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CIRCADIAN AND OTHER RHYTHMIC ACTIVITY OF NEURONES IN THE VENTROMEDIAL NUCLEI AND LATERAL HYPOTHALAMIC AREA

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SUMMARY

1. The frequency of firing was simultaneously recorded from single neurones of the ventromedial nuclei (VMN) and the lateral hypothalamic area (LHA) in urethane anaesthetized rats for many hours.

2. There were circadian changes of VMN and LHA neurone activity. The pattern of this circadian rhythm is as follows: throughout the day LHA neurones show higher activity than that of VMN, as indicated by higher frequency and more fluctuations in their rates of firing. In late afternoon the discharge rate of LHA neurones increases further, showing oscillations of short duration. In the early evening hours LHA neurone activity gradually goes down, as the VMN neurones become active. Throughout the night, VMN neurones are more active than those of LHA, just the opposite of the day period. In early morning hours VMN neurones gradually become quiet, while LHA neurones begin to show activity.

3. Superimposed on the circadian rhythm, at certain periods of the day, VMN and LHA neurones showed short duration oscillations in rate of firing, roughly every 7–15 sec and every 3–5 min.

4. Activities in neurones of the VMN and LHA were reciprocally related; a decrease in firing rate of one was associated with an increase in the other. This phenomenon was shown clearly by analysis of auto- and cross-correlation functions of firing patterns of VMN and LHA neurones.

5. The effects of stimulations of the prefrontal cortex and splanchnic afferents on VMN and LHA neurones depended on the basic firing frequency, thus they varied with the time of day. Definite relationships exist between basic firing frequency of a cell and the magnitude of changes evoked by these stimuli. Reactions of VMN and LHA neurones were the opposite in most instances. Septal stimulations (at more than 10/sec) always produced inhibition of LHA neurone activity.

6. Intravenous injection of glucose inhibited LHA neurones and

accelerated firing of VMN cells. This was true during the day period as well as at night when background activities of VMN and LHA neurones were different from that of the day.

7. Stimulation of the septal area with subthreshold pulses at a low rate (1–0·3/sec) suppressed or altered oscillations in firing frequency of LHA neurones. Severance of connexion between LHA and structures caudal thereto had no effect on LHA neurone firing rates or rhythms. Sections between the septal area and LHA, however, abolished or greatly altered the oscillatory rhythms of LHA cell activity, although spontaneous discharges continued at a somewhat lower rate for periods of hours.

8. Stimulation of suprachiasmatic nuclei with weak intensity and low frequency also changed oscillatory fluctuations in firing of LHA neurones.

9. Possible origins of circadian rhythm and oscillations of short duration in firing pattern of VMN and LHA neurones were discussed.

INTRODUCTION

Much has been published concerning the circadian rhythms observed to occur in mammals as well as in lower forms (Harker, 1964; Aschoff, 1965; Richter, 1965; Conroy & Mills, 1970; Mills, 1973; Hedlund, Franz & Kenny, 1975).

There are rhythmic changes in hormone secretion from endocrine glands and from the pineal body. The motor movements, the feeding behaviour and some other mammalian functional processes show 24 hr rhythms. Although analyses of neuronal circadian rhythms have been made in invertebrates (Lickey, Zack & Birrell, 1971; Strumwasser, 1973, 1974), such rhythmic changes in activity which occur in the individual neurones of the mammalian central nervous system have not been much studied. Only a few reports have appeared thus far (Johnson, Terkel, Whitmoyer & Sawyer, 1971; Schmitt, 1973*b*). In the course of studies of glucoreceptors in the liver which alter activities of neurones in ventromedial nuclei (VMN) and the lateral hypothalamic area (LHA), Schmitt made two observations of considerable significance (1973*a*, *b*). She found that not only do neurones in the LHA change their firing rate during the 24 hr day–night cycle but also their responsiveness to the injection of glucose into the liver portal system changes relative to the time of day. It was also observed that there are other neuronal oscillatory rhythms of shorter duration superimposed upon the slow circadian cycle.

Since feeding behaviour in rats is strongly linked to the 24 hr rhythm, the circadian fluctuation of LHA neurones found by Schmitt may constitute a part of the 'biological clock' and may not be simply induced by external environmental factors. For this reason it seems desirable to con-

tinue this study further and determine the pattern or nature of circadian and other rhythmic oscillatory fluctuations in cell activity. It is also important to ascertain where the rhythmic activities shown by hypothalamic neurones originate. Another problem is that of comparing the behaviour of cells in the VMN and LHA. Despite the vast amount of work done in the past, the exact role played by these two areas in control of feeding and the exact mechanism of interaction between is not clear. Many investigators have shown a reciprocal response of cells in the VMN and LHA to glucose administration (Anand, Chhina, Sharma, Dua & Singh, 1964; Oomura, Kimura, Ooyama, Maeno, Iki & Kuniyoshi, 1964; Brooks, Koizumi & Zeballos, 1966; Oomura, Ono, Ooyama & Wayner, 1969*a*). During feeding of unanaesthetized cats the reciprocity in firing rates of neurones in LHA and VMN has also been demonstrated (Oomura, Ooyama, Naka, Yamamoto, Ono & Kobayashi, 1969*b*). However, no comparison was made of the circadian rhythms occurring in two areas and their interrelationship. The immediate objectives of this study are to: compare the circadian rhythm and other oscillatory rhythms of short duration which occur in single neurones of the VMN and LHA; study changes in response patterns of neurones in both areas to neural and chemical stimuli applied at various times of the cycle; and conduct experiments for determining the origin of the rhythmic oscillation of neurone activity.

METHODS

Preparation. One-hundred-and-two male rats, weighing 300–450 g, were used in these experiments. They were kept for 1–2 weeks before the experiment in a normal animal room environment; exposed from 9 a.m. to 5 p.m. to artificial light and to 16 hr of darkness at a constant room temperature of 22° C and fed *ad libitum* (rat Purina pellets and water). All experiments were performed under the laboratory lighting conditions throughout a 24 hr period. The rats were anaesthetized with urethane (1·2 g/kg body weight) given intraperitoneally; additional doses of 0·2 g/kg were given usually every 4–5 hr. A trachea cannula was inserted, a splanchnic nerve was prepared for stimulation; its central cut end was mounted on a cuff electrode and the incision was closed. In most experiments the femoral artery or one carotid artery was used for blood pressure recording. The jugular vein was cannulated for injection of fluid and chemicals. The head was fixed in a rat stereotaxic instrument (David Kopf Instrument Co.) and the skull exposed. Several holes were made using a dental drill, in accordance with a stereotaxic atlas (Pellegrino & Cushman, 1967), for insertion of stimulating and recording electrodes. Rectal temperature was monitored continuously and kept at 36·5–37·5° C by a heating pad and an overhead lamp.

Since the anaesthesia and surgical procedure may influence the oscillatory cycle of hypothalamic neurones, experiments were started at different times of the day so that recordings began and ended in a different sequence in various experiments.

Stimulation and recording. A fine bipolar steel micro-electrode insulated except at the tip was inserted stereotaxically into the prefrontal area, or septal region,

depending on the need, and fixed to the skull by dental cement. Repetitive pulses of varied frequency and intensity were applied through a stimulus isolation unit (WP Instrument Co. 850). Splanchnic nerves were stimulated with repetitive pulses applied through a cuff electrode. Stimulus parameters are given for each occasion in the results section. For recording electroencephalogram (e.e.g.) a bipolar steel electrode was inserted to the parietal or temporal cortical region and fixed to the skull by dental cement.

Tungsten electrodes were used for recording from single cells. These were made by embedding fine tungsten wires in glass capillary tubes and etching their tips under microscopic observation to the desired size (Amassian, Macy, Waller, Leader & Swift, 1962). The best results were obtained by using electrodes having 15–20 μm wire tip exposure and a 1–2 μm tip size. Two such electrodes were mounted each on a separate micro-manipulator with remote controlled attachments (Narishige MO-15). Since the distance between VMN and LHA in rats is less than 1 mm, one electrode was inserted in VMN and the other in LHA of the contralateral side. Potentials were led through amplifiers (W-P Instruments 5-A) to oscilloscopes, to spike counters (Nicolet 1070 Series, SH-73 Multichannel Scaler) and to a polygraph (Grass 7A). Once a single cell was identified, the window discriminator was set for counting. Setting the upper limit of discrimination also eliminated the stimulus artifacts originating from stimulation of cortical areas which were of high magnitude. Occasionally but rarely two spikes, large and small, were picked up by one electrode and counted. Only when the difference between the two action potentials was very large, recordings of both units were made.

