Ph.D. Thesis

Projections of the Ventrolateral Periaqueductal Gray Matter to Various Areas of the Brainstem in Rats

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Introduction

It has been known for more than a quarter of a century that the midbrain periaqueductal gray (PAG) plays a crucial role in endogenous pain attenuation mechanisms of the central nervous system. Independent discoveries demonstrated that electrical or chemical stimulation of the ventrolateral subdivision of the PAG (vl-PAG) suppresses nociceptive reflexes and results in a profound analgesia. These studies also demonstrated that the PAG presents a high degree of anatomical and functional organization. The most important functions that are associated with the PAG such as defensive behaviour, cardiovascular functions or antinociception, are integrated by longitudinal columns of neurons that extend for varying distances along the rostrocaudal axis of the brainstem. From these longitudinally arranged cell assemblies the ventrolateral cell column is associated with pain attenuation mechanisms.

It is postulated that the PAG exerts its powerful inhibition on nociceptive spinal neurons through a disynaptic direct pathway. According to this theory, vl-PAG efferents form monosynaptic contacts with spinally projecting serotonergic and noradrenergic neurons in the RVM and pontine noradrenergic nuclei. The monosynaptically activated descending serotonergic raphe-spinal and noradrenergic coerulo-spinal pathways terminate and release serotonin (5HT) and noradrenaline (NE) in the spinal dorsal horn. The released 5HT and NE then produce a profound inhibition of nociceptive neurons in the spinal dorsal horn, resulting in a powerful attenuation of pain behaviour.

A growing body of experimental evidence, however, suggests that the attractive scheme, which outlines the neural basis of the PAG evoked analgesia as a straightforward, disynaptic pathway may require re-evaluation. It has been reported that after electric stimulation of the nociceptive specific areas of the PAG, no 5HT-like cells were monosynaptically activated in the RVM by single-pulse or train stimulation at antinociceptive intensities. In addition, a number of recent experiments have indicated that a non-serotonergic mechanism must also be involved in the control of dorsal horn nociceptive transmission by the RVM. These experiments suggest that monosynaptic excitation of serotonergic cells in the RVM is unlikely to be necessary for the antinociceptive effects of PAG stimulation.

Furthermore, the exact role played by the pontine noradrenergic nuclei in antinociception is also under doubt. A vast amount of conflicting data from various laboratories delineating the participation of the pontine noradrenergic nuclei in the

endogenous pain attenuating circuitry has been reported. Some reports demonstrated a prominent direct projection, whereas others could detect no significant projections from the PAG to the noradrenergic nuclei, indicating that similarly to serotonergic RVM cells, monosynaptic activation of noradrenergic cells in the pontine noradrenergic nuclei is also unlikely to play a substantial role in the mediation of signals from the vl-PAG to the spinal cord.

Therefore, if monosynaptic excitation from the PAG is not required for the activation of spinally projecting antinociceptive serotonergic and noradrenergic pathways the following questions have to be raised. By which mechanism can the PAG activate the spinally projecting antinociceptive serotonergic and noradrenergic pathways?

Objectives

Using both anatomical and electrophysiological techniques, we intended to investigate further the connection between the vl-PAG and the RVM and pontine noradrenergic nuclei:

- We examined the vl-PAG projections and their distribution within the brainstem, using tract tracers and computer-aided 3D-reconstruction.
- Using immunocytochemical (immunofluorescence, pre- and postembedding) techniques, we examined the immunoreactivity of neurons in various nuclei of the pons and medulla oblongata including the RVM, which received efferents from the vl-PAG, for serotonin, tyrosine hydroxylase, GABA, glycine and preproenkephalin.
- With *in vivo* electrophysiology, we intended to further analyse the response of RVM neurons, depolarisation/hyperpolarisation and response delay time, following electrical stimulation of the vl-PAG and sciatic nerve at noxious intensities.

