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Periaqueductal Gray neurons project to spinally projecting GABAergic neurons in the rostral ventromedial medulla

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Abstract

The analgesic effects of morphine are mediated, in part, by periaqueductal gray (PAG) neurons that project to the rostral ventromedial medulla (RVM). Although much of the neural circuitry within the RVM has been described, the relationship between RVM neurons and PAG input and spinal output is not known. The objective of this study was to determine whether GABAergic output neurons from the PAG target RVM reticulospinal neurons. Immunocytochemistry and confocal microscopy revealed that PAG neurons project extensively to RVM neurons projecting to the spinal cord, and two-thirds of these reticulospinal neurons appear to be GABAergic (contain GAD67 immunoreactivity). The majority (71%) of PAG fibers that contact RVM reticulospinal GAD67-immunoreactive neurons also contained GAD67 immunoreactivity. Thus, there is an inhibitory projection from PAG to inhibitory RVM reticulospinal neurons. However, there also were PAG projections to the RVM that did not contain GAD67 immunoreactivity. Additional experiments were conducted to determine whether the heterogeneity in this projection can be explained by the electrophysiological character of the RVM target neurons. PAG projections to electrophysiologically defined and juxtacellularly filled ON, OFF, and Neutral cells in the RVM were examined. Similar to the pattern reported above, both GAD67- and non-GAD67-immunoreactive PAG neurons project to RVM ON, OFF, and Neutral cells in the RVM. These inputs include a GAD67-immunoreactive projection to a GAD67-immunoreactive ON cell and non-GAD67 projections to GAD67-immunoreactive OFF cells. This pattern is consistent with PAG neurons producing antinociception by direct excitation of RVM OFF cells and inhibition of ON cells.

Keywords

antinociception; immunocytochemistry; pain modulation

INTRODUCTION

The periaqueductal gray (PAG) modulates nociception via a descending pathway that relays in the rostral ventromedial medulla (RVM) and terminates in the spinal cord [5]. The RVM is

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of particular interest because distinct cell classes in the RVM inhibit and facilitate nociception. RVM ON cells show an increase in activity associated with nociceptive reflexes and activation of these neurons facilitates nociception [7,9,20,21,34,50]. In contrast, OFF cells show a pause in activity associated with nociceptive reflexes, and activation of these cells by morphine administration or any other means produces antinociception [4,16,23,30,37,38,44,45]. Although morphine-induced antinociception is thought to be driven by a direct projection from the PAG to RVM OFF cells [44], the anatomical basis for this hypothesis has not been demonstrated.

Microinjection of morphine into the PAG appears to produce antinociception by inhibiting GABAergic neurons, thus leading to disinhibition of output neurons projecting to the RVM [48,49]. These PAG output neurons could produce antinociception by either exciting RVM OFF cells, inhibiting ON cells, or both [44]. Given that glutamate is excitatory and GABA is inhibitory in the RVM [24,25,30], the electrophysiological data cited above indicate that PAG neurons projecting to RVM ON cells should contain GABA because antinociception is associated with inhibition of ON cells [11,16,38,44]. Likewise, PAG projections to RVM OFF cells should contain glutamate because these neurons show an increase in activity following morphine administration [11,16,38,44]. The presence of GABA in a subset of PAG neurons projecting to the RVM support this hypothesis [33,42]. However, mu-opioid receptors are found on a subset of PAG neurons that project to the RVM [14,51], and opioid administration inhibits these neurons [39]. These data suggest that OFF cells also may receive GABAergic input from opioid-sensitive PAG neurons (inhibition of GABAergic input could also excite OFF cells). Given that both RVM ON and OFF cells project to the spinal cord [18], PAG neurons should contact RVM neurons projecting to the spinal cord as has been suggested previously by examining degeneration of PAG inputs to RVM neurons projecting to the spinal cord [12].

These hypotheses were tested in two studies. The first study used dual tract tracing methods (anterograde tracing from PAG and retrograde tracing from cervical spinal cord) combined with immunocytochemical detection of a synthetic enzyme for GABA (GAD67) to determine whether RVM neurons that project to the spinal cord receive input from the PAG. The second study combined anterograde tracing from the PAG with single unit recording and juxtacellular labeling of RVM ON, OFF, and Neutral cells to determine whether GABAergic PAG neurons target RVM ON cells specifically.

MATERIALS AND METHODS

Experimental animals

Male Sprague-Dawley rats (250 – 350 g; Charles River, Boston, MA) were used in all experiments. All procedures were conducted with the approval of the Institutional Animal Care and Use Committee in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS Policy) and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Guide). Animals were initially anesthetized in a Plexiglas chamber with 5% isoflurane. The head was shaved, and the rat was placed in a stereotaxic frame with a nose cone for anesthesia delivery (3% in oxygen). For all survival surgeries, incisions were closed with suture and the animal was kept on a warming blanket and carefully monitored during recovery from anesthesia.

Anterograde tracer injections in periaqueductal gray

The anterograde tracer *Phaseolus vulgaris* Leucoagglutinin (PHA-L; Vector Laboratories; Burlingame, CA) was used to examine projections from the PAG to RVM neurons. PHA-L (2.5% in 10 mM phosphate buffer) was iontophoretically injected into the left PAG (0.6 mm

lateral, 6.6 mm ventral to junction of the midline and interaural sutures) with positive current through a glass micropipette (5 – 7 μA; 7 second on/off cycles; total time 10 – 15 min). For tract tracing studies, rats also were injected with the retrograde tracer FluoroGold (FG) into the cervical spinal cord (see below). For electrophysiological studies, experiments were conducted at least 7 days after PHA-L injection into the PAG.

Retrograde tracer injections in cervical spinal cord

A retrograde tracer was used to identify spinally projecting RVM neurons. FluoroGold (2% in saline; Fluorochrome Inc.; Denver, CO) was microinjected into the left cervical spinal cord at C1 – C2 level (0.5 mm lateral; 0.5 mm ventral from the central artery). A series of microinjections (2 – 3 sites) were made in the rostrocaudal direction extending over approximately 0.7 mm (each microinjection was 35–50 nl) for a total volume of 100 120 nl. FluoroGold (FG) injections into the cervical cord and PHA-L applications to the PAG were both to the left side. Rats were perfused 7 days following injections.

