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Anatomical study of serotonergic innervation and 5-HT_{1A} receptor in the human spinal cord

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Serotonergic innervation of the spinal cord in mammals has multiple roles in the control of motor, sensory and visceral functions. In rats, functional consequences of spinal cord injury at thoracic level can be improved by a substitutive transplantation of serotonin (5-HT) neurons or regeneration under the trophic influence of grafted stem cells. Translation to either pharmacological and/or cellular therapies in humans requires the mapping of the spinal cord 5-HT innervation and its receptors to determine their involvement in specific functions. Here, we have performed a preliminary mapping of serotonergic processes and serotonin-1A (5-HT_{1A}) receptors in thoracic and lumbar segments of the human spinal cord. As in rodents and non-human primates, 5-HT profiles in human spinal cord are present in the ventral horn, surrounding motoneurons, and also contact their presumptive dendrites at lumbar level. 5-HT_{1A} receptors are present in the same area, but are more densely expressed at lumbar level. 5-HT profiles are also present in the intermediolateral region, where 5-HT_{1A} receptors are absent. Finally, we observed numerous serotonergic profiles in the superficial part (equivalent of Rexed lamina II) of the dorsal horn, which also displayed high levels of 5-HT_{1A} receptors. These findings pave the way for local specific therapies involving cellular and/or pharmacological tools targeting the serotonergic system.

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Spinal cord injury yields massive motor and sensory deficits in mammals, due to the lesion of descending and ascending fiber tracts. More specifically, severe thoracic lesions result in permanent paralysis of hind limbs in rodents, which have been extensively used as models for spinal cord injury in humans. Thanks to these animal models; locomotion has been found to result from the activation of a central pattern generator (CPG; for review see Grillner¹). This CPG has been described in many species, including lampreys,² it has been located in the lumbar cord in rats³ and has been suspected to be present in humans.⁴ Activation of the CPG can be achieved by the descending serotonin (5-HT) system in the lampreys⁵ and the rats.⁶ Indeed, the spinal cord is profusely innervated by 5-HT in the rats^{7,8} and the monkeys.⁹ Moreover, it has been shown that reflex locomotion could be restored in the paraplegic rats after complete thoracic spinal cord transection by transplantation of fetal 5-HT neurons below the level of the section. Furthermore, chronic microdialysis of the rat spinal cord has revealed a massive release of 5-HT during treadmill-driven locomotion.¹⁰ Thus, 5-HT appears to have a central role in motor control throughout phylogenies.

Similarly, the control of the genito-urinary tract has been found to be largely mediated by 5-HT^{11–13} as well as the

central command for ejaculation, which has been located at thoraco–lumbar and lumbo–sacral levels of the spinal cord.¹⁴

Interestingly, both locomotor and ejaculatory controls have been found to be mediated through the activation of 5-HT₁ receptors.^{11,15–17} Moreover, spinal control of nociception involves serotonergic projections to the dorsal horn of rats through 5-HT₁ receptors.^{18–20}

Experimental models may serve as a basis for therapeutic strategies aiming at repairing the injured spinal cord in humans. For instance, translation to clinics of repair of the locomotor drive requires that both the CPG and its serotonergic input are present in humans. Little is known about the serotonergic innervation of the human spinal cord²¹ and about the topography of 5-HT receptors.²² Such studies require a perfect preservation of the tissue such as what is achieved in experimental animals with a transcardiac perfusion of fixative. Necropsy pieces of human spinal cord are far from optimal in this respect.

In the course of an European Program (STREP RESCUE) aiming at studying the repair of spinal cord with stem cells, we had privileged access to spinal cords of brain-dead patients who agreed to donate their bodies for therapeutic and scientific purposes.²³ Due to the collaboration of clinical staff, dissection and fixation of specimen could be carried in the shortest delays compatible with the ethics rules. The objective

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Abbreviations: 5-HT, serotonin or 5-hydroxytryptamine; 5-HT_{1A}, serotonin-1A receptor; CPG, central pattern generator; T9–T10, thoracic levels 9 and 10; L3–L4, lumbar levels 3 and 4; PBS, phosphate buffered saline; BSA, bovine serum albumin; 8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; IML, intermediolateral; DH, dorsal horn; VH, ventral horn

