Materials and Methods

Materials:
I. Animal Model

Male Wistar rats weighing between 270–300 gm were used in this study. The rats were obtained from the Animal House Facility, JNU. Rats were maintained under 12:12 light: dark cycle with food and water ad libitum and were kept in polypropylene cages.

II. Chemicals

The chemicals used in this study, their function, route of administration and sources have been listed in table I.

III. Equipments used in the study

1. Stereotaxic Apparatus

The stereotaxic method (Greek *stereos*: three dimensional or spatial and *taxis*: arranged, ordered) uses a system of spatial coordinates to introduce accurately any thin object(s) (cannula, electrode) into a predetermined deep-seated structure of the brain or spinal cord. The essence of the stereotaxic technique is to locate a cerebral target on the basis of a system of coordinates incorporated in the stereotaxic device.

R.H. Clark and Victor Horsley built the first stereotaxic apparatus for neurophysiological experiments on animals (Clark and Horsley, 1906; Horsley and Clark, 1908). Clark and Horsley proposed the term "stereotaxis", which subsequently became firmly established in neurophysiology and neurosurgery (Clark and Horsley, 1906). However, seventeen years before the publication by these authors, both Russian and European literature carried reports by the Professor of anatomy at Moscow University, D.N. Zernov, who built a stereotaxic apparatus called encephalometer that was designed for both anatomic investigations and neurosurgical operations on human brain (1889). Zernov's stereotaxic apparatus, the first in the world was based on the polar system of coordinates whereas the first apparatus for experimental neurophysiology developed by Clark and Horsley (1906) was based on a rectangular system which was later used by Spiegel and Wycis (1947) in designing the first modern apparatus for human surgery.
i) Principle of stereotaxic method

Surgical stereotaxis is based on calculation of precise spatial relationship between any structure deep in the brain and a number of reference structures that can be intracerebral or external (to a considerably less extent) on the cranium. Based on such calculations, it is possible stereotaxically to reach surgically any structure situated in areas deep within the brain or spinal cord. These calculations are the basis of brain atlas. The purpose of an atlas is to provide stereotaxic coordinates for accurate placement of electrodes on the cortex or within the deep structures of the brain. To be of greatest use, it should be compatible with any or most of the existing stereotaxic instruments. The determination of the center of any deep-seated brain structure needs the identification of its position in space. From analytical geometry, it is known that the position of a point in space may be determined with the aid of Cartesian system of rectangular or polar coordinates. The coordinates of a point are the components of a tuple of numbers used to represent the location of the point in the plane or space. A coordinate system is a plane or space where the origin and axes are defined so that coordinates can be measured. The three dimensional coordinate system provides the physical dimensions of space — height, width and length, and this is often referred to as "the three dimensions". In the polar or circular coordinate systems a point is identified by distance from some fixed feature in space and one or more subtended angles.

Clark and Horsley's apparatus used the following baselines to define the three dimensional system of coordinates - three planes perpendicular to each other on animal skull:

- a line connecting the two auditory meati
- a line bisecting the cranium in the midline
- a line connecting the external auditory meati with the infraorbital ridge.

The line connecting the external auditory meati with the infraorbital ridge and the line connecting the two auditory meati together formed the horizontal plane. The sagittal plane passes through the line bisecting the cranium in the midline and is perpendicular to the horizontal plane. The coronal plane passes through the line connecting the auditory meati and is perpendicular to the sagittal and horizontal planes. Thus the coronal or
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Frontal plane passes through the centers of the external auditory meati, the horizontal plane through the auditory meati and the lower borders of the orbits and the sagittal plane through the anteroposterior midline on the skull.

**ii) Instrumental zero and alignment of animal head in the instrument**

The point where the three perpendicular planes intersect each other is considered as zero point. The instrument zero is reached through the following method: the external auditory meatus plugs (ear-plugs) are brought together so that their tips touch. The electrode carrier is set in its carrier at a 90° angle from the horizontal plane and brought down until its tip touches the point of contact between the tips of the external auditory meati plugs. The reading on the electrode carrier thus obtained represents "instrument zero". Instrument zero varies with the length of the electrode used. The line connecting the centers of the external auditory meati is known as interaural line and is taken as one of the reference points for stereotaxic surgery (Fig. XII). The interaural basal plane of the instrument (or the instrument zero) passes through the interaural line and the inferior orbital ridges, right and left. The zero horizontal plane for the animal or the animal zero is arbitrarily taken 10 mm above the interaural basal plane in the cat. In case of rats it is 5 mm above the basal plane (Fig. XII).

Although stereotaxic technique helps the experimenter in localizing the placement of electrodes in particular structures, complete accuracy of identification of these sites cannot be obtained with these techniques alone. This is due to a number of factors, in particular, the individual variability in cranial and cerebral dimensions. Such variability is especially prominent in humans (Spiegel and Wycis, 1952) and dogs (Hume and Ganong, 1956). Therefore, it is necessary to have histological verification of the locus of the electrode.

**iii) Brain atlas**

The atlas by Paxinos and Watson (1997) was used in this study. It is based on flat skull position of the rat in the stereotaxic apparatus. The flat skull position was achieved by lowering the incisor bar 3.3 ± 0.4 mm below horizontal zero. The atlas refers to bregma and interaural line as the reference points. Bregma is defined as the point of intersection of the sagittal suture with the curve of the best fit along the coronal suture.
Figure XII Dorsal and lateral views of the skull (290gm) of a male Wistar rat. The positions of coronal suture, sagittal suture, lambdoid suture, bregma, lambda and the plane of the interaural line are shown. The distance (3.3 mm) between the incisor bar and the horizontal plane passing through the interaural line is marked on the extreme left side. The right side shows the dorso-ventral distance of animal zero from the instrument zero that coincides with the horizontal plane. The distance between the bregma and the interaural line is 9.00 mm and the ventral extent of the brain from lambda is 10.0 mm.
When the two sides of the coronal suture meet the sagittal suture at different points, bregma usually fall midway between the two junctions. The anteroposterior position of the bregma is 9.0 ± 0.3 mm anterior to the coronal plane passing through the interaural line (Fig. XII).

Another reference point is Lambda, which is defined as the midpoint of the curve of best fit along the lambdoid suture. Lambda is located 0.3 ± 0.3 mm anterior to the interaural line. The top of the skull at the bregma and lambda is 10.0 ± 0.2 mm dorsal to the interaural zero plane (Fig. XII).

In this study, stereotaxic instrument manufactured by INCO (Mrs INCO Private Limited, Ambala, India) was used. The instrument (Fig. XIII) consisted of a standard "U" frame with rigid anteroposterior (AP) bars calibrated in millimeters and table fixing attachment. The AP bars carried the movable electrode carrier.

