Ph.D. Dissertation

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

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Contents

Abbreviations

5HT : Serotonin
ABC : Avidin-biotinylated peroxidase complex
CNS : Central nervous system
DAB : Diaminobenzidine
DLF : Dorsal lateral funiculus of the spinal cord
EAA : Excitatory amino acids
ENK : Enkephalin
GABA : γ-Aminobutyric acid
GAD : Glutamic acid decarboxylase
iRF : Intermediate subdivision of the ponto-bulbar reticular formation
NE : Norepinephrine
NRM : Nucleus raphe magnus
PAG : Midbrain periaqueductal gray matter
PB : Phosphate buffer
PBS : Phosphate buffered saline
PHA-L : Phaseolus vulgaris-leucoagglutinin
PPE : Preproenkephalin
Rgc : Nucleus reticularis gigantocellularis
Rgcα : Nucleus reticularis gigantocellularis pars alpha
Rmc : Nucleus reticularis magnocellularis
Rpg : Nucleus reticularis paragigantocellularis
Rpgl : Nucleus reticularis paragigantocellularis lateralis
RVM : Rostral ventromedial medulla
SIR : Serotonin immunoreactive
SPA : Stimulation produced analgesia
TH : Tyrosine hydroxylase
THIR : Tyrosine hydroxylase immunoreactive
TMB : Tetramethylbenzidine
vl-PAG : Ventrolateral periaqueductal gray matter

1. 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 [65, 124, 165]. 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 [68, 78, 115, 189]. 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 [12]. 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 [11, 31, 110, 159]. 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 [6, 15, 39, 41, 53, 100, 124]. 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 [68]. 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 [28, 173, 203]. 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 [11, 42, 55, 142, 183]. Some reports demonstrated a prominent direct projection [11], whereas others could detect no significant projections from the PAG to the noradrenergic nuclei [55, 183], 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?

2. Literature Review

2.1 Endogenous Pain Control System

The perception of pain is evoked by stimuli that are sufficient or nearly sufficient to produce tissue damage. At least in human psychophysical studies, there is a direct relationship between the stimulus intensity and the reported pain intensity. In addition, factors such as arousal, attention and emotional stress profoundly alter responses to noxious stimuli. For example, traumatic injuries sustained during athletic competitions or combat, are often initially reported as being relatively painless [132]. In other circumstances, similar injuries are extremely painful. The weight of evidence indicates that changes in pain responses due to arousal, attention and stress, which clearly involve CNS mechanisms, result from the action of modulatory networks that control the transmission of nociceptive messages in the CNS. An anatomically distinct and physiologically selective CNS pain modulatory network has been identified. This network has links in the hypothalamus and brainstem, controls nociceptive dorsal horn neurons and is sensitive to opioids.

As early as 1911, Head and Holmes explicitly postulated a modulatory influence on pain. They hypothesised that modulation of pain is a necessary part of the on-going process of discriminative sensation. However, the first real description of a specific pain modulatory system was clearly defined in 1965 by Melzack and Wall in the “Gate control theory of pain”. Where supraspinal influences on the “gate” was proposed, but there was limited evidence for the existence of a descending control of nociception.

The hypothesis that descending systems contribute to pain modulation was later strongly supported by the discovery of the phenomenon of “stimulation produced analgesia” (SPA) [128, 160]. SPA is a highly specific suppression of responses to noxious stimuli produced by electrical stimulation of discrete brain sites, such as the periventricular gray or PAG. During SPA, animals remain alert and active, although their responses to most environmental stimuli are unchanged, responses to noxious stimuli such as orientation, vocalisation, escape and defecation are absent. Thus, demonstrating that in animals SPA is a powerful, highly selective and robust phenomenon. More importantly, SPA is associated with the inhibition of reflex responses to noxious stimulation, such as the tail flick, which are mediated by intraspinal circuitry. This implicates the antinociceptive effect following PAG stimulation on descending pathways to the spinal cord [65].

The significance of SPA was confirmed by its demonstration in human subjects with chronic pain [27]. The specificity of the analgesia and the fact that it is consistently elicited from discrete brain sites that are homologous in a variety of species including humans is a powerful evidence for a specific endogenous pain-modulating system.

2.2 Components of the Endogenous Pain Control System

The Midbrain Periaqueductal Gray Matter

The PAG was the first brainstem region to be implicated in pain modulation [65, 124, 127, 160, 165]. Microinjection of opiates into or electrical stimulation of the PAG generates analgesia (via a pathway in the DLF) and inhibits the firing of the dorsal horn neurons [68, 78, 113, 115, 189]. This is consistent with the view that opiate analgesia and SPA operate via a common neural mechanism [128]. Experimental studies have demonstrated that the PAG is pivotally located to transmit (relay) cortical and diencephalic inputs to the lower brainstem.

The PAG is a cytoarchitectonically and chemically heterogeneous region, with a high degree of anatomical and functional organisation. Some of the important functions that are classically associated with the PAG, such as defensive reactions, analgesia and autonomic regulation, are integrated by overlapping longitudinal columns of neurons extending for varying distances along its rostrocaudal axis. Different classes of threatening or nociceptive stimuli trigger distinct coordinated patterns of skeletal, autonomic and antinociceptive adjustments by selectively targeting specific PAG columnar circuits [12]. The lateral and ventrolateral columns are in particular associated with antinociception. Stimulation of the lateral PAG column results in a defensive behaviour accompanied with hypertension, tachycardia and antinociception that is associated with strong emotional reactions (such as vocalisation, jumping and running). On the other hand, stimulation of the vl-PAG causes a cessation of on-going spontaneous activity (quiescence), profound hyporeactivity, hypotension and bradycardia. The animal neither orienting nor responding to its environment a behaviour usually seen in an animal following injury or social defeat. Unlike the lateral column, which is associated with cutaneous pain, the vl-PAG responds to deep somatic and visceral pain [104]. In contrast to superficial painful sensations, deep pain is both inescapable and usually impossible to control. A quiescent and hyporeactive reaction such as that mediated by the vl-PAG might represent one way for the animal to reduce

discomfort and limit interactions, which might increase pain [12]. Analgesia generated by the vl-PAG is regarded as the most effective [69], since the vl-PAG is the only PAG region that produces opioid sensitive analgesia [12].

Inputs to the PAG from more rostral brain centres are apparently critical for initiating the powerful descending control systems that act on spinal nociceptive neurons. The hypothalamus is a major source of afferents to the PAG [21]. Other important forebrain inputs arise from the frontal granular and insular cortex [21, 83], and the amygdala [79]. The vast majority of these inputs to the PAG utilise opioids as neurotransmitters, β-endorphin and enkephalin [65, 150]. Since excitation of the PAG output neurons is required to initiate descending control, it follows that opioids do not act directly upon the PAG output neurons. Given that the vast majority of enkephalin immunoreactive terminals in the PAG are presynaptic to dendrites [139], it was proposed by Fields H. (1984) that endogenous opioid peptides (ENK, dynorphin, β-endorphin) activate PAG output neurons by inhibiting inhibitory interneurons. A similar model has been proposed to account for the opiate excitatory effects on hippocampal pyramidal cells [141]. In the vl-PAG, μ-opioid receptor ligands are thought to act primarily on inhibitory (GABAergic) neurons to disinhibit PAG output rather than directly on medullary projecting PAG neurons [45, 147, 174]. Its been established that unlike 5HT and neurotensin neurons of the PAG, enkephalinergic neurons do not project to the medulla. This is consistent with the idea that they are interneurons that modulate projection neurons [22].

The PAG also receives diverse inputs from the brainstem. The majority of these inputs are derived from the adjacent nucleus cuneiformis, solitary tract, the pontomedullary reticular formation, the locus coeruleus, and other brainstem catecholaminergic nuclei [89]. In addition to the known direct spinal input to the PAG [24, 96], these regions probably provide a relay for the nociceptive input that activates PAG neurons. The locus coeruleus projection is of interest since it may contribute to the known norepinephrine antagonism of opiate and SPA [4]. The PAG is also reciprocally connected with neurons in the RVM [1, 21, 110, 118] that give rise to the bulk of pain modulatory fibres that project directly to the spinal dorsal horn.

Electrical stimulation in the PAG evokes excitation in many RVM neurons [119, 143, 154, 184]. Evidence that this effect is mediated at least in part by PAG neurons that

project to the RVM and utilise neurotensin and excitatory amino acids (EAA, glutamate and/or aspartate) as transmitters has been shown [2, 23, 194].

The ambiguous and periambigual region of the lateral medulla, and the caudal medulla receive a discrete projection from the vl-PAG, through which it may mediate, in part, some of the cardiovascular adjustments and vocalisation produced following stimulation of the PAG [31, 56, 88].

The Rostral Ventromedial Medulla

The RVM consists of the nucleus raphe magnus (NRM) and the adjacent reticular formation, including the nucleus reticularis paragigantocellularis (Rpg), the nucleus reticularis gigantocellularis pars α (Rgcα) and the nucleus reticularis paragigantocellularis lateralis (Rpgl) [15] and extends from the caudal pole of the facial nucleus to the level of the trapezoid body [15]. Cells throughout these nuclei are involved in pain modulation [21, 70, 118, 124, 136, 208].

The PAG and adjacent nucleus cuneiformis are the major sources of inputs to the RVM [1, 21, 22, 23, 31, 110]. Since the PAG has minimal direct projections to the spinal cord, the RVM is considered a necessary "relay" for the modulatory influences of the PAG upon the spinal nociceptive transmission [2, 15, 63, 158, 167, 182]. This hypothesis is supported by the observations that lesions of or local anaesthetic injections into the RVM abolish the analgesia produced by stimulation of PAG, [2, 20]. Electrical [61, 144] or chemical [19, 20] stimulation of the PAG that produce concomitant analgesia also evoke activation of putative pain inhibitory neurons in RVM [38]. Direct spinal projections to the RVM are sparse, but the RVM does receive a projection from the adjacent medullary nucleus reticularis gigantocellularis (Rgc) that in turn receives a large projection from nociceptive spinoreticular neurons [59].

The RVM consists of a very heterogeneous cell population, both in their neurochemical profile and physiological role. Among these, are neurons that contain 5HT, GABA, ENK, substance P, somatostatin and thyrotrophin-releasing hormones, all of which have been shown to project to the spinal cord [9, 28, 65, 140]. Three physiologically distinct classes of neurons (ON, OFF and Neutral) are identified within the RVM based upon changes in their firing pattern at stimulation evoked nociceptive reflexes (e.g. tail flick reflex) [63, 64, 65, 124]. ON cells are consistently excited, OFF cells inhibited and Neutral cells show no response associated with "tail flick" reflex

evoked by noxious stimuli applied over most of the body surface. Both ON and OFF cells are excited by electrical stimulation of the PAG [143, 185]. However, only OFF cells are excited by morphine administration [38, 62, 63]. Thus the activity of the OFF cells is most consistently related to suppression of nociceptive transmission. ON cells are inhibited by systemic, PAG or RVM opioid administration [38, 62, 65, 87]. Furthermore, during acute opioid abstinence when there is enhancement of withdrawal reflexes and dorsal horn responses to noxious stimuli, there is a marked increase in the firing of RVM ON cells [18, 103], indicating that ON cells are likely to facilitate nociceptive transmission at the level of the dorsal horn. ON and OFF cells tend to fire reciprocally under most conditions, when one group is active the other is silent. The tail flick threshold is significantly higher during periods of OFF cell activity (and thus ON cell silence) and lower during periods of OFF cell silence (and ON cell firing) [85]. These correlative results indicate that both classes of presumed nociceptive modulatory neurons in the RVM are involved in the modulation of nociception. Also, RVM cells of the same physiological class tend to fire simultaneously [85], suggesting that the network functions as a unit and exerts global, rather than topographically discrete control over dorsal horn pain transmission neurons.

The RVM projects to several brainstem and spinal cord sites, but its major descending projections are to the spinal (via the DLF) and trigeminal dorsal horns [14, 121]. An extensive body of experimental evidence implicates bulbospinal serotonergic projections in pain modulation [153, 171, 198]. The antinociceptive effects of 5HT are mediated by $5HT_2$ and $5HT_3$ receptors in the spinal cord [6, 47, 53], by a postsynaptic action. However, the complexity of the 5HT contribution is underscored by studies which demonstrated that acting via the $5HT_{1A}$ receptors, 5HT may produce either a facilitatory [207] or an inhibitory [51, 52, 53] action on spinal nociceptive processing. Similar responses have been reported following local application of 5HT into the RVM [65]. Supraspinal opioid microinjection evokes release of 5HT in the spinal cord [199]. Since OFF cells are the only RVM neurons activated by opioid administration, these data indicate, that at least a subset of OFF cells are serotonergic.