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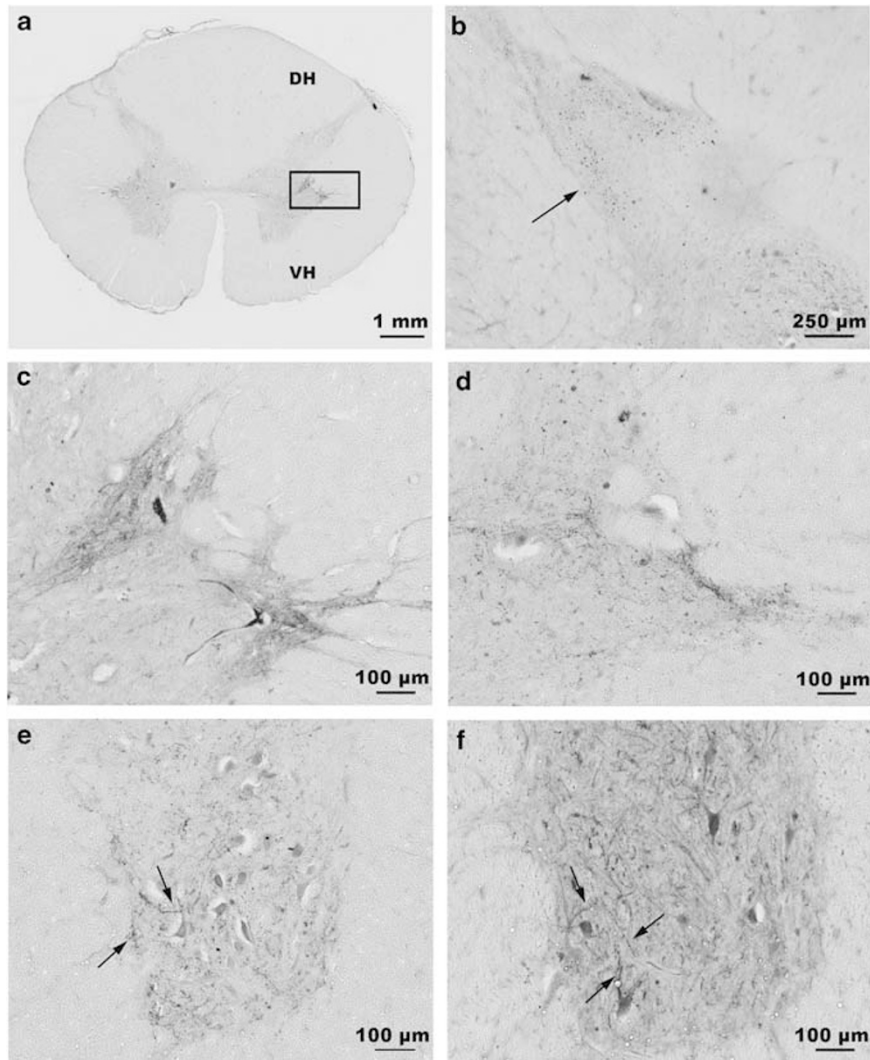


Figure 1 5-HT expression in adult human thoracic spinal cord. (a) Low-power photograph of a cross-section of the thoracic spinal cord illustrating the general topography of 5-HT projections. (b) In the dorsal horn, 5-HT appears as punctuate profiles (arrow) in a horseshoe-shaped area corresponding to the lamina II of Rexed. (c) In the IML area, 5-HT profiles concentrate around two groups of presumptive preganglionic neurons of the lateral most part of the IML. (d) In another specimen from another subject, the organization of the projection is slightly different with only one group of projections. (e) In the ventral horn, large neurons (arrows) and their dendrites are outlined by immunoreactive profiles. (f) In another subject, large dendrites are similarly labeled (arrows). DH: dorsal horn, VH: ventral horn. Box 1 corresponds to the area presented in (c). Scale bars: (a): 1 mm; (b): 250 μ m; (c–f): 100 μ m

of the present report is to give a preliminary description of the anatomy of serotonergic projections in the human spinal cord at thoracic and lumbar levels, together with the detection of serotonin-1A (5-HT_{1A}) receptors.

