

## Original Article

# Motor cortex-periaqueductal gray-rostral ventromedial medulla neuronal circuitry may involve in modulation of nociception by melanocortinergetic-opioidergic signaling

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**Abstract:** Previous studies documented that stimulation of the motor cortex and periaqueductal gray (PAG) was effective management for refractory neuropathic pain, however, our understanding about the neuroanatomical and neurochemical mechanisms of motor cortex and PAG stimulation is still limited. We used a modified retrograde multisynaptic pseudorabies virus tracer PRV-614 injection to the kidney with an attempt to provide morphological evidence of the neuroanatomical circuit among motor cortex, PAG, rostral ventromedial medulla (RVM), and kidney. The principal findings of this study are: (1) Spinal nerve injury induced an increase of c-Fos/MC4R-GFP dual labeled neurons in motor cortex. (2) The inoculation of PRV-614 into the kidney resulted in retrograde infection of neurons in RVM, PAG and motor cortex, and PRV-614/MC4R-GFP dual labeled neurons were detected in RVM, PAG and motor cortex. (3) MC4R-GFP/MOR dual labeled neurons were detected in PAG, and 50%-70% of the GFP-positive neurons in the ventrolateral PAG were MOR-immunoreactive. These results together demonstrate that MC4R signaling in motor cortex-PAG-RVM neural circuit may participate in the descending modulation of nociceptive transmission. The data provided by this study suggest that melanocortinergetic-sympathetic signaling of motor cortex-PAG-RVM neural circuit may be a potential target for the inhibition of nociceptive transmission.

**Keywords:** Motor cortex, periaqueductal gray, rostral ventromedial medulla, neural circuit, modulation of nociception, melanocortinergetic signaling, opioidergic signaling

## Introduction

Previous studies in animal and human documented that increasing motor cortex and periaqueductal gray (PAG) by means of electrical brain stimulation or neural mobilization technique was effective management for refractory neuropathic pain [1-6]. There is a strong rationale for exploring the mechanisms of brain stimulation or neural mobilization technique as a therapeutic candidate for neurological pain [6, 7]. It is noteworthy that PAG-rostral ventromedial medulla (RVM) pathway forms part of a descending nociceptive network that modulates nociceptive neurotransmission both at the supraspinal level and the spinal cord dorsal horn [8-10]. Though there exist obvious changes within brain after motor cortex and PAG stimulation, including blood

flow increase appeared in motor areas [11, 12] and the secretion of endogenous opioids [3, 6, 13], our understanding about the neuroanatomical and neurochemical mechanisms of motor cortex and PAG stimulation is still limited [14, 15].

Studies have shown that the melanocortin-4 receptor (MC4R) has been identified as significant analgesic targets and plays an important role in nociceptive behavior induced by nerve injury [16-18], but the anatomical mechanism of this action remains unclear. It has been extensively reported that opioids through interactions with receptors distributed in several regions, and activation of mu-opioid receptor (MOR) is also responsible for efficacious pain relief [6, 19, 20]. Evidences have showed that retrograde multisynaptic tracing technique of

pseudorabies virus (PRV) is used to characterize neuroanatomical circuits in the CNS [21-30]. The goal of the present study was to define whether stimulation originated from SNL induces the increase of c-Fos expression in the motor cortex, and elucidate whether motor cortex-PAG-RVM neuronal circuitry involves in opiodergic-melanocortineric signaling. We used transgenic recombinants of a PRV-Bartha derivative, PRV-614, to express a novel monomeric red fluorescent protein (mRFP1) [30-33] for direct visualization under fluorescence microscope, with an attempt to provide morphological evidence of the neuroanatomical circuit among motor cortex, PAG, RVM, and kidney. Immunohistochemical detection of the MOR was also incorporated into the experiments, so that we could provide direct neuroanatomical evidence for the central opiodergic-melanocortineric signaling in the PAG.

### Materials and methods

#### *Animals*

All experiments were performed in accordance with the guidelines of the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, the European Communities Council Directive of 24 November 1986 (86/609/EEC). This study was reviewed and approved in advance by the local Animal Care and Use Committee. Experiments were carried out on male transgenic mice, 4-8 weeks old (20-25 g), expressing GFP in a subset of MC4R-containing neurons, which were first obtained from Dr. Joel Elmquist and then bred to generate male and female mice. Mice were genotyped as described by Rossi et al [34]. Mice were kept under controlled conditions (24±0.5°C, 12 h alternating light-dark cycle, food and water *ad libitum*).

