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A microiontophoretic study of the actions of the putative sleep factor, piperidine, in the rat brainstem

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1 By means of microiontophoresis, we have compared the actions of a putative sleep substance, piperidine, with other neurotransmitters in the rat anaesthetized with urethane.

2 In the pons and midbrain, piperidine mimicked the actions of acetylcholine on more than 200 neurones. Piperidine- and acetylcholine-induced excitations were equally effectively antagonized by hexamethonium or atropine.

3 In 32 neurones piperidine showed no affinity for the receptors for the excitatory amino acid agonists, quisqualate and N-methyl-D-aspartate, piperidine-evoked excitations being unaffected by the antagonists glutamate diethylester or 2-amino-5-phosphonovalerate.

4 Similarly, piperidine-evoked excitations in 23 neurones were unaffected by α-methylnoradrenaline, suggesting that piperidine does not act at receptors for noradrenaline.

5 Twenty per cent of neurones responsive to piperidine were inhibited. These inhibitions in 12 neurones were insensitive to either strychnine or bicuculline indicating that piperidine does not act on receptors for glycine or γ-aminobutyric acid.

6 In a further 68 neurones, neither hexamethonium (4 out of 59 cells) nor atropine (0 out of 9 cells) was effective in antagonizing the inhibitions evoked by piperidine or by acetylcholine.

7 It is suggested that piperidine may exert its central hypnogenic effects by an action at cholinoceptors in brainstem areas involved in sleep regulation.

Introduction

Neurochemical investigations into sleep have concentrated upon two groups of substances: 'classical' neurotransmitters and sleep factors. The secondary amine piperidine is a possible candidate in both classes and particularly the latter.

Piperidine was first identified in cow's urine (von Euler, 1944) and subsequently it has been found widely within the mammalian central nervous system (CNS). The most reliable evidence has come from mass fragmentographic analysis which has demonstrated its presence in high concentrations in the striatum, pituitary and pineal of rats and rabbits (Miyata *et al.*, 1980). This amine has been shown to have cholinomimetic properties in the CNS (Kasé *et al.*, 1969; Miyata, *et al.*, 1974), and in the periphery (von Euler, 1944; Kasé *et al.*, 1967; Nishi *et al.*, 1979).

The central actions of piperidine are numerous; in dogs, rats and mice, when applied intra-cisternally, piperidine causes emesis by a direct action on the medullary chemoreceptor trigger zone with associated muscle relaxation, ataxia, ptosis and sedation (Kasé *et al.*, 1969; Miyata *et al.*, 1974). In cats it produces reduced EEG activity, hypotonia and ataxia and low dose microinjection of piperidine into the hippocampus or amygdala causes sedation while higher doses produce an increase in sexual behaviour and aggression (Miyata *et al.*, 1974).

Piperidine has been shown to exert a tranquillizing effect in schizophrenia (Tasher *et al.*, 1960) and to cause a prolongation of barbiturate-induced sleep (Kasé *et al.*, 1967). Additionally there are up to ten fold increases in the levels of piperidine in the brains of dormant, behaviourally asleep mice compared with awake animals (Stepita-Klauco *et al.*, 1974).

In the cat, perfusion of piperidine into the brainstem significantly reduces the onset latency of both rapid eye movement sleep (REMS) and slow wave sleep (SWS) and significantly increases REMS duration (Drucker-Colin & Giacobini, 1975).

The gigantocellular tegmental field (FTG) is a

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brainstem area which is believed to play a key role in the genesis of the SWS:REMS cycle (Hobson, *et al.*, 1974; Hobson *et al.*, 1975; McCarley & Hobson, 1975) and contains neurones which respond to cholinomimetics (George *et al.*, 1964). We have used the technique of microiontophoresis to investigate the actions of piperidine, a cholinomimetic, hypogenic amine, on single neurones in the FTG of the rat brainstem. Although our work concentrates upon the pontine FTG (nucleus reticularis pontis oralis and caudalis), for comparison, we also describe here the effects of piperidine in the midbrain reticular formation (nucleus cuneiformis and subcuneiformis). A preliminary account of this work has been given (Horton *et al.*, 1984).

