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TRANSMITTER CONTENT, ORIGINS AND CONNECTIONS OF AXONS IN THE SPINAL CORD THAT POSSESS THE SEROTONIN (5-HYDROXYTRYPTAMINE) 3 RECEPTOR

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Abstract—Recent evidence suggests that serotonin has pronociceptive actions in the spinal cord when it acts through 5-hydroxytryptamine (5-HT)$_3$ receptors. Cells and axon terminals which are concentrated in the superficial dorsal horn possess this receptor. We performed a series of immunocytochemical studies with an antibody raised against the 5-HT$_{3A}$ subunit in order to address the following questions: 1) Are axons that possess 5-HT$_3$ receptors excitatory? 2) Are 5-HT$_3$ receptors present on terminals of myelinated primary afferents? 3) What is the chemical nature of dorsal horn cells that possess 5-HT$_3$ receptors? 4) Do axons that possess 5-HT$_3$ receptors target lamina I projection cells?

Approximately 45% of 5-HT$_{3A}$ immunoreactive boutons were immunoreactive for the vesicular glutamate transporter 2 and almost 80% formed synapse-like associations with GluR2 subunits of the AMPA receptor therefore it is principally glutamatergic axons that possess the receptor. Immunoreactivity was not present on myelinated primary afferent axons labeled with the B-subunit of cholera toxin or those containing the vesicular glutamate transporter 1. Calbindin (which is associated with excitatory interneurons) was found in 44% of 5-HT$_{3A}$ immunoreactive cells but other markers for inhibitory and excitatory cells were not present. Lamina I projection cells that possessed the neurokinin-1 receptor were associated with 5-HT$_{3A}$ axons but the density of contacts on individual neurons varied considerably.

The results suggest that 5-HT$_3$ receptors are present principally on terminals of excitatory axons, and at least some of these originate from dorsal horn interneurons. The relationship between lamina I projection cells and axons possessing the 5-HT$_3$ receptor indicates that this receptor has an important role in regulation of ascending nociceptive information.

Key words: nociception, serotonin, glutamate, dorsal horn, confocal microscopy.

Serotonin (5-HT) acts through a variety of receptors which are present at both pre- and post-synaptic locations in the spinal cord and, for this reason, its actions of are complex. Although, traditionally 5-HT is associated with an antinociceptive action (e.g. see Millan, 2002) evidence is emerging which suggests that 5-HT also has pronociceptive actions when it acts through 5-HT$_3$ receptors (Green et al., 2000; Zeitz et al., 2002; Suzuki et al., 2002). Recent studies show that alterations in descending 5-HT control systems acting on 5-HT$_3$ receptors may contribute to the tactile allodynia that occurs in neuropathic pain states (Oatway et al., 2004; Suzuki et al., 2004) and clinical trials indicate that the 5-HT$_3$ receptor antagonist, ondansetron, is potentially a useful analgesic for the treatment of neuropathic pain (McCleane et al., 2003). As neuropathic pain is difficult to treat with standard analgesics, the role of 5-HT$_3$ receptors in pain states is clearly an issue of great importance.

The 5-HT$_3$ receptor is unique as it is a ligand-gated ion channel whereas all other 5-HT receptors that have been identified to date are metabotropic receptors. Two subunits of the receptor exist which have been termed 5-HT$_{3A}$ and 5-HT$_{3B.}$ All functional 5-HT$_3$ receptors are homomers of 5-HT$_{3A}$ subunits or heteromers of 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits (Davies et al., 1999; Dubin et al., 1999). However, there may be differences in the composition of receptors present in peripheral and central neurons. In the peripheral nervous system, heteromeric receptors are likely to be common whereas receptors in the CNS appear to be composed only of 5-HT$_{3A}$ subunits (Morales and Wang, 2002).

