Original Article
Central circuits regulating the sympathetic outflow to lumbar muscles in spinally transected mice by retrograde transsynaptic transport

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Abstract: Despite considerable interest in the mechanisms that control the hyperalgesia associated with muscle inflammation, the CNS descending pathways that coordinate autonomic circuits regulating lumbar muscles are not adequately understood. Here we used both pseudorabies virus (PRV)-614 retrograde transsynaptic tracing and spinally transected method in 33 C57BL/6J mice to map the polysynaptic pathways between lumbar muscle and CNS. Tissues were processed for dual-label immunocytochemical detection between PRV-614 and tryptophan hydroxylase (TPH) or tyrosine hydroxylase (TH)-expressing neurons in CNS. In intact mice, PRV-614 was transported to the intermediolateral column (IML) and ventral horn (VH) of spinal cord, with subsequent transport to many brain regions, including the medullary raphe nuclei, rostral ventrolateral medulla (RVLM), A5 cell group regions (A5), locus coeruleus (LC), the medullary and pontine reticular formation nucleus (MRN and PRN), paraventricular nucleus of the hypothalamus (PVN), and other central sites. However, PRV-614 in spinally transected mice produced retrograde infection of IML, with subsequent transport to main brain regions that have been shown to contribute to regulating sympathetic circuits, including RVLM, Lateral paragigantocellular reticular nucleus (LPGi), A5 cell group regions (A5), locus coeruleus (LC), the medullary and pontine reticular formation nucleus (MRN and PRN), paraventricular nucleus of the hypothalamus (PVN), and other central sites. However, PRV-614 in spinally transected mice produced retrograde infection of IML, with subsequent transport to main brain regions that have been shown to contribute to regulating sympathetic circuits, including RVLM, Lateral paragigantocellular reticular nucleus (LPGi), A5 cell group regions (A5), locus coeruleus (LC), the medullary and pontine reticular formation nucleus (MRN and PRN), paraventricular nucleus of the hypothalamus (PVN), and other central sites. However, PRV-614 labeling in VH and MRN was eliminated in almost every case. In above five brain regions, dual-labeling immunocytochemistry showed coexpression of PRV-614/TPH and PRV-614/TH immunoreactive (IR) neurons involved in these regulatory circuits. Our results reveal a hierarchical organization of central autonomic circuits controlling the lumbar muscles, thus providing neuroanatomical substrates for the central catecholaminergic and serotonergic system to regulate the lumbar muscles.

Keywords: Lumbar muscles, spinal transection, pseudorabies virus, tryptophan hydroxylase, tyrosine hydroxylase

Introduction

Muscle inflammation or injury clearly causes primary hyperalgesia (increased response to noxious stimuli at the site of injury) and secondary hyperalgesia (increased response to noxious stimuli outside the site of injury) [1-4]. Chronic musculoskeletal pain conditions such as repetitive strain injury are associated with peripheral tissue damage that includes inflammatory and non-inflammatory components of the muscle [5-7]. Previous literatures suggested that persistent low back muscle pain had developed a clinical problem [8-11]. Despite considerable interest in the mechanisms that control the hyperalgesia associated with muscle inflammation, the CNS descending pathways that coordinate autonomic circuits regulating lumbar muscles are not adequately understood.

The neurotropic pseudorabies virus (PRV) is used as a marker for synaptic connectivity in CNS by propagating retrogradely through chains of functionally connected neurons [12-15]. A number of studies have verified that this model system provides a highly specific method of mapping the motor and sympathetic pathways innervating a variety of targets [12, 16, 17]. PRV-614 strain used in this work is retrograde-specific, and expresses a novel monomeric red fluorescent protein (RFP) for visualization [18-21].
Central circuits controlling lumbar muscles and spinal transection

The goal of the present study was to elucidate the neuronal circuitry in the regulation of the hyperalgesia associated with low back muscle. The lumbar epaxial muscle is a typical representative of low back muscle, so PRV-614 was injected into the left lumbar epaxial muscle of each animal. To prevent PRV-614 from being transmitted to the brain via motor circuitry, a spinal transection was performed just below the L2 level, which is caudal to the majority of sympathetic preganglionic neurons (SPN), thereby allowing for a more selective uptake by SPN [17]. Immunohistochemical detection of tryptophan hydroxylase (TPH), the rate-limiting enzyme in serotonin production [22] and tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis [23-26], was also incorporated into the experiments, so that we could ascertain whether neurons infected by PRV-614 injections into the mouse muscle were at least partly serotonergic or catecholaminergic [27-30]. We seek to map the polysynaptic pathways between lumbar epaxial muscle and CNS (the brain and spinal cord), using PRV retrograde transsynaptic tracing and spinally transected method in mice.