Methods

Adult male and female Sprague-Dawley rats (250–300 g) were anaesthetized with urethane (0.8 ml:100 g of 20% w/v ethyl carbamate in isotonic saline solution) and following tracheal cannulation, were free to breathe spontaneously throughout the experiment. To expose the dorsal surface of the pons for access to the pontine recticular formation following a skin incision over the back of the skull and removal of muscle attachments, the occipital bone was carefully removed to a point 1–2 mm rostral to the occipital ridge and laterally as far as the attachments of the temporalis muscle. Bleeding from bone was stopped by using putty. The dura mater was then removed from the cerebellar surface and cerebellectomy was performed by aspiration. Subsequent bleeding was prevented by using calcium alginate (Calgitex) gauze or Sterispon soaked in thrombin.

Exposure of the cortex for access to the midbrain reticular formation was achieved by fixing the animal in a stereotaxic frame, exposing the vault of the skull by cutting away a piece of skin and by using a dental drill (Sterling) with a size one burr (Komet 1) to thin the temporal bone just lateral to the sagittal suture which could then be carefully removed and the underlying dura cut and reflected.

The animal was transferred to an earthed Faraday cage where it was fixed in a stereotaxic frame so that bregma was positioned 1 mm above lambda. This placement is that used for the stereotaxic atlas of the rat brain by Fifcova & Marsala (1967) and it was now possible to position accurately the microelectrode tip using the micromanipulator (Narishige-Japan) and hydraulic microdrive system (David Kopf Instruments). For experiments involving recording from the midbrain a perpendicular electrode penetration was made through the cortex. For the pontine preparation the floor of the fourth ventricle was exposed to reveal the obex which was used as a reference point for electrode penetration both rostrocaudally and laterally. Liquid paraffin was pipetted into the cavity to avoid drying of the brainstem surface and the multibarrelled microelectrode could then be positioned to enter the brainstem at 30 degrees, tip rostral.

The animal's body temperature was maintained at 37 ± 1°C by using a rectal probe and thermometer with the animal lying on a heater blanket.

Recording and iontophoresis

Five-barrelled glass micropipettes were used for recording the electrical activity of single neurones and for the iontophoretic application of the test drugs. The electrode tip was broken back under visual control until it was in the range 5–8 μm. Electrodes were selected with the recording barrel resistance less than 10 MΩ and the drug barrel resistances less than 50 MΩ when filled with the appropriate solution. The recording barrel was connected to a Neurolog a.c. preamplifier through a Neurolog high resistance probe (NL100). Recordings were made between this electrode and an indifferent electrode inserted into a nearby muscle. The signal was passed through a Neurolog filter-stage (NL125) set at 500 Hz (lower) and 5 kHz (upper) with a 50 Hz notch in place. One channel of a Tektronix 502A oscilloscope provided a visual display of single neurone activity. Action potentials were converted to constant amplitude rectangular pulses and counted in 5 s epochs in a spike processor (Digitimer D130) with an output to a chart penrecorder (Texas Instruments Servo/Riter II).

Iontophoretic currents were applied by a custom built microionotophoresis unit (Grayden Electronics). Retaining-currents of between 5–10 nA were used in all cases and ejection currents ordinarily ranged from 20–50 nA; a 10–80 nA range was chosen for examining the current-response relationship. Current controls (Na^+ ion ejection at 20 nA from one barrel containing pontamine sky blue in sodium acetate) were routinely conducted to see if the observed responses were due in total or in part to current rather than drug effects.

In most cases complete abolition of the agonist-evoked response occurred in the presence of an antagonist but occasionally a greater than 70% reduction in the response was considered as effective antagonism. On any one cell, agonists were applied at least twice during the application of the antagonist, which itself was applied more than once per cell. Pre- and post-antagonist control responses were recorded on every occasion.

In some instances there was a change in baseline firing during the application of the antagonists but the magnitude of the response and the complete abolition of the evoked-response by the antagonist ensured that

this change in background was not responsible for altering the response.

