The red nucleus and the rubrospinal projection in the mouse

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Abstract We studied the organization and spinal projection of the mouse red nucleus with a range of techniques (Nissl stain, immunoflurorescence, retrograde tracer injections into the spinal cord, anterograde tracer injections into the red nucleus, and in *situ* hybridization) and counted the number of neurons in the red nucleus (3200.96±230.80). We found that the rubrospinal neurons were mainly located in the parvicellular region of the red nucleus, more lateral in the rostral part and more medial in the caudal part. Labeled neurons were least common in the rostral and caudal most parts of the red nucleus. Neurons projecting to the cervical cord were predominantly dorsomedially placed and neurons projecting to the lumbar cord were predominantly ventrolaterally placed. Immunofluorescence staining with SMI-32 antibody showed that approximately 60% of SMI-32 positive neurons were cervical cord projecting neurons, and 24% were lumbar cord projecting neurons. SMI-32 positive neurons were mainly located in the caudomedial part of the red nucleus. A study of vGluT2 expression showed that the number and location of glutamatergic neurons matched those of the rubrospinal neurons. In the anterograde tracing experiments, rubrospinal fibers travelled in the dorsal portion of the lateral funiculus, between the lateral spinal nucleus and the calretinin positive fibers of the lateral funiculus. Rubrospinal fibers terminated in contralateral laminae 5, 6, and the dorsal part of the lamina 7 at all spinal cord levels. A few fibers could be seen next to neurons in the dorsolateral part of lamina 9 at levels of C8-T1 (hand motor neurons) and L5-L6 (foot motor neurons), which is consistent with a view that rubrospinal fibers may play a role in distal limb movement in rodents.

Key words: red nucleus; rubrospinal tract; midbrain; prosomere 1; GAD67; vGluT2; C1QL2; SMI-32

Introduction

The red nucleus is a distinct neuronal cluster in all vertebrates, and it has traditionally been considered to be located in the midbrain (Wild et al., 1979; ten Donkelaar et al., 1981; Prasada Rao et al., 1987; Nudo and Masterton, 1988). However, developmental and gene expression data show that this nucleus has two origins: the caudal part belongs to the midbrain but the rostral part belongs to the diencephalon (Puelles et al., 2011). The rostral part lies caudal to the fasciculus retroflexus, and the caudal part ends just rostral to the decussation of the superior cerebellar peduncle. The oculomotor nerve traverses only in the midbrain part of the red nucleus (**Fig. 1a**). Though the red nucleus is traditionally divided into the parvicellular part (RPC) and the magnocellular part (RMC), neurons of varying size are found in each part (Huber et al., 1943). Therefore the morphological division of the red nucleus does not by itself completely define the component parts of this nucleus.

Anatomical and physiological studies in species other than mice have shown that the red nucleus plays an important role in locomotion through its connections with the interneurons (Nyberg-Hansen and Brodal, 1964; Nyberg-Hansen, 1966; Warner and Watson, 1972; Wild

et al, 1979; Holstege et al, 1988; Küchler et al, 2002) and motor neurons in the spinal cord (Holstege 1987; Holstege et al 1988; Küchler et al, 2002), but species differences were also observed (Brown 1974; Küchler et al, 2002), indicating that rubrospinal neurons may play different roles in different species (Huber et al., 1943; Liang et al., 2011). Some spinal cord injury research on the impact of spinal cord injury focused on the degeneration and regeneration of the red nucleus neurons and rubrospinal axons (Guízar-Sahagún et al., 2005; Harvey et al., 2005; Cao et al., 2008; Chen et al., 2008; Jefferson et al., 2011). This indicates that it would be valuable to have a more detailed map of the organization of the red nucleus in rodents. We have attempted to produce such a map in the mouse, using Nissl staining, *in situ* hybridization, and immunofluorescence, in combination with retrograde and anterograde tracer injections. We found that large and small neurons were present in both the rostral and caudal parts of the red nucleus, and that the number and location of glutamatergic neurons matched those of the rubrospinal neurons. We conclude that rubrospinal neurons are probably all glutamatergic. Other major features of the rubrospinal neurons and their axons in the mouse were similar to those of other mammals. We believe that the present study provides a new perspective on the organization of the red nucleus in the mouse, and that the details will be of value to spinal cord injury research.