Materials and methods

Animal care and use

Adult male C57BL/6J strain mice weighing 25-30 g (n = 33 total) were housed in groups of three or four in a climate-controlled room under a 12 hr light/dark cycle. The use and handling of animals were approved by the Institutional Animal Care and Use Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology University and were in accordance with guidelines provided by the National Institutes of Health and the International Association for the Study of Pain.

PRV-614

The final titer was $2 \times 10^8$ plaque-forming units (pfu)/ml for PRV-614. Aliquots (20 µl) of the virus were kept in the freezer (-80°C). On each experimental day, an aliquot was thawed and kept on ice until immediately before injections.

Experimental groups

Experiment 1 (intact group, n = 12): aimed at optimizing viral infection of motor and autonomic pathways in the lumbar muscle. Experiment 2 (spinally transected group, n = 21): spinally transected to prevent infection of motor pathways before injection of PRV-614 into the lumbar muscle.

Surgical procedures

The L2 spinal cord was surgically transected in some mice using a technique described previously [17]. Briefly, animals were anesthetized with isoflurane inhalation via a nose cone, and the skin overlying the dorsal process of the 13th thoracic vertebra was incised, the fascia and back muscles were deflected laterally to expose the vertebra. Under a dissection microscope, the dorsal aspect of the vertebra was removed using an electrical drill to expose the upper lumbar spinal cord. Subsequently, the spinal cord was transected just below the L2 level using an electrocautery. Transection was made carefully under magnification and the transection site subsequently cleaned with saline-soaked swabs. After surgical procedures were completed, back muscles, fascia, and the overlying skin was closed with silk sutures.

Injection of PRV-614 into the lumbar muscle

Lumbar muscle injections were made as described previously [31-33]. Briefly, animals were anesthetized with isoflurane inhalation, and a midline longitudinal incision was made in the dorsal lumbar region after using aseptic procedures. The skin and connective tissue were dissected to expose the epaxial muscles at the level of the L5 vertebra, which was located using the depression between the lumbar and sacral vertebra as a landmark. Under a dissection microscope, a series of injections with PRV-614 ($2 \times 10^8$ pfu/ml in a total of 1 µl per injection at three injection sites) into the lumbar epaxial muscle were made using a 30-gauge needle connected to a Hamilton syringe (10 µl) under microscopic guidance. Injection sites

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Animal antibody Raised in</th>
<th>Dilution</th>
<th>Source</th>
<th>Lot no. or Ab no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPH</td>
<td>Sheep</td>
<td>1:1000</td>
<td>Chemicon, Temecula, CA</td>
<td>AB1541</td>
</tr>
<tr>
<td>TH</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Chemicon, Temecula, CA</td>
<td>AB152</td>
</tr>
</tbody>
</table>
Central circuits controlling lumbar muscles and spinal transection

were chosen at the level of the L5 vertebra at the following approximate distances from midline: 3, 8, and 12 mm. After the final injection, care was taken to swab the injection site to minimize leakage, and the muscle surface was rinsed twice with sterile saline-soaked swabs and dried repeatedly. The skin was closed with silk sutures and animals were returned to their home cages. Animals were allowed to survive 3-6 days. This survival period was chosen according to the results of preliminary experiments conducted in our laboratory. Mice were provided analgesia with an intramuscular injection of a mixture of ketamine (10 mg/kg) and ketoprofen (3 mg/kg) just before the surgery and every 12 h subsequently for a postsurgical period of 72 h. Mice infected with PRV-614 were euthanized either before or when they showed apparent trait of illness or distress (i.e., 5-7 d after inoculation). Afterward, all animal bodies were humanely destroyed and all cages were cleaned and sanitized.

**Tissue processing**

After a survival time of 3-7 d, deeply anesthetized animals were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde-borate fixative (pH 9.5) through the left ventricle of the heart. Brains and spinal cords were removed, and postfixed for 2 h in 4% paraformaldehyde-borate and overnight in a 30% sucrose solution at 4°C. Postfixed brains and spinal cords were blocked, sliced into 30 µm coronal sections on a freezing-stage sledge microtome, and collected into four serially ordered sets of sections. Tissue sections were stored at 4°C in cryoprotectant until they were processed for immunohistochemistry visualization.

