Central Autonomic Control of the Bone Marrow:
Multisynaptic Tract Tracing by Recombinant Pseudorabies Virus

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Abstract—Bone marrow is the primary place of hematopoiesis, where the development, survival and release of multipotent stem cells, progenitors, precursors and mature cells are under continuous humoral and neural control. Dense network of nerve fibers, containing various neurotransmitters is found in the bone marrow, however, the central neuronal circuit that regulates the activities of the bone marrow through these fibers remained unexplored. Transsynaptically connected neurons were mapped by virus-based transneuronal tracing technique using two isogenic, genetically engineered pseudorabies viruses, Bartha-DupGreen and Bartha-DupLac expressing green fluorescent protein and β-galactosidase, respectively. Bartha-DupGreen was injected into the femoral bone marrow of male rats and the progression of infection was followed 4–7 days post-inoculation. Virus-labeled cells were revealed in ganglia of the paravertebral chain and in the intermediolateral cell column of the lower thoracic spinal cord. Neurons were retrogradely labeled in the C1, A5, A7 catecholaminergic cell groups and several other nuclei of the ventrolateral and ventromedial medulla, the periaqueductal gray matter, the paraventricular and other hypothalamic nuclei, and in the insular and piriform cortex. Nerve transections and double-virus tracing from the bone marrow and the surrounding muscles were used to confirm the specific spreading of the virus. These results provide anatomical evidence for the CNS control of the bone marrow and identify putative brain areas, which are involved in autonomic regulation of the hematopoiesis, the release of progenitor cells, the blood supply and the immune cell function in the bone marrow. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: autonomic nervous system, sympathetic innervation, hypothalamus, brain stem, spinal cord, green fluorescent protein.

The bone marrow is a richly vascularized organ containing the principal hematopoietic tissue that generates various blood cells, which are constantly replaced throughout the life. Hematopoiesis occurs in association with a complex marrow stroma comprising a heterogeneous population of cells including fibroblasts, adipocytes, osteoblasts and other cellular elements (Mayani et al., 1992; Bianco and Riminucci, 1998). The bone marrow also contains cells that meet the criteria for stem cells of non-hematopoietic tissues (Prockop, 1997; Gronthos et al., 2003). These marrow stromal stem cells may be released in response to various challenges and are involved in the replacement or the repair, tissues of mesenchymal origin (Bianco et al., 2001; Huang et al., 2001; Hofstetter et al., 2002).

The proliferation and activity of bone marrow cells are regulated by local and systemic humoral factors. By presentation of cytokines, growth factors and membrane-bound adhesion molecules, stromal cells locally modulate leukocyte production, activation, migration and release (Yoder and Williams, 1995; van Buul et al., 2002; Cassese et al., 2003). It has also been shown that circulating steroid and peptide hormones affect blood cell proliferation, and thereby the immune function. The best known example is the immunomodulatory effect of stress-induced adrenal corticosteroids and catecholamines (Dhabhar et al., 1995; McEwen, 2000).

It has been clearly demonstrated that, in addition to hormonal regulation, the neural control plays a prominent role in the regulation of hematopoiesis (Maestroni and Conti, 1994a,b; Afan et al., 1997; Broome and Miyay, 2000; Maestroni, 2000a). The bone marrow, similarly to other lymphoid organs, is controlled by the autonomic nervous system. Myelinated and non-myelinated fibers originate from the nerves that enter the bone along with blood vessels through the nutrient foramina and give rise to smaller branches that spread over the periosteum while other branches penetrate into the bone marrow cavity. Nerve fibers may cross the parenchyma of the bone marrow or terminate on sinusoid walls, vascular elements and on perivascular stromal cells (Calvo, 1968; Yamazaki and Allen, 1990; Mach et al., 2002). Nerve fibers containing calcitonin gene-related peptide (CGRP), substance P, calcitonin gene-related peptide (CGRP), substance P; substance P; P2X receptors; tyrosine hydroxylase, substance P; and dopamine receptors.
(SP), neuropeptide Y (NPY), tyrosine hydroxylase (TH) and dopamine (DA) were identified in the bone marrow (Tabarowski et al., 1996; Elenkov et al., 2000; Mach et al., 2002) and neuropeptide/neurotransmitter receptors are present on the surface of several hematopoietic cell types (Liebl et al., 1991; McGillis et al., 1991; Santambrogio et al., 1993; Petito et al., 1994).

However, the exact localization and organization of the CNS circuit that provide relevant inputs to those cells that innervate the bone marrow remained unknown.

In order to reveal these circuits, we applied a multysynaptic tract-tracing method using pseudorabies virus (PRV), a neurotropic herpesvirus, which is a commonly used tool for delineation of hierarchically organized neuronal pathways (Card et al., 1990; DeFalco et al., 2001; Gerendai et al., 2001; Boldogkoi et al., 2002; Enquist and Card, 2003). After being uptaken by axon terminals, viral nucleocapsids travel retrogradely to the cell body, replicate, and the newly assembled mature virions infect functionally connected neurons across the synapses. We injected Bartha-DupGreen (BDG), a recombinant PRV strain with Bartha background (Boldogkoi et al., 2002, 2004) into the femoral bone marrow in order to identify the neuronal elements which are involved in the innervation of this structure.

