Projections from the paralemniscal nucleus to the spinal cord in the mouse

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Abstract: The present study investigated the projection from the paralemniscal nucleus (PL) to the spinal cord in the mouse by injecting the retrograde tracer fluoro-gold (FG) to different levels of the spinal cord and injecting the anterograde tracer biotinylated dextran amine (BDA) into PL. We found that the PL projects to the entire spinal cord with obvious contralateral predominance – 420 neurons projected to the contralateral cervical cord and 270 to the contralateral lumbar cord. Fibers from PL descended in the dorsolateral funiculus on the contralateral side and terminated in laminae 5, 6, 7, and to a lesser extent in the dorsal and ventral horns. A smaller number of fibers also descended in the ventral funiculus on the ipsilateral side and terminated in laminae 7, 8 and, to a lesser extent in lamina 9. The present study is the first demonstration of the PL fiber termination in the spinal cord in mammals. The PL projection to the spinal cord may be involved in vocalization and locomotion.

Key words: paralemniscal nucleus, spinal cord, fluoro-gold, retrograde tracing, anterograde tracing.

Introduction

As its name implies, the paralemniscal nucleus (PL) is situated close to the lateral lemniscus (ll) and the nuclei of the lateral lemniscus. Most of the nucleus lies medial to the intermediate (ILL) and ventral (VLL) nuclei of the lateral lemniscus (Franklin and Paxinos, 2008).

PL has been known as an important relay for startle response to acoustic, tactile, and vestibular stimuli (Sarno et al., 1993; Hammond et al., 1997). In the rat, injections of

excitatory amino acid antagonist into PL result in a depressed acoustic startle (Spiera and Davis, 1988). As the startle response involves limb and trunk movement, this suggests that PL is connected with the spinal cord. Electrical stimulation of PL induces stepping movement of the contralateral forelimb in thalamic cats (Shimamura et al., 1984). Injections of glutamate into PL mimic the response of electrical stimulation (Shimamura et al., 1990), therefore an amino acid mediated pathway may exist between PL and the spinal cord; this pathway could be monosynaptic or multi-synaptic. A retrograde tracing study showed that PL has contralateral projections to the cervical and upper thoracic cord in the rat (Leichnetz et al., 1978). In other mammals, PL is also intensely labeled after retrograde tracer injections in the upper cervical cord (Nudo and Masterton, 1988). In the mouse, our previous study showed that PL projects to the contralateral spinal cord (Liang et al., 2011). However, the fiber termination in the spinal cord has not been investigated. This present study was designed to reveal the details of the spinal cord projection pattern of PL neurons in the mouse with the use of retrograde and anterograde tracer injections. We found that PL projects to the entire spinal cord with an obvious contralateral predominance and its fibers mainly terminate in contralateral laminae 5 to 7 and ipsilateral laminae 7 and 8. The direct projection from the PL to the spinal cord might be involved in vocalization, locomotion, and the startle response.

Materials and Methods

Animals

Twenty eight C57/BL6 mice (10 to 12 weeks old, with a weight of 25 to 30 g) were used for this study. The mice were obtained from the Animal Resource Center in Western Australia. The experimental procedures were approved by the Animal Care and Ethics Committee of The University of New South Wales (11/75A).

Retrograde tracing

Fourteen mice were used for the retrograde tracing study. Mice were anaesthetised with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) before they were placed in a mouse stereotaxic head holder (Kopf Instruments, Tujunga, CA, USA).

A 5 µl Hamilton syringe (Hamilton Company, Reno, NV, USA) was mounted on a micromanipulator for spinal cord injections. The mouse adaptor was adjusted for optimal exposure of the vertebrae. Upper cervical and upper lumbar spinal cord segments were exposed by laminectomy at C2 and T11 to T12, respectively. The dura on the right side was incised with the tip of a 29-gauge needle and the 5 µl Hamilton syringe (the outer diameter is 0.711mm) was driven through this opening. An injection of 20 to 40 nl of fluoro-gold (Fluorochrome, Denver, Co, USA; diluted to 5% in distilled water) solution was made through multiple punctures into the right side of the spinal cord. The syringe was left in place for 10 minutes following the injections. Altogether, 10 mice were injected with fluoro-gold to the upper cervical and upper lumbar segments (5 mice in each group). The control group either received normal saline injections into the spinal cord (2 mice) or fluoro-gold injections into the cisterna magna (2 mice). At the end of the procedure, the soft tissue and the skin were sutured and topical tetracycline (Pfizer) was sprayed over the incision. To relieve the postoperative pain, subcutaneous buprenorphine (Tamgesic, Reckitt Benckiser) injections were applied.