**Table 2.** Quantitative analysis of PRV-614/TH double-labeled neurons 5 days after PRV-614 injection into the lumbar muscle

<table>
<thead>
<tr>
<th>Areas</th>
<th>PRV-614 infected neurons</th>
<th>TH-positive neurons</th>
<th>Double neurons/PRV-614-neurons</th>
<th>Double neurons/TH-neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVLM</td>
<td>++</td>
<td>+</td>
<td>16.7%</td>
<td>16.7%</td>
</tr>
<tr>
<td>LPGi</td>
<td>+</td>
<td>+</td>
<td>16.7%</td>
<td>33.3%</td>
</tr>
<tr>
<td>A5</td>
<td>+</td>
<td>+</td>
<td>33.3%</td>
<td>42.9%</td>
</tr>
<tr>
<td>LC</td>
<td>+++</td>
<td>++</td>
<td>33.3%</td>
<td>29.4%</td>
</tr>
<tr>
<td>PVN</td>
<td>+++</td>
<td>++</td>
<td>51.6%</td>
<td>36.4%</td>
</tr>
</tbody>
</table>

The number of positive neurons per region from a 1:4 subset of sections: +/-, 1-5, but not in all mice; +, 6-10; ++, 11-20; ++++, > 20.
Fluorescence immunohistochemistry

One of every four series from each animal was subjected to the double immunofluorescent staining procedure that was performed using standard procedures for visualization of putative neurotransmitter or enzymes in virally infected neurons [34-36]. PRV-614 infected neurons express the red fluorescent protein for direct visualization under fluorescence microscope. Briefly, free floating tissue sections were incubated at 4°C overnight in 0.02 M potassium PBS (KPBS; pH 7.4) containing 2% normal donkey serum, and 0.4% Triton X-100 (LKPBS). The tissue sections were treated further by washing with 0.01 M PBS (3 × 10 min) and then followed by incubation with a primary antibody solution (see list in Table 1 for antibody information including dilution and suppliers) in 0.02 M LKPBS for 24-48 h at 4°C. Sections were then rinsed thoroughly in 0.01 M PBS (3 × 10 min). All sections were mounted onto gelatin-coated slides, air dried overnight, and coverslipped with mounting media. In negative control incubations, the primary antiseraums were omitted from the immunohistochemical reaction. This procedure completely eliminated neuronal staining.

Tissue analysis

Immunoreacted tissue sections were examined and photographed using an Olympus X81 photomicroscope equipped with epifluorescence and filters that selectively excited Bodipy-FL, Cy3 or Fluor 350, and with a filter that allowed for the excitation of both fluorophores. The red fluorescence of Cy3 was used to identify cells infected after injection of PRV-614 into the lumbar muscle, whereas the green fluorescence of Alexa Fluor 488 was used to identify neurons which contained TH, and the blue fluorescence of Alexa Fluor 350 was used to identify TPH-containing neurons. Images were overlaid using Adobe Photoshop, and double-labeled neurons are presented as yellow or pink. High-magnification analysis was used to determine whether...
er overlapping yellow or pink images were due to colocalization in the same neuron or overlap of independently labeled neurons.

The TH-, TPH- and PRV-614-IR neurons were counted under the 20 × objective of a fluorescence microscope in both sides on all sections in each series. Double-labeled neurons were cautiously determined by switching red and green or blue filters under the 40 × objective. The number of neurons expressing PRV-614 and/or TH, TPH per section was assessed for each animal. The percentage of PRV-infected neurons in a region that was immunopositive for TH or TPH was determined in a similar manner. The regions in which infected cells were located were defined with reference to the atlases of Paxinos G and Franklin KB [37].

Results

Lumbar muscle injection of PRV-614 resulted in a consistent pattern of neuronal labeling in

<table>
<thead>
<tr>
<th>Areas</th>
<th>PRV-614 infected neurons</th>
<th>TPH-positive neurons</th>
<th>Double neurons/PRV-614-neurons</th>
<th>Double neurons/TPH-neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>RVLM</td>
<td>++</td>
<td>+</td>
<td>33.3%</td>
<td>20%</td>
</tr>
<tr>
<td>LPGi</td>
<td>+</td>
<td>+/-</td>
<td>16.7%</td>
<td>10%</td>
</tr>
<tr>
<td>A5</td>
<td>+</td>
<td>+/-</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>LC</td>
<td>+++</td>
<td>+/-</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>PVN</td>
<td>+++</td>
<td>++</td>
<td>26.1%</td>
<td>27.7%</td>
</tr>
</tbody>
</table>

The number of positive neurons per region from a 1:4 subset of sections: +/-, 1-5, but not in all mice; +, 6-10; ++, 11-20; ++++, > 20; *, no double-labeled neuron.
Central circuits controlling lumbar muscles and spinal transection

the spinal cord and brain of the intact and spinally transected mice. At progressively longer survival intervals, additional populations of cells were labeled sequentially, suggesting a hierarchical series of neuronal connections. The time course of PRV-614 neuronal tract tracing is presented within each level of the CNS.

