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Evidence for an excitatory amino acid pathway in the brainstem and for its involvement in cardiovascular control

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The source and possible role of excitatory amino acid projections to areas of the ventrolateral medulla (VLM) involved in cardiovascular control were studied. Following the injection of [^{3}H]D-aspartate ([^{3}H]D-Asp), a selective tracer for excitatory amino acid pathways, into vasopressor or vasodepressor areas of the VLM in rats, more then 90% of retrogradely labelled neurones were found in the nucleus of the solitary tract (NTS). Very few of the [^{3}H]D-Asp-labelled cells were immunoreactive for tyrosine hydroxylase, none for phenylethanolamine-*N*-methyltransferase or γ-aminobutyric acid. The density of labelled cells in the NTS was similar to that obtained with the non-selective tracers wheat germ agglutinin–horseradish peroxidase (WGA–HRP) and WGA–colloidal gold, but these tracers also labelled other cell groups in the medulla. Furthermore, the decrease in blood pressure, caused by pharmacological activation of neurones in the NTS of rats, or by electrical stimulation of the aortic depressor nerve in rabbits could be blocked by the selective *N*-methyl-D-aspartate (NMDA) receptor antagonist 2-amino-5-phosphonovalerate injected into the caudal vasodepressor area of the VLM. This area corresponds to the termination of [^{3}H]D-Asp transporting NTS neurones. These results provide evidence that a population of NTS neurones projecting to the VLM use excitatory amino acids as transmitters. Among other possible functions, this pathway may mediate tonic and reflex control of blood pressure via NMDA receptors in the VLM.

The medulla oblongata is the principal site for the integration of information regulating blood pressure. The nucleus tractus solitarii (NTS) in the dorsomedial medulla receives sensory afferents subserving many vegetative functions, including baroreceptor afferents contributing to cardiovascular control[18,22]. The major output of the NTS is directed to the ventrolateral medulla (VLM) where, among other homeostatic centres, a rostral vasopressor and a caudal vasodepressor area regulate cardiovascular function[7,8,11,18,22]. Since the activity of neurones in the VLM is strongly influenced by their input from the NTS[1], the nature of the transmitter mediating the output of the NTS is of major interest. As baroreflexes are blocked by excitatory amino acid antagonists injected into the VLM, excitatory amino acids have been implicated in neurotransmission in the VLM[10,12,15]. In this study we investigated the source of excitatory amino acid projections to areas of the VLM involved in cardiovascular regulation, using the retrograde transport of [^{3}H]D-aspartate ([^{3}H]D-Asp), a tracer that specifically reveals excitatory amino acid pathways[5]. Furthermore, extending previous reports in the rat[10,15] we examined the effect of 2-amino-5-phosphonovalerate (2-APV), a selective *N*-methyl-D-aspartate (NMDA) receptor antagonist injected into caudal VLM (CVLM), on the vasodepressor response evoked by the pharmacological stimulation of NTS neurons. The effect of 2-APV was also studied on the decrease of blood pressure evoked by electrical stimulation of the aortic depressor nerve (ADN) in rabbits, a species where aortic baroreceptors can be activated selectively. The results provide evidence that a population of NTS neurones projecting to the VLM uses excitatory amino acids as transmitters, and that

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NMDA receptors in the VLM may mediate the effect of these neurones on blood pressure.

