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Effects of cholecystokinin and related peptides on neuronal activity in the ventromedial nucleus of the rat hypothalamus

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1 An investigation into the effects of cholecystokinin octapeptide (CCK-8S) and its pentapeptide analogue, pentagastrin, on neurones located in ventromedial nuclei of rat hypothalamic slices maintained *in vitro* has been undertaken.

2 CCK-8S (0.01–1.0 μM) applied in the perfusion medium produced a concentration-dependent increase in firing rate. This effect could be mimicked by pentagastrin and was selectively blocked by L-364,718, a potent peripheral CCK receptor antagonist that has been shown to possess micromolar affinity for central CCK receptors.

3 Intracellular recordings from ventromedial nucleus neurones revealed two distinct populations with comparable resting membrane parameters but differing neuronal activity. One group fired tetrodotoxin (TTX)-sensitive action potentials spontaneously at resting membrane potential whilst the second group fired action potentials only on injection of depolarizing current and were otherwise silent.

4 Application of CCK-8S or pentagastrin to spontaneously active neurones produced a small depolarization concomitant with an increase in action potential firing rate but the peptides had no effect on membrane properties of 'silent' neurones.

5 These data suggest the existence of at least two populations of neurones in the ventromedial hypothalamus, only one of which is excited by CCK-8S and pentagastrin.

Introduction

A number of groups have made extracellular recordings of the spontaneous firing from neurones located in slices of rat brain containing discrete hypothalamic nuclei (Hatton *et al.*, 1978; 1980; Haller & Wakerley, 1980; Kow & Pfaff, 1984; Inenaga & Yamashita, 1986). Pharmacological studies of two regions in particular, the paraventricular (p.v.n.) and the ventromedial (v.m.n.) nuclei, have shown that these neurones are sensitive to a number of peptides, including arginine-vasopressin and oxytocin (Inenaga & Yamashita, 1986) and cholecystokinin octapeptide or CCK-8 (Kow & Pfaff, 1986). There is immunocytochemical evidence for a role for CCK-8 in the projection from the parabrachial nucleus (p.b.n.) to the v.m.n. (Zaborszky *et al.*, 1984; Fulwiler & Saper, 1985) and a recent study (Day *et al.*, 1986) has established that high levels of CCK receptor binding exist within the v.m.n. The peripheral CCK receptor antagonist, L-364,718 (Chang & Lotti, 1986) possesses approximately picomolar affinity peripherally but also has micromolar affinity for central CCK receptor binding sites, yet its pharmacological actions at central sites have still to be elucidated. We decided to investigate the effects of this compound on CCK-8S-induced increases in neuronal firing rate, recorded from within the v.m.n. of rat hypothalamic slices, in an attempt to ascertain whether the compound also acts antagonistically at central CCK receptors. The difference in selectivity shown by central CCK receptors compared to those found in peripheral tissue also led us to study the effects of the CCK analogue, pentagastrin, which has been found to be equipotent with CCK-8S at CA1 neurones of rat hippocampus (Boden & Hill, 1986).

Whilst there is a wealth of information from intracellular recordings of magnocellular neurones located in the supraoptic or paraventricular regions of the hypothalamus, little is known about the role

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of peptides in v.m.n. function. We therefore decided to study the effects of CCK analogues on cells characterized by use of intracellular recording techniques and labelled by fluorescent dye injection.

Methods

Adult male Wistar rats (50–100 g) were killed by a sharp blow to the back of the neck. The brain was removed and placed in cold artificial cerebrospinal fluid (ACSF) of a composition described previously (Boden & Hill, 1986) which had been pre-gassed for 1 h with a mixture of 95% O_2 : 5% CO_2. Coronal slices (350–400 μM) containing hypothalamic nuclei were cut with a Lancer Vibratome and placed immediately in ACSF at room temperature for 1 h. At the end of this period sections containing ventromedial nucleus (v.m.n.) of rat hypothalamus, identified by the presence of median eminence and arcuate nucleus, were placed on a Sylgard base in a Perspex recording chamber and perfused with ACSF at a temperature of 37°C. The dead space of the chamber was approximately 0.5 ml and the saline flow rate was 4 ml min^{-1}. Slices were held in position with a nylon mesh. An equilibration period of 1 h was allowed before recording was started.

