the manuscript two additional mutations in *ABCG8* were identified by sequencing: (i) del547C resulting in a premature stop codon at amino acid 191 in proband 7 and (ii) P231T (691 A>C) in proband 4. No additional mutations were identified in *ABCG5*.

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From Marrow to Brain: Expression of Neuronal Phenotypes in Adult Mice

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After intravascular delivery of genetically marked adult mouse bone marrow into lethally irradiated normal adult hosts, donor-derived cells expressing neuronal proteins (neuronal phenotypes) developed in the central nervous system. Flow cytometry revealed a population of donor-derived cells in the brain with characteristics distinct from bone marrow. Confocal microscopy of individual cells showed that hundreds of marrow-derived cells in brain sections expressed gene products typical of neurons (NeuN, 200-kilodalton neurofilament, and class III β-tubulin) and were able to activate the transcription factor cAMP response element–binding protein (CREB). The generation of neuronal phenotypes in the adult brain 1 to 6 months after an adult bone marrow transplant demonstrates a remarkable plasticity of adult tissues with potential clinical applications.

Until recently, the fate of adult cells has been thought to be restricted to their tissues of origin. Cells are well known to be capable of replenishing damage in tissues in which they reside, such as blood, muscle, liver, and skin. However, the finding that adult cells could be reprogrammed to express genes typical of differentiated cell types representing all three lineages (mesoderm, endoderm, and ectoderm) when fused to cells in heterokaryons was quite unexpected (*1–3*). This degree of plasticity demonstrated that the differentiated state is reversible and requires continuous regulation to maintain the balance of factors present in a cell at any given time (*4*). The cloning of frogs (*5*) and later sheep (*6*) further showed that previously silent genes could be activated in adult nuclei. Although remarkable, these examples of plasticity all involved extensive experimental manipulations. More recently, findings have been made that suggest that stem cells can assume diverse fates under physiologic conditions. Both transformed and primary neural cells can give rise to a range of phenotypes typical of their site of implantation within the central nervous system (CNS) (*7*, *8*). Bone marrow cells can yield not only all cells of the blood but also cells with a liver phenotype (*9*). Perhaps the greatest plasticity yet demonstrated is the "homing" of bone marrow–derived cells to damaged muscle in irradiated dystrophic *mdx* mice (*10*, *11*). Muscle-derived and CNS-derived stem cell–like populations have also been reported to reconstitute the blood and rescue lethally irradiated mice (*11*, *12*). Here we report that after lethal irradiation, bone marrow–derived cells administered by intravascular injection yielded cells that expressed genes specific to neurons (neuronal phenotypes) in the CNS. Moreover, both the sources and the recipients of these cells were adults.

To examine whether bone marrow–derived cells could give rise to cells in the brain, adult marrow was harvested from transgenic

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mice (8 to 10 weeks of age) that ubiquitously expressed enhanced green fluorescent protein (GFP) (*13*). GFP-expressing (GFP^+) bone marrow was administered by tail vein injection (6×10^6 nucleated cells per recipient) into lethally irradiated, isogenic adult (8- to 10-week-old) recipients (*14*). Brains harvested several months after the transplant (post-transplant) and examined by light microscopy revealed the presence of GFP^+ cells throughout the CNS, including the olfactory bulb, hippocampus, cortical areas, and cerebellum. No cells expressed detectable GFP in five age-matched bone marrow recipients (controls) transplanted with marrow that was not genetically engineered to express GFP. Thus, bone marrow–derived GFP^+ cells were clearly present in the brain.

A comparison by flow cytometry of GFP^+ cells derived from the bone marrow and the brains of transplanted animals revealed substantial differences (*15*). Isolated adult brains from transplanted mice were minced and treated with proteases as previously described (*16*) in order to obtain a single-cell suspension. Dissociated brain and bone marrow cells from the same mouse were stained with antibodies to the surface marker CD45 (*15*), which identifies all nucleated mature blood lineages (*17*), and to CD11b, which is expressed by all myelo-monocytic cells (*18*), including microglial cells in the CNS (*19*) (Fig. 1). Flow cytometry of whole bone marrow preparations obtained from recipients demonstrated that essentially all of the GFP^+ cells that engrafted also expressed CD45 (Fig. 1, A and B). By contrast, analysis of entire dissociated brains indicated that a significant subset (up to 20%) of the GFP^+ cells present in the CNS lacked both CD45 and CD11b (Fig. 1, A and C). These findings suggested that exposure to the CNS environment may have led a subpopulation of bone marrow–derived cells to acquire novel phenotypes. In order to identify such phenotypes unambiguously, we performed a single-cell analysis of brain sections.