Electrophysiological surgery

For electrophysiological recordings from RVM, rats were anesthetized with isoflurane in oxygen as described above. Body temperature was maintained by wrapping the rat in a 37oC water blanket. The interparietal bone was exposed and a hole drilled through the skull for insertion of the electrode. Prior to recording, anesthesia level was adjusted so tail withdrawal to hot water (52 – 54°C) could be elicited, but spontaneous movements were not present.

Extracellular recording

Glass capillary electrodes (1.5 mm) were pulled on a vertical PE-2 (Narshige Scientific Instrument Laboratories;Toyko, Japan). The tip was broken to a resistance of 18 – 20 MΩ and the electrode filled with Biotinamide hydrobromide (5% in 0.5 M sodium acetate; Invitrogen; Eugene, OR). Recordings were made along the midline 2.8 – 3.0 mm caudal to Lambda and 7.0 – 9.0 mm below the dorsal surface of the cerebellum. The electrode was advanced through the medulla in 1 μm steps (Micro Drive, F.H.C.; Brunswick, ME) until the spontaneous activity of a single neuron could be isolated from background noise. Neural activity was amplified (Axoclamp 2B, Axon Instruments; Sunnyvale, CA, and CyberAmp 380, Axon Instruments) and digitally converted for display and storage using Spike 2 software (Micro 1401, Cambridge Electronic Design; London, England).

Characterization of RVM neurons and juxtacellular labeling

Nociception was assessed by measuring the latency to withdraw the distal third of the tail from 52 – 54 °C water. The test was terminated if no response occurred within 20 s. At least 3 min separated each trial, and the tail was dried between trials. Neurons were characterized as ON, OFF, or NEUTRAL based on changes in activity associated with the tail withdrawal reflex [17]. Neurons that showed an increase in activity immediately before the tail withdrawal were classified as ON cells, and neurons that showed an abrupt decrease in activity were classified as OFF cells. NEUTRAL cells showed no change in activity associated with the tail withdrawal reflex. Each neuron was tested a minimum of two times to ensure physiological phenotype. Following characterization, the cell was juxtacellularly labeled by ejecting biotinamide hydrobromide from the electrode by passing a positive current (400 ms, 50% duty cycle). The current was increased from 2 to 7 nA until cell activity was entrained to the stimulation for up to 5 min. Data analysis was restricted to rats in which only one neuron was labeled with biotinamide.

Perfusion and tissue preparation

For dual tract tracing studies, rats were perfused 7 days following injections of FG to the spinal cord and PHA-L to the PAG. For juxtacellular labeling studies, rats were perfused at least 30 min, but not more than 4 hrs after the RVM neuron was juxtacellularly labeled. Rats were given a lethal dose of sodium pentobarbital (150 mg/kg, i.p.) and perfused transcardially through the ascending aorta with 10 mL of heparinized saline (1,000 units/ml), followed by 50 mL of 3.8% acrolein in 2% paraformaldehyde followed by 200 mL of 2% paraformaldehyde in 0.1 M phosphate buffer (PB). The brain was removed, and blocks of tissue containing the PAG, RVM, and spinal cord were placed in the final fixative for 30 min before transferring to 0.1 M PB. Tissue was sectioned on a vibrating microtome at 40 μm. Free-floating tissue sections were placed in 1% $NaBH_4$ (Sigma-Aldrich; Milwaukie, WI) for 30 min to bind remaining free aldehydes.

Immunocytochemistry

For dual tract tracing studies, RVM tissue sections were processed for retrograde FG, anterograde PHA-L and GAD67 immunoreactivity, by incubating in an antibody cocktail (rabbit anti-FG, 1:15000, Chemicon, Temecula, CA; goat anti-PHA-L, 1:10000, Vector Laboratories; and mouse anti-GAD67, 1:2000, Chemicon) for 48 hours at 4°C. In juxtacellular studies, RVM tissue was processed for PHA-L and GAD67 immunoreactivity by incubating in a similar antibody cocktail (goat anti-PHA-L, 1:10000, Vector Laboratories; and mouse anti-GAD67, 1:2000, Chemicon) for 48 hours at 4°C. In the first study, three Alexa Fluor-conjugated fluorescent secondary antibodies were used to visualize labeling (donkey anti-rabbit 488, donkey anti-goat 594 and donkey anti-mouse 647, 1:800; Invitrogen; Carlsbad, CA). In the second study, two secondary antibodies (goat anti-rabbit 647 and goat anti-mouse 488, 1:800; Invitrogen) and a streptavidin 594 (25μg/100μL; Invitrogen) cocktail were used to visualize PHA-L and GAD67 immunoreactivity and the biotinamide filled cell, respectively. All primary and secondary antibodies solutions were prepared in a 0.1% BSA solution; 0.25% Triton was also added to primary antibody solutions. Tissue was mounted on gelatin-coated slides, cover-slipped with ProLong Gold™ antifade reagent (Invitrogen) sealed and stored at −20°C to preserve labeling.

Microscopy and Analysis

Fluorescent markers were visualized using a Zeiss LSM 510 META confocal microscope. The single pass, multi-tracking format was utilized to allow multiple tracers to be individually excited with different lasers, and the emitted spectra collected separately to minimize overlap. AlexaFluor 488 was excited with a 488 nm (Argon/2) laser and emissions passed through a 500 – 550 nm band pass filter. AlexaFluor 594 was excited with a 543 nm laser (HeNe1) and emitted light passed through a 565 615 nm band pass filter. AlexaFluor 647 was excited with a 633 nm laser (HeNe2) and emitted light passed through a 650 – 710 nm band pass filter. Images were collected using an oil immersion objective (63x/1.4 Plan-Apochromat). For the dual tract tracing study, a total of 215 FG cells and their PHA-L input from the PAG were examined in the RVM of four animals. A single section through RVM was examined for each animal. The RVM section was based on anatomical location (Fig. 1) and verification of FG and PHA-L labeled cells. Cells were counted within medial, left and right RVM. A cell was scored as immunoreactive for FG if labeling was present in the cytoplasm and an unlabeled nuclear space was evident. A PHA-L varicosity was defined as profiles twice the diameter of the axon of origin and present in at least two optical slices. A PHA-L varicosity was scored as an apposition to a FG cell when pixels of each marker were directly adjacent in at least two optical slices.

