Kappa Opioid Receptor (KOR) and GAD67 Immunoreactivity Are Found in OFF and NEUTRAL Cells in the Rostral Ventromedial Medulla

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INTRODUCTION

Neurons in the rostral ventromedial medulla (RVM) are part of a network that modulates nociceptive transmission. The RVM receives glutamatergic input from the periaqueductal gray (PAG) (Aimone and Gebhart 1986; Beitz 1982; Carlton et al. 1983) and projects to the trigeminal nucleus caudalis and spinal cord (Basbaum and Fields 1979; Skagerberg and Björklund 1985). The PAG itself contains few direct projections to the spinal cord, making the RVM a necessary relay for PAG–spinal cord (Basbaum and Fields 1979; Skagerberg and Bjo¨rk-
KOR agonists could act either by direct hyperpolarization of off cells or by presynaptic inhibition of glutamatergic inputs to these cells (Ackley et al. 2001; Bie and Pan 2003; Meng et al. 2004; Pan et al. 1997). In vivo and in vitro studies support these findings (Bie and Pan 2003; Meng et al. 2004); for example, microinjection of KOR agonists into the RVM attenuate the ON cell burst just prior to the nociceptive withdrawal reflex suggesting a possible presynaptic site of action. KOR agonists in the RVM also inhibit the ongoing activity of a subset of off cells (Meng et al. 2004), suggesting that these cells contain postsynaptic KOR.

The neurotransmitters contained in ON and OFF cells are not known, but at least some of these neurons are thought to be GABAergic (Fields et al. 1991). Although these cells are located within the raphe nuclei, only neutral cells have been shown to contain serotonin (5-HT) (Potrebic et al. 1994). The present study determined if 5-HT and GABAergic markers are located in OFF, ON, or neutral cells and whether OFF cells contain KOR as indicated by previous electrophysiological and pharmacological studies. A juxtacellular recording and labeling technique was used to characterize and fill cells in the RVM. This is the first attempt to use this technique with these cells. The findings demonstrate the utility of combining these methods to reveal morphological details of functionally characterized individual neurons whose activity is related to specific behavioral responses in the intact animal.

METHODS

Experimental animals and surgery

All protocols were in accordance with the Institutional Animal Care and Use Committee at Oregon Health and Science University. Male Sprague-Dawley rats (n = 52; 250–375 g; Taconic Farms) were used. Animals were initially anesthetized in a Plexiglas chamber with 5% isoflurane in oxygen vaporized by an Isotec Tec3 (Datex-Ohmeda; Madison, WI). The head was shaved, and the rat was placed into a David Kopf stereotaxic frame with a nose cone for anesthesia delivery. The anesthesia level was lowered to 3% isoflurane in oxygen for the duration of the surgery. Body temperature was maintained with a 37°C water blanket (TPump; Gaymar; Orchard Park, NY). The interparietal bone was exposed and a hole drilled using a 1.8 mm burr for the insertion of the electrode. Prior to neuronal recording, the anesthesia level was adjusted (between 1 and 1.5%) so that tail flick latencies to a noxious heat stimulus were consistently between 10 and 20 s (see Nociceptive testing).

Extracellular recording

Glass capillary electrodes (1.5 mm) were made on a vertical electrode puller (PE-2, Narishige Scientific Instrument Laboratories, Tokyo, Japan), and the tips were broken to a resistance of 18–20 MΩ. Biotinamide hydrobromide (Molecular Probes; Eugene, OR) dissolved at 5% in 0.5 M sodium acetate was used to fill the electrodes. Electrode penetrations were made along the midline 2.8–3.0 mm caudal to Lambda and recordings were initiated at 7.0 mm below the dorsal surface of the cerebellum. The electrode was moved in 1-μm steps (Micro Drive, FHC; Bowdoinham, ME). Spontaneously active neurons were identified electrophysiologically and classified according to changes in activity temporally related to the tail-flick (see Off-line physiological analysis). Action potentials were recorded with an intracellular recording amplifier (Axoclamp 2B, Axon Instruments, Sunnyvale, CA), further amplified (CyberAmp 380, Axon Instruments) and digitally converted (Micro 1401, CED; Cambridge, U.K.). Digital information was displayed and stored for off-line analysis (Spike 2 v.5 software, CED).

