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ASCENDING AND DESCENDING PROPRIOSPINAL PATHWAYS BETWEEN LUMBAR AND CERVICAL SEGMENTS IN THE RAT: EVIDENCE FOR A SUBSTANTIAL ASCENDING EXCITATORY PATHWAY

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Abstract—Precise mechanisms are required to coordinate the locomotor activity of fore- and hind-limbs in quadrupeds and similar mechanisms persist to coordinate movement of arms and legs in humans. Propriospinal neurons (PSNs) are major components of the networks that coordinate these mechanisms. The b subunit of cholera toxin (CTb) was injected unilaterally into either L1 or L3 segments in order to label ascending and descending propriospinal pathways. Labelled cells were examined with light or confocal microscopy. Cells projecting to lumbar segments were evenly distributed, bilaterally throughout all cervical segments. However many more cells were labelled from L1 injections than L3 injections. Roughly 15% of cells in both sides of the C2 segment was found to be immunoreactive for calretinin and a small number (4%) was immunoreactive for calbindin. Axons projecting from L1 to cervical segments formed predominant ipsilateral projections to the cervical intermediate grey matter and ventral horn. Very large numbers of terminals were concentrated within the ventrolateral motor (VLM) nuclei of C7–8 segments but there was sparse innervation of the contralateral nucleus. The vast majority (85%) of these axon terminals in the ipsilateral VML was immunoreactive for the vesicular glutamate transporter 2 (VGLUT2) and the remaining 15% was immunoreactive for the vesicular GABA transporter (VGAT); many of these contained GABA and/or glycine. Inhibitory and excitatory terminals were also found in the contralateral VLM. Most of the terminals in the VLM made contacts with motoneurons. The major finding of this study is the existence of a substantial excitatory propriospinal pathway that projects specifically to the VLM. Motoneurons in the VLM supply muscles of the axilla therefore this pathway is likely to have a profound influence on the activity of the shoulder joint. This pathway may synchronise lumbar and cervical pattern generators and hence the coordination of locomotor activity in the fore- and hind limbs.

Key words: spinal cord, neuronal network, motor control, neurotransmitter, calcium-binding protein.

INTRODUCTION

In quadrupeds, precise mechanisms are required to coordinate the motor activity of fore- and hindlimbs (Miller et al., 1973, 1975; Jankowska et al., 1973, 1974; Alstermark et al., 1987a,b,c; Ballion et al., 2001; Juvin et al., 2005). In humans, similar mechanisms persist to coordinate movement of arms and legs during a variety of activities such as running, swimming and crawling (Wannier et al., 2001; Zehr and Duysens, 2004). Although little is known about coupling mechanisms involved in the coordination of these processes, it is certain that propriospinal pathways are major components of the networks that execute them. Several varieties of funicular intraspinal neurons exist but they may roughly be divided into three types according to the length of their axonal projections: propriospinal neurons (PSNs) with long axons; intersegmental interneurons with medium length axons that project through a limited number of segments; and intrasegmental interneurons with short axons confined mainly to a single segment (see Liu et al., 2010). PSNs give rise to ascending and descending pathways between cervical and lumbar enlargements and form a major communication between these enlargements. There have been a number of anatomical studies of PSNs in a variety of species including the cat, rat and monkey (Molenaar and Kuypers, 1978; Matsushita et al., 1979; English et al., 1985; Menétrey et al., 1985; Miller et al., 1998; Dutton et al., 2006; Reed et al., 2006). The majority of these studies involved retrograde tracing of the cells of origin of propriospinal pathways. These studies show that there are connections between lumbar and cervical enlargements which project both ipsilaterally and contralaterally and that the cell bodies that give rise to them are located principally in the intermediate grey matter and lamina VIII; hence they are located in regions of the grey matter that are important for motor coordination (Jankowska, 1992). In the rat, the most comprehensive study of descending PSNs to date was performed by Reed et al. (2006). This study revealed the

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Abbreviations: BDA, biotinylated dextran-amine; CCN, central cervical nucleus; CPG, central pattern generators; CTb, cholera toxin b subunit; ChAT, choline acetyltransferase; DAB, 3,3′-diaminobenzidine; GAD, glutamic acid decarboxylase; HRP, horseradish peroxidise; LCN, lateral cervical nucleus; LSN, lateral spinal nucleus; PB, Phosphate buffer; PBS, Phosphate buffer saline; PBST, phosphate buffer saline containing 0.3% Trition X-100; PSN, propriospinal neuron; VGAT, vesicular GABA transporter; VGLUT, vesicular glutamate transporter; VLM, ventrolateral motor nucleus.

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existence of extensive bilateral projections from all cervical levels to the L2 lumbar segment. Cells formed crossed and uncrossed projections which were distributed equally within medial laminae VII and VIII. It is important to have detailed maps of descending PSNs because these cells could form the basis of 'detour circuits' (Jankowska and Edgley, 2006) which contribute to recovery following spinal injury as they can provide alternative routes for information to pass from the cerebral cortex to lumbar segments (Bareyre et al., 2005). In the present study, our first aim was to extend knowledge of descending PSNs in cervical segments. In particular, we wished to produce comprehensive maps of the distribution of descending cervical PSNs that relate to the various laminae of Rexed (Molander et al., 1989).