Using laser scanning confocal microscopy, we determined definitively that individual cells coexpressed GFP and neuron-specific proteins (*20*). By using parameters that resulted in a calculated optical section thick-

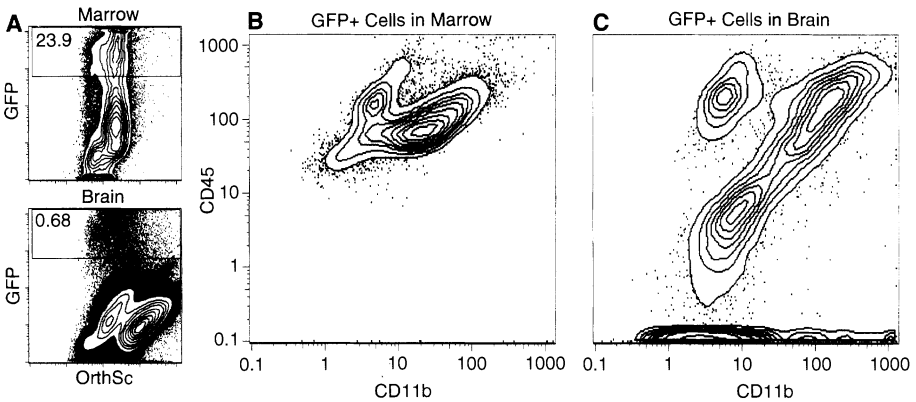

Fig. 1. Absence of hematopoetic markers on a subset of marrow-derived cells in the CNS of transplanted mice analyzed by flow cytometry. (**A**) Representative whole bone marrow (top) and brain (bottom) preparations from three mice transplanted with GFP^+ bone marrow. The GFP^+ cells included in the gates indicated are shown in (**B**) for marrow and (**C**) for brain. In (B) and (C), two antibodies were used: CD45, a marker of all mature nucleated hematopoetic cells visualized with an APC-conjugated antibody, and CD11b, a marker of myelo-monocytic and microglial cells visualized with a TC-conjugated antibody. The GFP^+ cells in transplanted mouse marrow were essentially all positive for CD45 (B) even when the marrow was subjected to the same dissociation protocol used for the brain preparation. By contrast, in the brain a GFP^+ population was readily identified that did not stain with CD45 or CD11b (C). No clear difference in the frequency of GFP^+ cells was observed between 2 and 12 months post transplant. In the brain preparation, two major populations are evident that differ in orthogonal scatter (A). Higher orthogonal scatter presumably represents debris resulting from the fragmentation of adult myelin, and is devoid of GFP^+ cells. The presence of a variable but sizable population of cells negative for both CD45 and CD11b detected only within the CNS of three different CD45 mice indicates that nonhematopoetic marrow-derived cells are present in this microenvironment.

Table 1. Laser scanning confocal microscopy was used to determine the morphologies of individual bone marrow–derived cells stained with a single antibody to neuron-specific proteins (*n*, total number of cells). The morphological classifications used were as follows: triangular (one end pointed, one end rounded), round, oval (ellipsoid, both ends rounded), rod (symmetrically cylindrical), spindle (both ends pointed, central thickening), and stellate (extensions and branching in all directions). Cells with a "triangular" morphology, the prevalent morphology of neuronal phenotypes, were subdivided into three classes: + (no extensions), ++ (single extension less then 10 μm), +++ (either single or branched extension of 10 to 70 μm, or more than one extension).