#### *c-Fos expression in motor cortex following spinal nerve ligation injury*

The MC4R-GFP transgenic mice were randomly divided into two groups: SNL group (subjected to left spinal nerve ligation injury, n=6) and control group (subjected to the same operation, but the spinal nerves were not ligated, n=6). Under pentobarbital sodium (50 mg/kg, i.p.) anesthesia, SNL injury was performed as previously described [35, 36]. Briefly, after a midline incision above the lumbar spine, deep dissection of the paraspinal muscles and exposure of the dorsal vertebral column from L4

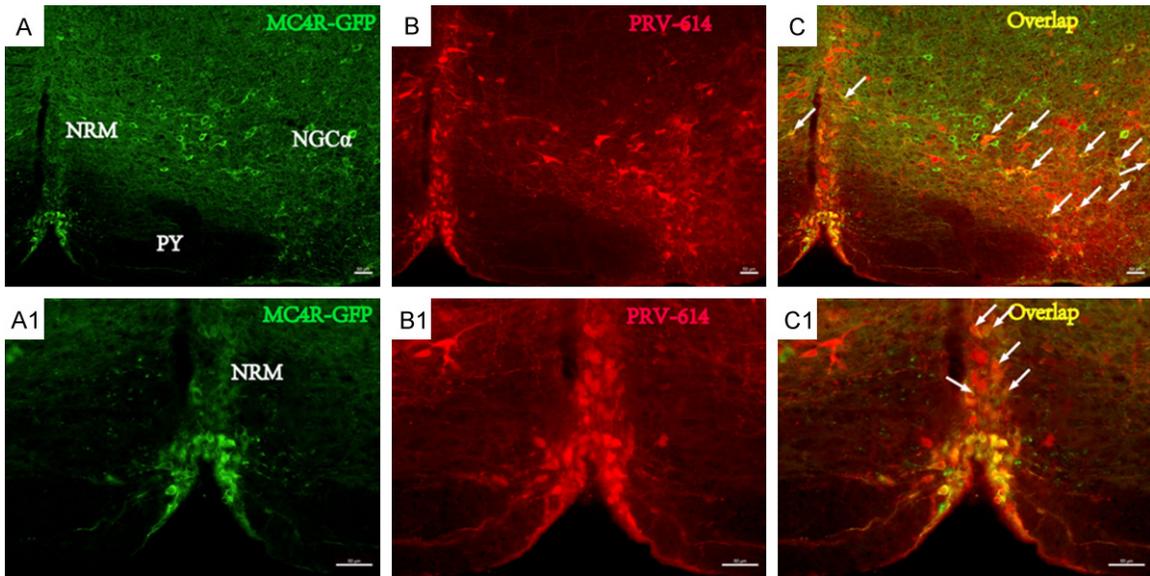
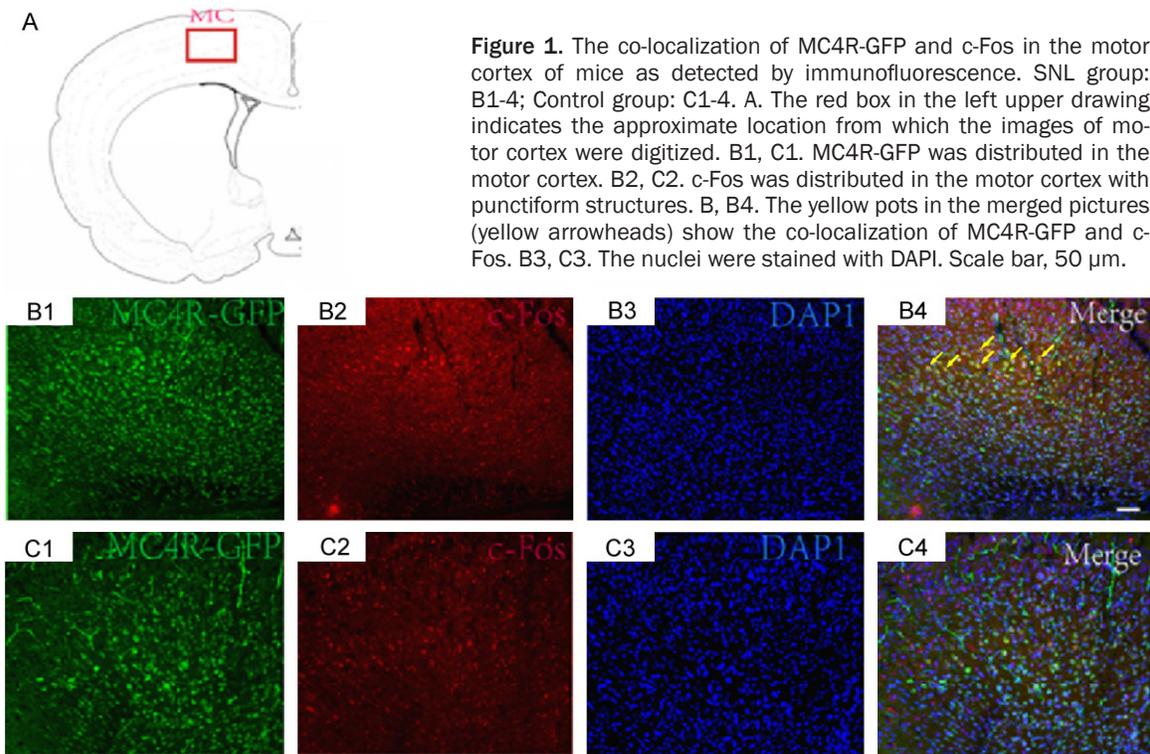
to S1, the left L5 and L6 spinal nerves were isolated and tightly ligated with a 4-0 silk suture distal to the DRG and proximal to the formation of the sciatic nerve. The incision was closed. 7 d after the surgery, mice were deeply anesthetized and perfused. Brains were post-fixed in 4% paraformaldehyde-borate. Coronal sections through the brain (25 µm) were collected into four series; tissue was either reacted immediately or stored in cryoprotectant at 4°C. Sections including motor cortex were processed to detect c-Fos by fluorescence immunohistochemical (IHC) in order to determine the relationship between neurons expressing MC4R-GFP and c-Fos. For MC4R-GFP and c-Fos IHC, the primary antibodies were mouse anti-GFP (AM1009a-ev; Abgent; 1:500) and rabbit polyclonal c-Fos antibody (sc-52, Santa Cruz, 1:200), and the corresponding second antibody included FITC-conjugated Goat-anti-Mouse IgG (H+L) (Jackson ImmunoResearch, 1:1000) and Cy3-conjugated Goat-anti-Rabbit IgG(H+L) (Jackson ImmunoResearch, 1:1000), respectively. Finally, sections were collected onto glass slides, covered with a coverslip, and examined under microscope by an investigator blinded as to treatment.