The electrode carrier can be moved in three-dimensions using three circular dials (marked with white star) corresponding to anterior-posterior, lateral and dorso-ventral directions. It can also be rotated through limited arcs. These AP bars have slots for removable ear bars that are used for mounting the animal in the head holder. The head holder or the earplugs consists of two external auditory meati plugs sliding on a transverse shaft. The incisor bar mounted on a vertical bar allows for the elevation or depression of the head position and to keep the animal head immobile in dorsoventral direction.

Fig. XIII Stereotaxic apparatus (INCO, Ambala, India)
Different views of the same stereotaxic apparatus
2. Polygraph

Polygraph was used for simultaneous recording of multiple electrophysiological (biological) signals on the chart paper. Grass Polygraph (Model 7H), a direct curvilinear recorder, was used for recording the electrophysiological parameters (EEG, EOG, EMG and hippocampal waves). It consisted of the following components:

i) Polygraph channels: There were eight separate channels for recording bioelectric signals. Besides the power switch and indicator light, each channel consisted of the following:

A) Differential AC preamplifier: Grass model 7 employed a high gain, wide band, combined driver amplifier and preamplifier (7P511L) with pen driving circuit. The eighth channel of the polygraph had a high gain DC preamplifier with pen driving circuit (7P122). Channels 1-7 were used for AC recordings as was done in this study. The preamplifier had the following features:

a) Inputs:

    • External input jack: There was one port of this type on each channel. Stereconnector with three active points was used for feeding the inputs from animal brain into the amplifier.

    • Electrode selector panel: The signal picked up from the animal brain was fed (using 2 mm EP pins) into a ‘Mini electrode board’ (Model IGMEB-NUM25). The Mini electrode board was mounted on the inside wall of the faraday cage. The mini electrode board had provision for 21 inputs and it sent output to the Electrode selector panel through an electronically shielded cable. The Electrode selector panel was a separate attachment fixed to the console of the polygraph. It had eight channels corresponding to the equal number of preamplifiers in polygraph and it transferred the input received from the animal brain through the ‘Mini electrode board’ to the polygraph. Each channel in the electrode selector panel had two rows of 1-21 push buttons and selecting one from each row, electrical parameter could be recorded between any pair of combinations of electrodes fixed on the animal brain. It also had a calibration switch that was common for all the channels and gave a voltage varying between 5 µV to 20 mV.
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- **J5 input jack:** This is used for receiving inputs from computer, telemeter etc for the purpose of recording on chart paper.

**b) Outputs:**
- **J6 output jack:** This is used for monitoring the signals amplified by the polygraph on oscilloscopes, computers etc.
- **J7 output jack:** This is also used for monitoring the signals amplified by the polygraph on oscilloscopes, computers etc. However, this jack is used whenever the signal to be taken out from the polygraph has high frequency response.

**c) Calibration switch:** A calibrator with five voltage values (5 μV, 50 μV, 0.5 mV, 5 mV and 50 mV) was provided on each channel of the polygraph for calibration.

**d) CAL-USE-EXT switch:** A switch was provided on each channel, which could be used to select between different calibration values (CAL) or to engage the electrode selector panel for recording (USE) or to record the signals using the external input jack through stereoconnectors (EXT).

**e) Sensitivity:** The amplified signal from the preamplifier reaches the galvanometers on which ink-pens were mounted. By increasing the signal amplification, even small signals could be made conspicuous enough for analysis. The amplification of the signal or the sensitivity was controlled at three levels:

- A switch provided an option to choose between μV/mm and mV/cm. Choosing μV/mm over mV/cm provides 1000X amplification of the input signal.
- An adjustable 12-position switch that displayed numbers (1, 1.5, 2, 3, 5, 7.5, 10, 15, 20, 30, 50 and 75), which controlled the level of amplification/sensitivity.
- A potentiometer was provided that had values from 1-10, which indicated the interval between two successive positions on the adjustable 12-position switch mentioned above.

**f) Frequency response:** The low and high frequency limit for any bioelectric signal being recorded was set using high and low frequency filters.
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- **Low frequency**: The low frequency filter had nine positions (0.03, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 Hz). It prevented the signals with frequency lower than that selected from being recorded.

- **High frequency**: The high frequency filter had seven positions (0.03, 0.1, 0.3, 1, 3, 10, 30 kHz). It prevented the signals with frequency higher than that selected from being recorded.

- **Line frequency** (50 Hz filter): It selectively rejected the line frequency i.e., 50 Hz.

**g) Baseline knob**: It was an in-built potentiometer. It gave DC signal that was used to neutralize stray DC, if any. Thus, it enabled to adjust the pen to record in a horizontal line. After the adjustment of baseline, the pens did not show any deflection when the polygraph was switched on in the absence of any input signal.

**B) Writer unit (Chart paper speed regulator)**

The basic components of the writer unit were the d’Arsenval galvanometer, the pen and the paper drive mechanism. The coil of each galvanometer was connected to the corresponding pen or stylus. The pens were connected through a small piece of polyethylene tubing to ink wells that were the reservoir for ink during recording. Paper speed could be set at speeds of 2.5 mm, 5.0 mm, 10.0 mm, 50.0 mm, and 100.0 mm/sec or mm/min by pressing respective push buttons. The speed of the paper in this study was set at 2.5 mm/sec.

**C) Time event and signal marker**

It gave tiny marks at 1 sec, 5 sec or 1 min on the paper during recording. The amplitude of marks made at different intervals differed to facilitate the identification of the marking. The signal marker gave upward or downward deflection depending on the mode selected from polygraph. The mark could also be given using the remote control or with the help of a switch provided on the polygraph.
3. **Infusion pump**

All the microinjections were done using an automatic digital infusion/withdrawal dual syringe pump (sp210i/w, WPI Inc., USA). The pump could be used for infusion as well as withdrawal of fluid. All control functions were performed automatically by the pump microcontroller and were based on linear motion of the pusher block associated with the syringe size (diameter) to deliver a known volume. The sp210i/w was designed to hold glass or plastic syringes of any make from 10 μL to 140 mL. The internal diameter of the syringe was used by the control program to calibrate the pump and deliver the chosen volume at selected flow rate. Two 10 μL glass Hamilton (1701, Gas tight) syringes were used for microinjections in this study.

4. **Microinjection using infusion/withdrawal pump**

The infusion/withdrawal pump (SP210 i/w pump, WPI Inc., USA) simultaneously drove two glass syringes. For withdrawal or refill operations, the withdrawal mode was selected from the MODE on the main menu and then the volume and the rate of the withdrawal of the solution was selected. The pump at this setting withdrew according to the rate and volume fed into the pump. Similarly, in the infusion mode the volume and rate settings for the microinjection of the chemicals were set as per the requirements.

