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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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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absence of donor bone marrow cells, PU.1 knockout mice lack macrophages, neutrophils, mast cells, osteoclasts, and B and T cells at birth (*15, 16*). These animals are born alive but require a bone marrow transplant within 48 hours after birth to survive and develop normally. There are no gross morphological differences in the brain cytoarchitecture of these mice versus wild-type mice. In the present study, PU.1 null mice were used as bone marrow recipients to optimize the number of cells derived from the donor and to permit an accurate estimation of the numbers of bone marrow cells that migrate into the nervous system.

NeuN, a nuclear protein that is found exclusively in neurons (*17–19*), was used as a neuronal marker. Specific NeuN immunoreactivity was not present in acutely isolated (*20*) bone marrow cells. Acutely isolated bone marrow cells were also examined for neural antigens in our transgenic mouse line in which oligodendrocytes and Schwann cells express LacZ (*21*). No LacZ-expressing or β-galactosidase–immunopositive cells were present, and there was no specific immunostaining for NG2 chondroitin sulfate proteoglycan or O4, antigens that are present in Schwann cells and oligodendrocytes (*22–24*). These results strongly suggest that the bone marrow cell preparations were devoid of neurons and glia at the time of transplantation. When adult bone marrow cells were grown in culture for several weeks, the neural stem cell antigen, nestin, was present in 18% of the population [see Web fig. 1 (*25*)], indicating that bone marrow can give rise to neural stem cells.