In the second study, 15 biotinamide-filled cells were examined, along with their PAG inputs, in 15 animals. Animals with ambiguous labeling, no cell, multiple cells, or a cell located outside

the PAG input area were not included in data analysis. Although these rigid criteria allowed data from only 15 of 51 rats, this approach limited confounds from false positive labeling of the wrong cell. PHA-L varicosities adjacent to biotinamide-filled cells were verified in at least 2 optical sections in order to be considered an apposition. Colocalization with GAD67 was determined in both biotinamide-filled cells and PHA-L varicosities. A cell was scored as GAD67-immunoreactive if labeling was present in the cytoplasm and an unlabeled nuclear space was evident. PHA-L varicosities were counted as colocalized if GAD67-immunoreactivity was contained within the boundaries of the PHA-L apposition and visible in at least two optical slices. Biotinamide-filled cells were not scored unless the surrounding field contained PHA-L immunoreactivity. Cell count, GAD-colocalization and PHA-L appositions were verified by two independent observers blind to the experimental condition.

RESULTS

Reticulospinal neurons and PAG efferent fibers are distributed bilaterally in the RVM

Injections of FG into the left cervical spinal cord resulted in retrogradely labeled reticulospinal neurons in the RVM (Fig. 1). Labeled neurons were equally distributed in the medial, ipsi- and contralateral sides of the medulla. Injections of PHA-L into the left ventrolateral PAG resulted in anterograde labeling in the RVM that also had a bilateral distribution (Fig. 1).

PAG inputs to RVM target reticulospinal neurons containing GAD67 immunoreactivity

Anterogradely labeled PAG varicose fibers contact retrogradely labeled reticulospinal neurons in the RVM (Fig. 2). Although PHA-L was administered to a relatively small region of the PAG, PHA-L varicosities were found in apposition to 72 of the 215 FG-labeled reticulospinal neurons that were analyzed (33%). Approximately half (53%) of the retrogradely labeled reticulospinal neurons contained GAD67 immunoreactivity (Fig. 3). PAG projections to reticulospinal neurons in the RVM often target these GAD67-immunoreactive cells (Fig. 4). Specifically, 45 of the 72 reticulospinal neurons (63%) that received appositions from PAG varicose fibers also contained GAD67-immunoreactivity. These data are summarized in Table 1.

PAG varicosities containing GAD67 immunoreactivity target reticulospinal neurons

Many of the PAG efferent fibers projecting to RVM also contained GAD67 immunoreactivity (Fig. 5). The PAG varicose fibers containing GAD67 immunoreactivity frequently target GAD67-immunoreactive reticulospinal neurons in the RVM. A subset of FG-labeled RVM cells contained GAD67 immunoreactivity and also received PAG input (45 cells). Of these, 32 (71%) received contacts from PHA-L varicosities that also contained GAD67 immunoreactivity (Table 1). In contrast, of the 27 FG cells that did not contain GAD67 immunoreactivity but did receive PAG input, only 13 (48%) received PAG input that contained GAD67 immunoreactivity (Table 1).

Biotinamide-filled RVM neurons contain GAD67 immunoreactivity and receive PAG input

Many of the RVM neurons that were characterized electrophysiologically and labeled juxtacellularly with biotinamide in the RVM contained GAD67 immunoreactivity. Of the 15 neurons filled, 60% (9/15) contained GAD67 immunoreactivity. This percentage is very similar to the 53% of retrogradely labeled reticulospinal neurons that were found to contain GAD67 immunoreactivity reported in the tract tracing study above. Appositions from PAG varicosities were evident on 80% (12/15) of the biotinamide-filled neurons, and included ON, OFF, and Neutral cells (Fig. 6, 7, & 8; Table 2). PAG input was more likely to be seen in biotinamide-filled cells than in the retrograde tracing study because of more extensive labeling of dendritic arbors in biotinamide-filled cells.

ON and OFF cells receive different PAG inputs

Although only small samples could be achieved with the combined anterograde tracing, juxtacellular labeling and immunocytochemical detection of GAD67, some intriguing results were observed. All three of the ON cells examined contained GAD67 immunoreactivity and input from PAG efferents. Only one cell received PAG input that contained GAD67 immunoreactivity (Fig. 7; Table 2).

Six OFF cells were examined, and 3 of these cells contained GAD67 immunoreactivity and also received appositions from PAG varicose fibers. None of the PAG inputs to GAD67 OFF cells contained GAD67 immunoreactivity (Fig. 8; Table 2). Of the remaining OFF cells, one did not contain GAD67 immunoreactivity and it received input from a PAG fiber containing GAD67 immunoreactivity, and the remaining 2 OFF cells did not contain GAD67 immunoreactivity and did not receive PAG input.

Neutral cells showed a mixed pattern of both GAD67 immunoreactivity and PAG input. Six Neutral cells were examined, and 3 contained GAD67 immunoreactivity. Five cells received PAG inputs, and 1 of those 5 cells received input from a GAD67-immunoreactive PAG varicose fiber (Table 2). Thus, no obvious pattern of PAG inputs to Neutral cells with regard to GAD67 immunoreactivity was observed.

DISCUSSION

The present data enhance understanding of the PAG/RVM circuitry underlying the modulation of nociception. Specifically, the data demonstrate that PAG neurons make direct contact with RVM neurons projecting to the spinal cord, that a subset of both PAG and RVM projection neurons appear to use GABA as a neurotransmitter, and that the PAG appears to modulate ON, OFF, and Neutral cells in the RVM via both GABAergic and non-GABAergic input. These data indicate that PAG neurons modulate nociception by directly inhibiting and exciting ON and OFF cells in the RVM.