Anterograde tracing

Fourteen mice were anaesthetised as described above and placed in the stereotaxic head holder. The skin on the skull was retracted and a hole was made in the skull over PL (drill from Fine Science Tools, North Vancouver, BC, Canada). The PL was injected with 10 to 20 nl of biotinylated dextran amine (BDA) solution (10,000MW, Invitrogen) (Bregma: -4.16~-4.72 mm, midline: +1.0~+1.25 mm, surface: -2.175~-2.5 mm; 6 mice) using the same Hamilton syringe as for retrograde tracer injection. Control animals received the same tracer BDA injections either into the cisterna magna (2 mice) or into the adjacent NA7 and oral pontine reticular nuclei in 6 mice. In each case, the syringe was left in place for 10 minutes after the injection. At the end of the procedure, the skin was sutured, buprenorphine was injected subcutaneously, and topical tetracycline was sprayed over the incision.

Tissue preparation

After survival times of 1 week (flouro-gold experiments) or 6 weeks (BDA experiments),

mice were anesthetised with a lethal dose of pentobarbital solution (0.1 ml, 200 mg/ml) and perfused through the left ventricle with 60 ml of 0.9% normal saline containing heparin (150 IU/mouse; Sigma). This was followed by 80 ml of 4% paraformaldehyde (Sigma) prepared in 0.1 M phosphate buffer and finally with 80 ml of 10% sucrose solution. The brain and spinal cord were removed and postfixed in 4% paraformaldehyde for 2h at 4^{o}C, followed by cryoprotection in 30% sucrose in 0.1 M PB solution overnight at 4^{o}C. Serial sections of the brain and spinal cord were cut at 40 µm using a Leica CM 1950 cryostat.

Immunohistochemistry

Brain sections from FG injected mice were washed in 0.1 M PB and treated with 1% H_2O_2 in 50% ethanol for 30 minutes at room temperature. The sections were rinsed in 0.1 M PB and then treated with 5% goat serum in 0.1 M PB to block the non-specific sites. The sections were incubated with the primary anti- FG antibody (Chemicon, 1:4000; raised in rabbit) overnight, rinsed in 0.1 M PB (3×), then treated with the secondary antibody (biotinylated goat anti-rabbit IgG; Sigma, 1:200) for 2h at room temperature. Sections were then washed in 0.1 M PB, and transferred to an extravidin peroxidase solution (Sigma, 1:1000) for 2h. After 3 rinses in 0.1 M PB, the sections were transferred to the 3, 3'-diaminobenzidine (DAB) reaction complex (Vector lab, Burlingame, CA, USA) until optimal color developed. At the end of the procedure, the sections were rinsed, mounted onto gelatinized slides, dehydrated in gradient ethanol, cleared in xylene, and coverslipped. Sections from the dextran injected mouse brains and spinal cords were treated with 1% H_2O_2 in 50% ethanol for 30 minutes at room temperature, followed by 5% goat serum in 0.1 M PB for 30 minutes. Then the sections were transferred to an extravidin peroxidase solution (Sigma, 1:1000) for 2 hours. Subsequent procedures were the same as for the FG immunostain.