Nociceptive testing

After locating a neuron, spontaneous baseline activity was recorded for ≥60 s. Next, a tail flick was evoked by placing the distal end of the tail into 52–54°C water. Both onset of tail immersion and the withdrawal response were identified with event markers in a separate channel on the computer during the recording session. Each neuron was tested at least twice to ensure correct classification. At least 1 min was allowed between nociceptive trials and noxious stimulation was discontinued if no response was elicited within 20 s. The tail was dried after each immersion into water.

Biotinamide fills

After nociceptive testing, positive-current pulses (400-ms duration, 50% duty cycle) of gradually increasing intensity (maximum = 15 nA) were applied to each cell through the bridge circuit of the recording amplifier until entrainment of cell discharge to the current pulse was achieved (Pinault 1996) (Fig. 1B). The duration of cell entrainment varied between 10 s and 4 min, but the quality of the cell fill was not directly related to the intensity of the entrainment pulse or to the duration of the entrainment. When possible, the response to noxious heat was again confirmed after the cell was entrained. Every cell that was tested after entrainment demonstrated the same response to noxious stimulation as it did prior to entrainment. In experiments involving labeling for KOR, a single RVM neuron was recorded and juxtacellularly labeled in each experiment; in some experiments involving immunocytochemical labeling for 5-HT, two neurons were recorded and filled in separate tracks. On rare occasions, two cells were filled during a single entrainment event in which the action potentials of a single neuron were observed. Also infrequently, a neuron was impaled during the advancement of the electrode, causing the cell to depolarize. We noted the dorsoventral locations of such events and observed cells lightly labeled with biotinamide at such sites. In neither of these circumstances were these cells used in the present analysis.

Perfusion and tissue preparation

Approximately 30 min after recording, rats were perfused transcardially through the ascending aorta with 10 ml of heparinized saline (1,000 units/ml), followed by aldehyde fixation. For 5-HT immunochemistry, labeling, 600 ml of 4% paraformaldehyde, 0.125% glutaraldehyde, and 0.2% sodium metabisulfite in 0.1 M phosphate buffer (PB), pH 7.4 was used. For KOR and GAD67 immunolabeling, 50 ml of 3.8% acrolein in 2% paraformaldehyde followed with 200 ml of 2% paraformaldehyde (in 0.1 M PB) was used. The brain was removed, and blocks of tissue containing RVM were placed in the final fixative for 30 min, then into 0.1 M PB. Tissue was sectioned on a vibrating microtome at 40 μm. Free-floating tissue sections were placed in 1% NaBH₄ (Sigma) for 30 min to bind remaining free aldehydes.

Immunocytochemistry

For 5-HT immunoactivity, tissue was incubated in a single primary antibody (rabbit α 5HT, 1:32,000, Immunostar) for 48 h at 4°C. For KOR and GAD67 immunoactivity, tissue was incubated in an antibody cocktail (rabbit α KOR, 554 μg/ml, gift from C. Chavkin; and mouse α GAD67, 1:2000, Chemicon). Bound 5-HT antibody and injected biotinamide were detected with a cocktail containing a single secondary antibody (Alexa Fluor 647 goat α rabbit, 1:800) and
streptavidin conjugated to Alexa Fluor 488 (6.25 μg/ml). Two secondary antibodies were used to visualize KOR and GAD67 (Alexa Fluor 488 goat α rabbit and Alexa Fluor 488 goat α mouse, 1:800 each), and streptavidin 546 (6.25 μg/ml) was used to detect the filled cell. All secondary antibodies and streptavidin conjugates were obtained from Molecular Probes. Tissue was mounted on gelatin-coated slides and cover-slipped with Prolong (Molecular Probes) to preserve labeling.

Neuronal location, confocal microscopy, and anatomical analysis

Neurons in animals with multiple fills were identified according to their recorded dorsal/ventral and rostral/caudal stereotaxic position. Low-magnification images were used to determine the location of each cell in relation to anatomical landmarks (ventral surface, etc) and the relative position between the two cells. Cells were then mapped onto the brain atlas at the appropriate level (Paxinos and Watson 1998).