The neural circuitry underlying nociceptive modulation has been well characterized both within the PAG and RVM. Previous studies reveal that opioids inhibit GABAergic neurons in the PAG and disinhibit output neurons [15,36,48,49]. Within the RVM, opioids inhibit ON cells and indirectly increase the activity of OFF cells [27,28]. Although PAG neurons synapse in the RVM [8,10,46] and RVM neurons synapse in the spinal cord [6,18,35], the relationship between this input and output, and the anatomical connection between PAG projections to ON, OFF, and Neutral cells has not been demonstrated previously.

Previous research has shown that GABA is present in a subset of PAG neurons terminating in the RVM [33,42], GABA terminals appear to synapse on RVM neurons that project to the spinal cord [12,54], a subset of RVM neurons with presynaptic GABA terminals also contain GABA [19], and a subset of RVM neurons projecting to the spinal cord contain GABA [2, 33,42]. The present data integrate these distinct findings and provide the first anatomical evidence for direct projections from the PAG to reticulospinal neurons in the RVM. These anatomical data are consistent with electrophysiological data suggesting direct projections from the PAG to ON and OFF cells [38,44]. Moreover, antidromic activation has shown that both ON and OFF cells project to the spinal cord [18]. Our finding that the PAG projects to both ON and OFF cells confirms that the relay from the PAG to spinal cord requires only two synapses.

Given that ON cells are silent when OFF cells are active and vice versa [3], these cell classes appear to have opposite functions. ON cells are active during periods of enhanced nociception such as secondary hyperalgesia, opioid tolerance, and morphine withdrawal [7,9,20,21,34,

50]. In contrast, manipulations that produce antinociception cause OFF cells to become continuously active [4,16,23,30,37,38,44,45]. Connections from the PAG to both ON and OFF cells suggest that the PAG coordinates the inverse firing pattern of these two cell classes as opposed to lateral inhibition between ON and OFF cells [13]. The present data showing both GAD67- and non-GAD67-immunoreactive inputs to the RVM suggests that the PAG modulates the activity of these neurons by both excitatory and inhibitory input.

The PAG also projects to a subset of RVM Neutral cells, half (3/6) of which had GAD67 labeling. The function of Neutral cells is not well understood, but probably represents a range of neuronal types. For example, a subset of Neutral cells is the only class of RVM neuron to contain serotonin [41,53]. GABA receptors have been found on both serotonin and non-serotonin containing neurons in the RVM [22] and a subset of these serotonin neurons project to the spinal cord [54]. Thus, like ON and OFF cells, our data along with these earlier studies show that Neutral cells receive direct input from the PAG and project to the spinal cord. Although electrophysiological analysis reveals no relationship between changes in nociception and activity in Neutral cells [11], pharmacological studies show that blocking serotonin receptors in the spinal cord attenuates antinociception mediated by the RVM [31,43]. Thus, serotonin does not appear to contribute to antinociception directly but may modulate nociception depending on the situation.

Although the present study focused on the neurotransmitter GABA, glutamate is the likely neurotransmitter in non-GABAergic input from the PAG to RVM. Glutamate receptors play an important role in driving the activity of RVM ON and OFF cells [26,29]. Moreover, disruption of this pathway by microinjection of glutamate receptor antagonists into the RVM disrupts antinociception evoked from the PAG [1].

The widely accepted model of PAG/RVM interactions includes an excitatory glutamatergic projection from the PAG to the RVM [1,47,52]. According to this model, opioids produce antinociception by inhibiting GABAergic neurons in the PAG that disinhibit glutamatergic output neurons projecting to RVM OFF cells [49]. Activation of OFF cells inhibits nociception at the spinal level. The present data showing non-GABAergic input from the PAG to RVM OFF cells provides excellent support for this model. However, PAG input to ON and OFF cells includes both GABAergic and non-GABAergic inputs suggesting a more complicated circuitry than described by this model. Mu-opioid receptors are found on many GABAergic neurons in the PAG [14,32], a subset of which project to the RVM [33,51]. Although these data indicate that microinjection of morphine into the PAG can excite RVM neurons by two distinct mechanisms (i.e., releasing PAG output neurons from tonic GABAergic inhibition and by inhibiting GABA input to RVM neurons) direct activation of PAG output neurons would excite and inhibit RVM neurons depending on whether the output neuron contained glutamate or GABA. This more complex model is consistent with the excitation and inhibition of OFF and ON cells, respectively, following direct excitation of PAG output neurons [44].

Although the relative importance of GABAergic and non-GABAergic input to ON and OFF cells for nociceptive modulation is impossible to know, the present data clearly demonstrate that these connections exist. However, the converse is not true. An inability to see input to a particular neuron does not mean that input does not exist. For example, only one third of the RVM neurons labeled from the spinal cord received input from the PAG, but that percentage is not an accurate reflection of contact between neurons because only a subset of neurons were labeled following injection of the tracers and the dendritic arbors of retrogradely labeled cells were not as completely defined as biotinamide-filled cells. The important point is that PAG neurons make direct contact with spinally projecting RVM neurons. Moreover, the pattern of labeling revealed in this study demonstrates that the PAG influences the activity of both ON and OFF cells directly.

Methodological problems limited the number of ON, OFF, and Neutral cells studied. These problems include finding and maintaining recordings of action potentials in lightly anesthetized rats subjected to multiple tail withdrawal trials. If the signal to noise ratio can be maintained and the cell is not damaged by the electrode, then biotinamide must be ejected in sufficient quantity to label the neuron, but not surrounding neurons. Additional problems arise in trying to find one and only one neuron after the tissue has been sectioned and visualizing all three labels. Although these are difficult experiments [40], the results are powerful in demonstrating specific anatomical and pharmacological relationships to physiologically defined cell classes. Given the potential for error, we included only those neurons in which we had high confidence that the recorded and labeled neuron were the same. Confidence in our labeling is bolstered by the close correspondence between our previous juxtacellular labeling study [53] and previous intracellular recordings [41]. Both studies reveal that serotonin labeling is found in only a subset of RVM Neutral cells

Given that PAG input to ON and OFF cells includes both GABAergic and non-GABAergic neurons, distinct subclasses of ON and OFF cells appear to exist. That is, OFF cells that receive GABAergic input from the PAG are distinct from OFF cells that do not receive GABAergic input. ON and OFF cells also can be subdivided on the basis of whether they contain GAD67 immunoreactivity. Although all three ON cells recorded in this study contained GAD67 immunoreactivity, a previous report from our lab found ON cells with and without GAD67 immunoreactivity [53]. Thus, a subset of both ON and OFF cells use GABA as a neurotransmitter. Presumably, GABAergic and non-GABAergic ON and OFF cells modulate nociception by projecting to distinct cell classes in the spinal cord. Overall the proportion of ON and OFF cells that received PAG inputs that were GAD67-immunoreactive was lower than the proportion of reticulospinal neurons that received GAD67 inputs from PAG. One possible explanation would be that a subset of the identified ON and OFF cells do not project to the spinal cord.