#### *Injections of PRV-614 and immunohistochemistry for MC4R*

Microinjection of retrograde tracer PRV-614 into the left kidney of the male transgenic MC4R-GFP mice (n=15) was performed in a manner similar to that described in previous reports [26-31, 37, 38, 52, 53]. The animals were anesthetized with isoflurane (3% induction, 2% maintenance), and the left kidney was exposed by ventral laparotomy. Under surgical microscope, 1 µl injections of PRV-614 (2×10<sup>8</sup> pfu/mL) was inserted into the left upper site of the exposed left kidney (0.2 µl per injection at 5 injection sites per one mouse) using a 30-gauge needle connected to a Hamilton syringe (10 µl). After PRV-614 injection, mice were allowed to survive for several days (3 days, n=5; 4 days, n=5; and 5 days, n=5) before being killed.

After a survival time of 3-5 days, the animals were humanly killed under deep anesthesia. The procedures of tissue treatment were similar to those described above. Sections including motor cortex, PAG and RVM were processed for rabbit anti-GFP (1:1,000; Molecular Probes, Eugene, OR) fluorescence immunohistochemis-

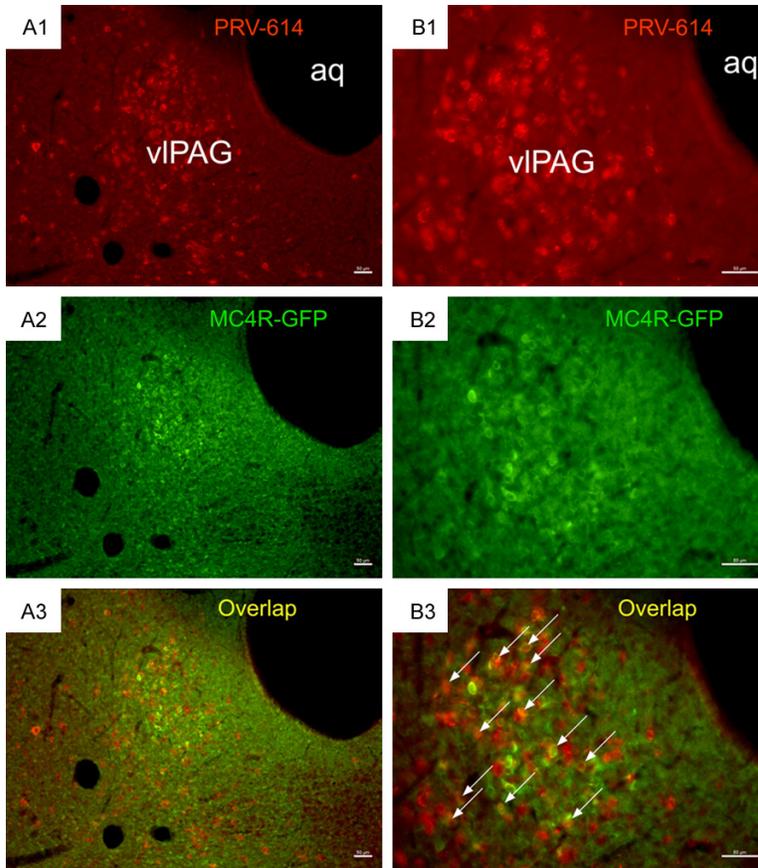
## Motor cortex-PAG-RVM neuronal circuitry



try according to standard protocols [37, 39]. PRV-614-infected neurons were directly visual-

ized under fluorescence microscope by a band pass filter for Cy3 [28, 30, 40-42].

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**Figure 3.** PRV-614/MC4R-GFP double-labeled neurons in the periaqueductal gray. A1 showed neurons infected with PRV-614, which send transsynaptic projections to the kidney; A2 showed MC4R-GFP positive neurons in the periaqueductal gray; A3 showed overlaid images of A1 plus A2. Images B1-3 amplified views of A1-3, respectively. aq, aqueduct; PAG, periaqueductal gray. Arrows indicate double-labeled neurons. Scale bars, 50  $\mu$ m.

