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SPATIAL CHARACTERIZATION OF THE MOTOR NEURON COLUMNS SUPPLYING THE RAT FORELIMB

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Abstract—Rats can generate a rich array of forepaw and forelimb movements that are similar, although not as complex, to those produced by human and non-human primates. When reaching for food for instance, rats display skilled movements of the forelimb and the paw, therefore, making them attractive models to validate strategies aimed at the recovery of fine motor control. Surprisingly however, few anatomical studies have been performed on the central control of forelimb movements in the rat. The current series of experiments examined the details of the segmental arrangement of motor neurons that supply the rat forelimb. The distribution of motor end plates across the rat forelimb was first visualized by means of acetylcholinesterase histochemistry, and this information was used to create a motor end plate map of the forelimb muscles. This map was subsequently used as a guide for multiple injections of retrograde tracers along the motor end plate regions of 11 forelimb muscles. The entire cervical region of the spinal cord was subsequently analyzed under epifluorescence. This tract-tracing analysis confirmed that motor neurons innervating the rat forelimb are arranged in columns within the cervical segments of the spinal cord. This anatomical investigation also supports the previous observation that, although discrete, some of the motor neuron columns lying in the cervical aspect of the rat spinal cord are inter-mingled. The length of these columns, and hence the overlap between them, appears to be greater than previously reported, particularly within the uppermost segments of the brachial plexus.

Key words: motor neurons, cervical spinal cord, retrograde tracers, rat, forelimb, motor end plate.

Knowledge regarding the central control of forelimb movement has emerged from research performed on monkeys, presumably because non-human primates have long been considered to be the most appropriate animal model of human motor dysfunction, (Kuypers, 1964, 1981; Lawrence and Kuypers, 1968a,b; Kuypers et al., 1976; Passingham et al., 1983; Lawrence et al., 1985; Lemon, 1993; Porter and Lemon, 1993). More recently, however, there has been a wealth of evidence that rats can generate complex forelimb and forepaw movements for reaching and grasping food targets (Whishaw et al., 1986, 1990, 1992, 1993, 1998; Whishaw and Pellis, 1991; Schrimsher and Reier, 1993; Whishaw and Gorny, 1994, 1996; Whishaw and Coles, 1996; McKenna and Whishaw, 1999; Gharbawie et al., 2005, 2007; Muir et al., 2007; Alaverdashvili et al., 2008; Stackhouse et al., 2008; Kanagal and Muir, 2009; Wu et al., 2009; Morris et al., 2011). In this regard, the commonalities between rat and primate prehension is so striking that there is growing evidence for homology of reaching behaviour between these two species (Iwaniuk and Whishaw, 2000; Cenci et al., 2002). Based on this evidence, rats are valuable models for the validation of therapeutic scenarios aimed at the restoration of motor control of the forelimb (Sacrey et al., 2009). Of particular clinical relevance is the development of rat models of spinal cord injury made at cervical levels (Anderson et al., 2007, 2009) and treatment approaches to encourage axonal regeneration and functional recovery (Liu et al., 2002; Ruitenberg et al., 2006; Koda et al., 2004, 2007; Tobias et al., 2005; Xiao et al., 2005, 2007).

Despite the growing use of the rat for the modelling of forelimb dysfunction, there has been little work on the origin and spatial distribution of the motor neurons that innervate the rat forelimb. Previous studies carried out in the rat have limited their scope to the organization of motor nuclei that supply the muscles of the neck and shoulder (Kitamura and Sakai, 1982; Brichta et al., 1987; Callister et al., 1987; Choi and Hoover, 1996; Sienkiewicz and Dudek, 2010). Other investigations have focused on the organization of the rat cervical motor neurons from a developmental perspective (Bennet et al., 1983; Gramsbergen et al., 1996; Curfs et al., 1996; Lowry et al., 2001). To our knowledge, there have been few investigations focusing on the distribution of motor neurons supplying the distal muscles of the rat forelimb (Bertelli et al., 1995; McKenna et al., 2000). The analysis by McKenna et al. (2000) has revealed that motor neurons supplying the rat forelimb are organized in longitudinally oriented columns that span across two or more spinal cord segments. This columnar organization exists throughout the spinal cord and has been reported in the lumbar segments of the rat spinal cord (Nicolopoulos-Stournaras and Iles, 1983) as well as in other species (Romanes, 1941, 1951, 1964; Rexed, 1964; Hollyday, 1980; McHanwell and Biscoe, 1981; Jenny and Inukai, 1983; Mutai et al., 1986; Hoover and Durkovic, 1991; Hörner and Kummell, 1993). However, the presence of some longitudinal overlap between the motor columns lying in the cervical aspect of the spinal cord was only incidentally mentioned and remains to be fully described (McKenna et al., 2000). The same authors have reported a surprisingly low number of motor columns spanning through C5-C6, that is, within the cervical enlargement. The aim of the present study was two-fold: first, it was to

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Abbreviations: PB, phosphate buffer.

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examine the details of the segmental distribution of the motor neuron columns that supply the rat forelimb, with specific emphasis on the degree of overlap that exists between them. Secondly, it was to re-evaluate the motor neuron columns within the upper segments of the brachial plexus. Such information is important for the development of therapies in rat models of forelimb dysfunction (e.g. cervical spinal cord injury, motor neuron disease).

EXPERIMENTAL PROCEDURES

Animals

All experimental procedures complied with the Animal Care and Ethics Committee of the University of New South Wales and were performed in accordance with the National Health and Medical Research Council of Australia regulations for animal experimentation. A total of 27 adult female hooded rats (*Rattus norvegicus*; Long-Evans) (Monash University, Victoria, Australia) weighing approximately 250 g at the time of surgery were used in this study. The rats were housed in groups of four in an animal holding room under 12-h light–dark cycle. Water and rat chow were continuously available throughout the experiment.

Acetylcholinesterase histochemistry of the rat forelimb

Acetylcholinesterase histochemistry was carried out to identify the pattern of motor end plates within the rat forelimb. The silver acetylcholinesterase method of Beerman and Cassens (1976) was adapted for the visualization of motor end plates in whole limb preparation. To minimize the use of animals, six lightly perfused forelimbs were obtained through tissue sharing. The forelimbs were dissected from the body, the skin and the fascia covering the limb were removed, and the limbs were immersed in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) overnight. The limbs were subsequently incubated for 4 h at 4 °C in 200 ml of PB to which 290 mg acetylthiocholine iodide, 600 mg glycine, and 420 mg copper sulfate were added. The limbs were subsequently washed for 2 min in saline and developed by quick immersion in a 10% ammonium sulfide solution (all reagents from Sigma-Aldrich, St. Louis, MO, USA). Analysis of the location of the motor end plates (Fig. 1) was transposed onto rat forelimb diagrams adapted from Greene (1935) (see Fig. 2).