Extracellular recordings were obtained by conventional means. Glass pipettes filled with 2 M sodium chloride were used (tip resistances 10–20 megohms) placed in the v.m.n., although in earlier experiments we obtained the same long-term recordings with similar electrodes filled with either 3 M KCl or ACSF, presumably because the resistance of the electrodes was high enough to minimise leakage from the electrode tip. Action potentials were recorded via an Axoclamp-2a amplifier and fed through a second filter amplifier (bandwidth 100–1000 Hz) for storage on magnetic tape (Racal-7DS). The events were replayed via an analogue to digital interface (1401, Cambridge Electronic Design, C. E. D.) linked to a Sperry XT microcomputer. Data analysis was performed by use of a programme that produced firing rate histograms together with the total number of action potentials recorded ('counts') for a given time period, before, during and after drug application ('Mrate', C. E. D.). All drugs were applied directly dissolved in the perfusing saline. Intracellular recordings were obtained by conventional techniques. Electrodes were filled with 3 M potassium acetate and had d.c. resistances of 100–150 megohm when measured in physiological saline. A period of 30 min was allowed for equilibration following impalement. Input resistances were derived from the slope of the current voltage plot obtained by measuring the electrotonic potential during current injection. For morphological studies electrodes were filled with 2% lucifer yellow (lithium salt) in 0.5 M potassium chloride and dye injected into neurones was later visualised on a Reichert-Jung Polyvar fluorescent microscope fitted with a B1 filter. CCK-8S was obtained from Bachem, pentagastrin (Boc Bala-CCK4) from CRB. Lucifer yellow and tetrodotoxin were from Sigma. L-364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-car-boxamide) was kindly donated by Merck, Sharp and Dohme.

All data are expressed as mean ± s.e. mean. Analysis of significance was by Student's t test, $P = 0.05$ being taken as significant.

Results

Extracellular recording

Concentration-response curves constructed from data obtained following analysis of neuronal firing rate showed that CCK-8S produced a dose-dependent increase in the spontaneous firing rate of cells contained within the ventromedial nucleus of slices of rat hypothalamus. We found that a large majority (22 out of 28) of neurones responded with an increase in firing rate and that this effect was reproducible when the same concentration of CCK-8S was re-applied 5 min after recovery from the first application. At a bath concentration of 1.0 μM CCK-8S produced a 375% (375 ± 30, $n = 5$) increase in firing rate. Neurones which were excited by CCK-8S were also excited by pentagastrin, and we were unable to detect any difference in potencies of the two peptides (Figure 1). Although in the presence of 10 μM L-364,718 the excitatory response to CCK-8S was reduced, concentrations of 30 μM L-364,718 were required to produce a statistically significant change (Figures 1b, 2). When 30 μM L-364,718 alone was applied to the preparation, no change in firing rate could be detected. These results could be repeated when pentagastrin was used in place of CCK-8S (Figure 1c) but responses to the amino acid L-glutamate (1 mM) which produced a 400% increase in spontaneous firing (400 ± 12%, $n = 3$) were unchanged in the presence of 30 μM L-364,718 (Figure 1d), indicating a selective CCK receptor antagonist role for L-364,718.

Intracellular recording

A total of 31 neurones were used in our intracellular study. Two distinct populations could be identified on the basis of long term (1 to 3 h) recordings of membrane parameters and neuronal activity. The first group ($n = 20$) possessed a mean resting potential of 68.8 ± 5.8 mV and input resistance of 232 ± 74 megohm and fired spontaneous action

