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# Autoradiographic and electrophysiological evidence for excitatory amino acid transmission in the periaqueductal gray projection to nucleus raphe magnus in the rat

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Selective retrograde labelling was used as an autoradiographic method to identify possible excitatory amino acid afferents to nucleus raphe magnus (NRM). Injections of 25–50 nl  $10^{-2}$  or  $10^{-3}$  M D-[<sup>3</sup>H]aspartate into the NRM resulted in prominent labelling of cells in ventrolateral mesencephalic periaqueductal gray (PAG). Electrophysiologically, stimulation in ventrolateral PAG excited cells in NRM with a latency of 2–12 ms. With many cells, microelectrophoretic application of the excitatory amino acid antagonists, kynurenate and  $\gamma$ -D-glutamyl-glycine, resulted in a reversible reduction of the PAG-evoked response. Selective antagonists of N-methyl-D-aspartate (NMDA) were less effective. It is suggested that neurones in the ventrolateral PAG projecting to NRM utilize an excitatory amino acid or structurally related compound as a transmitter, and that this transmitter acts on receptors of the non-NMDA type.

The periaqueductal gray (PAG) projection to the nucleus raphe magnus (NRM) is believed to play an important role in descending nociceptive control, opiate and stimulation produced analgesia (for review see ref. 3). Several transmitters have been implicated in the PAG–NRM projection. Beitz [6] demonstrated that some PAG neurones projecting to the NRM were immunoreactive for neurotensin. More recently, it was shown that many PAG cells stain with immunocytochemical markers for excitatory amino acids [9], and behavioural evidence implies excitatory amino

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acid transmission of the PAG–NRM projection [1]. Selective retrograde labelling with D-[<sup>3</sup>H]aspartate [20] has proved a useful method for predicting excitatory amino acid connections [4, 14, 15] (for review see ref. 10). In the present study, we have used this autoradiographic technique to identify possible excitatory amino acid projections from the mesencephalic PAG to NRM, and have subsequently used electrophysiological techniques to test the effects of excitatory amino acid antagonists on neurotransmission in this system.

*Neuroanatomical experiments.* Twelve Sprague–Dawley rats (250–300 g) were used. D-[<sup>3</sup>H]aspartate (D-[2,3-<sup>3</sup>H]aspartic acid, Amersham, 10–22 Ci/mmol) was evaporated under a stream of nitrogen, and redissolved in distilled water or saline to a concentration of 10<sup>-2</sup> or 10<sup>-3</sup> M. Rats were anaesthetized with pentobarbitone (40 mg/kg) (*n* = 5), or a mixture of ketamine (Imalgène, Rhône Mérieux; 65 mg/kg) and xylazine (Rompun, Bayer; 14 mg/kg) (*n* = 7), all i.p. Stereotaxic microinjections of 25 or 50 nl D-[<sup>3</sup>H]Asp solution were delivered over 10 min into the NRM through glass micropipettes (outer diameter 20–30 µm) glued to a 0.5 µl Hamilton syringe; pipettes were left in situ for another 5 min before withdrawal. After 6, 12 or 24 h survival, the rats were re-anaesthetized and perfusion fixed; blood was rinsed out with 100 ml Rheomacrodex–NaCl, followed by 1200 ml of 3.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, over 40 min. Dissected brains were kept overnight in the same fixative, and were transferred to 30% sucrose in the same buffer until they sank. Frozen sections were cut at 20 or 30 µm, every fifth section mounted on subbed slides, dried, defatted, and dipped in Kodak NTB-2 emulsion diluted 1:1 with distilled water. Autoradiograms were developed in Dektol after 4 or 8 weeks exposure, stained with Cresyl violet, coverslipped, and evaluated by bright and dark-field microscopy (for details, see ref. 10).

