Modulation of SCN Multiple Unit Activity (MUA) by Dorsal Raphe

“How can this clock be speeded up; slowed down; reset or stopped?”

Curt P Richter (1965)
1 INTRODUCTION

The property of rhythm generation in SCN is cell autonomous with a genetic basis (Welsh et al., 1995; Herzog et al., 1997; Antoch et al., 1997; King et al., 1997; Sun et al., 1997), implying the presence of multiple independent oscillators. However, the strengthening and stabilisation of the individual periods of these oscillators into a single unified circadian period is made possible by the circuit interactions among neurons (van den Pol and Dudek, 1993; Bouskila and Dudek, 1995; Zhang et al., 1995). The most common mode of interaction among neurons is by Na\(^+\) and Ca\(^{2+}\) dependent action potentials (van den Pol and Dudek, 1993).

1.1. ELECTROPHYSIOLOGY OF SCN NEURONS

Intracellular recordings have shown that SCN neurons have: an average membrane potential of -50/-59 mV, action potentials with amplitude ranging 58.2-83.8 mV with a high input resistance; short membrane time constants; and short duration action potentials followed by afterhyperpolarisations (Wheal and Thompson, 1984; Kim and Dudek, 1993).

SCN neurons, like other hypothalamic neurons, are known to display low spontaneous discharge rates: 0.2 - 2.2 Hz / 2 - 8 Hz / 0.1 - 11 Hz are the most common in vivo discharge rates with an irregular firing pattern; 3 - 9 Hz / 3 - 34 Hz are the most common in vitro firing rates in hypothalamic slices containing SCN (Meijer and Reitveld, 1989; Kim and Dudek, 1993), and 0 - 10.3 Hz in cultured SCN explants (Herzog et al., 1997). A function of the firing rate is the firing pattern (Kim and Dudek, 1993). In vitro extracellular single unit recordings revealed that firing patterns of SCN neurons can be regular, irregular, bursting (Meijer and Reitveld, 1989) or harmonic (Zhang et al., 1995), the last pattern thought to be essential for the rhythmic SCN discharge by acting as a filter.

The heterogeneity of SCN neurons in some basic as well as active membrane properties have enabled their classification into at least three clusters. Cluster I
and II cells fire spontaneously in a regular and irregular mode, respectively, and sustain prolonged spike trains. In contrast, cluster III cells have low firing rates but may adopt a burst-like firing mode when receiving appropriate input. While all clusters transmit output to target cells within and outside SCN, cluster III cells in particular are suggested to affect excitability of large numbers of SCN neurons by their extensive local network of axon collaterals (Pennartz et al., 1998). A collective measure of the electrical discharge rates of many (on the order of thousands) neurons is the multiple unit activity (MUA). MUA recorded from SCN shows distinctive characteristics in keeping with the circadian pacemaker function ascribed to this nucleus. These characteristics are (Inouye, 1996):

1. an exceptionally large amplitude of the MUA during the course of the day, the maximum MUA amplitude usually being more than ten times higher than the minimum activity at a different time of the day;
2. a unique phase relationship to the light/dark (L/D) cycles with broad peaks during subjective daytime (CT 6-8) and broad troughs during the night time; this phase relationship is invariant regardless of the experimental lighting regimen ie, L/D, LL or D/D;
3. the phase of MUA within SCN is opposite to that of the rest of the brain where it shows either no significant circadian variation or a small increase when the animal is behaviourally active;
4. the MUA of SCN is independent of sleep-wake stages of the animal in contrast to other brain regions where electrical activity is high when the animal is awake or in REM sleep, and low during slow wave sleep (SWS) at any time of the day. Thus, "pacemaker keeps a constant pace without being disturbed by external noises" (Inouye and Kawamura, 1982; Inouye, 1996; Meijer et al., 1996).¹

¹ However, spontaneous in vitro activity of SCN neurons in Trypanosome infected rats, displayed a reduced firing rate and phase advance of its circadian peak (Lundkvist et al., 1998).
(light activated) and the rest decreasing (light suppressed) (Meijer et al., 1996). These heterogeneously distributed light responsive cells (Meijer et al., 1996), possibly with heterogeneous intrinsic membrane properties (Kim and Dudek, 1993), enable the SCN to monitor enduring light intensities naturally occurring at dawn and dusk and thus mediate photic entrainment of circadian rhythms (Meijer et al., 1986).