Material and Methods

Morphological Investigations

Animals, Injection of Tracer, and Preparation of Tissue Sections

Experiments were carried out on Wistar-Kyoto rats, held under deep anaesthesia with sodium pentobarbital (35 mg/kg). The anterograde tracer *Phaseolus vulgaris*-leucoagglutinin (PHA-L; 2.5%), was iontophorically injected, unilaterally into the ventrolateral aspect of the PAG at the following coordinates: 0.6-0.8 mm from the

midline, 1.2-3.2 mm from the interaural line, and 4.6-5.1 mm from the upper surface of the brain. Each animal received two injections in a way that the two sites of injection were in a 0.5-1.0 mm distance from each other in the rostrocaudal direction.

Following a 3 week survival period, the animals were reanaesthetised, and perfused transcardially with Tyrode's solution, followed by one of the following fixatives: (i) 4% paraformaldehyde and 0.2% picric acid in 0.1M phosphate buffer (PB, pH 7.4); (ii) 2.5% glutaraldehyde, 0.5% paraformaldehyde and 0.2 % picric acid in 0.1M PB (pH 7.4); (iii) 4% paraformaldehyde in 0.1M PB (pH 7.4), for immunofluorescence. The brainstem was removed, postfixed in the same fixative for 1-2 hours, and then sectioned at 60 μm on a Vibratome.

Pre-embedding Immunocytochemistry

For immunocytochemical detection of PHA-L, free-floating sections were first incubated with biotinylated goat anti-PHA-L (diluted 1:1000). The sections were then transferred into a solution of avidin-biotinylated peroxidase complex (ABC; diluted 1:100). The immunoreaction was completed with a nickel-intensified diaminobenzidine (DAB) chromogen reaction.

To reveal whether 5HT- and NE-ergic neurons in the brainstem establish close appositions with axon terminals arising from the PAG, a double immunostaining procedure was performed in which the axonal tracing was combined with the immunocytochemical detection of 5HT and tyrosine hydroxylase (TH). First the sections were incubated in a mixture of biotinylated goat anti-PHA-L and rabbit anti-5HT (diluted 1:2000) or rabbit anti-TH (diluted 1:1000). Subsequently the sections were transferred into a mixture of ABC and goat anti-rabbit IgG (diluted 1:200). The PHA-L-labelled axons and axon terminals were visualized with a nickel-enhanced DAB chromogen reaction. The sections were then treated with a rabbit peroxidase anti-peroxidase complex (diluted 1:100), and the immunostaining for 5HT or TH was completed with a chromogen reaction using DAB alone. Sections were mounted on gelatin-coated slides and cover with Permount neutral medium.

Post-embedding Immunocytochemistry

Alternate sections were treated with 1% OsO_4, then dehydrated and flat embedded into Durcupan ACM resin on glass slides. Selected areas from the intermediate subdivision of the ponto-bulbar reticular formation (iRF), which contained

a high density of PHA-L labelled terminals, were re-embedded and serial ultrathin section were cut and collected on Formvar coated single-slot nickel grids.

Sections on consecutive grids were processed for GABA and glycine immunocytochemistry. Following incubations with anti-GABA (diluted 1:1000; Dr. J. Storm-Mathisen) or anti-glycine (diluted 1:2000; Dr. J. Storm-Mathisen) antisera, the sections were exposed to goat anti-rabbit IgG coupled to 20 nm colloidal gold particles (diluted 1:200). The sections were then counterstained with uranyl acetate and lead citrate.

Immunofluorescence and Confocal Microscopy

Free-floating sections of the brainstem were first incubated a solution containing the following primary antibodies: (i) biotinylated goat anti-PHA-L (diluted 1:500); (ii) mouse anti-glutamic acid decarboxylase (GAD; diluted 1:1000); (iii) rabbit anti-preproenkephalin (PPE; diluted 1:500; Dr. T. Kaneko). The sections were then incubated in a mixture containing Streptavidin-Alexa-Fluor 546 conjugate (diluted 1:2000), goat-anti-rabbit IgG-Alexa-Fluor 633 conjugate (diluted 1:500), and goat-anti-mouse IgG-Alexa-Fluor 488 conjugate (diluted 1:500). The sections were then mounted between two glass cover slips in a glycerol-based anti-fade medium. The sections were examined with a confocal laser-scanning microscope.