Surgical procedures

The animals were anesthetized with isoflurane (Provet, Sydney, NSW, Australia; 2–4% in O_2), and the skin area covering the targeted muscles was shaved and cleaned with ethanol. Incisions in the skin were made to expose the muscles of interest. Only the fascial sheaths covering the muscle(s) of interest were removed, and the muscles were targeted with the retrograde neuronal tracers Fluoro-Gold (Fluorochrome, Denver, CO, USA) or the dextran conjugate Fluoro-Emerald (Invitrogen, Carlsbad, CA, USA) (5% solution in distilled water). For each muscle under investigation, the tracers were injected through graded glass micropipettes (DKSH, Zurich, Switzerland) along the full length of the motor end plates as revealed by the whole limb acetylcholinesterase reactions described previously. The fasciae were removed over the muscle of interest to expose the direction of the muscle fibres, a critical landmark to locate the motor end plate region. It was also removed to ensure complete penetration of the tracer within the muscle of interest and to rule out any possibility that the tracer was caught between the fascia and the muscle. The injected area was then wiped with gauze to remove any tracer that had not penetrated the muscle. To avoid spurious labeling of motor neurons,

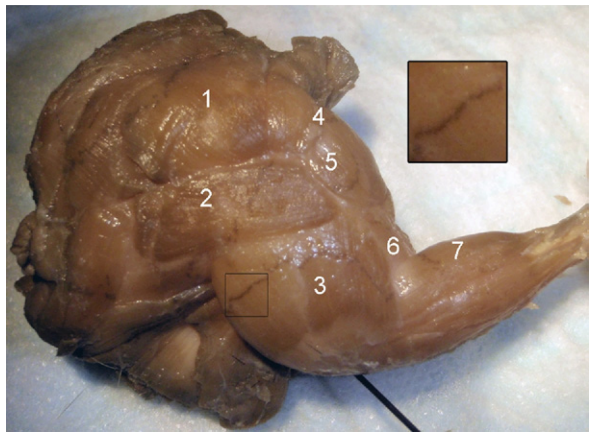

Fig. 1. A photograph of a lateral view of an acetylcholinesterase (AChE) reaction on the rat forelimb. The motor end plates are black speckles amongst the brown muscle fibres. The photograph also shows the muscle fibre direction. The inset region is a higher magnification of the motor end plate region. The muscles shown in this photograph are 1) Acromiotrapezius, 2) Spinodeltoideus, 3) Triceps brachii, 4) Levator claviculae, 5) Acromiodeltoideus, 6) Biceps brachii, and 7) Extensor carpi radialis.

great care was taken to keep intact the fasciae covering the surrounding muscle(s) for they act as a natural barrier to tracer leakage (Haase and Hrycyshyn, 1986). Special care was also taken to avoid damage to the blood supply. Bilateral injections of Fluoro-Gold were performed when only one pair of muscles was under investigation. In most cases, however, two pairs of muscles were targeted in the same animal. In such cases, the first pair of muscles to be subjected to tracer injections was targeted with one neuronal tracer in each forelimb (e.g. Fluoro-Gold on the left acromiotrapezius and Fluoro-Emerald on the right acromiotrapezius). For the second pair of muscles, the same tracers were used but, in this instance, with the opposite combination (e.g. Fluoro-Emerald on the left spinodeltoideus and Fluoro-Gold on the right spinodeltoideus). This tract-tracing protocol, with up to two pairs of muscles targeted per animal, minimizes the use of animals without compromising the integrity of the data as only one muscle on each forelimb is injected with a given neuronal tracer. A total of 76 injections were performed in the following muscles: acromiotrapezius (n=9), levator claviculae (n=11), spinodeltoideus (n=9), acromiodeltoideus (n=7), triceps brachii (n=7), biceps brachii (n=6), flexor digitorum profundus (n=6), flexor carpi radialis (n=5), palmaris longus (n=4), extensor carpi radialis (n=6), and pronator teres (n=6). For each muscle, a total of 4–8 μl of tracer was delivered into several small injections (1–2 μl) along the region of the muscle that contained the motor end plates. Immediately after the injections, the skin was closed with surgical clips and 4–6 ml of bupivacaine (Sigma-Aldrich, St. Louis, MO, USA) was administered along the skin incisions. The rats were kept for 12–15 days to allow for the tracers to be retrogradely transported to the spinal motor neurons.

Histological evaluation

At the end of the experiment, the rats were injected with a lethal dose of Lethabarb (Virbac, Sydney, New South Wales, Australia) and perfused through the heart with 0.1 M PB followed by a solution of 4% paraformaldehyde in 0.1 M PB. In each animal, the second cervical vertebra was first identified and then removed in order to expose the second pair of dorsal roots that were then colored with a marker. The third cervical vertebra was subsequently removed to expose the next pair(s) of roots and to color them. The pairs of roots were colored with alternate colors so that