### *Immunofluorescence staining for MC4R and MOR*

The transgenic MC4R-GFP mice (n=5) were transcardially perfused under anesthesia. The brain was postfixed and transferred to 30% sucrose. Double immunofluorescence staining was performed as described above with minor additions. Briefly, sections including PAG were incubated with a mixture of goat polyclonal MOR-1 (c-20) antibody (1:200, Santa Cruz, sc-7488) and rabbit anti-GFP (1:1000, Life technologies, A6455) diluted in PBS containing 0.1% Triton X-100 and 10% donkey serum overnight at 4°C. Subsequently, sections were incubated with a mixture of FITC-conjugated mouse-anti-Goat IgG (H+L) (1:1000, Jackson ImmunoResearch), Biotin-sp-conjugated affipure donkey anti-Rabbit IgG (1:2000, Jackson ImmunoResearch) and Cy3-

conjugated streptavidin (Jackson ImmunoResearch).

### *Tissue analysis*

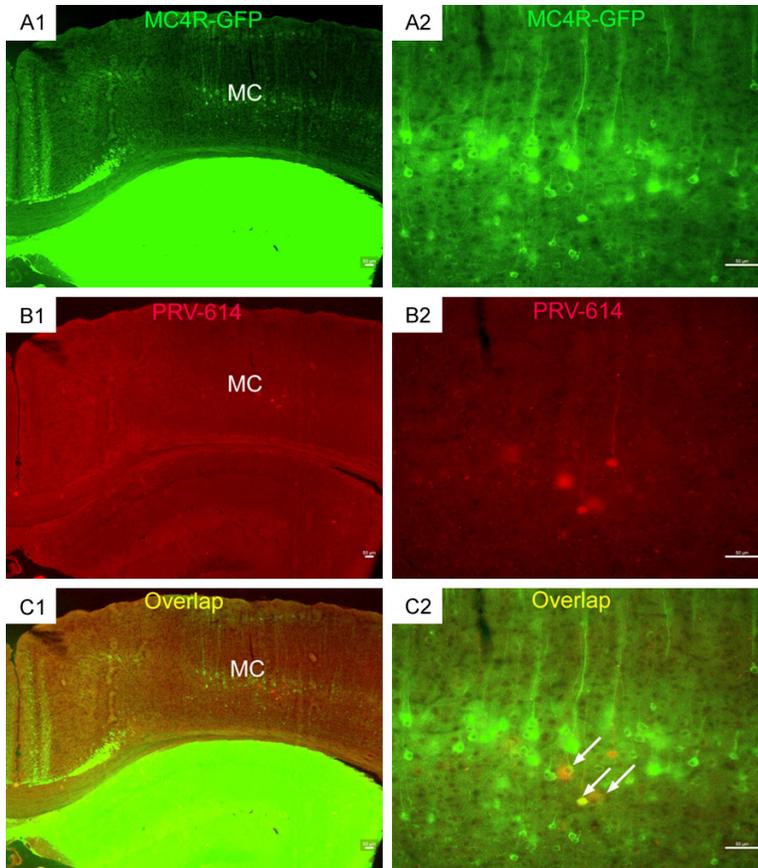
Immunofluorescence photomicroscope was visualized by an investigator blinded as to treatment, using an Two-Photon fluorescence microscopy or Olympus IX81 photomicroscope equipped with epifluorescence with a filter set for visualization of Alexafluor 488 (excitation range, 425-525 nm; emission range, 500-600 nm), Cy3 (excitation range, 500-560 nm; emission range, 560-650 nm) and Alexafluor 350 (excitation range, 300-400 nm; emission range, 440-490 nm). PRV-614-, MC4R-GFP-, DAPI-positive cells are identified with red, green, blue fluorescence, respectively. The nuclei were stained with DAPI. The regions in which positive cells were located were defined with reference to the atlases of Franklin KB and Paxinos G [43].

