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OPIOID SIGNALLING IN THE RAT ROSTRAL VENTROLATERAL MEDULLA

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SUMMARY

1. The present article reviews several aspects of opioid signalling in the rostral ventrolateral medulla (RVLM) and their implications for the neural control of blood pressure.

2. In the RVLM, preproenkephalin (PPE) mRNA is expressed by bulbospinal cells that are strongly barosensitive. These putative presympathetic neurons includes C1 and non-C1 neurons.

3. In the RVLM, PPE mRNA is also present in GABAergic neurons that do not project to the thoracic spinal cord.

4. Rostral ventrolateral medulla presympathetic cells receive enkephalinergic inputs and express μ-opioid receptors (MOR). Some of their synaptic inputs also contain MOR.

5. Pre- and post-synaptic modulation of RVLM presympathetic neurons by MOR agonists has been demonstrated in slices of neonate brain. The post-synaptic effect is inhibitory (increased gK). Presynaptic effects include disfacilitation (reduction of glutamate release) and possibly dishinhibition (reduction of GABA release).

6. In conclusion, opioid signalling plays a pervasive role in the medullospinal network that controls sympathetic tone and arterial pressure. Opioid peptides are made by the presympathetic, presumably excitatory, cells of the RVLM and by local GABAergic inhibitory neurons. In addition, RVLM presympathetic neurons are also controlled by opioid peptides at the pre- and post-synaptic level. μ-Opioid receptors are found post-synaptically, whereas presynaptic receptors probably include both μ and δ subtypes. Conditions that trigger the release of opioid peptides by presympathetic neurons or by inputs to these cells are not fully understood and may include decompensated haemorrhage and certain types of peripheral sensory stimulation related to acupuncture.

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INTRODUCTION

The autonomic regions of the thoracic and upper lumbar spinal cord receive a dense enkephalinergic innervation of supraspinal origin.[1,2] Based on immunohistochemical evidence from colchicine-treated rats, some of these enkephalin-immunoreactive (IR) fibres originate in the rostroventrolateral medulla (RVLM).[3] The pseudorabies virus experiments of Jansen *et al*.[3] also strongly suggested that some of the enkephalin-IR neurons of the RVLM may be presympathetic cells that control the sympathetic outflow to the heart and/or other structures innervated by the stellate ganglion. Consistent with this hypothesis, a few C1 neurons containing enkephalin (ENK)-like IR were found in the RVLM after treatment with colchicine.[4,5]

Some of the experiments summarized below[6] were designed to seek definitive evidence that RVLM presympathetic neurons express preproenkephalin (PPE), the precursor of the enkephalins. We examined whether the RVLM presympathetic cells that make PPE are C1 cells and we determined whether these cells are barosensitive as evidence that they regulate arterial pressure. To obviate the use of colchicine, PPE-synthesizing neurons were identified by the presence of PPE mRNA detected by *in situ* hybridization. The PPE mRNA-containing neurons will henceforth be called PPE-positive neurons.

Microinjection of various opioid receptor agonists into the RVLM produces profound hypotension.[7] This work suggests that the RVLM contains one or more types of opioid receptors whose overall activation results in the inhibition of the presympathetic neurons and a fall in blood pressure. Iontophoretic application of morphine also reduces the discharge rate of individual RVLM presympathetic neurons in anaesthetized rats, suggesting that some of these opioid receptors are in close proximity to the vasomotor cells.[8] Thus, one of our recent objectives has been to determine whether the presympathetic neurons that control arterial pressure[9,10] express MOR.[11]

Some of the C1 cells of the RVLM receive synapses that are IR for leu-ENK,[4] but the physiological role of these cells could not be determined in this anatomical study. Another objective of ours has been to extend this work by ascertaining that leu-ENK-IR terminals innervate the RVLM presympathetic bulbospinal cells that control arterial pressure.[12] The existence of enkephalinergic synapses and the presence of the cognate receptors on the post-synaptic side should provide strong evidence that opioid peptides are physiological modulators of the RVLM presympathetic cells that regulate arterial pressure. The last study summarized in the present review was designed to determine the effects of activating pre- and post-synaptic opioid receptors on the activity of RVLM presympathetic neurons in slices.[13]