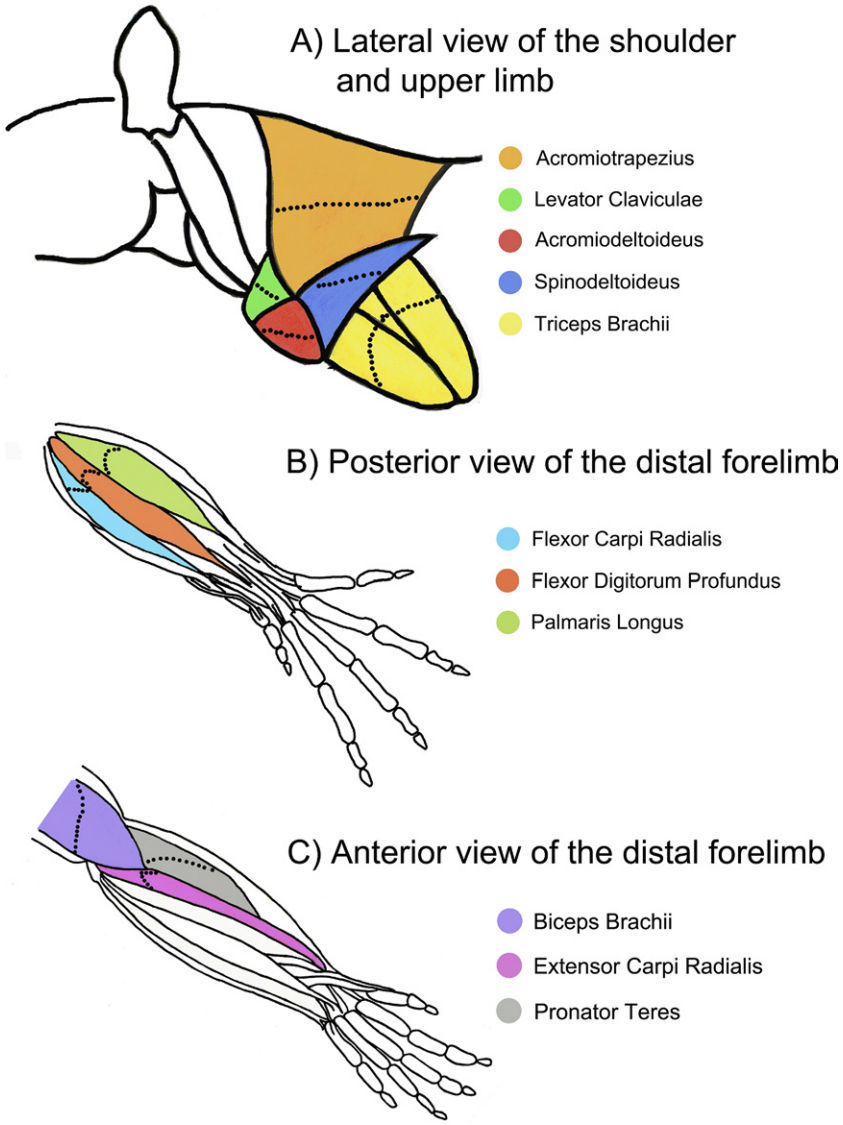

Fig. 2. Composite diagram representing the location of the muscles targeted with retrograde tracers. The dotted lines on each of these muscles indicate the position of the motor end plates. (A) Lateral view of the upper forelimb. The color-coded target muscles were Acromiotrapezius (orange), Levator claviculae (green), Acromiodeltoideus (red), Spinodeltoideus (blue), and Triceps brachii (yellow). (B) Posterior view of the distal forelimb. The color-coded target muscles were Flexor carpi radialis (light blue), Flexor digitorum profundus (terra cotta), and Palmaris longus (light green). (C) Anterior view of the distal forelimb. The color-coded target muscles were Biceps brachii (purple), Extensor carpi radialis (pink), and Pronator terres (grey).

C2, C4, C6, and C8 roots were colored in blue, and C3, C5, C7, and T1 were colored in black. The spinal cords were cut transversally *in situ* into two-segment blocks (i.e. C2−3, C4−5, C6−7, C8−T1). For each block, a fiducial mark was created between the two segments in the white matter of the left spinal cord, halfway between the upper and the lower roots. This fiducial mark represented the border between the two segments. The blocks were then dissected out, post-fixed overnight in a solution of 4% paraformaldehyde in 0.1 M PB, and cryoprotected in 30% sucrose (Sigma-Aldrich, St. Louis, MO, USA) in distilled water for three days at 4 °C. The blocks were cut longitudinally at 50 μm, and the tissue sections mounted onto gelatinized microscope slides. The slides were air-dried and subsequently coverslipped with an anti-fade medium containing DAPI (Invitrogen, Carlsbad, CA, USA).

Data analysis and presentation

The number of motor neurons supplying the different forelimb muscles under investigation was obtained by plotting the Fluoro-Gold- and Fluoro-Emerald-positive cells under epifluorescence on a schematic representation of the spinal cord. Motor neurons were considered positively labelled when both the soma and at least one attached axon/dendrite were labelled (Iliya and Dum, 1984; Vanderhorst and Holstege, 1997). Careful comparison of adjacent tissue sections was carried out to prevent double counting. For each tissue section from a given block, the data were transposed onto an Adobe Photoshop CS4-digitized image of the spinal cord with the labelled neurons accurately positioned in relation the fiducial marks and the root exit zones. The images were then stacked together using the fiducial marks in order to generate a 2-dimensional chart for

each spinal cord segment. Motor neuron plots for each muscle were extracted from these charts and transferred on outlines of the right cervical spinal cord for presentation. In this process, the neurons plots located on the left side of the spinal cord were transposed to the right side. The transverse reconstructions were created by using the fiducial marks, the location of the central canal, and the nerve root exit points. Individual data points from each tissue section were stacked to provide the location in both dorso-ventral and medio-lateral axis. The contours of the gray matter for the different spinal cord levels illustrated were adapted from Paxinos and Watson (2005).

RESULTS

Localization of the motor end plates in the rat forelimb

The location of the motor end plates in each muscle under investigation is shown in Figs. 1 and 2. Fig. 1 is a photograph of the lateral view of a rat forelimb processed with an aceytlcholinesterase reaction that reveals the motor end plate regions. On this photograph, the motor end plates appear as black speckles aligned on the brown muscle fibres. Moreover, Fig. 1 also shows the direction of the muscle fibres *in situ*. Analysis of the lateral, ventral, and dorsal views of the forelimbs used for this reaction was used to create the composite diagram shown in Fig. 2. For acromiotrapezius, levator claviculae, triceps brachii, flexor digitorum profundus, flexor carpi radialis, extensor carpi radialis, and palmaris longus, the motor end plates are located across the muscle fibres, in the thickest part of the muscle, the region referred to as the “belly” of the muscle. Contrary to the common view, however, the belly is not always located midway between the origin and insertion points of the muscles, at least in the rat forelimb. For example, the motor end plates of flexor digitorum profundus, flexor and extensor carpi radialis, and palmaris longus are located much closer to the origin of these muscles than to their insertion point. In some muscles, the motor end plates are distributed in a V-shaped line that is orthogonal to the orientation of the muscle fibres. This is the case for flexor carpi radialis, flexor digitorum profundus, and extensor carpi radialis. For other muscles such as spinodeltoideus, acromiodeltoideus, biceps brachii, and pronator teres, the motor end plates are spread across the muscle in a diagonal manner. In these muscles, however, the muscle fibres are also diagonally distributed so the motor end plates are still orthogonally distributed with regard to the orientation of the muscle fibres.

Success of intramuscular injections of Fluoro-Gold or Fluoro-Emerald

A total of 76 intramuscular injections of Fluoro-Gold or Fluoro-Emerald were performed in the shoulder and forelimb. Twenty-two of these injections produced weak and/or sparse labeling and are not mentioned henceforth. The remaining 54 injections produced intense retrograde labeling, and data from these injections have been included in the present analysis and are described later in the text. Fig. 3 gives an example of both Fluoro-Gold (Fig. 3A) and Fluoro-Emerald (Fig. 3B) positively labeled motor neurons. For each muscle under investigation, the labeled motor neurons were organized in columnar shape as demonstrated by Fig. 3.