Spikes from a unit were counted every 1 or 2 sec, depending on frequency of firing, the result displayed on an oscilloscope and photographed by a Polaroid camera, as well as recorded on an $X-Y$ plotter. The output from the counter was also fed continuously to the polygraph on which e.e.g. and the blood pressure were also recorded. A tape recorder was used on some occasions to store results for future computer analyses.

Proof that recording was from a single cell throughout the long period of experiment. The following criteria were used for identifying action potentials from single cells. The magnitude, the duration, as well as the shape of the potential had to be identical. This was determined by using very fast oscilloscope sweeps triggered by an action potential. In order to determine whether action potentials were from only one cell or from two adjacent cells we used the following reasoning. If two units rather than one are firing and showing exactly the same size of response there should be at least a few occasions when the two spikes would occur simultaneously thus producing a larger than normal action potential. When this is observed one knows that two units are contributing to the record. However, we rarely encountered such phenomena during recordings from the hypothalamus.

Sometimes even with one electrode only, recordings were made from more than one unit. In such instances one large spike appeared along with a number of small spike-like deflexions, which were of nearly the same magnitude and only slightly larger than the amplifier noise. The window discriminator was easily set to count spikes from one unit only. The difference in size of these multiple spikes was such that the slight fluctuations in amplitude which occurred during recordings of many hours duration did not interfere with this discrimination.

Throughout the experiment, action potentials recorded from micro-electrodes were displayed constantly on two oscilloscopes at fast and slow speed. With the latter recording, two discriminatory voltage levels of window discriminators were displayed. For each spike thus selected to be counted a pip also appeared on the same screen, so that one was certain that the action potentials of the same unit were being

counted (Fig. 1*C*). These techniques made it possible to recognize the firing of a single unit and to follow it throughout the experiment.

Identification of the site of stimulation and recording. At the end of each experiment DC current was passed through the electrode to make a lesion for later histological identification of electrode tip placement. Since stimulating electrodes were steel, the tip placement was recognized as a blue spot when the tissue was fixed in ferro-ferri-cyanide-formalin solution: 3 % ferro- and ferricyanide mixture, one part; and 10 % formalin solution, one part. To mark the site of recording electrodes which were made of tungsten, stronger current (50–75 μA) was passed so that an electrolytic lesion was still produced.

The brain was then fixed and frozen sections, 50 μm in thickness, were made and stained with thionin or cresylviolet. The sites of electrode positioning and recording were then determined microscopically.

Computer analysis of rhythms. Some records were stored on magnetic tapes and later auto-correlation functions of discharge pattern of single neurones in VMN and LHA as well as cross-correlation functions of neurones between the two areas were analysed by the use of PDP 11-45 computer.

RESULTS

Out of 102 rats, nine were used for preliminary experiments and results described in the following sections were derived from data obtained in the remaining experiments. Simultaneous recordings from both VMN and LHA were done successfully in fifty-six, in thirty-four only LHA neurones were studied, and in three only VMN cell activities were recorded.

Circadian changes of VMN and LHA neurone activity

Changes in rate of firing of single neurones in VMN and LHA throughout the day were studied by simultaneous recordings from the two hypothalamic areas. Firing frequency of the same neurone was followed for many hours continuously throughout different times of the day. For example, recordings started at 1 p.m. and continued into the evening, or started at 10 p.m. and continued throughout the night. In thirteen cases firing patterns of two neurones in VMN and LHA were successfully followed for 6–7 hr, in eight rats recordings were done for 7–11 hr at varied times of day. When all results were put together, an interesting picture emerged. This is illustrated in Figs. 1 and 2. In general, the pattern is as follows: throughout the day the activity of neurones in LHA is generally higher than that of VMN judging from the fact that the rate of firing of LHA neurone is either higher or fluctuates more than that seen in VMN cells. In late afternoon, the rate of firing of LHA neurone increases further and oscillations of short duration, roughly every 7–15 sec, become very distinct (Fig. 1*A*, 3 p.m.). The high activity period in the late afternoon is not related to the time after surgery or anaesthesia, since experiments were started at different times of the day, as stated previously. In the early or

late evening, at times somewhat different for each neurone, activity begins to decline. As LHA neuronal activity decreases the VMN neurones become active; their firing frequency shows an increase and great fluctuations (Fig. 1*A*, 7 p.m.). Throughout the night, the VMN neurone is more active compared to that of LHA; just the opposite of the day period (Fig. 1*B*,

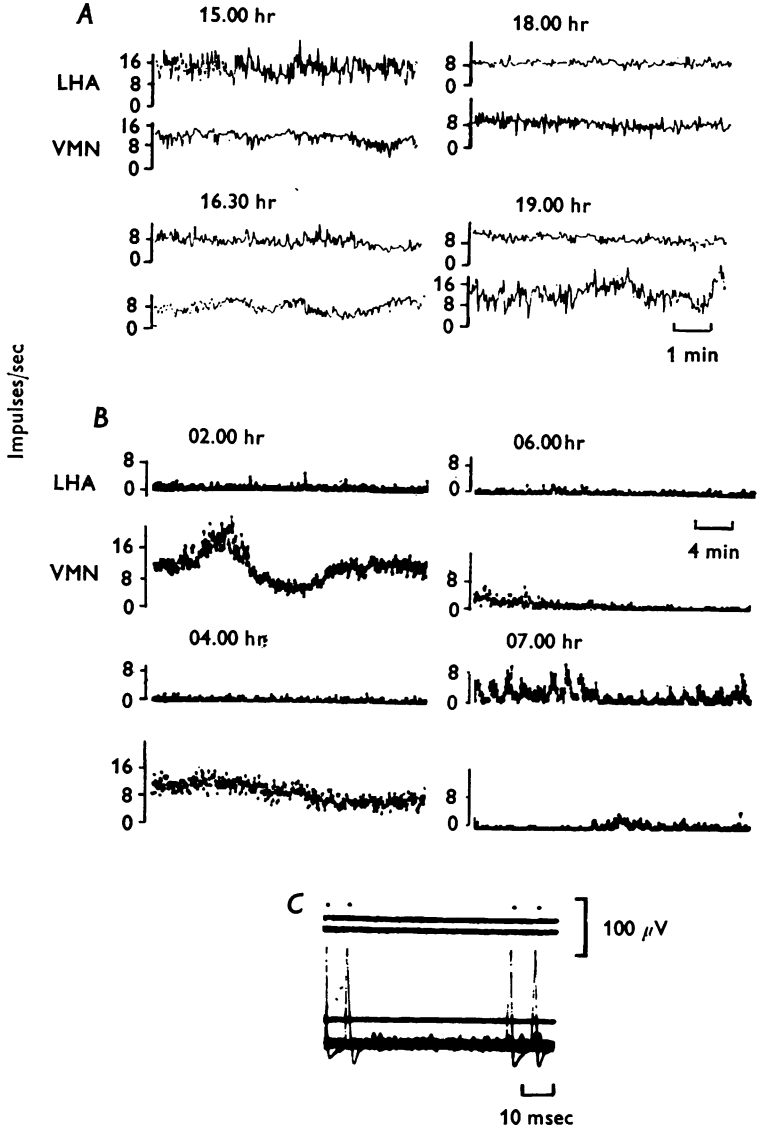

Fig. 1. For legend see opposite.

2–4 a.m.). These records shown in Fig. 1 *B* were taken from another experiment. In early morning hours, VMN neurones gradually become quiet (Fig. 1*B*, 6–7 a.m.), while LHA neurones begin to show some activity.