Recently it has been reported that evoked release of 5HT in the spinal cord is not necessary, for opioids to exert their analgesic action [125], and that a non-serotonergic mechanism must also be involved in the control of dorsal horn nociceptive transmission by the RVM [28, 68, 173]. Systemic administration of the 5HT agonist methysergide is only partially effective in blocking antinociception from the RVM [203], and a

significant proportion of RVM bulbospinal neurons do not contain 5HT. Indeed the observed conduction velocities of spinally projecting RVM ON and OFF cells are in the range of myelinated axons, whereas almost all serotonergic axons are unmyelinated [17, 185]. In addition to the raphe spinal projection from RVM, the descending effects of PAG stimulation are also mediated in part by other brainstem nuclei [70]. There is evidence showing that the spinal pathways mediating tonic or stimulation produced descending inhibition from PAG and RVM are separate [164]. A recent study demonstrated that there is a non-serotonergic raphe spinal inhibitory pathway, which releases glycine into the dorsal horn when the RVM is stimulated [173]. Also, its been demonstrated that GABAergic spinal projection neurons are concentrated in the RVM, some of which colocalise with 5HT [9, 135, 159]. It is well known that the RVM contributes to the analgesia elicited by stimulation of PAG [163]; therefore, it is conceivable that there is a bulbospinal GABAergic/glycinergic projection in addition to a descending antinociceptive pathway that activates local GABA and glycine interneurons in the dorsal horn [5, 179].

The Pontine Noradrenergic Nuclei

Antinociception produced by electrical or chemical stimulation of the vl-PAG can be blocked with intrathecal α-adrenoceptor antagonists [3, 57, 58, 134, 198]. Since neither the PAG nor the spinal dorsal horn contain noradrenergic neurons [48, 91], PAG neurons must either directly or indirectly activate spinally projecting noradrenergic neurons. Three noradrenergic cell groups, A5, locus coeruleus (A6) and locus subcoeruleus (A7) [48] project to the spinal cord and play a role in analgesia [30, 108, 134, 191, 192]. All three-cell groups are reported to receive inputs from the vl-PAG [11, 31].

The A7 cell group is considered as the most likely among the noradrenergic nuclei to mediate the antinociception produced by PAG stimulation. Since A7 neurons project to the superficial laminae of the rat spinal dorsal horn [40, 41, 108], where many second order nociceptive neurons are located. In addition, electrical or chemical stimulation of the A7 produces antinociception that can be reduced by intrathecal injection of α_2-adrenoceptor antagonists [201, 202]. The A7 region also projects to the RVM and via the DLF directly to the spinal dorsal horn [39, 40, 41, 49, 108].

However, the role played by the pontine noradrenergic nuclei in antinociception modulation is not very clear, since there are a lot of conflicting results from different laboratories. Some groups have reported that stimulation of these sites attenuates, while others reported that they facilitate pain [26, 77, 100, 101, 166, 190]. Further more, the pontine noradrenergic cell groups exhibit remarkably different spinal cord projections in different substrains of rats [39, 41, 42, 43, 156], adding more to the difficulty of the interpretation of experimental data collected till now.

The Spinal Dorsal Horn

Small calibre, unmyelinated, C- and medium calibre, myelinated Aδ primary afferent fibres, both of which are responsive to noxious (tissue damaging) stimuli, convey nociceptive information principally to the superficial (I/II) and deep (V/VI) laminae of the dorsal horn. On the other hand, larger calibre, myelinated, rapidly-conducting Aβ fibres transmit information concerning innocuous, mechanical stimuli to laminae III-VI. Primary afferent fibres either, directly stimulate projecting neurons, which relay their messages to the brain, or indirectly engage projecting neurons via excitatory interneurons. Comprising a brake on these actions, primary afferent fibres also target inhibitory interneurons, which interact with projecting neurons, excitatory interneurons or the terminals of the primary afferent fibres themselves. Descending pathways may, then modulate nociception via an interaction with several neuronal elements in the dorsal horn: (i) the terminals of the primary afferent fibres; (ii) projecting neurons; (iii) intrinsic excitatory and inhibitory interneurons; (iv) terminals of other descending pathways [66, 133, 200].

The RVM is the major brainstem source of axons that project to the spinal dorsal horn via the DLF. Electrical stimulation of the RVM selectively inhibits noci-responsive dorsal horn neurons, an effect that is blocked by DLF lesions [60, 195]. These projections terminate densely within laminae I-V of the dorsal horn, laminae that are targeted also by primary afferent fibres [14, 16, 50, 161]. RVM axon terminals directly contact spinothalamic relay cells and local circuit neurons in the dorsal horn [73]. They form axodendritic and axosomatic but not axoaxonic synapses, thus they do not exert direct conventional presynaptic control over the primary afferent terminals [16, 114]. Since the RVM is the primary source of 5HT containing terminals in the

dorsal horn, the demonstration that spinothalamic tract neurons receive a large serotonergic input provides a substrate for direct postsynaptic control by brainstem neurons. Recent evidence demonstrated that the spinal terminals of nociceptive primary afferents are postsynaptic to GABA containing synapses [8]. While results by Lin et al. (1994) suggested that glycinergic and GABAergic inhibitory interneurons in the spinal dorsal horn synapsing on spinothalamic tract cells are activated during PAG stimulation and contribute to the descending antinociceptive actions. The superficial dorsal horn contains large numbers of neurons with immunoreactivity for inhibitory neurotransmitters, GABA and ENK [74, 179]. Some are immunoreactive for both [180]. The existence of such a population of neurons provide the bases for the hypothesis that supraspinal pain modulation neurons produce inhibition by exciting inhibitory interneuron in the spinal dorsal horn. In view of the dense projection of RVM serotonergic neurons to the superficial dorsal horn and the evidence implicating 5HT in pain modulation, it is important that there is a population of superficial dorsal horn neurons that is excited by iontophoresis of 5HT [6].

ENK immunoreactive neurons in lamina II and I are directly contacted by 5HT terminals [75, 76], which are most likely derived from the RVM. Furthermore there are ENK immunoreactive contacts upon spinothalamic tract neurons in laminae I and V [162]. Since intrathecal naloxone antagonizes the analgesic action of morphine and RVM stimulation [209] it follows that there is an opiate link between the bulbospinal axons and the spinal nociceptors. Since 5HT is generally inhibitory to dorsal horn neurons, this synaptic arrangement raised a paradox. Inhibition of the ENK neurons by 5HT should disinhibit the spinal nociceptor. Conceivably another inhibitory interneurons, probably GABAergic is interposed between the opioid peptide neurons and the nociceptor. It is also possible that 5HT neither excites nor inhibits ENK neuron, but modulates other inputs to them.

Spinally projecting brainstem noradrenergic neurons are also critical to the regulation of nociceptive transmission [155, 193]. Acting through α_2-adrenergic receptors, iontophoretically applied NE inhibits the firing of nociceptive and projecting cells in the dorsal horn [65, 67, 193, 202]. In addition to this inhibitory effect, NE has been shown to excite a distinct population of inhibitory interneurons within the dorsal horn that respond only to innocuous peripheral stimuli. These inhibitory interneurons that are excited by NE are also excited by electrical stimulation of the PAG. This

suggests the NE inhibits nociceptive projecting neurons both directly and indirectly, by exciting local inhibitory interneurons [65].

Although questions have been raised as to the relative importance of spinal 5HT and NE to endogenous antinociceptive control mechanisms, there is no clear answer. Indeed, it appears that the two are concurrently activated by opioids administered supraspinally [181]. Moreover, there is considerable evidence that interactions occur in the dorsal horn at the level of the 5HT and NE terminals, and that the spinal nociceptive modulatory actions of 5HT and NE are interdependent [10, 198]. It must also be emphasized that although 5HT and NE are the major contributors to descending antinociceptive controls, many of the spinally projecting 5HT and NE neurons also contain other co-transmitters [28, 140] that may further modulate the primary effects of 5HT and NE.

3. 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.

We hope that results obtained from these studies will provide a better understanding of how the vl-PAG modulates the transmission of nociceptive messages.

4. Material and Methods

4.1 Morphological Investigations

Animals, Injection of Tracer, and Preparation of Tissue Sections

Experiments were carried out on Wistar-Kyoto rats (250-300, Gödöllő, Hungary). All animal study protocols were approved by the Animal Care and Protection Committee at the University of Debrecen, Hungary, and were carried out in accordance with the European Communities Council Directives. The skull was opened with a dental drill under deep anaesthesia (35 mg/kg sodium pentobarbital, i.p.), while the animal was held in a stereotaxic frame. Glass micropipettes with a tip diameter of 20-30 μm were filled with a 2.5% solution of *Phaseolus vulgaris*-leucoagglutinin (PHA-L, Vector Labs, Burlingame, CA, USA), the highly sensitive anterograde tracing substance [71]. The tracer was injected unilaterally into the ventrolateral aspect of the PAG by iontophoresis. The mediolateral, rostrocaudal and dorsoventral coordinates for the injection varied between 0.6-0.8 mm from the midline, 1.2 and 3.2 mm from the interaural line, and 4.6 and 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.

In three animals we eliminated the central tegmental tract of descending hypothalamic fibres by making a transverse lesion throughout the entire crossectional area of the PAG at the border of the diencephalon and mesencephalon 3 weeks before the tracer application.

After a 3 week survival period, the animals were reanaesthetised with an overdose of sodium pentobarbital (70 mg/kg), 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). The brainstem was removed, postfixed in the same fixative for 1-2 hours, immersed in 10% and 20% sucrose dissolved in 0.1M PB until it sank, freezed in liquid nitrogen, thawed in 0.1M PB at room temperature and sectioned at 60 μm on a Vibratome.

Pre-embedding Immunocytochemistry

For immunocytochemical detection of PHA-L, free-floating sections of the brainstem were first incubated with biotinylated goat anti-PHA-L (Vector Labs; diluted 1:1000) for 2 days at 4°C. Then the sections were transferred into a solution of avidin-biotinylated peroxidase complex (ABC; Vector Labs; diluted 1:100) for 4 hours at room temperature. The immunoreaction was completed with a nickel-intensified diaminobenzidine chromogen reaction [81].

To reveal whether serotonergic and noradrenergic 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), a good marker for catecholaminergic including noradrenergic neurons. First the sections were incubated in a mixture of biotinylated goat anti-PHA-L (Vector Labs; diluted 1:1000) and rabbit anti-5HT (Chemicon Inc.; diluted 1:2000) or rabbit anti-TH (Eugene; diluted 1:1000). The immunological and immunocytochemical characteristics of anti-5HT and anti-TH antibodies have been extensively tested and published earlier [54, 137, 187, 188]. Subsequently the sections were transferred into a mixture of ABC (Vector Labs; diluted 1:100) and goat anti-rabbit IgG (Vector Labs; diluted 1:200) and left overnight at 4°C. The PHA-L-labelled axons and axon terminals were visualized with a nickel-enhanced DAB chromogen reaction [81]. The sections were then treated with a rabbit peroxidase anti-peroxidase complex (DAKO; diluted 1:100), and the immunostaining for 5HT or TH was completed with a chromogen reaction using DAB alone. Before the antibody treatments, sections were kept in 20% normal goat serum for 50 minutes. All of the antibodies were diluted in 0.01M phosphate buffered saline (PBS, pH 7.4) to which 0.1% Triton X-100 and 1% normal goat serum were added. Between incubations in the antibody solutions, sections were rinsed three times for 30 minutes in the same buffer. Sections were mounted on gelatin-coated slides and covered with Permount neutral medium.

Post-embedding Immunocytochemistry

Alternate sections were treated with 1% OsO_4 for 45 minutes, then dehydrated and flat embedded into Durcupan ACM resin (Fluka) 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, while sections on every third grid were counterstained with uranyl-acetate and lead citrate. The immunostaining was performed according to the postembedding immunogold procedure described by Somogyi and Hodgson (1985). Briefly, the sections were etched with 1% periodic acid (8 min) and 5% sodium metaperiodate (10 min), and treated with 1% bovine serum albumin in 0.01M Tris-PBS (pH 7.4) for 30 minutes. Following incubations with anti-GABA or anti-glycine antisera for 90 minutes, the sections were washed and exposed to goat anti-rabbit IgG coupled to 20 nm colloidal gold particles (Amersham; diluted 1:200) for 2 hours. The sections were then counterstained with uranyl acetate and lead citrate and examined in a JEOL 1010 electron microscope.

The primary antisera used were kindly donated by J. Storm-Mathisen (Anatomical Institute, University of Oslo, Norway). The antisera were raised against amino acids conjugated to a carrier protein (bovine serum albumin) by glutaraldehyde [175]. In order to abolish possible weak cross-reactivities with other amino acids, the antisera were used as follows: the anti-GABA (code: 416; diluted 1:1000) was preincubated in 400 μM glutamate-glutaraldehyde conjugates, and the anti-glycine (code: 290; diluted 1:2000) was preincubated in 100 μM GABA- and glutamate-glutaraldehyde conjugates. All antisera have been characterised previously and tested for cross-reactivity [25, 146]. Antiserum specificity was also tested, by treating the diluted anti-GABA and anti-glycine sera with glutaraldehyde-GABA and glutaraldehyde-glycine conjugates, respectively. Sections were incubated according to the postembedding immunocytochemical procedure by using the anti-GABA and anti-glycine sera treated with glutaraldehyde-GABA and glutaraldehyde-glycine conjugates, respectively, as primary sera. Under these conditions specific immunostaining was completely abolished. To check the specificity of the immunostaining method per se, sections were treated by the immunocytochemical procedure described previously with the primary antiserum omitted or replaced with normal rabbit serum (diluted 1:100). No selective labelling was observed in these sections.

Immunoreactivities for GABA and glycine were evaluated by a quantitative criterion. Structures were considered labelled if their gold particle density was at least five times higher than the background.