The following solutions were used during the course of these experiments (in distilled water unless otherwise stated): NaCl solution for the recording barrel (4 M, pH 7.0), acetylcholine chloride (Sigma, 500 mM, pH 5.0), γ-aminobutyric acid (GABA) (Sigma, 200 mM, pH 8.9), atropine methonitrate (Burroughs Wellcome, 200 mM, pH 4.5), bicuculline methiodide (Sigma, 200 mM, pH 3.0, in 0.9% w/v NaCl), glutamic acid diethyl ester hydrochloride (GDEE) (Cambridge Research Biochemicals (CRB), 200 mM, pH 3.5), glycine hydrochloride (Sigma, 200 mM, pH 4.5), hexamethonium bromide (Sigma, 150 mM, pH 5.2), DL-homocysteic acid (DLH) (Sigma, 150 mM, pH 8.5), α-methylnoradrenaline hydrochloride (αMeNA) (Hoechst, 100 mM, pH 5.0), noradrenaline hydrochloride (Sigma, 50 mM, pH 5.0), piperidine hydrochloride (Sigma, 50 mM, pH 4.5), pontamine sky blue (PSB) (DIFCO, a 2.0% solution in 0.5 N sodium acetate, pH 7.7), sodium 2-amino-5-phosphonovalerate (APV) (CRB, 200 mM, pH 7.0), sodium N-methyl-D-aspartate (NMDA) (ICN/K and K Laboratories Inc. U.S.A., 200 mM, pH 7.0), sodium quisqualate (CRB, 200 mM, pH 8.0, in 0.9% w/v NaCl), strychnine sulphate (Sigma, 10 mM, pH 4.5).

Histology

During the course of these experiments neurones were regularly marked using pontamine sky blue (PSB) applied with d.c. current at 60 μA min through a constant current stimulator the output of which was monitored through the oscilloscope.

Once the experiment was completed the animal was killed and the brain and brainstem were removed and immersed in 10% formol saline. Conventional histological techniques were used to localize the electrode track and the blue spot resulting from PSB ejection. These were then matched with the stereotaxic atlas of Fifcova & Marsala (1967) to confirm the recording location.