Three-Dimensional Reconstruction of the Distribution of Anterogradely Labelled Axon Terminals and Immunostained Neurons

The distribution of PHA-L labelled axon terminals, and 5HT-immunoreactive (SIR) and TH- immunoreactive (THIR) neurons in the brainstem were investigated in serial sections. Keeping their consecutive order, every fourth of the sections were double stained for PHA-L and 5HT, whereas the consecutive ones were reacted for PHA-L and TH. By using the NEUROLUCIDA 3-D reconstruction system, the contours of these sections and the coordinates marking the location of the anterogradely labelled axon terminals as well as SIR and THIR neurons were fed into a computer. The number of labelled axon terminals and SIR and THIR neurons were counted. Close appositions between labelled axon terminals and immunostained neurons were also evaluated.

Physiological Investigations

Experimental Animals and Surgical Procedure

The experiments were carried out on anaesthetized and artificially ventilated male Sprague-Dawley rats, placed into a stereotaxic frame. The left sciatic nerve was dissected and prepared for electrical stimulation with bipolar hook electrodes. Following interparietal and occipital craniotomy, the cerebellar lobules were removed by suction in order to aid the positioning of microelectrodes into the right vl-PAG and the RVM.

Intracellular RVM Neuronal Recordings

Intracellular recordings were obtained from neurons in the RVM at the following coordinates 0.1-1.2 mm lateral to the midline, 3.0-4.0 mm rostral to the obex and 2.7-3.2 mm below the dorsal surface of the medulla. The recordings were made using single-barrel glass microelectrodes filled with a solution that contained 3% biocytin. Intracellular signals were recorded using an active bridge circuit and data analysis was carried out off-line using a digital data acquisition system and Spike2 and SigAvg software packages. Statistical differences between numerical data were determined using Mann-Whitney tests as appropriate. All data are expressed as means $\pm$ S.E.M. Mean values were considered significantly different if $P < 0.05$.

PAG Stimulation

In all experiments, bipolar stainless steel stimulating electrodes were positioned in the vl-PAG at the following coordinates 0.5-0.8 mm lateral to the midline, 0.7-1.4 mm rostral to the interaural line and 5.5-6.0 mm ventral to the dorsal surface of the brain. The position of the electrode within the vl-PAG was confirmed by eliciting a hypotensive response using a short train of stimulus. During intracellular recording from RVM neurons, a single pulse electrical stimulation of 100-300 μA was applied.

Peripheral Nerve Stimulation

The sciatic nerve was stimulated using bipolar silver wire electrodes. Following previously established methods, the intensity of sciatic nerve stimulation was set to 2 μA to activate both myelinated and non-myelinated fibres. During intracellular recordings, 0.2 ms wide, single pulse stimuli were delivered at 1 Hz.

Histological Processing of the Intracellularly Labelled Neurons

Following electrophysiological characterization of the recorded RVM neuron, biocytin was injected intracellularly, the animals were then perfused transcardially with normal saline, followed by a mixture of 4% paraformaldehyde and 2% glutaraldehyde in 0.1M PB (pH 7.4). Serial coronal sections of the medulla oblongata were then cut on a Vibratome at a thickness of 40 μm. The sections were then incubated with ABC. The labelled cells were visualized using a DAB stabilized TMB reaction. Reconstruction of the labelled neurons was performed with the aid of a NEUROLUCIDA 3-D reconstruction system.

Results

Distribution of PHA-L Labelled Axon Terminals in the Pons and Medulla Oblongata

Following unilateral PHA-L injections into the PAG, immunostained axons and varicose axon terminals were widely scattered in the pons and medulla oblongata. The number of axon varicosities found on the ipsilateral side always outnumbered those that were recovered contralaterally. The distribution of the terminals, were very similar in all the animals investigated in this study. Three animals were selected at random for quantitative studies and 3-dimensional reconstruction.