Morphologies of GFP+ cells in brain

Morphology	NeuN+	NF-H+	F4/80
n =	165	129	229
triangular	**62**	**61**	25
round	12	9	0
oval	9	11	4
rod	3	0	0
spindle	1	11	**36**
stellate	3	2	32
other	11	6	4

"Triangular" subcategories (%)

+	22	**57**	**72**
++	**56**	31	14
+++	23	12	14

Table 2. Number and location of individual bone marrow–derived CNS cells. Sections from five mice were stained with a single antibody to a cellular marker and were analyzed by laser scanning confocal microscopy. "Cells per section" indicates the average number of neurons, astrocytes, or microglial cells analyzed (±SD) in each coronal olfactory bulb section using antibodies specific to proteins for each cell type analyzed. The average number of neurons per OB section was determined using NeuN staining and was used to estimate neurons evaluated for NF-H staining. SAL, superficial axon layer; EPL, external plexiform layer; and IPL, internal plexiform layer.

	Neurons		Astrocytes	Microglia
	$NeuN^+$	$NF\text{-}H^+$	$GFAP^+$	F4/80
Mice analyzed	5	4	4	3
Sections analyzed	8	4	4	3
Cells per section	10,400 (± 600)		2000 (± 200)	550 (± 50)
	GFP^+ cells per OB layer from all sections analyzed			
Total	165	128	0	510
Layer				
SAL	105	66	0	312
Glomerular layer	30	41	0	114
EPL	14	10	0	56
Mitral cell layer	4	0	0	7
IPL	0	0	0	8
Granule layer	12	11	0	13

ness of 0.3 to 0.4 μm, this rigorous analytic method ensured that the colocalization of markers observed represented true coexpression of different proteins within the same cell. The olfactory bulb (OB) was selected for in-depth quantification because of its high frequency of ongoing neural regeneration relative to other parts of the brain (*21–23*). The olfactory bulbs of five recipients of GFP^+ bone marrow were analyzed in-depth.

Thousands of GFP^+ individual cells in the brain were subjected to confocal analysis in order to obtain quantitative data on morphology and gene expression (*20*, *24*). An average of 220 (SD ± 96) GFP^+ cells were observed per coronal OB section. The majority of GFP^+ cells in all sections expressed F4/80, which is indicative of a macrophage/microglial phenotype. However, because many GFP^+ cells in the population lacking F4/80 had morphologies consistent with those of neuronal cells, additional sections were stained with antibodies specific to neuronal proteins (Tables 1 and 2), including NeuN and the 200-kD isoform of neurofilament (NF-H) (*25*) as well as the astrocyte-specific marker, glial fibrillary acidic protein (GFAP) (*24*). GFP^+ cells were categorized according to their morphology and their expression of neuronal and non-neuronal proteins (Table 1 and Figs. 2 and 3). Neuronal gene expression was detected in round-, oval-, and rod-shaped cells, but was most abundant in triangular cells, many of which had one or more extensions of up to 70 μm.

A compilation of the number of individual cells analyzed by confocal microscopy that expressed the neuronal proteins, NeuN or NF-H, is presented in Table 2. A total of 165 NeuN-expressing and 128 NF-H–expressing cells were scored in eight and four OB sections, respectively. Given the total number of neurons estimated per section, about 0.2 to 0.3% of the total number of neurons were derived from the bone marrow by 8 to 12 weeks after the bone marrow transplant. Although this percentage is small, the total number of cells observed is substantial, providing strong evidence that cells derived from the bone marrow can express neuron-specific genes and assume a neuronal phenotype within the brains of adult animals.

The distribution within the OB of GFP^+ cells expressing neuron-specific genes was analyzed further. When coronal cross sections of the OB were analyzed, the majority of GFP^+ cells expressing neuronal markers were found in the superficial axon layer (SAL) and in the glomerular layer just adjacent to the SAL (Table 2). This finding is in good agreement with studies by others that indicate that the outer regions of the OB have a high degree of regenerative activity (*21–23*).

Although astrocytes expressing GFAP were readily detected in four OB sections (*24*), no GFP^+ cells were found in these sections that expressed GFAP (Table 2). This finding is in contrast to previous reports, which suggested that cells of the astrocytic lineage could be obtained from nonstromal bone marrow in vivo (*26*). This may be due either to a difference in experimental protocol or to the higher resolution made possible by using GFP-labeled cells in conjunction with confocal analysis.