Identified neurons and local 5-HT, KOR, and GAD67 immunoreactivity were visualized using a Zeiss LSM 510 confocal microscope. The single-pass, multi-tracking format was utilized to allow the tracers to be individually excited with different lasers, and the emitted spectra were collected separately to minimize overlap. Alexa Fluor 488 was excited with a 488 nm (Argon/2) laser and emissions passed through a 500- to 550-nm band-pass filter before collection. A 543-nm laser (HeNe1) was used to excite Alexa Fluor 546, and emissions were collected after passing through a 535- to 590-nm band-pass filter. Alexa Fluor 647 was excited with a 633 nm laser (HeNe2) and emitted light passed through a 650- to 710-nm band-pass filter before collection.

Filled cells were scrutinized for immunoreactive labeling both in the cell and the surrounding field. A cell was scored as immunoreactive if labeling was present in the cytoplasm and an unlabeled nuclear space was evident. Filled neurons were not scored unless the surrounding field contained immunoreactive cells. Somatic cross-sectional diameter, measured across the shortest distance between cellular membranes through the nucleus, was determined for all functionally characterized filled cells.

Off-line electrophysiological analyses

Extracellular single-unit recordings were stored digitally as Spike2 v.5 data files for off-line analysis. Cell class was determined by calculating the change in firing rate during a 5 s interval
surrounding the tail flick (“peri-flick” interval; 2.5 s on either side of the tail flick event marker) compared with a 30-s baseline period of spontaneous activity (ending 10 s prior to noxious heat). For each cell, two nociceptive trials were averaged to generate a mean change in activity. The percent change in activity was calculated as follows: \( \frac{[\text{peri-flick firing frequency} - \text{baseline frequency}]}{\text{baseline frequency}} \times 100 = \% \text{ change in activity} \). Neurons showing a 30% decrease in activity from baseline were considered OFF cells, whereas neurons with an increase of \( \geq 30\% \) were considered ON cells. Cells that were neither ON nor OFF were classified as NEUTRAL cells, whose activity was either weakly affected or unaffected by the noxious stimulus (Fig. 1A).

In addition to histological verification, a method for predicting serotonin-containing cells based on their spontaneous activity has been developed by Mason and colleagues (Gao and Mason 1997; Mason 1997). This method uses mean interspike interval (\( \bar{x} \), in ms) and the SD (\( s \), in ms) of the interspike intervals as independent variables in a multivariate discriminant function: \( y = 146 - \bar{x} + 0.98s \). For each neuron, if the discriminant function value is <0, the cell is likely to be serotonergic; if the value is >0, the cell is likely to be nonserotonergic. We calculated discriminant function values for all cells where 5-HT was assessed histologically; for each cell, 1-5 min of baseline activity was used for the analysis. This method has been shown to be \( \sim 90\% \) accurate (Mason 1997).

RESULTS

Physiology and anatomy of filled cells

A total of 52 spontaneously active cells were physiologically identified and successfully filled in 43 rats: 21 cells were identified as OFF cells, 14 as ON cells, and 17 as NEUTRAL cells. OFF cells had baseline firing frequencies ranging from 1 to 33 Hz (mean: 14 Hz), which decreased by an average of 52 \( \pm \) 4.5% during the peri-flick interval. ON cells had baseline frequencies ranging from 0.1 to 25 Hz (mean: 5.4 Hz), which increased on average by +1,800 \( \pm \) 870% during the peri-flick interval. NEUTRAL cells had baseline firing frequencies ranging from 0.8 to 44 Hz (mean: 8.7 Hz), which did not change significantly during the peri-flick interval (average of \( -2.7 \pm 3.3\% \)). There were no differences in basal firing frequencies among the three populations of RVM neurons; however, comparisons of the percent change of the peri-flick activity from the baseline activity indicated a significant difference (Kruskal-Wallis 1-way ANOVA on ranks, \( P < 0.001 \); post hoc Dunn’s multiple comparisons, \( P < 0.05 \)) among the peri-flick activities of ON, OFF, and NEUTRAL cells.