4. **Stimulator**

A dual pulse digital stimulator (Grass Model S8800, Grass Instrument Co., USA) was used in the present study to bilaterally stimulate substantia nigra pars reticulata (SNrpr). It was a constant voltage stimulator without in-built isolation unit and could give two independently regulated stimuli outputs (S1 and S2). This stimulator was capable of generating uni- or bi-directional square wave pulse with required voltage, duration and frequency. Using a voltage dial (VOLTS) provided for each output, the stimulus intensities of the pulse could be varied between 10 μV and 150 V. The pulse duration could be varied from 0.01 msec to 1 sec and the pulse frequency between 0.1–10 kHz. The stimulator had a voltage multiplier knob that could be set through several resistances at the output stage. The setting of the voltage multiplier knob was required for correct impedance matching between the stimulator and stimulation isolation unit (PSIU6 in this study). In this study the multiplier knob was set at 10 (×250 Ω) as the stimulus pulses...
were being fed into a photoelectric stimulus isolation unit (*Grass* PSIU6, *Grass Instrument Co.*, USA) before reaching the animal brain.

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1) *Stimulus artifact*

When a stimulus pulse is introduced into a preparation to evoke a response an electrical artifact appears in the recording. The source of artifact is either the spread of the stimulus current itself or the induced current in the recording electrodes. This artifact precedes the evoked response in time. The delay between stimulus artifact and the evoked response is dependent upon stimulation parameters and the characteristic properties of the preparation. Some stimulus artifact is useful as it indicates the time of stimulation. However, excessive stimulus artifact may obliterate the display of the desired response as is often the case with small evoked potentials, which are to be recorded after a stimulus pulse of excessive amplitude or duration. The size of the electrical stimulus, the proximity of the recording and stimulating electrode pairs to each other and the intervening medium contribute to artifact amplitude. The stimulus artifact was reduced in the following ways:

- Isolation of the stimulus pulse from the ground can reduce the circulating ground currents between the stimulator, preparation and recording instrument. A stimulus isolation unit (*Grass* PSIU6, *Grass Instrument Co.*, USA) was connected between the stimulator and the animal preparation to isolate the stimulus pulse from the ground. Depending upon the requirement of the animal preparation, the stimulus isolation unit can be either constant voltage or constant current type.

- By using as small a stimulator pulse with as short duration as is possible.

- By spacing stimulating and recording electrodes as far from each other as possible and positioning them for maximum cancellation of the field effect.

2) *Constant voltage Vs Constant current source for pulse stimulation*

While the principal factor responsible for electrical stimulation is current, the amount of voltage required to produce this current is the function of the impedance presented by the electrodes and the surrounding tissue.
“Constant voltage” inherently means a low impedance source, which can be relied upon to provide the source voltage at the electrode (metal-liquid interface) independent of cable and similar shunt capacitances.

“Constant current” on the other hand infers very high source impedance and its draw back is that it is difficult when cable, lead or similar shunt capacities are in real circuit. This is particularly true with currents below 10 μA and gets worse with smaller currents and high source and load impedances. Thus, “Constant current” sources are more practical with relatively larger currents (over 100 μA). For direct current (DC) stimulations “Constant current” is most advantageous and shunt capacities have no effect. One of the most important stimulus parameters is current density. Current density is the amperes per unit area at the specific responding tissue.

5. Photoelectric stimulus isolation unit (PSIU6)

i) The Photoelectric stimulus isolation unit (Grass PSIU6, Grass Instrument Co., USA) is a “constant current” unit. It has a transistorized optically coupled circuit. It was connected in series between the output of S8800 constant voltage stimulator and the input to stimulation electrodes. It isolates the stimulus from ground, thus reducing stimulus artifact and provided constant current to the preparation. A light emitting diode driven directly from the output voltage of the stimulator causes current to flow through the photosensitive diode, which is isolated from the ground. The current proportional to the stimulus voltage fed from the stimulator appears as an input signal to the constant current transistor. The PSIU6 receives operating power from two sources. Power for the output circuit and stimulus current is obtained from two replaceable batteries (42 V each, Grass BAT-21875, Grass Instrument Co., USA) located within the PSIU6 case. Modulating signal power for the input circuit (optical coupling) is obtained from the stimulator square wave output. The PSIU6 had output impedance of 2000 Ω. The instrument driving this isolation unit should have output impedance 5-10 times less than that of the PSIU6 input impedance (i.e., 200-400 Ω). The output multiplier switch of the S8800 stimulator was kept at X10 (SIU) during use for proper impedance matching. The PSIU6 comprised of the following component:
a) **Polarity Switch:** PSIU6 had a toggle switch to change the polarity of the pulses. In ‘Normal’ position of the switch the red output terminal was positive with respect to the black output terminal. In the ‘reverse’ position the black output terminal was positive with respect to the red terminal.

b) **Current Range:** The current range could be selected by a 5-position current selector switch. The ranges were 0.1 µA - 1.5 µA; 1.0 µA – 15 µA; 10 µA – 150 µA; 0.1 mA – 1.5 mA and 1.0 mA – 15 mA. The current that was obtained from the isolation unit depended on the setting in the VOLTS (continuous variable) dial of the stimulator (Model S8800) and the current setting range of the PSIU6. The amounts of output current of PSIU6 tracks numbers on stimulator VOLTS dial within 20%.

6. **Grass DCLM5 lesion maker**

A Grass DCLM5 lesion maker was used to produce electrical lesion at the site of stimulation. The lesion maker was operated by the AC supply of 240 V. The current strength could be adjusted between 0.1 and 50 mA, with the help of a knob and an amplifier (X0.1, X1 and X10) switch. The adjusted current could be read out from the displayed scale. The lesion maker passes anodal current through the stimulation electrodes. Anodal current implies flow of positive charge that cause electrolysis and hence release of ferric ions from the electrode at the site of stimulation. The deposition of ferric ions causes the lesion. Electrical stimulation implies deposition of negative charge at the site of stimulation that drives the neurons towards a depolarized state resulting in excitation.

7. **Electrodes**

i) **EEG electrodes:** Radiowires were soldered to small stainless steel EEG screws (0.86x1/16, Plastic One Inc., USA). These electrodes (Fig. XIV-A) were then screwed on the animal skull. EEG was recorded between 1–100 Hz with 50 Hz filter out.

ii) **EOG electrodes:** Radiowires were stripped on one end and a wire loop was made from the exposed surface (Fig. XIV-B). The loop end of the wire was tied to the external canthus muscle of the eye of the animal. EOG was recorded between 1–30 Hz with 50 Hz filter in use.
iii) **EMG electrodes:** One end of a thin insulated wire (COOGER WIRE, California, USA) was stripped and bent in the shape of a hook (Fig. XIV-C). The wire was passed through a hypodermic needle (20 G) with the hook-shaped end positioned at the tip of the needle. The needle along with the wire was inserted into the dorsal nuchal muscle. The hypodermic needle is gently withdrawn leaving the wire in place inside the muscle. EMG was recorded between 1–100 Hz with 50 Hz filter in.

iv) **Hippocampal electrodes:** Two thin (125 μm) straight formvar insulated (except at the tip) wires were glued together and using stereotaxic procedure was implanted into the CA1 region of hippocampus. The uninsulated tips of the glued wire were separated by about 0.5 mm (Fig. XIV-D). Hippocampal waves (Hipp) were recorded between 1–30 Hz with 50 Hz filter in.

v) **Stimulation electrodes:** Bipolar stimulation electrodes were made using two thin (125 μm) straight formvar insulated (except at the tips) wires that were glued together (Fig. XIV-D) and using stereotaxic procedure were implanted into SNrpr. The uninsulated tips of the glued wires at the implanted end were separated by about 0.5 mm.