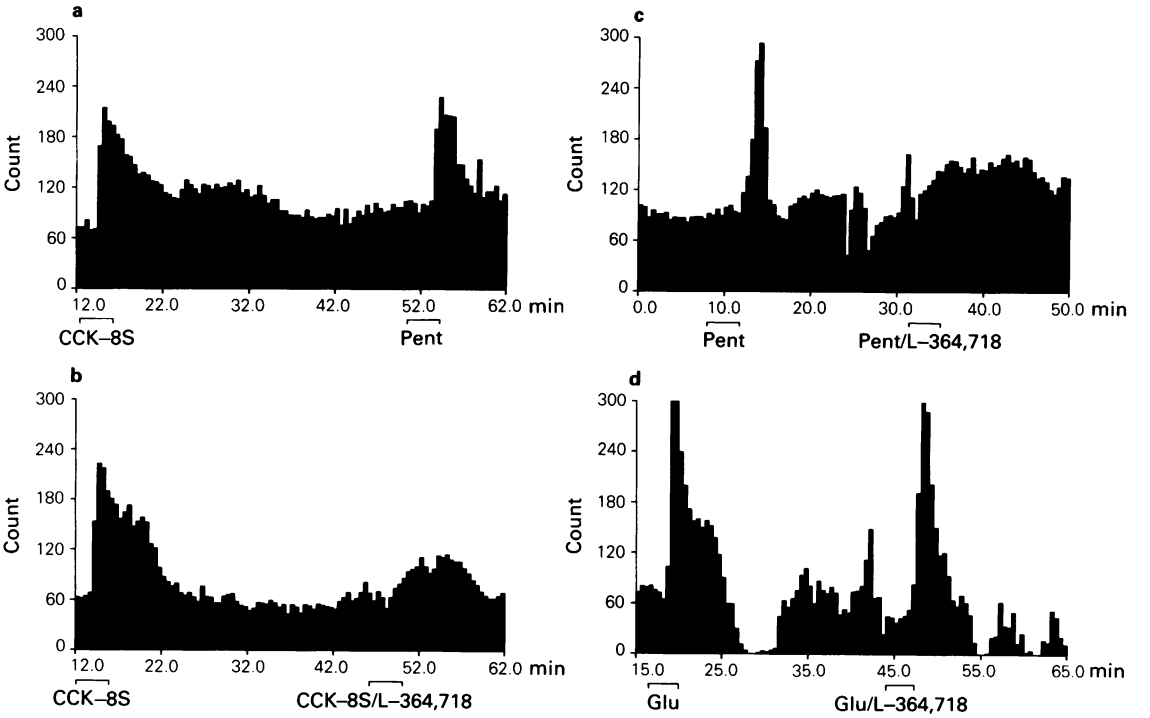

Figure 1 Ratemeter records of spontaneous firing rate taken from neurones located in the ventromedial nucleus (v.m.n.) of rat hypothalamic slices. Ordinate scale is 'count' recorded in consecutive 30 s time bins. The abscissa scale is time marked in min. Lines immediately beneath the abscissae indicate drug applications. Traces are from separate experiments with the exception of (d) which was recorded 10 min after trace (b). (a) Application of CCK-8S (1 μM) caused an increase in firing rate of a v.m.n. neurone which could be mimicked by exposing the neurone to pentagastrin (Pent, 1 μM) some 10 min after recovery from CCK-8S. (b) The change in firing brought about by CCK-8S (1 μM) was depressed by some 70% when the antagonist was present in the bathing solution. Note also that there was a slight increase in firing when the slice was finally returned to drug-free saline. (c) When the experiment in (b) was repeated using pentagastrin in place of CCK-8S an identical reduction in the peptide response was noted in the presence of L-364,718. (d) The excitatory amino acid L-glutamate (1 mM) clearly caused a large increase in firing rate which was not affected significantly by the presence of L-364,718 (30 μM).

potentials of less than 2 ms duration which were followed by a long lasting (80–150 ms) after hyperpolarization (a.h.p.) of some 20 mV at resting membrane potential (Figure 3a). Spontaneously active neurones characterized by injection of lucifer yellow ($n = 8$) were all found to lie with their nuclei within the v.m.n. but to possess extensive beaded processes with infrequent branching. The second group (mean resting potential 64.6 ± 8.9 mV, input resistance 192 ± 37 megohm, $n = 11$) only fired action potentials in response to depolarizing current injection and were otherwise silent. Action potentials from these neurones possessed an a.h.p. of similar magnitude but much shorter duration (20–50 ms) than that found in spontaneously active neurones. Unlike the spontaneously active cells, which continued to fire during sustained depolarizations (Figure 3c), silent neurones displayed rapid adaptation leading to a cessation of action potential firing (Figure 3d), which has been attributed to the activation of a calcium-dependent potassium current in hippocampus (Madison & Nicoll, 1984) and guinea-pig hypothalamus (Minami *et al.*, 1986b). We are unable at present from our limited lucifer yellow experiments to say whether these neurones are also morphologically different.