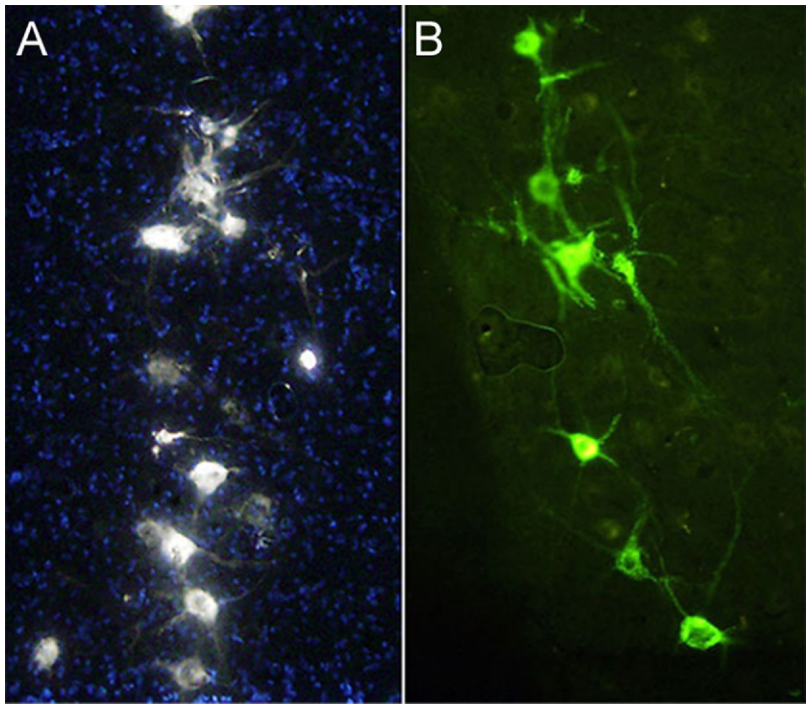

Fig. 3. Positively labeled motor neurons with (A) Fluoro-gold with DAPI mounting medium and (B) Fluoro-Emerald.

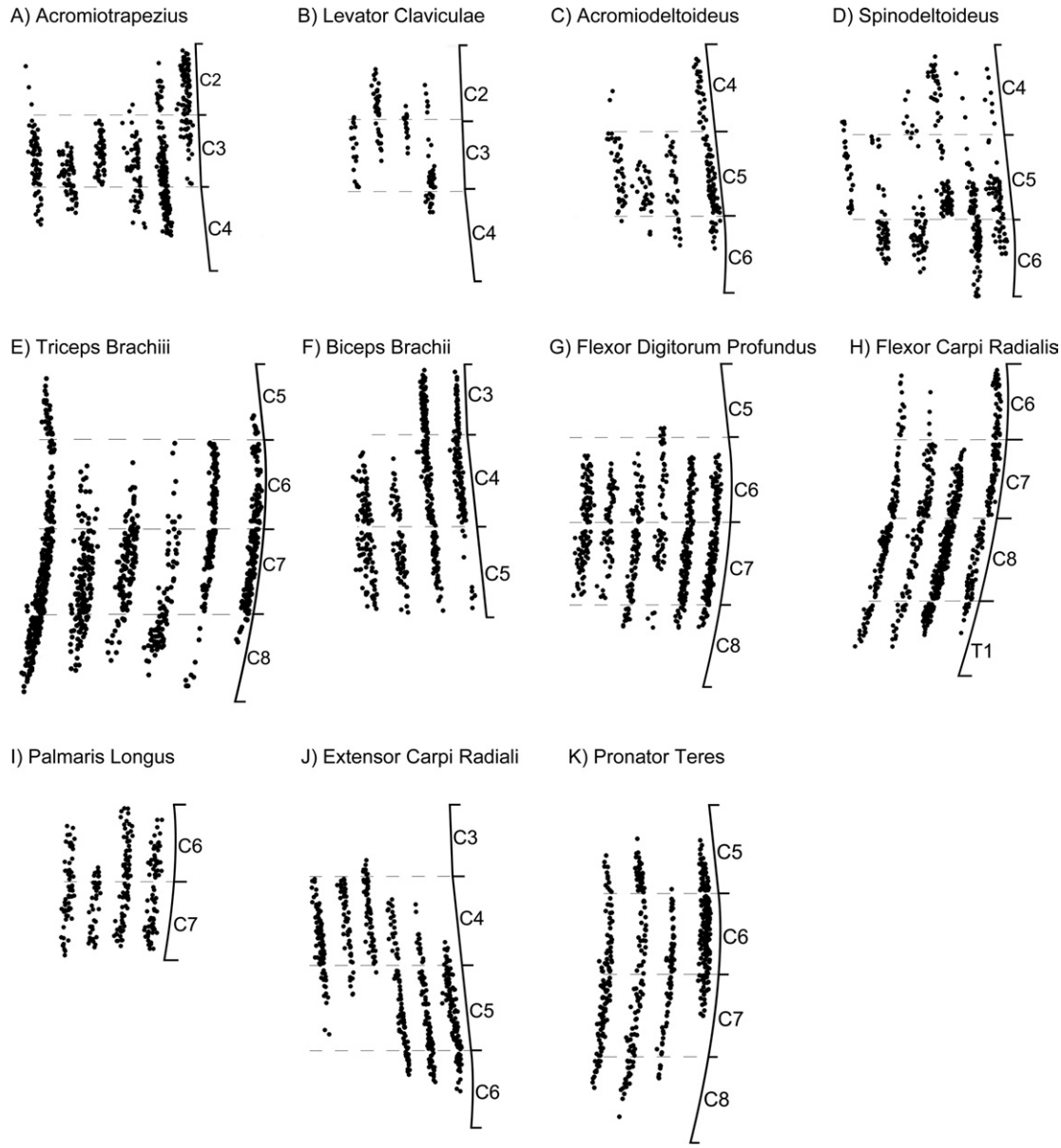

Fig. 4. Composite diagram illustrating the distribution of positively labelled motor neurons within the upper segments of the spinal cord in the following muscles: (A) Acromiotrapezius, (B) Levator claviculae, (C) Acromiodeltoideus, (D) Spinodeltoideus, (E) Triceps brachii, (F) Biceps brachii, (G) Flexor digitorum profundus, (H) Flexor carpi radialis, (I) Palmaris longus, (J) Extensor carpi radialis, and (K) Pronator teres. The spinal cord levels in which positively labelled motor neurons were present are indicated on the right side of each individual muscle plot.