Immunofluorescence and Confocal Microscopy

Free-floating sections of the brainstem were first incubated for 1 hour in phosphate-buffered saline (PBS) containing 10% normal goat serum and 0.9% NaCl. The sections were then incubated for 48 hours at 4°C in a solution containing the following primary antibodies: (i) biotinylated goat anti-PHA-L (Vector Labs; diluted 1:500); (ii) mouse anti-glutamic acid decarboxylase ($GAD_{65/67}$; Affiniti Ltd.; diluted 1:1000); (iii) rabbit anti-preproenkephalin (PPE; diluted 1:500; kindly donated by T. Kaneko, Dept. Morphological Brain science, Kyoto University, Japan). After thorough rinsing, the sections were then incubated overnight in a mixture containing Streptavidin-Alexa-Fluor 546 conjugate (Molecular Probes; diluted 1:2000), goat-anti-rabbit IgG-Alexa-Fluor 633 conjugate (Molecular Probes; diluted 1:500), and goat-anti-mouse IgG-Alexa-Fluor 488 conjugate (Molecular Probes; diluted 1:500). The sections were then mounted between two glass cover slips in a glycerol-based anti-fade medium (Vectashield; Vector Labs). The cover slips were sealed with nail varnish and the sections stored at –20°C. All antibody solutions were made up in PBS containing 1% normal goat serum. The primary antisera have all been previously characterised and tested [111, 126]. Also, controls carried out on sections showed that omission of the GAD and/or PPE antibodies abolished the corresponding type of fluorescent staining.

The sections were examined with a Zeiss LSM 510 confocal laser-scanning microscope. They were scanned sequentially with the 546, 488 and 633 nm lines in order to reveal the PHA-L-, $GAD_{65/67}$- and PPE-like immunoreactivity, respectively. No cross-detection of the three fluorochromes was observed. Sequential scans at a series of optical planes separated by 0.5 μm were performed with a 60 x oil immersion objective.

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

The distribution of PHA-L labelled axon terminals, and serotonin-immunoreactive (SIR) and tyrosine hydroxylase-immunoreactive (THIR) neurons in the brainstem were investigated in serial sections. Sections were cut from the site of PHA-L injection to the pyramidal decussation. 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 on-line digitising mode of a NEUROLUCIDA 3-D reconstruction system installed onto a Leitz Laborlux microscope with a motorized stage, 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 individual sections were merged in consecutive order, and the stack of the sections with labelled axon terminals and immunostained neurons were rotated around axes of the three-dimensional coordinate system. 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.

4.2 Physiological Investigations

Experimental Animals and Surgical Procedure

The experiments were carried out on anaesthetized and artificially ventilated male Sprague-Dawley rats (250-300 g, Bantin and Kingman, UK). Following the induction of surgical anaesthesia with Nembutal (60 mg/kg i.p.), the femoral artery, femoral vein and the trachea were cannulated, the animals were then placed into a stereotaxic frame. The left sciatic nerve was prepared for stimulation using bipolar silver hook electrodes. With an incision on the lateral surface of the left thigh, and blunt dissection of the superficial gluteal and femoral biceps muscles, the sciatic nerve was exposed underneath, on the surface of the adductor muscle. 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. During the experiments the animals were immobilized with gallamine (Flaxedil, 15 mg/kg/h, i.v., May and Baker, UK) and ventilated with oxygen enriched air (40%; Harvard Apparatus, USA). Core temperature was kept at 37 $\pm$ 1 °C with a combination of a heating blanket and an overhead lamp. During recordings, anaesthesia was supplemented by injections of α-chloralose (20-35 mg/kg/h i.v.) at regular intervals and as required. The level of anaesthesia was assessed by testing the cardiovascular responses, to noxious pinch of the hind paw at regular intervals.

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 that were filled with a mixture of 1.5M potassium methyl-sulphate and 3% biocytin ($R_{i,}$ 35-70 MΩ). Intracellular signals were recorded using an active bridge circuit (Axoclamp 2A, Axon Instruments, USA) and data analysis was carried out off-line using a digital data acquisition system and Spike2 and SigAvg software packages (CED, Cambridge, UK). 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 (100 µm OD, Harvard Apparatus, USA) 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 (Fig. 1) using a short train of stimulus (2 s, 0.5 ms, 50 µA, 30 Hz) [12, 97]. During intracellular recording from RVM neurons, a single pulse electrical stimulation of 100-300 µA (0.5 ms, 1 Hz) was applied. The location of the electrodes was marked for post-mortem identification by placing a small electrolytic lesion at the end of the experiments (2 mA DC, 1 min; Fig. 1).

Peripheral Nerve Stimulation

The sciatic nerve was stimulated using 75-µm bipolar silver wire electrodes that were fixed in position using dental imprint material (Coltene Ltd, UK). Following previously established methods, the intensity of sciatic nerve stimulation was set to 2 µA (2 V) to activate both myelinated and non-myelinated fibres [138, 176]. During intracellular recordings, 0.2 ms wide, single pulse stimuli were delivered at 1 Hz.

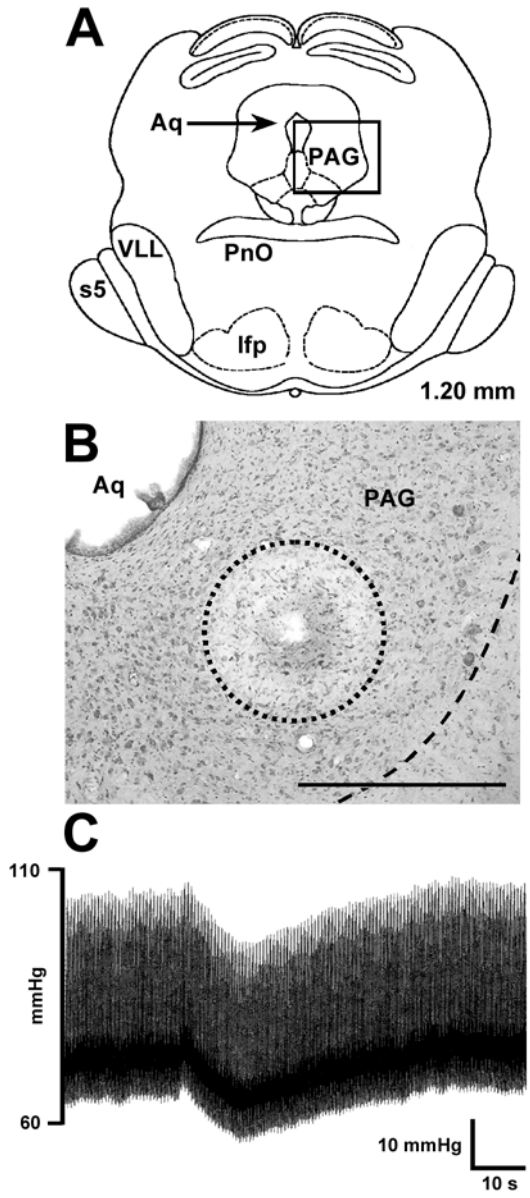

Figure 1: **A.** Camera lucida drawing of the mesencephalon at the level of the stimulation site in the vl-PAG. The rostro-caudal coordinate relative to the interaural line is indicated according to the atlas of Paxinos and Watson. **B.** A photomicrograph shows the stimulation site (area outlined in A.). A background staining with toluidine blue was applied, the stimulation site appears as a small necrotic area in the section. Bar: 500 µm. **C.** Blood pressure trace recording showing the hypotensive response evoked by the stimulation of the vl-PAG. Aq: cerebral aqueduct, lfp: longitudinal fasciculus of the pons, PAG: periaqueductal gray matter, PnO: pontine reticular nucleus, oral part, s5: sensory root of the trigeminal nerve, VLL: ventral nucleus of the lateral lemniscus.

Histological Processing of the Intracellularly Labelled Neurons

Following electrophysiological characterization of the recorded RVM neuron, biocytin was injected intracellularly using 1-3 nA constant negative current for up to 10 minutes. Thirty minutes after the dye injection, the animals were perfused transcardially with normal saline, followed with a mixture of 4% paraformaldehyde and 2% glutaraldehyde in 0.1M PB (pH 7.4). The brainstem was dissected and postfixed in the same fixative for 2 hours, then extensively washed in 0.1M PB. Serial coronal sections of the medulla oblongata were then cut on a Vibratome at a thickness of 40 μm. The sections were first treated with 45% ethanol for 45 minutes, then extensively washed in 0.1M PB and incubated with ABC diluted in 0.01M phosphate buffered saline containing 2% normal goat serum for 72 hours (VectaStain Elite kit, Vector Laboratories, USA; diluted 1:100). The labelled cells were visualized using a DAB stabilized TMB reaction [204], then incubated in 1% osmium tetroxide dissolved in 0.1M PB for 40 minutes, dehydrated in graded ethanol and flat-embedded in Durcupan ACM resin (Fluka, UK). Reconstruction of the labelled neurons was performed with the aid of a NEUROLUCIDA 3-D reconstruction system. The localization of the recorded and labelled cells was made with reference to the stereotaxic atlas of Paxinos and Watson (1998).

5. Results

Injection Site of PHA-L

The tracer was delivered into the ventrolateral area of the midbrain periaqueductal gray matter (Figs. 2, 3). In addition to the PAG, however, the tracer clearly spread into the adjacent midbrain tegmentum. As detected by the immunostaining many cells incorporated PHA-L within the site of injection, but labelled cell bodies were only occasionally found outside this area. The appearance of the labelled cells, as well as the injection site, was similar to those reported in previous studies [72, 196].

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 (Fig. 4) were widely scattered in the pons and medulla oblongata (Fig. 5). Numerous axons descended and terminated ipsilateral to the site of injection, whereas others crossed the midline at the level of the mesencephalon and projected to the contralateral side. The number of axon varicosities found on the ipsilateral side always outnumbered those that were recovered contralaterally. Although the numbers of the labelled axon terminals varied from animal to animal, presumably reflecting the differences in the sites and sizes of PHA-L delivery, the distribution of the terminals were very similar in all the animals that were investigated in this study. For quantitative studies and 3-dimensional reconstruction we have randomly selected three animals. Figure 3 illustrates sites of PHA-L injections in these animals, whereas figures 5, 7 and 9 show experimental data obtained from one of them.

In the rostral pons (Fig. 5c), most of the labelled axon terminals were observed in the noradrenergic cell groups. Within the confines of these nuclei, most of the terminals were distributed in the ventral and dorsal subdivisions of the locus subcoeruleus and in the locus coeruleus, whereas the density of labelling was lower in the alpha subdivision of the locus subcoeruleus and A5 cell group. In addition to this, 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.

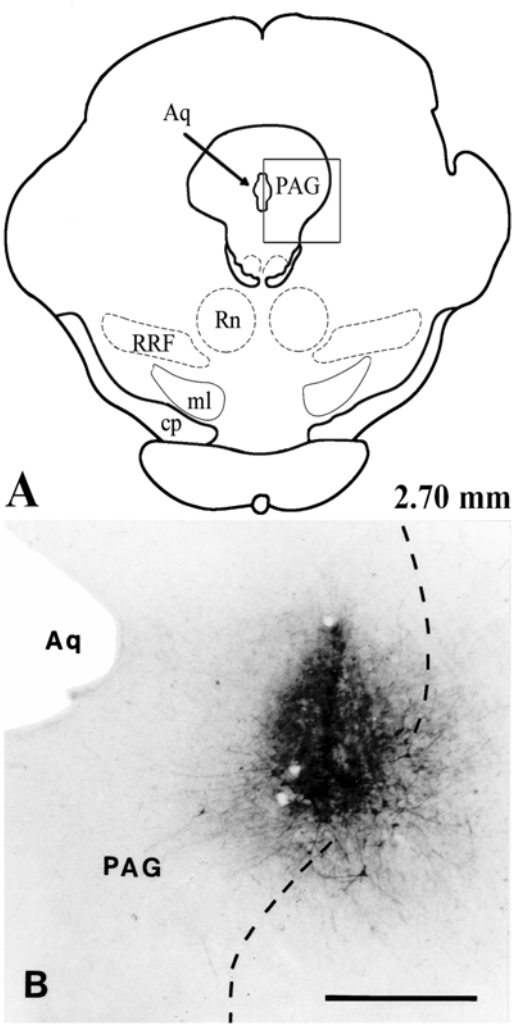

Figure 2: **A**. Camera lucida drawing of a transverse section of the mesencephalon at the level of the injection site of PHA-L. Rostro-caudal coordinate relative to the interaural line is indicated according to Paxinos and Watson (1998) **B**. The photomicrograph shows an injection site (the area outlined in **A**) of PHA-L in animal PAG1. Aq: cerebral aqueduct, cp: cerebral peduncle, ml: medial lemniscus, PAG: periaqueductal gray matter, Rn: red nucleus, RRF: retrorubral field. Bar: 500 μm.