In sum, while there was a general pattern of GAD67-immunoreactive projections from the PAG targeting GAD67-immunoreactive neurons in the RVM; functionally identified OFF cells show the opposite pattern. OFF cells containing GAD67 immunoreactivity received input that lacked GAD67 immunoreactivity. This input is likely to be excitatory and involved in the antinociceptive effects produced by morphine microinjection into the PAG. Together these findings indicate that the heterogeneous phenotype of PAG projections to RVM reticulospinal neurons may be partially explained by differential inputs to ON and OFF cells.

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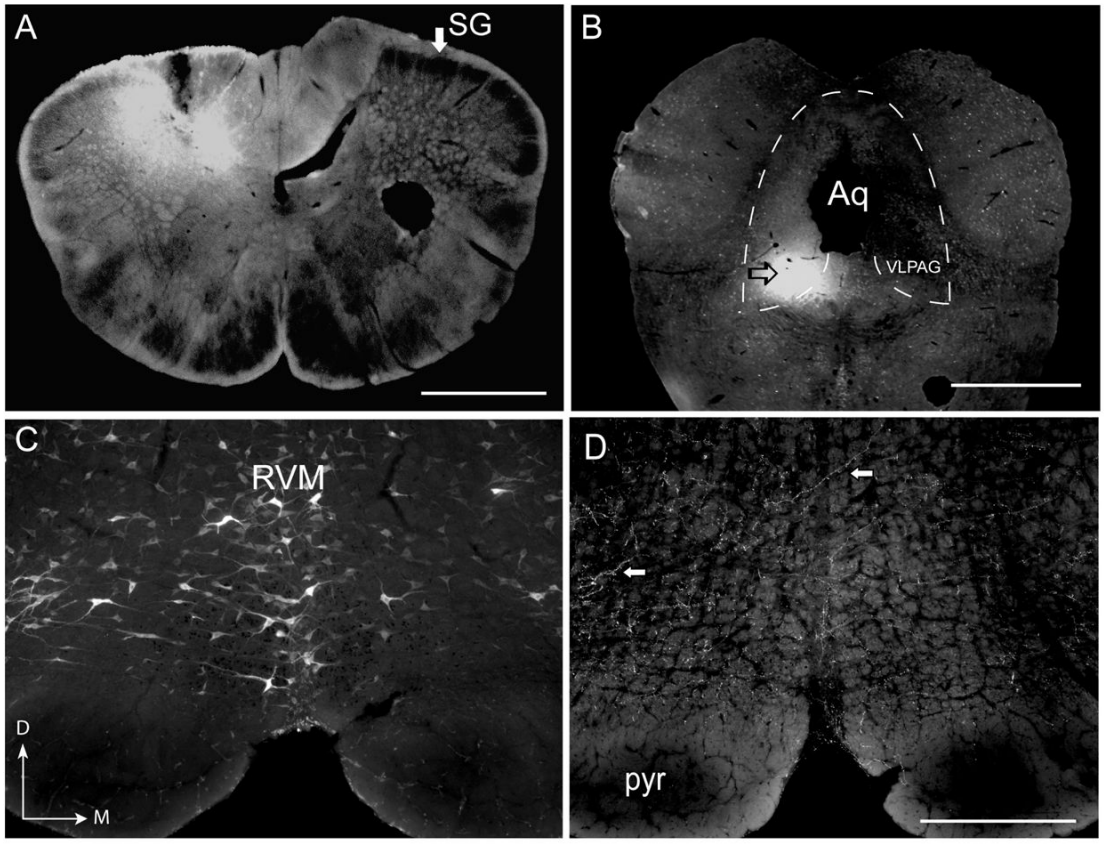

Figure 1.

Micrographs of representative injection sites into cervical spinal cord (A) and ventrolateral PAG (B), as well as micrographs of representative labeling of FG (C) and PHA-L in RVM (D). (A) Epifluorescent micrograph of FG injection into cervical spinal cord. Injection is apparent in the left dorsal horn region. Arrow indicates substantia gelatinosa (SG). (B) Epifluorescent micrograph of PHA-L injection site in ventrolateral PAG (VLPAG). Arrow indicates injection in the left VLPAG. Micropunches placed in tissue to distinguish left and right sides are visible in the lower right portion of tissue sections (A,B). The PAG is outlined by the dashed line. Aq = aqueduct. (C,D) Epifluorescent micrographs of FG (C)and PHA-L (D) labeling in RVM. Arrows indicate PHA-L fibers (D), which are abundant throughout RVM. pyr = pyramidal tract. Scale bars for A = 1.0 mm; B = 2.0 mm; C,D = 500 μm. Directional arrows indicate dorsal (D) and medial (M) for all panels.