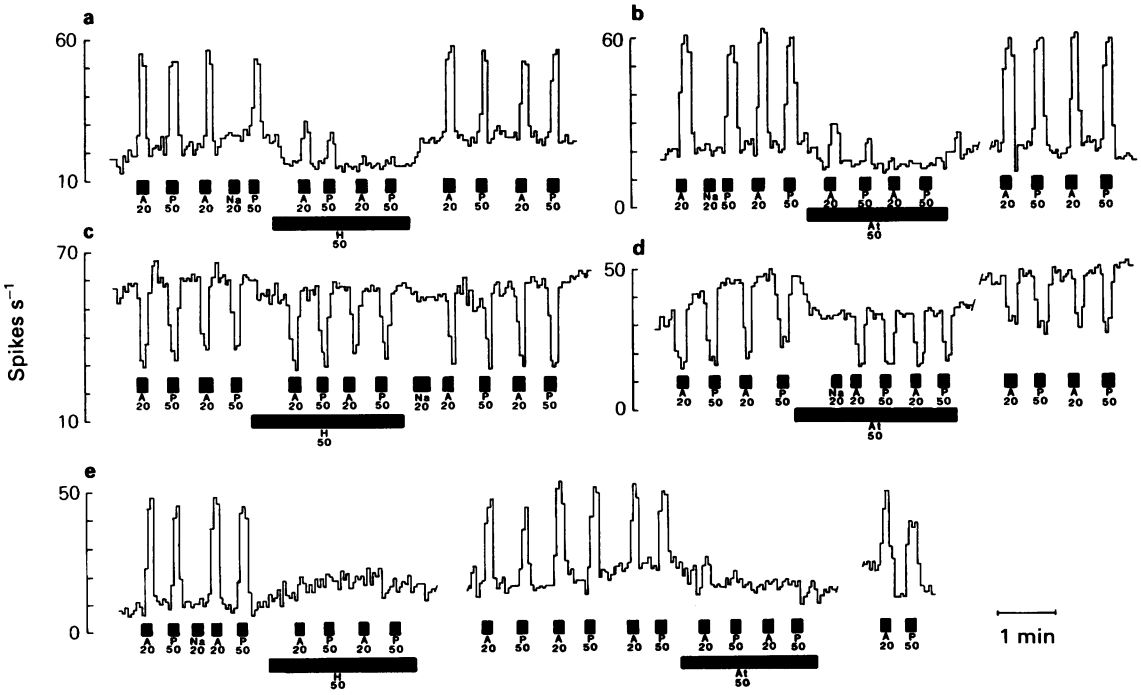

Figure 1 Ratemeter records from five different spontaneously active pontine neurones showing a comparison of the actions of piperidine (P) and acetylcholine (A) and the effects of hexamethonium (H) and atropine (At). Ordinate scale; spikes s^{-1}; Abscissa scale: time (min); solid bars represent the ejection intervals and the numbers below, the current in nA. (a and b) Short latency excitations induced by piperidine (50 nA) and acetylcholine (20 nA) are completely abolished in the presence of hexamethonium (50 nA) (a) and atropine (50 nA) (b). Control current ejections (Na^+, 20 nA) have no effect. (c and d) Short latency inhibitions induced by piperidine or acetylcholine are unaffected by hexamethonium (c) or atropine (d). (e) Discontinuous record from one neurone shows that the short latency excitations induced by piperidine and acetylcholine are completely abolished by hexamethonium and atropine indicating a receptor population with mixed nicotinic and muscarinic properties. The breaks in the record represent intervals of 2.5 and 3 min.

Results

The medial group of reticular nuclei in the pontine and midbrain formations were located by stereotaxic placement of the microelectrode and after recording this was checked by histological identification of the location of the PSB spots. Additionally, the receptive fields of the reticular cells were tested and these characteristically possessed broad receptive field properties showing non-specific polymodal responses to sensory stimuli below the neck.

Piperidine and cholinoceptors

Pons: Of 149 cells examined, 134 (90%) were responsive to piperidine, and also cholinoceptive. The great