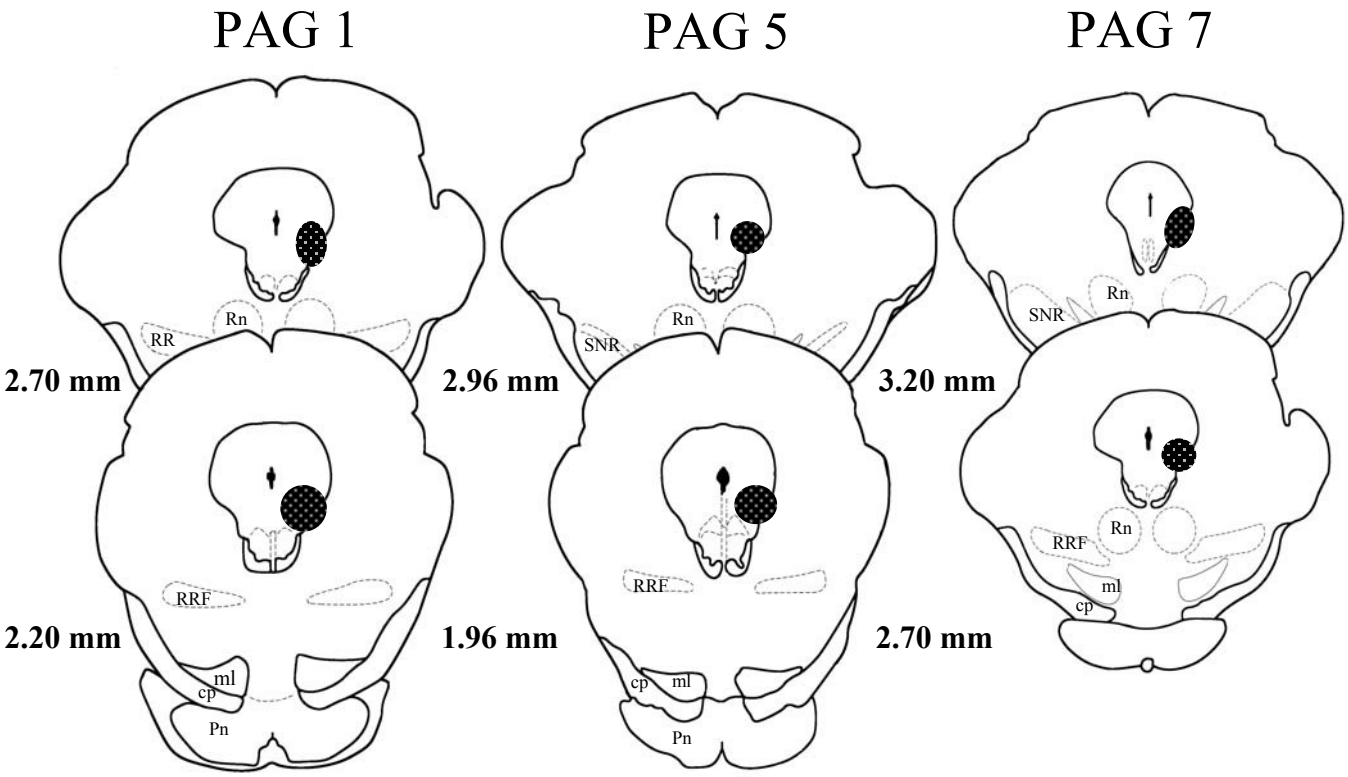

Figure 3: Camera lucida drawings of frontal sections of the mesencephalon showing the locations of PHA-L deposits in three animals. Rostro-caudal coordinates relative to the interaural line are indicated according to Paxinos and Watson (1998). cp: cerebral peduncle, ml: medial lemniscus, Pn: pontine nuclei, Rn: red nucleus, RRF: retrorubral field, SNR: substantia nigra.

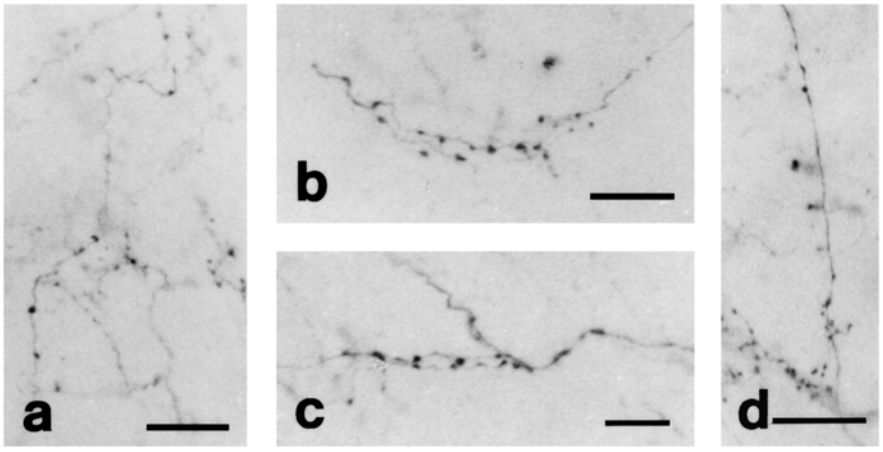

Figure 4: Photomicrographs showing PHA-L labelled axons and axon terminals that arise from the ventrolateral PAG and terminate in various parts **(a:** intermediate reticular nucleus**, b, c:** lateral paragigantocellular nucleus**, d:** dorsal paragigantocellular nucleus**)** of the pons and medulla oblongata. Bars: 50 μm.

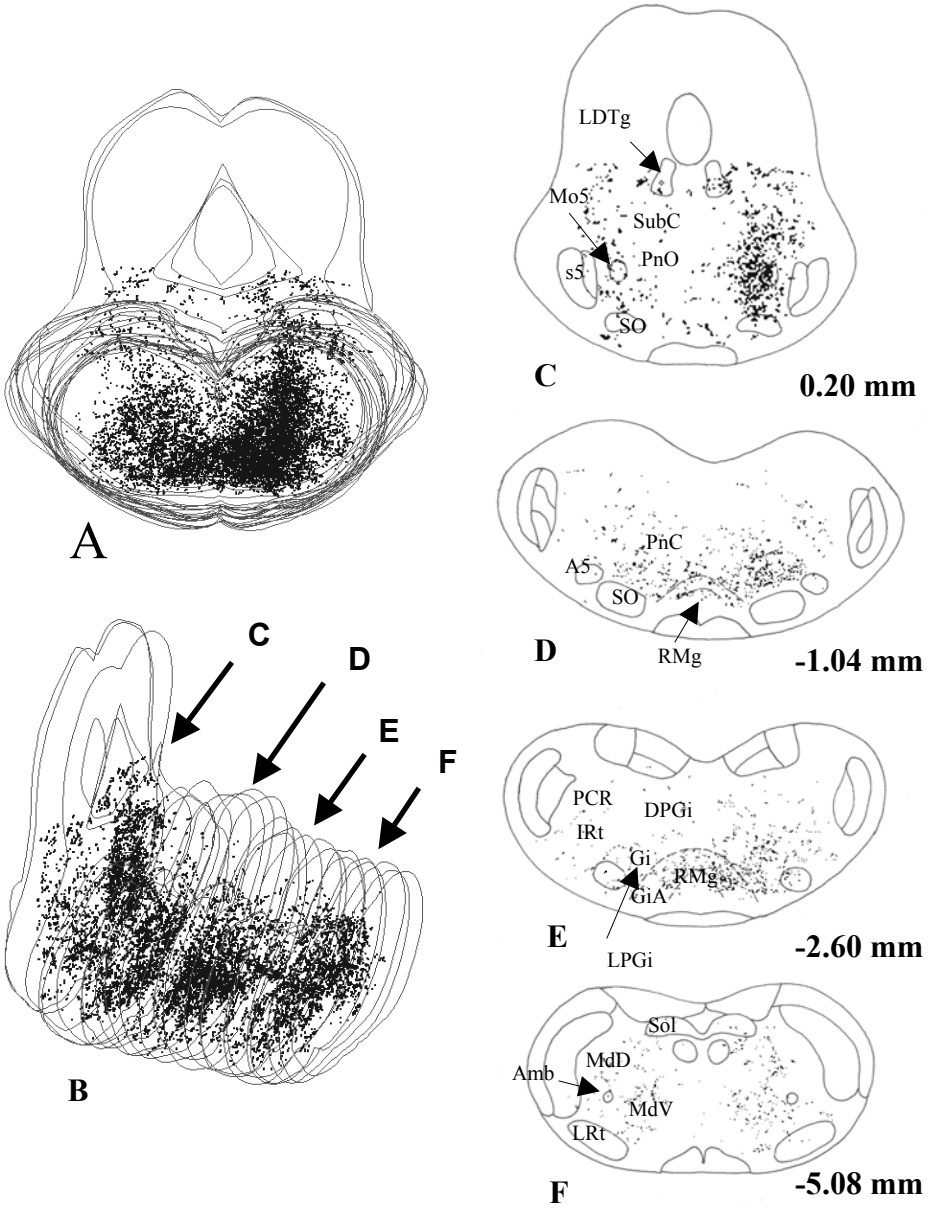

Figure 5: Schematic representation of the distribution of the PHA-L labelled axon terminals in 3-dimensional representations (**A**, **B**) and selected sections of the pons (**C**, **D**) and medulla oblongata (**E**, **F**). Each dot represents a labelled terminal. Data were obtained from animal PAG5. Rostro-caudal coordinates relative to the interaural line are indicated according to Paxinos and Watson (1998). Amb: ambiguus nucleus, A5: A5 noradrenergic cell group, DPGi: dorsal paragigantocellular nucleus, IRt: intermediate reticular nucleus, Gi: gigantocellular reticular nucleus, GiA: gigantocellular reticular nucleus pars alpha, LDTg: laterodorsal tegmental nucleus, LPGi: lateral paragigantocellular nucleus, LRt: lateral reticular nucleus, MdV: ventral medullary reticular nucleus, MdD: dorsal medullary reticular nucleus, Mo5: motor trigeminal nucleus, 7: motor facial nucleus, PCR: parvocellular reticular nucleus, PnC and PnO: pontine reticular nucleus (caudal and oral), RMg: raphe magnus nucleus, S5: sensory trigeminal nucleus, SO: superior olive, Sol: nucleus of the solitary tract, SubC: nucleus subcoeruleus.

In the caudal pons and rostral medulla oblongata (Fig. 5d, e), a substantial number of terminals were recovered in the RVM. Within the confines of the RVM, most of the terminals were concentrated in the Rgcα and Rpgl, whereas the NRM and Rpg were supplied only by a moderate number of terminals. 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 (Fig. 5f), 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 serotonin-immunoreactive Neurons

In addition to a heavy labelling of PAG efferents and their terminals, we have obtained a substantial immunostaining also for 5HT (Fig. 6). Most of the 5HT immunoreactive dendrites could be traced from the immunostained perikarya for a distance of at least 200-300 μm. In many cases, immunostained dendrites left the section that contained their somata and entered the consecutive section. Close appositions made by PHA-L labelled axon terminals were also investigated on these distal dendritic segments.

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 (Fig. 6). Most of the terminals were sitting on distal dendrites and only a few of them were apposed to proximal dendrites or somata of SIR neurons (Fig. 6). 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 (Fig. 7). In order to estimate the strength of PAG projection to the RVM and SIR neurons in particular, varicosities along the PHA-L labelled axons, regarded as potential synaptic sites of the terminals, were counted in every fourth section of the pons and medulla oblongata in three animals. From the 8663, 23138 and 25190 terminals counted in the individual animals, only 9.0%, 21.1% and 20.8% were found within the confines of the RVM, and even

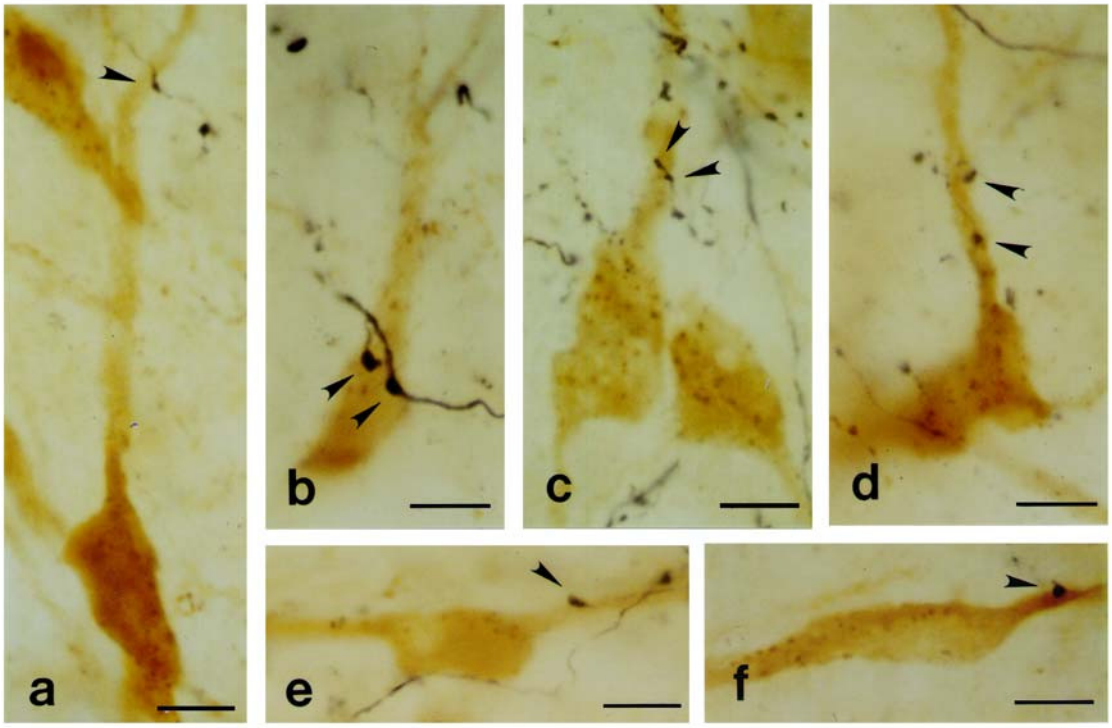

Figure 6: Photomicrographs of sections double-immunostained for the simultaneous visualization of PHA-L labelled axons and axon terminals that arise from the ventrolateral PAG (dark blue or black) and serotonin immunoreactive neurons (brown) that are located in the raphe magnus **(c, d),** gigantocellular reticular nucleus pars alpha **(a, b)** and lateral paragigantocellular nucleus **(e, f).** Arrowheads point to labelled axon terminals that are in close apposition to dendrites or somata of serotonin immunoreactive neurons. Bars: 20 μm.