**PRV-614 labeling in the spinal cord**

PRV-614 was injected into the lumbar muscle, and 80% of intact mice after 3 days survival showed PRV-614 cell labeling in spinal cord. Infected cells located within the ipsilateral intermediolateral cell column (IML) of the lower thoracic cord were heavily infected with PRV-614, whereas a few scattered cells were labeled the intercalated nucleus (IC) near the central canal, ventral horn (VH), and dorsal horn (DH) of the lower thoracic spinal segments ([Figure 1A](#)). However, infected neurons were not observed in spinal cord of transected mice 3d after PRV-614 injection in all spinally transected animals. When allowed to survive for 4

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**Figure 4.** Summary diagram showing that five common CNS areas containing neurons that project to both the autonomic circuitry and the sympathoadrenal outflow system. These areas are hypothesized to be involved in central control of blood flow to muscle.
Central circuits controlling lumbar muscles and spinal transection

to 6 d after PRV-614 injection, mouse had a selective effect, eliminating PRV-614 labeling in the VH of the lower thoracic cord, while sparing IML labeling in almost every case (Figure 1B).

**PRV-614 labeling in the brain of the intact animals**

After 3 d of PRV-614 injection (n = 3), two of the animals began to show infected cells in the rostral ventrolateral medulla (RVLM) and paraventricular nucleus of the hypothalamus (PVN) dorsal parvocellular (dpv) subdivision, and one animal began to show PRV-614 labeling in the raphe pallidus (RPa). After 4 d of PRV-614 injection (n = 3), labeled cells began to become more widespread, and were still apparent in the RVLM, RPa and PVN dpv as well as in several new regions. PRV-614 labeled neurons are concentrated in the rostral ventromedial medulla, the medullary and pontine reticular formation nucleus (MRN and PRN), the lateral brainstem [RVLM and A5 adrenergic cell group region (A5)], the dorsolateral pons [locus coeruleus (LC), nucleus subcoeruleus (SubC)], and hypothalamus [PVN, the ventromedial nucleus of the hypothalamus (VMH)]. After 5-6 d of PRV injection (n = 6), PRV-614 infected neurons were seen in all the same regions as in d 4, and new regions containing double-labeled neurons include the dorsal brainstem, midbrain (red nucleus, periaqueductal gray, Edinger Westphal nucleus), forebrain (motor cortex), and other central sites.

**PRV-614 labeling in the brain of the spinally transected animals**

In the 21 mice used in spinally transected experiment, the data of 12 mice were useful in understanding descending pathways that regulate sympathetic circuits. In 9 other mice, the spinal transection of 4 mice was incomplete, as indicated by a lack of the complete paralysis of the lower body, or made at the wrong level; the survival times of 2 mice were 3-48 h, and 3 mice were euthanized because they showed apparent trait of illness or distress during 4-6 d after inoculation.

Infected neurons were not observed in the brain 3 d after PRV-614 injection (n = 3). The survived 4 d mouse (n = 3) exhibited the infected neurons in RVLM and PVN. Comparison of labeling patterns in the intact mice, the survived 5-6 d mouse (n = 6) delayed the infection of several brainstem neurons, and the number of infected neurons was fewer in RVLM, LPGi, LC, A5 and PVN. Of the 9 animals with complete spinally transsection (including survived 4-6 d after muscle injection), 9 displayed labeled neurons in RVLM, 9 in PVN, 8 in LPGi, 8 in A5, and 7 in LC; whereas PRV-614 labeling in MRN was eliminated in almost every case.

The study of Experiment 2 spinally transected motor nerves before injection of PRV-614, thereby allowing for a more selective uptake by sympathetic preganglionic neurons. The presence of double labeling was confirmed that TH- or TPH-positive neurons in RVLM, PVN, etc., were involved in autonomic circuits.