A number of studies have reported that 5-HT$_3$ receptors are located principally on axon terminals in the spinal cord (Hamon et al., 1989; Kidd et al., 1993; Kia et al., 1995; Zeitz et al., 2002; Maxwell et al., 2003). Binding sites and immunoreactivity for 5-HT$_3$ receptors in the dorsal horn decrease following rhizotomy but are not completely abolished (Kidd et al., 1993; Kia et al., 1995) and therefore, not all 5-HT$_3$ receptors are located on terminals of primary afferent axons. Indeed, immunocytochemical and *in situ* hybridization studies confirm the presence of cells in the dorsal horn that express the 5-HT$_3$ receptor (Tecott et al., 1993; Kia et al., 1995; Maxwell et al., 2003) and these are likely to be the source of axon terminals that survive rhizotomy. We showed previously (Maxwell et al., 2003), that a small proportion (approximately 10%) of axon terminals in lamina II which are immunoreactive for the 5-hydroxytryptamine$_{3A}$ receptor subunit (5-HT$_{3A}$-IR) are also immunoreactive for calcitonin gene-related peptide and hence may originate from the peptidergic subgroup of unmyelinated primary afferent fibers. Very few 5-HT$_{3A}$-IR

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Abbreviations: ChAT, choline acetyltransferase; CTb, B-subunit of cholera toxin; CVLM, caudal ventrolateral medulla; GAD, glutamate decarboxylase; MOR-1, μ opioid receptor; NK-1, neurokinin 1 receptor; NOS, nitric oxide synthase; PV, parvalbumin; SST$_{2A}$, somatostatin receptor 2A; TSA, tyramide signal amplification; VGLUT 1, vesicular glutamate transporter 1; VGLUT 2, vesicular glutamate transporter 2; 5-HT, 5-hydroxytryptamine, serotonin; 5-HT$_{3A}$-IR, immunoreactive for the 5-hydroxytryptamine$_{3A}$ receptor subunit.

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axons were double-labeled with the IB4 lectin which is a marker for another subset of unmyelinated primary afferent fibers. A study performed by Zeitz et al. (2002) similarly concluded that only a small proportion of peptidergic and IB4-binding dorsal root ganglion cells contain 5-HT_{3A} m-RNA. Zeitz et al. (2002) also reported that approximately 80% of cells containing 5-HT_{3A} m-RNA were immunoreactive for N52 which is a marker for neurofilaments associated with myelinated primary afferents. As the greatest density of 5-HT_3 immunoreactivity and binding sites is found in the superficial dorsal horn, it likely that many of these fibers belong to the Aδ group of primary afferents that terminate in lamina I. Almost none of the 5-HT_{3A}-IR axons we examined in our previous study (Maxwell et al., 2003) contained 5-HT or glutamate decarboxylase (GAD). Furthermore, 5-HT_{3A}-IR axons did not possess the glycine transporter 2 (D.J. Maxwell, unpublished observation). These findings suggests that the 5-HT_3 receptor does not function as an autoreceptor on descending 5-HT axons and is not present in inhibitory cells since GAD is the synthetic enzyme for GABA and GABA or a combination of GABA and glycine is present in most dorsal horn inhibitory cells (Todd and Spike, 1993). In addition, ultrastructural examination of 5-HT_{3A}-IR axon terminals shows that they form asymmetric synaptic junctions which are indicative of an excitatory action (Uchinozo, 1965). These observations raise a number of issues concerning the nature and origin of dorsal horn axons that possess 5-HT_3 receptors. In the initial part of the present study, we performed a series of experiments in an attempt to answer the following questions: 1) Are 5-HT_3 receptors present on terminals of excitatory neurons that use glutamate as a neurotransmitter? 2) Are 5-HT_3 receptors present on terminals of myelinated primary afferents? 3) What types of dorsal horn cells possess 5-HT_3 receptors?