Data analysis

Mouse brain sections after FG injections into the spinal cord were scanned with an Aperio scanner (ScanScope XT) under 20× magnification. Scanned images were opened with Imagescope software and images of 4× magnification were extracted and opened

with Adobe Illustrator CS5. DAB stained neurons were mapped with black dots onto the diagrams of mouse brain atlas (Franklin and Paxinos, 2008). Cell numbers were calculated by counting the dots in every second section through the entire paralemniscal nucleus (3 series of sections from upper cervical and upper lumbar cord injections, respectively). Images of 20× magnification were extracted and opened with Image J software to measure the nuclear diameter of DAB labeled neurons. The estimated number of cells in the paralemniscal nucleus was calculated with the corrected Abercrombie formula (1946): $P = A\frac{M}{M+L}$. P is the estimated number of cells, A is the actual count, M is the section thickness, and L is the nuclear length (L was measured in every second section). Images of 10× magnification were also extracted from Imagescope to show DAB labeled neurons in the interested areas.

Sections from the BDA injected mouse brain and spinal cord were photographed with the Aperio scanner. Labeled fibers were compared with the spinal cord diagrams of the atlas of the mouse spinal cord (Watson et al., 2009). To establish the correspondence of the experimental sections with the atlas diagrams, adjacent Nissl sections were used as well as DAB stained sections that were counterstained with Nissl. For diagramatic images, lines representing BDA labeled fibers were drawn on the diagrams extracted from the mouse spinal cord atlas (Watson et al., 2009). We observed vesicular protuberances in association with the DAB labeled fibers in some areas of spinal gray matter. We have assumed that these structures were synaptic boutons, and we refer to them in the Results section as 'boutons'.

Results

Retrograde tracing

In mice with cervical injections of FG, intensely labeled neurons were found in the contralateral PL medial to the lateral lemniscus. The labeled neurons varied in size (21.40±6.46um × 12.99±3.84um). Most of the labeled cells were small and oval shaped, intermingled with some larger round (especially in the area adjacent to the epirubrospinal nucleus- ERS) or spindle shaped (more dorsally located) cells. After

cervical injections of FG, there were, in total, 420.2±15.0 (average nuclear diameter was 9.6±1.6um) labeled neurons. Medially these labeled neurons were continuous with small neurons in the epirubrospinal nucleus, which is dorsal to the rubrospinal tract (rs). FG labeled neurons of PL formed a column extending from the ventral border of the ventral nucleus of the lateral lemniscus (VLL) to the middle of the retrorubral nucleus (RR) dorsally, with most of these labeled neurons located in the caudal two thirds of the nucleus (**Fig. 1 a-c**). Labeled neurons were also found in the ipsilateral PL, but the number of labeled neurons was smaller than that of the contralateral counterpart (data not shown).

After lumbar injections of FG, the number of labeled neurons was much smaller (267.7±39.0) (average nuclear diameter was 9.3±1.2um). There were relatively fewer labeled neurons in the ventralmost and dorsalmost portions of the PL. The density of labeled neurons also decreased in the caudal two thirds of this nucleus. Most of these neurons were spindle shaped (18.67±4.22um × 10.83±2.21um) (**Fig. 2 a-c**).

Anterograde tracing

After injecting BDA into PL (**Fig. 3a-b**), labeled fibers travelled caudally and assumed a course immediately lateral to the pyramidal tract (in the caudal hindbrain lateral to the inferior olive) (**Fig. 3c**). Some fibers crossed the midline to the contralateral side at the level of the injection site. In the caudal hindbrain, contralateral fibers also took a similar course as those on the ipsilateral side, lateral to the pyramidal tract.

In the white matter of the spinal cord, labeled fiber tracts were seen in all segments. On the contralateral side, fiber tracts were observed in the dorsolateral funiculus (**Fig. 3d**), whereas ipsilateral fiber tracts were found in the medial part of the ventral funiculus (**Fig. 3e** and **f**). Some fiber tracts were also seen in the ipsilateral dorsolateral funiculus (**Fig. 3f**). These fibers might have arisen from the rubrospinal tract, which passes through the BDA injection site, since the rubrospinal tract crosses the midline in the midbrain and descends in the area medial to the lateral leminscus).