A substantial proportion of the marrow-derived cells assayed coexpressed multiple neuron-specific gene products. Sections from five different mice were analyzed by confocal microscopy for the coexpression of two neuronal proteins, 200-kD neurofilament (NF-H) and class III β-tubulin (TuJ1) (Fig. 2). Twenty-four of a total of 80 GFP^+ cells selected for analysis based on morphology were found to coexpress these two cytoskeletal proteins in addition to GFP. Two sections with groups of cells that coexpressed both NF-H and class III β-tubulin are shown in Fig. 3, A through E and F through I, respectively.

Bone marrow–derived neural gene expressing cells were well integrated in the microenvironment of the OB in a manner consistent with their neighbors (Fig. 4). With-

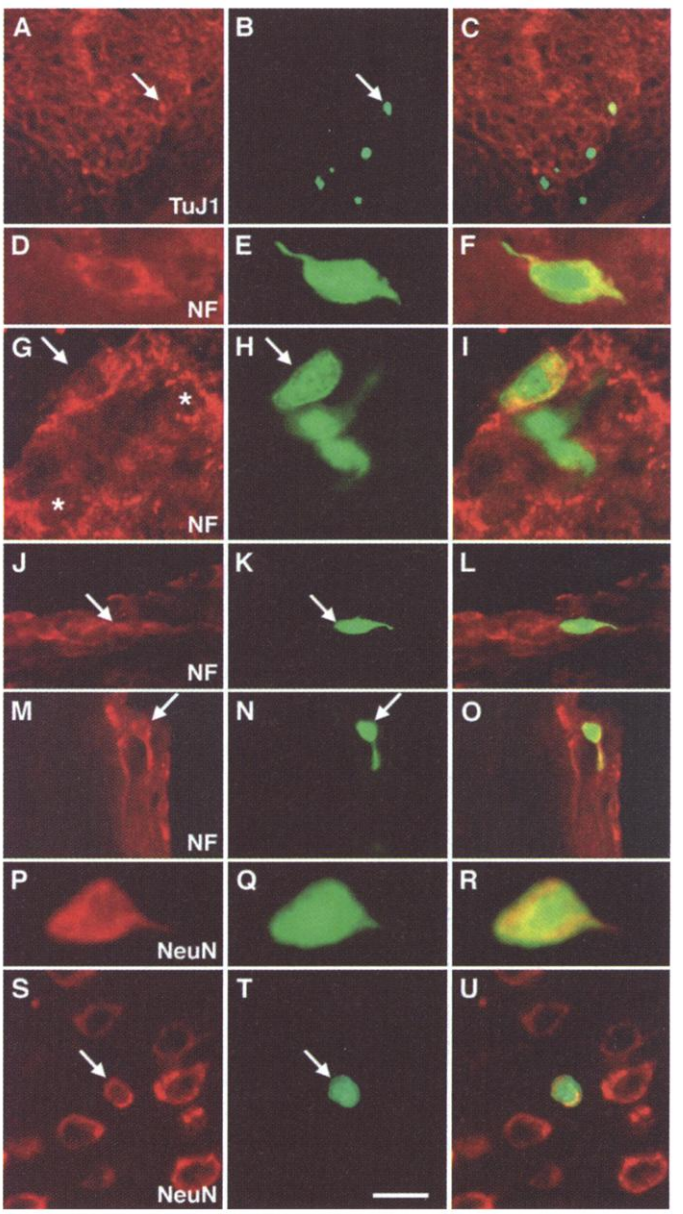

Fig. 2. Laser scanning confocal microscopic analysis of GFP^+ cells derived from bone marrow in adult brain sections. Marrow-derived cells (green in all images) from adult mice expressed neuronal-specific proteins. Morphological nomenclature is as described in Table 1. (**A** to **C**) A marrow-derived cell (arrow) expressing class III β-tubulin (TuJ1; red) in a group of five marrow-derived cells. (**D** to **F**) Cell with spindle morphology and a small extension expressing 200-kD neurofilament (NF-H; red). (**G** to **I**) Cell with oval morphology (arrow) expressing NF-H (red) next to two marrow-derived cells lacking expression of NF-H. Nuclear outlines (*) of non-marrow-derived neurons expressing NF-H (red) are visible. (**J** to **L**) A cell with triangular morphology (arrow) with a small extension expressing NF-H (red) in an olfactory bulb layer containing NF-H expressed by both marrow-derived cells and native neurons. (**M** to **O**) Cell with triangular morphology (arrow) with a small extension expressing NF-H (red) in the superficial axonal