In the rostral pons, most of the labelled axon terminals were observed in the noradrenergic cell groups. With the labelling density being highest in the ventral and dorsal subdivisions of the locus subcoeruleus and locus coeruleus, and lower in the alpha subdivision of the locus subcoeruleus and A5 cell group. Also, a moderate density of varicose fibres was seen in the parabrachial nucleus, laterodorsal tegmental nucleus, pontine reticular nucleus, motor and principal sensory nuclei of the trigeminal nerve, and superior olive.

In the caudal pons and rostral medulla oblongata, a substantial number of terminals were recovered in the RVM. In addition to the RVM, the intermediate reticular nucleus was also densely packed with labelled terminals, and a moderate number of axon varicosities were also seen in the parvocellular reticular nucleus and the motor nucleus of the facial nerve.

In the caudal medulla, labelled terminals were seen in almost equal densities in the following nuclei: nucleus of the solitary tract, nucleus of the Probst bundle, nucleus

ambiguous, nucleus retroambiguous, ventrolateral reticular nucleus, dorsal and ventral medullary reticular fields.

Relationship Between PHA-L Labelled Axon Terminals and SIR Neurons

Evaluation of sections double stained for PHA-L and 5HT showed that SIR neurons in the brainstem received close appositions from terminals of PAG efferents only in moderate numbers. Most of the 5HT-containing neurons that established close appositions with labelled terminals were seen within the confines of the RVM, but some of them were found also in the rostral pons and caudal medulla oblongata. Nine to twenty percent of the labelled terminals were found within the confines of the RVM, and even here most of these terminals were distributed in non-SIR territories. Only 0.8%, 1.0% and 0.7% of the total number of terminals were apposed to SIR neurons of the RVM.

Relationship Between PHA-L Labelled Axon Terminals and THIR Neurons

Close appositions between PHA-L labelled PAG efferents and neurons immunoreactive for TH were only occasionally found in the pons and medulla oblongata. Most of the THIR neurons that established close appositions with labelled terminals were seen in the ventral subdivision of the locus subcoeruleus and the A5 cell group. Ten to fourteen percent of the labelled terminals were found within the confines of the pontine noradrenergic cell groups (locus coeruleus, locus subcoeruleus and A5 cell group), where most of the terminals were distributed in non-THIR territories. Only 0.5%, 0.6% and 0.4% of the total number of terminals established close appositions with THIR neurons of the pontine noradrenergic cell groups in the individual animals.

Neurochemical Profile of the Postsynaptic Targets of the vl-PAG Terminals in the Intermediate Subdivision of the Pontomedullary Reticular Formation

Our anatomical studies showed that the iRF among the brainstem nuclei receives the most extensive innervation from the vl-PAG. Since this area have been shown to play a role in pain behaviour, the possibility that the iRF might be an intermediate relay station between the vl-PAG and the RVM was raised. Thus we examined the vl-PAG terminals and their postsynaptic targets within the iRF at the electron microscopic level. They were processed for the postembedding immunogold procedure to determine their GABA and glycine immunoreactivity. None of the PHA-L labelled vl-PAG terminals,

nor their postsynaptic targets that were examined displayed any immunoreactivity for GABA or glycine.

Using immunofluorescence and confocal microscopy we examined further the neurochemical profile of the postsynaptic targets of the vl-PAG terminals within the iRF for PPE and GAD (an enzyme needed for the biosynthesis of GABA). Confirming our electron microscopic results, none of the labelled vl-PAG terminals were seen to form close appositions with GAD immunoreactive elements. However, a number of PHA-L labelled vl-PAG axon terminals were seen to make close appositions with PPE positive neurons and dendrites within the Rgc and the dorsal medullary reticular formation.