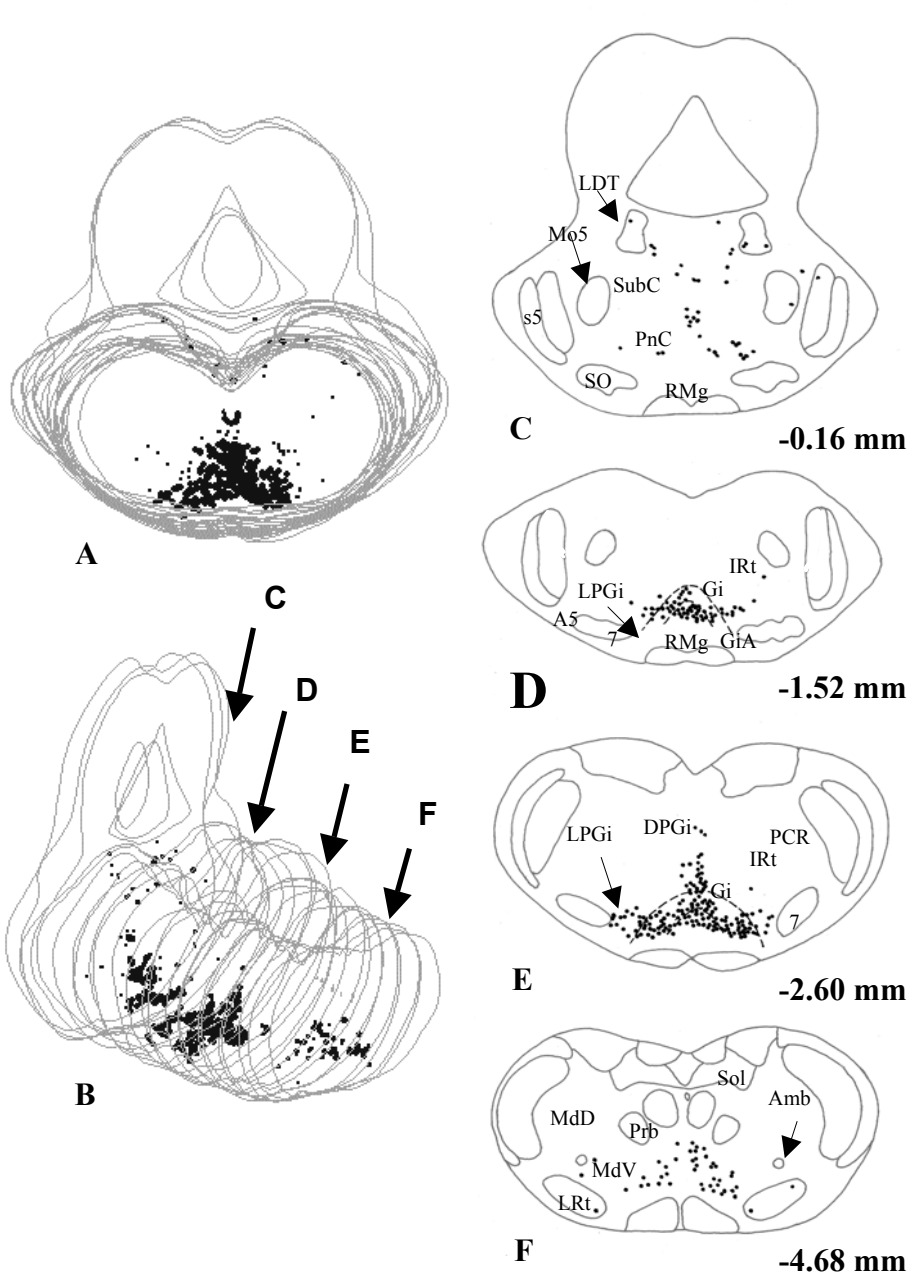

Figure 7: Schematic representation of the distribution of PHA-L labelled axon terminals arising from the ventrolateral PAG that established close appositions with serotonin immunoreactive dendrites or perikarya in 3-dimensional representations (**A**, **B**) and selected sections of the pons (**C**, **D**) and medulla oblongata (**E**, **F**). Each dot represents a PHA-L labelled axon terminal that established close apposition with a serotonin immunoreactive dendrite or perikaryon. Data were obtained from animal PAG5. Rostro-caudal coordinates relative to the interaural line are indicated according to Paxinos and Watson (1998). Amb: ambiguus nucleus, A5: A5 noradrenergic cell group, DPGi: dorsal paragigantocellular nucleus, IRt: intermediate reticular nucleus, Gi: gigantocellular reticular nucleus, GiA: gigantocellular reticular nucleus pars alpha, LDTg: laterodorsal tegmental nucleus, LPGi: lateral paragigantocellular nucleus, LRt: lateral reticular nucleus, MdV: ventral medullary reticular nucleus, MdD: dorsal medullary reticular nucleus, Mo5: motor trigeminal nucleus, 7: motor facial nucleus, PCR: parvocellular reticular nucleus, PnC: pontine reticular nucleus (caudal), Prb: nucleus of the Probst's bundle, RMg: raphe magnus nucleus, S5: sensory trigeminal nucleus, SO: superior olive, Sol: nucleus of the solitary tract, SubC: nucleus subcoeruleus.

here most of these terminals were distributed in non-SIR territories (Fig. 6). Only 0.8%, 1.0% and 0.7% of the total number of terminals (9.1%, 4.8% and 3.3% of those that terminated in the RVM) were apposed to SIR neurons of the RVM.

Relationship Between PHA-L Labelled Axon Terminals and Tyrosine hydroxylase-immunoreactive Neurons

Immunostaining for TH was even stronger than that we have obtained for 5HT. In addition to somata, a substantial compartment of the dendritic tree of stained neurons also showed a strong immunoreactivity. Most of the THIR dendrites could be traced from their perikarya for several hundreds of micrometers. Similarly to SIR dendrites, many of the TH immunostained dendrites also left the section that contained their somata and entered the consecutive section. Close appositions made by PHA-L labelled axon terminals were also investigated on these distal dendritic segments.

Close appositions between PHA-L labelled PAG efferents and neurons immunoreactive for TH were only occasionally found in the pons and medulla oblongata (Fig. 8). Stained PAG terminals were apposed mostly to distal dendrites, whereas axo-somatic appositions were only sporadically seen (Fig. 8). 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 (Fig. 9). In order to estimate the strength of PAG projection to the pontine noradrenergic cell groups and THIR neurons in particular, varicosities along the stained axons in the PHA-L/TH double stained sections were counted in the same way as it was done in the PHA-L/5HT double stained sections in three animals. Of the 10163, 14412 and 28141 terminals counted in the individual animals, only 14.0%, 13.3% and 10.9% 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 (Fig. 8). Only 0.5%, 0.6% and 0.4% of the total number of terminals (3.6%, 4.3% and 4.0% of those that terminated in the pontine noradrenergic cell groups) established close appositions with THIR neurons of the pontine noradrenergic cell groups in the individual animals.

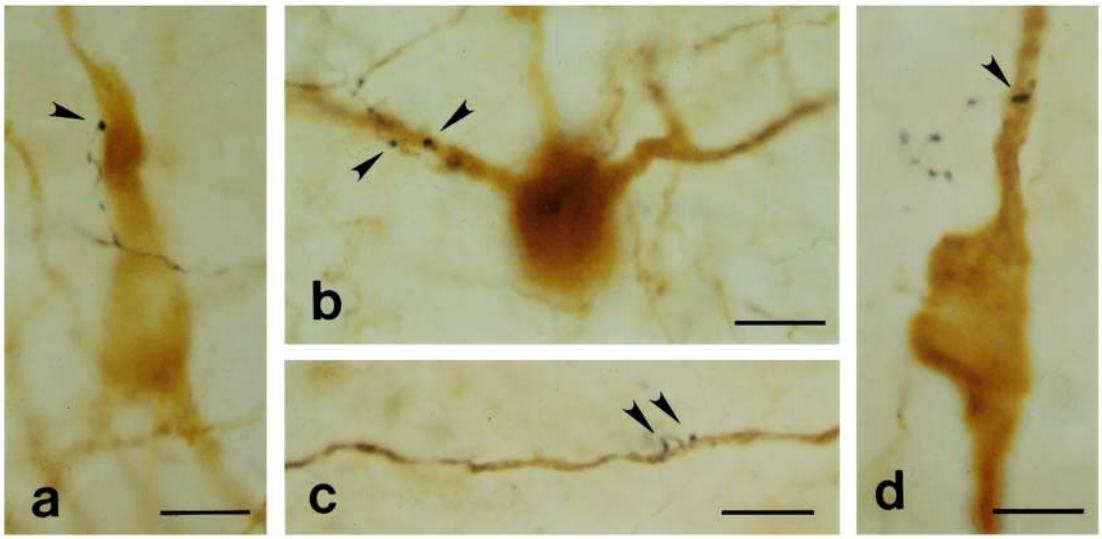

Figure 8: Photomicrographs of sections double-immunostained for the simultaneous visualization of PHA-L labelled axons and axon terminals that arise from the ventrolateral PAG (dark blue or black) and tyrosine hydroxylase immunoreactive neurons (brown) that are located in the locus subcoeruleus **(b, c, d)** and A5 nucleus **(a)**. Arrowheads point to labelled axon terminals that are in close apposition to dendrites or somata of tyrosine hydroxylase immunoreactive neurons. Bars: 20 μm.

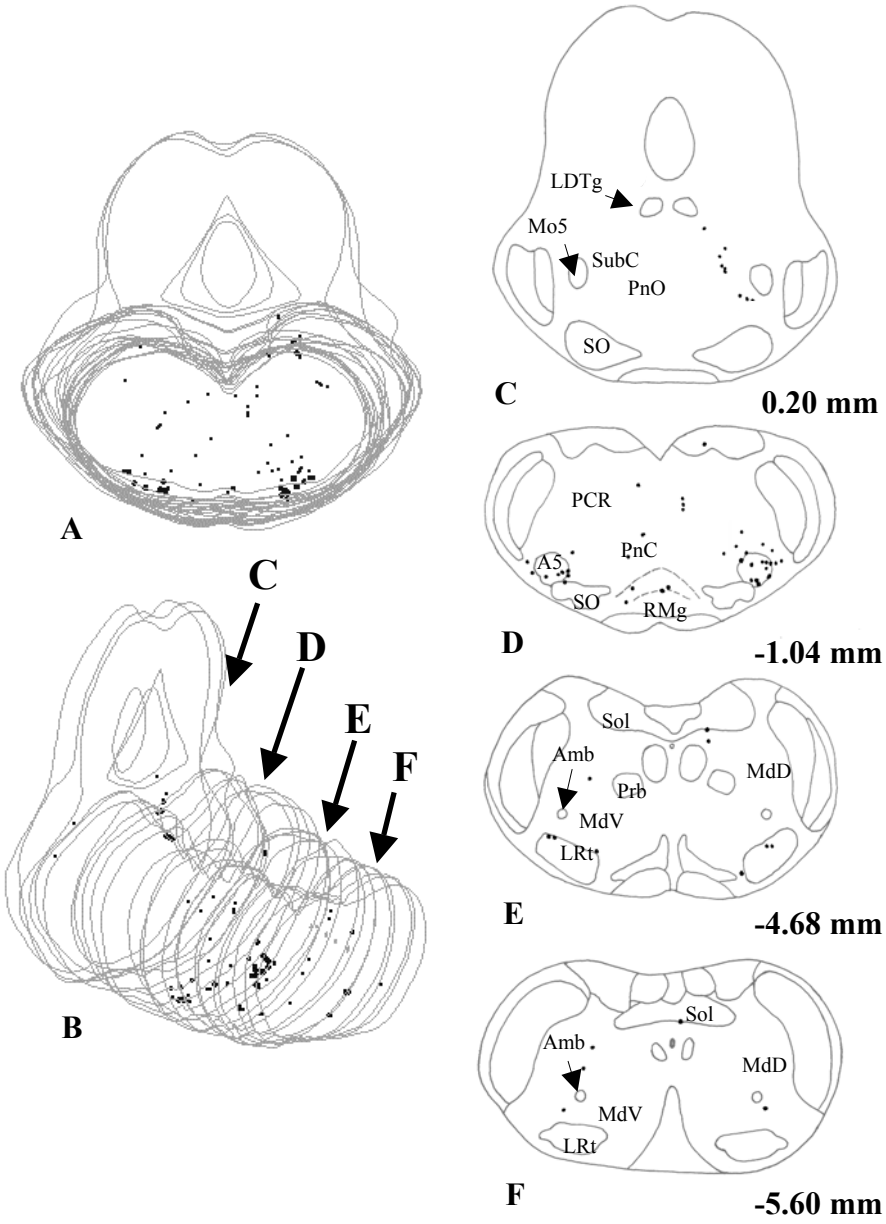

Figure 9: Schematic representation of the distribution of PHA-L labelled axon terminals arising from the ventrolateral PAG that established close appositions with tyrosine hydroxylase immunoreactive dendrites or perikarya in 3-dimensional representations (**A**, **B**) and selected sections of the pons (**C**, **D**) and medulla oblongata (**E**, **F**). Each dot represents a PHA-L labelled axon terminal that established close apposition with a tyrosine hydroxylase immunoreactive dendrite or perikaryon. Data were obtained from animal PAG5. Rostro-caudal coordinates relative to the interaural line are indicated according to Paxinos and Watson (1998). Amb: ambiguus nucleus, A5: A5 noradrenergic cell group, DPGi: dorsal paragigantocellular nucleus, LDTg: laterodorsal tegmental nucleus, LRt: lateral reticular nucleus, MdV: ventral medullary reticular nucleus, MdD: dorsal medullary reticular nucleus, Mo5: motor trigeminal nucleus, PCR: parvocellular reticular nucleus, PnC and PnO: pontine reticular nucleus (caudal and oral), Prb: nucleus of the Probst's bundle, RMg: raphe magnus nucleus, SO: superior olive, Sol: nucleus of the solitary tract, SubC: nucleus subcoeruleus.