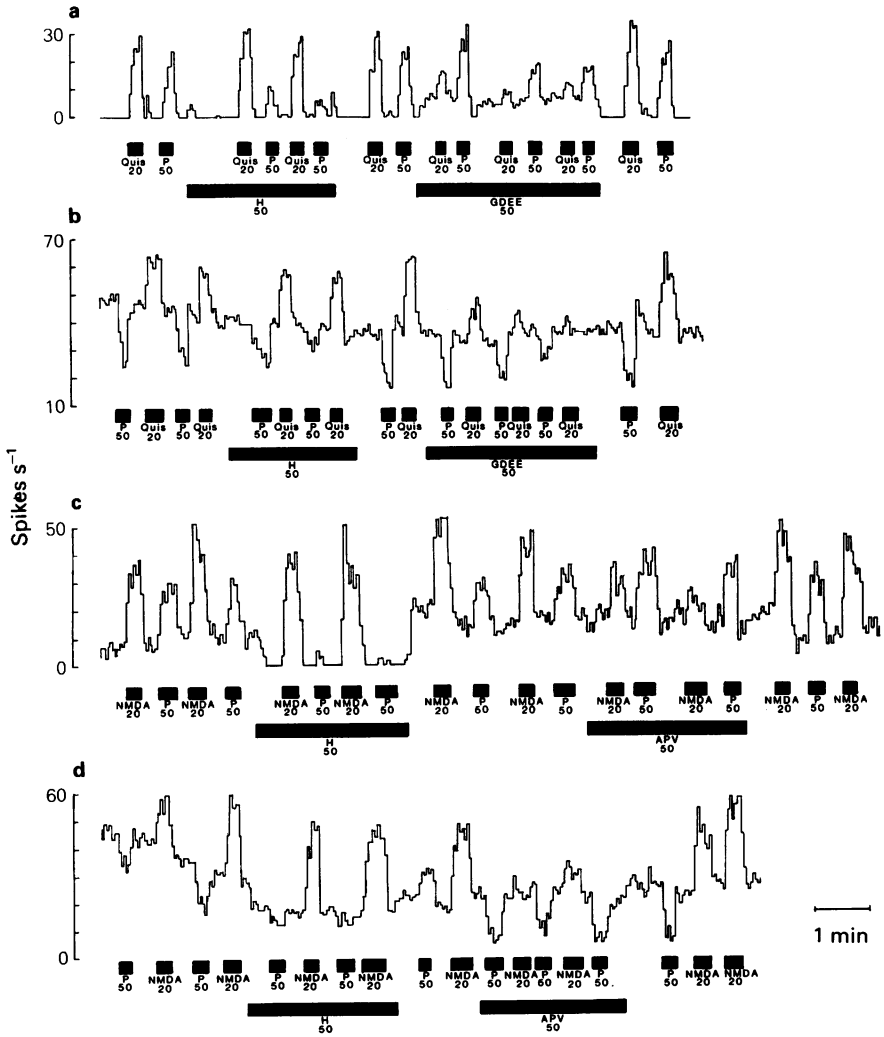

Figure 2 Ratemeter records from four different pontine neurones showing a comparison of the effects of piperidine (P) and the excitatory amino acid receptor agonists quisqualate (Quis) and N-methyl-D-aspartate (NMDA). Other details as in Figure 1. (a) Short latency excitations induced by piperidine (50 nA) which can be abolished completely in the presence of hexamethonium (50 nA) are only slightly reduced by glutamate diethyl ester (GDEE) (50 nA). Quisqualate responses (20 nA) are abolished by a similar dose of GDEE but unaffected by hexamethonium. (b) Similar data to (a) demonstrating the lack of effect of GDEE on the piperidine induced inhibitions. (c and d) These ratemeter records demonstrate that whilst 2-amino-5-phosphonovalerate (APV) (50 nA) antagonizes the NMDA-evoked excitations neither the piperidine-induced excitations (c) nor the piperidine-induced inhibitions (d) are affected by this substance.

majority of responsive cells (80%) were excited by piperidine and acetylcholine and the remainder were inhibited. Piperidine-and acetylcholine-induced excitations in 94 out of 97 (97%) cells were antagonized by hexamethonium (Figure 1a). In 19 out of 20 cells (95%) atropine similarly antagonized the excitations (Figure 1b). In the 17 cells which were inhibited by piperidine and acetylcholine these inhibitions were unaffected by hexamethonium (13 cells) (Figure 1c) and by atropine (4 cells) (Figure 1d).

In 8 cells, application of either atropine or hexamethonium completely abolished piperidine- and acetylcholine-induced excitations, suggesting that both nicotinic and muscarinic antagonists were acting on a single cholinoceptor population (Figure 1e).

Midbrain: Similar results to those found in the pons were obtained from the 87 neurones tested in this region of the brainstem. The excitations induced by piperidine and acetylcholine in 68 out of 68 of these cells were antagonized by hexamethonium. Atropine was effective in antagonizing piperidine- and acetylcholine-induced excitations in 12 out of 16 cells on which it was tested. Twenty-four cells were inhibited by both piperidine and acetylcholine but neither hexamethonium (2 out of 19) nor atropine (0 out of 5) was effective in antagonizing these inhibitions. In 6 cells showing excitations we again saw complete abolition of the response with either atropine or hexamethonium.

Piperidine and excitatory amino acid receptors

The pharmacology of excitatory amino acid receptors is complex and there may be as many as three pharmacologically distinct receptor types (Watkins & Evans, 1981). This difference of receptor type is particularly relevant to the present study in view of the fact that a piperidine derivative, *cis*-2,3-piperidine dicarboxylate (*cis*-2,3 PDA), has been shown to be a non-specific antagonist of responses evoked by excitatory amino acids (Davies *et al.*, 1981). We have examined the interactions of piperidine with the quisqualate receptor using glutamate diethyl ester (GDEE) as an antagonist and with the N-methyl-D-aspartate (NMDA) receptor using 2-amino-5-phosphonovalerate (APV) as an antagonist.