1.2. SEROTONERGIC (5-HT) MODULATION OF SCN

The response of SCN to L/D cycle and thus entrainment is modulated by several agents such as benzodiazepines, locomotor activity and scheduled voluntary exercise, activity inducing stimuli such as dark pulses, triazolam etc. (Turek et al., 1995). Several investigations have shown that 5-HT released from the terminals of the midbrain raphe nuclei (Azmitia and Segal, 1978; Moore et al., 1978; Steinbusch, 1981) could mediate non-photopic entrainment (Edgar and Dement, 1991) or modulate photic entrainment Bradbury et al., 1997).

1.2.1 Serotonergic System

The serotonergic (5-hydroxytryptaminergic, 5-HT) system, one of the most diffusely organised projection systems of the brain, consists of (1) a morphologically diverse groups of neurons the cell bodies of which are located primarily in the brainstem raphe nuclei and secondarily in some regions of reticular formation and (2) complex axonal systems which virtually innervate all regions of the central nervous system (CNS). The 5-HT cells throughout the brainstem are multipolar, though their size and orientation is remarkably different in different locations (Törk, 1990).

In the rat, the first 5-HT containing cells appear at E12, and additional 5-HTergic neurons appear for at least 3 more days. 5-HT cells appear in two distinct groups, a caudal one in medulla oblongata and in the caudal half of pons, and a rostral one in the midbrain and rostral pons (Törk, 1990). Immediately after they appear, 5-HT cells develop axons which from the rostral group ascend
towards the forebrain, and from caudal group descend to structures of brainstem and spinal cord; this high degree of polarity is unique to the 5-HTergic system and is maintained throughout life (Törk, 1990).

Serotonergic cell groups of rat brain stem have been classified by Dahlstrom and Fuxe (1964) into 9 different clusters (B1-B9), B1 being the most caudal [Fig.3.1.]. Rostral part of the 5-HT ergic system consists of caudal linear, median raphe, dorsal raphe nuclei and the extensive B9 group; the caudal part comprises raphe magnus, raphe pallidus, raphe obscurus, and 5-HT neurons of the medullary reticular formation and other non-raphe regions.

1.2.1.1. Dorsal Raphe (DR)

Located in the ventral part of the periaqueductal gray matter of midbrain, DR is the most prominent member of the brain stem 5-HT ergic nuclei containing the largest number of 5-HT neurons, and is highly bilaterally symmetrical appearing like the outlines of a fountain in midbrain cross sections. The neuronal population is quite diverse ranging from small through medium to large cells; smallest cells located on or near the midline, while the largest ones found primarily in the most lateral and dorsal regions of the nucleus. DR is composed of several subregions demarcated by differential cell density, cell morphology and projections:- interfascicular, ventricular (ventromedial), ventrolateral (lateral), dorsal (dorsolateral and dorsomedial), and caudal are the regions distinguished in most species (Törk, 1990).

1.2.2 Serotonergic modulation of entrainment

In rats, 5-HT containing axon terminals, distributed throughout the rostrocaudal extent of the VL SCN (van den Pol and Tsujimoto, 1985), make axosomatic and axo-dendritic synaptic contacts with VIP containing SCN neurons (Bosler and Beaudet, 1985). Serotonergic involvement in entrainment has been studied by different methods:- systemic administration of 5-HT agonists (Rea et al., 1994) and tryptophan induced increases in 5-HT production (Glass et al. 1995)
Figure 3.1 Distribution of serotonergic cell groups in Raphe Nuclei and in the brain stem Reticular Formation, according to Dahlstrom and Fuxe (1964)

Adapted and modified from Bradley (1988) and Tork (1990)
reduced behavioural phase advances in response to light; systemic injection of 5-HT antagonists enhanced photic phase shifts (Rea et al., 1995); and light induced phase delays in free running circadian rhythms during the subjective night was increased by lesion of the 5-HT containing terminals in suprachiasmatic hypothalamus (Bradbury et al., 1997). 5-HT pathway is also suggested to be involved in transmission, not only modulation, of photic information from the retina to the SCN (Moyer et al., 1997).