In vivo Intracellular RVM Neuronal Recordings

Intracellular recordings were obtained from 14 RVM neurons that responded to stimulation of the contralateral sciatic nerve. Action potentials could be elicited from all of them by either stimulation of the sciatic nerve or the vl-PAG. Of the 14 RVM neurons, 8 were excited and 6 were inhibited by single pulse electrical stimulation of the sciatic nerve. The average onset latencies of sciatic excitatory and inhibitory postsynaptic potentials were similar, 17.4 $\pm$ 3 and 13.3 $\pm$ 1.4 ms, respectively ($p < 0.28$). The excitatory responses had an average duration of 29.5 $\pm$ 5.6 ms, while the inhibitory responses were longer, 78.5 $\pm$ 22 ms. Responses to mechanical stimulation of the hind limb were also tested in 10 neurons using painful pinch of the left hind limb and/or toes. The responses evoked by noxious mechanical stimulus corresponded to those evoked by electrical stimulation of the sciatic nerve.

Stimulation of the Ventrolateral PAG

Stimulation of the vl-PAG excited 10 neurons (71%), elicited no visible response in 3 neurons (21%) and inhibited only 1 (7%). Regarding their onset latencies, the excitatory responses varied in a wider range and showed a strong correlation with the types of responses evoked by the stimulation of the sciatic nerve. Five neurons that were excited by sciatic nerve stimulation, received a fast excitatory input from the vl-PAG; the onset latency ranged from 1.8 to 6.9 ms (3.6 $\pm$ 0.9 ms average), with an average duration of 6.2 $\pm$ 1.5 ms. In the other 5 neurons the onset latency was much longer and ranged from 10.4 to 26.4 ms (14.8 $\pm$ 3 ms average; $p < 0.004$), with an

average duration of 17.5 $\pm$ 3.4 ms. All 5 neurons with delayed excitatory input from the vl-PAG were inhibited by the sciatic nerve stimulation.

Location and Morphology of the Intracellularly Labelled RVM Cells

The location and morphology of all 11 neurons receiving both sciatic and PAG inputs were identified. All labelled neurons were within the confines of the RVM. The orientation, distribution, size and shapes of the cells and their dendrites were similar to those reported in previous intracellular studies of the RVM.

Discussion

The Termination Pattern of Ventrolateral PAG Efferents in the Pons and Medulla Oblongata

The termination pattern of efferent fibres arising from the PAG has been extensively investigated most of the results obtained in the present experiment are in general agreement with the findings of previous observations. However, here we also presented data that advanced our understanding of possible functional links between the PAG and RVM, pontine noradrenergic nuclei as well as other territories of the brainstem. We have shown that in contrast to previous results PAG efferents make relatively few appositions with SIR and THIR neurons in the RVM and pontine noradrenergic nuclei, most of them terminate in non-SIR and non-THIR territories. We have also demonstrated that 20-30% of axon terminals arising from the vl-PAG project to the intermediate subdivision of the ponto-bulbar reticular formation, suggesting that efferent fibres to this area of the reticular formation may represent a functionally very important part of the projection system of the vl-PAG.

There is general agreement in the literature, that the vl-PAG projects to the RVM and pontine noradrenergic cell groups, through which it exerts profound effects on somatomotor, cardiovascular and nociceptive information processing mechanisms of the spinal cord. It has also been demonstrated, however, that the termination patterns of PAG efferents within the confines of the RVM and especially the noradrenergic nuclei show a wide variety among the different strains of rats. In the light of this, our present findings are quite unique since to our best knowledge this is the first account on this matter in Wistar-Kyoto rats. On the one hand, we found that the vl-PAG projects strongly to the ventral and dorsal subdivisions of the locus subcoeruleus and the locus

coeruleus, whereas the alpha subdivision of the locus subcoeruleus and A5 cell group is supplied weakly by terminals of the vl-PAG in Wistar-Kyoto rats. On the other hand, within the RVM most of the terminals were recovered in the n. reticularis gigantocellularis pars-α and n. reticularis paragigantocellularis lateralis, whereas the n. raphe magnus and dorsal paragigantocellular nuclei were supplied only by a moderate number of terminals.