**EXPERIMENTAL PROCEDURES**

**Viruses**

Two isogenic recombinant PRVs, BDG and Bartha-DupLac (BDL) (both are Bartha virus derivatives), with modified neuroinvasive- ness, were used in these experiments. The spreading characteristics of the viruses were altered by insertion of the LacZ (BDL) or the green fluorescent protein (GFP) (BDG) gene expression cassettes into the putative antisense promoter (ASP) located at the inverted repeat region of the virus. Both reporter genes were put under the control of the human cytomegalovirus major immediate early (IE) one promoter. These mutants label autonomic neurons in a slow manner and infect fewer neurons. Due to the slow spread of viruses, local immune cells effectively isolate infected neurons, whereby preventing local (non-specific) spread of infective viral particles that might be released from compromised cells at late stage of viral infection. The slow transsynaptic passage of BA-DupGreen enters the bone marrow and does not infect effectively any of the injected five animals. One out of four and two out of five animals were infected when rats were injected with inoculums containing 1.2×10³ and 2×10⁴ PFU of BDG, respectively. Finally, when 16 μl of 1.5×10¹⁰ PFU virus recombinants were injected into the bone marrow, 90% of the animals became infected. This dose was then further used to infect bone marrow in our mapping studies. The left femur was exposed, the bone surface was cleaned using 3% H₂O₂, and two holes were burred into the distal epiphysis. To avoid any viral contamination during injections, the femoral bone was isolated from the surrounding tissue using a cotton wool impregnated with 3% H₂O₂. A suspension (2×10⁸ PFU) of BDG virus (2.4×10⁸ PFU) was injected into the bone marrow using a 10 μl Hamilton syringe. The needle was kept in place for 5 min to avoid the reflux of the inoculums along the needle track. The place of injections was stained with Ethicon bone wax and the surface of the bone was wiped with 70% ethanol. The muscles surrounding the femur were sutured, and the skin was closed. Different survival times were used to evaluate temporal progression of the virus: 4 days (n=4), 5 days (n=6), 6 days (n=7), 7 days (n=5).

In four additional rats, the virus was inoculated into the bone marrow of the proximal epiphysis.

To infect the bone periosteum, 2 μl suspension of BDG virus (1.5×10¹⁰ PFU/ml) was dropped onto the isolated surface of the bone (n=3), kept in place for 10 min, and the wound was closed. We investigated in former studies whether this infection time and the amount of the inoculum is sufficient for the virus to invade nerve endings and to develop productive infection. The same amount (1.5×10¹⁰ PFU/ml) of BDG was dropped onto the surface of femoral muscles and wiped off 10 min later. Five days afterward, virus-labeled neurons were found in the brain stem and in the hypothalamic pre-autonomic nuclei (PVH). One animal was killed 6 days after virus injection because of visible signs of infection. These experiments verified that this infection timing is sufficient for the attachment of the virus particles to the nerve endings and resulted in productive infection in case of muscular elements.

An additional group of six rats was used to infect the femoral muscles. Ten times 1 μl BDG injections were given to different places of the femoral muscles (musculus vastus lateralis and musculus adductor brevis). The titer of the virus was 6×10⁸ PFU/ml (n=3) or 1.5×10¹⁰ PFU/ml (n=3).

To examine the specificity of BDG transport to the CNS following bone marrow injections, femoral, sciatic and obturator nerves were transected in six rats, immediately before inoculation of the ipsilateral bone marrow. The postinoculation time was 5 days.
**Double-virus labeling**

To differentiate between the neurons innervating the bone marrow and surrounding femoral muscles, five rats received 2×8 μl BDG injections into the femoral bone marrow. After 24 h the virus-injected leg was re-exposed and a sum of 10 μl of BDL (7×10^8 PFU/ml) was injected into the vastus lateralis and adductor brevis muscles at eight to 10 places. The animals survived 4 days after BDL injection (5 days after BDG injection). These experiments demonstrated that this kind of inoculation protocol resulted in synchronous infection by the two recombinants. A similar timing to match infections of two recombinant viruses after autonomic-motor injections was also used by others (Kerman et al., 2003).

**Tissue processing**

Animals were deeply anesthetized at various times after virus inoculation and perfused transcardially with 50 ml saline followed by 400 ml Zamboni’s solution (4% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate-buffered saline, pH 7.4). The brain and the spinal cord were removed, postfixed overnight in the same fixative and cryoprotected in a 20% sucrose solution at 4 °C. Serial coronal sections were cut on a freezing microtome at a thickness of 30 μm (brain) and 50 μm (spinal cord), with the frequency of 150 μm and 200 μm respectively. Spinal cord sections were cut and collected separately as four blocks: C1–T2, T3–T7, T8–T13, and the lumbar cord. All sections were collected into an antifreeze solution (containing ethylene glycol and glycine in phosphate-buffered saline) and stored at −20 °C until processing. The sympathetic chain was excised bilaterally from upper thoracic segments through the diaphragm, to the level of the aortas. After overnight fixation the tissue was washed in potassium phosphate-buffered saline, KPBS (pH 7.4) and before sectioning it was checked for GFP labeling using epifluorescent microscope. This procedure enabled us to evaluate the number and location of the infected ganglia in the sympathetic chain and to approximate number of GFP positive cells in each ganglion. Then, 10 μm sections were cut from the ganglia of the sympathetic chain on a cryostat.

**Immunohistochemical procedures**

Brain and spinal cord sections were rinsed in KPBS several times at room temperature. For PRV immunohistochemistry, endogenous peroxidase activity was blocked using 0.3% H2O2 for 10 min. After washing, sections were incubated in 2% normal goat serum for 1 h, diluted in KPBS with 0.3% Triton X-100. PRV-infected neurons were identified using a rabbit polyclonal antiserum (Rb 133, kindly provided by prof. Lynn W. Enquist, University of Princeton, NJ, USA), which recognizes all major viral envelope and capsid proteins (Card, 1994). Sections were incubated overnight in the anti-PRV serum at 1:10,000 dilution, then washed three times with KPBS. Following incubation in biotinylated goat anti-rabbit (1:1000, Vector Laboratories, Burlingame, CA, USA) for 1 h, sections were washed, and incubated in avidin–biotin–peroxidase complex (ABC-Elite kit, Vector). Diaminobenzidine (DAB) was used as chromogen. DAB reaction was intensified with 1.5% nickel–ammonium-sulfate. Sections were mounted onto gelatin-coated slides and coverslipped with Vectashield (Vector).

Geneva (1:1000, kindly provided by Dr. R. Corder, Geneva, Switzerland) antibodies for 48 h. After washing in KPBS, the sections were incubated in the mixture of donkey anti-mouse Alexa-488 and goat anti-rabbit Alexa-594 secondary antibodies (1:1000, Molecular Probes) for 1.5 h. Sections were mounted onto gelatin-coated slides and coverslipped with Vectashield (Vector).