Figure XIV: A. EEG electrode. B. EOG electrode. C. EMG electrode. D. deep brain recording/stimulation electrode
vi) Chemitrodes

❖ Guide cannula: Two 24 Gauge stainless steel cannula (15 mm) were soldered parallel at a lateral distance of 4 mm to form a bilateral guide cannula. The cannulae of the required length were made by cutting 24 Gauge hypodermic needles. Stylets made from stainless steel wire (30G) were inserted into the guide cannula.

The stylets were removed before inserting injector subsequently replaced back. The stylets prevented the el blocking the flow of tissue fluid that rises up the cannula due to capillary action (Fig. XV).

❖ Injector Cannula: 30 Gauge stainless steel cannula (17 mm) (Plastic One Inc., USA) was fitted with polyethylene (PE 20, Plastics One Inc., USA) tubing at one end. The tubing was connected to the Hamilton syringe mounted on the syringe pump for injection. The cannula end was inserted into the animal brain through guide cannula.

8. Cryostat

A Leica CM 1900 cryostat (Leica Instruments, Germany) was used to cut coronal sections of rat brain. The cryostat has a fixed place for positioning the blade that was aligned at a prefixed angle to the chuck holder. Tissue carrier plates or chucks are provided on which the tissue block is frozen and then placed in the chuck holder. The temperature of chuck holder was maintained at −15 °C. The temperature of the blade and the cryostat chamber was maintained at −30 °C. A section thickness knob was used to set the thickness of the cut sections. The blade was covered with an anti-roll plate that facilitated the formation of a ribbon of sections during sectioning. A handwheel was provided outside the cryostat chamber. Rotating the handwheel in a clockwise direction brings the chuck holder and hence the embedded tissue forward towards the blade with a vertical swiping motion. The sections were cut when the tissue swiped across the blade surface.
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Methodology

All the studies were conducted on chronically prepared freely moving normally behaving male Wistar rats (270-300 g) maintained on 12:12 light:dark cycle with food and water ad libitum. Institutional Animal Ethics Committee, JNU, approved the experiments and all possible care was taken to minimise pain and discomfort to the animals.

I. Pre-surgical procedure

1. The rat was anesthetised by intraperitoneal injection of combination of Ketamine hydrochloride (80 mg/kg body wt) and Xylazine hydrochloride (10 mg/kg body wt) (Paul et al., 1997).

2. Atropine sulphate was injected intramuscularly (0.6 mg/kg body wt) (Paul et al., 1997).

3. The hairs over the rat head were shaved and earplugs were fixed.

4. The rat was positioned in the stereotaxic apparatus.

II. Surgical procedure

1. Implantation of electrodes for sleep-wake recording

The rat head was cleaned using betadine solution and the scalp was incised to expose the cranium. The scalp muscles and tissue were pushed to the periphery of the cranium. Two screw electrodes were implanted on the skull for bilateral recording of EEG and a third screw electrode over frontal sinus to serve as animal ground. Insulated (except at the tip) flexible wires were connected bilaterally to dorsal neck muscles and muscles near external canthus of eyes to record bilateral EMG and EOG, respectively. A bipolar insulated (except at the tip) recording electrode was stereotaxically implanted unilaterally at coordinates, AP: Bregma -3.6 mm, L 2.0 mm, H 2.7 mm (Paxinos and Watson, 1997), to record hippocampal waves (Hipp) from CA1 region of the hippocampus. The free ends of these electrodes were soldered to a 9-pin D type electronic female connector, which was then fixed to the skull with dental acrylic.
2. **Implantation of the guide cannula**

A pair of bilateral stainless steel guide cannulae (24G) having indwelling stylets was stereotaxically implanted bilaterally through drill holes made on the skull so as to reach 2.0 mm above the PPT (AP: lambda +1.0 mm, L 2.0 mm, H 7.0 mm) or SNpr (AP: lambda +3.7 mm, L 2.5mm, H 6.1mm) (Paxinos and Watson, 1997). The cannula was fixed to the skull using dental acrylic.

3. **Implantation of the stimulation electrodes**

Two stimulation electrodes were implanted bilaterally in the SNpr at coordinates AP: Lambda +3.7 mm, L 2.5 mm, H 8.1 mm (Paxinos and Watson, 1997) through drill holes made in the skull. The free ends of the wires from stimulation electrodes were soldered into a 4-pin female connector, which was then fixed on the skull with dental acrylic. In addition to the stimulation electrodes in SNpr, guide canulae were also implanted in the PPT of the same animal. This group of rats was used for simultaneous microinjection of picrotoxin into PPT and electrical stimulation of SNpr.

**III. Post-operative care and habituation of the animal to recording set-up**

The animals were treated with adequate post-operative care that included:

1. Intramuscular gentamicin (antibiotic) injection for five days beginning a day prior to the day of surgery [(2.5 mg/kg body wt) (Deboer et al., 1998)].

2. Intramuscular dexamethasone (anti-inflammatory) injection [(2 mg/kg body wt) (http://ratguide.com/meds/)] for three days starting from the day of surgery.

3. 1 mL of sterile dextrose normal saline given intraperitoneally.

4. Daily cleaning with betadine (anti-microbial) solution till the wound healed.

5. Nebasul powder (anti-microbial) was applied till the wound healed.

6. The stylets of the guide cannula were cleaned daily with absolute alcohol.

A week was allowed for recovery from surgical trauma. The rats were habituated to the recording cables and the recording (faraday) cage during the recovery period.
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IV. Sleep-wakefulness recording

1. Microinjection study: The microinjection study was divided into following groups

   i) Sham injection: On day one of the recording schedule the rat was connected to the recording cable an hour before the start of the recording. Two injector cannulae (30G) fitted with collar so as to protrude 2.0 mm beyond the guide cannulae were bilaterally introduced through the guide cannulae to reach PPT or SNrpr but no injection was done. EEG, EOG, EMG and Hipp were recorded simultaneously in four separate channels of the polygraph for eight hours between 10:00 AM-7:00 PM. This group served as sham control for the microinjection study as no injection was done in this group. The values obtained for different stages of sleep-wake cycle for the eight-hour recording period were compared with the corresponding period of vehicle control group (positive control) and the treatment group (microinjection/co-injection of GABA-ergic or NA-ergic agonists/antagonists into PPT and glutamate injection into SNrpr). In one group of rats baseline recording was done without inserting injector cannulae into the site of injection. The baseline data obtained in this set of animals (data not shown) was comparable with the data obtained in sham injection and vehicle control group.