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 (which includes the pontine reticular nucleus, the parvocellular reticular nucleus of the pons, the intermediate and dorsal medullary reticular field) received the most extensive innervation from the vl-PAG in the brainstem [142]. Since these nuclei have been shown to send afferents to the RVM and play a role in pain behaviour [7, 90], the possibility that the iRF might be an intermediate relay station between the vl-PAG and the RVM was raised. Thus we intended to examine the targets of vl-PAG terminals within these territories.

Investigating the vl-PAG efferents at the electron microscopic level, we found that the vl-PAG terminals were primarily engaged in synaptic contacts with dendritic shafts with a diameter range of 1.3-2.4 μm (Fig. 10). Some of them also established synaptic contacts with dendritic spines, and only 3 of the 29 vl-PAG-iRF terminals investigated in this study were impinged upon somata. None of them were seen to engage in axoaxonic synaptic contacts. Investigating the GABA and glycine immunoreactivity of the labelled terminals, as well as their postsynaptic targets. None of the PHA-L labelled vl-PAG terminals was found to be immunoreactivity for GABA or glycine (Fig. 10). Dendrites, spines and somata postsynaptic to the labelled vl-PAG terminals were also negative for both GABA and glycine (Fig. 10).

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 (Fig. 11).

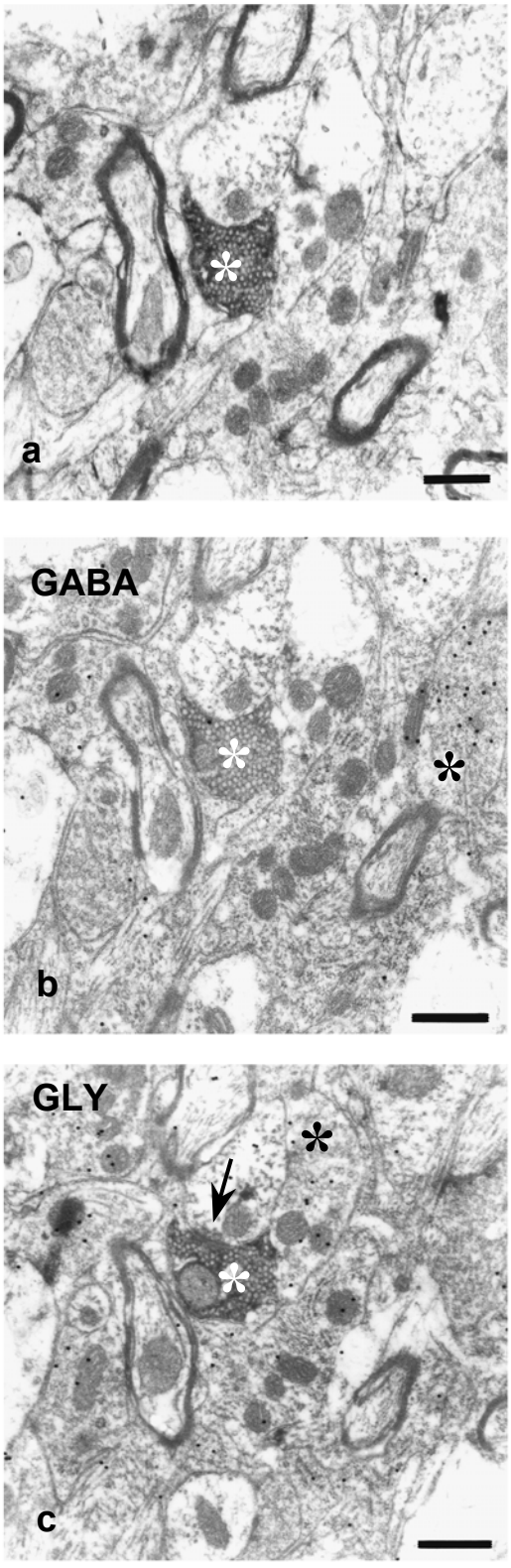

Figure 10: Electron micrographs of consecutive sections of a PHA-L labelled vl-PAG terminal (white asterisks) within the iRF. Sections are counterstained with lead citrate (**a**) or are immunostained by immunogold procedure for GABA (**b**) or glycine (**c**). As can be seen, the vl-PAG terminal and its postsynaptic target are immuno-negative for both GABA and glycine. The black asterisks represent a GABA (**b**) or a glycine (**c**) immunoreactive structure. The arrowhead points to the synaptic contact. Scale bars = 0.5 µm

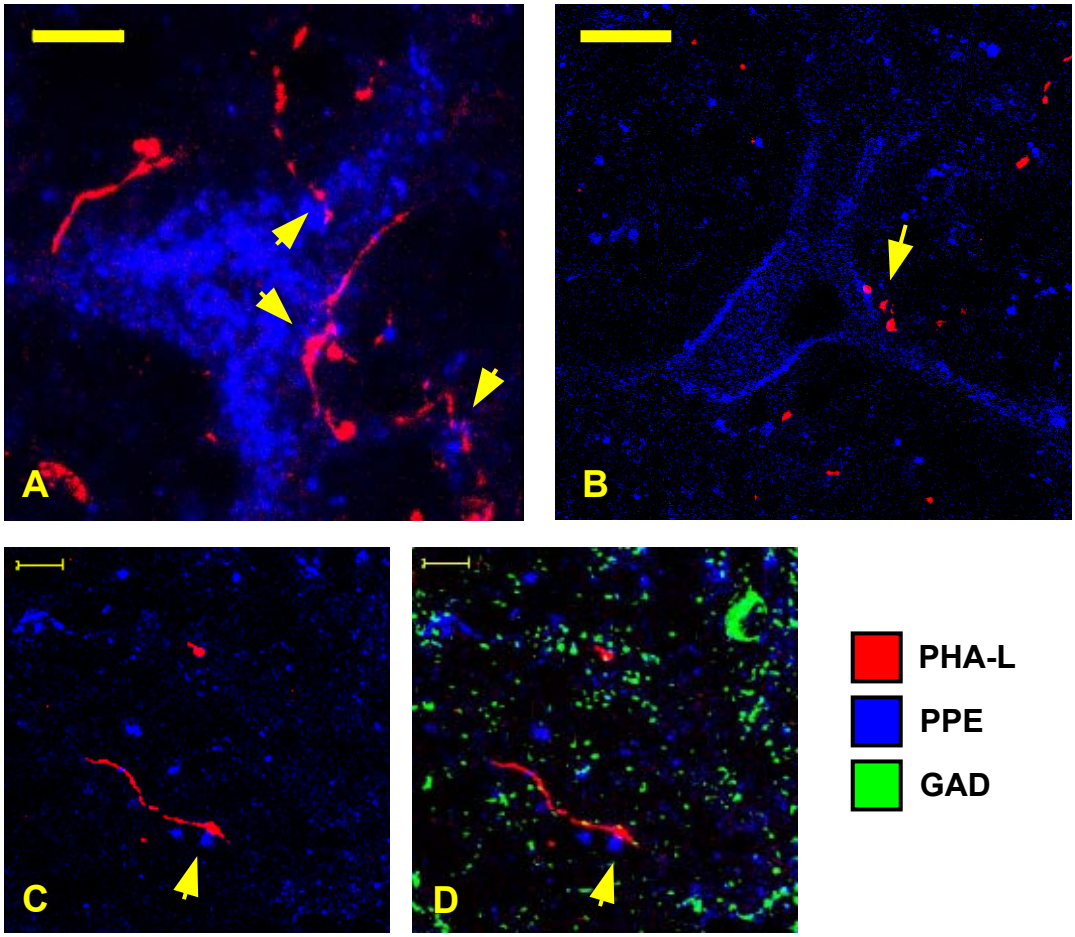

Figure 11: (**A**) A 3-dimensional reconstructed confocal image of a PPE immunoreactive neuron within the nucleus reticularis gigantocellularis. (**B**) A single confocal scan of a neuron, taken through a representative plane. These particular neurons receive a strong input from the vl-PAG. The arrowheads point to the PHA-L labelled vl-PAG axon terminals making close appositional contacts with the PPE positive cell body and dendrites. Images **C** and **D** show a PHA-L labelled vl-PAG axon and its terminal contacting a PPE immuno-positive structure (arrowheads), taken from the dorsal medullary reticular formation. As can be seen in image **D**, no colocalisation was observed in any of the structures. Also, none of the PHA-L labelled axon terminals was seen contacting a GAD positive element. Scale bars = 10 μm.

In vivo Intracellular RVM Neuronal Recordings

Intracellular recordings were obtained from 14 RVM neurons that responded to stimulation of the contralateral sciatic nerve. Neurons with a resting membrane potential of at least - 40 mV (average - 57 $\pm$ 3 mV) and ongoing firing rate of less than 40Hz were selected for the present study. Of the 14 neurons, nine exhibited ongoing discharges (average 8 $\pm$ 3 Hz), while the others were quiescent. Action potentials could be elicited from all of them by either stimulation of the sciatic nerve or the vl-PAG, or by injecting short positive current pulses (less than 0.6 nA, 0.2 ms) into the cell.

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. Excitatory postsynaptic potentials evoked by sciatic stimulation lead to a firing of 1 to 4 action potentials in every recorded neuron (Fig. 12). Following the evoked responses, 3 neurons that were excited, and 4 cells that were inhibited by sciatic nerve stimulation exhibited a longer latency, long-lasting inhibitory postsynaptic potential with an average onset latency of 97.3 $\pm$ 11 ms, similar to those described previously in the rostral ventrolateral medulla oblongata [206].

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, e.g. in neurons with an inhibitory sciatic response, the spontaneous firing rate was reduced following noxious mechanical stimulus, whereas in neurons with an excitatory sciatic response the ongoing firing rate increased in response to hind limb pinch (Fig. 13).

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%). The inhibitory vl-PAG response had an onset latency of 6.4 ms and duration of 90.0 ms. 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.

Each of the RVM neurons that received a fast excitatory input from the vl-PAG was excited by stimulation of the sciatic nerve, whereas all the RVM neurons that received a delayed input from the vl-PAG were inhibited by the sciatic nerve stimulation (Table 1; Fig. 12). When stimulation of the vl-PAG was applied 10 ms prior to the sciatic nerve stimulus, there was no visible attenuation or facilitation of the sciatic response in any of the recorded cells. The two responses appeared algebraically additive.