## Results

### *Co-localization of MC4R-GFP and c-Fos-positive cells in the motor cortex*

c-Fos expression in the motor cortex was clearly different for SNL group compared with Control group. As evident in the photomicrographs of **Figure 1**, pain stimulation with SNL group (**Figure 1**) elicited more c-Fos-positive cells medially compared with stimulation with Control group (**Figure 1**). The c-Fos-positive cells labeled in the transgenic mice were densely distributed in the dorsal region of the motor cortex but were sparse ventrally. The pattern of MC4R-GFP in the motor cortex was very striking. As illustrated in **Figure 1**, it was clear from visual inspection that relatively 50% c-Fos-positive cells in the motor cortex were MC4R-GFP positive, and the potential for double labeling was much more likely in the dorsal motor cortex (**Figure 1**).

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**Figure 4.** PRV-614/MC4R-GFP double-labeled neurons in the motor cortex. Images A-C were taken from an animal after injections of PRV-614. Image A1 showed MC4R-GFP positive neurons in the motor cortex; Image B1 showed neurons infected with PRV-614, which send transsynaptic projections to the kidney; Image C1 showed overlaid images of A1 plus B1. Images A2-C2 amplified views of A1-C1, respectively. Arrows indicate double-labeled neurons. Scale bars, 50  $\mu$ m.

After injection of the fluorescent microspheres PRV-614 into the left kidney, many neurons in the motor cortex, PAG and RVM regions were observed to contain retrograde tracer. These regions were examined contained single- and double-labeled cells. Initial analysis focused on qualitative characterization of areas that contained double-labeled neurons, which were co-expressed with PRV-614 and MC4R-GFP. PRV-614-infected neurons were retrogradely and bilaterally labeled in the motor cortex, PAG and RVM in late survival group, with approximately equal numbers of infected neurons on the ipsilateral and contralateral sides.

### *Specific expression of PRV-614 in the RVM, PAG and motor cortex*

At 3-4 d survival time (n=5), PRV-614 labeled neurons was located in the nucleus raphe mag-

nus (NRM), the ipsilateral and contralateral nucleus gigantocellularis pars alpha (NGC $\alpha$ ) (4 d survival time, **Figure 2**). In contrast to the RVM (NRM and NGC $\alpha$ ), we didn't detect PRV-614 labeled neurons in the motor cortex, suggesting that PRV-614-infected neurons were retrogradely labeled in the mice.

At 4-5 d survival time, PRV-614 positive neurons were observed in the ipsilateral and contralateral PAG. From 4 d to 5 d, the most substantial increase seen in the number of PRV-614-infected cells was in the ipsilateral and contralateral PAG (5 d survival time, **Figure 3**). Additionally, three of the animals surviving 5 d began to show PRV-614 labeling in the motor cortex.