PREPROENKEPHALIN mRNA IS EXPRESSED BY BLOOD PRESSURE-REGULATING PRESYMPATHETIC NEURONS OF THE RVLM

Except where indicated, all the experiments described in this section are the subject of a full report that should be consulted for experimental details.[6] *In situ* hybridization experiments performed with a digoxigenin-labelled cRNA probe confirmed that PPE mRNA is expressed by many neurons within the RVLM.[6,14,15] A fraction of the C1 cells were found to contain PPE mRNA, consistent with prior evidence that ENK-like IR is present in a small proportion of the C1 cells in colchicine-treated rats.[4,5] However, the number of PPE-positive cells in the RVLM vastly exceeded that of the C1 cells. Using a double *in situ* hybridization method, we found that up to 50% of these non-C1 PPE-positive neurons contain GAD_{67} mRNA and are thus GABAergic.[16]

In order to determine whether some of the PPE-positive neurons of the RVLM project to the thoracic spinal cord, neurons were labelled retrogradely with Fluoro-gold[17] injected bilaterally into segments T2 and T3. Rostral ventrolateral medulla sections were then processed for the simultaneous detection of PPE mRNA, Fluorogold and tyrosine hydroxylase (TH). Fluorogold was consistently detected in RVLM PPE-positive neurons. Almost all PPE-positive C1 neurons were bulbospinal, but the vast majority of bulbospinal C1 cells did not contain PPE mRNA and many RVLM bulbospinal PPE-positive neurons did not exhibit TH immunoreactivity.

To determine whether some of the PPE-positive bulbospinal neurons of the RVLM regulate arterial pressure, we tested whether they express c-Fos in conscious rats subjected to hypotension.[18–20] Fluoro-gold was injected into the upper thoracic cord of rats in order to label bulbospinal RVLM neurons and, 1 week later, animals were given the arterial vasodilator hydralazine intravenously while awake. Hydralazine produced immediate hypotension (mean arterial pressure (MAP) from 120 to 75 mmHg; $n = 7$) and tachycardia (from 370 to 480 b.p.m.) that were maintained throughout the 2 h recording period. Infusion of saline produced no change in MAP or heart rate in a group of control rats ($n = 7$). Hydralazine did not change the number of RVLM neurons that expressed PPE mRNA, but it greatly increased the number of Fos-IR neurons in the RVLM (8.5-fold increase relative to saline control; $P < 0.05$). At the more rostral level of the ventrolateral medulla, a large fraction of bulbospinal PPE-positive neurons were Fos IR ($55 \pm 5\%$) in hydralazine-treated rats, whereas none of these bulbospinal cells had Fos IR in control rats. According to the conventional interpretation of c-Fos data, this experiment suggested that the bulbospinal PPE-positive neurons of the RVLM are excited by hypotension. By extension, the data suggest that these cells are strongly inhibited by arterial baroreceptors under normal conditions, a hallmark of the arterial pressure-regulating presympathetic neurons of the RVLM,[9,10,21]

The next experiments were designed to provide definitive evidence that some of the vasomotor presympathetic cells are PPE positive. We performed extracellular recordings of barosensitive and bulbospinal RVLM neurons in anaesthetized rats[9] and labelled them with biotinamide[22,23] to later determine whether they were PPE positive. We also determined whether some of the recorded neurons were C1 cells,[21] by simultaneously detecting TH IR. Seventeen active and highly barosensitive RVLM bulbospinal neurons were filled with biotinamide in eight chloralose-anaesthetized rats. Ten of the 17 cells (59%) contained PPE mRNA and four of the PPE-positive neurons were TH IR (C1 cells). Most TH-negative bulbospinal and barosensitive cells were PPE positive (6/7), whereas a majority of the C1 cells (6/10) were PPE-negative. The PPE-positive neurons had a significantly higher ($P < 0.01$) axonal conduction velocity than the PPE-negative neurons (3.8 ± 0.6 *vs* 0.86 ± 0.18 m/s, respectively). This difference persisted when the cells were subdivided according to the presence or absence of TH IR. In agreement with our previous findings, the majority of bulbospinal barosensitive neurons devoid of TH IR (5/7) had axonal conduction velocities in the range of 3–6 m/s.[23]

In summary, PPE mRNA is expressed by a significant fraction of RVLM neurons with axonal projections to the thoracic spinal cord. Many of these cells express c-Fos during hydralazine-induced hypotension, suggesting that they are activated by baroreceptor unloading and may be vasomotor presympathetic neurons. This hypothesis was confirmed by identifying the phenotype of active barosensitive and bulbospinal neurons recorded electrophysiologically in anaesthetized rats. Altogether, the evidence demonstrates that PPE mRNA is expressed by RVLM sympathoexcitatory neurons that control highly barosensitive sympathetic efferents. We have not precisely identified the particular sympathetic efferents that are controlled by the PPE-positive RVLM barosensitive cells, but they are likely to include cardiac, visceral or skeletal vasoconstrictor efferents and efferents that control noradrenaline release from the adrenal medulla.