Material and methods

Animals

Thirty six C57/BL6 mice (10-12 weeks old, weight 25-30 g) were used for this study. 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 (08/48B).

Retrograde tracing

Twenty mice were used for the retrograde study. They were anaesthetised with an intraperitoneal injection of ketamine (67 mg/kg) and xylazine (10 mg/kg) before they were mounted 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 injection. 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/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 of the Hamilton syringe is 0.711 mm) 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, 16 mice were injected with fluoro-gold to the upper cervical and upper lumbar segments (8 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

Sixteen mice were anaesthetised with an intraperitoneal injection of ketamine (67 mg/kg) and xylazine (10 mg/kg), and then they were mounted in a mouse stereotaxic head holder (Kopf Instruments, Tujunga, CA, USA). A 5 µl Hamilton syringe (Hamilton Company, Reno, NV,

USA) (same as retrograde study) was mounted on a micromanipulator for tracer injection. After incising the skin, the skull was drilled with a bone drill (Fine Science Tools, North Vancouver, BC, Canada). 10-20 nl biotinylated dextran amine solution (Invitrogen) was injected into the red nucleus (Bregma: -3.16-4.16 mm, midline: +0.5~1.0 mm, surface: -3.75~4.25 mm; 8 mice). Control animals received the same tracer injections either into the cisterna magna (2 mice) or into the adjacent brain areas surrounding the red nucleus (5 mice). In each case the syringe was left in place for 10 minutes after the injection. At the end of the procedure, the soft tissue and the skin were sutured, Tamgesic was injected subcutaneously, and tetracycline was sprayed over the incision.

Tissue preparation

After a period of 7 days and 6 weeks survival time for the FG and BDA injections respectively, mice were anesthetised with a lethal dose of pentobarbitone sodium (0.1 ml, 60 mg/ml) and perfused through the left ventricle with 60 ml of 0.9% normal saline containing heparin (150 IU/mouse; Sigma), followed by 80 ml of 4% paraformaldehyde (Sigma) prepared in 0.1 M phosphate buffer (PB: Na_2HPO_4, 0.1 M; NaH_2PO_4, 0.1 M; pH = 7.4) and finally with 80 ml of 10% sucrose solution. The brain and spinal cord were removed and postfixed in 4% paraformaldehyde for two hours at 4^{o}C, followed by cryoprotection in 30% sucrose in 0.1 M PB solution overnight at 4^{o}C. 40 µm thick serial coronal sections were cut from the brain and spinal cord using a Leica cryostat CM 1950. Every third sections from FG injected mice were mounted directly to slides and coverslipped with anti-fade fluorescent mounting medium (Dako). The other two series of sections were stained by Nissl and neurofilament H non-phosphorylated (SMI-32) antibody respectively. Sections from BDA injected mice were washed in 0.1 M PB, and reacted with HRP conjugated extravidin (Sigma) for 2h at room temperature after deactivating the endogenous peroxidise with 1% H_2O_2. After a rinse in 0.1 M PB, the sections were transferred to the 3,3'-Diaminobenzidine (DAB) reaction complex (Vector lab, Burlingame, CA, USA) till optimal colour developed. Sections were rinsed, mounted onto gelatinized slides, dehydrated in gradient ethanol, cleared in xylene, and coverslipped. Some sections were counterstained with Nissl stain.

Immunofluorescence

The sections from 6 FG injected mouse brains (3 from cervical injections of FG, 3 from lumbar injections of FG) were incubated in the primary neurofilament H non-phosphorylated (SMI-32) monoclonal antibody (Covance, 1:1000; raised in mouse) and sections from 3 BDA injected mouse spinal cord were incubated in anti-calretinin polyclonal antibody (Chemicon, 1:2000; raised in rabbit) overnight after washing in 0.1 M PB. The mouse brain sections were then treated with the Alexa fluor 594 conjugated goat anti- mouse IgG (Invitrogen, 1:200), mouse spinal cord sections were treated with Alexa fluor 594 conjugated goat anti- rabbit IgG (Invitrogen, 1:200) and Alexa fluor 488 conjugated avidin (Invitrogen, 1:200) for 2 hours at room temperature. At the end of the procedure, the sections were rinsed, mounted onto slides, and coverslipped with anti-fade fluorescent mounting medium (Dako).

