**Cat's medullary reticulospinal and subnucleus reticularis dorsalis noxious neurons form a coupled neural circuit through collaterals of descending axons.**

Roberto Leiras*, Francisco Martín-Cora*, Patricia Velo, Tania Liste and Antonio Canedo.
Department Physiology; Faculty Medicine; University Santiago de Compostela; 15704 Santiago de Compostela; Spain

*RL and FM-C contributed equally to this work

**Corresponding author:**
Dr. Antonio Canedo
Dept. Physiology; Fac. Medicine
University Santiago de Compostela
15704 Santiago de Compostela
Spain

Email: antonio.canedo@usc.es
Phone: +34 881812292

**Running head:** noxious mMRF and SRD neurons form a coupled network

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Animals and human beings sense and react to real/potential dangerous stimuli. However, the supraspinal mechanisms relating noxious sensing and nocifensive behavior are mostly unknown. The collateralization and spatial organization of interrelated neurons are important determinants of coordinated network function. Here we electrophysiologically studied medial medullary reticulospinal neurons (mMRF-RSNs) antidromically identified from the cervical cord of anaesthetized cats and found that, 1) more than 40% (79/183) of the sampled mMRF-RSNs emitted bifurcating axons running within the dorsolateral (DLF) and ventromedial (VMF) ipsilateral fascicles, 2) more than 50% (78/151) of the tested mMRF-RSNs with axons running in the VMF collateralized to the subnucleus reticularis dorsalis (SRD) that also sent ipsilateral descending fibers bifurcating within the DLF and the VMF. This percentage of mMRF collateralization to the SRD increased to more than 81% (53/65) when considering the subpopulation of mMRF-RSNs responsive to noxiously heating the skin, 3) reciprocal monosynaptic excitatory relationships were electrophysiologically demonstrated between noxious sensitive mMRF-RSNs and SRD cells, and 4) injection of the anterograde tracer Phaseolus vulgaris leucoagglutinin evidenced mMRF to SRD and SRD to mMRF projections contacting the soma and proximal dendrites. The data demonstrated a SRD-mMRF network interconnected mainly through collaterals of descending axons running within the VMF, with the subset of noxious sensitive cells forming a reverberating circuit probably amplifying mutual outputs simultaneously regulating motor activity and spinal noxious afferent input. The results provide evidence that noxious stimulation positively engage a reticular SRD-mMRF-SRD network involved in pain-sensory-to-motor transformation and modulation.
**Key words**: medial medullary reticular formation, subnucleus reticularis dorsalis, dorsal and ventral bifurcating projections, synaptic interrelationships between noxious sensitive cells.

**INTRODUCTION**

The medullary gigantocellular and magnocellular reticular nuclei, here defined as medial medullary reticular formation (mMRF), receive input from supraspinal motor-related structures affecting spinal motoneurons and interneurons through its descending reticulospinal axons (Canedo 1997). The mMRF has been related to a variety of sensory, motor and autonomic functions (Mason 2005; Pfaff et al. 2012), receive noxious input (Wolstencroft 1964; Burton 1968; Casey 1969, 1971; Goldman et al. 1972; Leblanc and Gatipon 1974; Pearl and Anderson 1978; Willis et al. 1984; Sotgiu 1988; Fort et al. 1994; Li et al. 1998; Nagata et al. 2003), influence spinal motor and reflex functions (Chan and Barnes 1975; Siegel 1979; Peterson 1980; Shimamura et al. 1980; Drew et al. 1986), elicit aversive behavioral reactions (Casey 1971; Roberts 1991), and suppress nociceptive neuronal responses at the spinal dorsal horn (Hall et al. 1982; Pretel et al. 1988; Zhuo and Gebhart 1990). Dorsal and ventral reticulospinal tracts can inhibit and excite spinal motoneurons, respectively (Brown 1994). Imbalance in the descending activity favoring excitation, as after lesion of the dorsal reticulospinal tract, can induce motor overactivity leading to disordered co-contraction (Sheean 2002), and decrease afferent filtering leading to disordered motor control and spasticity associated pain.

The presence of double labeled mMRF reticulospinal neurons (mMRF-RSNs) after topical application of fluorogold in the dorsal horn and microinjection of cholera toxin B chain in the rat’s ventral horn (Lefler et al. 2008) raises the question as to whether individual mMRF-RSNs have descending axons bifurcating within the DLF and the VMF, which would entitle them to concurrently regulate motor output and afferent input. Consequently, the first aim of
the work was to electrophysiological test whether mMRF-RSNs project bifurcating axons within the DLF and the VMF.

The mMRF has been associated with nociceptive and escape motor responses (Casey 1971, Siegel 1979; Casey and Morrow 1989) and it has been suggested that together with the subnucleus reticularis dorsalis (SRD), a pure nociceptive structure (Villanueva et al. 1988, 1989), is potentially implicated in transforming and modulating painful information into motoric reactions (Velo et al., 2013), an issue not previously investigated. mMRF electrical stimulation evokes monosynaptic and antidromic responses on cat’s SRD (Soto and Canedo 2011; Velo et al. 2013) but the possibility that SRD stimulation reciprocate these responses on mMRF-RSNs was not tested. Accordingly, a second aim was to study whether mMRF-RSNs collateralize to the SRD. The fact that descending axons from mMRF-RSNs do emit collaterals to the SRD (see the Results section) prompted to study the SRD-mMRF reciprocal anatomical interconnections as well as the synaptic interrelationships between mMRF-RSNs and SRD neurons driven by noxious stimulation, which constituted the third aim of the work. The results demonstrated bidirectional SRD-mMRF excitatory synaptic interactions forming a reticular network probably modulating motor responses to pain and ascending spinal noxious transmission.

MATERIALS AND METHODS

General.

Successful electrophysiological experiments were conducted in 26 male cats. All procedures conformed to the International Council for Laboratory Animal Science, the European Union Council Directive (86/609/EEC), were approved by the University of Santiago de Compostela animal care Committee and were in accordance with the
guidelines of the International Association for the Study of Pain (Zimmermann 1983). All surgery was performed under anesthesia, and all efforts were made to minimize suffering. Electrophysiological data were obtained from cats weighing 2.5–4.5 kg, under anesthesia and neuromuscular blockade. Surgical anesthesia was induced with ketamine HCl (10–20 mg/kg i.m.) and continued with α-chloralose (60 mg/kg i.v.). Additional doses of anesthesia (1/2 of a full dose) were regularly administered every 5–7 h. The depth of anesthesia was evaluated by continuously monitoring the heart rate (maintained around 120 beats/min), the electrocorticogram (digitally filtered at a frequency band-pass of 1 to 50–100 Hz) and by observing the state of the pupil. High-amplitude and low-frequency electrocorticographic waves (recorded through an electrode inserted 1–1.5 mm deep in the lateral tip of the cruciate sulcus) were taken as sign of adequate anesthesia, and dilated pupils or pupils reacting rapidly to electrical stimuli were considered to reflect inadequate anesthesia in which case a supplementary half of a full dose of α-chloralose was immediately injected.

Tracheal and venous cannulae were inserted; the animal was positioned in a stereotaxic frame and artificially ventilated. After the electrocorticogram showed typical signs of deep general anesthesia, neuromuscular transmission was blocked using vecuronium bromide (0.2–0.3 mg/kg/ h i.v.) dissolved in a pH-balanced solution of 5% glucose in physiological saline which was continuously infused (4 ml/h) through a tail vein. A bilateral pneumothorax was routinely performed, the end-tidal CO2 was maintained at 4 ± 0.3% by adjusting the respiratory rate and the inspired volume, and the temperature was maintained near 37.5 ºC via a DC heating pad under control of a rectal thermoprobe.

**Electrical stimulation and mMRF recording**

In a first series of experiments (n=20), tungsten bipolar stimulating electrodes (NEX-200; Rhodes Medical Instruments, Summerland, CA, USA), were placed ipsilaterally in the DLF at C2 level (under visual guidance), in the VMF at C3 level (0.5 mm lateral to the midline, 5-
6 mm deep), and in the SRD at 1.5-2 mm below the more ventral part of the medial main
cuneate nucleus, where proprioceptive mass responses to passive movement and muscle
palpation were recorded while descending the later used SRD stimulating electrode.
Rectangular pulses of 0.01–0.15 ms (typically 0.05 ms) duration and gradually increasing
currents were applied to the stimulating sites although systematic strength-duration curves
were not accomplished as in West and Wolstencroft (1983). The current polarity between
both electrode terminals, oriented in a medial-lateral direction, was routinely varied to select
the stimulating cathode generating antidromic unitary responses with intensity currents
ineffective when applied through the other terminal. Stimulating currents below 0.5 mA
antidromically activated the DLF and VMF fibers (range, 0.1 mA to 0.45 mA; 0.1 mA being
the lower current intensity used). When antidromic responses were observed by stimulating
only one of the tracts (DLF or VMF), stimulation currents of up to 1 mA intensity applied to
the unresponsive tract (supposed to activate all the fibers in the tract) were also ineffective.
Bipolar stimulating needle electrodes with inter-tip separation of 5 to 8 mm were also
routinely thrust into all four central foot pads to stimulate receptors and afferent fibers
driving mMRF neurons, by passing rectangular current pulses of 0.5–1 ms duration and up
to 3-4 mA current intensity.
Antidromicity was determined by a discrete all-or-none response at threshold stimulating
currents, a constant latency response at stimulating currents of 1.5 threshold, and the ability
to follow a train at 100 to 500 Hz of at least three stimuli with constant latencies. Thresholds
were abrupt, with less than 0.15 ms change in latency with increasing amplitude for 0.05–
0.15 ms duration stimuli. All units fulfilling these criteria also collided with spontaneous or
orthodromically-evoked single-spikes at an interval equal or slightly shorter than the sum of
the antidromic latency plus the axonal refractory period. In the collision tests, the
spontaneous and/or orthodromically-evoked spikes were timed to occur before the
expected time of the antidromic spike by an interval greater than the refractory period of the cell. However, for fast conducting axons the intervals at which collision should occur were short and likely to be close to or overlapping the refractory period. In these cases, antidromicity relied on the rest of criteria. The silent neurons were orthodromically fired by peripheral or spinal cord stimuli to perform collision tests.