Peptide effects on membrane properties

When CCK-8S (0.5 μM) was added to the perfusing medium all spontaneously active neurones studied were depolarized (3.4 ± 1.8 mV, $n = 13$) and a large increase in firing rate was seen (Figure 4a). This precluded any measurement of concentration-

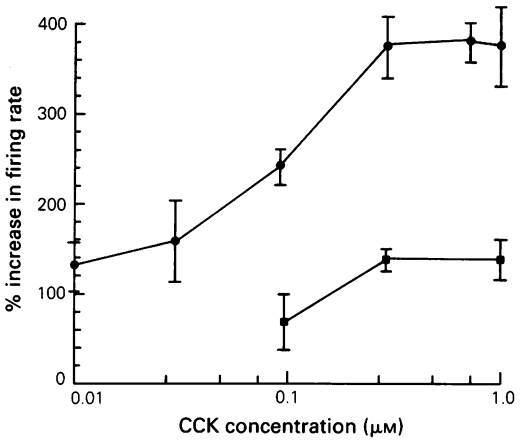

Figure 2 Concentration-response curves for the increase in firing rate produced by CCK-8S in the absence (●) and the presence (■) of the antagonist L-364,718 (30 μM). Abscissa scale is log. concentration (μM) and the ordinate scale is increase in firing rate expressed as a percentage of the firing rate recorded immediately prior to drug application. Data points are mean of at least three separate experiments; s.e. mean shown by vertical lines.

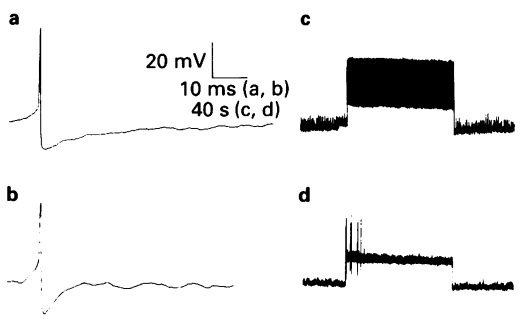

Figure 3 Differences in the action potential and firing pattern displayed by the two types of neurone used in the study. In (a) the typical action potential of a spontaneously firing neurone has an a.h.p. of some 20 mV amplitude with a decay time in excess of 100 ms, whilst (b) is the action potential recorded from a 'silent' type neurone, the a.h.p. of which is of the same magnitude but much shorter in duration (25 ms); (c) and (d) illustrate the differences of firing patterns of the two types following a step depolarization (0.05 nA) of 15 mV. Neurones with a long a.h.p. fired continuously at a high frequency (c) unlike the second type which rapidly returned to its inactive state (d). Traces (c) and (d) are from the same recordings as those of (a) and (b) respectively. Action potentials in (c) and (d) are truncated by the limited frequency response of the pen recorder. Calibration bars are ordinate scale, 20 mV, abscissa scale 10 ms (a, b), 40 s (c, d). Both neurones had a resting potential of −66 mV.

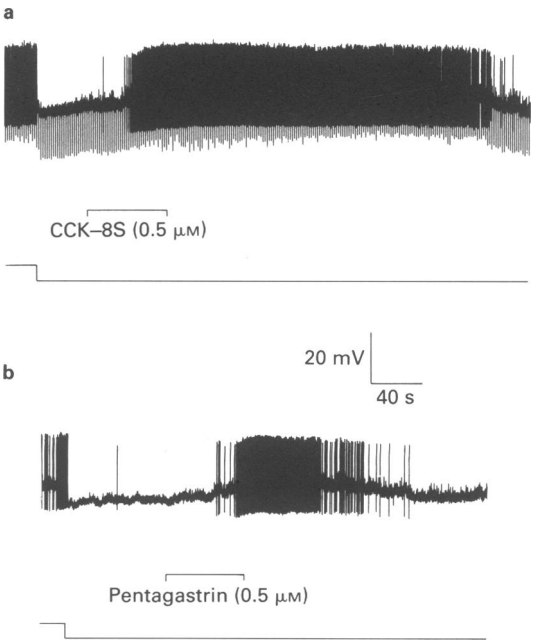

Figure 4 Both CCK-8S and pentagastrin depolarize spontaneously firing neurones and cause an increase in excitability. In (a) a spontaneously firing neurone was hyperpolarized by 5 mV (0.02 nA) to inhibit action potential firing. CCK-8S (0.5 μM) was then added, shown by the line. The neurone depolarized by 3 mV returning almost to its prehyperpolarized potential but the frequency of firing was enhanced considerably compared to that at the start of the experiment before hyperpolarization of the cell. The firing rate gradually returned to its original level on washing with drug-free saline and finally the neurone repolarized to that immediately prior to peptide application. In (b) the same protocol was used on another spontaneously active cell using pentagastrin (0.5 μM) instead of CCK-8S. Downward deflections (hyperpolarizing electrotonic pulses) were omitted from this experiment for clarity. The neurone depolarized by 4 mV in pentagastrin-containing saline and again a substantial increase in firing rate was found, both effects being fully reversed on returning the preparation to drug-free saline. Trace at the foot of each record is the current level showing the point at which the membrane was hyperpolarized in each case.