   ii) Vehicle control: On the second day of recording schedule, the rat was connected to the recording cable at least one hour before the start of recording. The 10 μL Hamilton glass syringe (1701, Gas tight) was manually filled with distilled water. Next, the polyethylene tubing (PE 20, Plastics One Inc., USA) with the injectors (30G) connected at one end was also manually filled with distilled water and connected to the glass syringe. The syringes were mounted on the pump and using the infusion mode, 4.00 μL of the distilled water was pushed out of the tubing. Next, using the withdrawal mode 1.5 μL of air was sucked into the tubing. Following this 2.5 μL of the chemical to be injected into the rat brain was sucked into the tubing. Therefore, now there was an air gap that separated the distilled water and the vehicle solution to be injected, which was filled up to the tip of the injector cannula. The pump was then set at the infusion mode and the volume (200 nL) as well as the rate of injection (100 nL/min) was fed into the pump. The injector cannulae were lowered into the bilateral guide and the tubing was fixed using
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cellophane tape to the recording cable (so that animal do not pull these out). The recording was continued for an hour.

After an hour of pre-injection recording, 200 nL of vehicle solution was injected into the PPT or SNpr at the rate of 100 nL/min. Saline served as the vehicle for (for dissolving) glutamate and all the agonist/antagonists, except prazosin. Prazosin was dissolved in 20% N, N-dimethyl Acetamide (N,N-DA) and hence was used as the vehicle control. The recording continued through the injections. The animals were not disturbed as the injections were done using remote controlled automatic pump. The recording was continued for eight post-injection hours. Saline/20% N,N-DA injection served as a positive control demonstrating that the effect of agonist or antagonist injection into the PPT and that of glutamate into SNpr was not due to the volume, pressure of injection or the vehicle (saline or N,N-DA) in which injected drugs were dissolved.

**iii) Injection of agonists/antagonists and glutamate:** On the day of recording, the rat was connected to the recording cable at least one hour before the start of recording and the injector cannulae lowered into the site of injection through the bilateral guide cannula as mentioned above. The drugs were filled in the tubing and the injector cannulae in a manner similar to that for the vehicle injection (described above). After an hour of pre-injection recording, 200 nL of the drug (agonist/antagonist/glutamate) was injected into either PPT or SNpr at the rate of 100 nL/min. The following drugs were injected:

**Microinjections into PPT**

1. **Single injections:**
   i. 0.86 mM Picrotoxin (GABA-A antagonist)
   ii. 3.5 mM Muscimol (GABA-A agonist)
   iii. 0.24 mM Prazosin (α1 antagonist)
   iv. 3.75 mM Clonidine (α2 agonist)
   v. 3.38 mM Propranolol (β antagonist)

2. **Co-injection**
   i. 0.86 mM Picrotoxin + 3.75 mM Clonidine

**Microinjections into SNpr:**

1. 0.86 mM Picrotoxin (along with simultaneous electrical stimulation of SNpr)
2. 5.35 mM glutamate
The animals were not disturbed as the injections were done using remote controlled automatic pump. The recording was continued for eight post-injection hours. There was a gap of at least one day between the vehicle and the drug microinjections. No rat received more than one drug injection.

**iv) Co-injection:** In one group of animals GABA-ergic antagonist (0.86 mM picrotoxin) and NA-ergic agonist (3.75 mM clonidine) were co-injected using the same procedure as mentioned above.

**v) Microinjections to confirm the site specificity of results:** In one group of rats, agonists/antagonists of GABA and NA were microinjected/co-injected at a site away from the PPT to confirm the site specificity of the results. Similar control studies were done using glutamate.

2. **Stimulation study:** The experimental set-up was divided into following groups:

   **i) Baseline group:** On day one of the recording schedule, the rat was connected to the recording set-up an hour before the start of the recording. EEG, EOG and EMG were recorded simultaneously in three separate channels of the polygraph for eight hours between 10:00 AM-7:00 PM. Hipp waves could not be recorded in the stimulation study because the antero-posterior coordinates for implantation of recording electrode overlapped with the antero-posterior coordinates for the implantation of stimulation electrodes. Therefore, very little space was left on the rat cranium to implant the electrodes to record hippocampal waves. The values obtained for different stages of sleep-wake cycle for the eight-hour recording period were compared with the corresponding period for stimulation group and the group in which stimulation was done in the presence of picrotoxin in PPT.

   **ii) Stimulation Group:** On day two of the recording schedule, the rat was connected to the recording cable at least one hour before the start of recording. After, pre-stimulation recording of one hour, bilateral stimuli consisting of electrical square wave pulses of 50-80 μsec duration, 60-110 μA amplitude and frequency of 2-5 Hz, were
delivered continuously through the recording period of eight hours. The voltage for the stimulation current was generated from a constant voltage stimulator and fed into photoelectric stimulus isolation unit to deliver constant current pulses into SNrpr. The eight-hour polygraphic recording along with the stimulation was followed by four hour of post-timulation recording, when the sleep-wake recording was carried out without stimulation. The stimulation at the above mentioned parameters did not cause any apparent and abnormal changes in the behavior of animal. The stimulation artifacts in the polygraphic recordings were either absent or minimal. However, stimulation of SNrpr at higher current strength (>110 µA) and increased pulse duration (>80 µsec duration) produced excessive artifacts in the polygraphic recording making it difficult to discriminate the different stages of sleep-wakefulness. Stimulation with lower current strength (<60 µA) and decreased pulse duration (<50 µsec) did not affect any of the sleep wakefulness stages.


**iii) Stimulation of SNrpr in the presence of picrotoxin in PPT:** After a gap of at least two days, SNrpr was stimulated along with simultaneous single injection of picrotoxin (0.86 mM) into the PPT. The rats were connected to the recording cables and the injector cannulae lowered into PPT through the guide cannula. After an hour of pre-injection and pre-stimulation recording, the stimulation and microinjection were started simultaneously. The microinjection of picrotoxin was over in two minutes whereas the stimulation was continued for eight hours. The microinjection procedure, volume and rate were same as mentioned earlier in the section on microinjection study. In addition to the eight-hour post-injection recording during which the SNrpr was continuously stimulated, four hour of post-stimulation recording was also done after stopping the electrical stimulation. A schematic diagram showing the experimental set-up for simultaneous electrical stimulation in SNrpr and microinjection of picrotoxin into PPT is shown in figure XVI.