Noxious stimulation was routinely applied through 9x9 mm square peltier probes fixed to the skin in the contralateral forelimb, the ipsilateral hindlimb and in the base of the tail (Fig.1). No thermally conductive gel was used between the peltiers and the skin. The peltiers were preheated and maintained at a basal temperature comprised between 30°C and 40 °C (usually around 35°C) and again heated up to a maximal peak transitory skin temperature of 63°C in 40-50s to test for thermal noxious sensitive cells. This slow rate of heating preferentially activates C-fiber nociceptors (Yeomans and Proudfit 1996). Heat stimuli were applied at intervals of at least 3 min (typically 5 min) which did not produce perceptible tissue trauma, inflammation or edema. Skin temperature changes under the base of the peltiers were measured by thermocouples type T of 0.3 mm external diameter (Omega Hyp-1; Omega Engineering; Manchester, Irlam; UK) contacting the peltiers and with the holder sub-epidermically thrusted. The thermocouple outputs were visualized online and stored on computer through analog converters with a linear 1mv/degree signal (Omega TAC80B-T). A stabilizing period of at least 5 minutes was allowed before initiating heating stimulation.

Extracellular mMRF recording was accomplished through epoxy-insulated tungsten microelectrodes of 12 MΩ resistance (AM Systems, Carlsborg, WA, USA) inserted at an angle of ≈40º through the cerebellar vermis. The unitary activity was amplified, digitized at 20 kHz through an analog to digital interface (CED 1401 Plus, Cambridge, UK) and stored.
on a PC for further analysis. CED spike2 v.7 software was used to process and analyze the
neuronal activity offline.

**mMRF and SRD simultaneous recording**

In a second series of successful experiments (n=6), the foramen magnum was exposed,
the posterior arch of the atlas and the occipital bone were resected, and the cerebellar
vermis was removed to insert recording/stimulating electrodes in the mMRF and the
ipsilateral SRD. Stimulating DLF and VMF electrodes as well as peltier probes were placed
as in the above experiments. After mMRF and SRD cells sensitive to noxious stimuli were
simultaneously recorded, the recording electrodes were alternately switched to stimulation
to pass cathodic pulses (0.05-0.5 ms pulse duration; 50-150 μA current intensity) relative to
an Ag-AgCl reference anode implanted in a nearby muscle. mMRF-RSNs and SRD cells
driven by noxious heat concurrently recorded were selected for further analysis when
recorded long enough to perform cross-correlograms, spike-triggered-averages (STA;
Canedo and Lamas, 1993) and alternate microstimulation (Leiras et al. 2010) through each
recording electrode.

**Statistics**

Mean antidromic conduction velocity estimates are expressed as arithmetic means ± SD
(Standard Deviation) and the statistical comparisons (significant P values ≤ 0.05) derived
from the Mann Whitney U test when two data sets were compared, and the Kruskal-Wallis
test followed by the Dunn’s test when comparing three or more data sets.

**Histology**

Following completion of the experiment, positive currents (20 μA for 20 s) were passed
through the stimulating and recording electrodes to mark their tip positions by electrolytic
lesions. Animals under deep anesthesia were killed by perfusion-fixation with 4%
paraformaldehyde and the neural tissues of interest were removed and postfixed.
Transverse 50 μm frozen sections were cut, serially mounted, stained with cresyl violet or neutral red, and the locations of recording and stimulating sites determined. The tips of the stimulating electrodes were located in the spinal white matter, within the DLF and the VMF, and the lesions made after recording were located within the SRD and the mMRF.

**Tracer injection and visualization**

To analyze the projections from the mMRF to the SRD and vice versa, we injected the anterograde tracer Phaseolus vulgaris leucoagglutinin (Pha-L) into the mMRF or the SRD and observed terminal labeling.

**Electrophoretic injection of Pha-L.** Six cats were used in this series.

Projections from the SRD were studied in three adult male cats, weighing 3.9–4.3 kg. Prior to surgery, animals received an injection of dexamethasone (0.25 mg/kg, IM) to limit tissue swelling, and atropine (0.05 mg/kg, SC) to reduce respiratory secretion. Following sedation with ketamine (30 mg/kg, i.m.), a catheter was placed in a foreleg vein. Cats were then anaesthetized with alpha-chloralose (60 mg/kg, IV), placed in a ventral-flexed position in a stereotaxic apparatus, the foramen magnum exposed and the rostral arch of the atlas bone resected to allow tracer injection into the left SRD. A 2.5% solution of Pha-L in 10mM phosphate buffer, pH 8 (L1110; Vector Labs, Burlingame, CA, USA) was ejected electrophoretically (+6μA current: 7 sec on/7 sec off; during 15 minutes) from glass micropipettes (20–30 μm outer tip diameter) which had been inserted perpendicular to the horizontal plane, 2–2.5 mm caudal to the obex, 2-2.3 mm lateral to the midline, and at a depth of 2.5-3 mm from the dorsal surface. Projections from the mMRF were studied in three adult male cats, weighing 2.5–4.3 kg. These cats were placed in a flat position in the stereotaxic apparatus and Pha-L was ejected into the left mMRF using micropipettes positioned at an angle of 25º to the vertical and aimed at Horsley-Clark coordinates P8, L 1.5, H-1.5 to -5.5 according with Snider and Niemer (1961). After completion of the
injections the micropipettes were left in place for 10 minutes before being slowly retracted.

Subsequently, gelfoam was used to fill the craniotomy, and the muscle and skin were reapproximated and secured with suture. Throughout the surgical procedure, core temperature was monitored and maintained within 37-38°C with a servo-controlled DC heating pad. Postoperatively, buprenorphine (0.01 mg/kg; SC) for analgesia, Ringers' lactate (SC) 50 ml for hydration, and penicillin G (20,000 IU/kg; i.m.) for prophylaxis were administered.

After 3-weeks survival, animals were heparinized (5000 IU; i.v.), deeply anaesthetized with sodium pentobarbital (60 mg/kg, IV) and transcardially perfused firstly with 2L of heparinized, phosphate-buffered saline (PBS; 0.1M; pH 7.4) containing sodium nitrite (0.5%) to facilitate vascular dilation, and secondly with 3L of 4% paraformaldehyde in PBS. After perfusion, brain and cervical spinal cord were removed and postfixed by immersion in the same fixative overnight, at 4ºC. Then tissues were cryoprotected in 20% sucrose solution in PBS until sunk at 4ºC. This was followed by a soak in 30% sucrose in PBS at 4ºC. Tissues were then blocked in the stereotaxic plane, rapidly frozen and stored at -80ºC.

Coronal sections at a thickness of 30 µm were cut on a freezing microtome (HM500, Microm Int. GmbH, Walldorf, Germany), collected in order in anti-freezer solution (30% sucrose and 30% ethylene glycol in PBS), and kept at -20ºC until immunohistochemistry.

**Visualization.** For immunohistochemical visualization of Pha-L, the sections were incubated for 1 hour in 10% normal horse serum, 3% normal goat serum and 0.3% triton X-100 in PBS (blocking solution; BS) and then incubated overnight with rabbit polyclonal anti-Pha-L antibody (AL-1801-2; E-Y Labs., San Mateo, CA, USA) diluted at 1:10000 in BS at 4ºC. On the next day, sections were incubated for 1 hour in a secondary horse biotinylated anti-rabbit- antibody (BA1100; Vectors Labs) diluted at 1:400 in BS. Afterwards, sections were incubated for four hours in Elite ABC complex solution (PK6100; Vectors Labs). The
final step for Pha-L detection consisted of nickel-enhanced diaminobenzidine precipitation detection using DAB Substrate Kit (SK4100; Vector Labs). The specimens were mounted onto gelatine-coated glass slides, air dried, dehydrated in a graded series of ethanol (50-100%), cleared with xylene, and coverslipped with Depex (VWR; Barcelona, Spain). For quantifying the number of Pha-L positive axon terminals appose/close to soma or proximal dendrites per neuron, a series of three sections separated by 300 µm were double-labeled for Pha-L and for the neuronal marker, NeuN. Sections were incubated for 1 hour in blocking solution (10% normal horse serum, 3% normal goat serum, 3% bovine serum albumin, and 0.3% triton X-100 in PBS) and then simultaneously incubated in 1:10000 rabbit polyclonal anti-Pha-L antibody and 1:1000 mouse anti Neu-N (MAB377 clone A60; Chemicon, USA) overnight at 4ºC. On the next day, sections were reacted to reveal grey-black Pha-L positive fibers using the procedure described above. Subsequently, sections were incubated for 1 hour in biotinylated anti-mouse-IgG produced in horse (BA2000, Vector Labs) diluted at 1:600 in blocking solution. Afterwards, sections were incubated for two hours in Elite ABC complex solution (PK6100; Vectors Labs), and finally were reacted with diaminobenzidine to produce a brown reaction product in the Neu-N labeled neurons. The counting target consisted of Pha-L immunoreactive terminal-like varicosities that are 0.5–3.5 µm in diameter close to or apposed to either soma or proximal dendrites of Neu-N immunoreactive neurons within the mMRF (see Figure 15F to I). The number of contacts per neuron was expressed as mean ± SD.

**Image processing.** The full extent of the injection sites was analyzed from a series of photomicrographs from tissue sections processed only with Ni-DAB. Images were captured (4X magnification) with a digital camera (DS-Fi1; Nikon) mounted on a light microscope (E800, Nikon), and stitched together into a single image per section using Nis-Elements-D software. Resulting images were used for measuring the area core injections. The area of
each slice was multiplied by 0.03mm -slice thickness- and added for estimating the volume of the injections. Those images were then used for drawing schematic representations of each section using Photoshop (Adobe) and later grouped on a standard drawing of a representative coronal section where the core of the injection was drawn as black and the halo was indicated as grey (Fig.12, insets in A and B; Fig.13, inset in A). The delineation and nomenclature of the schematic drawings was based on the atlases of Snider and Niemer (1961) and Verhaart (1964).

For photographic reconstructions of anterogradely Pha-L labeled fibers and terminals contained in a single tissue section, images corresponding to different focal planes of a large area of the tissue section were first captured at high magnification (40x).

Subsequently, the stacks of images were combined using ImageJ program (v1.48t; plug-in Stack Focuser). By employing this plug-in, several images corresponding to different focal planes were fused into a single focused image, including all the elements in each focal plane. Finally, since tissue sections were captured at high magnification, stitching of several of those focused images was performed. The resulting images were used for drawing each of the Pha-L labeled fibers and their terminals observed in the mMRF or the SRD on representative drawings of the sections. The results obtained from three consecutive serial sections were plotted on a drawing of a single section (see Figs. 12 C-D; 13 B-C). Four consecutive serial sections of the cervical spinal cord (C1-C2) were used for drawing a schematic reconstruction of descending axonal trajectories coming from the mMRF (see Figure 12 G). Pha-L labeled fibers and boutons in the vicinity of or overlying Neu-N labeled neurons were illustrated with high magnification microphotographs taken from single sections at several focal depths and transformed to a single image using ImageJ software (see Figs. 12 H-J; 13 E-I).
RESULTS

General

The mMRF sampled in the study included the gigantocellular and magnocellular nuclei, as defined by Berman (1968), from Horsley-Clarke coordinates P 6.5 to P 10, from 1mm to 3.5 mm lateral to the midline, and from 1.5 to 5 mm deep from the floor of the IV ventricle. In a first series of experiments, the potential axonal bifurcation of mMRF-RSNs within the DLF and the VMF was studied. A total of 183 mMRF-RSNs antidromically identified by VMF and/or DLF cervical cord stimulation were recorded, with 36 out of 136 tested (≈26%) changing firing frequency in response to noxiously heating the skin. More than 51% of the tested mMRF-RSNs (78/151) collateralized to or through the SRD.