The biotinamide juxtacellular labeling technique filled the somata and dendritic arbors of these cells extensively (Fig. 2); diverse somatic morphology and variable dendritic complexity were observed. While analyses of dendritic arborization were not conducted, there was no difference in cell soma size across the three classes of RVM neurons (mean diameter \( \pm \) SE): OFF cells (16 \( \pm \) 1.2 \( \mu \)m), ON cells (18 \( \pm \) 1.5 \( \mu \)m), and NEUTRAL cells (14 \( \pm \) 1.3 \( \mu \)m). These results are in contrast to previous studies suggesting differences in cell size between primary and secondary RVM neurons (Pan et al. 1990) but are in agreement with studies finding overlapping morphologies of ON, OFF, and NEUTRAL cells (Mason et al. 1990).

\[ 5-HT \text{ immunoreactivity in juxtacellularly labeled ON, OFF, and NEUTRAL cells} \]

Light microscopy revealed 5-HT immunoreactivity in neurons scattered throughout the RVM (Fig. 3A). We physiologically characterized, juxtacellularly filled and performed 5-HT immunocytochemistry on 24 RVM neurons (7 OFF cells, 6 ON cells, and 11 NEUTRAL cells). ON, OFF, and NEUTRAL cells were found throughout the dorsoventral extent of the RVM (Fig. 3B). Of the 11 NEUTRAL cells, 4 were 5-HT-immunoreactive (ir) (36%; Fig. 4), whereas none of the OFF or ON cells contained 5-HT immunoreactivity. The 5-HT-ir NEUTRAL cells were distributed throughout the RVM with no apparent anatomical segregation of 5-HT-ir NEUTRAL cells (Fig. 3B).

In addition to histological verification, a discriminant function developed to predict serotonin-containing cells by their firing characteristics, was calculated for each cell. The positive or negative value of this function indicates whether the cell is likely to be nonserotonergic or serotonergic, respectively (Mason 1997). Consistent with the prediction of the discriminant function, all four NEUTRAL cells histologically identified as being 5-HT-ir had negative discriminant function values, and the discriminant function values were positive for 7 OFF, 5 ON, and 5 NEUTRAL cells without 5-HT immunoreactivity. However, two NEUTRAL cells and one ON cell that lacked 5-HT immunoreactivity had negative discriminant function values. Overall, these data indicate that the discriminant function predicted 5-HT immunoreactivity with 88% accuracy (21 of 24 cells) in the present study. For some cells, only a few minutes of

FIG. 2. NeuroLucida reconstructions of representative biotinamide filled RVM cells. A: OFF cell. B: ON cell. C: NEUTRAL cell. We found no consistent morphological differences among cell types (see results). Scale bar = 250 \( \mu \)m.
spontaneous activity were examined, potentially lowering the accuracy of the algorithm.

**KOR and GAD67 immunoreactivity and colocalization in juxtacellularly labeled OFF, ON, and NEUTRAL cells**

We examined physiologically characterized OFF, ON, and NEUTRAL cells for the presence of KOR and GAD67. KOR immunoreactivity has been predicted for OFF cells (Meng et al. 2004), and both OFF and ON cells are thought to contain GABA (Fields et al. 1991). We examined 27 physiologically characterized (14 OFF cells, 8 ON cells, 5 NEUTRAL cells) and juxtacellularly labeled RVM neurons (Table 1). KOR-ir was observed in neurons throughout the RVM (Fig. 5A), and KOR-ir neurons were primarily OFF and NEUTRAL cells (Fig. 5B). KOR immunoreactivity was found in 12 of 14 (86%) OFF cells (Fig. 6, A and B), in 1 of 8 (13%) ON cells, and in 4 of 5 (80%) NEUTRAL cells. Most ON cells (Fig. 7A) did not contain KOR immunoreactivity (Fig. 7B). For OFF and ON cells, the presence or absence of KOR immunoreactivity was significantly related to cell classification ($P < 0.005$; Fisher exact test).

GAD67 immunoreactivity was found in 13 of 14 OFF cells (93%) (Fig. 6C), in 5 of 8 ON cells (63%), and in 4 of 5 NEUTRAL cells (80%) (Table 1). KOR immunoreactivity and GAD67 immunoreactivity were co-localized in 16 of 23 cells that were immunoreactive for at least one marker (Table 1). Both KOR immunoreactivity and GAD67 immunoreactivity were contained in 11 of 14 OFF cells (77%; Fig. 6D), 1 of 8 ON cells (13%), and 4 of 5 NEUTRAL cells (80%; Table 1). ON cells were more likely to be GAD67 immunoreactive (-ir) than KOR-ir but often did not contain either marker (Fig. 7, C and D; Table 1).