Results

Serotonergic innervation. At thoracic level, immunostaining of 5-HT appears clearly limited to three regions of the gray matter, namely the superficial part of the dorsal horn, the intermediolateral (IML) area and the ventral horn around motoneurons (Figure 1a). In the dorsal horn, 5-HT immunostaining appears as punctuate deposits strictly limited to lamina II of Rexed (Figure 1b, arrow) and Clark column. In the IML area, punctuate labeling and short varicose profiles are apparent in the lateral most part of the

gray matter, surrounding putative preganglionic neurons, as well as in a thin line extending to the central canal (Figures 1c and d). The ventral horn is by far the most intensely stained, with varicose profiles surrounding closely the perikarya and main dendrites (Figures 1e and f; arrows) of large cells, presumptive motoneurons.

At lumbar level, immunostaining of 5-HT appears most intense in the ventral horn, with more discrete profiles in the dorsal horn and the IML area (Figure 2a). In the dorsal horn, besides some punctuate deposits in layer II (not shown), a thin plexus of varicose fibers was apparent in the most superficial part of the gray matter, just below the pial surface (Figure 2b, arrow). In the IML area (Figures 2c and d), punctuate deposits and varicose fibers were apparent in the lateral most part. In the ventral horn, an accumulation of immunoreactive profiles clearly outlined a medial and a lateral group of motoneurons (Figures 2e and f). Quite often, immunoreactive