dependency or change in input resistance in the presence of the peptide. Pentagastrin (0.5 μM) depolarized six out of seven spontaneously active neurones (2.9 ± 1.7 mV) and also increased action potential firing rate (Figure 4b). One consistent feature of the peptide-induced responses was their relatively long onset time. Peptide-induced effects were not apparent for periods of up to 2 min follow-

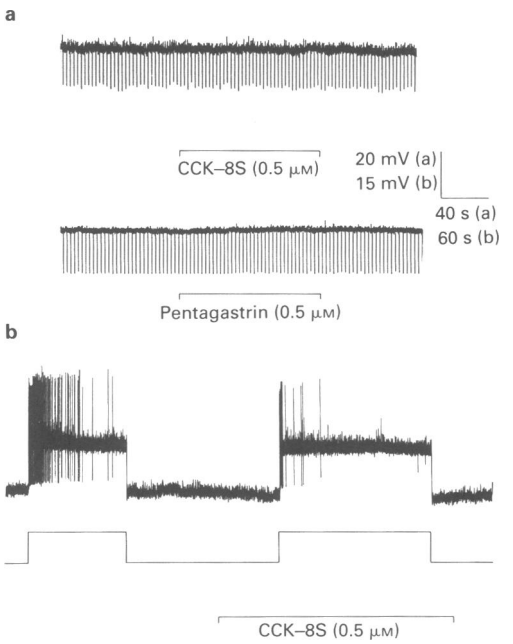

Figure 5 'Silent' neurones do not respond to peptide treatment: (a) was taken from a neurone which failed to demonstrate any change in potential or excitability during CCK-8S (0.5 μM) application. Higher concentrations were still unable to elicit a response. The same was true for pentagastrin shown in the lower trace. Trace (b) was taken from a neurone demonstrating rapid adaptation to step positive current injection of 0.05 nA producing a 15 mV depolarization. When CCK-8S (0.5 μM) was added, no effects on membrane parameters or excitability were evident, rather the neurone appeared to display more rapid adaptation.

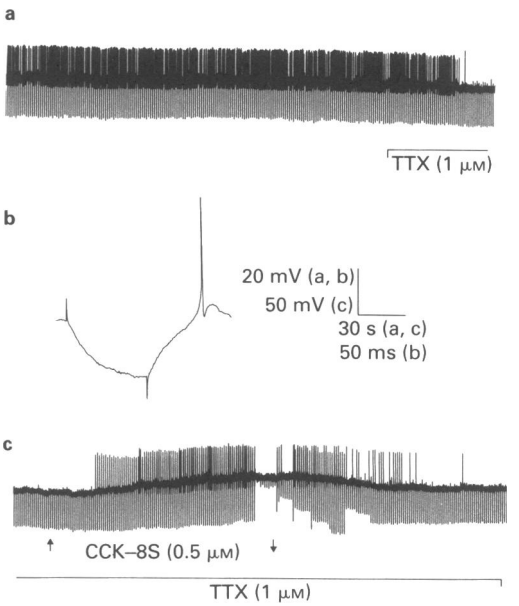

Figure 6 Effects of CCK-8S on a neurone bathed in tetrodotoxin (TTX) to prevent sodium-dependent action potentials. In (a), TTX (1 μM) was added to a spontaneously firing neurone (resting potential −68 mV), leading to abolition of spikes. Sodium-independent spikes could still be elicited (b) on the repolarizing phase of a large ca. 50 mV hyperpolarizing pulse. When CCK-8S (0.5 μM) was added to the TTX-treated neurone (↑) the cell membrane depolarized, an increase in input resistance was seen, and sodium-independent spikes were evoked. Changes in the size of electrotonic pulse seen during drug presence and at start of washout were for conductance measurement purposes only.

ing application of drug saline, unlike those to the excitatory amino acids which were seen within 30 s of exchange of bathing solution (not illustrated).

