dividing cells, and thereby neurogenesis, with the loss of hippocampal-dependent memories in the adult rat [11]; however, the techniques employed in the study leave the results open to interpretation [12, 13]. The present data provide direct evidence that neurons generated in the adult mammalian brain become functionally integrated into

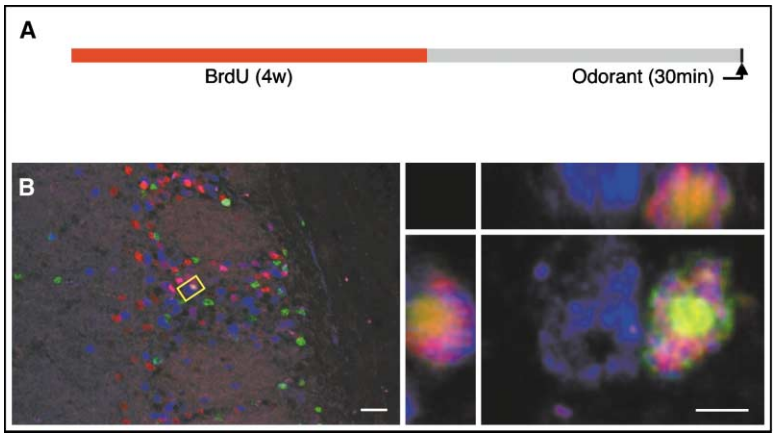

Figure 2. Activation of Adult-Generated Neurons by a Physiological Stimulus

(A) Odorant induction of c-Fos expression in adult-generated neurons. As previously, adult mice received a 4- week "pulse" of BrdU in their drinking water. Following a 3-week "chase" period, mice were exposed to an odorant cocktail for 30 min.

(B) Exposure to odorants induces widespread c-Fos expression in periglomerular neurons. Left: confocal image depicting a typical activated glomerulus (top of image), surrounded by many c-Fos-positive (red) neurons, adjacent to a less-activated glomerulus (bottom of image). BrdU incorporation is shown in green, and the neuronal marker NeuN is shown in blue (the scale bar represents 20 μm). Right: higher magnification confocal image showing a single periglomerular neuron (indicated by the small box at left) labeled for c-Fos induction (red), BrdU incorporation (green), and the neuronal marker NeuN (blue), with *z* axis projections showing colocalization of all three labels in all three planes (the scale bar represents 3 μm). c-Fos induction experiments were performed as previously described [10].

the existing synaptic circuitry and thus provide a cellular basis for the relevance of adult neurogenesis to brain function.

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