A more direct test of the function of 5-HT in the SCN has involved electrophysiological experiments in vivo and in vitro where 5-HT or its ligands (agonists/antagonists) were directly applied. In vitro administration of quipazine, a 5-HT agonist, produced phase advances during subjective day and phase delays during subjective night; and these phase shifts were not sensitive to interruption of synaptic transmission, most likely suggesting a direct effect of quipazine on clock cells having the appropriate 5-HT receptor (Prosser et al., 1992); phase advances mediated by 5-HT1A (Prosser et al., 1993) or 5-HT7 receptor (Lovenberg et al., 1993), and phase delays by a different uncharacterised 5-HT receptor (Miller et al., 1996).

5-HT and its agonist quipazine reduce the firing rate or field potentials of photically responsive or photically uncharacterised neurons in SCN in vivo and in vitro (Mason, 1986; Miller and Fuller, 1990; Rea et al., 1994), and this inhibitory effect is thought to be mediated by 5-HT1A receptor (Ying and Rusak, 1994).

Since all of the aforementioned studies investigated the effect of 5-HT on firing rate and/or phase of SCN neurons by the direct administration of 5-HT/its ligands into SCN (bath, microinjection or iontophoresis) and not by manipulating the 5-HT input (raphe) to SCN, an attempt was made to study the possible modulatory influence of the largest serotonergic nucleus (dorsal raphe) on SCN electrical activity.
2 MATERIALS AND METHODS

For all experiments, inbred male Wistar rats (250-350gms.) kept in polyethylene cages under 12hL:12hD cycle and provided with food and water *ad libitum* were used.

2.1. Materials used were:-

<table>
<thead>
<tr>
<th>No</th>
<th>Name</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Apparatus</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Stereotaxic apparatus (type SR-6)</td>
<td>Narishige Scientific Instrument Lab., Japan.</td>
</tr>
<tr>
<td>2</td>
<td>Polygraph (model 79 D)</td>
<td>Grass Instrument Co., Mass., USA.</td>
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<tr>
<td>3</td>
<td>Window Discriminator (model 121)</td>
<td>World Precision Instruments, USA.</td>
</tr>
<tr>
<td>4</td>
<td>Pulse Counter (with 1:1, 5:1 and 10:1 settings)</td>
<td>University Scientific Instrumentation Centre, JNU.</td>
</tr>
<tr>
<td>5</td>
<td>Rotary Microtome</td>
<td>Weswox Optik, Model MT-1090 A.</td>
</tr>
<tr>
<td>6</td>
<td>Lesion Maker</td>
<td>Grass Instrument Co., Mass., USA</td>
</tr>
<tr>
<td>7</td>
<td>Oscilloscope</td>
<td>1. Tektronix (model 5113)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Hewlett-Packard (model 1201B)</td>
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<tr>
<td>B</td>
<td>Accessories</td>
<td></td>
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<tr>
<td>1</td>
<td>Dental drill with burr head</td>
<td>Lear Avia Inc. Piqua, OH., USA</td>
</tr>
<tr>
<td>2</td>
<td>28G tubing (injector cannula or electrode)</td>
<td>Electromack, India.</td>
</tr>
<tr>
<td>3</td>
<td>20-22 G tubing (guide cannula)</td>
<td>Electromack, India.</td>
</tr>
<tr>
<td>4</td>
<td>28 G stainless steel wire(blocker)</td>
<td>Electromack, India.</td>
</tr>
</tbody>
</table>
5 Microsyringe (1μl) Top, India
6 9-pin D plug (male and female) Local
7 Dental Acrylic (Quick Ashvin) Dr.Jagdish Lal Sethi, Delhi.
8 Superglue Locktite
9 Fly wheel with axis (cutter) Electromack, India.