Table 1: Converging vl-PAG and sciatic nerve inputs on RVM neurons

vl-PAG Response	Sciatic Nerve Response	
	Excitatory	Inhibitory
Early excitatory	5	-
Delayed excitatory	-	5
Inhibitory	-	1
None	3	-

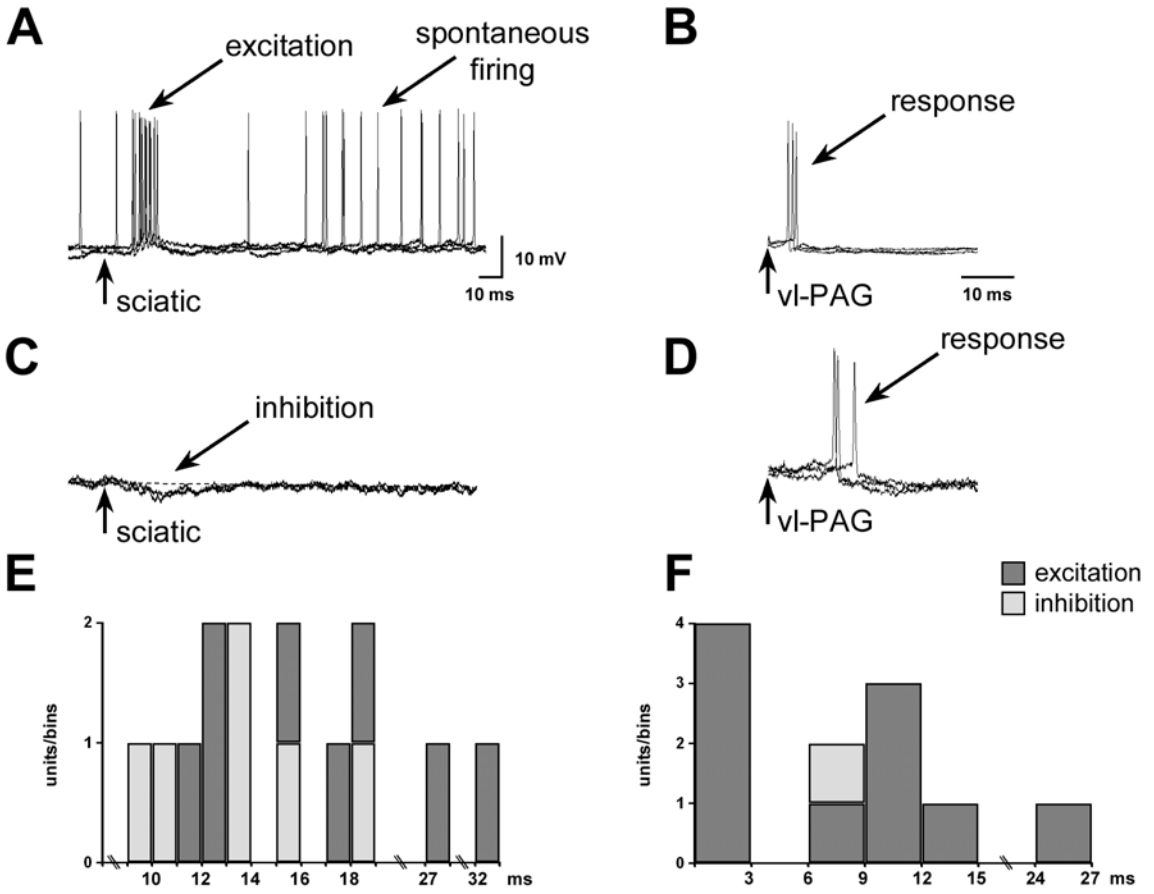

Figure 12: RVM cells that had an excitatory response to the sciatic nerve stimulation (**A**) had an early onset latency response to the vl-PAG stimulation (**B**). While cells with an inhibitory response to the sciatic nerve stimulation (**C**), had a delayed vl-PAG stimulus evoked response (**D**). Population histograms of the onset latencies of vl-PAG (**E**) and sciatic nerve evoked responses (**F**).

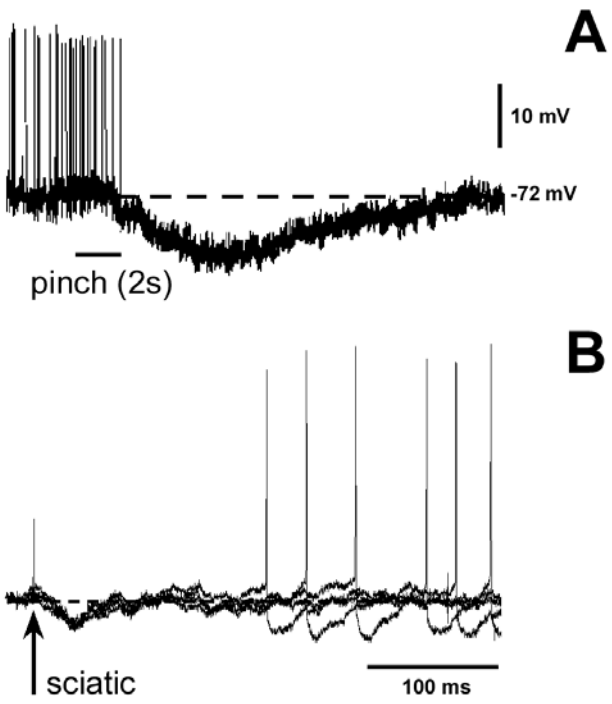

Figure 13: Recordings from an RVM neuron that responded with inhibition to both noxious mechanical stimulus of the hind limb (**A**) and electric stimulation of the sciatic nerve (**B**).

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 (Fig. 14), 4 within the NRM, 3 within the Rgcα, 2 in the Rpgl, and 2 in the Rpg.

Neurons that received fast excitatory vl-PAG inputs presented medium to large, multipolar, elongated cell bodies. The size of the somata varied from 41 x 11 to 82 x 32 μm (measured along the longest axis and at a right angle to it). They had four to eight primary dendrites with a base diameter ranging from 3 to 6 μm. Whereas the second group of RVM neurons with a delayed PAG response, tended to have small to medium (26 x 23 to 50 x 32 μm) sized, triangular cell bodies, with three to five primary dendrites, with a base diameter of 2 to 4 μm.

The primary and secondary dendrites of the labelled neurons could often be followed for several hundred microns from the cell body. The dendrites branched infrequently, forming a simple but extensive dendritic tree. The orientation, distribution, size and shapes of the cells and their dendrites were similar to those reported in previous intracellular studies of the RVM [117, 122, 205] (Fig. 14).

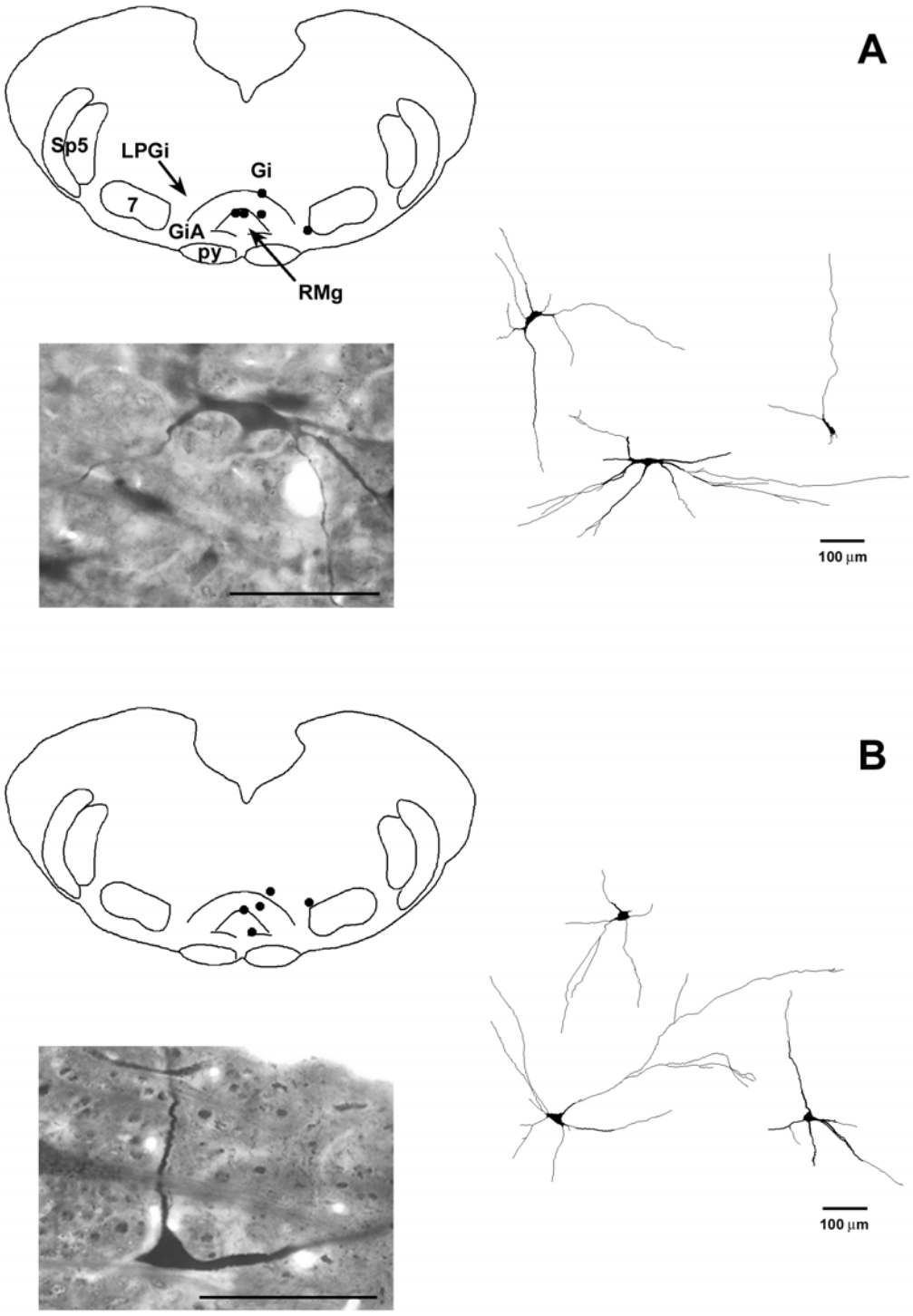

Figure 14: **A**: RVM neurons with early onset latency responses to vl-PAG stimulation, **B**: RVM neurons with delayed responses to vl-PAG stimulation. Gi: gigantocellular reticular nucleus, GiA: gigantocellular reticular nucleus, alpha part, LPGi: lateral paragigantocellular nucleus, py: pyramidal tract, RMg: raphe magnus nucleus, Sp5: spinal trigeminal nucleus, 7: facial nucleus. Bars on the photomicrographs represent 50 μm.

6. Discussion

Technical Considerations for Labelling of PAG Efferents with Phaseolus Vulgaris Leucoagglutinin

To label axon terminals of PAG efferent neurons in the pons and medulla oblongata, PHA-L, the highly sensitive anterograde tracer has been iontophoretically injected into the antinociceptive specific ventrolateral area of the PAG. A long line of experimental evidence indicates that after iontophoretic application PHA-L is internalised almost exclusively by perikarya and dendrites of neurons located within the region of the injection site [71, 72, 196, 197]. Consequently, most of the labelled axon terminals that we encountered in this study may represent terminals of neurons that are located within the confines of PHA-L injection sites. However, it has also been reported that some local axon terminals and fibres that originate outside the injection site and run through the area infiltrated by the tracer can also take up and transport PHA-L [37, 44]. Therefore in principle, in addition to axons of neurons that are located within the area infiltrated by PHA-L, axons of neurons in the posterior hypothalamus, that are reported to descend in the lateral/ventrolateral regions of the diencephalo-mesopontine periaqueductal gray [34, 186], could also take up and transport the lectin causing unspecific labelling in the brainstem. However, it is highly probable that this non-specific labelling was very weak in our experiments. First, the labelling of fibres of passage has always been found to be poor in the central nervous system of mammals [71, 72, 196, 197]. Second, in three animals in which we eliminated the central tegmental tract of descending hypothalamic fibres by making a transverse lesion throughout the entire crossectional area of the periaqueductal gray at the border of the diencephalon and mesencephalon 3 weeks before the tracer application, the distribution and density of the labelled axon terminals were identical to those that were obtained in animals with intact descending hypothalamic projections. Therefore, it seems that the unspecific labelling of fibres of passage does not interfere with the major findings of the present study.

In addition to the PAG, in some cases, the tracer clearly spread into the adjacent midbrain tegmentum. Therefore, it is necessary to raise the question whether the labelling of axon terminals of neurons in the midbrain tegmentum adjacent to the PAG might cause confusion in the interpretation of the results. A long line of experimental

evidence indicates that a partial labelling of the medial midbrain tegmental field should not result in any misinterpretation of the results obtained in this study. It has been demonstrated that the vl-PAG and the adjacent area of the midbrain tegmentum form a functionally homogeneous territory [24, 46, 78, 94, 105]. The electric stimulation of both fields evokes profound inhibition of nociceptive specific and wide dynamic range neurons in the spinal dorsal horn [32, 33, 78], and the two areas may also work together in the initiation of vocalization [94]. The spinal antinociceptive effects of the tegmentum, e.g. inhibition of heat evoked discharges of spinal neurons, are even stronger than that evoked by the activation of the vl-PAG [32]. In addition, the vl-PAG and the adjacent midbrain tegmentum receive identical ascending sensory inputs from the spinal cord [24, 46, 105]. It appears that the medial midbrain tegmental field can be regarded as an area that is functionally closely related to the vl-PAG, thus a partial labelling of this territory is unlikely to lead us to dubious conclusions concerning the distribution of the genuine population of PAG efferents in the pons and medulla oblongata.

Technical Considerations of Ventrolateral PAG Electrical Stimulation and Intracellular Recording of RVM Neurons

In addition to local neurons, electric stimulation of the vl-PAG may also activate axons that originate outside the vl-PAG and run through the stimulated area. Therefore, in principle, in addition to neurons in the vl-PAG axons of neurons in the posterior hypothalamus that are reported to descend in the lateral/ventrolateral regions of the diencephalo-mesopontine PAG [34, 186] could also be activated causing unspecific stimulation of brainstem neurons. In addition, due to current spread the possible simultaneous stimulation of the adjacent midbrain tegmentum and the ventral as well as the lateral cell columns of the PAG can neither be ruled out. However, it is highly probable that this unspecific current spread and stimulation of fibres of passage was very weak in our experiment. Previous studies have clearly shown that the current spread is negligible at electric stimulations with intensities up to 200 μA [2, 119, 120, 157], and our present observations indicate the same. In a number of cases we stimulated the vl-PAG initially with 100 μA, then the stimulus intensity was increased up to 300 μA. The increase of the intensity of the stimuli never caused any noticeable

change in the response pattern. Therefore, it seems that the unspecific stimulation of the neurons and axons in the vicinity of the vl-PAG was minimal.