Rats (Wistar–Kyoto, 250–350 g) were anaesthetised with sodium pentobarbitone (60 mg/kg)/chloral hydrate (100 mg/kg) i.p., cannulated through the femoral artery for arterial pressure monitoring, intubated and mechanically ventilated with O_2-enriched air. For CVLM injections the head was flexed to 45° in a stereotaxic frame and the dorsal surface of the medulla was exposed. For rostral VLM (RVLM) injections the incisor bar was set at +5 mm. Injections (WGA conjugated to 17 nm colloidal gold, prepared in house, was suspended in distilled water; WGA–HRP, Sigma, 2.5% in artificial cerebrospinal fluid [CSF]; [^{3}H]D-Asp, 10–20 μCi, 4.5 mM in CSF, Amersham, 22 Ci/mmol) were delivered in 5–20 nl steps over 30–60 min through micropipettes (tip 30–50 μm) placed in CVLM (approximately at AP –1.0 mm from obex, L 1.8 mm, DV –2.7 mm; head tilted 45° forward) or RVLM (approximately at AP –3.0 mm to the interaural line, L 2.0 mm, DV –9 to –9.5 mm from dura; incisor bar at +5 mm). To identify spinally projecting cells, animals with [^{3}H]D-Asp or WGA–gold injections in the medulla also received injections of WGA–HRP into the intermediolateral column of the spinal cord delivered via glass micropipettes (T_8–T_{10}, 5 × 100 nl, 2.5% in CSF, Sigma; coordinates L = 0.6–0.7 mm from midline, DV = 0.6–0.8 mm) one day prior to medullary injections. Although spinal cord injections were centered on the IML, the tracer probably spread to other areas as well, as shown by the widespread retrograde labelling of medullary neurones. Two to twenty hours after injections in the medulla animals were perfused with 3% glutaraldehyde in 0.1 M phosphate buffer. The brains were sectioned (60 μm) and using standard procedures alternate series of sections were reacted to visualize one or more of the following: HRP with Ni^{2+}/DAB histochemistry; gold with silver intensification (Janssen); [^{3}H]D-Asp with autoradiography (Ilford K5 emulsion, Kodak D19 developer); TH (rabbit antiseum to TH[25]) or, PNMT (rabbit antiserum to PNMT[14]) with peroxidase immunocytochemistry; Nissl substance with Cresyl violet.

Some sections from each [^{3}H]D-Asp-injected animal were dehydrated, embedded in epoxy resin, cut at 1 μm and reacted to visualize in serial sections either γ-aminobutyric acid (GABA) by postembedding immunocytochemistry[21] or [^{3}H]D-Asp by autoradiography.

Rats used only for physiological recording were anaesthetised and prepared as described above. The head was then flexed to 45°, and the dorsal surface of the medulla was exposed via the cisterna magna. Micropipettes (tip 30–50 μm) were stereotaxically placed in CVLM and in caudal NTS. The depressor area of CVLM was first identified using serial L-Glu injections (1 nmol in 10 nl) as the pipette was passed toward the ventral surface. L-Glu evoked short-lasting falls in arterial pressure and heart rate at several sites, with a strong response observed at AP –1.0 mm from obex, L 1.8 mm, DV –2.7 mm from the dorsal surface. Depressor sites were then localized in caudal NTS. At AP –0.2 mm, L 0.7 mm, DV 0.7 mm from the dorsal surface L-Glu always lowered arterial pressure and heart rate. 2-APV (0.5 nmol, in 20 nl) or vehicle was applied at the depressor site identified in CVLM. From 2 to 80 min after 2-APV injection L-Glu (1 nmol in 10 nl) was injected in the identified sites in NTS ipsilateral and contralateral to the CVLM injection.

Rabbits for physiological studies were anaesthetized with halothane (n = 4) or urethane (n = 4). The head was flexed in a stereotaxic apparatus, and the dorsal surface of the medulla was exposed. Arterial pressure was monitored with a femoral arterial catheter. Microinjections were made via a glass micropipette (tip 50–80 μm). Coordinates for injection in the CVLM were, from obex: AP –1.0 mm, ML 3.0 mm, DV –3.0 mm from the dorsal surface. The location of the pipette in the CVLM was verified for each site by injection of 5 nmol L-Glu dissolved in 50 nl saline. The aortic depressor nerve, identified electrophysiologically by its pulsatile discharge synchronized to the cardiac rhythm, was exposed on one side, placed on bipolar stimulating electrodes, and activated with a 10 s stimulus train (50 Hz, 0.1 ms pulse duration, 11 V). 2-APV (5 nmol in 100 nl) was injected bilaterally, and the ADN stimulated at 1, 5, 10 and 15 min after injection, and every 15 min thereafter for at least 90 min. Injection sites were localized histologically by injection of Pontamine sky blue following the experiment, and were found to be in the CVLM

depressor area as described previously[4].

Retrograde axonal transport of [^{3}H]D-Asp has been used extensively in the brain for the identification of pathways which use excitatory amino acids as transmitters (for review see ref. 5). Thus, to trace potential excitatory amino acid pathways to the VLM, [^{3}H]D-Asp was injected into the pressor region in the RVLM or into the depressor region in the CVLM of rats (Fig. 1). When injected into the RVLM [^{3}H]D-Asp evoked rises in blood pressure and elevation of heart rate, similarly to L-Glu (Fig. 1). In agreement with previous studies[6,17–19] the vasopressor injection sites contained neurones projecting to the spinal cord and/or positive for the adrenaline synthesizing enzyme phenylethanolamine-*N*-methyltransferase (PNMT). When injected into CVLM depressor region, either in the area containing tyrosine hydroxylase (TH)-positive cells, or dorsal to this area (Fig. 1) [^{3}H]D-Asp evoked depressor responses with associated decreases in heart rate. The depressor region dorsal to the TH-positive cells in the CVLM contained numerous spinally projecting neurones, and also neurones projecting to the RVLM.