**DISCUSSION**

We used a juxtacellular recording and filling technique, in combination with the tail-flick test, to characterize and identify three cell types in the RVM with distinct nociceptive responses. We combined this identification and labeling approach with immunohistological characterization for 5-HT, KOR, and/or GAD67. This technique is a novel approach to studying these neurons and is particularly valuable in an anatomically and functionally heterogeneous brain region such as RVM. We determined that 5-HT immunoreactivity was specific only to a subset (36%) of RVM NEUTRAL cells, consistent with previous studies (Potrebic et al. 1994). Additionally, we found KOR immunoreactivity was primarily in OFF and NEUTRAL cells as predicted by in vivo and in vitro physiological studies.
Finally, we found GAD67 immunoreactivity in all three classes of RVM neurons, suggesting that many of these cells are GABAergic; however, ON cells were the cell type least likely to contain KOR immunoreactivity or GAD67 immunoreactivity.

**Technical considerations**

The present juxtacellular labeling method allows identification of single, functionally characterized neurons in the brain (Pinault 1996). Advantages of this method include the ability to label single neurons without impaling the cell, thus preventing dialysis of cytoplasmic materials, to possibly recording from smaller neurons than with intracellular methods, and to relate neuronal morphology with discharge characteristics. Although it is possible to combine the juxtacellular labeling technique with determination of a neuron’s spinal projection (Aicher et al. 2001), the antidromic approach was not used in the present study. The present study examined only neurons that were spontaneously active, but silent neurons could be sampled for juxtacellular labeling if appropriate search stimuli or antidromic activation were used. With careful isolation of neuronal activity, single units are usually filled, although on occasion multiple cells are filled during entrainment; in many cases the activity of a second unit is detectable during entrainment (Pinault 1996). Entrainment and filling of multiple neurons occurred in 9% of cases in the present study and such cases were excluded from immunocytochemical analyses.

**Role of KOR ligands in the RVM**

In behavioral studies, KOR agonists have both anti- and pronociceptive effects, suggesting a complex role for this receptor in modulating pain transmission. Some of this complexity may be related to opposing effects of KOR ligands at different sites of action, such as the RVM and the spinal cord. The present studies suggest that within the RVM the KOR has a fairly consistent distribution in specific types of cells, thus emphasizing the importance of combining anatomical and functional methods. We found KOR in the vast majority of OFF and NEUTRAL cells in the RVM, consistent with observations in vitro that KOR ligands depolarize primary cells but only indirectly modulate the activity of secondary cells (Pan et al. 1997). Primary cells in the slice are defined according to responses to opioids, and this classification appears to include both OFF and NEUTRAL cells, whereas secondary cells in the slice appear to correspond to ON cells in the whole animal (Pan et al. 1990). Our data support the hypothesis that within the RVM, KOR ligands act postsynaptically on OFF and NEUTRAL cells (Meng et al. 2004). However, these findings do not preclude the possibility that KOR ligands also modulate presynaptic transmission in the RVM (Ackley et al. 2001; Bie and Pan 2003; Meng et al. 2004; Pan et al. 1997). A further complexity in the role of KOR ligands in nociception are findings that the effects of these agents are likely sex-related (Gear et al. 1996; Tershner et al. 2000). In rats, sex differences were seen in KOR immunoreactivity in both the dorsal horn of the spinal cord (Chang et al. 2000) and within the RVM (Drake et al. 2006). The net effect of KOR ligands within the RVM is therefore likely to be mediated by both pre- and postsynaptic receptors.

Our data support the conclusion that postsynaptic actions of KOR agonists are mediated through OFF and NEUTRAL cells. KOR is found in reticulospinal neurons (Kalyuzhnny and Wessendorf 1999), and the combination of a spinal projection and KOR immunoreactivity is likely to distinguish these OFF and NEUTRAL cells from other neurons within the RVM. At this time, there does not appear to be any histological distinction between OFF cells and NEUTRAL cells based on neurotransmitter or opioid receptor content.