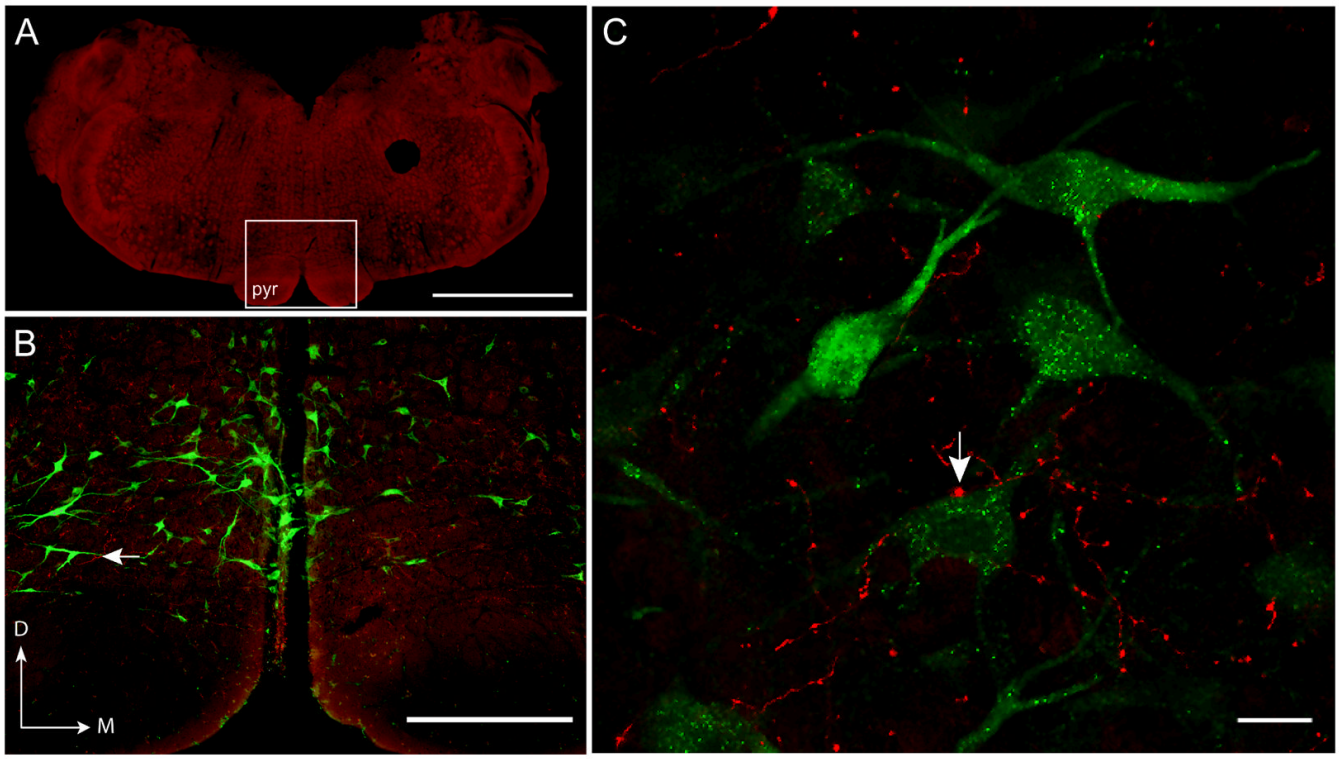

Figure 2.

Anterogradely labeled PAG fibers contact retrogradely labeled reticulospinal neurons in RVM. (A) Micrograph illustrating the level and area of RVM used for analysis, as indicated by the box. pyr = pyramidal tract. (B) Epifluorescent micrograph illustrating the overlapping distribution of retrogradely labeled FG neurons (green) and anterogradely labeled PHA-L fibers (red). Arrow indicates a PHA-L fiber. (C) Approximately one-third of the reticulospinal neurons identified in this study received appositions from PAG varicose fibers labeled in the same case (see text for detailed analysis). Arrow indicates a PHA-L varicosity (red) apposed to a FG neuron (green). The image is confocal Z projection of ten 0.8 μm optical sections with 0.4 μm of overlap forming a 4.4 μm thick stack. Scale bar for A = 2.0 mm, B = 500 μm; C = 20 μm. Directional arrows indicate dorsal (D) and medial (M) for all panels.

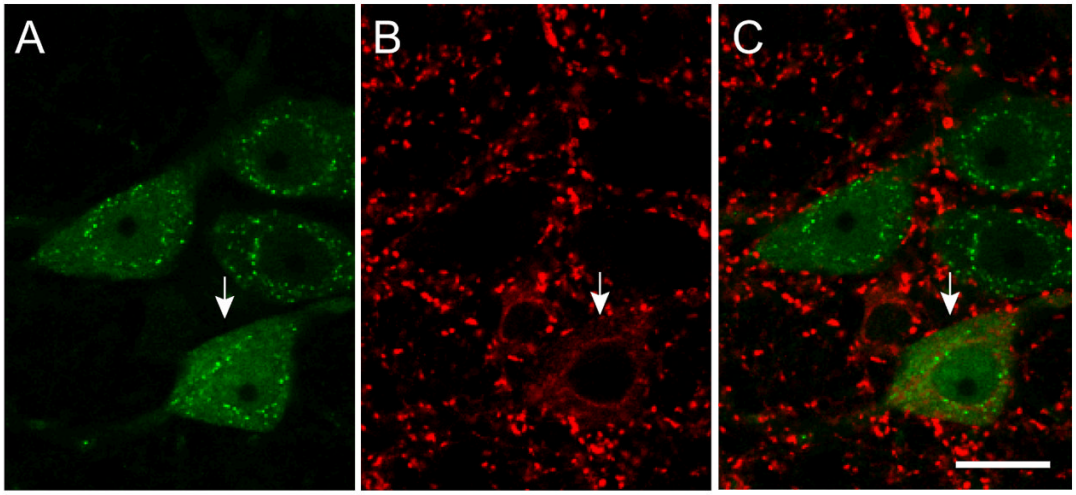

Figure 3.

GAD67 immunoreactivity is present in a subset of RVM reticulospinal neurons. RVM neurons are retrogradely labeled from the spinal cord with FG (A). (B) Immunostaining for GAD67 (red) in RVM. (C) An overlay of both markers shows clear colocalization between reticulospinal neurons (FG) and GAD67 immunoreactivity, as well as neurons that did not contain GAD67 immunoreactivity. The arrow indicates a reticulospinal neuron (A), GAD67 immunoreactivity of the same neuron (B) and the colocalization of both markers in the overlay (C). The image is a single confocal section, 0.8 mm thick. Scale bar = 20 μm.