Of all the RVM cells with somatosensory inputs, only one neuron exhibited an inhibitory response to vl-PAG stimulation. Because of the low incidence of inhibitory responses, it is necessary to raise the question whether this is a real representation of neurons receiving inhibitory vl-PAG inputs in the RVM? Extracellular and intracellular studies have always reported that the proportion of RVM cells with an inhibitory versus those with an excitatory response to vl-PAG stimulation is low [119, 154, 170, 185]. Similar results were also observed by Mohrland and Gebhart (1980), when they compared the effects of electrical stimulation of, and morphine microinjection into the PAG upon neurons in the medullary reticular formation. Thus, it appears that stimulation of the vl-PAG evokes primarily excitatory postsynaptic potentials on RVM neurons, although the unavoidable sampling bias towards larger cells when using intracellular electrodes must also be taken into consideration as a possible explanation for the lower proportion of cells with an inhibitory response.

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

The termination pattern of efferent fibres arising from the PAG has extensively been investigated in the pons and medulla oblongata using degeneration [80] and various anterograde tract-tracing methods [11, 31, 94, 118 130]. Most of the results obtained in the present experiment are in general agreement with the findings of these 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.

Similar to our results, it has been demonstrated that the vl-PAG innervates the parabrachial nuclei and suggested that this innervation functions as a behavioural state-dependent filter system that modulates ascending nociceptive information as it is relayed through the parabrachial nuclei to forebrain sites [106]. It has also been shown that a number of neurons in the vl-PAG send efferent fibres to the trigeminal sensory complex, presumably suppressing the activity of nociceptive neurons in the trigeminal system [112]. Confirming results of previous studies here we also demonstrated that the vl-PAG projects to the branchiomotor nuclei (motor nuclei of the trigeminal, facial, vagus and accessory nerves) and nucleus retroambiguous via which the PAG may produce excitation of motoneurons involved in vocalisation [56, 94, 95]. Previous anatomical and physiological studies have also shown, similarly to our present observations, that the vl-PAG innervates the rostral and caudal ventrolateral medulla through which it may regulate cardiovascular and respiratory functions [35, 36, 88, 183].

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 [98, 109], cardiovascular [12, 116] and nociceptive [12, 15, 65, 124, 165, 189] 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 [11, 31, 39, 41]. 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 Rgcα and Rpgl, whereas the NRM 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 serotonergic and noradrenergic neurons [11, 110, 159] and excite them through NMDA and AMPA receptor mechanisms [2, 194]. In support to this idea, 5HT receptor antagonists attenuate the antinociceptive effects of PAG

stimulation on dorsal horn cells [33, 149, 152, 198, 203]. Neurotoxic depletion of 5HT also results in the attenuation of PAG-evoked inhibition of dorsal horn neurons [33]. Combining experimental degeneration of PAG efferents with immunocytochemical detection of 5HT, however, Lakos & Basbaum (1988) found that in addition to serotonergic neurons non-serotonergic cells in the RVM also receive monosynaptic inputs from the PAG. In addition, a recent study by Gao et al. (1997), 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 serotoninergic-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 the findings of Gao et al. (1997). 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.

Here 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 using anterograde tracing with PHA-L [55, 183] or retrograde tracing with wheat germ agglutinin conjugated to horseradish peroxidase [55]. 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. However, we have to admit that the relationship between PAG afferents and noradrenergic cells in the pons does not seem to be so clear-cut as we suggested above. That is, it has recently been reported by Bajic & Proudfit (1999) that the vl-PAG projects strongly to the pontine noradrenergic cell groups and a substantial number of terminals arising from the PAG establishes close appositions with THIR neurons within these nuclei in the Sasco substrain of Sprague-Dawley rats. It is difficult to explain the differences between our results and those of Bajic & Proudfit (1999), but it is likely that the reported differences in the projections of

the vl-PAG to pontine noradrenergic cell groups may arise from at least two sources. First, injections made by Bajic & Proudfit (1999) were restricted to the vl-PAG, whereas in addition to the PAG the tracer clearly spread into the adjacent midbrain tegmentum in our experiments. The differences in the precise locus of PHA-L may well account for the reported differences in the termination fields of labelled axon terminals. Second, it may reflect real differences between neural circuits in the Sasco substrain of Sprague-Dawley and in other strains of rats. This notion is reinforced by other reports that document fundamental differences in the spinal projections [39, 41, 42, 156] and physiological functions [77, 190] of noradrenergic neurons in Sasco and other substrains of Sprague-Dawley rats.

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, the authors have not paid too much attention 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. The parvocellular reticular nucleus of the pons and the pontine reticular nucleus have been shown to send a strong projection to the RVM [90]. It has also been demonstrated that by making lesions in the dorsal medullary reticular field through local application of electric current or quinolinic acid formalin evoked pain behaviours can substantially be attenuated [7]. 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

Up till recently it has been widely thought that the vl-PAG exerts its modulatory control over the RVM neurons through a direct, monosynaptic pathway [15, 31, 110, 154, 167]. However, our morphological results, clearly demonstrated that only some of the vl-PAG efferents terminate in the RVM, most of them project to the intermediate subdivision of the ponto-bulbar reticular formation [142]. This, and recent reports that electrical stimulation of the vl-PAG does not lead to monosynaptic activation of spinally projecting RVM serotoninergic neurons [68], 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.

Considering the findings that short and long latency inputs from the vl-PAG are received by distinct types of RVM neurons that also displayed different responses to stimulation of the sciatic nerve, the possibility that the direct and indirect pathways between the PAG and RVM subserve different physiological or even behavioural roles must be considered [12, 65, 109, 116, 124].

Ventrolateral PAG Evoked Short Onset Latency Excitatory Responses in RVM Neurons

Assuming that the conduction distance between the vl-PAG and the RVM is approximately 4 mm, the synaptic delay is 1 ms, and the range of conduction velocities

of small myelinated fibres is 0.5-2 0 m/sec [154, 167], the short onset latencies (1.8 to 6.9 ms) could very well represent a direct, monosynaptic pathway from the vl-PAG to the RVM [15, 110, 117, 119, 142].

In this study we did not aim to identify the RVM cells as ON or OFF for various reasons, partly because of the differences in the experimental circumstances e.g. anaesthesia [145]. Nevertheless, 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 [63, 65, 124]. 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. Since the diameter and thus the conduction velocity of the axons of vl-PAG projection neurons has been reported to vary in a wide range, we have to raise the question whether the differences in onset latencies may merely be the result of variations in the conduction velocity of the different subsets of nerve fibres. Examining the conduction velocities of fibres projecting from the PAG to the RVM, Pomeroy and Behbehani (1979) and Shah and Dostrovsky (1980) found that the conduction velocities varied in the range of 0.5-2.0 m/sec. As they calculated, the maximum latency of a direct projection from the PAG to the RVM could not be longer than 9 ms. Thus it is highly probable that neurons that responded with onset latencies longer than 9 ms in our study were activated through 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 [63, 65, 124]. 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 really represent functionally different populations of RVM neurons.

It has been, postulated that OFF cells are activated through a disinhibitory mechanism [169]. 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 [65, 93, 169]. 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

The iRF consists of the pontomedullary intermediate reticular nucleus, the pontine reticular nucleus, the parvocellular reticular nucleus and the dorsal medullary reticular field [177]. All these reticular nuclei have been shown to have projections towards the RVM and the pontine noradrenergic cell group, particularly that of the A7 cell group [59, 90, 92]. Furthermore, physiological and pharmacological investigations suggest that these areas may participate in the processing of both nociceptive and cardiovascular information, acting as a central integrator for pain and cardiovascular related functions [7, 92, 107, 178]. In our anatomical studies, we found that this region 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 [65]. Experimental studies indicate that activation of a GABA containing input is responsible for the OFF cell pause [86, 122]. 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 [13]. It is also possible that the GABA containing cells that mediate the OFF cell pause are not intrinsic but extrinsic to the RVM [169]. 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 [62, 82, 87, 129, 148]. 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 [62, 86, 87, 122, 148]. The RVM contains a high density of ENK immunoreactive axon terminals that are received mostly by ON cells [123]. 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.

7. 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 [7] or more likely towards the RVM and pontine noradrenergic cell groups [90]. 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 [9, 29, 99, 102, 168], 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 [12, 15, 65, 110, 124, 159]. The spinally projecting neurons, both serotonergic and noradrenergic as well as non-serotonergic and non-noradrenergic [9, 29, 99, 102, 168], 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 [6, 9, 15, 39, 41, 53, 65, 67, 100, 124], and the released neurotransmitters may evoke inhibition in spinal neural circuits underlying nociceptive information processing, that results in analgesia and attenuation of pain behaviour [68, 78, 115, 189].

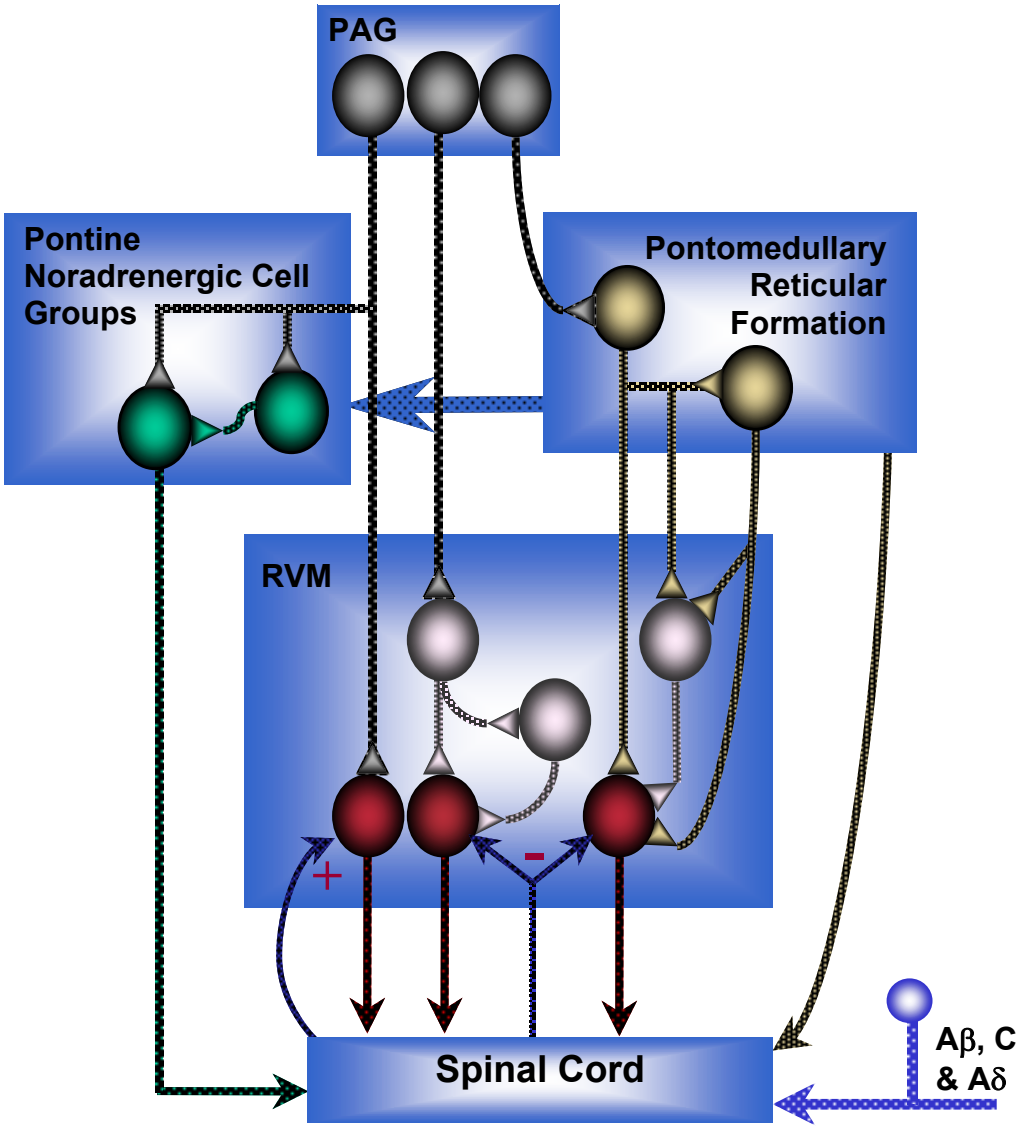

Figure 15: Schematic representation of our results and conclusions.

8. Summary

Summing up our results, we have shown the following:

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.

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11. 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

1. **Odeh, F.** and Antal, M.: The projection of the midbrain periaqueductal gray to serotonergic and noradrenergic nuclei of the pons and medulla oblongata in the rat. Neurobiology: 6. 235-236 (**1998**)
2. Antal, M. and **Odeh, F.**: The projection of the midbrain periaqueductal gray to serotonergic and noradrenergic nuclei of the pons and medulla oblongata in the rat. Eur. J. Neurosci. 10, Supp. 10, 218 (**1998**)
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8. Szűcs, P., **Odeh, F.**, Szokol, K. and Antal, M.: Electrophysiological and morphological characteristics of neurons in the intermediate gray matter (laminae V-VII) of the neonatal rat lumbar spinal cord in vitro. Neurobiology: (**2002**)
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10. Szűcs, P., **Odeh, F.**, Szokol, K. and Antal, M.: Electrophysiological and morphological characteristics of neurons in the intermediate gray matter (laminae V-VII) of the neonatal rat lumbar spinal cord in vitro. Eur. J. Neurosci. (**2002**)