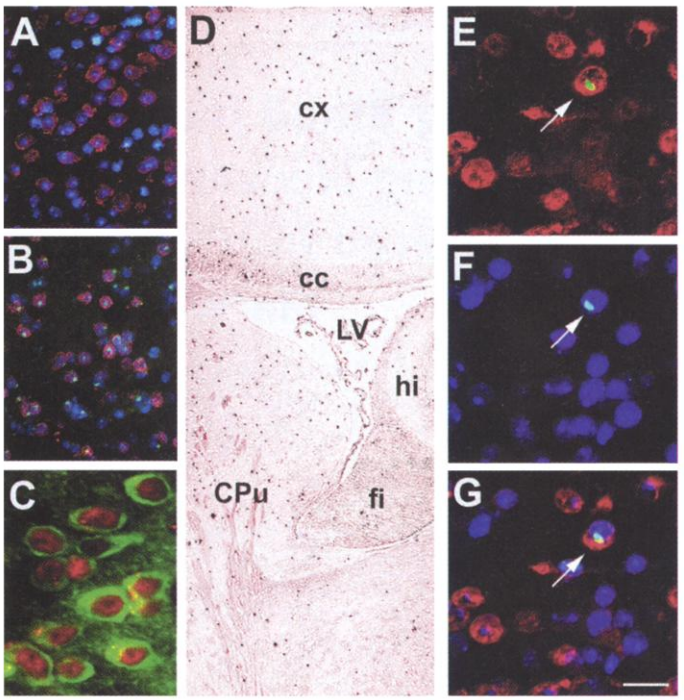

Fig. 1. Y chromosome staining in the CNS. Coronal sections from 4-month-old nontransplanted (**A**) female and (**B**) male brains were mounted and processed together. The panels show the overlay of the NeuN (red) immunostaining, Y chromosome nonradioactive ISH [visualized with tyramide-FITC conjugate (green)], and DAPI staining of cell nuclei (blue). The Y chromosome was restricted to the male brain, demonstrating hybridization specificity. (**C**) Confocal image of coronal sections from a 4-month-old recipient female striatum that was double-immunostained for the neuron-specific antigens NeuN and NSE. All NeuN-expressing cells (red) were also immunoreactive for NSE (green). (**D**) Sagittal section from a 1-month-old female PU.1 knockout mouse brain transplanted at birth with male bone marrow. The Y chromosome was visualized with BCIP/NBT (dark purple dots) to identify anatomical landmarks. cc, corpus callosum; cx, cerebral cortex; CPu, caudate putamen; fi, fimbria hippocampi; hi, hippocampus; LV, lateral ventricle. (**E** to **G**) Identical fields showing NeuN, Y chromosome, and DAPI nuclear triple staining in the hypothalamic dorsomedial nucleus of a 3-month-old female recipient. Colocalization of the Y chromosome [visualized with tyramide-FITC conjugate (green)] to a NeuN immunopositive (red) nucleus is shown in (E). In (F), DAPI staining identifies all cell nuclei (blue). Overlays of the NeuN, Y chromosome, and DAPI fluorescence are shown in (G). The arrow identifies a cell nucleus that contained both the Y chromosome (indicating the bone marrow origin) and NeuN. Scale bar in (G) represents the following sizes: 30 μm, (A) and (B); 10 μm, (C); 250 μm (D); and 12 μm, (E) to (G). Similar results were observed with three different animals for each experimental condition.

Within 24 hours after birth, PU.1 homozygous recipients were given intraperitoneal injections of bone marrow cells from wild-type mice (*20*). Seven transplant recipient mice and nontransplanted control littermates were examined between 1 and 4 months of age. To determine the efficiency of the transplantation, we analyzed different organ tissues for the presence of donor-derived cells. Y chromosome–positive male cells were identified in hematopoietic organs of female recipients by fluorescent in situ hybridization histochemistry. Greater than 90% of spleen cells, in both white and red pulp, and ~10 to 15% of liver cells were Y chromosome–positive. All brains were examined by using a combination of in situ hybridization (ISH) to detect the Y chromosome and immunohistochemistry to visualize the neuronal nuclear marker, NeuN. Brains from a 4-month-old nontransplanted female [Fig. 1A and Web fig. 2, A to E (*25*)] and a nontransplanted male [Fig. 1B and Web fig. 2, F to J (*25*)] were processed together and served as controls for the Y chromosome hybridization specificity and efficiency (*26*). There was no specific Y chromosome staining in the female brain. The Y chromosome was frequently localized at the periphery of the nucleus, which is characteristic of heterochromatin (*27, 28*). The NeuN immunostaining was predominantly localized to the nucleus, although some neurons [as reported by others (*19*)] also exhibited perinuclear staining [Figs. 1 and 2 and Web figs. 2 to 5 (*25*)].

Marrow-derived cells (i.e., Y chromosome–positive) were present in the CNS of all of the transplanted mice examined. Between 2.3 and 4.6% of all cells (i.e., all identifiable nuclei, including vasculature) were Y chromosome–positive (Table 1). The

Table 1. Quantitation of the number of donor cells in the forebrains of transplanted mice. A total of 21,682 cells was counted from seven animals. Ten to 20 random fields were photographed, and all DAPI-, NeuN-, or Y chromosome–positive nuclei were counted. Counts of cells represent an average from three independent investigators. The ratio of total cells to neurons was in good agreement with previous reports (*45, 46*).

Age (months)	DAPI-positive nuclei counted	NEU-positive nuclei counted	Y chromosome–positive cells	Y chromosome/NEU (double-labeled)	Neu-positive nuclei in all cells (%)	Y chromosomes in all cells (%)	Y of Neu-positive cells (%)
4	4831	1908	120	6	39	2.5	0.3
4	1322	221	60	5	17	4.5	2.3
3	3675	1483	130	16	40	3.5	1.1
3	4550	1825	105	15	40	2.3	0.8
2	3731	1039	162	16	28	4.3	1.5
1	1913	464	86	8	24	4.5	1.7
1	1660	380	76	7	23	4.6	1.8