**TABLE 1. Localization of KOR and GAD immunoreactivity in OFF, ON and NEUTRAL cells in RVM**

<table>
<thead>
<tr>
<th></th>
<th>KOR only</th>
<th>GAD67 only</th>
<th>KOR + GAD67</th>
<th>No ir</th>
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<tr>
<td>OFF</td>
<td>1</td>
<td>2</td>
<td>11</td>
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<td>ON</td>
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<td>4</td>
<td>1</td>
<td>3</td>
<td>8</td>
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<tr>
<td>NEUTRAL</td>
<td>0</td>
<td>0</td>
<td>4</td>
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<td>5</td>
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</table>

KOR only indicates KOR immunoreactivity, but not GAD67 immunoreactivity. GAD67 only indicates GAD67 immunoreactivity, but not KOR immunoreactivity. KOR + GAD67 indicates immunoreactivity for both KOR and GAD67. No ir indicates the number of physiologically characterized RVM neurons that did not contain either marker.
Role of 5-HT in nociception

Our results confirm that of the three RVM cell types identified (ON, OFF, and NEUTRAL) (Fields et al. 1983a), 5-HT was only found in a portion (36%) of the NEUTRAL cell population (Potrebic et al. 1994). Clearly, the NEUTRAL cell criterion is quite broad and is not sufficient to identify 5-HT neurons. We also tested the algorithm that has been developed to predict 5-HT content based on neuronal firing pattern alone (Mason 1997), and we found the algorithm to be 88% accurate. Incorrect predictions of the algorithm were only in the direction of predicting 5-HT content that was not verified histologically. This may reflect variability in the neuronal firing patterns or limitations of the immunocytochemical methods. These findings support the notion that analysis of discharge pattern (Mason 1997) is a better predictor of 5-HT content than responses to noxious stimulation.

The subset of NEUTRAL cells that contain 5-HT may contribute to the RVM serotonergic projection to the spinal cord (Potrebic et al. 1994; Wang and Wessendorf 1999). Evidence suggesting that this pathway may play a role in descending nociceptive modulation includes the observations that intrathecal administration of 5-HT can produce antinociception, (Schmauss et al. 1983) and that 5-HT antagonists reduce the antinociception produced by electrical stimulation of the RVM (Hammond and Yaksh 1984). However, the effects of 5-HT in the spinal cord are mediated by different classes of receptors; thus 5-HT may be anti- or pronociceptive in the spinal cord (Suzuki et al. 2002).

The high percentage of NEUTRAL cells that contained KOR immunoreactivity in the present study suggests that both serotonergic and nonserotonergic NEUTRAL cells are likely to...
neurons in the modulation of nociceptive pathways. It remains to be determined how these cells may modulate nociceptive transmission, particularly within the spinal cord. This approach may also be used in future studies to address the issue of whether RVM neurons may change their anatomical and/or functional phenotype under pathological conditions (Miki et al. 2002).

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FIG. 7. Many ON cells did not contain KOR or GAD67 immunoreactivities. A confocal Z projection of 4 0.8-μm optical sections with 0.4 μm of overlap forming a stack 2.0 μm thick shows an electrophysiologically characterized ON cell (red) in A, juxta-}

contain KOR. Although we were not able to directly test this prediction, the colocalization of KOR and 5-HT immunoreactivities in RVM has been previously reported (Kaluzhny and Wessendorf 1999) and 5-HT-ir neurons respond to KOR agonists (Marinelli et al. 2002). The functional roles of neutral cells and 5-HT RVM neurons in nociceptive transmission remain unclear (Gao and Mason 1997, 2001; Gao et al. 1998; Mason 1997), but distinct subpopulations may exist.

GABAergic neurons

The majority (82%) of neurons examined in the present study contained GAD67 immunoreactivity, suggesting that they are potentially GABAergic. The cell type least likely to contain GAD67 immunoreactivity was ON cells, whereas OFF cells were more likely to be GABAergic. These findings are consistent with models suggesting that OFF cells send inhibitory projections to the spinal cord. Our results suggest that ~2/3 of ON cells contain GABA, whereas one-third does not. In other studies, approximately one-third of ON cells have been shown to project to the spinal cord (Vanegas et al. 1984). Together these findings suggest two potential populations of ON cells that are distinct with regard to GABA content. Anatomically distinct subsets of ON cells have been described with regard to intra-RVM arborizations (Mason and Fields 1989), but the neurotransmitter content of these cells has not been confirmed. The existence of two populations of ON cells remains to be conclusively demonstrated.

In summary, our findings indicate that a combined analysis of cellular classification, morphological features, and potential neurotransmitter or receptor phenotype will greatly enhance our understanding of the functional role of RVM