Data analysis

After fluoro-gold injections to the spinal cord and BDA injections to the red nucleus, brain (including SMI-32 antibody stained sections) and spinal cord sections were examined with a Nikon Eclipse 80i microscope, with reference to a mouse brain atlas (Franklin and Paxinos, 2008) and a spinal cord atlas (Watson et al, 2009). Photomicrographs (including DAB stained sections and fluorescent sections) were taken with the same microscope. FG and SMI-32 antibody labeled neurons in the red nucleus were counted in every third section across the

entire red nucleus using the Stereoinvestigator software (MicroBrightfield, Williston, VT, USA). *In situ* hybridization data for the complement component 1, q subcomponent-like 2 gene (C1QL2), vesicular glutamate transporter 2 gene (vGluT2), glutamic acid decarboxylase gene (GAD67) were obtained from the Allen Brain Atlas website (http://mouse.brain-map.org). Positive neurons were counted in every fourth sections for C1QL2 gene and vGluT2 gene, and every eighth sections for GAD67 gene. Nuclear diameter was measured with the Stereoinvestigator software. For Nissl stained sections, only neurons with a visible nucleolus were counted. In cases of FG stained sections, a nucleolus is not visible and the nucleus looks like an empty dark area in the center of bright FG cytoplasmic staining. We simply measured the maximum diameter of the dark area and recorded it as the nuclear diameter. We found that the nuclear diameter of FG labeled neurons (12.05±2.18um) was very similar to that of Nissl stained neurons from the red nucleus (12.02±2.16um). In cases of in situ hybridization stained sections, intense staining is present not only in the nucleus of neurons but also in the cytoplasm. This made it virtually impossible to find a clear boundary between the nucleus and the cytoplasm. We therefore were forced to assume that the diameter of these neurons was the same as that measured in Nissl stained neurons from the same region. The counts of rubrospinal neurons, and SMI-32, C1QL2, vGluT2, and GAD67 positive neurons were corrected with the Abercrombie formula (1946). In this formula, the corrected count (A) is calculated by multiplying the total number of neurons counted (P) by a factor in which the section thickness is divided by the section thickness plus the diameter of the neuronal nucleus. The cap effect in neuronal counting is worth considering, but we feel for the purposes of this study that the application of the Abercrombie correction is sufficient to generate meaningful data.

Results

Neuronal populations in the red nucleus

The red nucleus in the mouse extends from the caudal midbrain to the rostral boundary of the first diencephalic prosomere (p1), just caudal to the fasciculus retroflexus (fr). The part which lies in the midbrain is predominantly magnocellular, whereas the rostral (diencephalic) part contains both small and large neurons according to Nissl stained sections (**Fig. 1a-c**). The midbrain part of the nucleus is traversed by the emerging fibers of the oculomotor nerve. We have attempted to further characterize the neuronal populations of the red nucleus in terms of their descending connections, Nissl histology, immunofluorescence, and gene expression.

FG injections into both the upper cervical (**Fig. 2a**) and lumbar cord (**Fig. 2i**) revealed labeled neurons in both the rostral and caudal parts of the red nucleus. The majority of labeled neurons in the rostral part were located in the ventrolateral portion of the contralateral red nucleus (**Fig.2b-d, j-l**). Labeled neurons were more medially placed in more caudal sections (**Fig. 2e-h, m-p**). A topographic organization of these rubrospinal neurons was observed. Neurons projecting to the lumbar cord were more ventrolaterally located than those projecting to the cervical cord. After counting every third section, the estimated number of labeled neurons on the contralateral side (the total number of labeled neurons) after cervical injections was 2117.3±154.8 and these neurons were approximately two thirds (66.2±9.2%) of the total neurons (3200.9±230.8, calculated from Nissl stained sections) in the red nucleus. Lumbar injections of FG resulted in 789.4±78.4 labeled neurons — approximately one quarter (28.0±4.1%) of the total number of neurons. By calculating the percentage of labeled neurons in each section (every third section) against the total number of labeled neurons (total number of every third sections), it could be seen that sections in the middle part of the red nucleus had the largest number of labeled neurons. Numbers of labeled neurons tapered in the

rostral and caudal parts of the red nucleus (**Fig. 2q-s**). Double staining with SMI-32 showed that 59.9±11.1% of the SMI-32 positive neurons (total number was 441.9±99.6) were cervical cord projecting neurons and 24.4±2.24% of SMI-32 positive neurons were lumbar cord projecting neurons. The most double labeled neurons were located in the ventromedial part of the red nucleus, and fewer neurons were located in the lateral part of the rostral half of the red nucleus (**Fig. 3a-c; Fig. 4a-c**). However, the overall percentage of double labeled neurons was higher in the caudal part of the red nucleus than in the rostral part (**Fig. 3d-f; Fig. 4d-f**).