C Chemicals
1 Saline (0.9% Na Cl) Local
2 4% Lignocaine hydrochloride(xylocaine) Astra-IDL Ltd., India.
3 Sodium barbitone May and Baker Ltd., UK.
4 Sodium pentabarbital S.D Fine Chemicals Ltd., India
5 Potassium ferrocyanide S.D Fine Chemicals Ltd., India
6 Ferric Chloride S.D Fine Chemicals Ltd., India
7 Paraformaldehyde S.D Fine Chemicals Ltd., India
8 Varnish (Dobeckot 505 C) Dr.Beck + Co. (India) Ltd.
9 Paraffin wax Sigma, MO., USA

2.2 Stereotaxic implantation

Rat was anaesthetised by intraperitoneal (i.p.) injection of sodium pentabarbital (35mg/Kg) or sodium barbitone (190mg/Kg). After anaesthetisation, scalp hairs were shaved off and the rat was fixed on the stereotaxic apparatus with the ear bars and incisor bar. A longitudinal incision was made on the skin of the skull and skull muscles were then scraped exposing bregma and lambda (as the reference points).

After cleaning the skull surface, four small watch screws were fixed on the skull for anchoring purpose and another screw electrode (watch screw with a thin
radiowire attached) was fixed in the midline over the frontal sinus which served as the animal ground.

A small oval window (2.00 mm × 2.00 mm) was made around the lambda with a dental drill, exposing the brain covered with duramater which was punctured, and a 20-22G guide cannula along with the blocker, previously held straight, was implanted approaching (1-1.5mm above the injection site) the dorsal raphe. The coordinates used (Paxinos and Watson, 1982) for DR injection site were:

- Antero-posterior (AP) referred to bregma (in mm) = - 7.8 ± 0.5;
- Medio-lateral (L) referred to sagittal plane (mm) = 0.0;
- Dorsoventral (DV) referred to skull surface (mm) = 6.5 ± 0.3.

The implanted guide cannula was fixed to the skull surface with dental acrylic. For electrode implantation, a square window (4.0mm × 2.00 mm) was made around the bregma and a set of bipolar electrodes (two 28G stainless steel wires - previously straightened by heating and pulling - coated four times with varnish (Dobeckot 505C) except their tips and glued together by Superglue) was lowered into the left SCN, the tips of the electrodes touching the SCN at the co-ordinates:

- AP = -1.3 ± 2.0
- DV = 9.4 ± 1.0
- L = 0.3 ± 1.0

Electrodes, like the guide cannula, were fixed to the skull with dental acrylic. The free ends of the electrodes and the tip of the ground electrode on the frontal were soldered to a 9-pin female D plug which also was fixed on the skull with dental acrylic.

After the surgery, the rat was allowed to recover from surgical trauma under post-operative care for at least three days, and the recording was started on the fourth day.

2.3. Recording and Collection of Data

The rat was anaesthetised with sodium barbitone (190 mg/Kg) before recording. After anaesthesia, the female D plug on the rat's head was connected to a recording plug attached to a low noise shielded cable, the other end of which was
connected to a polygraph (79 D, Grass Instruments, Mass., USA). Filter settings for the polygraph were:

-3 db Low = 300 Hz
-3 db High = 30 KHz.

The signals were fed from the output jack of the polygraph to a window discriminator (WPI, model 121, USA). Window levels were set (by observing them on an oscilloscope) in such a way that the signal to noise ratio was at least 2:1 with the least possible interference from white noise band. The discriminated output was routed to a pulse counter set to count 10 pulses as one (10:1) which, in turn, was fed into the input of the polygraph. The recording was done at a paper speed of 50mm/min for at least 36 hours (one and a half circadian cycle).