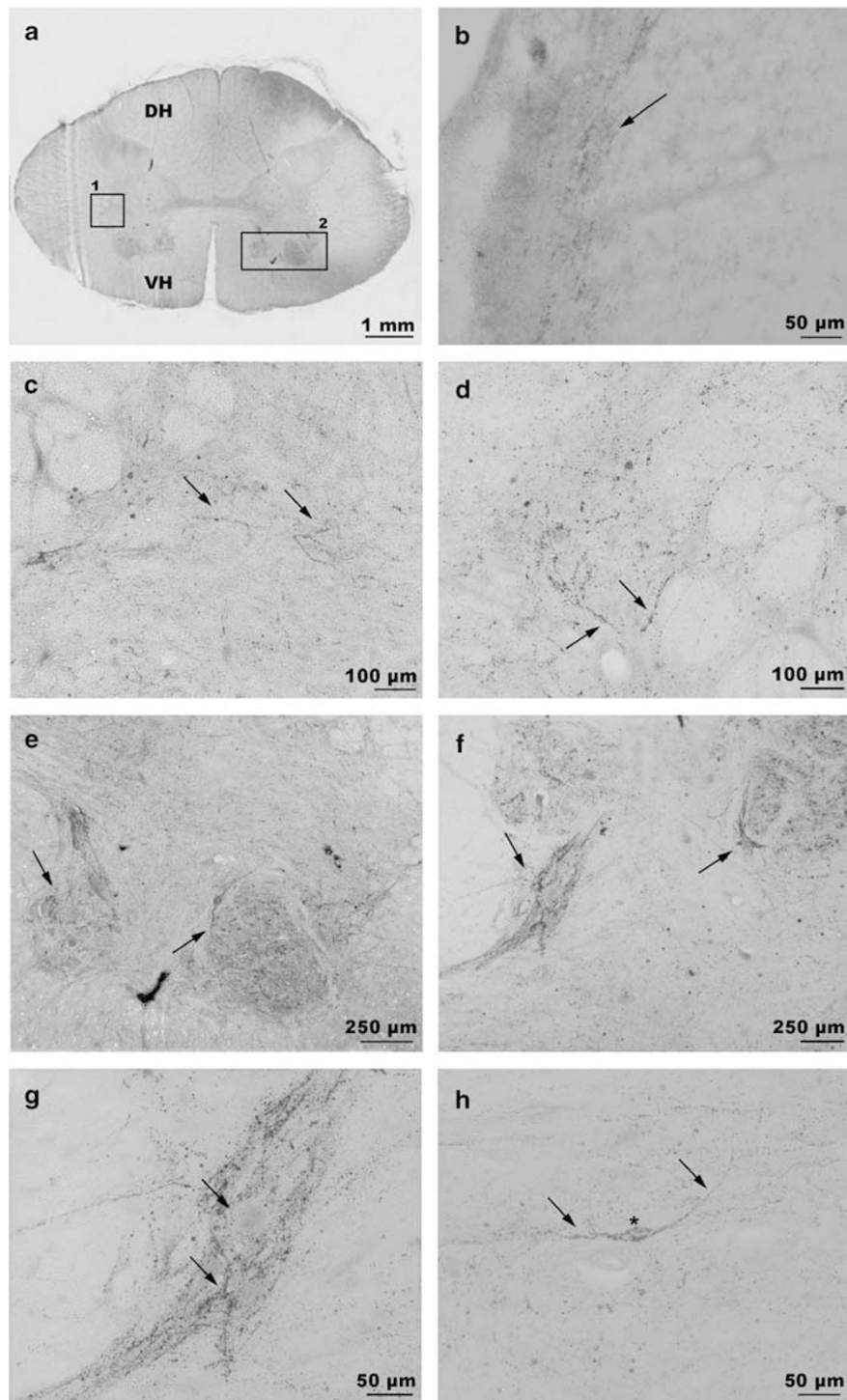


Figure 2 5-HT expression in adult human lumbar spinal cord. (a) Low-power picture of a cross-section of the lumbar spinal cord illustrating the general topography of 5-HT projections. (b) A thin plexus of 5-HT profiles (arrow) is present just beneath the pia. (c) In the IML area, thin profiles outline the perikarya and dendrites of presumptive preganglionic neurons (arrows). (d) In another specimen from another subject, several dendrites are also outlined (arrows). (e) In the ventral horn, two groups of large neurons are endowed by numerous immunoreactive profiles (arrows). (f) In another specimen from a different subject, again two groups of neurons are distinctly labeled (arrows). (g) On a higher magnification from (f), a large neuron and its primary dendrite are delineated by immunoreactive processes (arrows). (h) In the ventral horn, some medium-sized bipolar neurons are surrounded by 5-HT profiles (asterisk: perikarya; arrows: dendrites). DH: dorsal horn, VH: ventral horn. Box 1 corresponds to the area presented in (c); Box 2 corresponds to the area presented in (e). Scale bars: (a): 1 mm; (b, g and h): 50 μ m; (c and d): 100 μ m; (e and f): 250 μ m

profiles outlined perikarya and proximal dendrites of presumptive motoneurons (Figure 2g, arrows) and more rarely dendrites of small bipolar neurons (Figure 2h, arrows).

5-HT_{1A} receptors. Immunodetection of 5-HT_{1A} receptors at thoracic level (Figure 3a) evidences at low magnification a dense layer of immunoreactive profiles in Rexed lamina II of