Quantification of motor neurons innervating the rat forelimb muscles

The total number of motor neurons supplying the forelimb muscles under investigation was estimated by counting the number of positively labeled cells resulting from each intramuscular injection. It is important to note that cell count obtained with this tract-tracing technique could lead to some underestimation of the actual population of motor neurons innervating a given muscle, because the retrograde transport of neuronal tracers might not be always optimal. Therefore for each muscle under scrutiny, the maximum number of labelled motor neurons for each segment was presented next to the corresponding segment in Fig. 4.

Distribution of motor neuron columns supplying the rat shoulder

Fig. 2A shows a lateral view of the rat shoulder and upper forelimb and depicts the location of the muscles that were targeted with fluorescent retrograde neuronal tracers. The muscles of the shoulder that were under investigation are acromiotrapezius, levator claviculae, acromiodeltoideus, and spinodeltoideus. The rostrocaudal distribution of labeled motor neurons resulting from the intramuscular in-

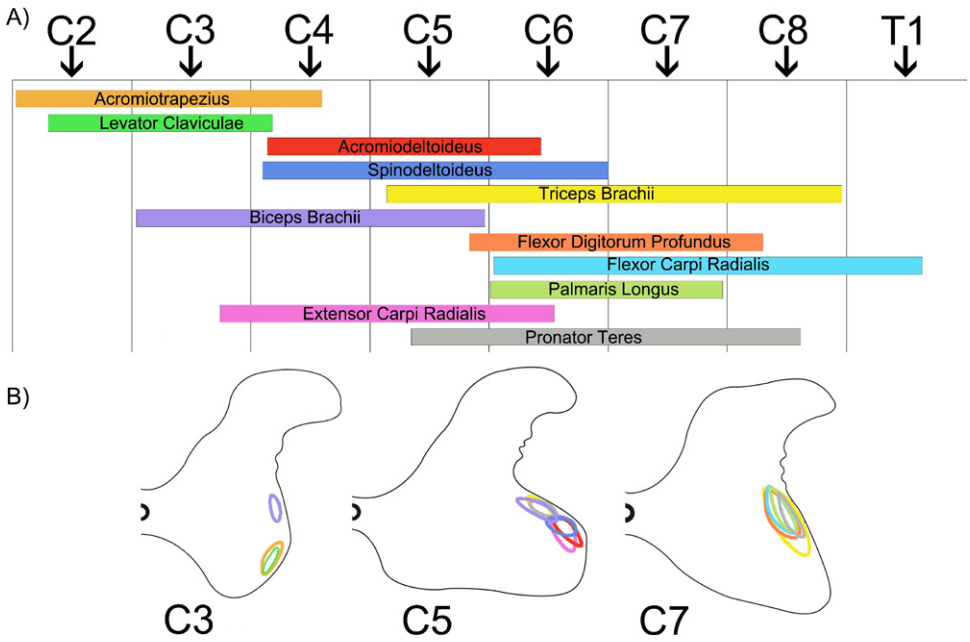

Fig. 5. Color-coded schematic reconstruction of the distribution, of the motor neuron columns for 11 muscles of the rat forelimb. The colors for these muscles correspond to that shown on Fig. 2. (A) Shows the motor neuron columns along the longitudinal axis. Arrows at the top of the figure indicate the positions of the root exit zones for C2-T1. (B) Shows the motor neuron columns in a transverse view of the cervical grey matter. Each motor column is encompassed within an individual color-coded boundary. Each segmental representation (i.e., C3, C5, and C7) is defined in correspondence with the dorsal root exit point.

jections of retrograde tracer in these shoulder muscles is illustrated in Fig. 4A–D. Together, these shoulder muscles are innervated by motor neurons spanning the entire C2–C6 region of the spinal cord.

Acromiotrapezius. The acromiotrapezius muscle, which is the dorsalmost shoulder muscle, is a large but thin muscle that abuts the dorsal aspect of spinodeltoideus (Fig. 2A). The function of acromiotrapezius is to draw the scapula medially (Wingerd, 1988). Nine injections of fluorescent neuronal dyes were made into acromiotrapezius, among which six injections gave rise to intense labeling in the ventral horn of the spinal cord. The six injections targeting acromiotrapezius highlighted motor neurons spanning cervical segments C2–C3, with labeling also observed in the uppermost half of C4 (Fig. 4A).

Levator claviculae. Levator claviculae is a small, thin triangular muscle that covers the shoulder and is located at the interface between the acromiotrapezius, spinodeltoideus, and acromiodeltoideus muscles that elevate the clavicle (Campbell et al., 1974) (Fig. 2A). Eleven injections of retrograde tracers were carried out in levator claviculae, with four injections giving rise to labeling in the spinal cord. These intramuscular injections produced a limited number of positively labeled motor neurons in C2–C4 segments of the spinal cord (Fig. 4B). The motor neuron column supplying levator claviculae completely overlapped with that innervating acromiotrapezius (see Fig. 5).

Acromiodeltoideus. The acromiodeltoideus muscle is another triangular muscle covering the glenohumeral joint that extends and rotates the humerus medially (Wingerd, 1988) (Fig. 2A). A total of seven injections of retrograde tracers were performed in acromiodeltoideus. Four of these injections produced satisfying labeling in the spinal cord and were therefore included in this analysis. These injections gave rise to labeled motor neurons within the entire length of C4–C5, with some labeling in the uppermost part of C6 (Fig. 4C).

Spinodeltoideus. The spinodeltoideus muscle is a flat muscle that adjoins acromiotrapezius and acromiodeltoideus, flexes and rotates of the humerus laterally (Wingerd, 1988) (Fig. 2A). Nine injections of fluorescent retrograde dyes were made into the spinodeltoideus muscle. Three injections were excluded from the analysis because they gave rise to faint labelling. The six injections that produced satisfactory levels of fluorescence and that were included in this analysis gave rise to positively labeled motor neurons spanning the entire C4–C6 region (Fig. 4D). The motor columns supplying acromiotrapezius, acromiodeltoideus, and spinodeltoideus overlapped with one another at the level of C4 (see Fig. 5).

Distribution of motor neuron columns supplying the rat upper forelimb

The muscles of the upper forelimb under investigation were triceps brachii and biceps brachii. The location of triceps brachii is illustrated in Fig. 2A, and the location of biceps brachii, which lies in the anterior part of the upper forelimb, can be seen in Fig. 2C. The rostrocaudal distribution of labeled motor neurons resulting from the intramuscular injections of retrograde tracer in these upper

forelimb muscles is illustrated in Fig. 4E, F. Together, biceps and triceps brachii gave rise to columns of labeled motor neurons that occupy the entire breadth of C3–C8.