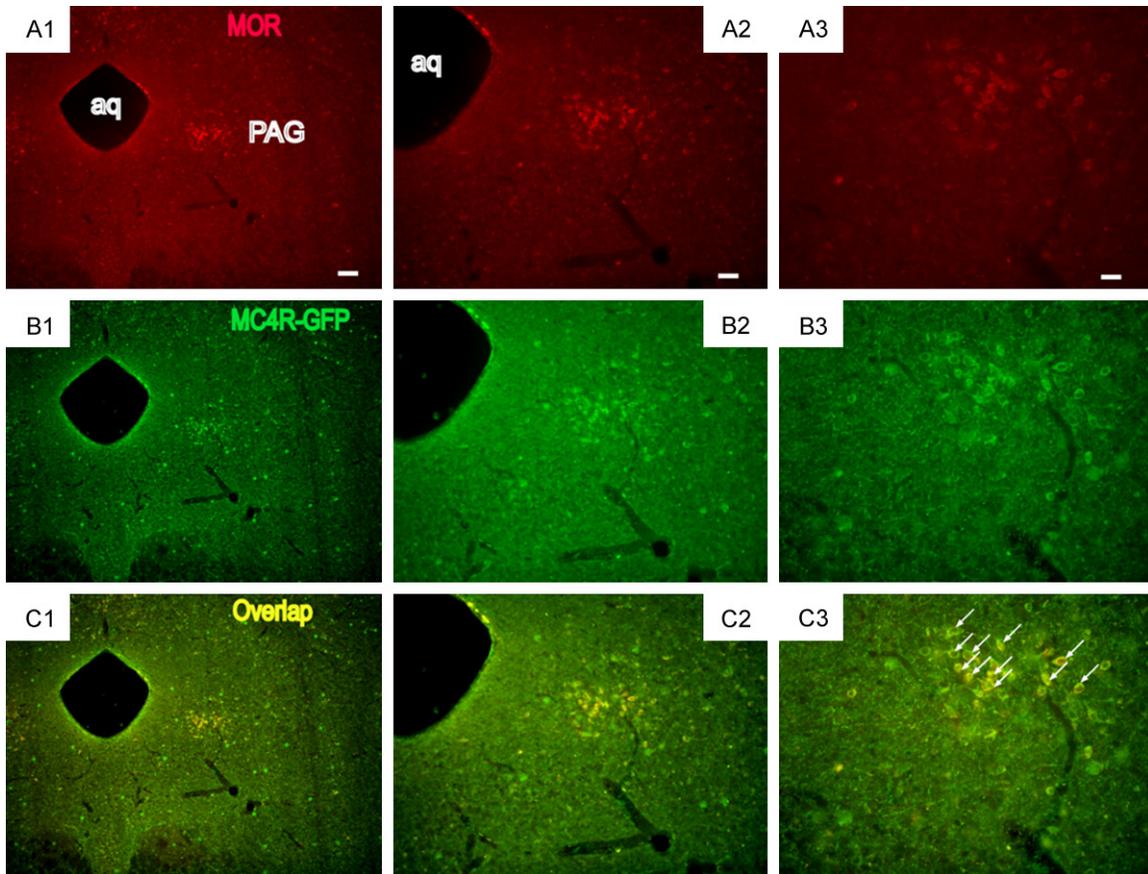
At 5-6 d survival time (n=5), PRV-614 labeling in the ipsilateral and contralateral motor cortex (6 d survival time, **Figure 4**) was significantly increased in all animals.

### *MC4R and PRV-614 co-expression in the RVM, PAG and motor cortex*

We assayed GFP expression in the MC4R-GFP reporter mouse by immunofluorescence staining, and found a large number of MC4R-GFP-ir cells in the RVM (NRM and NGC $\alpha$ ), PAG and motor cortex. We observed that double-labeled MC4R-GFP/PRV-614 cells were present in the RVM (4 d survival time, **Figure 2**), PAG (5 d survival time, **Figure 3**) and motor cortex (6 d survival time, **Figure 4**).

### *Co-localization of MC4R-GFP and MOR-positive cells in the PAG*

It is well known that the PAG anatomically comprises a number of columns. By immunofluorescence staining, we found that MC4R-GFP and MOR-positive cells were mainly located the column of the ventrolateral PAG (**Figure 5**). Double-labeled MC4R-GFP/MOR neurons were densely co-localized in the ventrolateral region



**Figure 5.** MOR/MC4R-GFP double-labeled neurons in the periaqueductal gray. A1 showed MOR positive neurons; B1 showed MC4R-GFP positive neurons in the periaqueductal gray; C1 showed overlaid images of A1 plus B1. Images A2-C2 amplified views of A1-C1, respectively. Images A3-C3 amplified views of A2-C2, respectively. aq, aqueduct; PAG, periaqueductal gray. Arrows indicate double-labeled neurons. Scale bars, 50  $\mu$ m for A1-C1, 100  $\mu$ m for A2-C2, 200  $\mu$ m for A3-C3.

of the PAG but sparsely distributed in the dorsal PAG, and 50%-70% of the GFP-positive neurons in the ventrolateral PAG were MOR-immunoreactive (**Figure 5**).