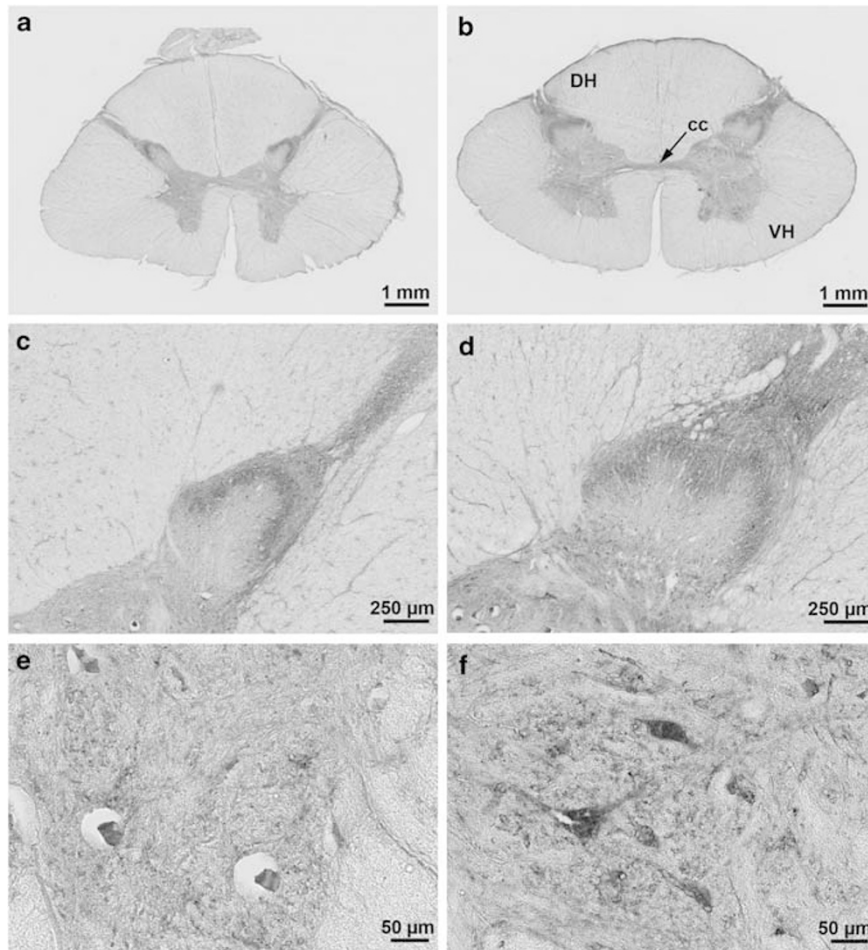


Figure 3 5-HT_{1A} expression in adult human spinal cord. (a, c and e) Adult human thoracic spinal cord. (b, d and f) Adult human lumbar spinal cord. (a) Low-power photograph of a cross-section of the thoracic spinal cord where stands out the horseshoe-shaped condensation of 5-HT_{1A} receptors in the dorsal horn. (c) High magnification of the dorsal horn. See for comparison Figure 1b. (e) A few immunoreactive profiles are detected in the ventral horn. (b) In this low-power view of the lumbar cord, the superficial part of the dorsal horn appears also heavily labeled. (d) Higher magnification of the dorsal horn. (f) In the ventral horn immunoreactive profiles appear more numerous than at thoracic level (4E). cc, central canal; DH, dorsal horn; VH, ventral horn. Scale bars: (a and b): 1 mm; (c and d) 250 μ m; (e and f) 50 μ m

the dorsal horn. At higher magnification (Figure 3c), these deposits match perfectly with those of 5-HT terminals in the same region (Figure 1b). In the ventral horn, rare punctuate deposits can be found on the dendrites and soma of motoneurons (Figure 3e).

At lumbar level, again, a dense layer of immunoreactive profiles is apparent in Rexed lamina II (Figures 3b and d), which matches the pattern of 5-HT immunoreactive profiles of Rexed lamina II (Figure 2a), but which is not in register with the submeningeal fiber plexus (Figure 2b). At variance with thoracic level, numerous immunoreactive profiles are found in the ventral horn on motoneurons soma and dendrites (Figure 3f). To better evaluate this difference, and for each cases, we carried out a semiquantification for the presence of 5-HT_{1A} receptors in motoneurons as well as 5-HT_{1A} profiles surrounding motoneurons, and compared lumbar and thoracic segments. In the lumbar segment, $94 \pm 11\%$ of motoneurons expressed the 5-HT receptor type 1A (5HT_{1A}) and $82 \pm 19\%$ of motoneurons were surrounded by at least one 5-HT_{1A} fiber as compared with $44 \pm 16\%$ and $19 \pm 17\%$, respectively, in the thoracic segment.

Discussion

Methodological considerations: technical conditions for the optimal preservation of human spinal cord samples have been obtained by lowering body temperature and maintaining blood circulation until 4 h before dissection.²³ Following immersion fixation, further processing was carried in the same conditions as those used in the rodent and primate spinal cord tissues.^{9,24} Thus, comparison of the anatomical organization of serotonergic projections and 5-HT_{1A} receptors in the spinal cord at thoracic and lumbar levels can be performed with a good confidence as to the quality of the immunodetection. However, the heterogeneity of the samples due to different conditions of death and processing (despite best efforts for standardization) precluded any quantitative appraisal.