The abundance of mMRF-RSNs collateralizing to or through the SRD demonstrated in the first series of experiments, together with the high collateralizing degree of spinally-projecting SRD axons to or through the mMRF previously demonstrated (Velo et al. 2013), prompted a second series of experiments using simultaneous extracellular unitary recording of SRD cells and mMRF-RSNs responding to noxious heat applied to the skin. Alternate microstimulation through each of these recording electrodes while recording from the other was also performed in an attempt to elucidate the possible unidirectional or bidirectional synaptic interactions among noxious responsive SRD cells and mMRF-RSNs. A total of 29 mMRF-RSNs and 17 SRD neurons sensitive to noxious stimulation (12 spinally-projecting) were simultaneously recorded and studied.

The results are reported in the sections below.

1. mMRF-RSNs with axons running in the DLF and/or the VMF.

Electrical stimulation at the DLF and VMF allowed separation of mMRF-RSNs sending axons through one of the fascicles (n=104) from those bifurcating into both (n=79) thus
establishing that a considerable percentage of mMRF-RSNs had bifurcating axons in the DLF and the VMF, exemplified by the data from one antidromically identified neuron illustrated in Fig.2A. Thresholds for antidromic activation were similar for DLF (0.31 ± 0.12 mA for DLF-only projecting, range 0.1 to 0.45 mA; 0.29 ± 0.14 mA for DLF and VMF projecting, range 0.1 to 0.4 mA ) and for VMF stimulation (VMF-only: 0.35 ± 0.19 mA , range 0.1 to 0.5 mA; VMF+DLF: 0.25 ± 0.1 mA, range 0.1 to 0.4 mA).

Comparison of conduction velocities of bifurcating and non-bifurcating fibers as well as those bifurcating dorsally and ventrally allows inferring whether there is a relation between collateralization and arrival time to target. Figure 2B resumes graphically the single and double projecting neurons as well as their antidromic conduction velocities.

Taking 0.4 ms as utilization time and subtracting it from the antidromic latency (see Results section 4), the corrected antidromic conduction velocities were 55.5 ± 24.9 (SD) m/s for the 109 mMRF-RSNs responding antidromically to DLF stimulation (median = 52.3 m/s, range 20 – 110 m/s), and 57.7 ± 33 m/s (median = 52.1 m/s, range 18 – 145 m/s) for the 153 mMRF-RSNs responding antidromically to VMF stimulation. These velocities were not significantly different from each other (Mann-Whitney U test) (Fig.2B1). Canedo and Lamas (1993) used a concentric bipolar electrode, 0.5 mm intertip spaced, to stimulate the VMF and found a mean antidromic velocity of 60 m/s and similar ranges as those reported here from a sample of 294 mMRF-RSNs intracellularly recorded.

Grouping the data into single and double projecting neurons shows that about 16% projected solely through the DLF, about 40% projected only through the VMF and about 43% had bifurcating axons within both fascicles (Fig.2B2, left).

The double-projecting axons were significantly faster conducting through the VMF than through the DLF (mean antidromic velocities of 72.9 m/s for VMF stimulation versus 55.7 m/s for DLF stimulation; Fig.2B2). Whereas the antidromic conduction velocity of single and
double projecting axons was not significantly different to DLF stimulation [55.7 ± 23.4 m/s for double projecting (range 23 – 110 m/s), and 54.8 ± 29.2 m/s (range 20 – 90 m/s) for single projecting (Fig. 2B2, left)], the double projecting axons were significantly faster conducting to VMF stimulation than the single projecting axons (72.9 ± 37.2 m/s, range 25 – 145 m/s for double projecting; 44.7 ± 19.3 m/s, range 18 – 95 m/s for single projecting).

Since conduction can be modulated by the axon membrane’s firing threshold which may depend on precedent activity (Schmied and Fetz 1987; Canedo and Lamas 1989), we tested whether the action potentials evoked by stimulating one branch of the bifurcating mMRF-RSNs affected the excitability of the other branch. Membrane potential cannot directly be measured in extracellular recordings but indirect evidence may be obtained through assessment of changes in axonal excitability measured by alterations in threshold currents to elicit antidromic action potentials. Variations in antidromic axonal excitability after a preceding action potential may provide some indication about variations in conduction velocity, invasion of bifurcating terminal branches and the probability of neurotransmitter release. The electrical excitability of mMRF fibers was quantified by the percentage of antidromic spikes generated at different delays from the conditioning antidromic response. Changes in the excitability of dorsal and ventral bifurcating axons were related to a previous impulse produced experimentally by activation of either collateral (Fig.3A1-A2). Threshold reduction in the test branch was only observed following conditioning spikes, suggesting that the axonal spikes and not the stimuli caused the threshold decrease, as reported in other descending tracts (rubrospinal: Canedo and Lamas 1989; corticospinal: Schmied and Fetz 1987). The increase of axonal excitability lasted longer when using DLF as suprathreshold conditioning stimulus and VMF as subthreshold test stimulus than when reversing the stimulating order, as it is shown by the mean ± SD values of Fig.3B for 6 different DLF and VMF bifurcating axons. The beginning
of the supernormal period occurred at a mean delay of $4 \pm 0.5$ ms and was evident up to an average delay of $20 \pm 4.2$ ms when conditioning stimuli were applied to the VMF, and up to a delay of $30 \pm 6.5$ ms when DLF stimulation was used as conditioning. The duration of the supernormal periods were uncorrelated with antidromic latency suggesting that they are independent of fiber size.

The single and double collateralizing mMRF-RSNs appeared intermingled within the sampled region without a specific distribution.

2. mMRF-RSNs with collaterals to or through the SRD.

A considerable percentage of mMRF-RSNs also responded antidromically to SRD stimulation (78 of 151 tested: ≈ 51%). Figures 4A1-A3 illustrate the identification criteria, and Fig.4B shows a graphical display of the 151 mMRF-RSNs tested to SRD stimulation, grouping them into different classes according to their antidromic responses to stimulation of only one (DLF or VMF) or both fascicles, and by separating the SRD-collateralizing from the SRD-non-collateralizing cells for each subgroup. None of the 20 mMRF-RSNs responding antidromically only to DFL collateralized to or through the SRD. On the contrary, more than 59% (78/131) of the VMF-antidromic mMRF-RSNs sent collateral branches to or through the SRD.

The VMF and DLF bifurcating axons had significant slower antidromic conduction velocity to DLF stimulation (no collaterals to SRD: $56.5 \pm 25.7$ m/s; collaterals to SRD: $46.4 \pm 25.3$ m/s) than to VMF stimulation (no collaterals to SRD: $70.3 \pm 25$ m/s; collaterals to SRD: $62.5 \pm 33.9$ m/s) and were significantly faster conducting than those that did not bifurcate (Fig.4B, left).

The antidromic conduction velocity of collaterals to or through the SRD were not significantly different for mMRF-RSNs with axons in the VMF and for DLF and VMF.
bifurcating axons (Fig.4B, right). These collaterals were significantly slower conducting than their reticulospinal parent axons: 21.65 ± 15.9 m/s for collaterals from mMRF-RSNs with VMF-only axons; 23.9 ± 14.9 m/s for collaterals from mMRF-RSNs with DLF and VMF bifurcating axons.

Again, the mMRF-RSNs collateralizing to the SRD appeared all over the region sampled without any specific distribution.

3. Effects induced on mMRF-RSNs by noxious stimulation.

Noxious receptive fields were routinely predefined by fixing three peltier thermodes over the shaved skin: one on the forelimb contralateral to the recording site, other on the hindlimb ipsilateral to recording, and the third on the base of the tail. This was done because some mMRF neurons receive noxious input (Wolstencroft 1964; Burton 1968; Casey 1969; Goldman et al. 1972; LeBlanc and Gatipon 1974) and can have large receptive fields (Pearl and Anderson 1978; Farham and Douglas 1985).

Noxiously heating the skin caused a response in about 26% of the tested mMRF-RSNs (36/136), 35 increasing and one decreasing discharge frequency. The silent mMRF-RSNs potentially inhibited by noxious heat could not be detected by extracellular recording. All noxious sensitive cells appeared intermingled with heat-insensitive mMRF-RSNs in a restricted region within the gigantocellular and magnocellular nuclei at stereotaxic coordinates from AP -7 to about AP-9, L 1 to 2.5, and at depths of about 2 to 5 mm from the floor of the IV ventricle (Fig. 5A1). The noxious responsive cells were not driven by visual (light flashes), auditory (clicks), proprioceptive (passive movement and muscle pressure) or cutaneous innocuous (brushing, gentle touch) stimuli. All 36 noxious sensitive mMRF-RSNs were fired by SRD stimulation, with more than 55% of them (20/36) having tri-furcating axons to the DLF, the VMF and the SRD; 9 with bi-furcating axons to the VMF and the SRD.
(25%), and the remaining 7 responded antidromically to VMF and monosynaptically
(faithfully responded to stimulation frequencies of 50-100 Hz with jitter of 0.5 ms or less:
Velo et al. 2013) to SRD stimulation. The tri-furcating axons were significantly faster
conducting to VMF stimulation than the bi-furcating ones and the SRD-monosynaptically
activated mMRF-RSNs (Fig.5A2).

The 36 mMRF-RSNs sensitive to noxious heat, exemplified by the data from the neuron of
Fig.5B1-B4, were also driven by electrical stimulation of fore-and-hind-limbs at current
intensities from 1.5 mA to 4 mA, presumed to activate peripheral receptors/fibers including
those within the noxious range (Fig.5B2). Fifteen of these cells (≈41%) were additionally
excited by intense mechanical stimuli, presumably noxious (≈ >5g), applied to the skin
through a feed-back controlled mechanical stimulator (Fig.5B3).