Y chromosome–bearing cells were evenly distributed throughout the different brain regions [Fig. 1D and Web fig. 2, K and L (*25*)], in both white and gray matter. The Y chromosome was present in 0.3 to 2.3% of the NeuN-immunoreactive nuclei (Table 1). Confocal microscopy confirmed the presence of the Y chromosome in NeuN-immunopositive nuclei [Fig. 2 and Web figs. 4 and 5 (*25*)]. Y chromosome staining was localized to NeuN immunopositive cells and was not associated with any other neighboring nuclei in the *x*, *y*, or *z* planes. In the CNS of transplanted female mice, all NeuN-immunopositive nuclei were found in neuron-specific enolase (NSE)–containing cells (Fig. 1C). In the brain, NSE is expressed exclusively in neurons (*29*), demonstrating that Y chromosome–bearing cells can express two neuronal antigens. Most of these cells were found in the cerebral cortex [Web fig. 3, A to F (*25*)]; however, they were also present in the hypothalamus (Fig. 1, E to G), hippocampus, amygdala [Web fig. 3, G to I (*25*)], periaqueductal gray, and striatum. We did not detect Y chromosome–positive large motor neurons in the spinal cord or brainstem. A substantial number of Y chromosome–positive cell nuclei were present in cells within the choroid plexus of the lateral ventricle, in the ependyma of the ventricular system, and in the subarachnoid space, suggesting the cerebrospinal fluid as a primary route of entry [Web fig. 6 (*25*)]. We did not observe an overall increased density of Y chromosome–positive cell nuclei in neurogenic regions, including the subventricular zone, olfactory migratory region, or hippocampus. Because mesodermal stem cells can differentiate into microglia (*8*) and all microglia in these recipient animals arise from the donor bone marrow and are also Y chromosome–positive, we could not determine regional differences in the distribution of Y chromosome–positive nuclei.

These studies demonstrate that bone marrow cells migrate into the brain and differentiate into cells that express neuron-specific antigens. In combination with previous in vivo studies (*9*, *12*, *13*), the present work suggests that the bone marrow can supply the brain with an alternative source of neural cells. Neurons and macroglia (oligodendrocytes and astrocytes) are thought to arise from pluripotent neural stem cells that are present both in the developing (*30*) and adult mammalian CNS (*31*–*35*). It has been estimated that, for every 2000 existing neurons, one new neuron is produced each day (*35*, *36*). In the rodent brain, there are two well-characterized neurogenic regions: one in the subgranular zone of the dentate gyrus and one in the forebrain subventricular zone (*37*–*41*). Two populations of neural stem cells have been identified in adult mammals: one in the ependymal cell layer lining the ventricles (*33*) and one in the subventricular zone [glial fibrillary acidic protein–immunoreactive cells (*34*), each of which gives rise to glial cells and neurons]. We suggest that, in addition to these sources of neural stem cells, there may be a continuous influx of bone marrow stem cells into the ependymal and subependymal zones that give rise to a variety of CNS neural cell types. An interesting possibility is that these entry routes might also serve as portals into the CNS for diseases that primarily originate in and affect the hematopoietic system (i.e., leukemia and AIDS).

Bone marrow is far more accessible than neural stem cells and has the added advantage of having inherent host compatibility, thereby obviating the need to screen for viral and foreign antigens. Although our study showed that only a small number of transplanted cells expressed neuronal antigens in the adult brain, there may be factors that promote the differentiation of bone marrow cells into distinct neural cell types. Once these factors are identified, bone marrow cells might be expanded in vitro and provide an unlimited source of cells for the treatment of CNS disease and injury. Because at least two different types of stem cells have been isolated from bone marrow (hematopoietic and stromal), characterizing the potential for each population will be an important step toward optimizing regenerative therapies.