Fig. 2 illustrates changes in activity of LHA and VMN neurone during a 24 hr period. The average firing frequency of a neurone was determined every 20–30 min and plotted, so that short period oscillatory changes in cell activity are not shown in this Figure, as in Fig. 1. Fig. 2*A*, shows records obtained from four experiments in which single cells in both LHA and VMN were followed for many hours throughout different times of the day. As stated previously, the Figure shows the general pattern of circadian rhythm, i.e. during the day, activity of neurones in VMN is less than that of those in LHA but during the night their activity increases, while LHA cells show a low activity period in the morning and a period of high activity in the afternoon. In the early evening their (LHA) activity gradually decreases and continues to be low throughout the night. In Fig. 2*B* the firing frequencies of all neurones studied are averaged for each hour. Although changes in firing frequency of neurones occur at slightly different times, and the peaks and the troughs of their activity curves do not exactly coincide so that averaging makes the hour to hour changes in cellular activity seem much less, the Figure nevertheless indicates a general circadian pattern of VMN and LHA neurone activity.

Reciprocity in activity between VMN and LHA neurones

In addition to the circadian rhythm described above, the firing rate of VMN and LHA neurones showed several rhythmic fluctuations of shorter duration. The most clear and frequently seen rhythms showed fluctuations

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Fig. 1. Circadian changes in firing rates of neurones in ventromedial nucleus (VMN) and lateral hypothalamic area (LHA) recorded simultaneously. Each strip taken at a specific time of day as shown by time notations which indicate the beginnings of each of the paired tracings. *A*, records of discharge rates counted and displayed on an *X–Y* plotter in the late afternoon, 3–7 p.m. *B*, another experiment showing neurone activity in the early morning hours, 2–7 a.m. Polaroid pictures of oscilloscope display of a spike counter. Note that in the late afternoon LHA cellular activity begins to decrease, as that of VMN increases. During the night VMN activity is high but in the early morning, as VMN neurone becomes quiet, LHA neurone activity increases. *C*, a polaroid picture of oscilloscope display of individual spikes of a LHA neurone; three superimposed tracings. The top tracing shows pips indicating when spikes are counted. The second and third tracings are the upper and the lower voltage limits of a window discriminator. Any spikes appearing between these two lines are counted. Note the similarity in size and shape of spikes. Voltage calibration applies only to action potentials.

which occurred every 3–5 min and every 7–15 sec. During such rhythmic fluctuation in firing rates of VMN and LHA neurones, recorded simultaneously, the activity of two units was opposite, that is, when one unit's rate of firing increased that of the other decreased. Fig. 3 shows four

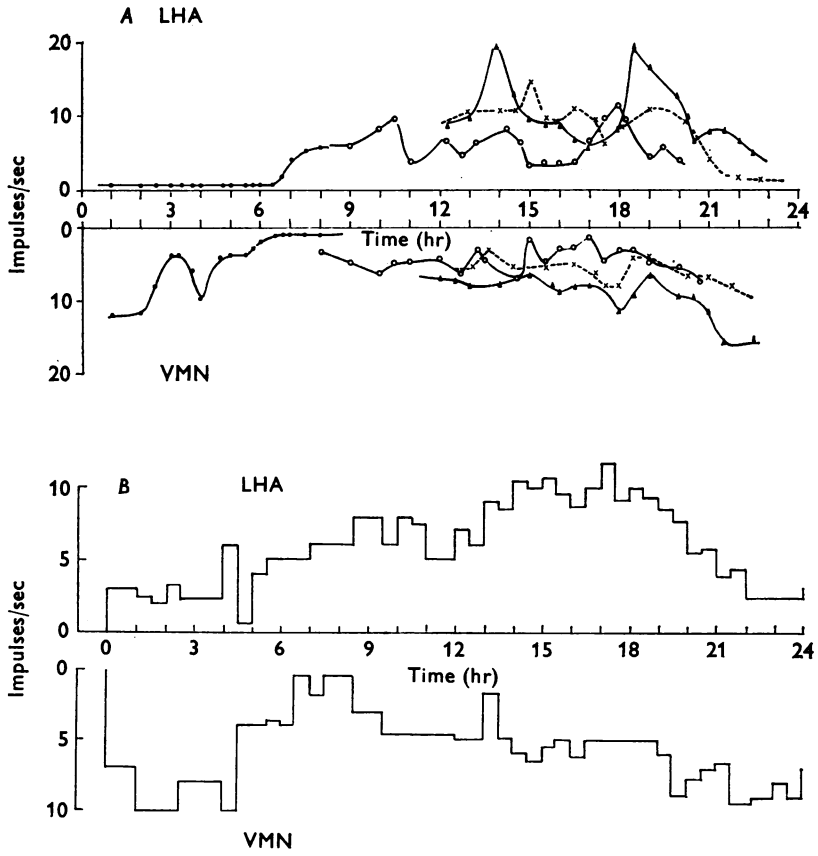

Fig. 2. Circadian changes in discharge frequencies of neurones in LHA and VMN. *A*, result from four different experiments in which single neurones from both areas were recorded simultaneously. Average frequency in every 20–30 min interval is plotted so that short period oscillatory changes in cell activity are not shown. *B*, compiled data from all experiments (thirty-three). Firing frequencies of all neurones are averaged for each hour. Note a general circadian pattern of VMN and LHA neurone activity.

examples. All are the $X-Y$ plot of firing frequency against time. In Fig. 3*A* there were slow and fast oscillatory changes in cell activity; the fast occurred every 5–7 sec, the other at 3–5 min. In both cases units in VMN and LHA changed in opposite directions.

As stated previously, the firing rates of LHA neurones were generally higher than those of VMN neurones and they also showed a greater oscillatory fluctuation during the day when most of these experiments were done. However, if one observes for only short periods the relationship may

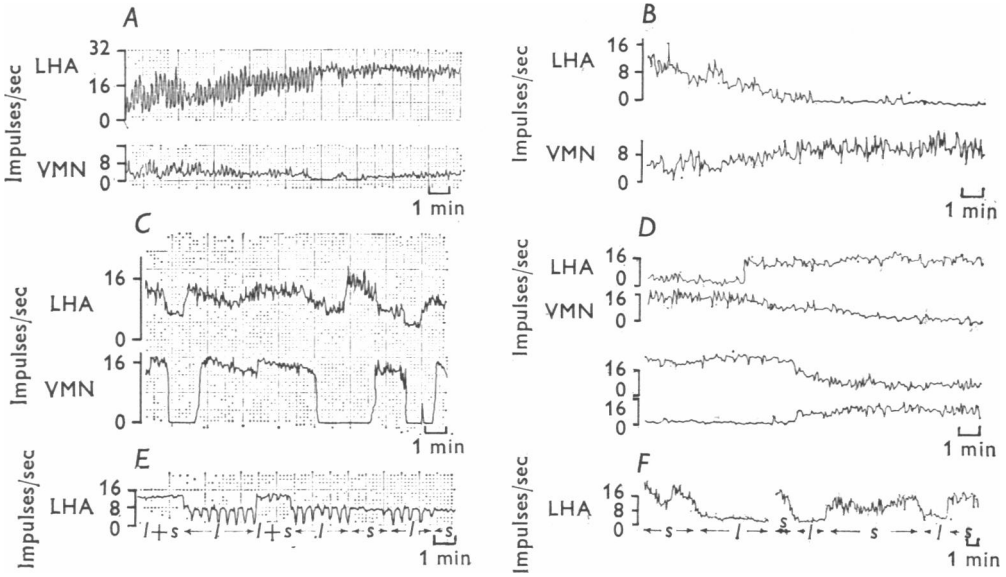

Fig. 3. Rhythmic fluctuations of short duration in activity of VMN and LHA neurones recorded simultaneously. All records are $X-Y$ plots of firing frequency counted every second against time. *A*, fast oscillatory changes occurring roughly every 5–7 sec. Slow changes, every 3–5 min, are in opposite direction in LHA and VMN neurones. *B*, slow oscillatory changes occurring every 5–8 min, again in reverse direction in the two neurones. *C*, changes in the activity of LHA and VMN neurones are in the same direction. *D*, the top and the bottom paired tracings are a continuous record. Slow oscillatory changes occurring every 15–18 min but in opposite direction in LHA and VMN neurones. *E*, behaviour of two LHA neurones, large (*l*) and small (*s*) spikes recorded from the same electrode: $l+s$ means both spikes counted together; *l*, large spikes alone; *s*, small spikes alone. Note that larger spikes show rhythmic oscillations but small spikes fire at steady rates. *F*, similar to *E* but from another LHA neurone. In this instance small spikes show higher and more fluctuations in rates of firing, while large spikes show low and steady rates.

appear to be different: during a time of low LHA cell activity and of high VMN cell activity the rate of firing of the VMN cell might be twice as greater as that of the LHA neurone. During another period LHA neurones might be seen to fire at a high rate, while the VMN neurones were almost quiescent.