layer. (**P** to **R**) Prototypical triangular cell with a small extension expressing NeuN (red) which is known to be nuclear and perinuclear in the cell body (*33*). (**S** to **U**) Bone marrow–derived cell (arrow) expressing NeuN (red) in layer 2/3 of the parietal cortex. Left panels [(A), (D), (G), (J), (M), (P), and (S)], expression of neuron-specific proteins (TuJ1, NF, or NeuN); middle panels [(B), (E), (H), (K), (N), (Q), and (T)], GFP^+ marrow-derived cells in CNS; right panels [(C), (F), (I), (L), (R), (O), and (U)], colocalization of markers in left and middle panels. Scale bar in (T) corresponds to 31 μm for (A) through (C), 7.5 μm for (D) through (F), 10 μm for (G) through (I), 20 μm for (J) through (L), 18 μm for (M) through (O), 6 μm for (P) through (R), and 14 μm for (S) through (U). TuJ1, class III β-tubulin; NF, 200-kD neurofilament, NF-H.

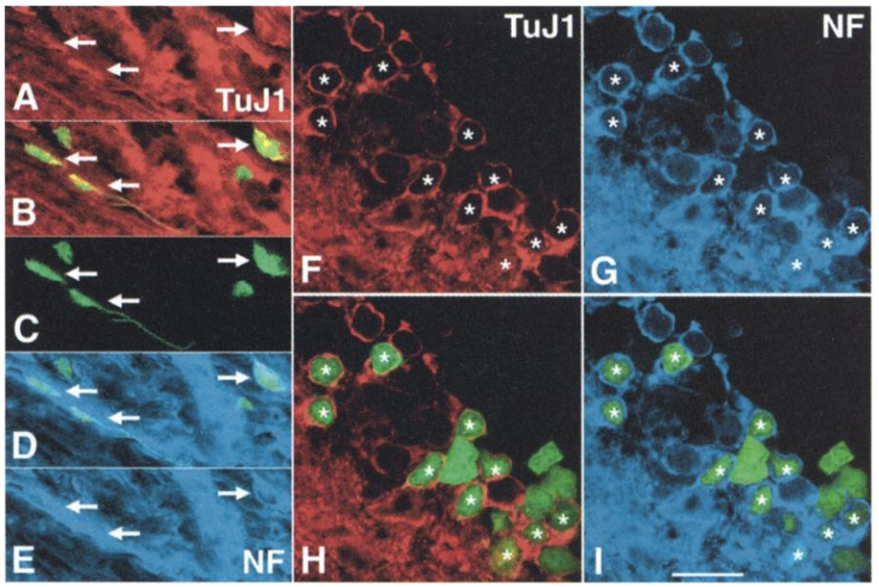

Fig. 3. Laser scanning confocal analysis of two groups of marrow-derived cells (green) coexpressing the neuronal proteins class III β-tubulin (TuJ1, red) and 200-kD neurofilament (NF-H, blue). (**A** to **E**) Three marrow-derived cells (arrows) coexpressing both TuJ1 and NF-H, one of which exhibits a 70-μm process, in the glomerular layer of the OB. (**F** to **I**) Ten bone marrow–derived cells coexpressing both TuJ1 and NF-H (*) clustered with native cells expressing both neuronal proteins. Several marrow-derived, GFP^+ cells not expressing neuronal proteins are also present. A rotating three-dimensional view of this group (*34*), constructed from 35 optical sections, better demonstrates that both marrow-derived and endogenous cells expressing neuronal proteins are surrounded by positive staining for both TuJ1 and NF-H. Scale bar in (I) corresponds to 35 μm for (A) through E) and 14 μm for (F) through (I). TuJ1, class III β-tubulin; NF 200-kD neurofilament.