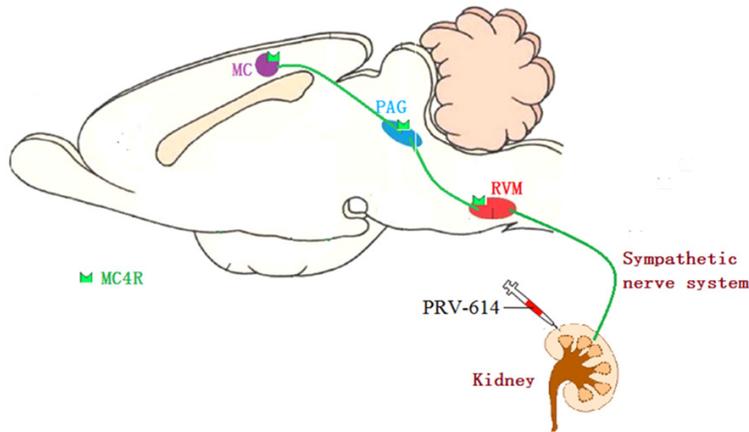
### Discussion

The principal findings of this study are: (1) Spinal nerve injury induced the increase of c-Fos/MC4R-GFP dual labeled neurons in motor cortex. (2) The inoculation of PRV-614 into the kidney resulted in retrograde infection of neurons in RVM, PAG and motor cortex, and PRV-614/MC4R-GFP dual labeled neurons were detected in RVM, PAG and motor cortex. (3) MC4R-GFP/MOR dual labeled neurons were detected in PAG, and 50%-70% of the GFP-positive neurons in the ventrolateral PAG were MOR-immunoreactive. These results together suggest that MC4R signaling in motor

cortex-PAG-RVM neural circuit may participate in the descending modulation of nociceptive transmission. This study suggests that melano-cortinergetic-sympathetic signaling of motor cortex-PAG-RVM neural circuit may be a potential target for between the inhibition of nociceptive transmission and the prevention and reversion of neuropathic pain.

The immediate early gene c-fos is rapidly and transiently expressed in excited neurons in response to noxious stimulation [44-46]. In the present study, one of the most interesting results that algescic stimulation originated from SNL induced an express pattern of c-Fos in the motor cortex. In particular, there was a dense concentration of c-Fos in the dorsal region of the motor cortex following stimulation with SNL, suggesting that these c-Fos-positive neurons may involve in the circuit for processing

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**Figure 6.** Summary diagram showed that the melanocortin-sympathetic pathway between the MC and kidney. Neurons within the MC send projections to PAG and RVM, which in turn projects to the sympathetic nervous system, which control renal activity regulated by melanocortin-sympathetic signals. MC, motor cortex; MC4R, the melanocortin-4 receptor; PAG, periaqueductal gray; PRV-614, pseudorabies virus-614; RVM, rostral ventromedial medulla. Some drawings were taken from HB Xiang (Brain 2013).

noxious stimuli that lead to this painful response. The presence of MC4R in motor cortex reported by Liu and others fits with the functional organization of the autonomic homeostasis system [47]. We confirmed that spinal nerve injury induced the increase of c-Fos/MC4R-GFP dual labeled neurons in motor cortex, indicating that these dual labeled neurons in motor cortex may be part of the circuit for the modulation of nociception by melanocortineric signaling.

The finding presented here indicated that the neurotropic viruses PRV-614 were able to replicate in the neuronal cell body and were then retrogradely transported to adjacent neurons via functionally active synapses. Our results showed that injections of PRV-614 into the kidney resulted in retrograde infection of neurons in the RVM, PAG, and motor cortex that was very similar to that observed in the rat [48, 49]. This transsynaptic retrograde tracing method can be used to show the CNS neuronal circuit innervating the site of PRV-614 injection. Because there is no evidence that the motor and parasympathetic pathway provides any innervations to kidney, motor cortex, PAG, and RVM were infected with PRV-614 only via the sympathetic pathway. So, this study provided direct mapping of motor cortex, PAG, and RVM neurons innervating the kidney, suggesting that motor cortex-PAG-RVM neuronal

circuitry appears to play a substantial role in regulating sympathetic outflow to kidney.