**Double labeling for PRV-614/TH in the brain of the spinally transected animals**

The location of PRV-614 infected neurons was similar to what we had previously observed in brain sections processed for single PRV-614 labeling, with most PRV-614-positive cells located in PVN, LC, RVLM, LPGi, and A5 (Table 2). Most TH-positive cells were found in the PVN and LC areas (Table 2, Figure 2). Double-labeled PRV-614-/TH neurons were mainly located in the PVN, and 33.3% of the virally infected neurons in the A5 and LC were TH-immunoreactive, and double-labeled neurons were also detected in the RVLM (16.7%), and LPGi (16.7%) (Table 2, Figures 2, 3).

**Double labeling for PRV-614/TPH in the brain of the spinally transected animals**

In retrogradely labeled PRV-614 immunoreactive neurons, we found that most TPH-positive cells were in PVN, RVLM and LPGi. Double-labeled PRV-614/TPH neurons were mainly located in the RVLM, and 26.1% of the virally infected neurons in PVN were TPH-immunoreactive cells (Table 3, Figures 2, 3).

**Discussion**

The retrograde transsynaptic tracer pseudorabies virus (PRV) is used as a marker for synaptic connectivity in the CNS. In the present report, a spinal transection, as indicated by a lack of neurons immunopositive for PRV-614 in the VH of spinal cord, blocked the transport of
peripherally administered neural tracers PRV-614 into somatic motoneurons, but not the autonomic circuitry, suggesting that the brain neurons were infected with PRV-614 via the autonomic nervous system.

The finding of this study is that we have localized the CNS sites that innervate the autonomic circuits of the lumbar muscle. The most striking observation is there are five areas of the brain that appear to regulate autonomic outflow systems. These common areas include the RVLM, LPGi, A5, LC, and PVN. These same CNS areas innervate sympathoadrenal preganglionic neurons [38, 39], suggesting the possibility that they serve a common function - the central regulation of blood flow to muscle. Because this is a vital function, two parallel neural/endocrine systems exist (Figure 4). One of these pathways provides excitatory inputs to the lumbar muscle via autonomic circuits; another excitatory pathway involves excitatory inputs to the sympathoadrenal neurons triggering the release of adrenaline into the blood stream [39, 40]. In addition, 15-45% of the neurons in these regions that were labeled for the presence of PRV-614 were also immunopositive for TH or TPH, showing that descending catecholaminergic and serotonergic circuits are mainly involved in regulating the autonomic circuits innervating lumbar muscle.

The exact location of CNS sites that regulate blood flow to muscle remain unknown, but several studies have emphasized the importance of descending sympathoadrenal pathways originating in the medulla oblongata [17, 40-42]. Some studies using synaptic tracers have suggested that RVLM, LC, and SubC neurons, including noradrenergic cells, located in these regions, have collateralized projections to the spinal cord [41, 43]. In animals in which the hindlimb was completely sympathectomized, double-infected neurons from adrenal gland and muscle were observed in the A5 and RVLM, suggesting direct connections exist both sympathoadrenal neurons and muscle, and projections from these regions are believed to multisynaptic pathways. The data presented here suggest that two main regions of the medulla oblongata (RVLM and LPGi) may be involved to sympathetic circuits. These areas innervate IML and sympathoadrenal preganglionic neurons [38, 39], raising the possibility that central control of two neural/endocrine circuits, sympathoadrenal and sympathetic, maybe regulated by parallel systems under certain conditions, such as during the noxious stimulus [44].

Our results also showed that, after spinal transection, spinal cord-related autonomic neurons underwent physiological changes that significantly retard PRV-614 uptake in the brain, which is consistent with previous reports suggesting host cell-viral interactions and/or axonal transport in sympathetic circuits is altered after spinal cord injury [13]. Recent studies had shown that spinal lesion had been linked to the development of a number of debilitating conditions including autonomic dysreflexia [45, 46], which may be the result of altered synaptic inputs to sympathetic preganglionic neurons below the injury site [47, 48].

Collectively, the present data demonstrated that there are five CNS sites that project to autonomic nerve systems of the lumbar muscle, as well as the sympathoadrenal system. Our results revealed a hierarchical organization of central autonomic circuits controlling the lumbar muscles, thus providing neuroanatomical substrates for the central serotonergic and catecholaminergic system to regulate the lumbar muscles.

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Disclosure of conflict of interest

None.

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Central circuits controlling lumbar muscles and spinal transection

References


[23] Manger PR, Fahringer HM, Pettigrew JD and Siegel JM. The distribution and morphological characteristics of catecholaminergic cells in the brain of monotremes as revealed by tyro-


Central circuits controlling lumbar muscles and spinal transection