In the dorsal horn, the specific projection in lamina II is in register with the equivalent structures in the rodents and the primates.^{9,24} There is a perfect matching with the mapping of 5-HT_{1A} receptors, as seen in the present study as well as in the radio-autographic detection with tritiated DPAT²² in the aged humans and the adult rats,⁷ and with specific antibodies.²⁵

There is ample indirect evidence that this projection is involved in the control of the transmission of nociceptive signals both in animals,²⁶ and humans.²⁷ Moreover, the involvement of 5-HT₁ receptors in this control has been well documented in humans.¹⁸ However, 5-HT release in this region of the spinal cord may not be related only to nociception, as it has been shown with microdialysis in the rat cord that it was also related to locomotor exercise.²⁸ In rats, most of these projections to the superficial layers of the dorsal horn occur through a non-synaptic modality,²⁹ and the so-called volume transmission is, at least, partly mediated by receptors present on astrocyte membranes. One could hypothesize that this non-synaptic projection corresponds to the nociceptive pathway, whereas the minority of synaptic contacts, present mostly in deepest layers, could be involved in locomotion. More studies are thus needed on human spinal cord samples to decide whether the ultrastructural organization is similar to that of the rat.

One unexpected finding in the human is the presence of a narrow bundle of thin serotonergic immunoreactive profiles at lumbar level coursing at the surface of the dorsal horn, just below the meninges. The absence of a significant detection of 5-HT_{1A} receptors at this level would suggest that another subtype of receptor is operating at that level.

In the IML region, again the architecture of the 5-HT projection is similar to that found in other species.³⁰ Briefly, at thoracic level, this projection is organized as a continuous bundle of thin neurites and punctuate deposits extending laterally in-between the central canal and the lateral most part of the gray matter, where it spreads in a fan-like fashion around presumptive preganglionic neurons. In the rat, there is little evidence of a similar organization at lumbar level, at variance with the human where we have seen here that a similar architecture exists, with however a more discrete projection. This could suggest that many preganglionic neurons are also present throughout lumbar level in the humans. Interestingly, we have not detected any significant expression of 5-HT_{1A} receptors in this area, at both levels.

Control of sexual behavior through 5-HT has long ago been described in rats.³¹ Since then, it has been shown that spinal 5-HT_{1A} receptors are involved in this function^{12,32} in both rats and humans. As we have not detected these receptors in the IML column of the human spinal cord, it is thus likely that this function is controlled by specialized motor nuclei of the lumbar cord such as Onuf's nucleus⁹ (see below, ventral horn 5-HT innervation). Similarly, the control of micturition through 5-HT_{1A} receptors in the thoracic and lumbar cord is likely to occur through the ventral horn.³³ Future studies will have to identify the type of 5-HT receptors present in the human IML region and to determine their function through comparison of preclinical and clinical data.

We have found here that, as in rodents³⁴ and non-human primates,^{9,31} the ventral horn of the human spinal cord is profusely innervated by 5-HT terminals, some of which are apposed on presumptive postsynaptic structures. At variance with Marlier *et al.*⁷ in rats and Laporte *et al.*²² in humans, we have found a significant presence of 5-HT_{1A} receptors in the ventral horn, mostly at lumbar level. This discrepancy may be due to the different techniques used (immunodetection *versus* binding) or to the fact that Laporte *et al.*²² analyzed only aged

human spinal cords. Ventral horn 5-HT innervation has been long ago implied in the control of locomotion.^{35,36} Further studies with microdialysis¹⁰ and transplantation of 5-HT neurons³ have substantiated this point, and the latter has shown that 5-HT input to the CPG of locomotion located at lumbar level in the rat was mandatory for locomotion. In humans, Dimitrijevic *et al.*⁴ and Minassian *et al.*³⁷ have further found that the electrical stimulation of the lumbar cord elicited alternative leg movements in paraplegic patients as a proof of concept of the existence of the CPG in humans. Interestingly, Antri *et al.*¹⁵ and Guertin *et al.*³⁸ found that in spinal-cord-transected rats, treatment with the 5-HT_{1A} agonist 8-OH-DPAT elicited reflex locomotion on a treadmill. Thus, one can hypothesize that these receptors in the human lumbar spinal cord could take part in the triggering of the CPG in humans.