**Response properties of mMRF-RSNs to noxious thermal stimulation.** More than
half of the noxious sensitive mMRF-RSNs were silent at rest (20/36: 12 antidromic to DLF
and VMF, 8 antidromic to VMF) and some became persistently active after electrical
stimulation for antidromic identification (n=5; 3 antidromic to DLF and VMF) or after noxious
stimulation (n=4; all antidromic to DLF and VMF). The majority of the spontaneously active
heat-sensitive mMRF-RSNs spiked irregularly at a mean of less than 3 Hz (n=11/16; 7
antidromic to DLF and VMF, 4 antidromic to VMF) with the remaining 5 (1 antidromic to
DLF and VMF, 4 antidromic to VMF) discharging regularly-spaced single spikes at 25-55
Hz. Most of the tested mMRF-RSNs showed spatial (10 of 15 tested) and temporal (8 of 12
tested) summation to noxious heat and to peripheral electrical stimulation in different limbs
and/or the base of the tail.

All 5 heat-sensitive mMRF-RSNs showing regular spontaneous firing at rest had a similar
behavior. Figure 6 illustrates one of these cells with a descending bifurcating axon that also
collateralized to or through the SRD (Fig.6A). Electrical stimulation at the VMF and at the
SRD (but not at the DLF) induced silenced firing following the antidromic responses (Fig.6B). Thermal noxious stimulation converted the spontaneous single-spike activity into spike doublets (Fig.6C).

Stimulation of the excitatory receptive field at the pre-established locations showed summation to noxious heat as well as to electrical stimuli in 5 of 8 mMRF-RSNs tested (Fig.7), implying that more dorsal horn nociceptive neurons sending convergent inputs to these neurons were recruited as stimuli were applied to distant, including bilateral, regions.

Low-frequency repetitive electrical and noxious stimulation produced wind-up (enhanced neuronal responses) in various spontaneous (n=6/16) and silent at rest (n=10/20) heat sensitive mMRF-RSNs. To test silent cells for wind-up, consecutive 1-min-separated-trains of electrical stimuli at 1 Hz were applied to the limbs and/or the DLF. Wind-up was evident following the second and successive trains. The data shown in Fig.8A-D illustrate the behavior of a silent mMRF-RSN with its axon descending in the VMF. The responses to 5 successive trains applied to the DLF shown in Fig.8C illustrate that the second component of the response (presumably due to C-fiber activation) developed wind-up from the second train of stimuli and beyond (note the rasters below each train). This same cell also responded to noxious stimulation with increasing post-discharges to stimulus repetition (Fig.8D). Fig.8E illustrates the windup phenomenon developed by a different mMRF-RSN in response to DLF stimulation (left) and by 5 distinct mMRF-RSNs (right).

4. mMRF-RSNs and SRD neurons activated by peripheral noxious stimulation are synaptically interconnected.

In this series of experiments, paired mMRF and SRD recordings were performed to study the potential interactions between mMRF and SRD heat-sensitive neurons by compiling crosscorrelograms, STA, and microstimulation. The experimental strategy consisted to
isolate a single SRD heat-sensitive cell and maintain the SRD recording electrode in place while searching for heat-sensitive mMRF-RSNs. In so doing, 17 SRD (12 spinally projecting) and 29 mMRF-RSNs heat-sensitive cells were simultaneously recorded long enough to complete the experimental protocol (Table I).

The mMRF recordings were circumscribed to a region extending from P7 to P9 (Fig.5A1), and the SRD recordings were obtained from neurons located under the main cuneate nucleus from 0.5 to 3 mm posterior to the obex and from 2.5 to 5 mm below the floor of the IV ventricle (Soto et al. 2008; Velo et al. 2013). The heat-sensitive mMRF-RSNs had descending axons running in the DLF (n=6), the VMF (n=13) and axons bifurcating into both fascicles (n=10) (Table I). Except for 5/13 of the VMF-only descending axons, the remaining 24 mMRF-RSNs collateralized to the SRD.

The finding that 5/12 SRD noxious cells sent descending axons bifurcating in the DLF and the VMF was unexpected since, to our knowledge, SRD cells with spinal dorsal and ventral bifurcations have not been previously reported. All 12 SRD cells emitting axons to the spinal cord collateralized to the mMRF.

STAs were compiled off-line for each pair of SRD/mMRF cells with at least 4,000 recorded action potentials. Activity was averaged from 4 to 8 ms preceding the trigger spike and up to 12 ms following it, including activity before, during, and after heat stimulation.

Presumed unidirectional and bidirectional monosynaptic interactions were found as follows

  i) monosynaptic excitation:
  a) 2 SRD non-spinal projecting cells on 2 mMRF-RSNs (antidromic: 1 DLF+VMF; 1 VMF only),
  b) 3 other mMRF-RSNs (all 3 antidromic to VMF only) on 3 SRD cells (all 3 antidromic to DLF only),
c) 2 mMRF-RSNs (both antidromic to DLF and VMF) on 2 SRD cells (antidromic: 1 DLF; 1 DLF+VMF).

ii) bidirectional monosynaptic excitation:

2 SRD-mMRF pairs, both pairs with DLF and VMF bifurcating axons.

Examples in Figs.9-11 show antidromic identification, responses to heat stimulation, crosscorrelograms, microstimulation, and STA. Figure 9A1 illustrates the antidromic identification of a DLF and VMF bifurcating mMRF-RSN simultaneously recorded with a non-spinally projecting SRD neuron, both driven by noxious heat stimulation (Fig.9A2). This pair showed random, asynchronous activity, even during heat stimulation as shown by the crosscorrelogram and STA (Fig.9A3).

An example of a presumed unidirectional SRD to mMRF monosynaptic excitation is shown in Fig.9B between a non-spinal projecting SRD neuron and a DLF and VMF bifurcating mMRF-RSN (Fig.9B1). Both cells were driven by noxiously heating the base of the tail (Fig.9B2, upper). The crosscorrelogram showed a clear synchronization around zero time suggesting a common input, and a peak in the first bin of 5ms at the right of zero time (Fig.9B2, lower) pointing to a monosynaptic SRD to mMRF effect.

Microstimulation through the SRD recording electrode antidromically activated the mMRF-RSN (1.9 ms onset latency: Fig.9B3, upper right inset). Computation of STA (SRD spikes as triggers), displayed a narrow peak (2 ms onset latency) with a duration of about 1 ms (Fig.9B3, lower), superimposed on a broader effect. We interpret the broader effect as a common input and the narrow peak as a monosynaptic response. If a synaptic delay of 0.5 ms is assumed, the SRD to mMRF conduction time would be 1.5 ms (conduction velocity of about 7 m/s, estimating a SRD-mMRF distance of 11 mm). Since the antidromic latency was 1.9 ms, the stimulus utilization time should be around 0.4 ms which, accordingly, was the value taken to compute the antidromic conduction velocities described in previous
sections. Compilation of STA using the mMRF spikes as triggers produced a broad effect around zero time, suggestive of a common input (Fig.9B4).

An example of a DLF-only projecting SRD neuron presumably exerting a monosynaptic excitation on a VMF-only projecting mMRF-RSN is shown in Fig.10 (antidromic identification in Fig.10A1 and A2). Both neurons were driven by noxiously heating the base of the tail (Fig.10B, upper) as well as the ipsilateral hindlimb (Fig.10B, lower) generating rhythmic firing. A similar behavior was reported for some mMRF-RSNs from intact cats walking on a treadmill that generated bursting activity modulated in time with locomotor rhythms (Drew et al. 1986). The crosscorrelogram (SRD spikes as triggers) showed a narrow peak in the first 2 ms bin after zero time superimposed on a broader effect (Fig.10C), suggesting a common input and a SRD to mMRF monosynaptic excitation.

In fact, the STA (SRD spikes as triggers) showed a clear peak at an onset latency of 1.5 ms, superimposed on a broader effect (Fig.10D). Microstimulation through the SRD recording electrode induced a response in the mMRF-RSN with little jitter and a minimal latency of 2 ms (Fig.10D, upper right inset). This presumed monosynaptic excitatory effect was unidirectional (SRD to mMRF) since mMRF microstimulation (not shown) was ineffective, and STA (mMRF spikes as triggers) displayed a broad effect during heating, ascribable to a common input (Fig.10E).

Finally, an example illustrating presumed bidirectional monosynaptic excitatory effects is shown in Fig.11 where both simultaneously recorded SRD and mMRF neurons projected bifurcating DLF and VMF axons (Fig.11A-B) and were driven by noxiously heating the ipsilateral hindlimb (Fig.11C). The crosscorrelogram showed two narrow peaks at the first 3 ms bins at both sides of zero time, superimposed on a much broader effect (Fig.11D); which we interpret as evidence for mutual activation and common excitation, respectively. This interpretation appears coherent with the STA and microstimulation data of Fig.11E.
Using the SRD spikes as triggers to compile the mMRF average revealed a presumed monosynaptic response at a rather imprecise initial latency of 0.6-0.9 ms with a peak latency of 1.4 ms (Fig.11E1, upper). By stimulating through the SRD recording electrode with gradually increasing currents (from 50 to 100 μA), an antidromic mMRF response at an onset latency of 1.9 ms was first displayed at lower currents whereas higher stimulating currents induced an orthodromic response with a minimal latency of about 1 ms which obliterated the antidromic one either by collision or refractoriness (Fig.11E1, lower left inset). The assumption is made that the orthodromic response was induced via one SRD axon collateral as depicted in the schematic diagram of Fig.11E1 (lower, right inset), representing the presumed circuitry that would explain these data. The longer latency antidromic response relative to the orthodromic one suggests that the activated mMRF and SRD collaterals differed from each other by their myelination, running course and/or diameter’s size.

Using the mMRF spikes as triggers to compute the SRD average, a presumed monosynaptic response with an onset latency of 1.8 ms was revealed (Fig.11E2, upper). Microstimulation (150 μA) through the mMRF recording microelectrode induced an antidromic spike with onset latency of about 1 ms followed by an orthodromic response with first spike onset latency of 2.1 ms (Fig.11E2, lower left inset). Again, the suggestion is made that this orthodromic response was produced by activation of a collateral branch entering the SRD from the trifurcating mMRF-RSN axon, as schematized in Fig.13E2 (lower, right inset).

This interpretation appears coherent with the neuronal latencies observed. Thus the SRD collateral to the mMRF had a minimal onset orthodromic latency of 1 ms to electrical microstimulation (Fig.11E1, lower left inset), and a STA onset latency of 0.6-0.9 ms (Fig.11E1, upper). Stimulation of the mMRF collateral to the SRD produced a response with
minimal onset orthodromic latency of 2.1 ms (Fig. 11E2, lower left inset) and a STA peak with onset latency of 1.8 ms (Fig. 11 E2, upper). The small discrepancies in latencies from both methods were probably due to the time taken by the electrical stimuli to generate action potentials (utilization time) and the likely different sites at which the action potential was picked up to compile STA and where it was initiated by electrical stimulation.