Our recent work[6] also confirms that a group of RVLM C1 cells express the PPE gene. Furthermore, these C1 cells are highly barosensitive and belong to a group of lightly myelinated C1 cells previously shown not to express neuropeptide Y (NPY) mRNA.[24] Thus, PPE and NPY may be mutually exclusive in bulbospinal C1 cells. Finally, this study[6] shows that PPE mRNA is present in a large majority (6/7) of RVLM bulbospinal and barosensitive neurons that are devoid of TH IR. Single-cell labelling experiments revealed that these neurons do not contain GAD_{67} mRNA ($n = 3$; PG Guyenet and RL Stornetta, unpubl. obs., 2000). These neurons may be glutamatergic.[25]

RVLM VASOMOTOR PRESYMPATHETIC CELLS ARE CONTROLLED PRE- AND POST-SYNAPTICALLY BY OPIOID SYNAPSES

Two anatomical studies were performed at the electron microscopic level.[11,12] In each case, we recorded from a single RVLM barosensitive bulbospinal cell in an anaesthetized rat[9] and we labelled

the recorded cell with biotinamide using the juxtacellular labelling method.[22,23] This process was then repeated on the contralateral side. In one study, biotinamide and leu-ENK IR were simultaneously detected by histochemical procedures[12] and, in the other, biotinamide and MOR IR were identified.[11] The tissue was examined at the light and electron microscopic levels.

At the light microscopic level, close appositions between leu-ENK-IR terminals and biotinamide-labelled presympathetic cells were commonly found, especially on cells with lightly myelinated axons (conduction velocity > 3 m/s). Up to several hundred leu-ENK IR close appositions were mapped on some of these biotinamide-labelled cells using computer-assisted reconstruction.[12] At the electron microscopic level, leu-ENK-IR synapses were identified on all cells examined.[12]

μ-Opioid receptor IR (gold particles) was detected on at least some of the processes from each biotinamide-labelled neuron examined ($n = 9$).[11] μ-Opioid receptor IR was located intracellularly and on the plasma membrane. It was also found in a few terminals making synapses onto the biotinamide-labelled neurons.[11]

In summary, the barosensitive presympathetic neurons of the RVLM receive a dense enkephalinergic input. The anatomical origin of this enkephalinergic input is uncertain. By reason of proximity, the numerous PPE-positive neurons that are present in the RVLM may be a source of input to the nearby presympathetic cells. As mentioned above, some but not all, of these PPE-positive cells are GABAergic.[16] Preliminary evidence suggests that in the RVLM synapses leu-ENK IR is colocalized with either GABA or glutamate.[12] Other putative sources of ENK-IR terminals in the RVLM include, but are not limited to, projections from the nucleus tractus solitarius and, possibly, recurrent collaterals of the presympathetic cells.[26] Most, if not all, barosensitive presympathetic neurons also express MOR, consistent with prior evidence that MOR agonists produce post-synaptic inhibition of RVLM bulbospinal neurons by activating a potassium conductance.[13] μ-Opioid receptor IR is also found on inputs to the barosensitive presympathetic neurons. Again, this evidence is consistent with prior electrophysiological evidence for presynaptic inhibition of glutamate release on these cells *in vitro*.[13] In combination, anatomical and electrophysiological evidence strongly suggests that enkephalins are physiological modulators of the activity of the RVLM presympathetic neurons that regulate arterial pressure. There is also good evidence that MOR are involved pre- and post-synaptically; however, δ-opioid receptors are also very probably involved in the presynaptic control of these cells.[27] The presynaptic regulation is complex given that opioid agonists inhibit the release of both glutamate and GABA from different afferents to RVLM presympathetic neurons.[13] Thus, dependent on which input to the cell is active, the presynaptic effect of opioid agonists in the RVLM may cause either disfacilitation or dishinhibition.