It has recently been suggested that 5-HT_3 receptors are important elements of an ascending-descending spinobulbo-spinal pathway which regulates spinal cord excitability (Suzuki et al., 2002). Lamina I projection cells which possess the neurokinin 1 (NK-1) receptor are likely to form the origin of this pathway. These cells provide an indirect positive drive to neurons in the nucleus raphe magnus which, in turn, project to the spinal cord and increase excitability. A mechanism of this sort may underlie the hyperalgesia which is associated with central sensitization (see above). Therefore the final aim of this study was to investigate the relationship between axons that possess 5-HT_3 receptors and projection cells.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were conducted according to British Home Office legislation and were approved by the University of Glasgow Ethical Review Committee. Steps were taken to minimize the numbers of animals used in the study and to prevent any suffering. Altogether 12 male Sprague–Dawley rats (250–350 g; Harlan, Bicester UK) were used in the experiments. Seven animals were deeply anesthetized with sodium pentobarbitone (1 ml i.p.) and perfused through the left ventricle with saline followed by a fixative containing 4% formaldehyde in phosphate buffer pH 7.6. To label myelinated primary afferents we injected the B-subunit of cholera toxin (CTb) into the left sciatic nerve of three further rats. CTb is specifically taken up by these afferents and transported to their central terminations (LaMotte et al., 1991). Animals were deeply anesthetized with halothane and the left sciatic nerve was exposed under strict aseptic conditions. A micropipette containing a 1% solution of CTb (Sigma-Aldrich Ltd., Poole, UK) in sterile distilled water was inserted into the perineurium and 3–4 μl was pressure injected into the nerve. Following 3 days' survival, these rats were anesthetized with pentobarbitone and perfused as described above. To label lamina I projection neurons retrogradely, two rats were deeply anesthetized with a mixture of ketamine and xylazine (7.33 and 0.73 mg/100 g i.p.) and 200 nl of 1% aqueous solution of CTb was injected stereotaxically within the left caudal ventrolateral medulla (CVLM; co-ordinates=AP −4.8; DV −0.6; ML +2.1; Paxinos and Watson, 2003). Following 3 days' survival, operated animals were anesthetized with sodium pentobarbitone and perfused as described above. Mid-lumbar segments were removed from all animals and postfixed in the same fixative for 8 h at 4 °C. Horizontal or transverse sections (60 μm thick) were cut with a Vibratome and treated with 50% ethanol (30 min) to enhance antibody penetration.

Aim I: are 5-HT_3 receptors present on terminals of excitatory neurons that use glutamate as a neurotransmitter?

In order to determine if 5-HT_{3A}-IR axons are likely to be glutamatergic we performed a series of experiments where 5-HT_{3A}-IR axons were double labeled with antibodies raised against either the vesicular glutamate transporter 1 (VGLUT1) or the vesicular glutamate transporter 2 (VGLUT2). VGLUT1 and VGLUT2 are known to be reliable markers for glutamatergic neurons (see Todd et al., 2003 for further details). Transverse sections from three rats were incubated in a cocktail of rabbit anti-5-HT_{3A} (diluted 1:200; Oncogene Research Products, Boston, USA) and guinea-pig anti-VGLUT1 (1:5000; Chemicon International, Harlow, UK) or guinea-pig anti-VGLUT2 (1:5000; Chemicon) antibodies. All antibodies used in this study were diluted in phosphate-buffered saline containing 0.3% Triton X-100. Sections were rinsed and incubated in solutions containing species-specific secondary antibodies (all raised in donkey and diluted 1:100) coupled to fluorophores: fluorescein isothyoanate, to identify 5-HT_3 immunoreactivity, or Rhodamine Red to identify the VGLUT marker (Jackson ImmunoResearch, Luton, UK). Sections were mounted in a glycerol-based antifade medium (Vectashield, Vector Laboratories, Peterborough, UK) and examined with a BioRad MRC 1024 (BioRad, Hemel Hempstead, UK) confocal laser scanning microscope. Fields from the superficial dorsal horn (laminae I and II) were scanned systematically from sections cut in the transverse plane. It was apparent that there was no colocalisation between VGLUT1 and 5-HT_{3A}-IR and therefore the following procedure was adopted to quantify the extent of colocalisation between 5-HT_{3A}-IR and VGLUT2 only. Each field consisted of six optical sections which were gathered sequentially with a ×40 oil-immersion lens at a zoom factor of 3 and at 0.5 μm intervals from both left and right dorsal horns. In total 80 fields were gathered from the three animals (24, 28 and 28 respectively). The extent of colocalisation was quantified by using Neurolucida for Confocal (MicroBrightfield, Colchester, VT, USA). Forty 5-HT_{3A}IR axon terminals were selected at random from the third optical section of each scanned field. Sampled boutons 5-HT_{3A}-IR were assessed individually to determine if they contained immunoreactivity for VGLUT2. The numbers of double-labeled profiles were then counted for each animal and the final overall mean percentage value (±standard deviation) was calculated for the three animals.