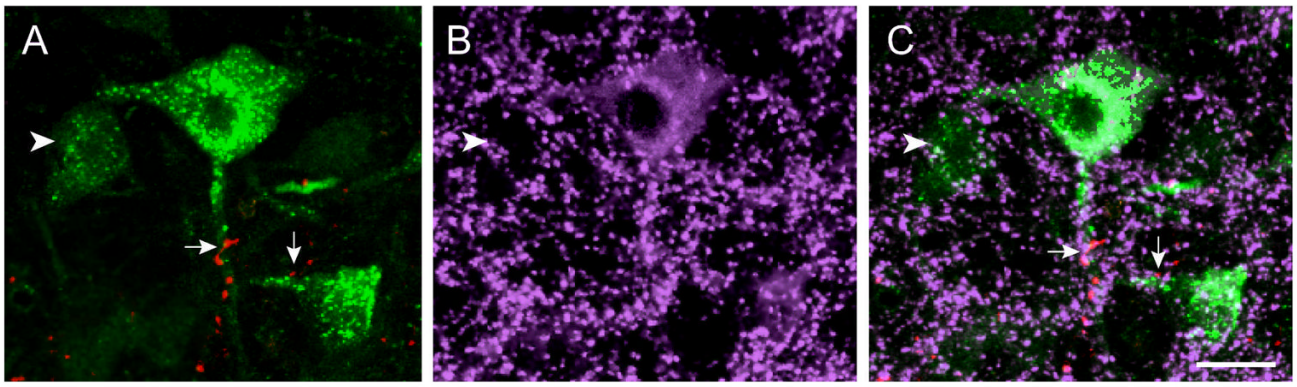

Figure 4.

PAG fibers often target neurons in RVM that contain GAD67 immunoreactivity. (A) PHA-L varicose fibers (red) appose retrogradely labeled FG reticulospinal neurons (green), as indicated by the two arrows. (B, C) GAD67 immunoreactivity (purple) is apparent in the region and both FG neurons that receive PAG input are also GAD67 immunoreactive. The arrowhead in all three panels indicates a FG-labeled reticulospinal neuron that does not contain GAD67 immunoreactivity nor receives input from PAG. Of the 72 FG cells that received PAG input, 63% (45/72) of the target neurons contained GAD67 immunoreactivity. Images are a confocal Z projection of twelve 0.8 μm optical sections with 0.4 μm of overlap forming a 5.2 μm thick stack. Scale bar = 20 μm.

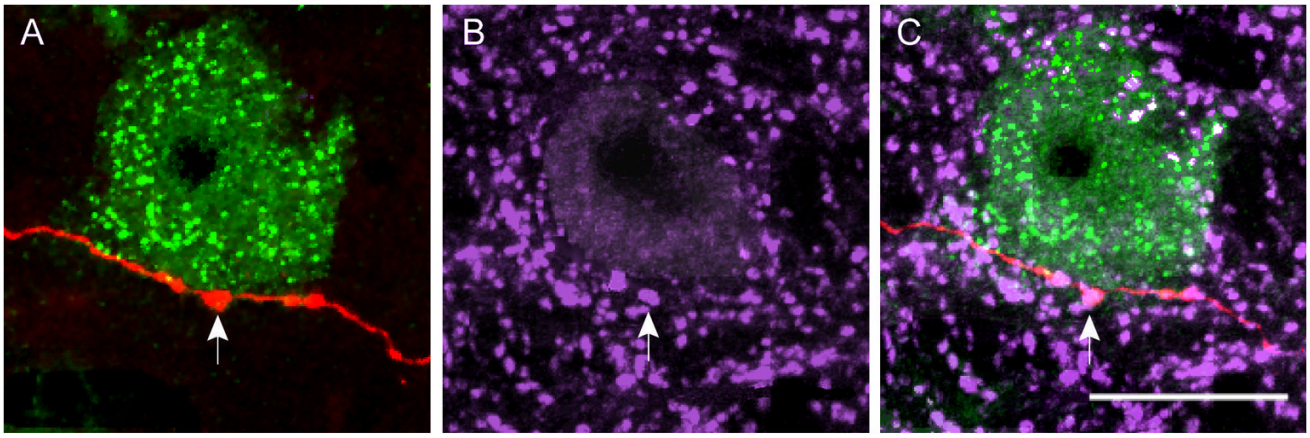

Figure 5.

PAG fibers containing GAD67 immunoreactivity target GAD67 immunoreactive reticulospinal neurons in RVM. (A) A PHA-L varicose fiber (red) is apposed to a reticulospinal FG neuron (green; arrow). (B) GAD67 immunoreactivity (purple) is seen in the same area. (C) GAD67 immunoreactivity is colocalized within both the reticulospinal neuron and the PHA-L varicosity (arrow), is apparent in the overlay. Of the 45 FG and GAD67 immunoreactive cells that received PHA-L input, 32 (71%) received PAG input colocalized with GAD67 immunoreactivity. In contrast, of the 27 FG-only cells that received PAG input, only 13 (48%) received PAG input colocalized with GAD67 immunoreactivity. Image is a confocal Z projection of eight 0.8 μm optical sections with 0.4 μm of overlap forming a 3.6 μm thick stack. Scale bar = 20 μm.

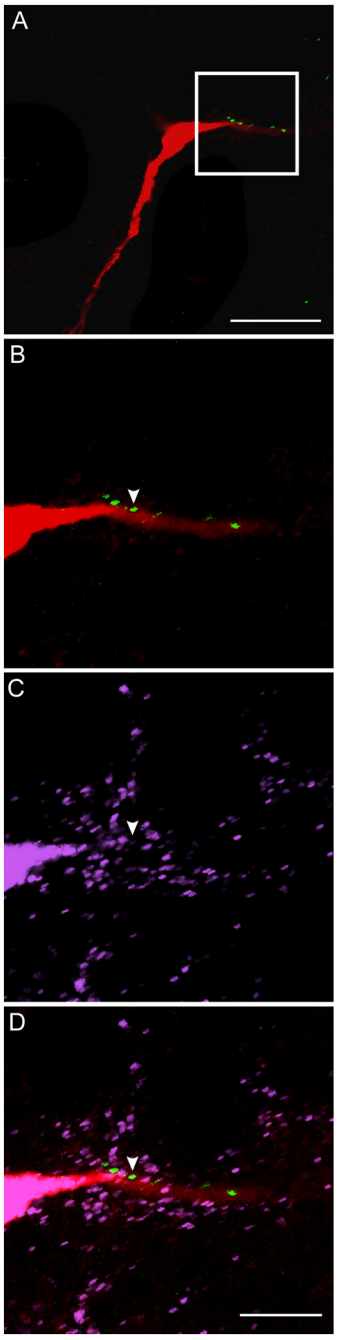

Figure 6.