Because the above results point to strong synaptic interrelationships between heat sensitive mMRF-RSNs and SRD neurons, the next step was to study these potential anatomical connections as described below.

5. Injection of Pha-L substantiated the electrophysiological data.

Anterograde markers were ejected in the mMRF in three cats and in the SRD in another three different cats. The results showed labeled fibers from the mMRF and the SRD synapsing on the soma and proximal dendrites of SRD and mMRF neurons, respectively (Figs. 12 and 13).

The data reported and illustrated in figures 12 and 13 are based on representative injection sites. A systematic study of all mMRF and SRD projections is not described since this study was devoted to analyze the projections from mMRF to SRD and vice versa.

**Pha-L injection sites and projections from the mMRF to the SRD.** Panels A and B of Figure 12 show two examples of Pha-L injections within the mMRP at a similar AP level. In case E258 (Fig. 12A), the injection area was ventrally located whereas it was dorsally located in case E323 (Fig. 12B). In both cases, serial sections showed that the injection sites were well within the mMRP, were almost oval, and occupied similar volumes (E258, 0.059 mm$^3$; E323, 0.050 mm$^3$). In both cases, fibers and terminals were observed in the SRD bilaterally, but with an obvious ipsilateral predominance. The distribution of the labeled terminals corresponded well with that of the labeled fibers as shown in Fig. 12C and 12D.
Examples of labeled fibers and terminals in the ipsilateral SRD of cases E258 and E323 are shown in figures 12 E-E1 and 12 F-F1. The descending axonal trajectories coming from the mMRF were studied at the upper cervical cord. Representative results and examples are shown in panels G, G1 and G2 (Fig.12). On the schematic drawing (Fig.12G), identified fibers in four upper cervical cord sections were plotted. Most labeled fibers from the mMRF descended ipsilaterally mainly through the VMF and in minor numbers in the DLF. Representative photomicrographs of labeled fibers within the VMF and the DLF are shown in Figs. 12G1 and 12G2, respectively. Pha-L-labeled mMRF terminals within the SRD were observed in close association with the Neu-N-labeled SRD neuronal soma and proximal dendrites. These close associations are demonstrated in the photomicrographs of Fig.12H-J. Pha-L-labeled terminal puncta were seen in close association with the soma (arrowheads; Fig.12H-I) and proximal dendrites (arrows; Fig.12I-J) of Neu-N SRD labeled cells.

**Pha-L injection sites and projections from the SRD to the mMRF.** The photomicrograph in Figure 13A shows a representative Pha-L injection site in the SRD (case E284). The injection area was almost cylindrical in shape (see lower right inset in Fig.13A; estimated Pha-L-injected tissue volume was 0.005 mm$^3$). Labeled fibers and terminals within the mMRF were unevenly and bilaterally distributed with ipsilateral predominance (see schematic drawings Fig.13B and 13C). Microphotographs illustrating the presence of labeled fibers and terminals in the ipsilateral mMRF are shown in Figs. 13D-D1. Most labeled terminal swellings were either distributed along the terminal fibers or tightly aggregated along fine terminals (arrowheads in Fig.13D1) with lesser labeled fibers lacking boutons (arrows in Fig.13D1). Terminal Pha-L-labeled swellings closely apposed to the soma and proximal dendrites were observed in the mMRF (Fig.13 E-I). In some sparse cases, single terminal swellings with short stacks were given off directly from some fibers.
(see arrowhead in Fig. 13E). It was more frequently observed that several terminal swellings were closely apposed to the soma (arrowheads in Fig 13F) or proximal dendrites (arrows in Fig 13F-I) of medium to large sized multipolar mMRF neurons. The number of boutons apposed per neuron ranged from 1 to 10 (mMRF cells studied: ipsilateral = 344; contralateral = 193). The number of axodendritic contacts per neuron was significantly higher than the axosomatic ones for both ipsilateral and contralateral neurons (Ipsilateral: axodendritic = 4.8 ± 0.6, n=263; axosomatic = 1.5 ± 0.3, n=209. Contralateral: axodendritic = 4.1 ± 0.4, n = 154; axosomatic = 1.6 ± 0.3, n=110). The number of axodendritic contacts was similar for ipsilateral and contralateral neurons, and the same was true when ipsilateral and contralateral axosomatic contacts were compared (see above). Finally, it is worth to mention that SRD fibers were seen to cross the mMRF to terminate in the parvocellular reticular nuclei with part of them having non-terminal swellings within the mMRF (not shown).

DISCUSSION

General

This study describes new properties of the cat’s mMRF and SRD neurons: i) VMF and DLF bifurcating axons were issued by cells from both structures, including neurons sensitive to noxious heat, ii) more than 50% of the mMRF-RSNs tested (78/151) collateralized to the SRD, percentage that increased to more than 81% (53/65) when globally considering the noxious responsive mMRF-RSNs, iii) unidirectional and bidirectional monosynaptic interactions among noxious sensitive mMRF-RSNs and SRD cells were electrophysiologically demonstrated and anatomically confirmed by injection of Pha-L showing SRD to mMRF and mMRF to SRD connections with labeled fibers contacting the soma and proximal dendrites within each structure.
**mMRF-RSNs collateralization**

About 43% (79/183) of the sampled mMRF-RSNs emitted DLF and VMF bifurcating axons and about 51% (78/151) of the tested mMRF-RSNs collateralized to or through the SRD.

When identifying bifurcating VMF and DLF axons, the possibility exists of misinterpreting as main collaterals the potential activation, by current spread, of trunk terminals within the grey matter. If so, the antidromic conduction velocity to stimulation of one of the fascicles should be substantially faster than that of the second fascicle whose stimulation activated terminals from the first since terminals are expected to be thinner than the parent axons. For example, the conduction velocity of the bifurcating axons should be significantly faster to DLF than to VMF stimulation if spreading current from the VMF electrode activated DLF terminals in the grey matter, which was not the case (Fig.2B2, right).

Also, activation of VMF terminals by the DLF electrode is unlikely since, i) the stimulating electrodes were histologically verified to be located within each tract and the stimulating currents were limited to intensities firing cells antidromically through one of the electrode terminals but not through the other when reversing polarity (less than 1mm separation between both electrode terminals), ii) the current thresholds through the DLF electrode for antidromic activation of only-DLF and DLF+VMF bifurcating fibers were similar, as were those to antidromically fire single and double projecting fibers through the VMF electrode.

To activate VMF terminals within the grey matter by DLF stimulation, significantly higher thresholds should have been necessary to fire the thinner (probably unmyelinated) and more distant VMF terminals than to activate larger myelinated and closer trunk axons, and iii) axon terminals are less excitable than trunk axons due to their low Na+/K+ conductance ratio producing a shunting effect by K+ channels (Kawaguchi and Sakaba 2015), probably to prevent generation of aberrant action potentials in the terminals (Dodson et al. 2003), which possibly would made the terminals unable to follow the stimulating frequencies used...
for antidromic identification. Accordingly, the VMF collaterals from bifurcating mMRF-RSNs
had, in fact, a significantly faster mean conduction velocity than the DLF collaterals
(Fig.2B2, right).

The finding that about half of the dorsal (DLF) and ventral (VMF) double bifurcating mMRF-
RSNs also collateralized to the SRD, a pure nociceptive region (Villanueva et al. 1988,
1989; Soto et al. 2008), that also sends descending fibers all along the spinal cord (Velo et
al. 2013), points to a conjoint mMRF and SRD modulation of spinal motor and nociceptive
circuitry acting in response to noxious stimuli. If part of the mMRF axons running in the DLF
terminate at the dorsal horn (Basbaum et al. 1978; Martin et al. 1985; Wei et al. 1999) and
axons in the VMF terminate at the ventral horn (Canedo 1997), the trifurcating mMRF-
RSNs (to DLF, VMF and SRD) would influence motor output, nociceptive ascending
transmission through the dorsal horn nociceptive neurons (Almeida et al. 1993, 2000;
Tavares and Lima 1994; Villanueva et al. 1996; Lima and Almeida 2002), and the SRD
output (Soto et al. 2008; Soto and Canedo, 2011; Velo et al. 2013). If the SRD descending
axons not only terminate in the superficial and deep laminae of the dorsal horn (Tavares
and Lima, 1994; Lima and Almeida, 2002) but also in the ventral horn, parallel descending
fibers from the mMRF and the SRD may concurrently modulate the nociceptive ascending
transmission as well as the spinal circuitry involved in the generation of aversive motor
responses to painful stimuli. The combined mMRF-SRD modulation would probably depend
on mMRF axons running in the VMF since none of the 20 mMRF-RSNs responding
antidromically only to DFL collateralized to the SRD. The bifurcating (DLF+VMF) and
trifurcating (DLF+VMF+SRD) fibers showed mean antidromic conduction velocities
significantly faster to VMF stimulation than to DLF stimulation, suggesting that the VMF
putative effect on ventral horn motor output will be exerted in advance of the postulated
modulatory effect of DLF fibers on the dorsal horn. These time differences would however
tend to be equated in relation to movement initiation given the extra delays imposed by the muscle electromechanical coupling and viscoelastic properties.

The mean antidromic conduction velocity of mMRF-RSNs to cervical stimulation observed in this study was similar to the velocity obtained by Wolstencroft (1980) using a stimulating array of electrodes placed around the cord at lower thoracic or upper lumbar levels but slower than the mean velocity reported by others for reticulospinal axons reaching the lumbar cord (Eccles et al. 1975; Peterson et al. 1975; Drew et al. 1986; Noga et al. 2003). A conduction delay in rubrospinal (Canedo and Lamas 1989), SRD-spinal (Velo et al. 2013) and reticulospinal (Eccles et al. 1975; Peterson et al. 1975) fibers appears to occur within the brain stem; but axons of RSNs terminating at the cervical cord were significantly slower conducting than those reaching thoracic and lumbar segments (Peterson et al. 1975) as were SRD-spinal axons (Velo et al. 2013). Accordingly, mMRF-RSNs conduction time might be tuned to fiber length to reach their different spinal targets at nearly the same time to generate multisegmental coupled synergies. Noxious sensitive mMRF-RSNs are presumably implicated in fast, urgent movements for simultaneous and rapid postural adjustments of the limbs, the body, and the neck. To accomplish this simultaneity of action it is to be expected that axons running longer distances will have faster conduction velocities. Also, the trifurcating axons (VMF+DLF+SRD) were the fastest suggesting that they originated from larger mMRF-RSNs if axonal conduction velocity relates to soma size.