In the contralateral cervical spinal cord, labeled fibers entered the gray matter from the lateral part of laminae 5, 6, and dorsal lamina 7 and extended towards the central canal (**Fig. 3d**). The largest number of labeled fibers and boutons was seen in these three laminae (**Fig. 3d**). In addition, a small number of fibers and boutons was seen in laminae 4, 8, 9 (adjacent to motor neurons in the ventral horn of the cervical cord), and 10. The ipsilateral projection was smaller than the contralateral one and labeled fibers and boutons were mainly found in laminae 7, 8 and 9 where fibers were adjacent to motor neurons. In laminae 5 and 6, a small number of labeled fibers was seen. They might be fibers arising from inadvertent labeling of the rubrospinal tract.

In thoracic segments, labeled fibers were more medially located in the white matter on the contralateral side, whereas ipsilateral fibers occupied a similar location as those in the cervical cord. In the gray matter, contralateral fibers and boutons were similarly distributed as those in the cervical cord. Labeled fibers were mainly found in laminae 5 and 7, and to a lesser extent in lamina 8 and the dorsal horn (predominantly in lamina 4). Ipsilaterally labeled fibers were seen in ventral lamina 7 and lamina 8. The termination in lamina 5 can be expected to be from the inadvertent contamination of the rubrospinal tract, but it is most likely that the fibers in laminae 7 and 8 originate from the paralemniscal nucleus (**Fig. 3e**).

In the lumbar and sacral cord, the contralaterally labeled paralemniscospinal fiber tract took a superficial position in the white matter (**Fig. 3f**). In the gray matter, the majority of labeled fibers and boutons were found in laminae 5 and 6 (**Fig. 3g**). A few fibers and boutons were also observed in the dorsal commissural nucleus at sacral levels (**Fig. 3g**). A small number of sparsely distributed fibers was seen in laminae 7, 8, and in the lateral part of lamina 9 (where motor neurons are located) (**Fig. 3h**). On the ipsilateral side, fibers and boutons were found in lamina 8 and the central part of lamina 7, and to a lesser extent in lamina 9. Some fibers in lamina 7, as well as labeled fibers in laminae 5 and 6, might have arisen from the rubrospinal tract (**Fig. 3i**).

When BDA was injected to the adjacent noradrenergic group 7 (NA7) (which is caudal to PL) (**Fig. 4a**), labeled fibers were found bilaterally in the spinal cord with an ipsilateral predominance (**Fig. 4b**). The fiber tract was mainly located in the dorsolateral funiculus. In the gray matter, labeled fibers were predominantly distributed in the ventral lamina 5, laminae 6 and 7 and, to a lesser extent in lamina 8 (**Fig. 4c, d**). In the thoracic region of the spinal cord, some fibers terminated on the intermediolateral column (**Fig. 4b, c**).

When BDA was injected to the oral pontine reticular nucleus (PnO), which is medial to PL (**Fig. 5a**), fiber tracts were seen in similar locations as those from PL (**Fig. 5b**). Contralaterally labeled fiber tracts were also found in the dorsolateral funiculus in a more medial location (**Fig. 5b**) and their fibers were mainly found in medial laminae 7, 8, 10, and to a lesser extent in the lateral part of lamina 6 (**Fig. 5c**). Ipsilaterally labeled fiber tracts occupied a wider space in the ventral funiculus and in the ventrolateral funiculus (**Fig. 5b** and **d**). A large number of fibers terminated on laminae 7, 8, 9, and 10 (**Fig. 5d**).

Discussion

The present study found that PL projects to the spinal cord bilaterally with a contralateral predominance. Its fibers mainly terminate in laminae 5, 6, and 7, and to a lesser extent in the dorsal and ventral horns on the contralateral side. On the ipsilateral side, sparsely labeled fibers mainly terminate in laminae 7, 8 and 9.

Spread of spinal cord tracer injections across the midline

In the retrograde tracing study, FG was injected to the right half of the spinal cord. However, some spread of FG to the left side was impossible to control. This complicates data interpretation. For this reason, cell counting was only conducted on the contralateral side. However, anterograde tracer BDA injections to PL revealed that fibers from the PL terminate bilaterally in the spinal cord with a contralateral predominance, and these anterograde tracings do not suffer from problems of contralateral contamination.