Triceps brachii. Triceps brachii is a large heterogeneous muscle on the lateral aspect of the upper brachium consisting of three distinct heads that extend the elbow (Wingerd, 1988) (Fig. 2A). Seven injections of retrograde neuronal tracers were carried out into the three heads of triceps brachii, with six injections producing intense labeling in the ventral horn of the spinal cord. These injections revealed that the motor column supplying triceps brachii spans the full length of C5–C8 (Fig. 4E).

Biceps brachii. Biceps brachii, located anterior to triceps brachii on the upper aspect of the forelimb, is composed of a long lateral head and a short medial head that together flex the antebrachium (Wingerd, 1988) (Fig. 2C). A total of six injections of retrograde neuronal tracer were performed in biceps brachii, with four injections producing strong retrograde labeling in segments C3–C5 of the spinal cord (Fig. 4F). The motor neuron column innervating biceps brachii is located considerably more rostrally than that supplying triceps brachii. However, these columns overlap with each other at cervical level C5 (see Fig. 5).

Distribution of motor neuron columns supplying the posterior aspect of the rat distal forelimb

Fig. 2B shows the muscles that can be seen on the dorsal surface of the rat lower forelimb and that were targeted with fluorescent dyes. These muscles are flexor digitorum profundus, flexor carpi radialis, and palmaris longus. The rostrocaudal distribution of labeled motor neurons resulting from the intramuscular injections of retrograde tracer in these lower forelimb muscles is illustrated in Fig. 4G–I. Together, the motor columns supplying these three lower forelimb muscles span segments C5–T1 (Fig. 5).

Flexor digitorum profundus. This flexor muscle, composed of four heads, is located between flexor carpi radialis and palmaris longus on the flexor surface of the distal forelimb that flexes digits 2–5 and the carpus (Greene, 1955; Hebel and Stromberg, 1976) (Fig. 2B). A total of six injections of fluorescent retrograde tracer were performed in flexor digitorum profundus, and all injections were included in this analysis. Motor neurons supplying flexor digitorum profundus were found to be organized in a column that occupies the entire cervical segments C6–C7, as well as the rostralmost part of C8 (Fig. 4G). In one case, a few labeled motor neurons were present in C5.

Flexor carpi radialis. This long superficial flexor muscle is found between flexor digitorum profundus and pronator teres that flexes the carpus (Greene, 1955; Hebel and Stromberg, 1976) (Fig. 2B). Five injections of fluorescent dyes were carried out in flexor carpi radialis, four of which gave rise to intense labeling in the spinal cord. In these four cases, motor neurons innervating flexor carpi radialis were observed to span across the C6–T1 region (Fig. 4H).

Palmaris longus. Palmaris longus is another long superficial muscle found on the dorsal aspect of the distal forelimb that flexes the manus and the wrist (Greene, 1955) (Fig. 2B). Four injections of fluorescent tracer were performed in palmaris longus, and all of them resulted in intense retrograde labeling in the ventral horn of the spinal cord. These four injections were therefore included in this analysis and gave rise to a short column of labeled motor neurons spanning cervical segments C6 and C7 (Fig. 4I). Together, the motor columns supplying the three flexor forelimb muscles under investigation overlap with each other at the level of C6–C7 (see Fig. 5).

Distribution of motor neuron columns supplying the anterior aspect of the rat distal forelimb

Fig. 2C depicts the location of the muscles lying on the anterior surface of the rat lower forelimb that were subjected to fluorescent dye injections. These muscles are extensor carpi radialis and pronator teres. The rostrocaudal distribution of labeled motor neurons resulting from the intramuscular injections of retrograde tracer in these distal forelimb muscles is illustrated in Fig. 4J, K. Together, a pool of motor neurons that occupies the C4–C8 region innervates these two muscles.

Extensor carpi radialis. Both extensor carpi radialis longus and brevis were targeted together and presented as one. These extensor muscles are located on the anterior surface of the distal forelimb and extend the carpus (Greene, 1955) (Fig. 2C). The six injections of retrograde tracer that were carried out in extensor carpi radialis resulted in intense labeling in the ventral horn of the spinal cord, and all were therefore included in the analysis. These injections revealed that the column of motor neurons supplying extensor carpi radialis occupies the whole length of cervical segments C4 and C5 as well as the upper half of C6. In one case only, a few labeled motor neurons were also found in C3 (Fig. 4J).

Pronator teres. This muscle is found on the anterior surface of the distal forelimb, between extensor carpi radialis and flexor carpi radialis, and pronates the antebrachium (Greene, 1955; Hebel and Stromberg, 1976) (Fig. 2C). Six injections of fluorescent neuronal tracer were carried out in pronator teres, with four injections resulting in intense labeling in motor neurons that span the caudal half of cervical segment C5, C6, C7 as well as the rostral half of segment C8 (Fig. 4K). The two motor neuron columns supplying extensor carpi radialis and pronator teres overlap with each other at cervical segments C5 and C6 (see Fig. 5).

Overall organization of the different motor columns innervating the rat forelimb

The rostro-caudal overlap between motor columns innervating individual muscles has been described in the previous section and is shown in Fig. 5A. Overall, there is a consistent overlap between motor columns throughout C2 to T1. There is also a topographic relationship between the motor columns and the muscles they innervate. The motor

columns innervating the shoulder muscles span across C2–C6; the motor columns innervating the upper forelimb muscles span across C3–C8, whereas the majority of the motor columns innervating the lower forelimb muscles span across C5–C8, with a minor distribution in C3–C4 and T1. An overlap also exists between the motor columns in both the dorso-ventral and the medio-lateral axes as demonstrated at three different levels (i.e. C3, C5, and C7) in Fig. 5B. As is the case for the rostro-caudal axis, there is a topographic relationship between the muscles and the motor columns they supply on the dorso-ventral and the medio-lateral planes. For instance, the motor columns innervating the shoulder occupy the ventral aspect of the ventral horn, whereas those controlling the distal forelimb are located in the dorsal aspect of the ventral horn.

DISCUSSION

The aim of this research was to characterize the details of the spatial organization of the columns of motor neurons that supply the musculature of the rat forelimb. Particular attention was given to identify the degree of overlap between these motor columns that lie in the cervical segments of the spinal cord and that has been incidentally reported (McKenna et al., 2000). Special emphasis was also placed on the number of motor neuron columns spanning the cervical enlargement. The results of this anatomical tract-tracing investigation confirmed that the motor neurons that control the muscles of the rat forelimb are organized into columns within the cervical segments of the spinal cord. Transverse reconstruction of the spinal cords revealed the dorso-ventral and medio-lateral organization of these motor neuron columns, with more dorsally located columns supplying distalmost muscles. This anatomical investigation also supported the previously reported observation that, although discrete, the motor neuron columns lying in the cervical aspect of the rat spinal cord are intermingled.