In situ hybridization against the mRNA of complement component 1, q subcomponent-like 2 gene (C1QL2), a landmark for red nucleus neurons, shows that C1QL2 positive neurons are mainly ventrolaterally distributed in the rostral part and ventromedially distributed in the caudal part of the red nucleus (data from Allen Brain Atlas- http://mouse.brain-map.org) (**Fig. 5a-c**). This suggests that the majority of C1QL2 positive neurons are located in the conventionally recognized magnocellular part of the red nucleus with a total number of 2244.2±187.2, which is close to the number of cervical projecting red nucleus neurons.

However, *in situ* hybridization against the mRNA of glutamic acid decarboxylase 1 gene (GAD67) and solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6 gene (Slc17a6, also known as vesicular glutamate transporter 2-vGluT2) (data from Allen Brain Atlas- http://mouse.brain-map.org) shows that the caudal part of the red nucleus is positive only for vGluT2, whereas the rostral part is positive for both vGluT2 and GAD67 genes (**Fig. 6a-d**). By counting the number of vGluT2 and GAD67 positive neurons, we find that 2181.7±12.1 neurons are positive for vGluT2 and their location is very similar to that of the rubrospinal neurons. We find that 2353.7±65.5 neurons are positive for GAD67 gene. The total of vGluT2 gene and GAD67 gene positive neurons is approximately 4500, which is greater than the total number of neurons counted in Nissl stained sections (3200.96±230.80). However, it has been shown that GABA and glutamate can coexist in adult mouse neocortex neurons (Hill et al., 2000). Therefore, the higher number may be explained by the possibility that neurons expressing both GABA and glutamate are common in the rostral part of the red nucleus.

Anterograde labelling

Injections of BDA into the rostral red nucleus (**Fig. 7a)** showed that labeled fibers crossed the midline at the level of the ventral tegmental decussation and then travelled caudally. In the rostral hindbrain, labeled fibers were located medial to the intermediate nucleus of the lateral lemniscus (**Fig. 7b**) and the motor root of the trigeminal nerve. At the level of the motor trigeminal nucleus (5N), fibers went through between the motor trigeminal nucleus and the principal sensory trigeminal nucleus (Pr5) and then became applied to the ventrolateral surface of the hindbrain, ventromedial to the facial nerve (7n) (**Fig. 7c**). At the level of the facial nucleus (7N), fibers moved to a location medial to the spinal trigeminal nerve (sp5) and ventrolateral to the facial nucleus. These fibers maintained this location in the rest of the hindbrain.

In the spinal cord, the rubrospinal tract was seen in the dorsolateral part of the lateral funiculus (**Fig. 7d, e, g, h**) in the entire spinal cord on the contralateral side, ventral to the lateral spinal nucleus. The rubrospinal fibers were located between the lateral spinal nucleus and the calretinin positive fibers in the lateral funiculus (**Fig. 7f**). This tract formed a wedge shape with the tip going towards the gray matter. In the gray matter of the spinal cord, densely labeled fibers were mainly seen in laminae 5, 6, and the dorsal portion of lamina 7

(**Fig. 7d, g, h, l**). Fewer fibers were found in lamina 8 and 10 after rostral red nucleus injections. Some fibers crossed the area 10 and extended to the other side (**Fig. 7m**). On the ipsilateral side, the rubrospinal tract could also be seen, but with fewer fibers, and projections of these fibers to the gray matter were very sparse in laminae 5, 6, and dorsal part of lamina 7 (**Fig. 7i and j).** Ipsilateral fibers were not seen in the lumbar cord and lower segments. Since the injection site in one case also involved a portion of the ventral tegmental decussation, it is possible that these ipsilateral fibers might not originate from the ipsilateral red nucleus, but from the red nucleus contralateral to the injection site. An injection of BDA into the rubrospinal tract below the decussation (data not shown) confirmed that fibers of passage could be labeled with BDA.