Neither peptide was found to be effective in depolarizing 'silent' neurones (Figure 5a). No apparent increase in excitability could be detected since neurones still displayed adaptation in peptide-containing solution (Figure 5b). Some neurones appeared to adapt more rapidly in the presence of peptides but we considered this to be nonspecific since the effect could also occur when two successive depolarizing steps were elicited in control conditions.

Peptide responses in tetrodotoxin-treated slices

The experiments were repeated on a total of four neurones in the presence of tetrodotoxin (TTX) at a concentration of 1 μM to block sodium-dependent action potentials (Figure 6a). Sodium-independent action potentials could be elicited in TTX-treated preparations following large (ca. 50 mV) hyperpolarizing steps (Figure 6b). We still did not find that the depolarization was related to CCK concentration but were able to detect an increase in input resistance in the presence of the peptide (Figure 6c).

Discussion

Our finding that CCK-8S is a potent excitant of these neurones is in agreement with other published observations (Kow & Pfaff, 1986; Pan *et al.*, 1986) and suggests a major neuromodulator (or neurotransmitter) role for the peptide in this region. Additionally, the two CCK analogues used in this

study were both found to be excitants of v.m.n. neurones. We were unable to establish any difference in potency between them from analysis of firing rate records and this in conjunction with our intracellular data leads us to conclude that the structural criteria for activation of this central CCK receptor are not as stringent as those for the peripheral counterpart. A similar finding has already been described in CA1 neurones of rat hippocampus (Boden & Hill, 1986), and is in agreement with radioligand binding studies (Clark *et al.*, 1986).

The results obtained with the CCK antagonist L-364,718 show that this compound is able to block selectively the excitatory effect of CCK-8S on ventromedial hypothalamic neurones. The concentrations required suggest that L-364,718 is, to date, the most potent available antagonist at central CCK receptors with a concentration of 30 μM producing a reduction of some 70% in the CCK response. We also noted that there was always a period of increased firing when the agonist/antagonist mixture was removed but are unable to conclude whether this merely reflects the difference in diffusion time for the two compounds or is a result of rapid dissociation from the receptor of the antagonist. We have been unable to demonstrate any antagonist action of the purported gastrin receptor antagonist benzotript in our model at concentrations up to and including 30 μM (unpublished results).

Our intracellular data indicate the existence of at least two types of physiologically distinct neurones in the ventromedial nucleus. These could be those described in *in vivo* studies by Murphy & Renaud in 1969 although Minami *et al.* (1986a, b) found that three neuronal types could be distinguished in ventromedial nuclei of guinea-pig hypothalamic slices. Two differences were always apparent between the two classes of neurone found in our study. Spontaneously firing neurones displayed long (80–150 ms) a.h.p.s whilst silent neurones had a.h.p.s which were considerably shorter in duration (20–50 ms). These neurones also exhibited rapid adaptation during depolarizing current injection, unlike spontaneously firing neurones which fired at a maintained frequency when step-depolarized. The peptides used in this study were both found to be excitants of only one population of neurones in the v.m.n., those which are spontaneously firing at resting membrane potential. The finding that in TTX-treated slices the predominant membrane effect of CCK-8S was one of a decrease in resting conductance would imply that a block of potassium channels is the major ionic mechanism by which the peptide exerts its action and this would substantiate findings from studies of CCK on hippocampal CA1 neurones (Boden & Hill, unpublished observations).

The evidence from lucifer yellow staining is that the CCK-sensitive neurones are most akin to one of the three types described by Millhouse (1973a), possessing few collaterals but with beading throughout the entire axonal system which may extend for several hundred microns, and afferent input to these neurones would appear to originate at least partly from axons of the lateral hypothalamus (Millhouse, 1973b) an area suggested as being linked in the regulation of food intake by a negative feedback loop with the v.m.n. (Oomura *et al.*, 1967; Sutin & Eager, 1969). Whether these features are also common to silent or CCK-insensitive neurones has yet to be established.

References

BODEN, P. & HILL, R.G. (1986). Evidence for the cholecystokinin (CCK) fragments CCK-8S, CCK-4 and pentagastrin sharing a common site of action on rat hippocampal CA1 neurones *in vitro*. *J. Physiol.*, **381**, 48P.