Fig. 3*B* and *D* show still slower fluctuations in rates of firing, one which occurred every 15–18 min and another every 5–6 min, nevertheless, the change was opposite in VMN and LHA neurones. Occasionally the rhythmic fluctuations in activity of neurones in the two areas were in the same rather than reciprocal direction, as seen in Fig. 3*C*.

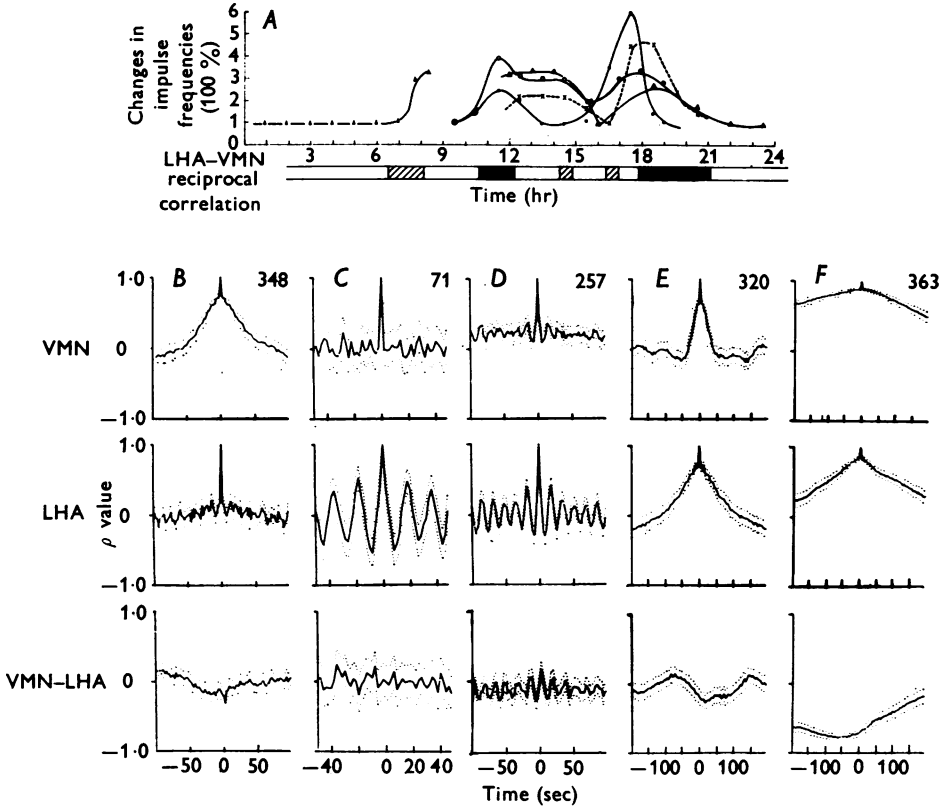

Fig. 4. Changes in discharge patterns of neurones in LHA and VMN. *A*, graph shows changes in firing frequency of six LHA neurones in 24 hr. The column at the bottom indicates the period when firing patterns of LHA and VMN neurones show reciprocal relationships. Periods when reciprocal relationships are the clearest (filled bars) correspond to the peak of high activity of neurones. Hatched bars also indicate similar occurrences but reciprocal relationships are less clear. *B–F*, computer analyses of five paired recordings of VMN and LHA neurones. Auto-correlation functions of VMN and LHA neurones shown on the upper two rows. The bottom row shows cross-correlation functions between VMN–LHA neurones. ρ values are correlation coefficients. Numbers indicate number of counts entered in the computer. Each vertical column of three graphs (*B–F*) taken from five different experiments. See text for further explanation.

During many hours of observation neurones in VMN and LHA underwent periods of steady firing, mixed with periods of rhythmic fluctuation during which rates in the two areas waxed and waned mostly in a reciprocal fashion. The latter occurred when LHA neurones showed high activity. This is shown in Fig. 4*A*. Alterations in firing rates of six different LHA neurones for 8–10 hr are calculated as % changes from their lowest activity period. They are plotted on the top tracings while periods of reciprocal changes in the activity of both VMN and LHA neurones are indicated below. The filled areas show times when reciprocal relations of the two neurone types were very clear, hatched areas show somewhat less correlation but nevertheless such occurrences were present. This was compiled from thirty-three preparations.

Fig. 4*B–F* shows five examples of auto- and cross-correlation functions of VMN and LHA neurones analysed by a computer. Autocorrelation functions of VMN neurones are shown on the top row, that of LHA neurones on the middle row, and cross-correlation functions of VMN *vs.* LHA neurones are represented in the bottom row. Fig. 4*C* and *D* show clearly the fast rhythmic patterns of LHA neurone firing at a rate of approximately every 15 sec, while *B* and *F* show slower rhythm of VMN and in *E* and *F* LHA neurones exhibit slow oscillations occurring at 2–6 min. The reciprocal relationship between activity of VMN and LHA neurones is shown as a negative peak in cross correlation functions, as shown in Fig. 4*B* and *F*. In Fig. 4*B*, it is seen that the VMN neurone discharge is likely to lead that of the LHA by 1 sec, while in Fig. 4*F*, which shows the slow oscillations in both VMN and LHA neurones, the rhythmic firing of LHA cell seems to lead that of the VMN by nearly 1 min.

Another interesting observation is that neurones which lay side by side did not necessarily have the same rhythm. When activity of two neurones, one with large spikes and the other with small spikes, were recorded by an electrode simultaneously, one showed rhythmic fluctuations in firing frequency, while the rate of the other was steady. Fig. 3*E* and *F* show such examples. In Fig. 3*E*, a cell having large spikes discharged at a slower frequency and with a rhythmic pattern, but the other neurones having small spikes fired at a steady frequency. In Fig. 3*F*, taken from another experiment, the relationship was opposite: a neurone with large spikes fired at low, steady level, while another neurone with small spikes fired at faster rates and showed more fluctuations.

Response of neurones to external stimuli

In order to study the difference in behaviour of neurones in both VMN and LHA to the same external input, two stimuli were chosen. One was stimulation of splanchnic afferent fibres as representing impulses from

visceral organs, the other was stimulation of prefrontal cortex or septal area as examples of parts of the higher centres influencing the hypothalamic regions under study. Fig. 5 shows examples in which the same external stimuli produced the opposite effect in VMN and LHA neurones. The stimulation of the prefrontal area of the cortex at 1/sec produced a decrease in firing rates of the LHA neurone under study, while the VMN neurone increased its firing rates following the same stimulus (Fig. 5*A*). On