Peripheral and/or central stimulation, including noxious heat, is expected to activate mMRF-RSNs of different sizes and if threshold is determined by cellular size, cells receiving uniform inputs will be orderly recruited from smallest to largest (Henneman et al. 1965). The earlier activity of the smaller fibers within each descending tract will enhance the excitability of the later firing neighboring larger fibers lowering their thresholds (Fig.3) and thus increasing their conduction velocity, a mechanism that will tend to homogenize the target's
arrival time of larger and smaller fibers within a given tract. The axonal hyperexcitability will also favor the invasion of action potentials over axonal bifurcations aiding to reduce action potential attenuation or failure at the terminals and enhancing activity-dependent vesicular release over widespread terminals as, for example, the mMRF terminals within the SRD (Fig.12H-J).

Pain decreases the activity of low threshold spinal motoneurons and recruits different units of those recruited during non-painful movement, probably because of uneven distribution of synaptic input to the motoneuron pool (Tucker et al. 2009). Noxious sensitive reticulospinal cells could play some role on this reordered recruitment.

**Receptive fields and properties of noxious mMRF-RSNs**

The sampled noxious sensitive mMRF-RSNs had large, usually bilateral, receptive fields; and were not driven by visual, auditory, proprioceptive or innocuous cutaneous stimulation in accord with prior work describing mMRF noxious-sensitive cells mostly devoid of other sensory input (Wolstencroft 1964; Casey 1969; Pearl and Anderson 1978; Willis et al. 1984; Farham and Douglas 1985). If spontaneous noxious sensitive mMRF-RSNs (about 45% in our preparation) are also present in the alert animal, they could participate in a tonic modulation of dorsal horn nociceptive ascending transmission and/or tonic modulation of flexion reflexes. The silent cells at rest (about 55% in this work, but probably less abundant in the non-anesthetized animal) can mediate not only the coordination of postural reactions but also limb movement (Schepens and Drew 2004, 2006; Buford and Davidson 2004; Davidson and Buford 2006; Schepens et al. 2008; Riddle et al. 2009; Riddle and Baker 2010; Baker 2011; Soteropoulos et al. 2012; Ortiz-Rosario et al. 2014) in response to transient noxious stimuli, coordination that would become continuous in conditions of chronic pain (Hodges 2001).
The noxious sensitive mMRF-RSNs showed spatial summation to distant, including bilateral stimulation sites (Fig.7), implying the recruitment of more dorsal horn nociceptive neurons as stimuli were applied and suggesting central neurons to integrate the outputs of dorsal horn cells. Temporal summation and prolonged postdischarges following repetitive stimulation were common in mMRF-RSNs (Fig.8) as it would be expected if they elicit robust movement and escape responses. Summation improves sensitivity but decreases spatial acuity and precise localization. The increased sensitivity will trigger prompt reactions to low intensity noxious stimulation over large portions of the skin and thus the mMRF-RSNs will mostly deal with the total heat and not with its accurate distribution. The SRD also encodes pain intensity (Villanueva et al. 1989). A prompt motor reaction to a noxious input is linked to a pain sensation and the mMRF could also be implicated in painful sensations through ascending projections to the medial/intralaminar thalamus (Bowsher et al. 1968; Peschanski and Besson 1984; Steriade et al. 1984; Vertes et al. 1986; Matsuyama et al. 1988; Krout et al. 2002) which is also targeted by fibers from the SRD (Krout et al. 2002). Even some mMRF neurons emit bifurcating ascending and descending fibers (Eccles et al. 1975; Steriade et al. 1984; Martin et al. 2011) although it is unknown if part of them have noxious receptive fields. The nociceptive-specific medial/intralaminar thalamic neurons also have bilateral large receptive fields (Dong et al. 1978; Dostrovsky and Guilbaud 1990) sending onward projections to sites related to the subjective and emotional perception of pain (Vogt and Sikes 2000; Vogt 2005). If simultaneous noxious signals from different sources are evaluated to produce a coherent percept, then spatial convergence and summation should be maximized, a role that the mMRF would aid to perform.

Summation and postdischarges allow the mMRF cells to generate a strong motor reaction and send an ascending amplified signal of potential/real tissue damage to
medial/intralaminar thalamic cells that would, i) discriminate noxious intensity (Bushnell and Duncan 1989) sending this information to widespread cortical regions and the basal ganglia (Royce and Mourey 1985; Royce et al. 1989; Nakano et al. 1990; Groenewegen and Berendse 1994; Percheron et al. 1994; Smith et al. 2004), and ii) play a role in widespread clinical pain (Staud et al. 2004).

**SRD and mMRF reciprocal relations**

Anatomical data showed that fibers from the SRD make synaptic contact on basal dendrites and soma of mMRF cells as do fibers from the mMRF on SRD neurons, thus allowing a strong reciprocated excitatory monosynaptic effect that could be picked up electrophysiologically. The electrophysiological data suggest that the mMRF-SRD network might be constituted mostly by connections through collaterals of mMRF-RSNs descending in the VMF, and by collaterals of SRD spinally projecting axons. Reciprocal direct connections cannot be discarded since the collateralizing axons were significantly slower conducting than their parent fibers and thus presumably of smaller diameter and less likely to be filled with Pha-L. Antidromic identification of DLF and VMF bifurcating SRD fibers was an unexpected finding and points to a motor function of SRD axons within the VMF. Accordingly, noxious responsive neurons forming the SRD/mMRF network could modulate their own activity through reciprocal connections and, through functionally coupled parallel descending systems, would affect the activity of dorsal and ventral horn neurons to regulate nociceptive initiated movements, reflex coordination and ascending noxious information.

Nociceptive integration and modulation involves regulation of temporal neural responses over the entire nervous system. The rapid spinal protective reflex would be complemented through the mMRF-SRD network and, on the sensory side, the mMRF would amplify
ascending painful information to the medial thalamus not only from the spinal cord but also from the SRD (Fig.14) since SRD-thalamic fibers are uncommon in the cat (Velo et al. 2013).

The spinal circuitry mediating reticulospinal influences is complex and constituted by different spinal networks. Although most reticulospinal fibers are glutamatergic, some are GABAergic, some glycinergic and still some others may release other neurotransmitters (Holstege and Bongers 1991; Antal et al. 1996; Holstege 1996; Jordan et al. 2008; Du Beau et al. 2012). The inhibitory descending axons give the reticulospinal system a unique property among the descending tracts, the potentiality to monosynaptic inhibit or disynaptic disinhibit spinal neurons.

In the proposed forward model schematized in Fig.14, noxious input is assumed to modulate the activity of the SRD-mMRF network whose outputs are used as new entries, thus maintaining active the reverberating circuit. The network amplifies noxious input through temporal summation (windup) sending outputs to the spinal cord and to the thalamus. Once noxious input disappears or it is unable to generate windup, the activity in the network recovers to basal level. This implies that the SRD-mMRF network descending system will become more active in modulating spinal nociceptive and motor processes during the development of persistent pain. Thus, the network would function phasically in response to transient noxious stimuli but tonically during chronic painful input as after spinal dorsal horn sensitization.

Endogenous pain modulatory systems use multiple parallel descending pathways including one from the midbrain periaqueductal grey (PAG) through its projections to nucleus raphe magnus (NRM) that via raphespinal fibers regulate ascending nociceptive transmission at the spinal cord dorsal horn (Basbaum et al. 1976, 1978; Bebehani and Fields 1979). Both NRM and mMRF are primary medullary targets of projections from the PAG (Basbaum et
al. 1978; Fields and Basbaum 1978; Gallagher and Pert 1978; Bebehani and Fields 1979; Abols and Basbaum 1981; Mantyh 1983). NRM and mMRF are simultaneously involved in mediating descending PAG effects and must be simultaneously blocked before the efficacy of PAG stimulation is affected (Gebhart et al. 1983; Sandkühler and Gebhart 1984). Since the NRM does not appear to receive direct spinal cord inputs (Abols and Basbaum 1981), the neuronal pathway linking spinoreticular neurons with the NRM may involve the mMRF (Braz et al. 2009). Therefore, descending fibers from the NRM and the mMRF may perform similar/complementary roles in relation to pain and motor modulation. A phasic motor role has been recently proposed for the NRM by Hellman and Mason (2012) who emphasized its role in withdrawals and in modulating nociception only when necessary. As stated above, the mMRF-SRD network could also serve such a phasic role in response to transient stimuli although via separate descending pathways impinging on different spinal neurons (McCreery et al. 1979; Gebhart et al. 1983). If the NRM and the SRD are interrelated as are the SRD and the mMRF (this work), all three structures SRD, NRM and mMRF will form a reticular network granting their near simultaneity of action on spinal modulation.

One of the multiple possible mechanisms that reticulospinal fibers may employ to regulate muscle activity and noxious ascending transmission is to activate spinal GABAergic interneurons to decrease transmitter release from presynaptic terminals of primary afferents. GABA binds to GABA-A receptors in the presynaptic terminals to open ionotropic chloride channels, leading to presynaptic depolarization (PAD) that diminishes neurotransmitter release (Rudomin and Schmidt 1999) thus limiting pain transmission and regulating motoneuronal excitability by, for example, restricting the truncation of monosynaptic reflexes produced by Iβ disynaptic inhibition (Fig.14). Reticulospinal stimulation decreases PAD in Iα terminals and increases PAD in Iβ terminals (Rudomin and
Schmidt 1999) and, as depicted in Fig.14, whereas DLF fibers would be mostly implicated in filtering noxious and IB afferent input, VMF axons would be mostly related to movement mainly through excitatory interneurons.

Presynaptic inhibition of “I₈” afferents decreases in spasticity (Delwaide and Oliver 1988; Morita et al. 2006; Dietz and Sinkjaer 2007), and thus disruption of reticulospinal fibers inducing PAD on “I₈” afferents should increase motoneuronal excitability and may play a role in the clasp-knife response seen in spastic patients. PAD is an inhibitory process in normal conditions but can be transformed into excitation when the depolarization is strong enough to generate action potentials (dorsal root reflex) as it occurs in persistently active nociceptor terminals (Rees et al. 1995; Lin et al. 2000). Indeed, mMRF stimulation can induce excitatory and inhibitory effects on noxious heat ascending transmission (Haber et al. 1980; Zhuo and Gebhart 1990, 1992). In normal conditions, the sign of effects would probably depend not only on the behavioral context (Seki et al. 2003) but also on the priority of pain in relation to other behaviors. Even the same spinal circuitry, differently modulated, could serve distinct behaviors (Dyson et al. 2014).