After either RVLM or CVLM injections, more then 90% of the neurones that selectively accumulated [^{3}H]D-Asp in the medulla were in the NTS, predominantly ipsilaterally, and in the area post-

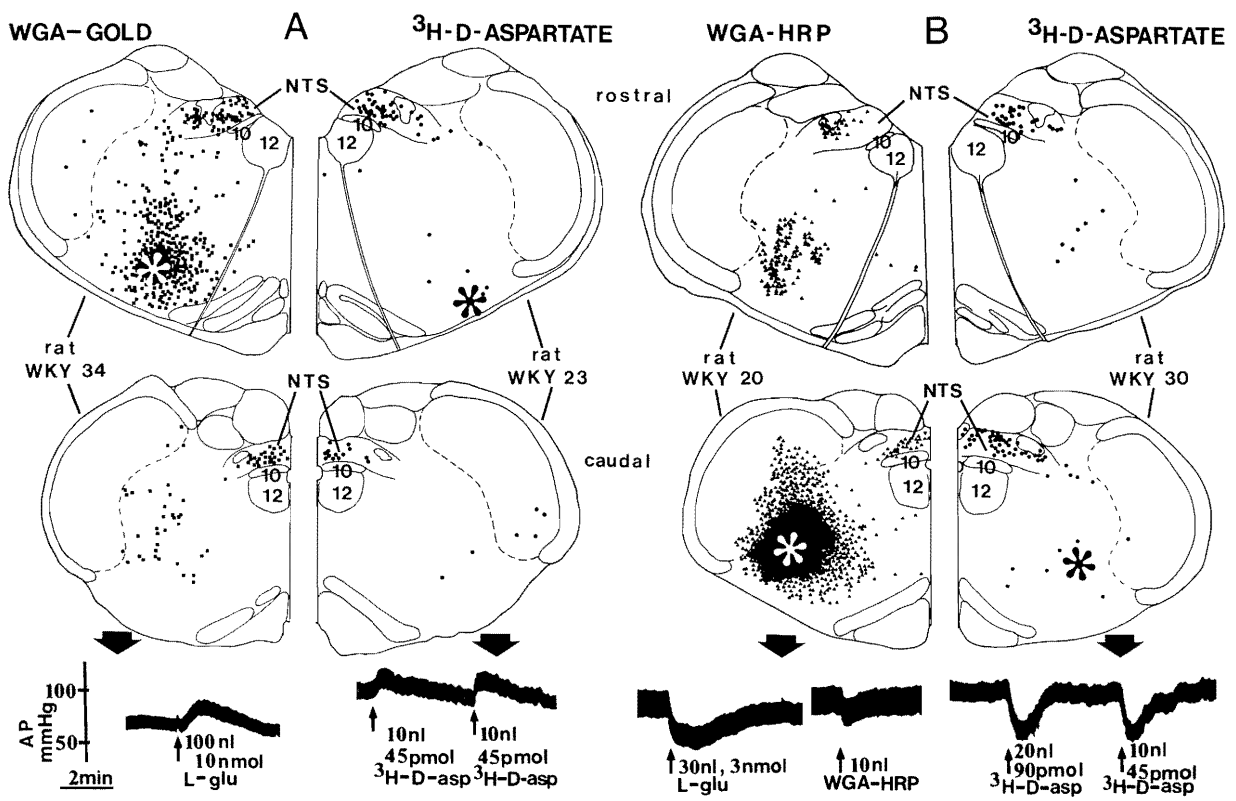

Fig. 1. Selective retrograde labelling of neurones in the NTS by [^{3}H]D-Asp. A and B: distribution of neurones labelled by WGA–gold (squares, A), WGA–HRP (triangles, B) or [^{3}H]D-Asp (dots, A,B) following injections (asterisks) into the rostral pressor area (A) or the caudal depressor area (B). The pressor area contained neurones projecting to the spinal cord and/or immunopositive for PNMT, the depressor area contained neurones projecting to the spinal cord but negative for PNMT. All tracers labelled numerous cells in the NTS but, in contrast to WGA–gold and WGA–HRP, the majority of [^{3}H]D-Asp-labelled cells were in the NTS. Bottom: the effect of the tracers on blood pressure, at sites showing a pressor or depressor response to L-Glu. 10, dorsal motor nucleus of the vagus; 12, hypoglossal nucleus.