Injections of dextran into the nuclei that lie dorsal to the red nucleus, including the periaqueductal gray (PAG), interstitial nucleus of Cajal (InC), nucleus of Darkschewitsch (Dk), and the mesencephalic reticular nucleus (mRt) resulted in distinctly different distribution patterns. Projections from these nuclei mainly travelled in the ipsilateral ventromedial funiculus and spread mainly in lamina 7, 8, and 9. Their fibers also extended towards the dorsal horn, and could therefore be differentiated from the terminating pattern of rubrospinal fibers (**Fig. 7m**).

Injections to the middle part of the red nucleus resulted in densely labeled fibers in the ventral part of lamina 5, lamina 6, and 7 in the cervical cord. In C8 and T1 segments, some fibers were seen in the dorsolateral hand motor neuron group (see Watson et al., 2009). The thoracic and lumbar segments had fewer labeled fibers in laminae 5, 6, and 7. When dextran was injected to the dorsal part of the caudal red nucleus, more densely labeled fibers were found in lamina 5 in the lumbar cord (**Fig. 7k, l**). In the latter case, the injection site spread to the lateral periaqueductal gray, and labeled fibers were mainly seen in lamina 5 of the ipsilateral spinal cord (data not shown).

When the caudal most part of the red nucleus was injected (**Fig. 7n**), densely labeled fibers were seen in the laminae 5, 6, and 7 of lumbar and sacral segments (**Fig. 7q and r**). A few fibers were also observed in lamina 7 in segments of the thoracic cord. In T1 and L5-L6, a small number of fibers was seen in the dorsolateral motor neuron group (**Fig. 7o-r**).

Discussion

Subdivisions of the red nucleus

We have studied the organization of the mouse red nucleus with a variety of techniques. We have found that the red nucleus is not a homogeneous neuron group; it contains a number of discrete populations of neurons. Rubrospinal neurons occupy the ventrolateral portion of the rostral part of the red nucleus and the dorsomedial portion of the caudal part of this nucleus. vGluT2 gene and C1QL2 gene positive neurons are present in the same regions as rubrospinal neurons. The distribution of SMI-32 positive neurons is more limited: they are mainly located among rubrospinal neurons in the ventromedial part of the red nucleus; approximately 60% of SMI-32 positive neurons are double labeled by FG after cervical injections of FG, 24% are double labeled by FG after lumbar injections of FG.

It has been customary to divide the red nucleus into a rostral parvicellular part and a caudal magnocellular part (Huber et al., 1943). However, gene expression data on the development of the red nucleus show that the rostral part of the red nucleus belongs to prosomere 1 of the diencephalon, whereas the caudal part of the red nucleus, traversed by the oculomotor nerve,

belongs to the midbrain (Puelles et al., 2011).

Our retrograde tracing experiments show that rubrospinal neurons are found in both the rostral and the caudal parts of the red nucleus, and there is no clear boundary to separate these two spinal projecting neuron groups. In fact, there were actually more rubrospinal neurons in the rostral diencephalic part than in the caudal midbrain part of the red nucleus (see Fig. 2Q-S). However, the correlation of retrograde labelling and *in situ* hybridization data shows that the rubrospinal neurons are very likely to be vGluT2 positive. Surprisingly, the rubrospinal neurons located in the area that lacks GAD67 expression constitute approximately 36% of the total rubrospinal neurons, and those in the GAD67 expression area constitute 64%. Note that the area lacking GAD67 expression contains the minority of the vGluT2 expressing neurons, and represents the midbrain (m1) part of the nucleus. No sharp boundary is observed between the diencephalic and midbrain parts of the red nucleus, but it must be pointed out that the present study provides evidence for the segmentation of the red nucleus.