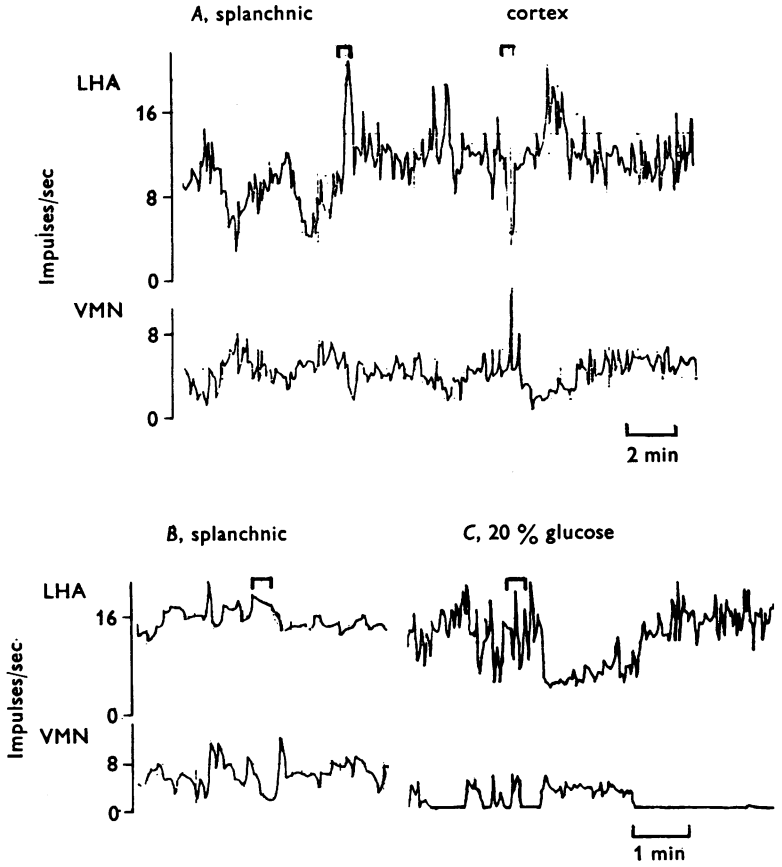

Fig. 5. Effects of stimuli on neurones of VMN and LHA, simultaneously recorded. *A* and *B* show effects of stimulation of splanchnic afferents (splanchnic: 20/sec, 0·3 msec, 10 V) and of prefrontal cortex (cortex: 5/sec, 0·2 msec, 10 V). Note reciprocal effects on frequency of firing of two neurones. The time of stimulus indicated by a mark (⊓). *C*, effects of an intravenous injection of 20 % glucose, 0·4 ml. Note the latency of this response is much larger than that of other stimulations. *X–Y* plotter was used for recording counted pulses every second.

the other hand, splanchnic afferent stimulation at 20/sec caused an increase in LHA neurone activity and inhibited the VMN neurone (Fig. 5*A* and *B*).

Another important finding was that responses to these external stimuli changed; what occurred depending on the time of day, or on the different levels of background firing rates. Fig. 6*A–E* shows another example. When an LHA neurone's discharge rate was around 4–5/sec, as occurs

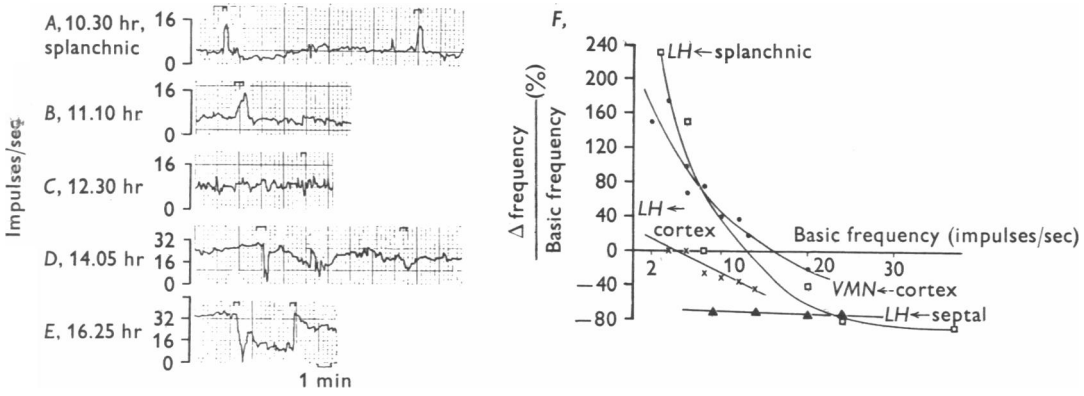

Fig. 6. *A–E*, changes in nature of response to splanchnic afferents stimulations (20/sec, 0·3 msec, 10 V; marked by ⌐) during the day. *X–Y* plotting of counted spikes from a single LHA neurone is shown. *A* and *B*, accelerations occurred in the morning; *C*, the activity and responsiveness were low about noon; *D*, in the late afternoon activity was at a high level and splanchnic stimulation had an inhibitory action. *E*, a second stimulation was given when cell activity was still depressed from the first stimulus. Note that an increase in firing was produced. *A–E*, the time of the beginning of each record indicated on the left. *F* shows relationships between basic firing frequency and effects of various stimuli. Each curve was obtained from single neurones of VMN and LHA in different experiments. Basic firing frequency at the time of stimulation was plotted against % changes in rates of discharge: □—□, *LH* ← splanchnic, responses of LHA neurone to splanchnic afferent stimulation; × — ×, *LH* ← cortex, prefrontal cortex stimulation effect on LHA neurone; ●—●, *VMN* ← cortex, prefrontal cortex stimulation effect on VMN neurone; ▲—▲, *LH* ← septal, septal stimulation on LHA neurone. All curves are calculated to be the best fit for the recorded points (□—□ and ●—●, exponential; × — ×, linear regression; ▲—▲, quartic curves).

during the low activity period of the late morning (10.30 a.m.), splanchnic nerve stimulation increased the rate of firing to 15/sec. The repeated test made 15 min later gave an almost identical response (Fig. 6*A*). At 11 a.m. when firing rate was still at the same level the nerve stimulation produced a similar augmented response in cellular activity. At the midday period, however, the neurone activity was becoming slightly higher, approximately 8/sec, the same stimulus produced a very slight reduction (to

5/sec) in firing rate (Fig. 6*C*). Fig. 6*D* and *E*, show the effect of the same nerve stimulation given during a period of high activity in the late afternoon. Shortly after 2 p.m. when firing rate of the cell was over 20/sec, splanchnic nerve stimulation caused a sharp decrease in rate and a nearly quiescent state (Fig. 6*D*). After that activity of the cell became more variable but the second trial still reduced it, from 19 to 10/sec. At 4.30 p.m. when the cell was most active, firing at over 30/sec, the nerve stimulation produced a profound depression, to zero activity temporarily, and the rate subsequently remained at the low level of 10–14/sec for 4 min. At this time again the same stimulus was given. The rate of firing of the cell increased instantaneously to 35/sec, then gradually fell to 25/sec. It might be argued that the splanchnic stimulation merely aided the recovery of the cell activity from the previous stimulation but certainly the stimulus did not produce further decrease in firing.

The graph in Fig. 6*F* gives a clear picture of relationship between the basic firing frequency and the effect of stimulations of splanchnic nerves and prefrontal cortex. Each curve was taken from one neurone in the various experiments. Splanchnic stimulations augmented the activity of an LHA cell when the basic rate was low, less than 8/sec in this particular instance, while inhibited it when basal activity was higher than this level. A similar tendency was observed in the response of a VMN neurone to prefrontal cortex stimulation. A curve expressing the effects due to stimulation of splanchnic afferents on VMN neurone could not be constructed, since most experiments of this type were done during the day when VMN activity was usually low. Splanchnic nerve stimulation only mildly inhibited the cell. It was interesting to find that septal stimulation at higher frequency (10–20/sec) always inhibited LHA neurones regardless of the basic frequency of discharge.

The relationships between basic firing frequency and the responses to external stimuli shown in this graph (Fig. 6*F*) support a general conclusion that when basic firing rate is low, responses are greater and that they are reduced when basal rates are high. The exact slope of the curve and the point where a curve crosses the basic frequency line differ from one neurone to another and one cannot predict exactly at what frequency the activity of a cell will be augmented or depressed by a given stimuli. However, when the activity of a neurone is followed for many hours, and if one knows its high- or low-activity period, it is almost always possible to estimate whether a stimulus will evoke an increase or a decrease in rate of firing.

The graph also explains why certain external stimuli produced opposite effects in VMN and LHA neurones, since firing frequency of VMN cells is generally low and that of LHA neurones is high during the day. It also shows that, at the same frequency of firing, stimulation of prefrontal cortex

tended to augment VMN neurones, while inhibiting LHA neurones. Thus, there is some intrinsic difference in external influence on the two areas, but the result of the stimulations is markedly altered by basal activity and hence responsiveness of a cell. Therefore, the influences of external stimulus depend on the time of the day as well as the period during which shorter rhythmic fluctuations are most obviously superimposed on the circadian rhythm.