Retrogradely labeled neurons in the PL

The present retrograde study showed that PL predominantly projects to the contralateral spinal cord. The PL fibers reach at least as far caudal as the lumbar cord, as demonstrated by the presence of labeled neurons in this nucleus after FG injections into the lumbar cord. This finding is consistent with previous studies (in rat: Leichnetz et al., 1978; Watkins et al., 1981; Leong et al., 1984; Reiner et al., 2008; in rat and cat: Basbaum and Fields, 1979; in monkey: Carlton et al., 1985; in many species: Nudo and Masterton, 1988). However, in the present study a small ipsilateral projection was also observed. The ipsilaterally labeled neurons were similarly distributed in PL as their contralateral counterpart, mainly concentrated in the caudal two thirds of the ipsilateral PL. This is consistent with the finding of a retrograde tracing study in the monkey (Carlton et al., 1985). In that study, PL neurons were labeled after the retrograde tracer HRP was injected into the ventral half of the spinal cord, thus showing that PL fibers descend in the ipsilateral ventral funiculus as well.

Termination of PL fibers in the spinal cord

Although there are minor problems with PL injection sites overlapping the edge of the oral pontine reticular nucleus and the rubrospinal tract. We believe that we have enough evidence to define the spinal projection of PL (see the drawing of PL, PnO, and rs fibers in **Fig. 6**). The spinal projection of PL has the following characteristics:

1. The descending fibers form a contralateral tract in the dorsal part of the lateral funiculus and an ipsilateral tract in the medial part of the ventral funiculus.
2. The contralateral tract terminates mainly in laminae 5 to 7 with fewer fibers in laminae 4, 8, 9, and 10.
3. The ipsilateral tract terminates chiefly in the medial part of laminae 8 and 9.
4. Both the contralateral and ipsilateral tracts extend as far as sacral levels of the spinal cord.

If we accept that this is the definitive pattern of termination of the paralemniscospinal tract, it can be contrasted with the pattern of termination of the adjacent rubrospinal tract and the projection from PnO, since almost all injections of PL may encroach to some degree on these neighboring structures. In the case of the rubrospinal tract, the distinction

is relatively easy since the tract is already crossed at the point when it is close to PL; this means that its tract in the dorsal part of the lateral funiculus and its spinal gray projections will be completely ipsilateral.

In the case of the projection from PnO, the situation is more difficult, because these fibers travel mainly in the ipsilateral ventral funiculus and contralateral dorsolateral funiculus and terminate in similar areas of the spinal cord as those from PL. However, the area occupied by the ipsilateral PnO tract is more extensive than that taken by the ipsilateral paralemniscospinal tract, and PnO terminals are more widespread in the medial part of the ventral horn. The contralateral fiber tract is located in the dorsolateral funiculus. Fibers from this tract predominantly terminate in laminae 7, 8 and, to a lesser extent in laminae 9 and 10. This is consistent with previous studies on the reticulospinal terminals originating from PnO (in rat: Sirkin and Feng, 1987; in cat: Nyberg-Hansen 1965). In the present study, when an injection was made into the rostral part of PL, where pontine reticular neurons are less likely to project to the spinal cord, densely labeled fibers were found in the contralateral dorsolateral funiculus and laminae 4, 5, and 6. Fiber bundles in the ventral funiculus are less widely distributed and the majority of these fibers terminate in laminae 7, 8, and to a lesser extent in lamina 9. This confirms that the distribution of PL fibers in the spinal cord is bilateral.

In the present study and in a previous study (Liang et al., 2011), we showed that the epirubrospinal nucleus (ERS) has contralateral spinal cord projections. In the present anterograde study, ERS is likely to be often involved in BDA injections, therefore, some of the fibers found in the contralateral spinal cord might arise from this nucleus.

BDA injections did involve some other adjacent nuclei that also have spinal cord projections, but the discrete distribution pattern of paralemniscal fibers differentiates them from descending fibers of other nuclei. Fibers from the adjacent NA7 take a position in the dorsolateral funiculus and mainly terminated in laminae 5, 6, 7 and, to a lesser extent in lamina 8 with an ipsilateral preference. Some fibers also terminated bilaterally on the intermediolateral column (**Fig.4c**). This finding is partially consistent

with a previous study on NA7 terminals in the spinal cord (Fritschy and Grzanna, 1990) in that we noted that fibers from NA7 mainly terminate on the intermediolateral column.