**Conclusion**

The main conclusion from the present work is that noxious mMRF-RSNs and SRD cells form an interrelated network through reciprocal neuronal projections mainly via collaterals of spinally descending axons. A single descending axon from a neuron in either structure can branch to innervate neurons in the other, as well as neurons in the dorsal and/or ventral columns of the spinal cord. This network would probably intervene in rapid spinal-reticular-spinal reactions providing a sensorimotor representation of nociceptive input, leading to adaptive adjustments of behavior through parallel efferent projections simultaneously regulating prompt motor responses and nociceptive afferent input.
GRANTS
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DISCLOSURES
The authors declare no competing financial interests.

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Current address Roberto Leiras: Department of Neuroscience; Karolinska Institutet; Retziusväg 8; 17177 Stockholm; Sweden.

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**FIGURE LEGENDS**

**Figure 1**

General experimental arrangement. Extracellular single neuronal recordings were obtained from the SRD and/or the mMRF. Descending reticulospinal axons were antidromically identified by ipsilaterally stimulating the DLF and the VMF at cervical 2-3 levels. Peltier cells (9x9 mm) were fixed to the contralateral forelimb, the ipsilateral hindlimb and the tail base in experiments studying noxious sensitive neurons.

Abbreviations: C2-C3, cervical spinal cord at 2-3 segmental level; Cru, cruciate sulcus; Cu, cuneate nucleus; DLF, dorsolateral funiculus; ECoG, electrocorticogram; Gr, gracile nucleus; IC, inferior colliculus; ICP, inferior cerebellar peduncle; IO, inferior olive; mMRF, medial medullary reticular formation; SRD, subnucleus reticularis dorsalis; VMF, ventromedial funiculus.

**Figure 2**

mMRF reticulospinal axons bifurcate dorsally and ventrally. A, antidromic identification of a mMRF reticulospinal neuron. A1, schematic drawing representing one mMRF-RSN with its
descending axon bifurcating in the DLF (blue) and the VMF (red). An orthodromic (ortho.)
spike travels down invading both bifurcations while one antidromic spike (Anti.) elicited by
electrically stimulating each axon collateral travels up towards the soma also invading the
other collateral. Collision between orthodromic and antidromic spikes occurs when an
orthodromic action potential precedes stimulation out in the axon by a time-period shorter
than the “collision interval” (latency to the stimulating site plus the axon’s refractory period).
Two colliding spikes annihilate because they enter into unexcitable membrane regions
immediately after their collision. A2-A4, same neuron with a sharp threshold to VMF (A2,
upper) and to DLF stimulation (A3, upper) and that had the ability to follow trains of VMF
(A2, lower left) and DLF (A3, lower) stimuli at 200 Hz with constant latencies. Collision of a
suprathreshold VMF-evoked antidromic spike with a previous orthodromic one is shown in
A2 (lower right). The reciprocal collision-extinction of suprathreshold antidromic spikes
elicited by stimulating VMF and DLF branches was obtained by gradually shortening the
delay between the two stimuli until causing a failure of the second stimulus (A4), indicating
that both action potentials arose from collaterals that shared the same parent axon. ISI:
interstimulus interval. The asterisks signal the stimulus artifacts. B, Antidromic conduction
velocity of mMRF-RSNs to DLF and VMF stimulation both as a population (B1) and
grouped according to their descending projection through one or both fascicles (B2).
ns, statistically non-significant difference (P>0.05). *= p < 0.05, ** = p < 0.01, *** = p <
0.001.

Figure 3

Antidromic conditioning-test interactions showed that axonal activation increased the
excitability of bifurcating mMRF reticulospinal fibers. A, same mMRF neuron whose
antidromic activation from the VMF collateral (A1) or the DLF collateral (A2) changed
subthreshold testing of the other collateral to suprathreshold at diverse interstimulus intervals. The numbers over each set of superimposed traces indicate the interstimulus intervals. Stimulus artifacts signaled by black arrowheads (VMF) and asterisks (DLF). B, Variation in antidromic excitability for 6 different mMRF-RSNs cells. Excitability was calculated as the percentage of antidromic spikes elicited by at least 10 consecutive test stimuli at each interstimulus interval.

**Figure 4**

mMRF-RSNs collateralize to the subnucleus reticularis dorsalis (SRD). A1, just threshold antidromic responses to SRD (left) and VMF (right) stimulation. A2, in one of the two superimposed traces, a spontaneous spike (sp) collided with one suprathreshold SRD-induced antidromic spike (left). The superimposed sweeps at right show that SRD suprathreshold stimulation elicited antidromic spikes (upper) that were suppressed when VMF suprathreshold stimulation elicited antidromic spikes within the collision interval (lower). The asterisks mark the stimulus artifacts. A3, the cell faithfully followed 200 Hz VMF stimulation. B, Graphical display of antidromic conduction velocities of mMRF-RSNs with and without SRD collaterals (left) as well as antidromic conduction velocities to SRD stimulation (right).

**Figure 5**

mMRF-RSNs activated by noxiously heating the skin. A1, schematic diagram showing the location of histologically reconstructed noxious sensitive cells plotted on a single, representative, frontal brainstem section at about P8.5. A2, antidromic conduction velocity of trifurcating (DLF+VMF+SRD), bifurcating (VMF+SRD) and single fibers (VMF) to VMF stimulation.
IO, inferior olive; NRGc, nucleus reticularis gigantocellularis; NRM, nucleus raphe magnus; NRPC, nucleus reticularis parvocellularis; PT, pyramidal tract.

B1-B4, same cell with a bifurcating axon (VMF+SRD) whose SRD suprathreshold antidromic spike collided with a spontaneous spike (sp) (B1, left) and SRD-VMF collision (B1, right). B2, unitary recordings and peristimulus histograms illustrating the response of the neuron to electrical stimulation of the ipsilateral forelimb (iFL, upper) and contralateral hindlimb (cHL, lower). B3, ON-OFF neuronal responses to mechanical stimuli of about 25 gr and 2 s duration applied through a feedback mechanical stimulator. Note that the cell did not respond to small-force stimuli (about 5 gr). Histograms showing the ON-OFF responses to 50 consecutive stimuli are shown in the lower panels. B4, responses to noxious heat applied to the skin.

Figure 6

Noxious stimulation converted regularly spaced single-spike spontaneous activity of mMRF-RSNs to regularly spaced spike doublets. A, antidromic identification of cell with a DLF and VMF bifurcating axon that collateralized to the SRD, as indicated over each vertical panel of two single sweeps in A1 through A4. Antidromic collisions of spontaneous spikes (sp) with suprathreshold evoked antidromic responses are illustrated in the lower records of each panel. Stimulus artifacts marked by asterisks (VMF), black ovals (DLF) and black arrowheads (SRD). B, Neuronal silences followed the antidromic spikes to VMF and to SRD stimulation. C, noxious stimulation of contralateral hindlimb (cHL) and ipsilateral forelimb (iFL) converted most of tonic single spikes into tonic doublets relative to control and recovery (autocorrelation histograms are shown at right for each condition).

Figure 7
Spatial summation of noxious receptive fields. **A**, silent cell at rest with an axon bifurcating through the DLF and the VMF. VMF (left) and DLF (right) suprathreshold stimulation evoked antidromic responses that collided with heat evoked action potentials in one of the two superimposed traces in each panel. Stimulus artifacts marked by asterisks. **B**, concurrent contralateral forelimb and ipsilateral hindlimb noxious stimulation produced spatial summation (autocorrelations below each recording condition). **C**, autocorrelations of unit activity to separate and simultaneous noxious stimulation (means of 5 cells) in the forelimb and hindlimb. **D**, poststimulus histograms showing the mean responses of 5 different neurons to electrical stimulation in the contralateral forelimb and the ipsilateral hindlimb, when applied separately (left) and simultaneously (right).

**Figure 8**
Heat-sensitive mMRF-RSNs developed windup. **A-C**, same neuron silent at rest. **A**, the cell projected an axon through the VMF (A1, a DLF-evoked orthodromic spike collided with the suprathreshold VMF-evoked antidromic response, lower) and responded orthodromically to DLF and SRD stimulation (**B**). Stimulus artifacts marked by asterisks in **A**. **C**, repetitive trains of electrical stimuli applied once per minute developed windup from the second train and beyond as more clearly seen in the raster plots below each train. **D**, repetitive noxious stimulation gradually increased postdischarges. **E**, a different neuron developing windup to 1 Hz DLF stimulation (left), and progression of windup with DLF stimulus number for 5 different mMRF-RSNs (right).

**Figure 9**
Noxious sensitive mMRF-RSNs are monosynaptically activated by non-spinally projecting SRD neurons also receiving noxious input. **A**, neuron with its descending axon bifurcating
in the DLF and the VMF. Threshold stimulation is shown in A1, left panel. One spontaneous spike (sp) collided with one suprathreshold VMF evoked antidromic spike (A1, middle panel) and the antidromic spikes elicited by suprathreshold VMF and DLF stimulation collided with each other (A1, right; lower superimposed traces). Stimulus artifacts marked by asterisks. Noxious stimulation at the ipsilateral hindlimb activated this neuron as well as a non-spinally projecting SRD cell simultaneously recorded (A2). Both cells displayed random activity in relation to each other (A3). B, a different mMRF-RSN also with an axon bifurcating in the DLF and the VMF (B1) simultaneously recorded with another non-spinally projecting SRD cell (B2). In one of the superimposed traces in the left panel of B1, spontaneous spikes collided with suprathreshold DLF (upper) and VMF (lower) evoked antidromic responses. VMF-DLF collision is shown at the right vertical panel (lower).

Stimulus artifacts marked by asterisks. Both cells were activated by noxious heat applied to the base of the tail (B2, upper) and had cross-correlated activities with a narrow peak after zero time (B2, lower). Electrical stimulation through the SRD electrode antidromically activated the mMRF cell (B3, upper right inset; a spontaneous spike in one of the superimposed traces collided with the SRD-antidromic response). The spike-triggered average (STA) using SRD spikes as triggers displayed a narrow peak at about 2 ms latency (B3, lower) suggesting a monosynaptic SRD to mMRF excitation. Compilation of STA using the mMRF spikes as triggers produced a broad peak straddling zero time, suggesting a common input (B4).

**Figure 10**

Noxious sensitive mMRF-RSNs are monosynaptically activated through collaterals of spinally-projecting SRD neurons also sensitive to noxious stimulation. A, mMRF and SRD simultaneous recording allowed to antidromically identify a mMRF-RSN with an axon.
projecting in the VMF and a SRD cell projecting in the DLF (collision between spontaneous 
(sp) and suprathreshold elicited antidromic spikes in lower records of A1 and A2). Stimulus 
artifacts signaled by asterisks. B, both mMRF and SRD cells were activated by noxious 
heat applied to the base of the tail and to the ipsilateral hindlimb. Note the rhythmic activity 
induced by noxious stimulation on both mMRF and SRD cells. C, the crosscorrelogram 
displayed a narrow peak near zero time, suggesting a monosynaptic influence of the SRD 
cell over the mMRF neuron. D, electrical microstimulation through the SRD electrode 
induced a monosynaptic response on the mMRF cell (upper right inset; the asterisk mark 
the stimulus artifacts), effect that was confirmed by the STA (lower). E, the STA compiled 
with the mMRF spikes triggering the SRD average showed a broader effect spanning zero 
time during noxious heating, suggestive of a common input (upper) but a non-significant 
effect before heating (lower).