The intravenous injection of glucose always produced augmentation of the activity in VMN neurones, while inhibiting the LHA neurones. An example is shown in Fig. 5*C*. The Figure shows the usual glucose effect after a considerable latency (10 sec) and the reversal of response or overshoot which generally occurs at the end of the positive reactions. Two minutes after the end of injection, LHA neurone increased its firing rate to or above the level maintained previous to the injection, while VMN neurone became completely quiescent. The above mentioned effect of glucose on the hypothalamic cells has been reported previously by many investigators (Anand *et al.* 1964; Oomura *et al.* 1964, 1969*a*; and Brooks *et al.* 1966). Since such tests were made during the day, when activity of LHA neurones is high and that of VMN neurones is low, we investigated the glucose effect in similar experimental conditions during the night when VMN neurone activity was high and LHA neurones fired at slow and steady rates. The result from one such experiment is illustrated in Fig. 7. Fig. 7*A* shows that at 8–8.30 p.m. LHA neurone is firing at a low and fairly steady level, while the VMN cell is more active compared to its firing level during the day. Some waxing and waning in firing frequency is apparent but the injection of glucose inhibited the LHA neurone to such a degree that the cell became almost quiescent. Conversely the frequency of VMN neurone firing increased greatly for several minutes. One hour later, at 9.30 p.m., glucose was again injected at a time when LHA neurone showed some periodic increase in firing rate. Essentially the same results were obtained. Thus, the action of glucose on these neurones does not seem to be affected by the circadian changes of neurone activity.

The origins of oscillations in LHA neurones

The first attempt to determine the origin of the LHA neurone's rhythms of firing consisted of making surgical sections around the recording electrode without losing a unit. Only oscillations of short duration were studied since it was not possible to investigate over many hours the long-term changes in neurone activity. In such instances one cannot state with certainty whether alterations in firing rhythm of a single cell are caused by experimental procedures or occur naturally. All the studies described in this section were done on LHA neurones alone, and not on

VMN cells, since the latter showed less rhythmic fluctuations in short duration activity.

In five experiments the tissue surrounding the electrode was severed by inserting a fine razor blade. It was found that a section could be made caudal to the electrode, or rostral thereto, or both, but never could cuts be made completely surrounding an electrode, so as to make an island, without silencing or losing the unit from which recordings had been made for hours previously. A section caudal to the electrode did not produce any

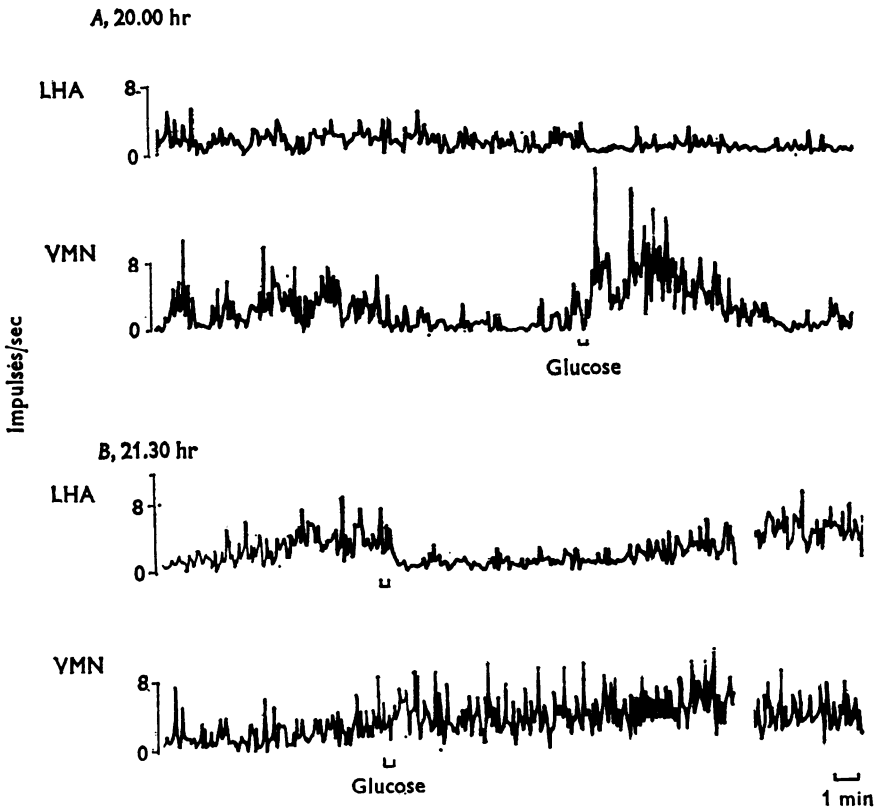

Fig. 7. Effect of 20 % glucose (0·2 ml.) injected intravenously on LHA and VMN neurones recorded simultaneously. $X-Y$ plot of counted spikes every second against time. Records were taken at night when LHA neurone activity was low while VMN cell activity was high. *A*, at 8 p.m. glucose injection produced a greatly increased VMN cell discharge which continued for several minutes. LHA neurone activity was depressed and firing almost ceased for 2 min. *B*, at 9.30 p.m., glucose was again injected when LHA neurone showed some waxing tendency. Glucose greatly decreased its activity and a gradual recovery began after several minutes. VMN cellular activity was augmented. The break in tracings indicates 5 min period.

change in the cell's oscillatory activity but a rostral section, though not stopping the firing, always abolished the rhythmic changes of at least those of short duration.

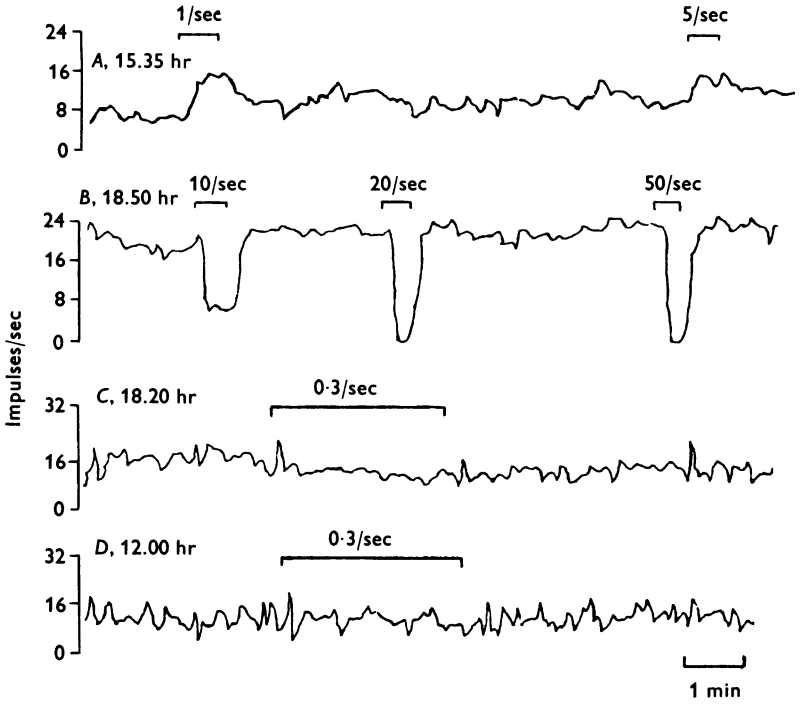

Fig. 8. *A* and *B*, effect of septal stimulation (0·3 msec, 10 V; marked by ⌐) on frequency of firing of a LHA neurone. *X–Y* plotting of spikes counted every second against time. Note that responses differed with frequency of stimulation. *C* and *D*, effect of septal stimulation at a subthreshold intensity and low frequency: *C*, 0·1 msec, 3 V; *D*, 0·1 msec, 5 V. Note previous oscillations in firing rates were depressed or altered by the stimulus. *A* and *B*, the same cell; *C* and *D*, each from different experiments. Numbers indicate time of day.

Since the septal area has major connexions with the hypothalamus, we first focused our attention on the role of this region. Septal nuclei, if stimulated by a strong single pulse, produced an action potential with a 4 msec latency in LHA neurones followed by inhibition of spontaneous discharges. Changes in the frequency of stimulation of the septal area produced different effects on spontaneous firing of LHA cells; Fig. 8 shows examples of these effects. Stimulation of the septal area at a rate of 1–5/sec evoked augmentation (Fig. 8*A*), while 10/sec stimulation produced mainly a depression of LHA neurone activity (Fig. 8*B*). As the frequency was increased to 20 and 50/sec the inhibitory effect became greater (Fig. 8*B*).

When the stimulus intensity was reduced much below the threshold for direct excitatory and inhibitory effect on LHA neurones, stimulation of the septal area disturbed the oscillations in firing rate of LHA neurones. Fig. 8*C* and *D* illustrates this. More specifically, when the parameters of the applied stimulus were changed to less than half of threshold intensity, to a pulse duration a third of that previously used and to a stimulus rate of once every 3·3 sec, stimulation disturbed the oscillatory tendency of the cell (Fig. 8*C*). In another animal such a stimulus produced a different and slower rhythm during its application to the septal area, as seen in Fig. 8*D*.