**RESULTS**

**Virus labeling pattern in bone marrow-inoculated rats**

Sympathetic chain and spinal cord labeling. The first infected neurons in the ganglia of the sympathetic chain at the lumbar level were identified 4 days following BDG inoculation to the bone marrow. Before sectioning, each ganglion was examined for GFP expression as it was described in the “Experimental Procedures.” At 4 days, one to four infected ganglia at the lumbar level of the sympathetic chain were revealed with the average number of five to 10 visible, GFP-positive cells/ganglia. Because of the relatively large thickness of the ganglia, this method was used to evaluate the number of the infected ganglia and the spreading of infection in later survival times rather than the determination of the exact number of the compromised neurons. Labeled cells were found marginally and characterized by oval cell bodies and processes running parallel to the axis of the elongated ganglia. By 5 days, the infection spread up to the lower thoracic levels and the number of the infected neurons in the lumbar ganglia was slightly increased.

In the spinal cord, the first virus infected neurons were found at 4 days, bilaterally in the intermediolateral cell column (IML), at the T8–L1 levels. Scattered cells were observed in the central autonomic nucleus (CAN, lamina X) on the two sides of the central canal.

At day 5 post-infection, a large number of compromised cells was found in the spinal cord. In the T10–L1
levels, groups of infected neurons were observed bilaterally in the IML and CAN, demonstrating the focus of primary infection, and individual PRV-positive cells appeared in the T4–L1 levels. In the lumbar and sacral levels, scattered labeling was found in the central gray matter around the central canal (lamina X), in the lateral funiculus and in the parasympathetic preganglionic neurons in the sacral spinal cord (Fig. 1.). In addition, laminae I–II of the thoracic dorsal horn contained groups of compromised cells and cells in laminae IV, V, VIII, IX were also positive to viral labeling in some sections, bilaterally.

**Brainstem labeling.** At 4 days post-infection only one of the four examined rats showed moderate PRV-immunoreactivity (-ir) in the rostral ventrolateral medulla.

Most of the animals displayed brainstem labeling at 5 days postinoculation following BDG injection into the femoral bone marrow (Table 1). In these animals, infected neurons were detected bilaterally in the C1 and A5 catecholaminergic cell groups of the rostral ventrolateral medulla (Fig. 2). In the ventromedial medulla, PRV-ir cells were revealed in the raphe magnus, the gigantocellular (Gi), and the lateral paragigantocellular nucleus (LPGi). Scattered labeling was also present in the parapyramidal and ambiguous nuclei, the nucleus of the solitary tract (NTS) and in the C3 adrenergic cell group. In the caudal medulla some infected cells were found marginally, in the lateral reticular nucleus including the C1 adrenergic cells, and the nucleus raphe pallidus.

At the level of the pons and midbrain, compromised neurons were found in the locus coerules (LC) and the subcoerules region, in the A7 catecholaminergic cell group and in the nuclei of the pontine reticular formation. In two out of six animals, few virus-infected cells were observed in the pedunculopontine tegmental nucleus, and in the deep mesencephalic nucleus. The periaqueductal gray matter (PAG) displayed PRV-positive cells in all animals examined, mainly in its ventrolateral subdivisions. All observed neuronal labeling were bilateral in the brainstem, with no obvious dominance to either side. The list of brain sites exhibiting virus labeling is summarized in Table 1.

Six days following BDG injection the progression of viral infection was obvious in all animals examined. In A5 and C1 cell groups that contained the highest number of infected neurons at 5 days’ survival, the number of infected neurons significantly reduced and signs of neuronal degeneration were observed. Other areas, such as the nuclei of the ventromedial medulla contained more labeled cells. By 6 days, labeled cells appeared in the area postrema, the pedunculopontine and laterodorsal tegmental nuclei, the parabrachial nuclei. Cells along the ventral PAG on the
Table 1. Distribution of BDG-infected neurons in the brain following inoculation of the femoral bone marrow

<table>
<thead>
<tr>
<th>Area</th>
<th>PRV labeling</th>
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<td></td>
<td>4 Days (n=4)</td>
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<tr>
<td>Forebrain</td>
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<td>Infraorbital cortex</td>
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<td>Prelimbic cortex</td>
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<td>Cingulate cortex</td>
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<td>Motor cortex</td>
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<td>Somatosensory cortex</td>
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<td>Insular cortex</td>
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<td>Piniform cortex</td>
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<td>Entorhinal cortex</td>
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<td>Nucleus of the diagonal bond</td>
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<td>Septum</td>
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<td>Hippocampus</td>
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<td>BNST</td>
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<td>Substantia innominata</td>
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<td>Amygdala</td>
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<td>Median preoptic nucleus</td>
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<td>Medial preoptic nucleus</td>
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<td>Medial preoptic area</td>
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<td>Lateral preoptic area</td>
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<td>Subfornical organ</td>
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<td>Supraoptic nucleus</td>
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<td>PVH, anterior</td>
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<td>PVH, dorsal parvocellular</td>
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<td>PVH, ventral parvocellular</td>
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<td>PVH, dorsomedial parvocellular</td>
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<td>PVH, magnocellular</td>
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<td>PVH, posterior</td>
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<td>Periventricular nucleus</td>
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<td>Suprachiasmatic nucleus</td>
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<td>Perifornical nucleus</td>
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<td>Arcuate nucleus</td>
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<tr>
<td>Lateral hypothalamic area</td>
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<tr>
<td>Ventromedial hypothalamic nucleus</td>
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<tr>
<td>Dorsomedial hypothalamic nucleus</td>
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<td>Posterior hypothalamic area</td>
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<tr>
<td>Mamillary nuclei</td>
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<td>Zona incerta</td>
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<td>Midbrain-pons</td>
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<td>PAG</td>
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<tr>
<td>Paraoculomotor nuclei</td>
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<tr>
<td>Deep mesencephalic nucleus</td>
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<td>Nucleus of Darkschevitsch</td>
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<td>Red nucleus</td>
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<td>Ventral tegmental area</td>
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<td>Substantia nigra</td>
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<td>Parabralinal nuclei</td>
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<td>Laterodorsal tegmental nucleus</td>
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<tr>
<td>Pedunculopontine tegmental nucleus</td>
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<tr>
<td>A7 region</td>
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<td>A5 region</td>
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<td>Pontine reticular nucleus</td>
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<td>LC</td>
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<tr>
<td>Medulla</td>
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side of the oculomotor nuclei and in the nucleus commissuralis of Darkschevitsch (Table 1) became also virus-immunoreactive by 6 days. Only a few, if any BDG-labeled neurons were identified in motor-associated nuclei such as the substantia nigra or the red nucleus compared with those experiments when BDG was injected into the femoral muscles (see below). By the seventh day postinoculation, most of the virus-infected neurons disappeared from