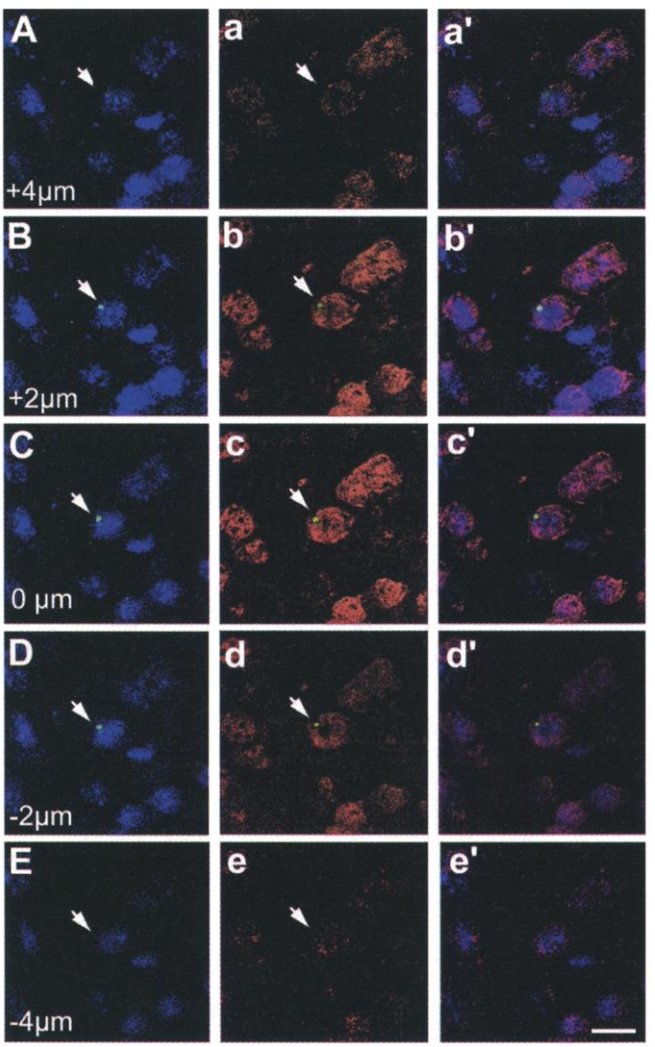

Fig. 2. A NeuN- and Y chromosome–positive cell in the cingulate cortex (1.2 mm behind the bregma) of a 3-month-old homozygous female PU.1 knockout transplanted at birth with male bone marrow. The images were obtained with a Zeiss confocal microscope. (**A** to **E**) Five different levels through the section (1 μm thick each), overlaying the Y chromosome [visualized with tyramide-FITC (green) and DAPI (blue) staining]. (**a** to **e**) Overlays of the corresponding NeuN (red) and Y chromosome staining. (**a′** to **e′**) Overlays of the corresponding NeuN, Y chromosome, and DAPI fluorescence. The Y chromosome hybridization was localized to a NeuN-immunopositive cell (arrow) and was not associated with any neighboring nuclei in the *x*, *y*, or *z* planes. Scale bar, 10 μm. These results were observed with five independent Z series from three different animals.