As soon as this pre injection recording was over, the rat was disconnected from the polygraph and 0.4 μl of saline (as control) or xylocaine (a local anaesthetic for inactivating the neurons: vide box below) was injected (at 12 O' clock, noon) into the DR with the help of an injector cannula connected by a polyethylene tubing

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**Xylocaine (Lidocaine)**

Lidocaine was the first amino amide type of local anaesthetic and introduced into clinical practice in mid-1940s. Its physicochemical and pharmacological properties are (Covino, 1993):

- Molecular weight: 234
- pKa value (at 36°C): 7.8
- Onset of action: Fast
- Duration of action: Moderate

![Chemical configuration of Lidocaine](image)

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Xylocaine, like other local anaesthetics, exert a direct action on specific binding sites of sodium channels to inhibit the flux of Na+ ions and prevent membrane depolarisation and thus action potentials (Strichartz, 1993).

to the needle of a 1μl microsyringe. The chemical was injected at the rate of 0.1μl/min so as to give enough time for the diffusion of chemical and to reduce tissue damage. After the injection, the injector cannula was retained in the same position for at least one minute to prevent backward flow of the injected chemical due to capillary action. The injector cannula was then removed and the blocker replaced. This single injection procedure took approximately 4-5 minutes. Anaesthesia at a dose of 150mg/Kg (and 1.0 ml of dextrose saline) was given every 6 hours. Baseline recording was also done in freely moving rats.

After injection, the recording was continued for at least 48 hours (two circadian cycles). A total of 2 groups of rats, with 5 rats in each group (one for saline and the other for xylocaine) where injection and recording were within the desired sites, was studied.

On completion of experiments, under deep barbitone anaesthesia (200 mg/Kg), 0.4μl (same as the quantity of chemical injected in the same rat during the experiment) of 2% ferric chloride solution was injected into the DR, and a lesion was made in SCN by passing 100mA of DC current for 20 seconds. After about 20-30 minutes, the brain of the rat was intracardially perfused with 100 ml of saline followed by 200 ml of fixative containing paraformaldehyde, saline and 3% potassium ferrocyanide. After perfusion, the brain was taken out and preserved in the same fixative until further use.

A small piece of the fixed brain tissue including either the injection site and its surrounding areas or the lesion site with the adjoining areas were cut and embedded in paraffin block, which was then cut into 10μm thick sections with a rotary microtome. Ribbons of sections mounted on glass slides were dehydrated by
passing through different grades of alcohol and xylene. They were stained with crystal violet so as to give contrast to the injection/lesion site visible as Prussian blue colouration. Stained sections were mounted in DPX and examined under a microscope. The presence and extension of Prussian blue colour indicated the site and spread of the injected chemical/lesion. Data for analysis was taken only from those rats where the Prussian blue colour was within the limits of SCN [Fig.3.2.] and DR [Fig.3.3.].

2.4 Data Analysis

After the experiment, the whole record was divided into bins of 6 minutes and spikes in each bin (represented as vertical lines on the paper, each line representing ~10 action potentials since the pulse counter was set at 10:1) were visually counted.

The counted spikes were entered into the data sheet of SigmaPlot (version 3.0) and plotted as dots, each dot representing the number of action potentials for every 6 minutes. The firing frequency (represented as dots) was plotted against zeitgeber time: 0 or 24 and 12 on the abscissa representing "lights on" (ZT0; 7.00) and "lights off" (ZT12; 19.00) respectively. To determine the overall pattern of electrical activity including the rhythmicity, a complex cosinor function was fitted to the neuronal firing rate according to the method of Puchalski et al. (1996). Elements of the fitted function are a constant, a cosine and a sine term with 24 h as the fundamental period as well as a cosine and a sine term with the first harmonic (12h) as the period tested. The function used for fitting was

\[ y = a + b \sin (p^* h/12) + c \cos (p^* h/12) + d \sin (p^* 4/6) + e \cos (p^* h/6) \]

From this fitted function, the acrophases of the firing (ie the point on the abscissa - representing time - corresponding to the peak of the fitted curve) in the baseline/saline controls and after xylocaine injection were determined. The plots and curves for pre injection/saline and post injection periods were made separately and later superimposed to display the phase shifts, if any. Since MUA recordings from within SCN were found to show a circadian pattern, action potentials in the pre injection period were recorded for only 24 hrs. and double plotted (i.e. values recorded for 24 hrs were copied and plotted for 48 hrs). To determine the
Figure 3.2. Rat brain (coronal section) :- (1) Location of SCN denoted by arrow above the optic chiasma. Map (512² pixels resolution) accessed from http://loni.ucla.edu.; (2) Photomicrograph of SCN; (3) Photomicrograph showing position of the recording electrode (Prussian blue colouration). OC optic chiasm; SCN suprachiasmatic nucleus; 3V third ventricle; Ti tractus infundibularis.
Figure 3.3. Rat brain (coronal section):- (1) Location of Dorsal Raphe indicated by arrow. Map (512² pixels resolution) accessed from http://loni.ucla.edu.
(2) Photomicrograph showing the site of injection (Prussian blue) in dorsal raphe.
significance of alteration in mean firing rate and acrophase (manifested as a phase shift), Mann-Whitney test was used.