### Table 1. Continued

<table>
<thead>
<tr>
<th>Area</th>
<th>PRV labeling</th>
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<tr>
<td></td>
<td>4 Days (n=4)</td>
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<tr>
<td>LPGi</td>
<td>+</td>
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<tr>
<td>Dorsal paraganglionic nucleus</td>
<td>+</td>
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<tr>
<td>Raphe nuclei</td>
<td>+/++</td>
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<tr>
<td>Ventrolateral medulla [C1/A1]</td>
<td>+/+1</td>
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<tr>
<td>Parapyramidal nucleus</td>
<td>+</td>
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<tr>
<td>Nucleus ambiguous</td>
<td>+</td>
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<tr>
<td>C3 region</td>
<td>+</td>
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<tr>
<td>Nucleus of the solitary tract</td>
<td>+</td>
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<tr>
<td>Area postrema</td>
<td>+/2</td>
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<tr>
<td>Spinal cord</td>
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<tr>
<td>Cervical</td>
<td>+</td>
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<tr>
<td>Thoracic</td>
<td>+</td>
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<tr>
<td>Lumbar</td>
<td>+</td>
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<tr>
<td>Sacral</td>
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Plus signals correspond to the approximate number of infected neurons according to the following scale: +: 1–3, ++: 4–8, +++: 9–15, ++++: >16 infected cells/section. In the cases, when an infected brain area was positive to PRV in some of the animals only, at the given time point, the number of animals exhibited positive labeling was indicated after the + sign.

**Fig. 2.** Virus-infected cells in the brainstem at 5 days post-inoculation. PRV-positive neurons are found in C1 and A5 cell groups of the ventrolateral medulla, in the LC and subcoeruleus area as well as in the nuclei of the rostral ventromedial medulla (RVM). py, pyramidal tract; 4v, fourth ventricle; 7n, facial nerve. Scale bar = 100 μm.
the infected areas of the brainstem, only a few PRV-positive cells were found in the PAG (in two animals), and in the LC (in one animal).

**Forebrain labeling.** At 5 days, PRV-positive neurons were already apparent in some forebrain areas. At this time point, PRV-labeled cells in the diencephalon were concentrated in the PVH, where most of the labeling was confined to the autonomic-related dorsal- and ventromedial-parvocellular subdivisions. In the PVH, an obvious contralateral dominance was observed in PRV labeling. Virus-infected neurons were additionally found in some other hypothalamic areas in all animals examined, such as the retrochiasmatic and the lateral hypothalamic areas. In one case, PRV-positive cells were observed in the mamillary nuclei, the zona incerta, the dorsomedial hypothalamic nucleus, and two cases in the ventromedial hypothalamic nucleus and in the preoptic area.

At 6 days, several forebrain areas displayed positive staining to the virus antigens. In the PVH, the number of infected cells increased and some virus containing neurons were revealed in the magnocellular, as well as in the dorsomedial, anterior and posterior parvocellular subdivisions of the nucleus. Disappearance and degeneration of previously infected neurons was also observed in the hypothalamus as it was seen in the brainstem with a complete absence of labeled neurons by day 7 (Fig. 3). By 6 days, the periventricular, perifornical, dorsomedial, ventromedial hypothalamic nuclei, the zona incerta and the posterior hypothalamic area became PRV positive in all investigated animals ($n=7$). In addition, scattered labeling was observed in the dorsal tuberomamillary, premamillary, ventral and lateral mamillary nuclei in some animals. The supraoptic, suprachiasmatic, arcuate nuclei, the substantia innominata and the circumventricular organs, such as the organum vasculosum of lamina terminalis (OVLT) and the subfornical organ were slightly labeled in two to three animals (Table 1). Large number of virus-infected cells was seen in the medial, median, ventrolateral preoptic nuclei as well as in the medial and lateral preoptic area caudal to the level of OVLT (Fig. 4).

PRV-positive profiles were detected in limbic structures, such as the central and medial nuclei of the amygdala, bed nucleus of the stria terminalis (BNST) (Fig. 5) and in the lateral and medial septal nuclei. In the cerebral cortex, most of the PRV-positive cells were found in the insular and piriform cortex $+1.70$ to about $-2.56$ mm from the level of the bregma (according to Paxinos and Watson, 1997). In addition, some infected cells were observed in the infralimbic cortex in two cases and in the prelimbic and cingulate cortex as well as in the motor and sensory cortical regions in three cases.

Seven days after virus inoculation, the earlier infected forebrain areas were devoid of PRV positive cells, while the infection progressed up to the cortical regions and
increasing number of virus-labeled neurons was observed in the insular and piriform cortical areas. Scattered single cells or small clusters of PRV-positive cells were characteristic in the rostral agranular insular cortex, while labeled profiles formed bigger clusters in the pyramidal layers at the level of the bregma – 1.80. At this postinoculation time, PRV-infected neurons of the BNST and amygdala started to degenerate, that was accompanied with partial reduction of their antigen-positivity. In addition, small, glia-like profiles were recruited to the site of the infection and dilated blood vessels were also observed at the site of the infection (Fig. 5).