Several observations suggest that the MC4R is an essential mediator for nociceptive behavior induced by nerve injury [50-54]. Pharmacological studies have shown that MC4R play a pivotal role in the regulation of sympathetic signal [55, 56]. Chu et al intrathecally treated with MC4R antagonist HSO14 or p38-MAPK inhibitor SB203580 in rats given chronic constriction injury (CCI), and reported that both HSO14 and SB203580 reduced CCI-induced hyperalgesia, suggesting that MC4R mediates the mechanisms of

neuropathic pain by p38MAPK signaling [18]. We discovered that PRV-614/MC4R-GFP dual labeled neurons were detected in the RVM, PAG and motor cortex. Thus, motor cortex-PAG-RVM neuronal circuitry involved in modulation of melanocortineric-sympathetic signaling.

Accumulating reports highlight the opioidergic nature of the PAG. The  $\mu$ -opioid receptor (MOR) has been characterized as the main mediator of opioid signaling in neuronal cells. Furthermore, the importance of the PAG in the opioid inhibitory system is well characterized in studies with the administration of mu-opioid agonist DAMGO, indicating that PAG is a region with high levels of MOR express and one of the most important areas for opioid-mediated anti-nociception [6, 36-38, 48-53, 57-59]. Pharmacological evidence supports the involvement of MOR-expressing cells in the PAG circuitry [60]. Fonoff et al reported that motor cortex stimulation induced the increase in nociceptive threshold, which is abolished by opioid receptors blockade [13]. Our results showed that double-labeled MC4R-GFP/MOR neurons were mainly co-localized in the ventrolateral PAG, and 50%-70% of the GFP-positive neurons in the ventrolateral PAG were MOR-immunoreactive, indicating that these dual labeled neurons in PAG may be involved in the modulation of nociception by melanocortineric-opioidergic signaling.

The motor cortex emerges as an important structure involved in the processing of nociceptive response. In the last years, experimental evidence was in line with this idea, showing that antinociceptive effect elicited by motor cortex stimulation may be mediated via the activation of descendent inhibitory pain pathway [13, 61]. In previous studies, we demonstrated that motor cortex-PAG circuitry involved in modulation of nociceptive behavior by melanocortinerbic-sympathetic signaling [15]. The study from Pagano et al showed that neurocircuitry during the antinociception induced by motor cortex stimulation involved an inhibition of GABAergic interneurons of the PAG which activated special neurons responsible for the descending pain inhibitory control system [7]. Furthermore, Franca et al demonstrated that motor cortex stimulation increased the nociceptive threshold in rats by disinhibiting the neurons in PAG [62]. Together, these studies suggest that motor cortex has strong anatomical connections to the PAG, suggesting that there exists motor cortex-PAG neurocircuitry participated in the regulation of nociceptive behavior (**Figure 6**).

It is known that PAG-RVM circuit played an important role in modulation of nociception [63]. Farkas et al studied the descending efferent PAG projections by using the anterograde axonal marker Phaseolus vulgaris leucoagglutinin (PHA-L), and found that PAG projections to sympathetic premotor neurons in the RVM [64], indicating that there exist a sympathetic pathway from PAG to RVM. In addition, reports from Fields et al showed that the PAG is responsible for the projection of neuron groups in the region of the RVM in nociceptive modulatory circuits [65]. Braz et al found that neurons of the PAG that project to the RVM were not postsynaptic to 5-HT neurons, suggesting that there is non-5-HT descending control pathway in PAG-RVM neuronal circuitry [66]. There is growing evidence that PAG is an important supraspinal site involved in opioid-mediated analgesia [67]. A previous report considered that stimulation targeting the PAG produced analgesia through the action of endogenous opioids on PAG  $\mu$ -opioid receptors [20, 68, 69]. Findings from Marinelli et al indicated that functional opioid receptor subtypes existed on spinally projecting RVM neurons [8]. Based on our findings, we speculate that PAG-

RVM neuronal circuitry may involve in the regulation of opioidergic-sympathetic pathway (**Figure 6**).

Based on all these findings, our results confirm and extend previous findings that the motor cortex-PAG-RVM circuit plays a crucial role involved in the regulation of nociceptive transmission by melanocortinerbic-sympathetic and melanocortinerbic-opioidergic pathway. Advances in understanding the motor cortex-PAG-RVM circuit are pointing toward new preventive and symptomatic treatments of chronic pain through endogenous analgesic modulation. Further investigation of the mechanisms involved in this circuit may contribute to the improvement of the clinical treatment of persistent pain.

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### Disclosure of conflict of interest

None.

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