**V. Confirmation of the site and spread of microinjection**
After all the recordings were completed, rats were anaesthetized with Ketamine-Xylazine combination and 200 nL of 2% pontamine sky blue dye (also known as Chicago
Figure XVI Representative rat brain sections showing simultaneous bilateral microinjection and electrical stimulation in PPT and SNpr, respectively.
blue dissolved in 0.5 M Sodium acetate) was injected using the same injector cannulae. The injection procedure was same as mentioned earlier. Thereafter the rats were perfused intracardially. The rat brain was cryosectioned into 40 µm coronal sections and stained with 2% neutral red dye. Some of the sections through PPT were stained immunohistochemically with anti-ChAT antibody. The injection sites and spread of injected agonists/antagonists were identified by the presence and extension of pontamine blue dye coloration in the histological sections.

VI. Confirmation of the site of electrical stimulation

The rat was anaesthetized with Ketamine-Xylazine combination and using a lesion maker, anodal current (500 µA, 20 sec) was delivered to the site of stimulation through the electrode that was used for stimulation. The anodal current was delivered through the electrode pole that was used for the stimulation. Therefore, the pole that served as cathode during stimulation was made anode for the purpose of lesion. The passage of current caused the iron ions to leech out from the tip of the electrode into the surrounding brain tissue. These metallic ions (ferric ions) were rendered visible by perfusing the animal with 4% paraformaldehyde containing 2% potassium ferrocyanide. Potassium ferrocyanide reacted with iron and gave a Prussian-blue colour. The reaction is known as Prussian blue reaction. Prussian Blue was the principal pigment of the dyes used in German army uniforms, hence the name. The brain was sectioned and stained 2% neutral red dye.

\[
4\text{FeCl}_3 + 3\text{K}_4\text{Fe(CN)}_6 \rightarrow \text{Fe}_2[\text{Fe(CN)}_6] + 12\text{KCl}
\]

{Prussian blue}

VII. Histology and immunohistochemistry

1. Brain perfusion

Brain perfusion was done to serve two purposes:

i) The blood in the brain capillaries would interfere with the staining procedure and hence needed to be washed out.
ii) Since brain is a soft tissue, fixing it inside the cranial cavity made it hard enough to be taken out without any distortion and damage. Also, fixation prevented the brain tissue from decomposition by either putrefaction or autolysis (by the enzymes present within the cells). Fixation and embedding cause antigen masking, but also better retention of labile proteins, nucleic acids and small peptides. Paraformaldehyde stabilizes the tissue proteins by forming methylene cross-links between them.

The rat was anaesthetised with overdose of Ketamine-Xylazine combination. The thoracic cavity was opened and ribs were removed to expose the heart. Using a hemostatic scissor, the descending aorta was clamped that restricted the flow of blood to the lower part of the animal’s body. This reduced both the time needed and the chemicals required to perform the brain perfusion. Flexible polyethylene tubing connected to a reservoir containing 0.1 M phosphate buffer saline (PBS) was introduced into the left ventricle and a cut was made on the right auricle. Next, 100 mL of 0.1 M PBS was passed through the left ventricle. The PBS entered the systemic circulation through the left ventricle and pushes the blood out through the right auricle. PBS is followed by 100ml of 4% paraformaldehyde through the same tubing. After the perfusion was completed, the cranium was carefully broken piece by piece and the brain was taken out. The brain was allowed to remain overnight in 4% paraformaldehyde at 4°C for proper fixation to occur and then either shifted to 30% sucrose, if sectioning was to be done or to 0.1 M PBS for storage

2. Cryoprotection

The brain was transferred to 30% sucrose solution and kept for about two days till it sank into the solution. Sucrose is a good cryoprotectant. It enters the interstitial spaces within the brain tissue and displaces water, thus, preventing the formation of ice crystals on cooling to very low temperature (-15 °C). The formation of ice crystals damages the tissue. Also, since sucrose is inert it does not react with the tissue.
3. **Sectioning**

Using Leica Cryostat, 40 µm thick sections were cut from the rat brain through PPT and SNrpr. The brain was cut into small block containing the desired area and then put onto a tissue carrier plate the chuck. The brain tissue embedded on the chuck using tissue-embedding fluid (OCT, *Tissue-Tek, Miles Inc.*, USA). The OCT compound firmly holds the frozen specimen block onto the cryostat chuck or the block holder providing stability and protection to the tissue from the sudden impact with the knife's cutting edge. The chuck was then placed inside the chuck holder. The temperature of chuck holder was maintained at -15 °C and was aligned at a prefixed angle to blade. The temperature of the blade and the cryostat chamber was maintained at -30 °C. The section thickness knob was set for 40 µm. The anti-roll plate was positioned and sectioning was done by rotating the handwheel in a clockwise direction. The movement of the handwheel cause the chuck and hence the embedded tissue to move in the direction of blade with a vertical swiping motion. The sections were cut when the tissue swiped across the blade surface. A ribbon of sections was obtained, which was picked up with a moist brush and placed in vials containing PBS or directly on glass slides. The coronal sections (40µm) cut through PPT and SNrpr were stained with 2% neutral red dye. Some of the sections through PPT were immunostained using anti-ChAT antibody (Table II).

4. **Neutral red staining:** The tissue sections were taken directly onto the subbed slides after cutting on the cryostat. The sections were allowed to dry and stick properly onto the glass slide for 2-3 days. Thereafter, the staining was done as follows:

i. The glass slide with sections was rinsed in distilled water.

ii. Slide kept in neutral red stain for 5-10 minutes.

iii. The sections were dehydrated in ascending alcohol grades (30%, 50%, 70%, 90% and 100%) and cleared in xylene.

iv. Mounted in DPX.

5. **Immunostaining**

Immunohistochemistry (IHC) is in situ-detection of antigens in tissue sections and cells by lectins, monospecific monoclonal and polyclonal antibodies. The basic principle of immunohistochemistry is the use of enzyme-linked antibodies to detect tissue antigens.
The colorless substrate is converted by the enzyme into a colored product that precipitates on the slide at the site of the reaction.

The tissue sections were incubated with primary antibody that was raised in goat or sheep and directed against the tissue antigen under study. In order to prevent non-specific staining, it was essential to block the endogenous peroxidases present in the tissue, which otherwise act on 3,3′ di-amino benzidine (DAB) that was used as the substrate for horse raddish peroxidase (HRP) tagged to the secondary antibody. This was prevented by incubating the tissue sections with 4% hydrogen peroxide (H₂O₂) in 90% methanol for 1 to 1.5 hrs. Binding of the primary antibody to non-specific antigenic sites was prevented by incubating the tissue sections with normal serum from the species in which the secondary antibody was raised.

Triton X-100 was added along with the primary antibody as it helped in reducing the surface tension in washing solutions, allowing the slides or vials to remain wet. Once cells are fixed they no longer can select intracellular traffic of molecules however, the antibodies can easily cross cell membranes (cytoplasmic, nuclear) in fixed cells.