**Neurochemical phenotype of BDG infected neurons following bone marrow injection.** To characterize the neurochemical phenotype of some infected neurons in the brainstem and spinal cord, we colocalized the virus marker GFP with TH, the rate-limiting enzyme of the catecholamine biosynthesis. At 5 days' survival in the C1 area, 21% of the PRV-infected neurons were positive to TH. In the A5
and A7 cell groups this ratio was 59% and 66% respectively. Up to 81% of infected LC neurons were found double-labeled (Fig. 6A). In the spinal cord, no double-labeled cells were found.

In the sympathetic chain, colocalization of NPY with virus marker GFP revealed that only some of the PRV-infected neurons express NPY (Fig. 6B).

**Control experiments**

To investigate that the distribution of virus-labeled neurons following bone marrow infections is characteristic to the bone marrow, we also injected BDG into the proximal epiphysis of the left femur (n=4). PRV-labeled neurons appeared first in the T8–L1 spinal cord sections, at 4 days’ survival. At 5 days, neurons in the C1, A5, LC cell groups and nuclei of the ventromedial medulla were found labeled, similarly to those cases when BDG was injected into the distal epiphysis. One animal was left to survive for 7 days. The spreading kinetics of the virus and the distribution of the infected neurons in the forebrain structures were similar to those found in previous experiments when the virus was inoculated into the distal femoral epiphysis. Quantitative analysis of the infected neurons in the pre-autonomic nuclei of the medulla and pons, confirmed these observations. The average number of infected cells/section in the C1 (6±2.4), the A5 (4.6±2.5) and the RVMM (7.5±3.5) following BDG injection into the distal epiphysis was comparable to cell numbers seen after BDG administration into the proximal epiphysis (5±1.6; 5.7±2.7 and 4.5±2.5 cells/section respectively).

To exclude the possibility that PRV transneuronal labeling originates from the sensory fibers innervating the periosteum during the virus inoculation of the bone marrow, some animals were prepared where BDG was applied onto the surface of the periosteum. In these animals, scattered PRV-positive cells were revealed only in the A5 (2.1±0.7 cells/section) and C1 (1.4±0.4 cells/section) cell groups and in the NTS (2.3±0.7 cells/section). (Compared with the bone marrow-injected rats, we never found labeled profiles in the NTS at 5 days’ survival). This result may indicate the involvement of the viscerosensory pre-autonomic neurons in the innervation of the periosteum. No other brain areas displayed labeled neurons in these rats at 5 days’ survival.

Three out of six animals with transection of the major trunks of the sciatic, femoral and obturator nerves, showed no signs of virus-labeling in the brainstem and spinal cord 5 days after BDG inoculation into the bone marrow. Al-
though the distribution of the infected areas in the rats with incomplete denervation was similar to that of intact rats, the number of PRV-immunoreactive cells was significantly reduced. Only a few neurons have been visualized in the T8–L1 spinal cord segments, much less than in intact rats. The number of infected cells in nerve transected animals C1 (1.1 ± 0.4 cells/section), A5 (1.3 ± 0.6 cells/section) and RVMM (3.6 ± 3 cells/section) was significantly lower than in the non-transected, sham-operated controls (6 ± 2.4; 4.6 ± 2.5 and 7.5 ± 3.5 cells/section respectively).

Another set of control animals was assigned to compare the patterns of transneuronal labeling in CNS neurons after BDG injections to femoral bone marrow and the surrounding femoral muscles. Those rats that were given 10 μl of BDG from the 6 × 10⁸ PFU/ml virus suspension (n = 3), survived more than 5 days. Compared with injections exclusively into the bone marrow, animals following muscular inoculation exhibited much more labeled neurons in the substantia nigra than it was observed in some BDG-injected animals. We found infected neurons in the red nucleus and the striatum, which areas were unlabeled after bone marrow injections. Following muscular inoculations, a high number of virus-labeled cells were seen in the motor cortex, compared with the bone marrow inoculations, while the labeling in the insular as well as in the piriform cortex was less abundant. However, the brainstem and diencephalic patterns of PRV-positive cells were similar in bone marrow- and in muscle-inoculated animals.

To differentiate at the cellular level between neurons that innervate the bone marrow and the surrounding muscles in these areas, we performed double virus tracing. Using two isogenic forms of the PRV, BDG, expressing the GFP was injected into the bone marrow, and BDL, expressing β-gal was inoculated into the femoral muscles surrounding the femur. To provide matching of the infection of the two recombinants, several types of protocols were tested. In our first experiments when BDG or BDL was injected into the vastus lateralis and adductor brevis muscles, the spreading of the infection to the spinal cord and brainstem was significantly faster than following injection of these viruses into the bone marrow. In addition, the spreading kinetics of BDG was identical to BDL in case of inoculation of each recombinant virus into muscular elements. The kinetics of the infection following injection of BDL into the femoral muscles was the same as in case of double-virus labeling experiments. When BDG and BDL

Fig. 7. Double virus labeling. Distribution of infected neurons following co-inoculation of BDG (expressing GFP) into the femoral bone marrow and BDL (expressing β-gal) into the femoral muscles. Reporter proteins were visualized as green (GFP) or red (β-gal) colors by double immunofluorescence. (A) In the left (L) and right (R) PVH, retrogradely labeled neurons from different targets are segregated in the dorsal and ventral parvocellular subdivisions. Note the absence of double-labeled neurons in this nucleus and the contralateral dominance of the labeling originating in the bone marrow. (B) In the ventrolateral and ventromedial medulla, scattered neurons expressing both recombinants (arrowhead) are found. This indicates the capacity of these neurons to be infected by both recombinants. 3V, third ventricle. Scale bar = 100 μm.
were injected in the same time, at 5 days’ survival, a high number of β-gal-positive cells were found in the brainstem and the hypothalamus compared with the number of GFP expressing cells. For these reasons, in further experiments BDL was injected 1 day after BDG administration to balance the temporal pattern of infections with both recombinants.