These observations seem to suggest that the septal area may impose some kind of rhythmic fluctuation on the LHA neurones since the sections made between the hypothalamus and septal area eventually abolished or altered the pattern of activity in LHA neurone. Fig. 9 shows that the severance of connexion between the two areas abolished oscillations seen previously in LHA neuronal activity (Fig. 9*B–E*). Septal stimulation after this procedure showed no effect on LHA cell activity, as expected, although it had a mild augmenting effect on the LHA cell before the section. A low voltage high frequency e.e.g. was usually observed at least for some time after the section was made, as during the period when the picture of Fig. 9*C* was taken. This could be due to injury of the cortical tissues since a fine razor blade had been used to cut through all structures below the septum. However, some 38 min later, at the time when Fig. 9*D* was taken, the e.e.g. pattern returned to its previous state showing a low frequency, high amplitude pattern.

Another example of the effects of stimulations and brain sections on LHA cell rhythms is shown in Fig. 9*F–J*. The particular cell of the LHA from which these recordings were made showed oscillatory changes in firing rate which occurred at 3–4 min intervals in the late afternoon period (Fig. 9*F*). When a splanchnic nerve was stimulated the rate of firing was reduced and the slow oscillatory waves also disappeared, as seen in Fig. 9*F* and *G*. After some 10 min of this state the septal area was stimulated at a rate of 5/sec. It not only increased the firing rate but also the slow oscillatory fluctuation in cell activity returned, as seen in Fig. 9*G* and *H*. Following the sectioning of the brain between septal region and the hypothalamus, the characteristic oscillations disappeared and were replaced by random changes of small magnitude. Again, during the period Fig. 9*I*, though not shown here, the e.e.g. pattern changed to a low voltage, high frequency pattern but returned to the previous level of high amplitude, slow wave during Fig. 9*J*.

Another area which exerts strong influence on rhythmic fluctuation is that of the suprachiasmatic nuclei of the hypothalamus. The role of suprachiasmatic nuclei in the control of circadian rhythm of pineal enzyme

and adrenal corticosterone levels has been discovered recently (Moore, 1974). Also the influence of light on neurones of the suprachiasmatic nuclei has been studied (Nishino, Koizumi & Brooks, 1976). As shown in Fig. 10*A*, stimulation of this nucleus at rates of 5–20/sec inhibited neurones

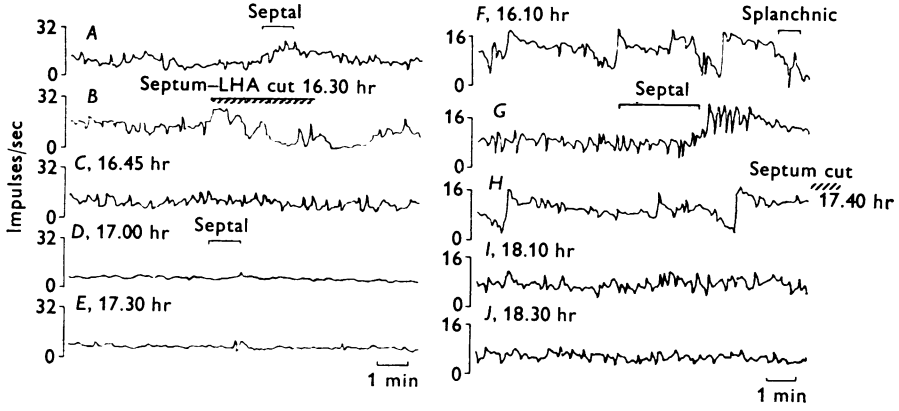

Fig. 9. Changes in frequencies and rhythms of discharge of an LHA neurone. *A–E*, *X–Y* plotting of counted spikes from the same cell and *F–J*, from another cell. *A* and *B*, continuous recording showing effects of septal stimulation (2/sec, 0·3 msec, 7V) and effect of transecting connexions between septum and LHA at 4.30 p.m. *C*, LHA activity 15 min after transection and at a time when low voltage high frequency e.e.g. was observed (not shown). *D*, the low level of activity and failure to respond to a septal stimulation (as in *A*), 30 min after transection. At this time e.e.g. showed high voltage, low frequency pattern, similar to the period before transection. *E*, low frequency of discharge 1 hr after severance of connexions between LHA and septum. *F*, another cell. Control, or basic state, showing slow (3–5 min) oscillatory changes in firing rates. Splanchnic stimulation (20/sec, 1 msec, 10 V) reduced frequency and abolished major slow oscillations. *G*, continuation of effects of splanchnic stimulus until septal stimulation (5/sec, 0·3 msec, 7 V) after which frequency increased and major slow oscillations reappeared as shown in *H* (*F–H* a continuous recording). *H*, connexions between septum and LHA cut at 5.40 p.m., end of period. *I*, frequencies and rhythms recorded after transection and during a time when e.e.g. showed a low voltage high frequency pattern. *J*, state of activity in LHA neurone 50 min after transection. E.e.g. pattern (not shown) returned to that observed before the transection.

in LHA. However, when the intensity and the frequency of stimulus was much reduced, suprachiasmatic nuclei stimulation only inhibited the LHA neurone rhythm, as seen in Fig. 10*B* and *C*. Since electrical stimulation is an artificial means of producing excitation of neurones, the above finding does not necessarily prove the existence of a direct physiological influence

of suprachiasmatic nuclei on LHA neurones but it indicates this possibility and suggests that various regions probably exert influences on LHA, thus creating various rhythms of neurone activity therein.

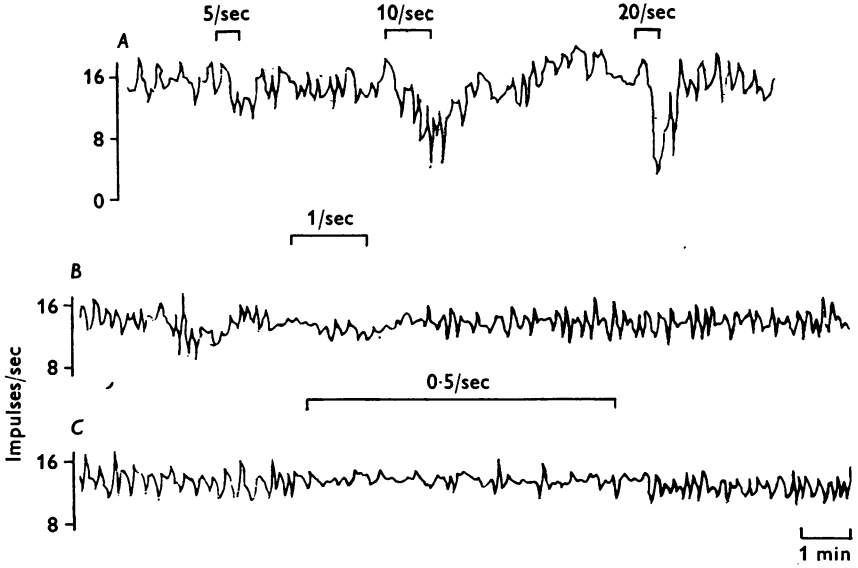

Fig. 10. Effect of stimulations of suprachiasmatic nucleus on LHA neurones. $X-Y$ plot of discharge frequency counted every second against time. A, inhibitory effects on firing frequency produced by high intensity and frequency of stimulation (0·5 msec, 10 V). B and C, in contrast with A stimulation at a subthreshold intensity and low frequency (0·2 msec, 4 V) produced only changes in pattern of discharge of LHA neurone. B, fast oscillations were depressed. C, changes in oscillation frequency occurred. B and C, are from two different experiments.