According to the most widely accepted theory most of the PAG efferents that project to the RVM and pontine noradrenergic cell groups form monosynaptic contacts with spinally projecting 5HT- and NE-ergic neurons and excite them through NMDA and AMPA receptor mechanisms. However, its been shown, that in addition to 5HT-ergic neurons non-5HT-ergic cells in the RVM also receive monosynaptic inputs from the PAG. In addition, a recent study, in which the effects of the PAG stimulation was tested on RVM neurons provided little evidence for the existence of monosynaptic connections between PAG and 5HT-ergic-like cells in the RVM. No SIR cells were activated by single pulse or train stimulation of the PAG at antinociceptive intensities. The results of the present experiment appear to be in a good agreement with these findings. We have observed most of the PAG efferents in non-SIR territories of the RVM, and only less then 10% of the labelled terminals recovered within the confines of the RVM were seen to establish close appositions with SIR neurons. This suggests that monosynaptic activation of serotonergic cells in the RVM is unlikely to be necessary for the nociceptive modulatory effects of PAG stimulation, at least in Wistar-Kyoto rats.

We have also demonstrated that the projection of the vl-PAG to pontine noradrenergic cell groups and THIR cells is even weaker than that to the RVM and SIR neurons. As we found, less than 5% of the PAG efferents that terminate in the pontine noradrenergic cell groups may establish close appositions with THIR, presumably noradrenergic neurons. Confirming our results, several other reports did neither find any significant projections from the vl-PAG to locus coeruleus in the rat. This indicate that similarly to serotonergic cells in the RVM, monosynaptic activation of noradrenergic cells in the pontine noradrenergic cell groups is also unlikely to play a substantial role in the mediation of signals from the vl-PAG to the spinal cord.

Although the illustration of previous reports unequivocally show that in addition to the nuclei discussed above the PAG projects also to various territories of the ponto-bulbar reticular formation, not too much attention has been paid to these areas till now. In contrast to this, here we would like to emphasis that according to our findings the vl-

PAG sends a substantial projection to the ponto-medullary intermediate reticular nucleus, and also innervates the pontine reticular nucleus, the parvocellular reticular nucleus of the pons and the dorsal medullary reticular field. Since we found 20-30% of the PHA-L labelled axon terminals in these reticular fields, it appears to be highly probable that efferent fibres to these areas of the ponto-bulbar reticular formation may represent a functionally very important part of the projection system of the vl-PAG. These areas are known to have a role in pain attenuation and give projections to the RVM and pontine noradrenergic cell groups. These observations suggest that the areas of the ponto-bulbar reticular formation that receive direct inputs from the vl-PAG might turn out to be organic parts of the descending somatomotor, cardiovascular and antinociceptive pathways. Volleys arising from the vl-PAG might activate the neural circuits of these areas, and in case of suprathreshold activation they may influence the spinal somatomotor, cardiovascular and nociceptive apparatus either directly by sending descending fibres to the spinal cord or indirectly by activating neural circuits in the RVM.

In vivo Intracellular Investigation of Ventrolateral PAG Evoked Postsynaptic Responses in RVM Neurons

Our morphological results, clearly demonstrated that only some of the vl-PAG efferents terminate in the RVM, most of them project to the iRF. This, and recent reports that electrical stimulation of the vl-PAG does not lead to monosynaptic activation of spinally projecting RVM serotoninergic neurons, raised the possibility that the vl-PAG activates a complex pontobulbar neuronal assembly that may send both mono-, di- and polysynaptic inputs to the RVM.

Using in vivo intracellular recording, we studied the vl-PAG evoked postsynaptic responses in RVM neurons with somatosensory input. Over 90% of neurons that responded to PAG activation had an excitatory response to the vl-PAG stimulation. Based on the range of onset latencies of the vl-PAG evoked responses, we divided the recorded RVM cells into two groups. One group had an early and the other a delayed response to vl-PAG stimulation. Neurons in the different groups also differed in their responses to electrical stimulation of the sciatic nerve. Neurons that exhibited short latency vl-PAG responses were all excited by stimulation of the sciatic nerve at nociceptive intensities. In contrast to this, neurons that exhibited longer latency vl-PAG responses were all inhibited by sciatic nerve stimulation. The difference in the onset

latencies of the vl-PAG evoked responses between the two subgroups is consistent with the existence of a monosynaptic and a di- or polysynaptic PAG-RVM pathways.