In the spinal cord, BDL-infected alpha motoneurons were found in the L2–S3 segments, the focus of the infection was observed in L4–L5 segments, where two to three neurons/sections were labeled. No double-labeled neuron was found among the motoneurons in the ventral horn. In the brainstem, the patterns of infection of the two recombinants were similar in some areas of the ventromedial medulla such as the LPGi and the C1 cell group in the rostral ventrolateral medulla (RVLM), where some co-infected neurons were found with an average of one to two cells/sections (Fig. 7B). Other areas, like the A5 or A7 regions contained double-labeled cells only very rarely. Within the PVH, the distribution of cells infected by the two recombinants was completely separated. Although single-labeled cells were revealed in the autonomic-related dorsal and ventral parvocellular subdivisions, no co-localization of BDG and BDL markers was observed (Fig. 7A). GFP-staining originating from the bone marrow infection displayed contralateral dominance, while β-galactosidase staining following muscle infection appeared mainly on the ipsilateral side.

DISCUSSION

The present study revealed, for the first time, the web of central autonomic premotor neurons that is in the position to regulate the bone marrow. We infer the brain areas found infected following virus inoculation into the femoral bone marrow belong to a hierarchically organized circuit that provide efferent inputs to the sympathetic preganglionic neurons (SPN), which may regulate the hematopoiesis, the blood supply and the immune cell function in the bone marrow (Fig. 8).

Methodological considerations

Genetically modified PRV strains, BDG and BDL were used as trans-synaptic tracers (Boldogkoi et al., 2002), to localize peripheral and CNS sites involved in innervation of the femoral bone marrow.

Factors that affect the primary and transneuronal infections are (i) the injection volume, (ii) the titer of the virus, (iii) the number of nerve endings and (iv) axonal PRV receptors in a given structure (Card et al., 1999; Aston-Jones et al., 2001). In our pilot studies with these genetically modified viruses, when $10^8 - 10^9$ PFU/ml ($10^5 - 10^7$ injected virions) was injected into the bone marrow, the rate of infection was only 20–40%. To achieve productive in-
fections in all of our animals we have used higher virus titers ($1.5 \times 10^9$ PFU/ml, $3 \times 10^9$ injected virions) compared with other virus tracing studies (Jansen et al., 1997; Cano et al., 2001). However, our BDG and BDL virus recombinants used in these experiments are characterized with a reduced neuroinvasiveness due to the insertion of the reporter GFP or lacZ genes into the ASP region (Boldogkoi et al., 2002). These mutant viruses label central autonomic neurons in a slower and much more restricted (labeling fewer neurons) manner than the parent strain of PRV (Boldogkoi et al., 2002). Although the bone marrow is densely innervated by sympathetic fibers (Mach et al., 2002) and high density of terminal fields allows faster achievement of the threshold for the onset of the virus replication within the neurons (Card et al., 1999), there is no information about the number of axonal PRV receptors in the bone marrow.

To rule out the possibility that our virus injections accidently infected the surrounding tissues, we have compared infection patterns following periosteal and intramuscular virus injections. From the periosteum the infection rate was very low, and the PRV-positive neurons were restricted to the brainstem. Intramuscular injections of the virus revealed special motor-associated structures in the forebrain. However, the distribution of virus-labeled cells in brainstem and some diencephalic structures was similar to that of the bone marrow infections, suggesting that the same hypothalamic/medullary neuron population provide efferents to the bone marrow and to the femoral muscles. Timed double-infection studies, however, revealed that only a small subpopulation of these neurons was co-infected with both recombinant viruses. In addition, several infected, large motoneurons were revealed in the ventral horn of the spinal cord following intramuscular virus injection. This may indicate monosynaptic transport of the viral particles from the place of inoculation and results in faster delivery of the virus to the brainstem than in case of virus injection into the bone marrow. Since muscular elements also contain dense network of blood vessels as well as dense sympathetic vascular innervation, these results indicate that the majority of labeled neurons following virus-injection into the bone marrow are not identical with those innervating muscular elements and may represent both perivascular and parenchymal tissues which are characteristic to the bone marrow. In addition, double-labeled neurons may be the part of a command autonomic-motor system, whose existence was revealed recently by two different groups (Kerman et al., 2003; Krout et al., 2003).

Similarly to other reticular organs, the bone marrow is also innervated by sympathetic nerves and contains blood vessels, which have their own autonomic innervation. The nerve fibers that enter the femur through the nutrient foramina are components of the lumbar plexus and also contain the sympathomotor neurons that contact the blood vessels in the bone marrow. In addition, the walls of the sinusesoids in the bone marrow are also innervated and TH as well as NPY-positive nerve fibers form free linear profiles among hematopoietic and lymphopoietic cells that are not associated with the vasculature (Calvo, 1968; Felten et al., 1985, 1988; Tabarowski et al., 1996; Mach et al., 2002). Afferent (sensory) nerves containing SP and CGRP also innervate the precursor and accessory cells in the bone marrow (Broome and Miyan, 2000; Broome et al., 2000) and in lesser part also are responsible for the delivery of the virus to the spinal cord. Some uncertainty still exists, however, as to whether viral labeling seen after inoculation of the bone marrow originates from the supplying bone marrow blood vessels, parenchyma, or both. Recently available track-tracing strategies in general, and virus tracing studies in particular, do not differentiate between perivascular and parenchymal innervation, which is an inherent confounding factor in all PRV-based retrograde transneuronal virus tracing studies (Cano et al., 2001). However, the blood supply of the bone marrow is tightly associated with its hematopoietic function and noradrenaline, released non-synaptically from any sympathetic axon terminals, affects immune cells in their close proximity (Elenkov et al., 2000).

Dissection of the sciatic, femoral and obturator nerves that was assigned to separate peripheral fibers of the first-order neurons, was effective in preventing viral labeling in the CNS only in half of the operated animals. Despite our best efforts, because of the large number of small accessory branches from the lumbar plexus innervating the femoral region including the bone marrow, complete denervation is difficult to be carried out. However, the marked reduction of the infected neurons in animals with incomplete nerve transection indicates that major branches of sciatic, femoral and obturator nerves have significant contribution to the innervation of the bone marrow.