Double staining with SMI-32 antibody in FG stained sections after spinal cord injections shows that 60% of SMI-32 positive neurons are cervical cord projecting neurons, and the majority of them are in the medial portion of the caudal 2/3 of the red nucleus. Therefore, SMI-32 serves as a useful signal for rubrospinal neurons, especially for those in the caudal part. In a primate spinal cord injury study, SMI-32 antibody was used as a marker to label rubrospinal neurons (Wannier-Morino et al., 2008). After unilateral section of the spinal cord, this study showed that the number of SMI-32 positive neurons was decreased. It is interesting to note that the total number of SMI-32 positive neurons in this monkey study ranged from 39 to 174. In contrast, the mouse has more than 400 SMI-32 positive neurons, and 60% of these neurons project to the cervical cord. Considering the fact that the mouse is a very small mammal, this is consistent with a view that the rubrospinal projection is considerably more important for limb movement in the mouse than in the monkey.

C1QL2 expression is also a useful marker for mouse red nucleus neurons, and the number of C1QL2 positive neurons is similar to that of FG labeled neurons. However, their location, compared to the results of retrograde labelling, suggests that some of them are not spinal cord projecting neurons, especially in the rostral part of the red nucleus. This is further evidence that the red nucleus contains overlapping populations of different neuron types.

The rubrospinal tract

The mouse rubrospinal tract crosses the midline in the ventral tegmental decussation and travels in the ventrolateral hindbrain. In the spinal cord this tract occupies a dorsolateral position, taking a wedge shape. In the gray matter of the spinal cord, fibers are mainly distributed in laminae 5, 6, and the dorsal part of lamina 7. Some fibers are also observed in the dorsolateral motor neuron groups in C8-T1 and L5-L6. An ipsilateral rubrospinal tract is also present but with fewer fibers terminating in laminae 5, 6, and the dorsal part of lamina 7. Our result is consistent with previous studies on other species (rat: Brown, 1974; Antal et al., 1992; Yasui et al., 2001; Küchler et al., 2002; pigeon: Wild et al., 1979; cat: Nyberg-Hansen and Brodal, 1964; Nyberg-Hansen 1966; Gibson et al., 1984; McCurdy et al., 1987; Holstege 1987; Fujito and Aoki, 1995; tree shrew: Murray et al., 1976; monkey: Shapovalov et al., 1971; Ralston et al., 1988; marsupial-possum: Warner and Watson, 1972).

Our results confirm that the dorsolateral motor neuron group in the C8-T1 is innervated by the rubrospinal fibers as reported in some other studies (Shapovalov et al., 1971; Holstege

1987; McCurdy et al., 1987; Ralston et al., 1988; Fujito and Aoki, 1995; Küchler et al., 2002), but also show that the dorsolateral motor neuron group at L5-L6 receives rubrospinal projections. This group supplies the foot muscles (Watson et al., 2009). This latter observation has previously been reported in physiological studies (Hongo et al., 1969; Shapovalov et al., 1971) and in one anatomical study on the monkey (Holstege et al., 1988). The present study is the first one to show the anatomical evidence of probable monosynaptic connections between the red nucleus and distal limb motor neuron groups in the mouse. This suggests the important role of the red nucleus in the distal limb movement.

Ipsilateral rubrospinal tract and fibers were observed in the present study, but they are few in number. Our retrograde tracing studies showed that labeled neurons are present in the ipsilateral red nucleus. This is similar to findings in the rat (Antal et al., 1992), cat (Holstege 1987), and a marsupial (Warner and Watson, 1972). In the cat, ipsilateral fibers were shown to terminate only in the cervical and upper thoracic cord (Holstege, 1987). In the present study, a few ipsilateral fibers were seen in the cervical and thoracic cord in one case. However, in this case the injection site spread into the ventral tegmental decussation, and it is possible that these fibers of passage might have been labeled by the injection. In a separate experiment in which the injection site was centred on the rubrospinal tract caudal to the decussation, labeled fibers were found in the ipsilateral spinal cord, showing that BDA can be taken up by fibers of passage. A further possibility is that rubrospinal fibers that cross the midline in the spinal cord might have been labeled in retrograde tracing experiments (Liang et al, 2011), and this label might be transmitted to neurons in the ipsilateral red nucleus (after recrossing in the ventral tegmental decussation).