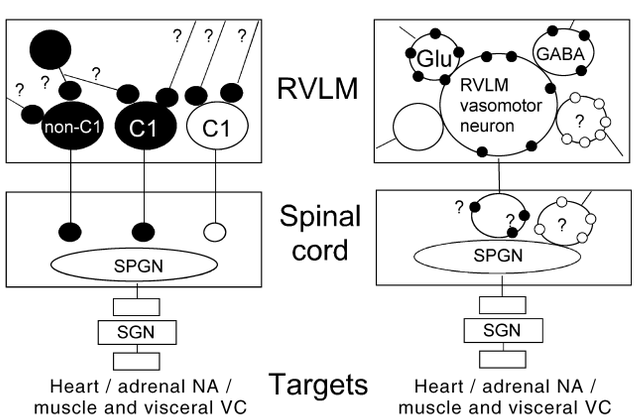

Fig. 1 Sites of enkephalin synthesis or release and location of opioid receptors in the rostral ventrolateral medulla and intermediolateral cell column. (a) Sites of synthesis or release of preproenkephalin (PPE)-derived opioid peptides (composite from previously published data[1–4,6,12]). These sites include a significant portion of the presympathetic neurons (a few C1 and most non-C1 cells) and many of the afferents to the same cells. The origin of these afferents is uncertain (question marks) and may include local GABA interneurons (Pelaez *et al*.[16]). (●), enkephalinergic neuron or terminal. (b) Location of μ-opioid (MOR; ●) and δ-opioid receptors (DOR; ○) in the same system (composite after previously published data[11,13,32,33]). In this portion of the figure, all subtypes of presympathetic cells have been regrouped under the heading of vasomotor neurons. Question marks denote uncertainty as to the presence of the receptor (e.g. MOR in spinal terminals of presympathetic neurons) or major transmitter content of the neuronal elements bearing the receptors (e.g. all terminals bearing DOR). Glu, glutamate; NA, noradrenaline; SGN, sympathetic ganglionic neuron; SPGN, sympathetic preganglionic neuron; VC, vascoconstrictor post-ganglionic neurons.

SUMMARY AND CONCLUSIONS

This brief review, focused on PPE-derived opioids and MOR, illustrates the pervasive nature of opioid signalling within the bulbospinal network that controls sympathetic vasomotor efferents. The most salient conclusions are represented in schematic form in Fig. 1. Added layers of complexity may exist due to the presence in the medulla oblongata of endomorphins,[28] preprodynorphin-derived peptides[29] and a multiplicity of other opioid receptors,[7,27] the synaptic relationships of which with the cells that regulate arterial pressure remain largely unknown. The role of opioids is probably equally complex within other brainstem regions that modulate the sympathetic outflow, such as the nucleus tractus solitarius and the caudal ventrolateral medulla.

From a cell biological point of view, the most intriguing observation is that, at least in the ventrolateral medulla, PPE mRNA and, presumably, the derived peptides are present in both inhibitory GABAergic neurons[16] and in excitatory neurons, such as the putatively glutamatergic presympathetic cells of the RVLM.[25] Because opioid peptides seem to exert uniformly inhibitory effects in the RVLM, both pre- and post-synaptically,[13] their corelease with GABA could be viewed as a mechanism that reinforces the inhibitory effect of the principal transmitter GABA. Opioid peptides may be released in the RVLM by electrical stimulation of the median nerve, a procedure that normally attenuates sympathetic nociceptive reflexes.[30]

The release of opioid peptides by excitatory cells such as the presympathetic neurons of the RVLM poses a more complex interpretative challenge. Given the scant information on the effect of opioids in the intermediolateral cell column (IML),[31] all interpretations must remain speculative. Perhaps the opioids released by RVLM bulbospinal neurons subserve an autoregulatory role (i.e. they limit the release of excitatory transmitter released by these cells in the IML). Although this view is consistent with the presence of MOR in the soma of the presympathetic cells,[11] there is no evidence yet that this[32] or any other opioid receptor is actually present in the terminals of these cells within the IML. Alternatively, the opioids released by RVLM presympathetic cells may inhibit other synaptic

inputs to the preganglionic neurons. This second interpretation is consistent with the presence of presynaptic δ-opioid receptors in the IML.[33] Because these δ-opioid receptors are present neither in TH- nor in ENK-IR terminals,[33] they have to be expressed by cells other than the barosensitive RVLM presympathetic neurons. The third possibility is that some of the preganglionic neurons express opioid receptors, but no conclusive supporting evidence could be found in the literature.[31–33] The physiological conditions that lead RVLM bulbospinal neurons to release enkephalins in the IML are not known. Because intrathecal administration of the opioid receptor antagonist naloxone attenuates the hypotension caused by severe haemorrhage,[34,35] endogenous opioids may be released in the spinal cord under these conditions, conceivably by the RVLM presympathetic neurons. However, other evidence suggests that RVLM presympathetic neurons are inhibited rather than activated during the decompensated phase of haemorrhage.[36] To reconcile the two pieces of evidence, one would have to assume that opioid release from RVLM neurons can be regulated by factors other than action potential frequency.

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