Very little is known about the neurochemical properties of long descending PSNs. Marsala et al. (2004) showed that some PSNs in the dog projecting from lower lumbar regions to caudal cervical segments contained nitric oxide synthase (NOS). Sherriff and Henderson (1994) found evidence for a population of cholinergic short intersegmental interneurons projecting up to six segments but PSNs with longer axons were not cholinergic. We also showed recently that there were numerous short intersegmental interneurons that contained the calcium-binding proteins calbindin and calretinin (Liu et al., 2010) and therefore our second aim was to extend this analysis to PSNs.

Few studies have attempted to examine axon terminals and target neurons of PSNs. Classical studies using degeneration techniques in combination with the Nauta method have revealed topically organised projections from various levels of the spinal cord to cervical motor nuclei (Sterling and Kuypers, 1968; Matsushita and Ueyama, 1973). The latter study revealed the presence of a substantial ipsilateral pathway in cats, rats, rabbits and dogs that projected via caudal thoracic segments which specifically targets the ventrolateral motor (VLM) nucleus in C7–T1. According to Miller (1970) this nucleus consists of motoneurons that supply the axillary muscles, in particular, pectoralis major and latissimus dorsi. Evidence supporting this comes from observations that VML motoneurons do not show chromatolysis following sectioning of the nerves that form the brachial plexus (Matsushita and Ueyama, 1973) nor are they labelled from muscles supplying the knee, ankle or digits of the rat forelimb (McKenna et al., 2000). This pathway is of particular interest because it could have a fundamental role in interlimb coordination in quadrupeds. Miller et al. (1973) showed that motoneurons in the VML were monosynaptically excited by ascending PSNs. More recently, a model based on studies of neonatal rat spinal cord was proposed by Juvin et al. (2005) which predicts that there is a powerful ipsilateral excitatory pathway from the rostral lumbar cord to caudal cervical cord and that this pathway has a strong effect on forelimb extensor motoneurons. Therefore the final aim of our study was to test the hypothesis that PSNs projecting to the C8 VLM nucleus are excitatory and form direct contacts on motoneurons.

EXPERIMENTAL PROCEDURES

Surgical procedures and labelling of PSNs

Experiments were performed on five adult male Sprague–Dawley rats (designated animals 1–5; 250–350 g; Harlan, Bicester, UK). All procedures were conducted according to British Home Office legislation and were approved by the University of Glasgow Ethical Review Committee. Rats were deeply anaesthetised with isoflurane. The procedure for injection of tracer into the spinal cord was similar to that reported in Liu et al. (2010). The thirteenth thoracic vertebra was identified according to the location of the last rib and a small dorsal midline incision was made at this level. A hole with a diameter of 1 mm was made adjacent to the midline in the laminar surface of the caudal part of the Th13 or L1 vertebrae to expose the dorsal surface of L1 or L3 segments of the spinal cord. The tip of the injection needle was inserted into the spinal cord to a depth of up to 1.5 mm from the surface at an angle of 15°. A 100 nl volume of 1% b-subunit of cholera toxin (CTb; Sigma–Aldrich, Co., Poole, UK) in distilled water was microinjected at the target site. The needle was left in place for 5 min following the injection to prevent backflow of tracer. The wound was sutured and animals recovered uneventfully. Following a 6-day survival period, rats were reanaesthetised with pentobarbitone (1 ml i.p.) and perfused through the left ventricle with mammalian Ringer's solution followed by 1 L of a fixative containing 4% formaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) at room temperature. The spinal cord and brain were removed and post-fixed for 8 h at 4 °C. The spinal cord was then divided into segments (C2–C8). Segments and injection sites were cut into 50-μm thick transverse sections with a Vibratome (Oxford Instruments, Technical Products International, Inc., USA). All sections were treated with an aqueous solution of 50% ethanol for 30 min to enhance antibody penetration.

Identification of injection sites

Injection sites were visualised by using 3,3′-diaminobenzidine (DAB) as a chromogen. Sections were incubated in goat anti-CTb for 48 h followed by a reaction with biotinylated anti-goat immunoglobulin gamma (IgG) for 8 h. Sections were then incubated overnight in avidin-horseradish peroxidase (HRP). Finally, hydrogen peroxide plus DAB was applied for a period of approximately 10 min to show immunoreactivity at injection sites. Following dehydration in a series of ethanol solutions, all sections were mounted on gelatinised slides, observed under a microscope and photographed with a digital camera. The segmental locations of injection sites were finally assessed and confirmed using the stereotaxic atlas of Paxinos and Watson (1997) according to the shape of the grey matter.

Light microscopy of cervical segments

Six transverse sections (50-μm thick) from each cervical segment (C2–C8) from all five animals were reacted and processed as described above for injection sites. Every fifth section was processed to avoid the possibility of double cell counts, Sections were examined under a light microscope (Nikon Eclipse E600, Technical Instrument, Burlingame, CA, USA) and photographed using an AxioCam digital camera and AxioVision 3.1 software (Carl Zeiss, Inc., Germany). A photomicrograph of each section was superimposed on templates of C2–C8 segments (taken from the stereotaxic atlas of Paxinos and Watson, 1997) with the graphics software Xara Xtreme 2.0e (Xara Group Ltd., Hemel Hempstead, UK) and the locations of CTb-labelled cells was recorded on the corresponding site on the template as a dot. The templates were used to estimate the number of contralateral and