CHANG, R.S.L. & LOTTI, V.J. (1986). Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist. *Proc. Natl. Acad. Sci., U.S.A.*, **83**, 4923–4926.

CLARK, C., DAUM, P. & HUGHES, J. (1986). A study of the cerebral cortex cholecystokinin receptor using two radiolabelled probes: evidence for a common CCK 8 and CCK 4 cholecystokinin receptor binding site. *J. Neurochem.*, **46**, 1091–1101.

DAY, N.C., HALL, M.D., CLARK, C.R. & HUGHES, J. (1986). High concentrations of cholecystokinin receptor binding sites in the ventromedial hypothalamic nucleus. *Neuropeptides*, **8**, 1–18.

FULWILER, C.E. & SAPER, C.B. (1985). Cholecystokinin-immunoreactive innervation of the ventromedial hypothalamus in the rat: possible substrate for autonomic regulation of feeding. *Neurosci. Letts*, **53**, 289–296.

HALLER, E.W. & WAKERLEY, J.B. (1980). Electrophysiological studies of paraventricular and supraoptic neurones recorded *in vitro* from slices of rat hypothalamus. *J. Physiol.*, **302**, 347–362.

HATTON, G.I., ARMSTRONG, W.E. & GREGORY, W.A. (1978). Spontaneous and osmotically stimulated activity in slices of rat hypothalamus. *Brain Res. Bull.*, **3**, 497–508.

HATTON, G.I., DORAN, A.D., SALM, A.K. & TWEEDLE, C.D. (1980). Brain slice preparation: hypothalamus. *Brain Res. Bull.*, **5**, 405–414.

INENAGA, K. & YAMASHITA, H. (1986). Excitation of neurones in the rat paraventricular nucleus *in vitro* by vasopressin and oxytocin. *J. Physiol.*, **370**, 165–180.

KOW, L-M. & PFAFF, D.W. (1984). Suprachiasmatic neurones in tissue slices from ovariectomized rats: Electrophysiological and neuropharmacological characterization and the effects of estrogen treatment. *Brain Res.*, **297**, 275–286.

KOW, L-M. & PFAFF, D.W. (1986). CCK-8 stimulation of ventromedial hypothalamic neurons *in vitro*: a feeding relevant event? *Peptides*, **7**, 473–479.

MADISON, D.V. & NICOLL, R.A. (1984). Control of the repetitive discharge of rat CA1 pyramidal neurones *in vitro*. *J. Physiol.*, **354**, 319–331.

MILLHOUSE, O.E. (1973a). The organization of the ventromedial hypothalamic nucleus. *Brain Res.*, **55**, 71–87.

MILLHOUSE, O.E. (1973b). Certain ventromedial hypothalamic afferents. *Brain Res.*, **55**, 89–105.

MINAMI, T., OOMURA, Y. & SUGIMORI, M. (1986a). Electrophysiological properties and glucose responsiveness of guinea-pig ventromedial hypothalamic neurones *in vitro*. *J. Physiol.*, **380**, 127–143.

MINAMI, T., OOMURA, Y. & SUGIMORI, M. (1986b). Ionic basis for the electroresponsiveness of guinea-pig ventromedial hypothalamic neurones *in vitro*. *J. Physiol.*, **380**, 145–156.

MURPHY, J.T. & RENAUD, L.P. (1969). Mechanisms of inhibition in the ventromedial nucleus of the hypothalamus. *J. Neurophysiol.*, **32**, 85–102.

OOMURA, Y., OOYAMA, H., YAMAMOTO, T., NAKA, F., KOBAYASHI, N. & ONO, T. (1967). Neuronal mechanism of feeding. *Prog. Brain Res.*, **27**, 1–33.

PAN, J-T., KOW, L-M. & PFAFF, D.W. (1986). Single-unit activity of hypothalamic arcuate neurons in brain tissue slices. *Neuroendocrinology*, **43**, 189–196.

SUTIN, J. & EAGER, R.P. (1969). Fiber degeneration following lesions in the hypothalamic ventromedial nucleus. *Ann. New York Acad. Sci.*, **157**, 610–628.

ZABORSZKY, L., BEINFELD, M.C., PALKOVITS, M. & HEIMER, L. (1984). Brainstem connection to the hypothalamic ventromedial nucleus in the rat: a CCK-containing long ascending pathway. *Brain Res.*, **303**, 225–231.

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