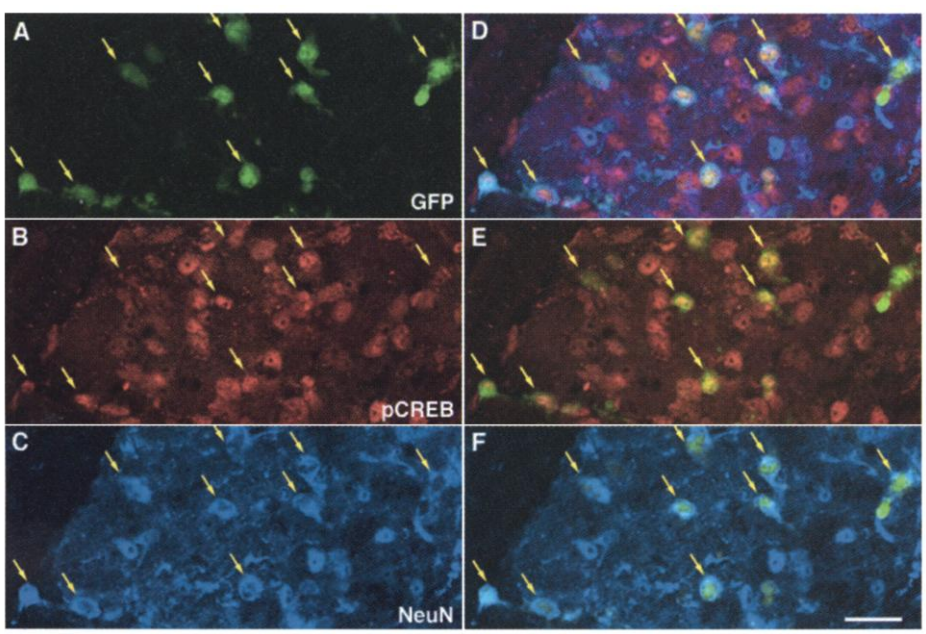

Fig. 4. Integration of marrow-derived GFP^+ cells expressing NeuN and activated CREB in the microenvironment of the OB. Laser scanning confocal microscopy demonstrated that all three markers were present in the same cell. Clusters of GFP^+ cells (green) with neuron-specific gene expression were frequently observed within the glomerular and superficial axonal layers. In this panel, nine GFP^+ marrow-derived cells [green in (**A**)], which have activated the transcription factor CREB [red in (**B**)] and express a neuron-specific gene, NeuN [blue in (**C**)], are indicated (arrows). Overlays of all three markers (**D**), of GFP and pCREB (**E**), and of GFP and NeuN (**F**) are also shown. Many (but not all) of the surrounding cells exhibit phosphorylation of CREB, suggesting that these neuronal phenotypes are well integrated into their environment and are capable of responding to extracellular stimuli in a manner consistent with neighboring cells. Note that NeuN has been shown in neurons in vivo to stain both nuclei and cytoplasm (*33*). Scale bar in (F) is 25 μm.

out the GFP marker, the cells are not easily distinguishable from their neighbors. This is true not only with respect to neuronal gene expression, for example NeuN, but also with respect to their ability to phosphorylate the transcription factor CREB. Indeed, nine cells in the field in Fig. 4 exhibited all three markers: GFP, NeuN, and pCREB (*27*). CREB is a transcription factor that is activated by phosphorylation (pCREB) in a number of diverse cell types. In neurons of the brain, CREB is activated in response to extracellular stimuli such as glutamate, neurotrophins, trauma, or stress (*28*, *29*). The GFP^+ cells from the marrow exhibited phosphorylation of CREB in a manner similar to the cells surrounding them. We observed several such regions in the outer layers (glomerular and superficial axonal layers) of the OBs of all three transplanted mice that were stained for NeuN and pCREB. These cells were in layers in which the majority of neuronal regeneration has been documented to occur in adult mice (*21*–*23*). In the OB, the cells that phosphorylate CREB most strongly are neurons (*30*); however, the existence of $NeuN^+$ cells that are lacking pCREB is not surprising, as the OB contains diverse types of neurons (*31*). Taken together, the CREB phosphorylation shown here indicates that a major signal transduction pathway present in neurons is also intact in bone marrow–derived cells that express neuron-specific genes in the brain. These results suggest that the cells are able to respond to cues in their environment in a manner consistent with the surrounding cells.