The present anterograde tracing study showed that fibers from PL project to the spinal cord bilaterally. We believe that this is the first anterograde study to show the termination of PL fibers in the mouse spinal cord. It confirms the contralateral predominance of PL projections in retrograde studies and the presence of ipsilateral fibers in the ventral funiculus. Furthermore, the present study provides the detailed termination pattern of PL fibers in the spinal cord. In the rat, Reiner et al (2008) found more labeled neurons in the PL after dorsal horn injections with FG. This is consistent with our findings that PL fibers terminate on the dorsal horn as well.

Functional significance

The PL has been shown to be involved in vocalization in physiological (Covey 1993) and pharmacological studies (Shimamura et al., 1990). This is consistent with the fact that PL has connections with the periaqueductal gray, an area involved in vocalization (Marchand and Hagino, 1983; Schuller et al., 1997; Hardy et al.,1983; Hannig and Jürgens, 2006), facial nucleus (Li et al., 1997; Schuller et al., 1997; Panneton and Martin, 1983), and ambiguus nucleus (Metzner 1996). However, the paralemniscal nucleus seems to be involved only in echolocation (Fenzl and Schuller, 2002) and appears to be sufficient but not necessary for call emission (Pillat and Schuller, 1998).

PL also participates in other physiological functions. One pharmacological study showed that excitatory amino acid injected to PL could depress the acoustic startle response (Spiera and Davis, 1988). Another study demonstrated that nociceptive stimuli could change the firing rate of the paralemniscal neurons (Hardy et al., 1983). Furthermore, it has been shown that glutamate injections to PL could induce the contralateral forelimb extension in the cat (Shimamura et al., 1990) and this could be mimicked by electrical stimulation of this nucleus.

Since the vocalization and startle responses must be executed by laryngeal muscles and limb movements, the descending projection of PL to the spinal cord might serve as a pathway mediating the execution of limb movements.

Captions

Fig. 1 Labeled neurons in the PL after cervical cord injections of FG. **a-c**. Diagram of FG labeled neurons in the PL at 3 rostrocaudal levels. Labeled neurons are predominantly found in the caudal 2/3 of the PL. Photomicrographs show labeled neurons after anti-FG immunohistochemical stain. The photomicrograph in the lower right shows the injection site. The scale bar for photomicrographs is 100 μm. The scale bar for the injection site is 200 μm. The right is the ipsilateral side and the left is the contralateral side. This convention applies to all the figures.

Fig.2 Labeled neurons in the PL after lumbar cord injections of FG. **a-c**. Diagram of FG labeled neurons in the PL at 3 rostrocaudal levels. The distribution of labeled neurons in PL is similar to the pattern seen after cervical cord injections. However, there are fewer labeld neurons after lumbar injections of FG. Photomicrographs show labeled neurons after anti-FG immunohistochemical stain. The photomicrograph in the lower right shows the injection site. The scale bar for photomicrographs is 100 μm. The scale bar for the injection site is 200 μm.

Fig. 3 Fiber distribution in the spinal cord after BDA injections to the PL. **a-b**. Injection site of BDA as shown by yellow dashed circle rostrocaudally. **c.** Descending fibers from PL are lateral to the pyramidal tract in the hindbrain. **d**. A section from C5 shows BDA labeled fibers. The contralateral fiber tract travels in the dorsolateral funiculus (the red arrow heads). Fibers and boutons are present mainly in laminae 5, 6, and dorsal 7 (arrows). A smaller number of fibers and boutons is present in laminae 8 and 9 (arrows). **e**. A section from T4 shows BDA labeled fibers and boutons. The fiber tracts in the contralateral dorsolateral funiculus and ipsilateral ventromedial funiculus are indicated with arrowheads. Labeled fibers and boutons are similarly distributed in the thoracic cord as those in the cervical cord. **f**. A section from L4 shows BDA labeled fibers and boutons. Labeled fiber tracts (arrow heads) are similarly distributed as those in cervical and thoracic cord. **g**. A higher magnification photo of the rectangular area in upper left **f** shows labeled fibers and boutons in laminae 4, 5, 6, and dorsal part of 7 (arrows). **h**. A

