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NEUROTRANSMITTER PHENOTYPES OF DESCENDING SYSTEMS IN THE RAT LUMBAR SPINAL CORD

A. DU BEAU, S. SHAKYA SHRESTHA, B. A. BANNATYNE, S. M. JALICY, S. LINNEN AND D. J. MAXWELL *

Spinal Cord Group, Institute of Neuroscience and Psychology, College of Medicine, Veterinary Medicine and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK

Abstract—Descending systems from the brain exert a major influence over sensory and motor processes within the spinal cord. Although it is known that many descending systems have an excitatory effect on spinal neurons, there are still gaps in our knowledge regarding the transmitter phenotypes used by them.

In this study we investigated transmitter phenotypes of axons in the corticospinal tract (CST); the rubrospinal tract (RST); the lateral component of the vestibulospinal tract (VST); and the reticulospinal tract (ReST). They were labelled anterogradely by stereotaxic injection of the b subunit of cholera toxin (CTb) into the motor cortex, red nucleus, lateral vestibular nucleus and medial longitudinal fascicle (MLF) to label CST, RST, VST and ReST axons respectively. Neurotransmitter content of labelled axons was investigated in lumbar segments by using immunofluorescence; antibodies against vesicular glutamate transporters (VGLUT1 and VGLUT2) were used to identify glutamatergic terminals and the vesicular GABA transporter (VGAT) was used to identify GABA- and glycinergic terminals.

The results show that almost all CST (96%) axons contain VGLUT1 whereas almost all RST (97%) and VST (97%) axons contain VGLUT2. Although the majority of ReST axons contain VGLUT2 (59%), a sizable minority contains VGAT (20%) and most of these terminals can be subdivided into those that are GABAergic or those that are glycinergic because only limited evidence for co-localisation was found for the two transmitters. In addition, there is a population of ReST terminals that apparently does not contain markers for the transmitters tested and is not serotoninergic.

We can conclude that the CST, RST and VST are 'pure' excitatory systems whereas the ReST consists of a heterogeneous population of excitatory and inhibitory axons. It is anticipated that this information will enable inputs to spinal networks to be defined with greater confidence. © **2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: motor control, descending tracts, neuroanatomy, immunocytochemistry, tract tracing, neurotransmitters.

INTRODUCTION

Sensory and motor processes within the spinal cord are greatly influenced by descending systems from the brain. Although we know that many descending systems excite spinal neurons there are gaps in our knowledge regarding the neurotransmitter phenotypes used by them. In this study we examined terminations of four descending systems to determine if they contain markers for excitatory and/or inhibitory neurotransmitters. These systems were: (1) the corticospinal tract (CST); (2) the rubrospinal tract (RST); the lateral component of the vestibulospinal tract (VST) and a component of the reticulospinal tract (ReST) passing through the medial longitudinal fascicule (MLF). Excitatory actions of the CST on spinal neurons are well established (see Lemon, 2008). It is known that CST terminals are enriched with glutamate (Valtschanoff et al., 1993) and that pyramidal cells contain vesicular glutamate transporter 1 (VGLUT1) mRNA (Fremeau et al., 2001). Axons of the pyramidal tract are immunoreactive for VGLUT1 but not the VGLUT2 (Persson et al., 2006). Hantman and Jessell (2010) found that VGLUT1 was present in some CST axons that were labelled with the genetic marker Emx1 in young mice but it was unclear if all terminals of CST axons contained VGLUT1. Likewise it is known that the RST and the lateral component of the VST are excitatory but direct evidence for the presence of neurotransmitter pools of glutamate in the terminals of these tracts is lacking. Beitz and Ecklund (1988) reported that a proportion of RST cells in the red nucleus was immunoreactive for glutaminase but not the gamma-aminobutyric acid (GABA) synthesizing enzyme glutamic acid decarboxylase (GAD) and Fremeau et al. (2001) provided evidence that many cells in the red nucleus contained VGLUT1 mRNA. In addition, Antal et al. (1992) showed that RST terminals are not immunoreactive for GABA or glycine and hence are likely to be glutamatergic. Cai et al. (2008) reported that efferent projections from vestibular nuclei contain either VGLUT1 or VGLUT2 but according to Valla et al. (2003)

*Corresponding author. Tel: +44-0141-330-6455.
E-mail address: David.Maxwell@Glasgow.ac.uk (D. J. Maxwell).

Abbreviations: CTb, cholera toxin B subunit; CST, corticospinal tract; DAB, 3,3′-diaminobenzidine; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; GLYT2, glycine transporter 2; HRP, horseradish peroxidase; MLF, medial longitudinal fascicle; ReST, reticulospinal tract; RST, rubrospinal tract; SD, standard deviation; VGAT, vesicular GABA transporter; VGLUT, vesicular glutamate transporter; VST, vestibulospinal tract.