Most PAG fibers apposed to a Neutral cell in the RVM do not contain GAD67 immunoreactivity. (A) A biotinamide-filled neuron in RVM, electrophysiologically characterized as a Neutral cell. The following three panels are magnified images of the boxed area. Scale bar = 50 μm. (B, C, D) The arrowhead in the three panels indicates a PHA-L varicose fiber (green) apposed to the Neutral biotinamide-filled cell (red) and, while GAD67 immunoreactivity (purple) is present in the region, there is no colocalization between the PHA-L varicosity and GAD67 immunoreactivity. Colocalization between the biotinamide-filled cell and GAD67 immunoreactivity is apparent in the overlay (D). The images are a confocal Z

projection of ten 0.8 μm optical sections with 0.4 μm of overlap forming a 4.4 μm thick stack. Scale bar = 20 μm.

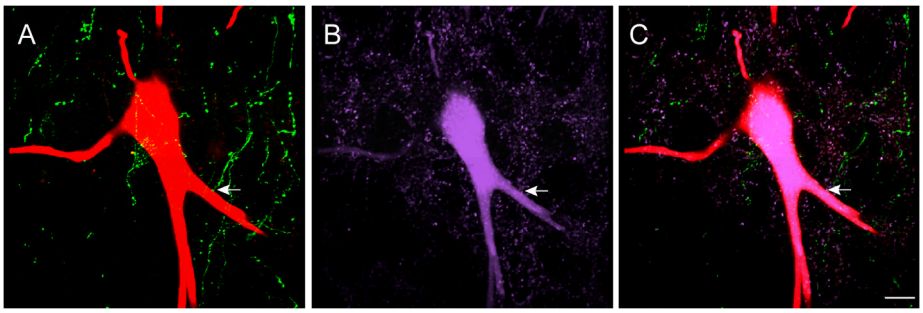

Figure 7.

PAG fibers apposed to an ON cell in the RVM. Both the ON cell and the PAG fibers contain GAD67 immunoreactivity. (A) A biotinamide-filled neuron (red), functionally characterized as an ON cell, as well as PHA-L fibers (green; arrow). (B) GAD67 immunoreactivity is present in the same region (purple); the arrow points to the GAD67 immunoreactivity of the previously indicated PHA-L apposition (A). (C) Colocalization between GAD67 immunoreactivity and both the ON cell as well as the PHA-L apposition (arrow) is apparent in the overlay. Of the three functionally characterized ON cells, all of them contained GAD67 immunoreactivity and received PAG input. Images are a confocal Z projection of twenty 0.8 μm optical sections with 0.4 μm of overlap forming an 8.4 μm thick stack. Scale bar = 20 μm.

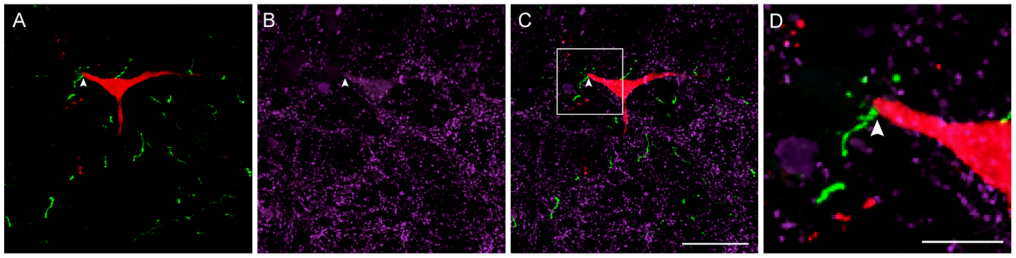

Figure 8.

PAG fibers apposed to an OFF cell in the RVM. The OFF cell contains GAD67 immunoreactivity, but the PAG fibers do not. A PAG varicose fiber (green) apposes a biotinamide-filled OFF cell (arrowhead; A). GAD67 immunoreactivity (purple) is in the same region (B). GAD67 immunoreactivity is found in the OFF cell (C; inset D), but not in the PHA-L varicosity (arrowhead). Of the three functionally characterized OFF cells containing GAD67 immunoreactivity, all three received PAG input; however, none of the PAG inputs contained GAD67 immunoreactivity. Image is a confocal Z projection of twenty-four 0.8 μm optical sections with 0.4 μm of overlap forming a 10 μm thick stack. Scale bars for A, B, C = 50 μm; D = 15 μm.

Table 1

Summary of PAG input and GAD67 labeling for RVM neurons retrogradely labeled with FluoroGold (FG)

<table>
<tr><td colspan="4">FG reticulospinal neurons (n=215)</td></tr>
<tr><td colspan="4">FG neurons containing GAD67 (n=114/215; 53%)</td></tr>
<tr><td colspan="4">FG neurons with PAG input (n=72/215; 33%)*</td></tr>
<tr><td colspan="2">FG neurons with PAG input; GAD67+ (n=45/72; 63%)</td><td colspan="2">FG neurons with PAG input; GAD67-(n=27/72; 38%)</td></tr>
<tr><td>PAG input GAD67+ (n=32/45; 71%)</td><td>PAG input GAD67− (n=13/45; 29%)</td><td>PAG input GAD67+ (n=13/27; 48%)</td><td>PAG input GAD67− (n=14/72; 51%)</td></tr>
</table>

*Note: Does not represent all PAG inputs because PHA-L was administered to a small region in the ventrolateral PAG.

Table 2

PAG Inputs to Physiologically Characterized RVM neurons

	ON Cells (n = 3)		OFF Cells (n = 6)		Neutral Cells (n = 6)	
Input	**GAD+**	**GAD−**	**GAD+**	**GAD−**	**GAD+**	**GAD−**
PHA-L only	2	0	3	0	2	2
PHA-L + GAD67	1	0	0	1	1	0
No PAG Input	0	0	0	2	0	1