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26. Tissues were collected as follows. Reconstituted and normal mice were killed at the appropriate age with carbon dioxide gas. Tissues were collected and immediately stored at −80°C until used. ISH, histochemistry, and immunohistochemistry were performed as follows. Fresh frozen brain sections (12 μm thick) or acutely isolated bone marrow cells were fixed with 2 to 4% paraformaldehyde and immunostained with the neuronal nuclear marker NeuN [monoclonal immunoglobulin G1, 1:1000 dilution (Chemicon, Temecula, CA)]. The antibody was detected by using the Mouse on Mouse kit (Innogenex, San Ramon, CA) and subsequent deposition of biotinylated tyramide preceding the ISH. After the ISH, streptavidin-546 Alexa dye conjugate (Molecular Probes, Eugene, OR) was added to bind the biotin. Immediately following the deposition of the tyramide, nonradioactive ISH was performed on the same sections to detect the Y chromosome by using a 1.5-kb RNA probe, pY3531B, that was generated against a repeat sequence of the mouse Y chromosome (*17*) and labeled with digoxigenin–uridine 5′-triphosphate (for technical details, see http://intramural.nimh.nih.gov/lcmr/snge/Protocol.html). After several washes, the digoxigenin was developed using an antibody to digoxigenin conjugated to either alkaline phosphatase (1:1500 dilution) or peroxidase (1:400 dilution) (Roche Pharmaceuticals, Indianapolis, IN). The antibody to digoxigenin was then visualized with either 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) as substrate (purple precipitate with light microscopy) or tyramide–fluorescein isothiocyanate (FITC) (NEN, Boston, MA) (green fluorescence). Subsequently, cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (blue fluorescence). Representative sections from transplanted mice were double-labeled with NeuN and NSE [polyclonal, 1:10,000 dilution (Polysciences, Warrington, PA)] antibodies. Primary antibodies were visualized with an Alexa 594 antibody to mouse (NeuN, 1:1000 dilution, Molecular Probes) or Alexa 488 secondary antibodies to rabbit (NSE, 1:500 dilution, Molecular Probes).
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47. E.M. dedicates this report to the memory of János Szentágothai (1912–94), anatomist, statesman, romantic, artist, and mentor, who helped me understand the difference between looking at tissue sections and seeing the secrets they hold. The authors would like to express their sincere thanks to R. Dreyfus for his help with the conventional microscopy and C. L. Smith and R. Cohen for their help with the confocal microscopy. We are also grateful to M. Brownstein, R. Cohen, H. Gainer, L. Hudson, and M. Palkovits for their helpful suggestions and support throughout the work. These studies were supported by NIH grant AI30656 to R.A.M.

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Coding the Location of the Arm by Sight

Michael S. A. Graziano,* Dylan F. Cooke, Charlotte S. R. Taylor

Area 5 in the parietal lobe of the primate brain is thought to be involved in monitoring the posture and movement of the body. In this study, neurons in monkey area 5 were found to encode the position of the monkey's arm while it was covered from view. The same neurons also responded to the position of a visible, realistic false arm. The neurons were not sensitive to the sight of unrealistic substitutes for the arm and were able to distinguish a right from a left arm. These neurons appear to combine visual and somatosensory signals in order to monitor the configuration of the limbs. They could form the basis of the complex body schema that we constantly use to adjust posture and guide movement.

Without an accurate sense of the position of the limbs, head, and torso, we would be unable to guide movement, process the spatial location of nearby objects, or distinguish our own body parts from external objects. People with damage to their parietal lobes can have difficulty in all of these dimensions (*1, 2*). Studies in normal humans show that the body schema is not simply a representation of joint angles, but a complex integration of vision, proprioception, touch, and motor feedback (*3–6*). Although a great deal is known about the processing of joint angle and muscle stretch in the somatosensory system (*7*), little is known about how different sensory modalities are combined by neurons in the parietal lobe or elsewhere to construct the body schema (*8, 9*).

The present set of studies focused on the coding of static arm position. The sense of arm position depends on many sources of information, including proprioception and vision (*3–6, 10–12*). Here we show that neurons in parietal area 5 of the monkey brain, but not in the primary somatosensory cortex, respond in relation to the seen position of a false arm. They are also sensitive to somatosensory signals, responding in relation to the felt position of the monkey's actual arm. These somatosensory and visual signals are combined in individual neurons to provide a possible code for static limb position.

Responses of single neurons in area 5 were studied in two monkeys (*13*). The recording site in monkey 1 is shown in Fig. 1A, and the apparatus is shown in Fig. 1B. The arm contralateral to the recording electrode was outstretched, and the ipsilateral arm was held close to the body (not shown). The arms were covered with a black plastic plate. On top of the plate, a realistic false arm was placed in the monkey's view. This false arm was from a monkey of the same species and had been prepared by a taxidermist. The cut end was covered from view by a portion of

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