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some VST neurons may contain GAD thus raising the possibility that they exert direct inhibitory actions on spinal neurons. There are several components of the ReST; in this study we have focused upon axons that project via the MLF because in electrophysiological studies it is possible to activate many reticulospinal fibres by electrical stimulation within this region which produces profound effects on networks involved in motor control (Jankowska et al., 2003; Edgley et al., 2004). It has been reported previously that reticular descending systems are heterogeneous in the neurotransmitters they contain, for example it is well established that many of the descending systems originating from the medulla are serotoninergic (Bowker et al., 1981) and may also contain a number of co-transmitters such as amino acids and peptides (Bowker and Abbott, 1990; Maxwell et al., 1996). There is evidence in the rat (Vetrivelan et al., 2009) and mouse (Martin et al., 2011) that spinally projecting neurons surrounding the region of the MLF contain VGLUT2 mRNA thus corroborating electrophysiological evidence in cats that many ReST neurons have strong excitatory actions on spinal neurons (e.g. see Wilson and Yoshida, 1969a; Jankowska et al., 2003; Edgley et al., 2004; Hammar et al., 2011). However, some ReST axons are GABAergic (Holstege, 1991; Antal et al., 1996) or glycinergic (Holstege and Bongers, 1991) and recent evidence suggests that these transmitters may be co-localised in the same neurons (Hossaini et al., 2012).

Vesicular glutamate transporters (VGLUT1 and VGLUT2) are present in terminals of glutamatergic neurons and in the spinal cord but large diameter primary afferents contain VGLUT1 whereas VGLUT2 is predominantly associated with terminals of interneurons (Olivera et al., 2003; Todd et al., 2003; Alvarez et al., 2004; Persson et al., 2006). Similarly, the vesicular GABA transporter (VGAT) is found in inhibitory axon terminals that contain GABA and/or glycine (Chaudhry et al., 1998) and therefore it is possible to use VGLUT and VGAT to determine whether a synaptic terminal is likely to have an excitatory or inhibitory action. In view of uncertainty in the literature surrounding the transmitter phenotypes of descending systems we performed a systematic analysis of the neurotransmitters associated with these four descending pathways. Descending axons were labelled anterogradely by stereotaxic injection of the b-subunit of cholera toxin (CTb) into the motor cortex, red nucleus, lateral vestibular nucleus and medial longitudinal fascicle (MLF) to label CST, RST, lateral VST and ReST axons respectively. Neurotransmitter content of labelled axons was investigated by using a combination of immunofluorescence and confocal microscopy. Antibodies against VGLUT1 and VGLUT2 were used to identify glutamatergic terminals and the VGAT was used to identify GABAergic and glycinergic terminals.

There were two principal aims: (1) to determine if each of the four systems is predominantly excitatory and, if so, if they express either VGLUT1, VGLUT2 or both; (2) to determine if any component of the four systems is inhibitory and expresses VGAT. As only ReST axons were found to be immunoreactive for VGAT we went on to determine if inhibitory ReST axons are glycinergic, GABAergic or both. We also investigated the possibility that ReST axons emerging from the MLF contain serotonin.

EXPERIMENTAL PROCEDURES

Surgical procedures

All animal procedures were carried out according to British Home Office regulations and were approved by the Glasgow University Ethics committee. Fourteen (three for CST, VST and RST experiments and five for ReST experiments) adult male Sprague Dawley rats (250–350-g) were deeply anesthetized with an intraperitoneal injection of Ketamine and Xylazine (2:1 0.1 ml/100 g) and placed in a stereotaxic frame under strict aseptic conditions. The skin at the back of the head was cut in the midline, the skull was exposed and a small burr hole was then made. The stereotaxic coordinates for each tract are given in Table 1 (see Paxinos and Watson, 1997). For RST, VST and ReST tracts injections were made according to inter-aural co-ordinates but for CST injections Bregma was used because we found that this was the most effective way to target the hind limb region of the sensorimotor cortex (see Neafsey et al., 1986). A glass micropipette with a tip diameter of 20 μm filled with 1% cholera toxin B subunit (CTb; Sigma–Aldrich, Co., Poole, UK) in distilled water was aligned with the burr hole and inserted into the brain. CTb (200 nl) was injected by pressure with a Pico injector (World Precision Instruments, Sarasota, FL, USA) except for the CST where 3 × 200 nl injections were made into the sensorimotor cortex. The micropipette was left in place for 5 min after injection to prevent backflow of tracer and then removed. The exposed surface was then sutured and the animals recovered from anaesthesia usually within 2–4 h, displaying exploratory behaviour and starting to drink. Following a six-day postoperative survival period, the animals were re-anesthetized with Pentobarbitone (1 ml of 200 mg/ml; i.p.) and perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) through the left ventricle. The spinal cord and brain were removed and post-fixed in the same fixative for 8 h at 4 °C. The brain was kept in a fixative containing 30% sucrose for 1–2 days and cut coronally by using a freezing microtome at 100-μm intervals for histological examination of the injection site.

Identification of injection sites and test sections of spinal cord

Injection sites and a small number of test transverse sections of the spinal cord from the L4 segment were visualized by using 3,3′-diaminobenzidine (DAB) as a chromogen. Sections were incubated in goat anti-CTb for 48 h followed by biotinylated anti-goat immunoglobulin gamma (IgG) for 3 h at room temperature. Sections were then incubated in avidin-horseradish peroxidase (HRP) for 1 h and hydrogen peroxide plus DAB was applied for a period of approximately 15 min to reveal immunoreactivity. Sections were finally mounted on gelatin-coated slides, dehydrated, cleared and a coverslip was applied. Injection sites and spinal cord sections were viewed with transmission light microscopy and photographed digitally (AxioVision 4.8 software, Zeiss, Germany). The location of the injection site was determined with reference to the stereotaxic rat brain atlas of (Paxinos and Watson, 1997).

Immunohistochemical procedures

L3–L5 segments of rat spinal cord were cut into 50-μm thick transverse sections with a Vibratome (Oxford instruments, Technical products international Inc. USA) after which they