*Electrophysiological experiments* were performed on 15 rats anaesthetized with pentobarbitone (*n* = 4) or pentobarbitone supplemented with α-chloralose (*n* = 11). Two Tungsten stimulating electrodes, spaced 2 mm apart and mounted on a micro-manipulator, were lowered into the PAG at a ventrocaudal angle of 45° using a dorsal approach; they were positioned initially 1–2 mm anterior and 5 mm dorsal to the interaural line, and were subsequently tracked to obtain the site requiring the lowest stimulation strengths to elicit responses in NRM neurones. Stimulation parameters were 30–200 µA and 0.2–1.0 ms at 1 or 2 Hz. Multibarrel micropipettes were inserted into NRM using a ventral approach. The centre barrels of pipettes contained 3.5 M NaCl and were used for extracellular recording of unitary NRM activity. Side barrels contained combinations of the following drugs for microelectroretic administration; kynurenate Na (100 mM in 100 mM NaCl), γ-D-glutamylglycine Na (DGG; 200 mM), (±)-2-amino-5-phosphonovalerate Na (AP5; 50 mM in 150 mM NaCl), quisqualate Na (5 mM in 195 mM NaCl), kainate (5 mM in 195 mM NaCl) and *N*-methyl-D-aspartate (NMDA; 100 mM in 100 mM NaCl), all at pH 7.5–8.0; ketamine HCl (50 mM in 150 mM NaCl, pH 5) and Pontamine sky blue (2% in 500 mM sodium acetate, pH 7.7) which was ejected at selected recording sites. In some experiments the effect of intravenous ketamine (4 mg/kg) was tested. Tests were recorded as oscilloscope records, as peristimulus histograms (bin width 0.1–1 ms) and as counts of

evoked spikes. Histograms were of 128 or 256 sweeps and were constructed automatically every 2–3 min throughout recording periods. On some cells tests were performed (not illustrated) to check the selectivity, between amino acid analogues, of AP5 and ketamine at the doses tested on the evoked responses. At the end of experiments, the position of stimulating electrodes was marked by electrolytic lesioning. The dissected brains were fixed in 4% formaldehyde, sectioned on a freezing microtome, and positions of stimulating and recording sites identified.

The D-[<sup>3</sup>H]Asp injection sites were usually well centred in the NRM, and were characterized by a central zone of high grain density which progressively decreased towards the periphery (Fig. 1A). In all experiments, retrogradely labelled cells appeared in the mesencephalic PAG (Fig. 1B, C). The number of labelled PAG cells and their labelling intensity depended on the amount of D-[<sup>3</sup>H]Asp injected into NRM; in cases injected with 10<sup>-2</sup> M solution several hundred labelled cell profiles were detected in each series of autoradiograms. The labelled PAG cells were of small to moderate size, and often demonstrated labelling of a few primary dendritic processes (Fig. 1C). The labelled cells were concentrated in the ventrolateral portion of