Table 1 Summary of the interactions of piperidine with neurotransmitter receptors in the pons and midbrain

Antagonists	*Piperidine-evoked* *Excitations*	*Inhibitions*
Hexamethonium	248/252*	4/59
Atropine	31/36	0/9
GDEE	0/14	0/2
APV	0/13	0/3
αMeNA	0/23	—
Bicuculline	0/21	0/7
Strychnine	0/16	0/5

*represents number of responsive-neurones antagonized/total number of responsive-neurones. GDEE: glutamic acid diethylester; APV: sodium 2-amino-5-phosphonovalerate; αMeNA: α-methyl-noradrenaline.

In experiments on 16 neurones where we compared the effects of piperidine and quisqualate on the glutamate-preferring receptor, GDEE antagonized 86% of the quisqualate-induced excitations leaving the piperidine excitations mainly unaffected (Figure 2a and Table 1). However, although on occasions GDEE partially reduced the responses to piperidine it was never able to abolish the response completely (see Figure 2a).

In similar experiments with NMDA, the aspartate-preferring receptor agonist, piperidine-induced excitations were completely unaffected by APV whereas NMDA excitations were abolished in 15 out of 16 cases by this substance (Figure 2c and Table 1). Piperidine inhibitions were unaffected by GDEE and by APV (Figures 2b and 2d and Table 1). Hexamethonium was ineffective as an antagonist of either quisqualate- or NMDA-induced excitations whilst in the same cells it was always effective in abolishing the excitations induced by piperidine (Figure 2). We rarely encountered cells in which piperidine had no effect; thus it was difficult to test whether piperidine could act as an antagonist of the excitatory amino acids.

Piperidine and noradrenaline receptors

Noradrenaline-induced excitations are specifically antagonized by α-methylnoradrenaline (αMeNA) (Boakes *et al.*, 1971) and used together with piperidine and hexamethonium we found that on the 23 neurones (11 in the pons and 12 in the midbrain) on which it was tested, α-MeNA had no effect on piperidine-induced excitations (Figure 39). Hexamethonium which antagonized 23 out of 23 piperidine excitations left the responses to noradrenaline unaffected.

Piperidine and inhibitory amino acid receptors

Since piperidine was capable of producing inhibitions in 20% of all neurones studied, we compared the actions of piperidine with those of the inhibitory amino acids glycine and GABA using their respective antagonists, strychnine and bicuculline. The data are summarized in Table 1. For the 13 neurones tested in the pons, glycine produced 13 inhibitions whilst in the same cells piperidine produced 11 excitations and only 2 inhibitions. Thus it seemed from the start that piperidine was acting on different receptors from