The tissue was then incubated with the secondary antibody directed against the primary antibody. The secondary antibody was bound to the antigen-primary antibody complex on the tissue. The secondary antibody is usually raised in rabbit against IgG of the host species (goat or sheep used for raising primary antibody. The secondary antibody was diluted in the serum of the species in which it was raised (rabbit).

In order to enhance the sensitivity of the immunostaining so that even antigens present in low amounts could be detected, the secondary antibody was conjugated with biotin. Biotin is a small molecular weight vitamin that could be easily recognized by avidin. Avidin is a high molecular weight (68,000) glycoprotein that has an extremely high affinity for biotin (10¹⁵ M⁻¹). Because this affinity is over one million times higher than that of antibody for most antigens, the binding of avidin to biotin (unlike antibody-antigen interactions) is essentially irreversible. Avidin has four binding sites for biotin and most proteins (including antibodies and enzymes) can be conjugated with several molecules of biotin. Therefore, a preformed macromolecular complex between avidin and biotinylated enzyme, which still retains biotin-binding sites (Vectorstain Elite ABC kit, Vector Laboratories, Burlingame, CA, U.S.A.) was used to amplify the signal from
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immunostaining. A preformed macromolecular complex present in the Vectorstain Elite ABC kit contained Avidin DH and biotinylated horseradish peroxidase (HRP) H reagents. The HRP is revealed with hydrogen peroxide as substrate and DAB as electron donor. The reaction causes rapid oxidation and polymerization of DAB resulting in the formation of amorphous osmiophilic polymers, which are insoluble in water or lipid. This ABC complex bound to the biotinylated secondary antibody. The chromogen used was DAB with H$_2$O$_2$ as the substrate reagent (Vector Laboratories, Burlingame, CA, U.S.A.) that resulted in a brown precipitate at the antigenic site of the tissue.

6. Subbing

The coating of glass slides with gelatin is known as “subbing”. Gelatin acts as an adhesive and prevents the tissue sections from getting washed off during mounting and staining procedure. Glass slides were dipped into subbing solution a few times and then left overnight for drying.

**Composition of Subbing Solution:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>1 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>200 mL</td>
</tr>
<tr>
<td>Chrom Alum</td>
<td>0.1 g</td>
</tr>
</tbody>
</table>

The above compounds were mixed and heated to 60°C under continuous agitation till it was completely dissolved

7. Dehydration and mounting

The sections mounted on subbed slides were left for about two days to dry. Thereafter, the sections (now glued to the slide) were dehydrated in alcohol and cleared in xylene. In order to prevent tissue shrinkage on contact with absolute alcohol, the sections were dehydrated gradually by using ascending grades of alcohol (30%, 50%, 70%, 90%, 100%). Dehydration is required as the mounting medium is generally a resinous medium. The resinous media are dissolved in organic solvents such as xylene and are not miscible in water or alcohol. Distrene Plasticiser Xylene (DPX) was used as a mounting medium.

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VIII. Sleep scoring and analysis

The sleep-wakefulness records were scored in the epochs of 10 seconds into active wakefulness (AW), quiet wakefulness (QW), slow wave sleep1 (SWS1), slow wave sleep2 (SWS2) and REM sleep (Timo-Iaria et al., 1970; Singh and Mallick, 1996). The AW was identified by the presence of desynchronized EEG accompanied by high EMG tone with or without phasic muscle movement, presence of eye movements in the EOG and hippocampal theta waves. The QW was characterized by the presence of occasional (<25% of the epoch time in the EEG) spindling, no active muscle movement, reduced eye movements and non-continuous hippocampal theta waves. The SWS1 was identified by the presence of EEG synchronization up to 50% of the epoch time, reduced EMG tone, reduced eye movements and absence of hippocampal theta waves. The SWS2 was characterized by the presence of synchronized EEG (>75% of the epoch time), significantly reduced muscle tone, no eye movements and absence of theta waves. The REM sleep was characterized by EEG desynchronization accompanied by muscle atonia, rapid eye movements and continuous hippocampal theta waves.

Total time spent in each of the five stages of AW, QW, SWS1, SWS2 and REM sleep during the entire recording period was calculated. The mean frequency of occurrence of REM sleep episodes per hour and the mean duration of REM sleep per episode per hour were calculated for the entire recording period. The effects of antagonists/agonists/glutamate microinjected either individually or in combination on these parameters were statistically compared with that of the sham injection and vehicle control groups. Additionally, to evaluate the temporal extent of the effect of injection of antagonists/agonists/glutamate on REM sleep, the post-injection data was divided into the bins of two-hour each and statistical comparison between the control (sham and vehicle injection) and treatment group (antagonists/agonists/glutamate) was done.

Similarly, for the experiments involving bilateral stimulation of SNrpr alone and in the presence of picrotoxin in PPT, total time spent in each of the five stages of sleep-wakefulness during the entire recording period was calculated. The mean frequency of occurrence of REM sleep episodes per hour and the mean duration of REM sleep per episode per hour were also calculated for the entire recording period. The effects of stimulation of SNrpr alone and in the presence of picrotoxin microinjection into PPT were
statistically compared with that of the baseline group. Additionally, to evaluate the temporal extent of the effect of stimulation of SNrpr alone as well as in the presence of picrotoxin microinjection into PPT, data from baseline group as well as from both the stimulation and combined stimulation and microinjection groups were divided into bins of two-hours and statistically compared with each other. The post-stimulation data was also similarly analysed.

All the statistical comparisons were done using Repeated Measures One Way analysis of variance (ANOVA) coupled with Student-Neuman-Keuls post-hoc test using statistical software package Graphpad (Demo version) and significance levels determined. One Way or One Factor Repeated Measures ANOVA tests for differences in the effect of a series of experimental interventions on the same group of subjects by examining the changes in each individual. Examining the changes rather than the values observed before and after interventions removes the differences due to individual responses, producing a more sensitive (or more powerful) test. The design for a One Way Repeated Measures ANOVA is essentially the same as a Paired t-test, except that there can be multiple treatments on the same group. The null hypothesis is that there are no differences among all the treatments. One Way Analysis of Variance is a parametric test that assumes that all treatment effects are normally distributed with the same standard deviations (variances).