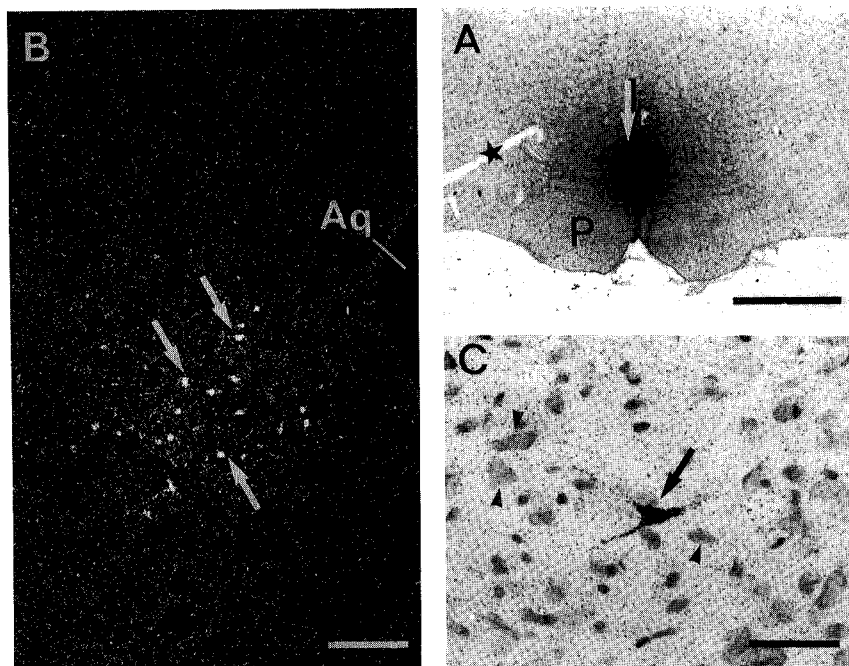


Fig. 1. Autoradiographic labelling observed after injection of 50 nl D-[<sup>3</sup>H]Asp, 10<sup>-2</sup> M, into the nucleus raphe magnus. A: low-power bright-field view of the injection site (arrow) at the level of the facial nucleus. P, pyramid; asterisk, cut in tissue for identification of left hand side. Bar = 1 mm. B: low-power dark-field view of retrogradely labelled cells (arrows) in the ventrolateral periaqueductal gray. Aq, cerebral aqueduct. Bar = 200  $\mu$ m. C: high-power bright-field micrograph showing a well labelled cell in PAG (arrow), and several neighbouring unlabelled cells (arrowheads). Bar = 50  $\mu$ m.

PAG. Comparison with the careful mapping of serotonergic cells by Descarries et al. [12], indicated that a few D-[<sup>3</sup>H]Asp-labelled cells were situated within the more medial region of PAG that contain serotonergic cells. In contrast, very few labelled cells were found in the dorsomedial part of PAG, which is also known to project to the NRM [8]. Other mesencephalic regions known to project to the NRM, such as the cuneiform nucleus and superior colliculus [8], showed no or very few labelled cells.

In electrophysiological experiments, cells in the NRM which responded to PAG stimulation were found readily. Most of the marked stimulation sites were in the ventrolateral PAG, corresponding to the region where an accumulation of labelled cells had been observed in the D-[<sup>3</sup>H]Asp tracing experiments. The latency of evoked responses varied between cells from 2 to 12 ms.

Forty-five NRM cells responding to PAG stimulation were tested with microelectrophoretic administrations of antagonists of varying selectivity for the 3 commonly recognized receptors for excitatory amino acids [2, 21]. Fig. 2 illustrates the effect of kynurenate, an excitatory amino acid antagonist which does not distinguish between different subclasses of glutamate receptors [19], on the PAG-evoked responses of an NRM neurone. Kynurenate reduced the shorter latency component of PAG responses, while the later responses were less affected. Another non-selective excitatory amino acid antagonist, DGG, also decreased PAG-evoked responses but higher electrophoretic currents were usually required than with kynurenate. In all, about one quarter of the cells tested with kynurenate (10–100 nA) demonstrated a marked reduction ( $\geq 50\%$ ) of the PAG-evoked responses, another quarter of the cells showed a less pronounced effect, while half of the cells showed no or very little effect