Ventrolateral PAG Evoked Short Onset Latency Excitatory Responses in RVM Neurons

It is quite interesting that, all RVM neurons that received short onset latency inputs from the vl-PAG, responded with excitation to sciatic nerve stimulation and noxious pinch of the hind limb, a behaviour characteristic of ON cells. This finding strongly suggests that the monosynaptic vl-PAG input is mostly, if not exclusively, received by ON cells in the RVM.

Ventrolateral PAG Evoked Long Onset Latency Excitatory Responses in RVM Neurons

Some RVM neurons responded to vl-PAG stimulation with an onset latency that was nearly four times longer than that of other recorded neurons. This longer onset latency suggests that the vl-PAG-RVM projection is complex and in addition to a monosynaptic projection may also include a di- or polysynaptic pathway.

RVM neurons with short onset latency responses to vl-PAG stimulation displayed excitatory responses to sciatic nerve stimulation. In contrast to this, RVM neurons that showed long onset latency responses to vl-PAG stimulation, were all inhibited by electric stimulation of the sciatic nerve and noxious pinch of the hind limb, a behaviour associated with OFF cells. The finding that cells with different response properties to sciatic nerve stimulation proved to be also different in regard to the way of how they receive inputs from the vl-PAG suggests that these two cell groups may represent functionally different populations of RVM neurons.

It has been, postulated that OFF cells are activated through a disinhibitory mechanism. It is highly conceivable that the long latency vl-PAG input may be the one that utilises this disinhibitory pathway to activate RVM neurons. Projection fibres arising from the vl-PAG may excite enkephalinergic neurons either in the reticular formation or within the RVM, and the activated enkephalinergic neurons will inhibit GABAergic neurons that keep the RVM cells under tonic inhibition. The release of the GABAergic tonic inhibition will then result in the increased activity of the OFF cells.

The Postsynaptic Targets of the Ventrolateral PAG Efferents in the Intermediate Pontomedullary Reticular Formation

In our anatomical studies, we found that the iRF receives a very extensive projection from the vl-PAG. Thus, considering its connection with the RVM and the pontine noradrenergic cell group, the possibility of the iRF being a relay station, through which the vl-PAG may modulate the neuronal activity within the RVM and the noradrenergic nuclei was raised.

RVM ON cells facilitate and OFF cells inhibit nociceptive transmission in the dorsal horn. These two cell populations tend to fire reciprocally under most conditions, when one group is active the other is silent. Experimental studies indicate that activation of a GABA containing input is responsible for the OFF cell pause. Considering the reciprocal firing patterns of the two groups, it has been proposed that a subset of ON cells are GABAergic interneurons responsible for the inhibition of OFF cells. It is also possible that the GABA containing cells that mediate the OFF cell pause are not intrinsic but extrinsic to the RVM. However, our results indicate that this is unlikely since the postsynaptic targets of the vl-PAG axon terminals within the iRF turned out to be negative for the inhibitory amino acids, GABA and glycine.

In addition to GABA, opioids may also inhibit OFF cells. However, just to the contrary, it has widely been demonstrated that RVM OFF cells are activated by opioids, while ON cells are inhibited. It is likely that the activation of OFF cells by opioids is an indirect, secondary effect by inhibition of an inhibitory input, most likely a GABAergic input to OFF cells. The RVM contains a high density of ENK immunoreactive axon terminals that are received mostly by ON cells. Thus it appears to be likely that OFF cells in the RVM are activated by an ENK-GABA mediated disinhibition. ENK containing neurons that are located outside the confines of the RVM might project to GABAergic ON cells that in turn project to OFF cells. ENK-ergic inhibition of GABAergic ON cells, thus may lead to disinhibition, that is excitation of OFF cells. The ENK-ergic neurons that are involved in this mechanism may be located in the iRF since we found the PPE immunoreactive neurons in the iRF to receive a massive innervation from the vl-PAG. Thus it is likely that the iRF can be regarded as a relay station between the vl-PAG and the RVM that may modulate nociceptive information processing and pain behaviour, at least partly through disinhibitory mechanisms.