The figures in the results section show the raw data in the graphical as well as tabular form.
### Materials and Methods

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Action</th>
<th>Route of injection</th>
<th>Dose/Volume injected</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketamine hydrochloride</td>
<td>General Anesthesia</td>
<td>IP</td>
<td>80 mg/kg body wt</td>
<td>Neon Labs, India</td>
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<tr>
<td>Xylazine hydrochloride</td>
<td>Sedative, analgesic, Muscle relaxant</td>
<td>IP</td>
<td>20 mg/kg body wt</td>
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<tr>
<td>Atropine sulphate</td>
<td>Mixed cholinergic blocker</td>
<td>IM</td>
<td>0.6 mg/kg body wt</td>
<td>Seth Pharmaceuticals</td>
</tr>
<tr>
<td>Normal saline</td>
<td>Physiological solution</td>
<td>IP</td>
<td>Upto 1 mL</td>
<td>Core Healthcare Ltd</td>
</tr>
<tr>
<td>Dextrose normal saline</td>
<td>Physiological solution</td>
<td>IP</td>
<td>Upto 1 mL</td>
<td>Rathi Labs India</td>
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<tr>
<td>Dexamethasone</td>
<td>Anti-inflammatory</td>
<td>IM</td>
<td>2 mg/kg body wt</td>
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<td>Gentamicin</td>
<td>Anti-Biotic</td>
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<td>2.5 mg/kg body wt</td>
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<td>Betadine solution</td>
<td>Anti-septic</td>
<td>Topical</td>
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<td>Win-Medicare Ltd</td>
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<tr>
<td>Hemostatic gel</td>
<td>Foamgel to stop bleeding during surgery</td>
<td>Topical</td>
<td>--</td>
<td>Sri Gopal Krishna Labs Pvt Ltd, India</td>
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<td>Picrotoxin</td>
<td>GABA-A antagonist</td>
<td>Intracerebral local microinjection</td>
<td>0.86 mM 200 nL</td>
<td>Sigma Chemicals</td>
</tr>
<tr>
<td>Muscimol</td>
<td>GABA-A agonist</td>
<td>Intracerebral local microinjection</td>
<td>3.5 mM 200 nL</td>
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<td>α1 noradrenergic antagonist</td>
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<td>Glutamate (monosodium)</td>
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<td>Pontamine sky blue dye</td>
<td>To mark site and spread of microinjection</td>
<td>Intracerebral local microinjection</td>
<td>2% 200 nL</td>
<td>Sigma Chemicals</td>
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</tbody>
</table>
**Materials and Methods**

**Table II**

<table>
<thead>
<tr>
<th>CHOLINE ACETYL TRANSFERASE (ChAT)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagents</strong></td>
</tr>
<tr>
<td>Phosphate buffer saline (PBS 0.1 M, pH 7.4)</td>
</tr>
<tr>
<td>90% methanol (Qualigens) and 4% H₂O₂ (Qualigens)</td>
</tr>
<tr>
<td>Normal goat serum (Sigma Chemical)</td>
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<tr>
<td>Triton X-100</td>
</tr>
<tr>
<td>Primary antibody (rabbit anti-ChAT, polyclonal, Chemicon)</td>
</tr>
<tr>
<td>Secondary antibody (goat anti-rabbit, Vector)</td>
</tr>
<tr>
<td>Elite ABC kit (Vector)</td>
</tr>
<tr>
<td>DAB kit (Vector)</td>
</tr>
</tbody>
</table>

**Method**

1. Free floating (40 µm) sections were treated with 4% H₂O₂ in 10 mL of 90% methanol for 1.5 hrs in the dark.
2. Sections were washed in PBS (3 x 10 mins).
3. Blocked in 10% NGS and 0.5% Triton X-100 in PBS for 2 hrs at room temperature.
4. Incubated with primary antibody (1:2000 dilution) in PBS with 5% NGS and 0.5% Triton X-100 for 3 days at 4 degree Celsius.
5. Washed in PBS (5 x 10 mins).
6. Incubated with secondary antibody (1:200 dilution) in PBS with 1% NGS for 18hrs (4 degree Celsius).
7. Washed in PBS (3 x 10 mins).
8. Incubated with ABC (1: 50 dilution) in PBS for 2 hrs at room temperature.
9. Washed in PBS (3 x 10 mins) and then in distilled water.
10. Treated with DAB and H₂O₂ in DAB substrate (tris) buffer for 3 mins.

**Observation**

Brown colored ChAT positive neurons in PPT were visible on a yellow background. Other non-cholinergic neurons were not visible.
## Summary of the experiments conducted

1. Microinjection experiments:

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Group</th>
<th>No. of Rats</th>
<th>Treatment</th>
<th>Volume (nanoliters) nL</th>
<th>Molarity</th>
<th>Area</th>
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<tbody>
<tr>
<td>1</td>
<td>Sham Control</td>
<td>92</td>
<td>Baseline recording</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>Vehicle Control 1</td>
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<td>Saline</td>
<td>250</td>
<td>6.4 mM</td>
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<tr>
<td></td>
<td></td>
<td>89</td>
<td></td>
<td>200</td>
<td>6.4 mM</td>
<td>PPT</td>
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<tr>
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<td>20% N-NDA</td>
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<td>PPT</td>
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<td></td>
<td>3</td>
<td></td>
<td>200</td>
<td></td>
<td>PPT</td>
</tr>
<tr>
<td>4</td>
<td>Dose Response 1</td>
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<td>Picrotoxin</td>
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<td>0.172 mM</td>
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<td></td>
<td>200</td>
<td>0.172 mM</td>
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<td>200</td>
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<tr>
<td></td>
<td>Experimental 1</td>
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<td></td>
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<td>Muscimol</td>
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<td>3.75 mM</td>
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<tr>
<td>7</td>
<td>Dose Response 3</td>
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<td>Propranolol</td>
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<td>PPT</td>
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<td>Experimental 4</td>
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<td>Site Specificity 4</td>
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<td>200</td>
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<td>8</td>
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<td>200</td>
<td>0.24 mM</td>
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<tr>
<td>9</td>
<td>Experimental 6</td>
<td>5</td>
<td>Co-injection Picrotoxin (0.86 mM) + Clonidine (3.75 mM)</td>
<td>200</td>
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<tr>
<td></td>
<td>Site Specificity 6</td>
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<td>200</td>
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<td>Experimental 7</td>
<td>5</td>
<td>Glutamate</td>
<td>200</td>
<td>5.35 mM</td>
<td>SNrpr</td>
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<td></td>
<td>Site Specificity 7</td>
<td>3</td>
<td></td>
<td>200</td>
<td>5.35 mM</td>
<td>Outside SNrpr</td>
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2. Electrical stimulation of substantia nigra par reticulata in the absence and presence of picrotoxin in PPT:

<table>
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<tr>
<th>S. No</th>
<th>Group</th>
<th>No. of Rats</th>
<th>Treatment</th>
<th>Volume (nanoliter) Molarity</th>
<th>Area</th>
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<tr>
<td>2</td>
<td>Experimental</td>
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<td>Electrical stimulation</td>
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<td>SNrpr</td>
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<tr>
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<td>Experimental</td>
<td>5</td>
<td>Electrical stimulation</td>
<td>50-80 μsec, 60-110 μA 2-5 Hz,</td>
<td>SNrpr</td>
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<td>Picrotoxin microinjection</td>
<td>200nl, 0.86 mM</td>
<td>PPT</td>
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<tr>
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<td>Site specificity</td>
<td>3</td>
<td>Electrical stimulation</td>
<td>-</td>
<td>Outside SNrpr</td>
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<td>control</td>
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