3 RESULTS

Baseline recording (without any injection) in anaesthetised rats showed a peak of MUA during day time and a trough at night; on the contrary, baseline recording in free moving rats showed two peaks, one during day and the other at night [Graph 3.1.]. In anaesthetised rats, the maximum, minimum and mean values of neuronal firing were 614.0 ± 44.67, 10.0 ± 0.00 and 138.16 ± 4.32 respectively. The acrophase of firing was 29.0 ± 0.56.

Injection of 0.4 µl of saline into DR did not cause any significant change in the firing rate or phase compared to baseline [Graph 3.2.]. The maximum, minimum and mean values were 532.50 ± 68.36, 10.00 ± 0.00 and 141.18 ± 3.73 respectively, and the acrophase was 29.5 ± 0.293.

0.4 µl of xylocaine injection into DR resulted in a phase advance of 6.7 ± 0.05 hours compared to baseline (p = < 0.0001; Mann-Whitney test) and 7.2 ± 0.29 hours compared to saline (p = < 0.0001; Mann-Whitney test); and significantly reduced the firing rate [Graph 3.2.] compared to either baseline or saline injection (p = < 0.0001; Mann-Whitney test). The reduced maximum and mean firing rates were 494.0 ± 16.92 and 105.63 ± 3.58 respectively.

Table 3.1. Alterations in MUA of SCN after xylocaine inactivation of Dorsal Raphe

<table>
<thead>
<tr>
<th></th>
<th>Maximum</th>
<th>Minimum</th>
<th>Mean</th>
<th>Acrophase</th>
<th>Phase shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>614.0 ± 44.7</td>
<td>10.0 ± 0.00</td>
<td>138.16 ± 4.3</td>
<td>29.0 ± 0.56</td>
<td>----</td>
</tr>
<tr>
<td>Saline</td>
<td>532.5 ± 68.4</td>
<td>10.0 ± 0.00</td>
<td>141.18 ± 3.7</td>
<td>29.5 ± 0.293</td>
<td>0.5 ± 0.0851</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(compared to baseline)</td>
</tr>
<tr>
<td>Xylocaine</td>
<td>494.0 ± 16.9</td>
<td>10.0 ± 0.00</td>
<td>105.63 ± 3.5</td>
<td>22.3 ± 0.19</td>
<td>7.2 ± 0.292</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(compared to saline)</td>
</tr>
</tbody>
</table>

1 p = < 0.177; 2 p = < 0.0001 (Mann-Whitney rank sum test).
Graph 3.1. MUA recorded from rat: (1) outside SCN under anaesthesia, (2) inside SCN in free moving, and (3) inside SCN under anaesthesia. Black bars represent D of L/D cycle ("lights off").
Graph 3.2. Superimposed curve fits (1) baseline (vs) saline, (2) baseline (vs) xylocaine (phase advance $6.7 \pm 0.049$ hr), and (3) saline (vs) xylocaine (phase advance $7.2 \pm 0.29$ hr); black bars represent "lights off", and vertical lines in (2) and (3) represent acrophases.
4 DISCUSSION

The pre injection (baseline) MUA recording of SCN is similar to the MUA recordings reported by Inouye and Kawamura (1982), Inouye (1996) and Meijer et al., (1996; 1996a). In the present study, DR inactivation effects were studied in anaesthetised preparation since movement artefacts could confound the circadian rhythmicity determinations as was demonstrated in free moving rats - two peaks in one circadian cycle [Graph.3.1.-] and suggested by Inouye and Kawamura (1982). The sharp peaks and broad troughs observed in the anaesthetised preparation of this study could be ascribed to the action of sodium (penta) barbitone on GABA neurons of SCN where it might have enhanced the dual action (excitatory during day and inhibitory at night) of GABA on SCN EPSCs and IPSCs as reported by Wagner et al., (1997). This possibility is supported by the finding that MUA recorded from outside the SCN does not show such characteristic peaks and troughs; instead, they are more gradual and sloping; besides, extra SCN MUA rhythm is in antiphase to the electrical activity within SCN, and the fitted curve showed no circadian rhythmicity, corroborating the earlier studies of Inouye and Kawamura (1982) [Graph.3.1.].