rema, with few labelled cells in the VLM (Figs. 1, 2). For comparison, and to demonstrate the selectivity of [^{3}H]D-Asp, we also injected the non-specific retrograde tracers WGA–gold or WGA–HRP into the RVLM or CVLM. The density of cells in the NTS retrogradely labelled with the non-specific tracers was very similar to that with [^{3}H]D-Asp (Fig. 1), indicating that the majority of NTS cells projecting to the VLM also accumulate [^{3}H]D-Asp. However, in contrast to [^{3}H]D-Asp, WGA–gold and WGA–HRP labelled numerous cells around the injection sites, as well as neurones retrogradely in both caudal and rostral areas of the VLM. The connections between rostral and caudal areas of the VLM have been shown previously with non-selective tracers[3,18,20], and the paucity of labelling by [^{3}H]D-Asp suggests that most of these cells do not use excitatory amino acids as neurotransmitters. Indeed physiological studies suggest that some of the neurones projecting from CVLM to RVLM use GABA as transmitter[2,24,26].

Immunocytochemical demonstration of PNMT or TH in the same sections prior to autoradiography, or of the inhibitory amino acid GABA in serial semithin sections (Fig. 2C,D) showed that apart from very few TH-positive cells, none of the other cells that accumulated [^{3}H]D-Asp were labelled for these transmitter markers. In contrast, both WGA–HRP and WGA–gold retrogradely labelled a number of catecholaminergic neurones in the NTS and area postrema as shown by their immunoreactivity for PNMT or TH. These results confirm that uptake of [^{3}H]D-Asp is selective, and show that neurones that accumulate [^{3}H]D-Asp are a neurochemically distinct population in the NTS.

The functional interpretation of these results depends to a great extent on the location and role of the area in VLM from which the retrograde labelling took place. The anatomical delineation of the uptake zone (effective in producing retrograde labelling) is not possible because the size of the area containing autoradiographically detectable [^{3}H]D-Asp in VLM depends on postinjection survival time and on the exposure time of the autoradiograms. In addition the labelled substance at the delivery site is not in the extracellular space, indicating the zone where nerve terminals could take it up, but it is in glial cells, nerve terminals and axons. Only the location of the pipette tip can be determined unequivocally. Therefore, we used as criteria for characterizing the [^{3}H]D-Asp injection sites the presence or absence of spinally projecting and/or PNMT- and/or TH-immunoreactive neurones around the pipette tip, as well as the physiological response to the tracer as detected by measuring the blood pressure and heart rate. Naturally at the injection sites in the brainstem reticular formation of the rat, the presence of neurones involved in functions other than cardiovascular regulation cannot be excluded.

The large number of NTS neurones that can be selectively labelled by [^{3}H]D-Asp transported from the VLM suggests that these neurones use excitatory amino acids as transmitters. To test this hypothesis we investigated the effects of an excitatory amino acid antagonist delivered into the CVLM on the physiological response to activation of neurones in the NTS. The selective *N*-methyl-D-aspartate (NMDA) receptor antagonist 2-APV[9] was used. Depressor areas of CVLM and NTS were first localized by injections of L-Glu, which evoked similar decreases in arterial pressure on injection in either areas (41 ± 4 and 35 ± 3 mm Hg, respectively; mean ± S.E.M.; Fig. 3A). Decrease was also observed in heart rate (69 ± 14 and 34 ± 6 bpm, n = 10). Unilateral injection of 2-APV into the depressor area of CVLM caused a small rise in arterial pressure (10–15 mm Hg) lasting approximately 10 min in some rats and was without effect on resting pressure in other animals. In all rats, 2-APV in CVLM abolished the depressor effect of L-Glu injected into the ipsilateral NTS (Fig. 3A). The effect of L-Glu in the NTS was actually reversed by 2-APV in the CVLM, becoming a pressor response (10 ± 2 mm Hg). Similar result have been reported following the depression of neuronal activity in the CVLM[23,24]. The time course of the 2-APV antagonism was variable, some rats showing a progressive recovery of the depressor L-Glu response, beginning approximately 30 min after 2-APV. Depressor responses evoked from the contralateral NTS were not affected by injection of 2-APV ipsilaterally in CVLM, supporting evidence for a predominantly unilateral projection from NTS to CVLM, as demonstrated by the [^{3}H]D-Asp transport. Injection of vehicle in CVLM had no effect on resting arterial pressure or on the effect of L-Glu in NTS.