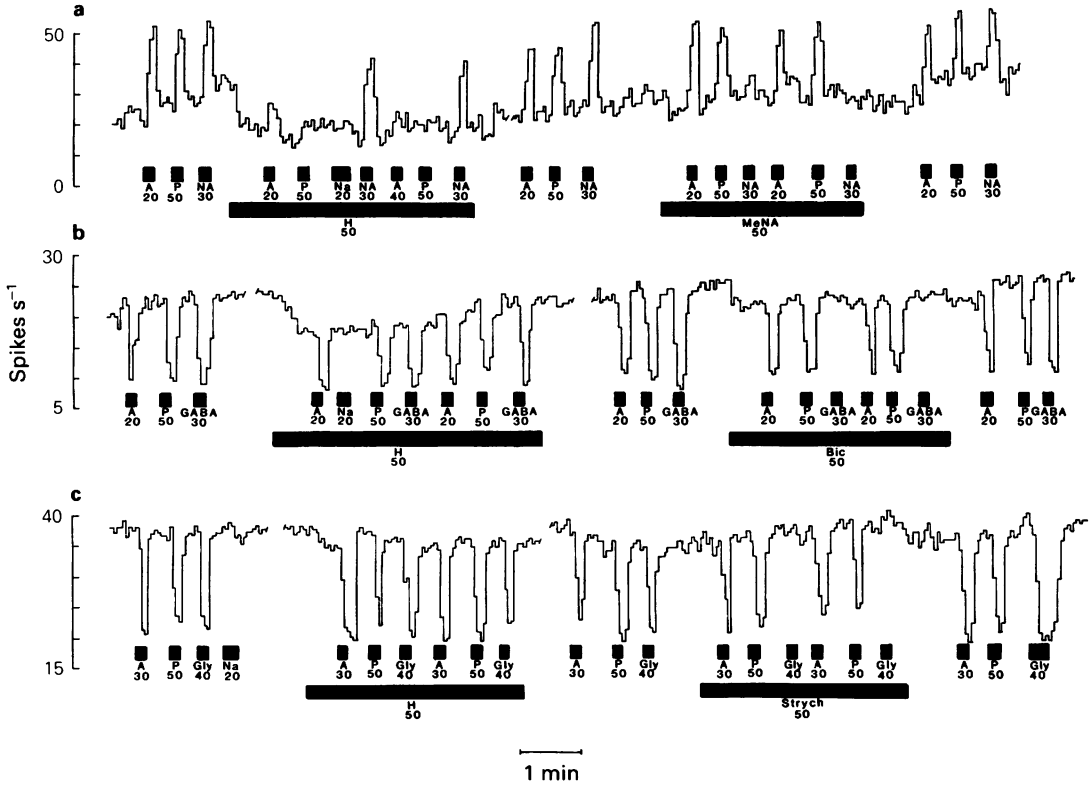

Figure 3 Ratemeter records from three pontine neurones demonstrating a comparison of the actions of piperidine (P) and acetylcholine (A) with noradrenaline (NA) (a), with GABA (b) and with glycine (c). Details as for Figure 1. (a) Excitations induced by piperidine and acetylcholine are unaffected by α-methyl noradrenaline (αMeNA) (50 nA) at doses that successfully antagonized the effects of noradrenaline (30 nA). (b) Inhibitions produced by piperidine (50 nA), acetylcholine (20 nA) and GABA(30 nA) are unaffected by hexamethonium (H)(50 nA), whereas bicuculline (Bic) (50 nA) selectively antagonizes the GABA effects leaving the piperidine and acetylcholine responses untouched. (c) Similar data to (b), this time demonstrating selective antagonism of the glycine-evoked inhibitions by strychnine (Strych) without affecting the piperidine- or acetylcholine-induced inhibitions.

glycine. This was confirmed by experiments which demonstrated that in all the cells tested, strychnine which consistently blocked responses to glycine, had no effect on the piperidine responses (Figure 3c).

Similarly in the 18 cells that were inhibited by GABA, piperidine evoked 15 excitations and 3 inhibitions. We found that bicuculline had no blocking action on piperidine-induced inhibitions or excitations but consistently antagonized all of the GABAergic inhibitions. (Figure 3b).

The results obtained from the 18 cells studied in the midbrain are qualitatively identical with those described above. Strychnine and bicuculline always antagonized the responses to glycine and GABA whilst they never affected the responses to piperidine.

Discussion

Our results suggest that piperidine exerts its central excitatory effects by acting at cholinoceptors and not at the receptors for excitatory amino acids or for noradrenaline. Furthermore the inhibitory effects of piperidine are not produced by actions on the receptors for the inhibitory amino acids GABA and glycine. Moreover, we have found no evidence to suggest that there is a population of specific piperidine receptors within the rat CNS since it was impossible to separate the effects of piperidine from those of acetylcholine. Previous studies have shown that piperidine possesses potent nicotine-like actions in peripheral tissues (Kasé *et al.*, 1967; Nishi *et al.*, 1979) and in the CNS (Kasé *et*