higher magnification photo of the rectangular area in lower left **f** shows labeled fibers and boutons in laminae 7, 8, and 9 (arrows). Note some boutons terminate next to motor neurons in the central and lateral lamina 9 (arrows). **i**. A higher magnification photo of the rectangular area in lower right **f** shows labeled fibers and boutons in ipsilateral laminae 7, 8, and 9 (arrow). The scale bar for **a-b** and **f** is 200 μm, the scale bar for **c-e** is 100 μm, the scale bar for **g-i** is 50 μm.

Fig. 4 Fiber distribution in the spinal cord after rostral A7 injections of BDA. **a**. Injection site of rostral A7 (the Kölliker-Fuse nucleus) (red dashed circle). **b**. Lower magnification of labeled fiber tracts in bilateral dorsolateral funiculi (arrows) of T2. Note there are more fibers on the ipsilateral side than the contralateral side. **c**. Higher magnification of labeled fibers and boutons in the rectangular area of **b**. Labeled fibers and boutons are found in laminae 5 and 7 (arrows) with an ipsilateral predominance. Note boutons terminating on bilateral intermediolateral columns (arrows). **d**. A section showing labeled fibers and boutons in S3 (arrows). Boutons are similarly distributed as those in the thoracic cord. Note the fiber and boutons in the sacral dorsal commissural nucleus (arrow). Scale bar for **a** and **b** is 200 μm. The scale bar for **c** and **d** is 100 μm.

Fig. 5 Fiber distribution in the spinal cord after BDA injections to PL and the lateral PnO. **a**. Injection site in PL and the lateral PnO (black circled area). **b**. A section showing labeled fiber tracts in bilateral C5. Note there are more fibers in the ipsilateral ventral funiculus and these fibers are more widely spread than those from PL (thick arrows). The contralateral fiber tract (thick arrows) and the ipsilateral rubrospinal tract (star) are also seen. **c**. Contralaterally labeled fibers and boutons in the left rectangular area of **b**. Fibers are mainly located in the central and medial parts of the gray matter, including laminae 7, 8, 9, and 10 (arrows). Note the contralateral fiber tracts (thick arrow) **d**. Ipsilaterally labeled fibers and boutons in the right rectangular area of **b**. Fibers are mainly located in the medial part of the gray matter, including laminae 7, 8, 9, and 10 (arrows). Note the ipsilateral rubrospinal tract is also seen (star). The scale bar for **a** and **b** is 200 μm, the scale bar for **c** and **d** is 100 μm.

Fig. 6 A diagram of fiber projections from PL, PnO, and the red nucleus in the spinal cord. **a**. PL fiber terminal distribution in the spinal cord. The ipsilateral fiber tract is located in the medial part of the ventral funiculus and its fibers are found in the medial laminae 7 and 8, and to a lesser extent in laminae 9 and 10. The contralateral fiber tract is located in the dorsolateral funiculus and its fibers are predominantly found in laminae 5 to 7 with a smaller number of fibers in the dorsal horn (especially in lamina 4) and medial ventral horn. The density of the contralateral fibers is higher than that of the ipsilateral fibers. **b**. PnO fiber terminal distribution in the spinal cord. The ipsilateral PnO tract occupies a wider space in the ventral funiculus than the PL fiber tract. Its fibers terminate on laminae 7, 8, 9, and 10. The contralateral fiber tract is also in the dorsolateral funiculus and its fibers are found in medial laminae 7, 8, 9, and 10, and to a lesser extent in the lateral part of lamina 6. **c**. Rubrospinal fiber distribution in the spinal cord. The ipsilateral rubrospinal tract is located in the dorsolateral funiculus and its fibers are found in laminae 5, 6, and 7.

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