Our results clearly show that adult cells from the marrow can gain access to the adult brain and assume characteristics of CNS neurons. Hundreds of GFP^+ cells in the OB expressed neuron-specific genes. Of these, only a few had long axon-like extensions (Figs. 2 and 3), whereas short nonbranching extensions were prevalent. However, neuronal cells with this morphological characteristic are not unexpected in the OB, as they appear to be typical for class III β-tubulin expressing "neuroblasts" migrating into the OB from the subventricular zone (SV2) (*22*, *23*). To determine definitively the origin of cells in the OB will require marking studies and time courses as described by others (*22*, *23*). Many factors remain to be explored that could increase the frequency at which cells derived from marrow produce neuron-like cells in the brain: for example, the influence of donor and recipient age, the relative role of transient or chronic injury, the time period after transplantation, and the availability of specific trophic factors. Nonetheless, the results reported here reveal an unexpected degree of plasticity of adult bone marrow–derived cells. Recently, marrow-derived cells were reported to express

neuronal markers in tissue culture (*32*); however, their ability to yield neuronal phenotypes in response to physiological signals in vivo has not previously been shown. Thus, our findings are not only of fundamental interest but also, once more robust, could have application as a cell-mediated therapy. Not only could neurons be contributed to the adult brain, but, if genetically engineered, they could be a potentially useful tool for treating disorders characterized by defective neuronal function or a loss of neurons such as Parkinson's disease, lysosomal storage disorders, psychiatric disorders, trauma, and other types of CNS injury.

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14. Marrow was isolated in a sterile environment from 8- to 10-week-old, male transgenic mice that ubiquitously expressed enhanced green fluorescent protein (GFP) and non-GFP control mice. After lethal irradiation, 8- to 10-week-old C57BL/6 mice (Stanford) received 6×10^6 cells by tail vein injection.
15. Isolated brains were minced with a razor blade, dissociated with proteases, washed, stained with TriChrome (TC)–conjugated rat antibody to mouse CD11b and allophycocyanin (APC)–conjugated rat antibodies to mouse CD45, and analyzed by flow cytometry.
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Turning Blood into Brain: Cells Bearing Neuronal Antigens Generated in Vivo from Bone Marrow

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Bone marrow stem cells give rise to a variety of hematopoietic lineages and repopulate the blood throughout adult life. We show that, in a strain of mice incapable of developing cells of the myeloid and lymphoid lineages, transplanted adult bone marrow cells migrated into the brain and differentiated into cells that expressed neuron-specific antigens. These findings raise the possibility that bone marrow–derived cells may provide an alternative source of neurons in patients with neurodegenerative diseases or central nervous system injury.

Neural stem cells, the self-renewing precursors of neurons and glia, are the focus of intensive research aimed at developing transplantation strategies to promote neural recovery in the diseased or injured nervous system (*1, 2*). Recently, Bjornson *et al.* (*3*) demonstrated that neural stem cells could also differentiate into a variety of hematopoietic cells, including the myeloid and the lymphoid cell lineages, as well as more immature blood cells. Circulating T cells, B cells, and macrophages enter the brain (*4–7*). Rodent bone marrow cells migrate into the brain and differentiate into microglia and astrocytes when transplanted into previously irradiated recipients (*8, 9*). Recent evidence suggests that, under experimental culture conditions, human and rodent bone marrow stromal cells can differentiate into cells bearing neuronal markers (*10, 11*). When transplanted into the lateral ventricle or striatum of mice, cultured marrow stromal cells migrate into the brain and differentiate into astrocytes (*12, 13*). There is evidence that other types of mesodermal-derived cells can also differentiate within the mammalian nervous system. For example, luteinizing hormone-releasing hormone (LHRH)–producing neurons originate from outside the central nervous system (CNS) and migrate into the hypothalamus (*14*). In the present study, we show that bone marrow–derived cells enter the brain and differentiate into cells that express neuronal markers, supporting the idea that mesodermal-derived cells can adopt neural cell fates.

Mice homozygous for a mutation in the *PU.1* gene were used as bone marrow transplant recipients. PU.1 is a member of the ETS (DNA binding domain) family of transcription factors and is expressed exclusively in cells of the hematopoietic lineage. In the

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