The post injection recording showed a drastic reduction in firing rate and a significant phase advance [Graph.3.2.]. In view of the findings that 5-HT/its agonist induced reduction in firing rate (Ying and Rusak, 1994), and phase advance (Prosser et al., 1990) of SCN neuron activity was attenuated or blocked by treatment with the respective antagonists, the expected observations would have been increased firing rate and phase delay after DR inactivation (because 5-HT transmission to SCN was temporarily interrupted) which is opposite to the observations of this study.

The effects observed, though quite unlike those produced by 5-HT antagonist (or 5-HT depletion) on SCN, are similar to those produced by NPY. NPY is known to induce phase advance (Rusak et al., 1989; Medanic and Gillette, 1993; Gribkoff et al., 1998) and reduction in firing rate (Medanic and Gillette, 1993; van den Pol et al., 1996; Gribkoff et al., 1998) of SCN neurons. Since DR also sends its projections to IGL (Azmitia and Segal, 1978; Villar et al., 1988), the source of NPY afferents to SCN,
xylocaine administration might have caused an inhibition of 5-HT release at IGL which in turn will facilitate NPY release in SCN (disinhibition of NPY neurons of IGL). This disinhibition can precipitate phase advance and inhibition of SCN neurons.

Although DR inactivation will presumably interrupt the 5-HT neurotransmission to SCN as well as IGL, this effect can be even more for the IGL at this particular injection time (ZT5; noon 12 O' clock) since this structure has been reported by Mason (1986) to show much more sensitivity to the inhibitory action of 5-HT than SCN during day. This can imply that the primary action of DR mediated 5-HT inhibition during day is through IGL rather than directly on SCN, as is suggested by the findings of this study.

Since NPY is known to induce long term depression (LTD), a cellular basis of learning, in glutamatergic neurons of SCN (van den Pol et al., 1996), the observed phase advance and decrease in the firing could be the population expression of neuronal learning. LTD induced by NPY afferents to VL SCN (where they make synapses with VIP and glutamatergic neurons, among others) might be sufficient to induce depression in the entire SCN, because LTD induced on the glutamatergic synapses of hippocampus is known to spread laterally, forward and backward ie., not strictly synapse specific (Fitzsimonds et al., 1997). This NPY induced learning might be the possible function of the preferential activation (by disinhibition) of NPY containing IGL neurons, an aspect which will further widen the role played by IGL (and GHT) in the integration of photic and non photic inputs. Synaptic depression is also known to enable a neural network to detect synchronous firing rate changes in a population of high and low frequency afferents with equal sensitivity; in addition, it plays an important role in the genesis of periodic spontaneous activity ie., rhythmogenicity (O'Donovan and Rinzel, 1997).

The possibility that the effects observed after DR inactivation are non specific can be ruled out by the following observations :-

(1) The observed effects were drug specific ie., they were induced only by xylocaine and not by saline;
(2) The effects were site specific *i.e.*, induced only when the site of injection was within DR and not anywhere outside or near it; and also only when the recording electrode was within SCN;

(3) Injection to DR did not damage its structural integrity since a few animals for a repeat experiment (though the results were not included in analysis) showed reproducible results.

5 CONCLUSION

Xylocaine - induced inactivation of DR caused a significant phase advance and reduced the firing rate of SCN. These effects are similar to those of NPY (not of the depletion of 5-HT, the major DR neurotransmitter) on SCN; possibly due to the preferential activation of NPY containing IGL neurons. This preferential activation could be significant for the learning (expressed as LTD) of the SCN clock.