al., 1969; Miyata *et al.*, 1974). Our data are consistent with these observations since we have observed that hexamethonium always antagonized the excitatory effects of ACh on brainstem neurones. However, we were also able to antagonize the excitatory actions of piperidine equally effectively with atropine thus suggesting that piperidine may be equally effective as a muscarinic agonist. Our observations in 14 neurones on which it was shown that both hexamethonium and atropine were able to abolish completely the excitations induced by piperidine or ACh suggest that rather than dealing with two distinct receptor populations we may be dealing with a homogeneous receptor population, possessing mixed nicotinic and muscarinic properties as has been reported in previous iontophoretic studies (Andersen & Curtis, 1964; Bird & Aghajanian, 1976; Segal, 1978). However, we are aware of the problems in interpreting data obtained using the technique of microiontophoresis and the limitations imposed on a method that is not based on standard, quantitative receptor pharmacology. Thus we cannot rule out the possibility that ACh or piperidine are acting at sites presynaptic to the neurone from which we are recording and the observed responses merely reflect some complex neuronal integration. Neither can we ignore the possibility that atropine or hexamethonium is acting on cholinoceptor-related ion channels (see Rang, 1982) and not acting as a pure cholinoceptor antagonist. Either possibility would be an equally valid conclusion to our observations.

The failure of piperidine to stimulate excitatory amino acid receptors can be explained by the proposed requirements for activation of the receptors. Successful binding to, and activation of, these receptors appears to require two carboxyl groups and an amino group in a particular stereochemical configuration (Watkins & Evans, 1981). Such a requirement is partially fulfilled by the *cis*-2,3 dicarboxylated derivative of piperidine (*cis*-2,3 PDA) which acts as an antagonist at these receptors (Davies *et al.*, 1981) but not by piperidine. In some experiments GDEE partially antagonized the effects of piperidine and this is most probably explained by the fact that GDEE has some anticholinoceptor activity (Curtis *et al.*, 1972; Davies & Watkins, 1979; Kemp & Sillito, 1982) rather than by piperidine having an affinity for the quisqualate receptor.

Although piperidine shares some structural similarity with GABA, in our study it did not interact with the receptors for this inhibitory amino acid. However it does inhibit [^{3}H]-GABA uptake in rat brain synaptosomes (Nomura *et al.*, 1978) and interestingly, piperazine, the diamino unsaturated analogue of piperidine, has been reported to be a good agonist for GABA receptors in rat cortex and pallidum (Perkins & Stone, 1982). Pipecolic acid, the immediate metabolic precursor of piperidine (Kasé *et al.*, 1967) also inhibits [^{3}H]-GABA uptake (Nomura *et al.*, 1978) and depresses the firing rate of hippocampal neurones when applied microiontophoretically (Kasé *et al.*, 1980).

Piperidine has been demonstrated to affect neural mechanisms involved in a variety of behavioural paradigms and in particular it has been suggested that this amine may play an important role in sleep regulation (Giacobini, 1976). Moreover, its distribution (Miyata *et al.*, 1979; 1980), synaptic actions (Kasé *et al.*, 1969; Miyata *et al.*, 1974), subcellular localization, uptake and storage mechanisms (Nomura *et al.*, 1980) suggest it may be worthy of consideration as a neurotransmitter.

In this study we have demonstrated that there is no evidence for pharmacologically-distinct piperidine receptors in rat FTG and that the most likely explanation for the sleep-producing effects of piperidine is by an action on cholinoceptors in the pons and midbrain. Previous work has shown that microinjection of cholinomimetics into these areas in the rat and the cat produces a REMS-like state of atonia and cortical desynchronization which can be antagonized by atropine (George *et al.*, 1964, Amatruda *et al.*, 1975). However, it is noteworthy that the levels of piperidine in nervous tissue have been shown to fluctuate with respect to the loss and the recovery of the righting reflex (Miyata *et al.*, 1982). Also, in urethane anaesthesia, piperidine levels are elevated in certain brain regions (Miyata *et al.*, 1981). No information is available, as yet, as to whether there is a change in the sensitivity of neurones to piperidine which accompanies these changes in levels under urethane anaesthesia but previous studies have shown that urethane does not affect the responsiveness of brainstem neurones to iontophoretically-applied ACh (Bradley & Dray, 1973).

Thus, although piperidine has yet to be established as a neurotransmitter it may act as an endogenous sleep substance by its interaction with acetylcholine receptors in brain areas known to play a key role in sleep mechanisms.

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