The neuronal circuit that innervates the femoral bone marrow

The genetically modified virus strains used in these experiments retain their ability to travel primarily in a retrograde fashion (Boldogkoi et al., 2002). Therefore, neurons labeled from the femoral bone marrow were infected retrogradely via paravertebral ganglia and preganglionic neurons in the spinal cord. Lumbar ganglionic cells of the sympathetic chain were thereby considered to be the first virus-labeled neurons following BDG inoculation into the femoral bone marrow. Because the number of infected cells in the lumbar ganglia was significantly higher than the number of labeled neurons in the spinal cord at 4 days and the infection of the lumbar sympathetic chain was evident in each animal examined, we did not investigate earlier survival time points. In addition, our results are consistent with the physiological observation that the stimulation of the lumbar sympathetic trunks resulted in the release of reticulocytes and neutrophils into the circulating blood (Depace and Webber, 1975). SPN are found in four different compartments of the spinal cord: in the IML, the CAN, the lateral funiculus and intercalated nucleus, whose contribution to innervation of various organs is different (Strack et al., 1988, 1989). Following infection of the bone marrow, the majority of primarily infected PRV-positive cell bodies were found in the IML and to a lesser extent in the CAN,
suggesting that these cell groups give rise to preganglionic fibers to the bone marrow.

Neurons in the sympathetic chain and preganglionic neurons in the spinal cord are topographically organized with respect to their tissue/organ targets. Indeed, the focus of the PRV-infected neurons shortly after the inoculation of the bone marrow was found in the lower thoracic and upper lumbar segments.

In contrast to the strict topographical organization of the circuit in the spinal cord, the autonomic premotor areas impinge on the preganglionic cell groups are widely distributed throughout the neuraxis. Consistent with previous studies that also used retrogradely transported viruses for tracing autonomic circuits regulating various peripheral organs, two main brain autonomic pre-motor regions were found labeled in our study: the brainstem and the hypothalamus (Fig. 8).

Direct projections to IML arise from several brainstem cell groups, including the A5 group in the ventrolateral pons-medulla, the Kölliker-Fuse nucleus in the parabrachial complex, A2 noradrenergic neurons in the caudal part of the NTS, the ventromedial medulla with the nucleus raphe magnus, and the C1, C3 adrenergic cell groups in the rostral ventrolateral medulla (Saper, 1995).

Relatively high number of neurons in the A5 cell group became infected at early time points following virus inoculation of the bone marrow, identifying this cell cluster as an important source of afferent inputs to the bone marrow-regulating preganglionic neurons. The A5 group represents a special subgroup of noradrenergic neurons (Everitt et al., 1984) with high terminal density at the SPNs in the IML cell column as well as at the sensory projecting neurons in the dorsal horn.

Neurons within the LC and subcoeruleus area also contribute to the innervation of the bone marrow, since these areas always contained PRV-infected neurons following virus inoculation to the femoral bone marrow. Retrograde tracing experiments revealed that noradrenergic axons from these neurons descend in the pontomedullary reticular formation and the lateral spinal funiculus and innervate the spinal cord. Large multipolar cells in the ventral part of the LC and the subcoeruleus area project to the spinal cord (Loughlin et al., 1986a,b). LC neurons are thought to regulate states of attention and vigilance as well as activity of the sympathetic nervous system (Nestler et al., 1999) and might synchronize behavioral states with autonomic functions.

C1 adrenergic cell group in the rostral ventrolateral medulla was also shown to send descending efferent projections to the preganglionic neurons of the spinal cord (Ross et al., 1981, 1984b; Tucker et al., 1987). Sympathoexcitatory neurons in the C1 cell group play an important role in the reflex adjustments of sympathetic activity to internal and external stimuli, while non-adrenergic cells in the C1 area are rather involved in the maintenance of basal sympathetic tone (Morrison et al., 1988; Guyenet et al., 1989; Schreihofer et al., 2000). Our present virus-tracing experiment identified catecholaminergic neurons in the A5, LC, C1 and C3 areas as 3rd order elements in the multisynaptic circuit regulating the bone marrow. Most PRV tracing studies revealed infected cells in these areas following peripheral virus-inoculation, which may be involved in the control of local parenchymal elements and/or the general vascular sympathetic tone in the target organ. Despite the possible representation of both (perivascular and parenchymal) innervation in the labeling pattern in case of the bone marrow, functionally, these processes are tightly coupled. For example, several observations have shown that the blood flow in the bone marrow is modulated according to the hematopoiesis turnover rate (Iversen et al., 1992, 1993). In addition, the modulation of the autonomic responses by specified neurons of the A5, LC, C1 and C3 cell groups may result in altered non-synaptic noradrenaline release to hematopoietic cells in the bone marrow, which mechanism has been found to be very similar in case of other immune organs such as the spleen and the thymus (Elenkov et al., 2000).

Other cell groups in the rostral medial medulla (lateral and dorsal paragigantocellular nucleus, gigantocellular reticular nucleus and nucleus raphe magnus) were also identified as parts of the brainstem autonomic circuit regulating the bone marrow. Early appearance of PRV-ir in these brain regions following bone marrow infection is compatible with their direct input to the spinal preganglionic neurons. Such connections have already been described by monosynaptic tracing methods (Ross et al., 1984a; Hermann et al., 2003; Babic and Ciriello, 2004).

In the hypothalamus two major regions were revealed, which provide efferents to the bone marrow: the paraventricular nucleus and the lateral hypothalamic area. Retrogradely transported virus tracers consistently labeled both areas.

Descending projections originating from the ventral medial, dorsal and lateral paraventricular subdivisions of the PVH were identified as sources of afferent inputs to the spinal preganglionic neurons innervating the bone marrow. These autonomic premotor neurons expressed virus proteins early on the progression of the infection, and their distribution within the PVH was similar to that reported previously using various classic retrograde tracer injections into the IML of the spinal cord (Saper et al., 1976; Zhang et al., 2000).

About one fourth of these autonomic projection neurons in the PVH was identified to express oxytocin, corticotropin-releasing hormone, vasopressin, somatostatin, or met- and leu-enkephalin (Swanson and Sawchenko, 1980; Sawchenko and Swanson, 1982; Cechetto and Saper, 1988). In addition, other transmitters such as angiotensin II, dopamine and neurotensin were also found in this cell cluster, however the whole list of neuroactive substances, which are contained within paraventriculo-spinal pathway remains to be ascertained.