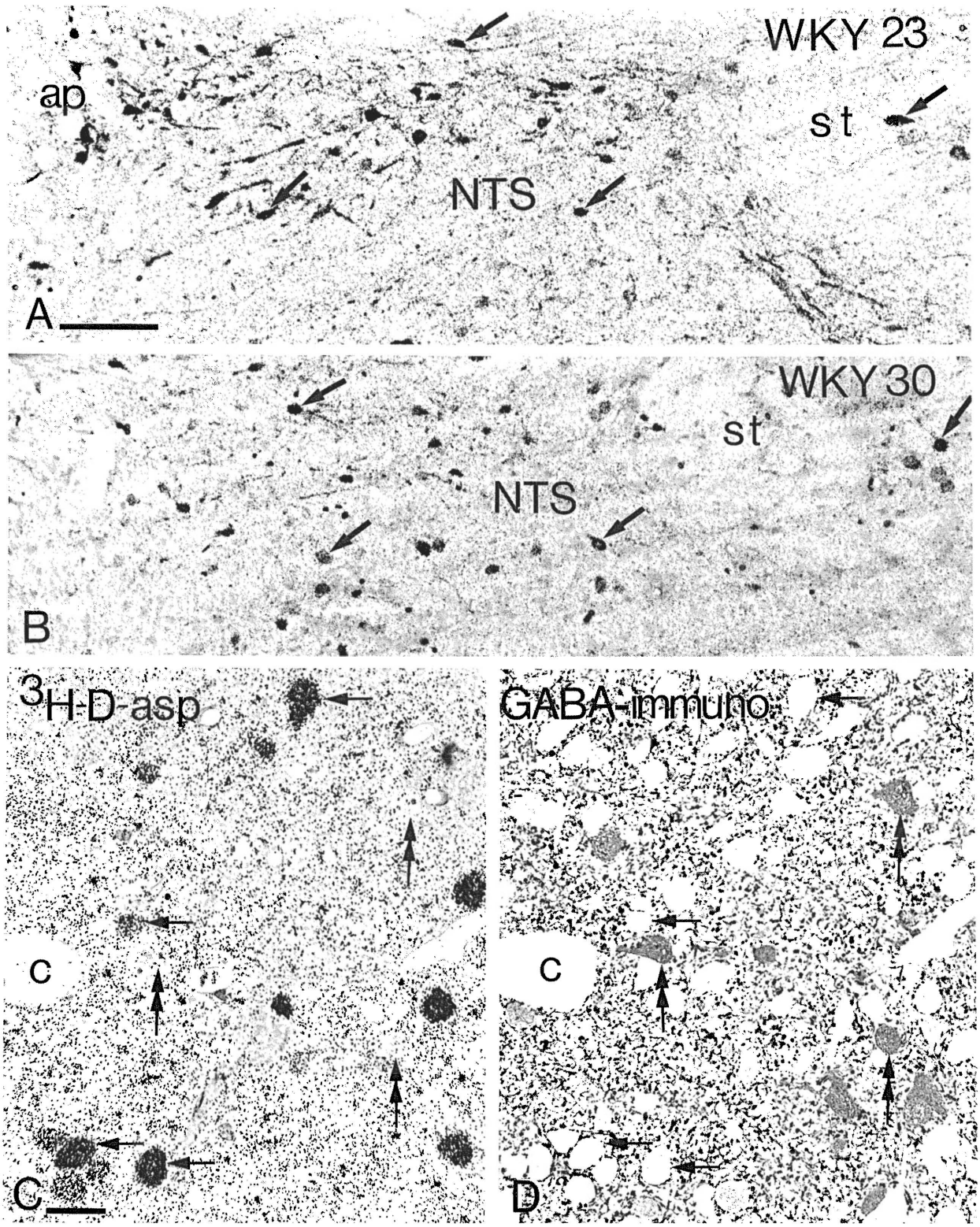

Fig. 2. Retrogradely [^{3}H]D-Asp-labelled neurones (e.g. arrows) in autoradiograms of the NTS and area postrema (ap), following RVLM (A), or CVLM (B and C) injections. WKY 23 and 30, same animals as in Fig. 1. (C and D) Semithin (1 μm) sections showing that GABA immunopositive (e.g. double arrows) and [^{3}H]D-Asp transporting (e.g. arrows) neurones are two separate populations in the NTS. st, solitary tract; c, capillary. Scales: A and B, 100 μm; C and D, 20 μm.