In the mouse spinal cord, calretinin antibody staining reveals a negative area between the calretinin positive fibers in the dorsolateral funiculus which corresponds with the location of the rubrospinal tract (Watson et al., 2009). We found that the rubrospinal tract is located between the calretinin positive fibers in the dorsolateral funiculus, with a small number of rubrospinal fibers intermingled with the calretinin positive fibers. Therefore, calretinin antibody staining may serve as a useful landmark for studies that focus on the degeneration and regeneration of rubrospinal fibers after spinal cord injury.

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Figure legends

Fig. 1
a. A diagram showing the location of the red nucleus in a horizontal section of the mouse brain. The diencephalic (p1) part is caudal to the fasciculus retroflexus and rostral to the oculomotor nerve fibers. The midbrain (m1) part is traversed by the oculomotor nerve fibers and rostral to the decussation of the superior cerebellar peduncle. p1: prosomere 1, m1: mesomere 1. The thick dashed line indicates the contour of the red nucleus. The thin dashed line indicates the probable location of the boundary between the p1 and m1. **b.** A Nissl-

stained coronal section through the rostral part of the red nucleus. Large (star) and small (arrow) neurons are seen in the red nucleus, but the majority are small neurons. **c.** A Nissl-stained coronal section through the caudal part of the red nucleus. Large (star) and small (arrow) neurons are seen in the red nucleus at this level, but the majority are large neurons. con: contralateral, ip: ipsilateral, ml: medial lemniscus, mlf: medial longitudinal fasciculus. The scale bar is 50μm.

Fig. 2
Labeled neurons in the red nucleus after spinal cord injections. **a.** An injection site in the upper cervical cord. **b-h.** Labeled neurons in the red nucleus after the injection shown in **a**. Labeled neurons are placed more medially in more caudal sections in the red nucleus. **i.** An injection site in the upper lumbar cord. **j-p.** Labeled neurons in the red nucleus after the injection shown in **i**. The distribution of labeled neurons is similar to that seen after cervical injections, but their position is more ventral and lateral. **q and r.** The percentage of labeled neurons in each section (every third section) compared to the total number of labeled neurons in every third sections in the red nucleus after cervical and lumbar injections respectively. The largest proportion of labeled neurons is in the middle sections of the red nucleus. The distribution of rubrospinal neurons rostrocaudally is similar. **s.** A diagram showing the distribution of labeled neurons (black dots) in each section. D: dorsal, L: lateral. The scale bar for injection sites is 100 μm. The scale bar for photographs of the red nucleus is 50 μm.

Fig. 3
Double labelling with SMI-32 antibody in FG stained sections after cervical injections. **a-b.** A rostral section stained with FG (**a**) and SMI-32 antibody (**b**). Double-labeled neurons (arrows) are 50.0% of SMI-32 positive neurons. **c.** Merged photo of FG and SMI-32 antibody staining. **d-e**. A caudal section stained with FG (**d**) and SMI-32 antibody (**e**). Double labeled neurons (arrows) are 80.0% of SMI-32 positive neurons. **f.** Merged photo of FG and SMI-32 antibody staining. Note the oculomotor nerve fibers (star) traversing in the red nucleus. D: dorsal, L: lateral. Scale bar is 50 μm.

Fig. 4
Double labelling with SMI-32 antibody in FG stained sections after lumbar injections. **a-b.** A rostral section stained with FG (**a**) and SMI-32 antibody (**b**). Double labeled neurons (arrows) are 15.0% of SMI-32 positive neurons. **c.** Merged photo of FG and SMI-32 antibody staining. **d-e.** A caudal section stained with FG (**d**) and SMI-32 antibody (**e**). Double labeled neurons (arrows) are 50.0% of SMI-32 positive neurons. **f**. Merged photo of FG and SMI-32 antibody staining. D: dorsal, L: lateral. Scale bar is 50 μm.

Fig. 5
Images of sections of *in situ* hybridization preparations to show expression of C1QL2 gene from Allen Brain Atlas website (http://mouse.brain-map.org). **a.** A rostral section of the red nucleus. C1QL2 positive neurons are located in the lateral portion of the red nucleus. **b.** A middle section of the red nucleus. C1QL2 positive neurons are located in the ventral and dorsomedial portions of the red nucleus. **c.** A caudal section of the red nucleus. C1QL2 positive neurons are located in the medial portion of the red nucleus. The scale bar is 211 μm.
We are indebted to the creators of Internet-based collections of brain anatomy whose sections we have used in the preparation of the present paper. It is the Allen Institute for Brain Science (Lein et al., 2007 and the Allen Brain Atlas [Internet], 2008 — http://mouse.brain-map.org/welcome.do).