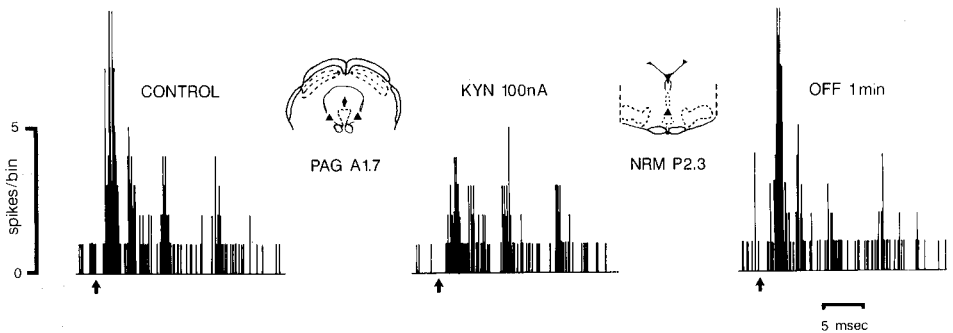


Fig. 2. Responses of an NRM neurone to stimulation of the PAG. NRM neurone location 2.3 mm posterior to the interaural line, as indicated by the insert diagram. PAG electrode sites shown on insert diagram; electrical stimuli were of 160  $\mu$ A and 0.5 ms repeated at 2 Hz. The evoked responses were averaged in a peristimulus time histogram (0.1 ms bins) collected over 256 consecutive sweeps. The arrows under the histograms indicate the onset of the stimulus. The record is suppressed during the period of the stimulus and resulting artefact. After the control period (left histogram) kynurenate was ejected microelectrophoretically at 100 nA for 1 min before the start of the next accumulation (centre histogram). One min after the end of the kynurenate ejection the right hand histogram collection was started. Note that the shorter latency part of the evoked response was reduced preferentially during the kynurenate ejection.

of the antagonist. The responses of most NRM cells were resistant to the selective NMDA antagonists ketamine or AP5.

The selectivity of retrograde labelling with D-[<sup>3</sup>H]Asp depends on how selective the axon terminal uptake mechanisms are for neurones which utilize excitatory amino acid (or related) neurotransmitters [10]. Since this uptake system fails to distinguish between L-Glu, L-Asp and D-Asp [11], the method does not indicate whether glutamate or aspartate is more likely to be the endogenous transmitter. Moreover, it has been claimed that a dipeptide active at excitatory amino acid receptors, *N*-acetyl-aspartyl-glutamate, may be the transmitter of certain neuronal pathways. Recently, it has been suggested that such neurones may demonstrate glutamate uptake [7]. It is, therefore, possible that neurones utilizing this dipeptide could be labelled retrogradely with D-[<sup>3</sup>H]Asp.

The characteristics of the PAG-evoked responses recorded electrophysiologically in NRM were similar to those reported previously [5, 16–18]. The spread of latencies of PAG-evoked responses is consistent with the conduction velocities of PAG–NRM axons in rats which vary between 0.4 and 9.6 m/s [18] so that stimulation in the PAG would evoke monosynaptic potentials with a latency range as wide as 0.5–15 ms; monosynaptically evoked spikes are therefore likely to have a variable latency. Our autoradiographic experiments with D-[<sup>3</sup>H]Asp indicate that there is a direct excitatory amino acid projection from PAG to NRM, but electron-microscopical investigation of identified PAG terminals would be necessary to demonstrate if they synapse directly on the NRM cells and their dendrites. The preferential sensitivity of the earlier components of evoked responses to the amino acid antagonists is however supportive of amino acid mediation of the PAG projection neurones.

The susceptibility of responses to kynurenate and DGG provides no evidence on the amino acid receptor types mediating the response. Selective NMDA antagonists were, however, markedly less effective, whether administered electrophoretically (AP5 and ketamine) or intravenously (ketamine) at NMDA-blocking doses [2]. These observations therefore suggest that the excitatory amino acid (or structurally related) neurotransmitter acts on receptors of the non-NMDA type.

Interestingly, the region of the PAG which was preferentially labelled in our autoradiographic experiments, and stimulated in our electrophysiological tests, seems to correspond to the 'pure analgesic region' in the ventrolateral PAG described by Fardin et al. [13] in their topographic analysis of stimulation produced analgesia. Moreover, Aimone and Gebhart [1] have demonstrated in behavioural experiments that the antinociceptive effects of stimulating the ventrolateral PAG can be reduced by microinjections of excitatory amino acid antagonists into the NRM. These combined observations indicate that the component of the PAG–NRM projection identified by the present study plays a significant role in descending nociceptive control.

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