Conclusion

Our results suggest that neural activities of the vl-PAG may influence the nociceptive information processing mechanisms of the spinal cord through an intricate interneuronal circuit. Most probably signals from the vl-PAG substantially influence the excitation level of the medial subdivision of the ponto-medullary reticular formation including the intermediate reticular nucleus, pontine reticular nucleus, parvocellular reticular nucleus of the pons and the dorsal medullary reticular field. Here the PAG signals may activate neural circuits that forward neural activities towards the spinal cord or more likely towards the RVM and pontine noradrenergic cell groups. A second group of PAG efferents terminate within the RVM and pontine noradrenergic cell groups on non-serotonergic and non-noradrenergic neurons. Some of these neurons may represent cells of origin of raphespinal and coerulospinal pathways, others might be local interneurons. The activated interneural circuits presumably further process the incoming volleys, and then transmit the signals to spinally projecting neurons. A third group of efferent fibres may establish monosynaptic contacts with serotonergic and noradrenergic neurons among which there might be some spinally projecting cells. The spinally projecting neurons, both serotonergic and noradrenergic as well as non-serotonergic and non-noradrenergic, then presumably integrate the monosynaptic inputs and signals coming from the activated intra- and extranuclear interneural circuits, and in case of suprathreshold activation they may conduct volleys to the spinal dorsal horn. In the superficial dorsal horn, the terminals of the descending fibres may release 5HT, NE, GABA, glycine and various neuropeptides including ENK, and the released neurotransmitters may evoke inhibition in spinal neural circuits underlying nociceptive information processing, that results in analgesia and attenuation of pain behaviour.

Summary

1. Direct monosynaptic inputs from the vl-PAG to the RVM are not as strong as previously reported. Only 10-20% of the vl-PAG efferents terminate within the confines of the RVM, and only less than 5-10% may make direct contacts with SIR neurons in the RVM.

2. In contrast to previous reports, we have demonstrated that the A5, A6 and A7 noradrenergic cell groups receive a weak projection form the vl-PAG. Only 10-14%

of the vl-PAG efferents are located within the confines of the pontine noradrenergic cell groups, and only less than 5% establish contacts with THIR neurons.

3. We showed that the intermediate subdivision of the pontomedullary reticular formation, an area known to play a role in pain behaviour and project to the RVM and pontine noradrenergic nuclei, receives a substantially large proportion of the vl-PAG efferents. Thus, the possibility of the iRF being a part of an alternative di- and/or polysynaptic pathway between the vl-PAG and the RVM and pontine noradrenergic nuclei, was raised.
4. We have demonstrated that RVM neurons receive very heterogeneous but predominantly excitatory inputs from the vl-PAG. This supports our anatomical results, indicating the presence of an alternative di- and/or polysynaptic pathway between the vl-PAG and the RVM. ON cells may receive direct monosynaptic inputs from the vl-PAG, whereas OFF cells may be innervated indirectly (di- or polysynaptically) by the vl-PAG.
5. Our data support the idea that the iRF may be a relay station through which the vl-PAG may modulate the neuronal activity within the RVM and pontine noradrenergic nuclei, partly through disinhibitory mechanisms.

List of Publications

Peer-reviewed articles related to this study

- **Odeh F.** and Antal M.: The projections of the midbrain periaqueductal gray to the pons and medulla oblongata in rats, Eur. J. Neurosci. 14, 1275-1286, **2001**, **IF: 3.919**
- **Odeh, F.**, Antal, M. and Zagon, A.: Heterogeneous Synaptic Inputs from the Ventrolateral Periaqueductal Gray Matter to Neurons Responding to Somatosensory stimuli in the Rostral Ventromedial Medulla of Rats, Brain Res., (in press) **2002**, **IF: 2.489**

Other peer-reviewed articles

- Szűcs P., **Odeh F.**, Szokol K. and Antal M.: Neurons with distinctive firing patterns, morphology and distribution in laminae V-VII of the neonatal rat lumbar spinal cord, Eur. J. Neurosci. (in press) **2002**, **IF: 3.919**

Abstracts

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