These preliminary data on the functional anatomy of serotonergic projections in the human spinal cord not only validate some translations from preclinical models to the interpretation of clinical data, but also show that the architecture of the projections is largely similar with those of rodents and non-human primates, and thus validate the use of these species for preclinical models. Some subtle differences await further studies to evaluate their importance.

They also pave the way for a more accurate design of pharmacological, cellular or gene therapies in spinal cord injured patients. They should contribute to a better definition of clinical trials aiming at restoring pain control, genito-urinary functions and ultimately locomotion in paraplegic patients.

Materials and Methods

Human spinal cord samples. Human thoracic (T9–T10) and lumbar (L3–L4) spinal cords were obtained from nine brain-dead organ-donor patients (17–74 years old, mean age: 52 ± 18; 2 females and 7 males) under the approval of the French Institution for Organ Transplantation. Patients died from stroke ($n=2$), ruptured aneurysm ($n=4$) or traffic accident ($n=3$). Body temperature was lowered with ice, and blood circulation and ventilation were maintained until 4 h before spinal cord removal. This short-time interval permitted good preservation of the tissue, as indicated by the near absence of morphologically altered cells at both light and electron microscopic levels.²³ After organs removal for therapeutic purposes, T8–L5 vertebral bloc was isolated and spinal cord segments were removed and immediately processed for histology.

Histology and immunodetection. For light microscopy, spinal cord samples were placed in refrigerated paraformaldehyde 4% for 24 h, cryoprotected (30% sucrose 24 h at 4 °C), frozen and stored at –80 °C. 'Floating' 40-μm thick cryosections of thoracic and lumbar segments were collected for all of the nine cases and placed in phosphate buffered saline (PBS) in a 24-well plate. Section were washed twice in PBS (5 min), treated for 30 min in PBS containing lysine (20 mM, pH7.4) and for 20 min in 3% H₂O₂. Tissue sections were then permeabilized and blocked for 30 min with PBS containing bovine serum albumin (BSA, 10%) and Triton X-100 (0.1%) and incubated over night at 4 °C with primary antibodies.

For 5-HT_{1A} semiquantification three sections of both thoracic and lumbar segments were analyzed.

Antibodies characterization. Rabbit anti-5-hydroxytryptamine (1:30 000; Immunotech, Marseille, France) and rabbit anti-5-HT_{1A} (1:500; a gift from Dr. Michel Hamon INSERM U677 Neuropsychopharmacology Unit, Paris, France) primary antibodies were used. The preparation and characterization of rabbit polyclonal antibody intended for the specific immunocytochemical visualization of the 5-HT_{1A} receptor in rat CNS tissue has already been described in detail.²⁵ This antibody was raised against synthetic peptides corresponding to residues 243–268 from the predicted amino-acid sequences of the third inner cytoplasmic loops of the two receptors in the rat. This particular sequence was chosen for its high selectivity

among other receptors of the protein G-coupled superfamily. The preparation and characterization of rabbit polyclonal antibody intended for the specific immunocytochemical visualization of the 5-HT have already been described in detail.²⁵ Specificity tests included pre-immune serum (no staining), cross-reactivity with chemically similar compounds (negligible), and adsorption with the 5-HT-BSA conjugate (no staining).²⁵ This antibody has been used and published in many studies with results consistent with the present ones.^{26,39}

Secondary peroxidase-conjugated antibodies (Immunotech) were used at a 1 : 500 dilution. The sections were scanned and analyzed (Nano Zoomer Digital Pathology System, Hamamatsu Photonics, Hamamatsu, Japan). The controls were done in the absence of the primary antibody and were negative in all cases (Supplementary Figure).

Upon inspection with the light microscope of hematoxylin- and eosin-stained cross-sections, the preservation of the tissues appeared adequate in all the specimens, with an easy identification of neurons and glial cells in the gray matter, and of glial cells and myelinated fibers in the white matter.

Conflict of interest

The authors declare no conflict of interest.

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