Fig. 6
Images of sections of *in situ* hybridization preparations to show expression of vGluT2 gene and GAD67 gene from Allen Brain Atlas website (http://mouse.brain-map.org). **a.** A section shows that vGluT2 positive neurons are present in the prosomere 1 part of the red nucleus (p1R). **b.** A section adjacent to **a** shows that GAD67 positive neurons are present also in p1R. **c.** A caudal section shows that GAD67 positive neurons are absent in the mesomere 1 part of the red nucleus (m1R), but present in the pararubral nucleus (PaR). **d.** A section adjacent to **c** shows that vGluT2 positive neurons are present in the m1R. Dk: nucleus of Darkschewitsch, 3N: oculomotor nucleus, IP: interpeduncular nucleus, mlf: medial longitudinal fasciculus. The scale bar is 314 µm. We are indebted to the creators of Internet-based collections of brain anatomy whose sections we have used in the preparation of the present paper. It is the Allen Institute for Brain Science (Lein et al., 2007 and the Allen Brain Atlas [Internet], 2008 — http://mouse.brain-map.org/welcome.do).

Fig. 7
Labeled rubrospinal fibers in the spinal cord after BDA injections to the red nucleus. **a.** Injection site in the rostral red nucleus which involves the interstitial nucleus of Cajal (star). **b.** Crossed rubrospinal fibers (arrow) in the rostral hindbrain, medial to the intermediate nucleus of the lateral lemniscus (arrow). **c.** Rubrospinal fibers in the hindbrain, medial to the facial nerve. **d.** Rubrospinal fibers in the contralateral C3 (arrow). Note the ipsilateral fibers, which arise from the interstitial nucleus of Cajal. **e.** Rubrospinal fibers in the contralateral C7. The rubrospinal fibers are present in laminae 5, 6, and 7 (arrows). **f.** Alexa fluor 488 labeled rubrospinal fibers (arrows) and Alexa fluor 594 labeled calretinin fibers (stars) and neurons in the contralateral C4. Note some rubrospinal fibers mix with calretinin positive fibers (lower arrow). **g.** Labled rubrospinal fibers in the contralateral T5. Fibers are present in lamina 5, dorsal lamina 7, and the intercalated nucleus (arrows). Note some fibers cross the midline and extend into the ipsilateral side. Ipsilateral fibers from the interstitial nucleus of Cajal are present in laminae 5, 7, and 8. **h.** Dense fibers in lamina 5 in L4 (arrow). **i.** Injection site to the medial part of the caudal red nucleus (arrow). Note the crossing fibers ventral to the injection site. **j.** Ipsilateral rubrospinal fibers in laminae 5 and 6 in C4 (arrows). Some fibers in laminae 7 and 8 are from the interstitial nucleus of Cajal. **k.** Injection site in the dorsal portion of the caudal red nucleus (star). **l.** Densely labeled rubrospinal fibers in laminae 5 and 6 (arrow) in the contralateral L5. Note some fibers in the dorsolateral motor neuron group. **m.** Labeled fibers from the interstitial nucleus of Cajal. Fibers are present in laminae 5, 6, 7, and 8 (arrows). Note the fibers from the contralateral side. **n.** Injection site in the caudal most part of the red nucleus (star). **o.** Labeled rubrospinal fibers in the contralateral T1. **p.** Higher magnification of fibers in the dorsolateral motor neuron group in the rectangular area in **o**. Note the boutons on two possible motor neurons. **q.** Labeled rubrospinal fibers in the contralateral L6. **r.** Higher magnification of the rubrospinal fibers in the rectangular area in **q**. Note the dense fibers in laminae 6 and 7, boutons on two possible motor neurons (arrows). 7n: facial nerve, fr: fasciculus retroflexus, PAG: periaqueductal gray, Pn: pontine nucleus, Pr5: the principal sensory trigeminal nucleus, rs: rubrospinal tract. The scale bar for **a**-**d**, **i**, **k**, **n**, **o**, **q** is 200 µm. The scale bar for **e**-**h**, **j**, **l**, **m**, **p** and **r** is 50 µm.

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