The activation of cells in NTS by L-Glu is non-selective, involving neurones that may participate in different physiological functions and reflexes. Whether the excitatory amino acid pathway from NTS to CVLM is involved specifically in baroreflexes was studied in rabbits, a species in which peripheral baroreceptor afferents running in the aortic depressor nerve (ADN) can be activated selectively, evoking a depressor response (Fig. 3B). Bilateral injection of 2-APV (5 nmol in 100 nl) into the CVLM region of rabbits (n = 8) produced an increase in arterial pressure (from 81 ± 6 to 126 ± 12 mm Hg; Fig. 3B), which reached a peak after approximately 15 min, and gradually recovered over about 30–40 min. Moreover, 2-APV abolished (6 animals) or attenuated (2 animals) the ADN depressor response within 5–10 min. Saline injection had no effect on either basal pressure or ADN response.

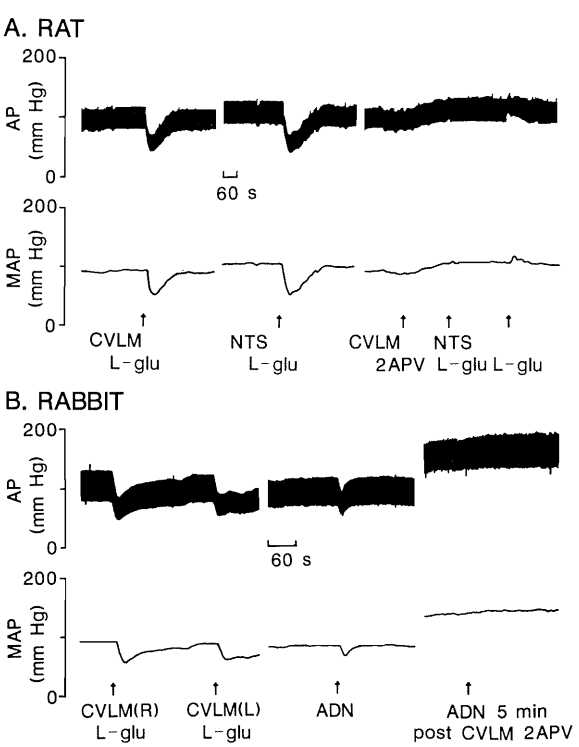

Fig. 3. A: unilateral injection of the NMDA receptor antagonist 2-APV (0.5 nmol in 20 nl) into CVLM blocks the depressor response evoked by L-Glu (1 nmol in 10 nl) injected into the NTS. B: 2-APV (5 nmol in 100 nl) injected bilaterally into CVLM blocks ADN stimulation evoked depressor response in rabbit. The depressor areas in CVLM were identified first by L-Glu (5 nmol in 50 nl) injections.

The results of this study provide evidence that excitatory amino acids are major transmitters in the pathways from NTS to the VLM. Furthermore, in agreement with previous reports[10,12,15,24], the physiological studies in CVLM suggest that these projections participate in the tonic and reflex regulation of the cardiovascular system. The activation of depressor neurones in the CVLM by NTS occurs predominantly via NMDA receptors. These CVLM cells may use GABA as transmitter[2,24,26] and participate in baroreflexes by suppressing the tonically active sympathoexcitatory neurones that form a major descending pathway from the RVLM to preganglionic neurones in the spinal cord[6,17,19].

Given that the vasopressor neurones are tonically active, it may appear paradoxical that both the pressor and depressor areas of the VLM receive excitatory amino acid input from the NTS. One explanation may be that some of the terminals in RVLM contact cardiomotor parasympathetic neurones in the nucleus ambiguus. Another possibility is that the distribution of depressor neurones overlaps with the pressor neurones in the RVLM. These rostral depressor neurones, synthesising GABA and acting on the pressor neurones[16], may be the targets of the excitatory amino acid pathway from the NTS since depressor responses are also evoked at sites dorsal to the pressor sites in RVLM[13]. Finally, the excitatory amino acid pathway to RVLM may in fact be a pressor pathway whose activation may be responsible for the reversal of the effects of NTS stimulation from a depressor to a pressor response after muscimol[23] or 2-APV (present study) injection in the CVLM, or after bicuculline injection into RVLM[24]. These possibilities can only be clarified by the identification of the specific neurones receiving synaptic input from this excitatory amino acid pathway.

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