The main findings of this series of experiments are (1) the characterization of the motor end plates for selected rat forelimb muscles. To our knowledge, such map has not been published before. (2) The upper part of the cervical enlargement (C5–C6) contains a greater amount of motor neuron columns than previously reported. (3) The length and therefore the overlap between some motor neuron columns is greater than previously described. (4) Triceps brachii is innervated by the greatest number of motor neurons, which reflects the size of the muscle. These findings were confirmed by quantitative analysis. Our results therefore expand on previous work and provide a greater understanding of the organization of the motor neuron columns supplying the rat forelimb.

Topographic organization of the motor neurons supplying the rat forelimb

The topographic organization between the skeletal muscles and the motor neurons that innervate them has long been recognized (Romanes, 1941; Rexed, 1964; Nicolopoulos-Stournaras and Iles, 1983; Vanderhorst and Holstege, 1997; McKenna et al., 2000). Such topographic organization has been described between the position of the motor columns on the rostro-caudal axis in the cervical spinal cord and the proximo-distal position of the muscles that they supply (Romanes, 1941; McKenna et al., 2000; Küchler et al., 2002). For instance, motor neurons innervating proximally located forelimb muscles cluster in columns that lie within the rostralmost aspect of the cervical spinal cord, whereas motor neurons supplying more distal muscles are found in more caudal columns (McKenna et al., 2000; Küchler et al., 2002). Overall, data obtained in the present series of experiments support the general principle of nerve-muscle rostro-caudal topographic organization. Indeed, the results of the present study have shown that motor neurons supplying the proximal muscles of the shoulders span throughout the upper cervical segments of the spinal cord, that is, C2–C6. This analysis has also revealed that neurons controlling the muscles of the upper forelimb are distributed in columns that lie throughout cervical segments C3–C8. Finally, the current series of experiments have highlighted that motor neurons innervating the muscles of the lower forelimb have distribution predominantly throughout segments C5–C8, as well as minor distribution in C3–4 and T1.

A topographic organization was also found in both the dorso-ventral and medio-lateral axis within the spinal cord gray matter. The medial motor group, present throughout the whole spinal cord, is located ventro-medially within the ventral horn and contains motor neurons innervating the muscles of the trunk (Romanes, 1941; Rexed, 1964; Vanderhorst and Holstege, 1997). The lateral motor column, which is primarily evident within the cervical and lumbosacral enlargements, contains neurons that innervate the muscles of the forelimb and hindlimb, respectively (Romanes, 1941; Rexed, 1964; Nicolopoulos-Stournaras and Iles, 1983; Coonan et al., 2003). On the transverse plane at cervical levels, the lateral motor group can be divided into a dorsal and a ventral region (see Fig. 5B). The ventral subdivision within the lateral motor group, which lies in the rostralmost segments of the cervical spinal cord, is populated by neurons that supply the muscles of the shoulder. Conversely, the dorsal subdivision within the lateral motor group is located in the caudalmost segments of the cervical and first thoracic spine and contains neurons that innervate the more distal muscles of the manus and digits (Hollyday, 1980; Coonan et al., 2003).

Our data indicate a spatial organization within the lateral motor group. First, the motor columns innervating the proximal muscles of the forelimb are located in the ventral division of the lateral motor group (e.g. acromiotrapezius and levator claviculae; see C3 in Fig. 5B). Conversely, the motor columns innervating the distal muscles of the forelimb lie in the dorsal division of the lateral motor group (e.g. palmaris longus and flexor carpi radialis; see C7 in Fig. 5B). One exception to this organization is that of biceps brachii and triceps brachii; biceps brachii is located more rostrally and triceps brachii is located more caudally (see C3 and C5 in Fig. 5B). This exception has been reported by McKenna et al. (2000). This spatial organization con-

curs with that shown in previous studies (McKenna et al., 2000; Coonan et al., 2003).

In summary, the spatial organization of the motor columns innervating the forelimb can be observed in the rostro-caudal, the dorso-ventral, and the medio-lateral axes. In general, motor columns supplying proximal muscles tend to be located in rostral segments, as well as ventrally within the lateral motor group. This relationship is most evident when looking at the shoulder muscles (Fig. 2A). For instance, acromiotrapezius and levator claviculae, which are the most proximal muscles acting on the shoulder joint, are supplied by motor columns located rostrally at C2–C4. Furthermore, these columns are also located ventrally within the lateral motor group (see Fig. 5B). On the other hand, spinodeltoideus and acromiodeltoidous, which are located immediately distal to acromiotrapezius and levator claviculae, are supplied by motor columns located caudally (C4–C6) and dorsally with regards to the previously described motor columns (Fig. 5). This "caudo-dorsal shift" is in keeping with previous studies of motor columns supplying the rat forelimb (McKenna et al., 2000; Küchler et al., 2002).

Length of the motor neuron columns of the rat forelimb

Characterizing the forelimb motor end plates has helped maximize the initial uptake of the tracers within the muscles, as well as their retrograde transport into the spinal cord. As a consequence, we have found that many motor columns span across a greater portion of the cervical spinal cord than previously observed (see McKenna et al., 2000). Moreover, eight motor neuron columns were found to span the upper part of the cervical enlargement (i.e. C5–C6). This finding is in sharp contrast with the report of only two motor neuron columns in the same region of the cervical enlargement (see McKenna et al., 2000). These results, however, better reflects the nature of the cervical enlargement where motor neurons supplying the numerous muscles of the forelimb are particularly abundant. Additionally, we found that triceps brachii contains the greatest number of motor neurons that span C5–C8, a finding that reflects the size of the muscle. Methodological differences, particularly the choice of non-lipophilic neuronal tracers and multiple injections along the entire motor end plate region, between the present study and that of McKenna et al. (2000) can, at least partly, explain these discrepancies.