In addition to the autonomic projection neurons in the PVH, later on the infection progression (6 days), some PRV-positive cells were detected in the hypophyseotropic paraventricular and magnocellular compartments as well. Morphological and electrophysiological studies already suggested interactions between parvo- and magnocellular
neurons within the PVH (Leranth et al., 1983). Our recent findings also confirm this connection that may function to coordinate autonomic-and neuroendocrine responses to various challenges.

The lateral hypothalamic area also sends direct projections to the thoracolumbar SPNs in the IML cell column of the spinal cord (Cechetto and Saper, 1988; van den Pol, 1999). Orexinergic fibers, originating in the lateral hypothalamic area were found around the SPNs (van den Pol, 1999), where expression of both types of orexin receptors was confirmed (van den Top et al., 2003). Electrophysiological studies also support the direct excitatory and synchronizing role of orexin A and orexin B on the SPNs that results in augmentation of sympathetic outflow (Dun et al., 2000; Antunes et al., 2001; van den Top et al., 2003). Recently, some of these orexinergic neurons in the lateral hypothalamic area were identified as central sympathetic command neurons that provide input simultaneously to diverse sympathetic targets (Geerling et al., 2003).

In addition to these sites, some labeled cells were consistently found in the medial retrochiasmatic area and later on in the arcuate nucleus. These neurons also give rise direct descending projections to the SPNs in the spinal cord and may be involved in mediating leptin’s effect on stimulating the sympathetic outflow (Elias et al., 1998).

The circuit is complicated by the fact that in addition to their direct projections to the spinal cord, most of the infected hypothalamic areas innervate brainstem areas involved in the sympathetic regulation. Among others, such indirect projections were described between the PVH and A5, PAG, LC, and the ventrolateral medulla (Swanson and Sawchenko, 1983; Byrum and Guyenet, 1987; Shafton et al., 1998). The lateral hypothalamic area also sends efferent fibers to the rostral ventrolateral medulla especially its C1 region (Cechetto and Chen, 1992).

Medullary and hypothalamic autonomic premotor neurons receive various inputs from limbic and cortical areas. Among those brain sites that became infected at longer survival time after virus inoculation of the bone marrow were: the BNST, the amygdala, insular, piriform and other cortical areas. This complex cortical and subcortical network may initiate autonomic responses to emotional challenges such as stress, fear or anxiety. Westerhaus and Loewy (2001) reported distinct pattern of cortical representation of the sympatho-adrenal, cardioboth sympathetic and gastroenterosympathetic systems. In assessing the possible role of various cortical areas in the autonomic regulation of the bone marrow, we suppose that insular and piriform cortices are involved in the sympathetic control, and indicate a weaker contribution of the infralimbic cortex. Infected cells in these cortical areas are not considered to be specifically related to the regulation of the bone marrow, but may be involved in the modulation of the autonomic processes that give rise to the homeostatic alteration of the hematopoietic environment. Despite the possible involvement of these neuroanatomical connections in the regulation of the bone marrow, we did not identify unique cell groups that are exclusively characteristic to the bone marrow innervation.

**Functional considerations**

Within the bone marrow, efferent noradrenergic/peptidergic (NPY-containing) fibers innervate blood vessels and are found directly adjacent to the stromal and hematopoietic cells. This anatomical scenario allows coordinated regulation of the blood flow, the immune cell development and function in the bone marrow. The effect of the autonomic nervous system on the immune cells is mediated by norepinephrine released locally from non-synaptic varicosities while neurotransmitter receptors are present on several hematopoietic cell types.

Several lines of evidence support the importance of the neural control of the bone marrow. (i) Surgical denervation of the femur in mice results in a decreased number of cells in the bone marrow with a concomitant increase of circulating progenitors (Afan et al., 1997). (ii) Patients with complete spinal cord injury display impaired progenitor growth and reduced lymphocyte activity compared with normal controls (Iversen et al., 2000). (iii) Local sympathetic nervous activity in the early phase of stimulation of bone marrow-derived dendritic cells influence the T helper Th1 and Th2 balance (Maestroni, 2000b). (iv) Increased sympathetic load during chronic social stress significantly alters leukocyte trafficking and lead to a gradual redistribution of immune cells in bone marrow, peripheral blood and spleen (Engler et al., 2004).

The basic organization of the central circuit outlined here to innervate the bone marrow shows similarities with those that have been described to provide sympathetic control to various peripheral targets i.e. adrenal (Strack et al., 1989); brown and white adipose tissues (Bamshad et al., 1998, 1999); kidney (Schramm et al., 1993); mammary gland (Gerendai et al., 2001); pancreas (Janssen et al., 1997); testis (Gerendai et al., 2000) or other immune organs such as spleen (Cano et al., 2001).

Double virus tracing strategies revealed a common set of sympathetic command neurons in the brainstem and in the hypothalamus that provide a dual input to the SPNs regulating cardiac and adrenomedullary functions (Janssen et al., 1995). In addition, neurons were recently identified that are responsible for coordinated and complementary activation of motor and autonomic outflows (Kerman et al., 2003).

Functional anatomical studies also identified autonomic command neurons. It has been revealed that only a relatively small subset of hypothalamic and medullary autonomic premotor neurons became activated during immune (Zhang et al., 2000) or metabolic (Elias et al., 1998) challenges. These neurons through their widespread connections to the preganglionic neurons at different levels of the spinal cord are in the position to coordinate sympathetic outflow to relevant targets. In addition, the command neurons through their rich intrahypothalamic and intramedullary connections might be involved in the adjustment of sympathetic activation to ongoing behavioral and endocrine responses.

Although there are overlaps between CNS structures that provide relevant efferents to the spleen, the thymus...
and the bone marrow, it is not yet clear whether "immuno-
sympathetic" command neurons may exist within the rat
brain to orchestrate neuroimmune functions.

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