DISCUSSION

Circadian rhythm of neurone activity in VMN and LHA

There are many 24 hr rhythms in body functions and their peaks occurring at different times (Moroji, Yamazaki, Okada, Saito, Asano & Yamashita, 1973; Hedlund *et al.* 1975). The well known periodicity in corticosterone secretion by the adrenal cortex, in rats, has its highest peak in the afternoon. Plasma 17-hydroxycorticoid and adrenocorticotrophin (ACTH) levels are highest before periods of maximum activity (around 6–8 p.m. in rats, in the early morning in man). ACTH releasing factor activity within the hypothalamus is also known to have a circadian periodicity. There is a 24 hr rhythm in pineal melatonin and *N*-acetyl-transferase levels, high peaks occurring in the darkness or at night. A circadian pattern of the pituitary growth hormone release has a peak around 6–9 p.m. The

pituitary prolactin content is highest during the periods of light, while plasma prolactin level is high during darkness. Many known neurotransmitters, acetylcholine, norepinephrine, 5-hydroxytryptamine, all have been reported to have a 24 hr periodicity in their levels in various regions of the central nervous structures. These observations indicate that the hypothalamus is very much involved in circadian activity.

Papers describing the circadian rhythm of neurone activity in mammals are scarce. There is an abstract reporting that multi-unit activity of the rat's pre-optic area shows a circadian rhythm (Johnson *et al.* 1971). Higher levels of activity have been found during the night and this rhythm of change remained unaffected by gonadal hormone levels. Work from this laboratory (Schmitt, 1973 *a*, *b*) showed that neurones in the rat's LHA have a period of general low activity in the late morning which extended to the early afternoon hours and a high activity period which occurs in late afternoon. The present work reports for the first time a linked circadian rhythm of both LHA and VMN neurones simultaneously recorded. The results show that during the day time the activity of LHA neurones is generally higher than that of VMN cells; during the night the reverse occurs, i.e. VMN neurones are more active than LHA neurones. In the early evening and early morning a shift in neurone activity of both areas take place and, as a neurone in one nucleus become gradually active, a cell in the other nucleus become quiet, as though the one area imposes some influence on the other.

The identification of the factors which produce or influence the circadian rhythm in VMN and LHA neurones poses a difficult problem. Recently the importance of the suprachiasmatic nuclei in control of circadian rhythms of pineal enzyme levels as well as of adrenal corticosterone levels and of other various behaviours has been demonstrated, since a lesion in this area abolishes the above mentioned cyclic changes (Moore, 1974; Rusak & Zucker, 1975). We have previously shown an interrelationship between the optic system and suprachiasmatic nuclei neurones, as well as the latter's influence on the cervical sympathetic nerves which innervate the pineal gland (Nishino *et al.* 1976). All these data suggest that the suprachiasmatic nuclei may influence the circadian rhythm of LHA and VMN cells and we indeed showed that stimulation of this area with very low intensity and frequency did alter rhythmic activity of short duration in LHA neurones.

However, this was not the only area found to influence rhythmic fluctuations of LHA neurone activity. The septal area also had a strong effect on LHA activity and stimulating the area with low frequency pulses tended to 'drive' LHA neurones. The severence of connexions between the septal area and LHA abolished certain rhythmic fluctuations in LHA cell activity. It has been shown that activity of septal neurones in rats is

affected by light, e.e.g. arousal and sleep (Yamaoka & Hagino, 1974). Since recordings have not been taken throughout the entire day in their work, it is not known whether septal neurones possess a circadian rhythm but it is interesting that septal neurones have a close relationship to the hippocampus (Macadar, Roig, Monti & Budelli, 1970; Yokota, Reeves & MacLean, 1970; Morales, Roig, Monti, Macadar & Budelli, 1971), which is said to be involved in diurnal variation of ACTH release (Mason, 1958; Moberg, Scapagnini, de Groot & Ganong, 1971). More studies will be needed to find the source of circadian and other rhythmic fluctuations of LHA neurones. However, the results presented have shown that LHA cells of the hypothalamus themselves do not possess an extensive intrinsic rhythmicity of activity under normal conditions, at least under conditions which we were able to maintain and which are considered normal.

Short period oscillations of neurone activity

Oscillations of short duration in discharge rates of neurones have been described also by others. A periodicity of from 7 sec to 2 min was found in the slow potential recorded from the surface of the cortex and from the hypothalamus of rabbits (Aladjalova, 1964). The author suggested its origin to be related to humoral factors operating within the hypothalamus. Oomura *et al.* (1969*b*) found that some cells in the LHA and VMN of freely moving cats exhibited a periodicity, activity increasing every 7–15 sec but these cells were mostly not those responding to behavioural changes, such as transition from sleep to the alert state ('non-specific cells'). These authors suggested that these non-specific cells may be responding to humoral factors and thus acting as 'tonic neurones'. Their activities were thought to be related to the long term regulation of food intake. In the work described in this paper we found that there were every 7–15 sec oscillatory rhythms as well as slower, 3–5 min periodicities in rat LHA neurones. Occasionally slower waves of 15–18 min duration were also recorded. These oscillations were less prominent in VMN cells.

It is interesting that in unanaesthetized cats most VMN cells (over 70%; were aperiodic during the waking period but during sleep their rates of discharge increased and short term oscillations were present. On the other hand LHA neurones increase their firing when conditions of the cat change from the sleeping state to wakefulness (Oomura *et al.* 1969*b*; Oomura, 1973). Such behaviour of VMN and LHA neurones in sleeping, unanaesthetized cats resembles what we observed in anaesthetized rats during the night. Since rats are considered to be more active during the night than the day, the two states do not have behavioural correlates. The common feature for both states, however, is the lack of input to the optic system. Thus this change in activity may be related to changes in

pineal hormones or other humoral factors. More study is needed before we can offer any explanation of this point.

Reciprocal relationships between VMN and LHA

Another intriguing problem is the interaction between VMN and LHA neurones. Reciprocal relationships between neurones of the two areas were observed in their circadian rhythmicity as well as in shorter oscillatory changes which occur in neurone activity. Oomura and his associates (1969*b*) have shown that in cats under chronic conditions over 60% of neurones in VMN and LHA show reciprocal responses in states of sleep, alertness and eating, if the cat is in the 'food deprived' state. Responses of VMN and LHA neurones to glucose are also reciprocal, as stated previously. Morphologically there seems to be ample connexions between VMN and LHA. Axons of VMN neurones project to LHA in addition to other areas in hypothalamus, afferents from the LHA neurones reach VMN (Millhouse, 1973*a*, *b*). It has been shown that VMN stimulation inhibits LHA neurone activity or vice versa (Oomura, Ooyama, Yamamoto & Naka, 1967; Oomura, 1973), although such inhibitory influences could not be demonstrated by others (Murphy & Renaud, 1969).

Our study of cross-correlation functions between oscillatory activities of VMN and LHA neurones indicates that the LHA cell may lead VMN neurone in one occasion, in another the reverse occur and that peaks of oscillatory waves of neurone activity of VMN and LHA can be separated by a second to as long as 50 sec. A similar observation also has been reported (Oomura *et al.* 1967). Thus, it is yet difficult to know whether VMN or LHA neurones exert direct influence on the other area, or whether both areas are affected differently by another driving source in the central nervous system.

Responses of VMN and LHA neurones to external stimuli

One of the important findings of this paper is that the response of a LHA or VMN neurone to the same external stimulus varies with the basic firing frequency of the neurones at the time of stimulation and thus varies with the time of the 24 hr cycle. Schmitt (1973*a*) also found that responses of LHA neurones to activation of 'glucoreceptors' in the hepatic portal area were different at various times of the day. It is not clear why effects of certain stimuli on hypothalamic neurones are dependent on the basic firing frequency of the cell, while other stimuli such as stimulation of the septal area or glucose administration have similar effects regardless of the basic activity of the cell.

The mechanisms by which the same afferent impulses evoke depolarization or hyperpolarization of a neurone, depending on the state of the

membrane is not known. There may be such a phenomenon as occlusion of impulses converging on a cell membrane so that there is a certain limit of firing frequency, or these neurones possess recurrent collaterals. The existence of both inhibitory and excitatory axon collaterals in VMN has been reported (Renaud & Martin, 1975). It is possible that when cells are firing at high frequency afferent impulses will bring the cell to such a state that inhibitory recurrent collaterals begin to operate; this would result in depression of cellular activity. On the other hand, in slowly firing cells the same input only evokes an increase in firing rates without much affecting the recurrent collateral system. Whatever the reason, one must be cautious in offering explanations when studying the response of hypothalamic neurones to external stimuli.

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