Figure 11

mMRF-RSNs and spinally projecting SRD cells activated by noxious input monosynaptically 
influence each other through collaterals of their spinally-projecting axons. A, antidromic 
identification of a mMRF neuron with its descending axon bifurcating in the DLF and the 
VMF showing threshold (upper left two panels), DLF and VMF antidromic collisions with 
spontaneous spikes (in one of the two superimposed traces of the upper right two panels, 
suprathreshold evoked DLF and a VMF antidromic responses collided with spontaneous 
(sp) spikes), and VMF-DLF collision (lower right panel). B, antidromic identification of a 
simultaneously recorded SRD cell with its descending axon also bifurcating in the DLF and 
the VMF showing the latencies to VMF and DLF (upper left), axonal reciprocal collisions 
(upper middle and right) and suprathreshold DLF and VMF antidromic responses colliding 
with spontaneous (sp) spikes. Stimulus artifacts in A and B marked by asterisks. C, both
neurons were activated by noxious heat applied to the ipsilateral hindlimb. D, crosscorrelogram showing two narrow peaks at both sides of zero time suggesting reciprocal monosynaptic excitation. E, compilation of STA using the SRD spikes as triggers displayed a monosynaptic excitation (E1, upper) corroborated by electrical SRD microstimulation (E1, lower left inset. Asterisk marks the stimulus artifacts). Note that gradually increasing the electrical SRD microstimulation revealed an antidromic response at a lower threshold than another orthodromic response with a shorter latency (E1, lower left inset). The diagram at the lower right inset in E1 illustrates the circuitry that would explain the data. The compiled STA using the mMRF spikes as triggers also exhibited a monosynaptic excitation (E2, upper) that was confirmed by mMRF electrical microstimulation (E2, lower left inset. The asterisk signals the stimulus artifacts). The circuitry that would explain these data is illustrated by the diagram shown at the lower right inset in E2.

**Figure 12**

mMRF neurons project to the SRD and the spinal cord. Pha-L labeled fibers and terminal boutons were found bilaterally in the SRD after tracer injection in the left mMRF. A-B, photomicrographs of injection sites in two cats (cases E258 and E323); tracer injection involved primarily the ventral portion of the mMRF in case E258 (A) and the dorsal one in case E323 (B); the approximate AP level, according to the atlas of Snider and Niemer (1961), is indicated at the lower left of panels A and B. Lower right inset drawings in A and B show the full extent of the tracer deposit in black and the area of diffusion in grey. C-D, schematic representations of the distribution of Pha-L labeled immunoreactive fibers (lines in C) and terminals (dots in D) in the SRD. E-F, low magnification photomicrograph of left coronal hemisections at the level of the caudal medulla. E1 and F1 are enlargements of the
boxed areas marked in E and F, showing the presence of labeled fine fibers (arrows) and fibers with terminal boutons (arrowheads) in the SRD. G, schematic drawing of Pha-L immunoreactive axons descending from the mMRF through the spinal white matter of the upper cervical cord. G1 and G2 are higher magnification images of the regions delineated in G, showing axons (arrows) coming from the left mMRF running down in the dorsolateral (G1) and ventromedial (G2) ipsilateral funiculi. H-I-J, high magnification photomicrographs showing the close relationship between Pha-L labeled mMRF boutons (black) and Neu-N labeled SRD neurons (brown). Note the presence of Pha-L labeled fiber boutons contacting cellular somas (arrowheads in H and I) and proximal dendrites (arrows in I and J).

**Figure 13**

The SRD projects to the mMRF. Injection of Pha-L into the SRD anterogradely labeled axonal fibers and terminals in the mMRF. A, photomicrograph showing the injection site of Pha-L centered on the SRD of a representative case (E284), and schematic drawing (lower right inset) showing the full extent of the central core of Pha-L deposit (black) and the halo region (grey). B-C, schematic representations of Pha-L immunoreactive fibers (B) and terminals (C) through the mMRF. D, low magnification photomicrograph of left coronal hemisections at the level of the mMRF (approximate Horsley-Clarke coordinate P8). D1, higher magnification of the boxed area in D. Note the presence of labeled fibers (arrows) and terminal boutons (arrowheads) in the mMRF. E-I, high magnification photomicrographs of mMRF neurons labeled with Neu-N showing nearby preterminal fibers and boutons anterograde labeled from the SRD. Note in E-F the axosomatic relationship (arrowheads) of Pha-L-labeled axonal boutons and in F-H, Pha-L-labeled axonal boutons in close association with proximal dendrites (arrows). Note also in I, a Pha-L-labeled axon with en passant puncta (arrows) closely associated to a dendrite.
Schematic wiring diagram illustrating one of the possible SRD-mMRF-spinal networks underlying regulation of ascending noxious information and motoneuronal excitability. Noxious information in the anterolateral system reaches the SRD and the mMRF activating reticulospinal cells in both structures that establish mutual SRD-mMRF interconnections through collaterals of descending DLF and/or VMF fibers. Reticulospinal fibers activate GABAergic interneurons producing presynaptic inhibition by depolarizing the terminals of primary afferents (PAD), and excitatory interneurons that co-activate alfa (αMn) and gamma (µMn) motoneurons. It is postulated that while DLF fibers would be mostly related to presynaptic inhibition, VMF fibers would be mostly motor-related. Muscle contraction activates IB afferents that disynaptically inhibit motoneurons projecting to the activated muscle and its agonists, and disynaptically excite motoneurons projecting to antagonists (not represented). Nevertheless, many details of the functional organization and interrelationship(s) of reticulospinal systems on the modulation of spinal circuitry remain to be resolved.
A. Antidromic Identification

A1. Schematic Diagram

A2. VMF Stimulation

A3. DLF Stimulation

A4. Reciprocal Collisions

B. mMRF-RSNs Sampled (n=183)

B1. Total Sample

B2. Single and Double Projecting
A1. DLF Subthreshold

17.5 ms

16.5 ms

VMF

DLF

A2. VMF Subthreshold

29 ms

25 ms

VMF

18 ms

VMF

B. Conditioning-Test Antidromic Excitability

n = 6

Antidromic Excitability (%) vs. Interstimulus Intervals (ms)

+/− SD

VMF (Conditioning) - DLF (Test)

DLF (Conditioning) - VMF (Test)
A1. Thresholds

A2. Collisions

A3. VMF Stimulation (100 Hz)

B. Reticulospinal Collaterals to the SRD (n = 78 of 151 tested)

B1. Reticulospinal Antidromic Velocity (m/s)

B2. SRD-mRF Antidromic Velocity (m/s)

Corrected (0.4 ms)

d = 11 mm

21.65 +/- 1.59
23.90 +/- 1.49
A1: Antid.r. (DLF+VMF+SRD)
○: Antid.r. (VMF+SRD)
*: Monosynaptic to SRD

B1. Collisions
SRD
* 2 ms
sp

B2. Skin electrical stimulation

B3. Skin mechanical stimulation

B4. Noxious heat stimulation

A2: VMF Antidromic Velocity (m/s)

![Graph showing VMF Antidromic Velocity (m/s)]

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<th>Condition</th>
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<td>20</td>
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<td>VMF + SRD</td>
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<td>VMF</td>
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Note: **p < 0.01, ***p < 0.001
A. Antidromic Identification

A1. VMF (*)    A2. DLF (*)    A3. SRD (●)

Field Potential

sp

4 ms

sp

sp

4 ms

sp

sp

A4. Reciprocal Collisions

VMF (*) - SRD (●)    VMF (*) - DLF (●)

4 ms

B. Silences following antidromic spikes

Silences:
129 +/- 18 ms

VMF Stimuli

34  35  36  37  38  39  40  s

Silences:
117 +/- 23 ms

SRD Stimuli

294  296  298  300  s

C. Noxious Heat Stimulation

C1. Control

40℃

iFL

chL

Spike/ms

60 ms

C2. Noxious Heat (chL)

40℃

iFL

chL

Spike/ms

60 ms

C3. Noxious Heat (iFL)

40℃

iFL

chL

Spike/ms

60 ms

C4. Recovery

40℃

iFL

chL

Spike/ms

60 ms
A. Collisions

B. Noxious Heat

C. AUTO: Means of 5 cells

D. Electrical Stimulation: Means of 5 cells
A. Antidromic Identification

C. DLF Stimulation, five consecutive trains

D. Increased Postdischarges to Stimulus repetition

E. Wind-up to DLF Stimulation
A1. Antidromic Identification (mMRF Recording)

Threshold
Collision
Reciprocal collision

DLF
VMF

A2. Noxious Heat Stimulation

°C
Ipsilateral Hindlimb

SRD Recording

mMRF Recording

Time [s]

A3. Unrelated SRD and mMRF Activity

Crosscorrelogram
n = 20,414
5 ms bin

mMRF Count

mMRF [μV]

SBP [μV]

Time [ms]

B1. Antidromic Identification (mMRF Recording)

Collisions
Reciprocal collision

SP
VMF

B2. Noxious Heat Stimulation

Base of Tail

mMRF [Hz]

Mean Frequencies

Time [s]

B3. SRD-mMRF STA

Trigger SRD
n = 21,066
Lat = 2 ms

mMRF [μV]

B4. mMRF-SRD STA

Trigger mMRF
n = 24,013
A. Antidromic Identification

A1. mMRF Recording
A2. SRD Recording

B. Noxious Heat Stimulation

B1. Base of Tail
B2. Ipsilateral Hindlimb

C. Crosscorrelogram

C1. Trigger SRD
n = 10,338
2 ms bin
Mean (all spikes included)
SD

D. SRD-mMRF STA

D1. Electrical Microstimulation
SAD (100 µA)
Minimal latency: 2 ms

E. mMRF-SRD STA

E1. During heating
n = 13,135

E2. Before heating
n = 4,265
Table I. Paired SRD-mMRF recordings. SRD cells projecting in the DLF (n=7), the DLF and the VMF (n=5), or that did not project to the spinal cord (nSPr, n=5) were simultaneously recorded with mMRF-RSNs projecting in the DLF and/or the VMF, as indicated.

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