Overlap between the motor columns of the rat forelimb

Previous analysis has reported that, although the motor columns supplying the rat forelimb are discrete, their spatial distribution is such that many overlap with each other, particularly in the caudal aspect of the cervical spinal cord (McKenna et al., 2000). A similar observation was made with regard to the distribution of motor neuron columns in the lumbar spinal cord of the rat (Nicolopoulos-Stournaras and Iles, 1983). Our data have confirmed the existence of an overlap between the motor columns that supply the rat forelimb. For instance, the shoulder muscles under scrutiny were shown to be innervated by columns of motor neurons spanning the entire C2–C6 region of the spinal cord. Among these motor columns, those supplying acromiotrapezius and levator claviculae overlap, both rostro-caudally (Fig. 5A) and on a transverse axis (C3 in Fig. **5B**). The overlap is also noticeable between the motor columns innervating acromiodeltoideus and spinodeltoideus, again on both planes (Fig. 5A and C5 in 5B). Interestingly, triceps and biceps brachii found in the upper forelimb and both acting on the elbow joint are supplied by motor columns that occupy the entire breadth of segments C3–C8 and that overlap at level C5. This overlap on the transverse plane is in keeping with the spatial organization mentioned above, with the flexors (e.g. biceps brachii) located more medially, and the extensors (e.g. triceps brachii) located laterally (see C5 in Fig. 5B). A similar arrangement of motor columns can be observed between flexor carpi radialis and extensor carpi radialis. The relationship between functionally antagonistic pairs of muscles acting on the same joint, but with noticeable difference in distribution, has also been mentioned previously (McKenna et al., 2000). Finally, the motor columns supplying the lower aspect of the forelimb span interruptedly throughout all the segments comprising the cervical enlargement, that is, from C5 to thoracic segment T1, exhibiting a great degree of overlap with each other. The overlap is noticeable in both rostro-caudal and transverse planes (see Fig. 5A and C7 in Fig. 5B).

Methodological considerations

All animals received bilateral intramuscular injections, with Fluoro-Gold and Fluoro-Emerald delivered to the same pairs of muscle. For example, Fluoro-Gold was injected in the left biceps brachii and Fluoro-Emerald in the right biceps brachii. As two pairs of muscles were usually under scrutiny in the same rat, this bilateral injection procedure was then applied to the second pair of muscles but in the reverse order, for example, Fluoro-Gold injected in the left biceps brachii and Fluoro-Emerald in the right biceps brachii followed by Fluoro-Emerald injected in the left triceps brachii and Fluoro-Gold injected in the right triceps brachii. Two intramuscular injections per limb with retrograde tracers of different light absorption and emission spectra ruled out any possible confusion about the relationship between the injected muscles and the resulting pools of labeled neurons. Although this method was primarily adopted to minimize the use of experimental animals, it also provided a systematic comparison of the retrograde properties of the two fluorescent tracers. After pressure injections at the motor end plates of the muscles, both Fluoro-Gold and Fluoro-Emerald produced excellent labeling in the ventral horn of the spinal cord.

An important methodological issue when performing intramuscular injections of neuronal tracers is the risk of tracer leakage outside the muscle of interest and into surrounding muscles. To avoid this problem, great care was taken to not damage the blood supply and the fasciae covering the surrounding muscles as they act as a natural

barrier to prevent tracer leakage (Haase and Hrycyshyn, 1986; Vanderhorst and Holstege, 1997). All the muscles targeted in the present analysis had well-developed fasciae, and it was difficult to pierce the fasciae covering them with the glass micropipettes containing the tracers, thereby giving confidence that only the muscles of interest were targeted and not the muscles beneath them. However, as Fluoro-Gold and Fluoro-Emerald were sometime injected into nearby muscles (e.g. Fluoro-Gold in acromiotrapezius and Fluoro-Emerald in spinodeltoideus), labeled motor neurons were always scrutinized under both UV and FITC filters to detect the presence of any double-labeled neurons. Double labeling neurons, which would indicate leakage of the tracer from one muscle to the other, were never observed. This control ensured that selective retrograde labeling was achieved.

Comparisons of the number of labeled motor neuron for the individual cases revealed that injections targeting the same muscle gave rise to variability in the numbers of positively labeled motor neurons (see Fig. 4). It is noteworthy that such variability has also been reported by others (Hollyday, 1980; Nicolopoulos-Stournaras and Iles, 1983; McKenna et al., 2000). One drawback of the use of retrograde neuronal tracers is that they give, at best, only an approximation of the total number of neurons projecting to the site of injection. For any given muscle, the level of variability in the numbers of positively labeled neurons resulting from injections performed on different animals was comparable with that obtained within the same animals, that is, when bilateral injections of the same muscle were performed. Thus, we are inclined to think that the variability in the number of labeled neurons arising between injections in the same muscle is related to a large extent to differences in intake and/or transport of retrograde tracers. We therefore concur with others that, when based on retrograde transport, the population of motor neurons supplying the skeletal musculature is likely to be underestimated (see Nicolopoulos-Stournaras and Iles, 1983).

Significance for the treatment of spinal cord trauma and other spinal degenerations including motor neuron disease

The organization of the motor neurons supplying the rat forelimb described in the present series of experiments may have significant implications for the development of therapies in rat models of forelimb dysfunction including models of spinal cord injury and motor neuron diseases. For instance, evidence has been gathered that implantation of cells that are genetically modified to secrete therapeutic molecules (e.g. neurotrophins) are neuroprotective and elicit axonal elongation in the injured spinal cord (e.g. Baumgartner and Shine, 1997, 1998a,b; Boulis et al., 2003). With this approach, however, elongating axons tend to stay in the rich environment provided by the cell implants rather than to seek out for and reconnect with their former post-synaptic targets (e.g. Liu et al., 1999), a process that is essential for the recovery of motor function. One way to circumvent this problem is to avoid cellular implants altogether and to deliver these therapeutic compounds into motor neurons located at the level of the spinal cord lesion. This intervention could be achieved via viral vector-mediated gene transfer (Baumgartner and Shine, 1998b; Koda et al., 2004), for example, the intramuscular injection (and resulting retrograde transport through the peripheral nerve) of viral vectors containing the coding sequence for a therapeutic compound could result in the expression of the compound in the corresponding motor neurons to restore motor function. This is best instantiated by Kaspar et al. (2003) wherein thoracic muscle/motor neuron topography was used to specifically rescue respiratory motor function in a mouse model of motor neuron disease. In light of these findings, knowledge regarding the precise relationship between motor neurons and the muscles that they supply, provided by the present study, is critical for studies aiming to restore motor function in specific muscle or muscle groups.

CONCLUSION

This anatomical investigation supports previous observations that, although discrete, some of the motor neuron columns lying in the cervical aspect of the rat spinal cord are inter-digitized. The length of these columns, and hence the overlap between them, appears to be greater than previously reported, particularly within the uppermost segments of the cervical enlargement. This map constitutes a valuable guide for the selection of appropriate muscle(s) for the delivery of therapeutic genes into specific segments of the cervical spinal cord. The organization of the motor neurons supplying the rat forelimb may have significant applications for the development of therapies in rat models of forelimb dysfunctions including models of motor neuron diseases and spinal cord injury.

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