**p-CPA Pretreatment Reverses the Changes in Sleep and Behavior Following Acute Immobilization Stress Rats**

Rakesh Kumar SINHA

Department of Biomedical Instrumentation, Birla Institute of Technology, Mesra (Ranchi), Jharkhand–835215, India

**Abstract:** The effects of *p*-CPA (para-chlorophenylalanine) pretreatment was studied on the sleep-wake parameters and patterns of behavioral activities in an animal model of acute immobilization stress. For the experiments, young male Charles Foster rats were divided into three groups, subjected to (i) acute immobilization stress for four hours on specially designed wooden boards, (ii) a similar model of acute immobilization stress after pretreatment of *p*-CPA (injected through i.p. route), and (iii) control rats (*p*-CPA untreated and unstressed). Three channels of electrographic signals, i.e., EEG (electroencephalogram), EOG (electrooculogram), and EMG (electromyogram) were recorded continuously for four hours for all three groups of rats to analyze the changes in sleep-wake stages. The assessment of behavior was performed just after the stress on separate groups of rats in Open-Field (OF) and Elevated Plus-Maze (EPM) apparatuses. The significant changes in total sleep time (*P* < 0.05), total time for rapid eye movement sleep (*P* < 0.01), and total time in wakefulness (*P* < 0.01) following acute immobilization stress were found reversed in the *p*-CPA (a serotonin inhibitor) pretreated group of rats. Simultaneously, the results of the present work also revealed that the changes in grooming behavior (*P* < 0.05) in OF and the total time spent on the center of EPM (*P* < 0.05) were observed altered in *p*-CPA pretreated group of rats.

**Key words:** immobilization stress, *p*-CPA, sleep, behavior, open-field, elevated plus-maze.

It has been established that when animals are subjected to acute stress, a wide range of physiological alterations take place, and a short single intense exposure to stress can produce profound changes in an animal’s stress response and behavior [1]. Immobilization is one of the most widely used methods to produce emotional stress. Evidences proposed that acute immobilization stress triggers corticosterone secretion, which is responsible for the alterations in sleep-wakefulness of mammals [2, 3]. Except for this, there have been many studies also showing significant correlations between the secretion of neurohormones and behavior [4–7] and neurohormones and sleep [8]. A review of literature suggests that acute immobilization stress causes great elevation in brain neurohormones in mice and rats [9, 10].

Apart from the regulatory activity of the body temperature, the hypothalamus is also considered as the chief regulating center of sleep and behavioral expression of the animals [11] in different psychophysiological stress. Brain serotonin is believed to be an important factor for the distribution and regulation of ions in the hypothalamus. It has also been established that dorsal raphe nucleus contains the highest density of the serotonergic neurons and extends serotonin fibers to the LHA (lateral hypothalamic area) [12]. Thus serotonin has significant correlation with hypothalamic activities. Apart from these findings, laboratory experiments also suggest that dissimilar psychophysiological stress causes significant increase in the serotonin activity in brain, which is finally responsible for the changes in sleep and behavior of the subjects [5, 8–11].

The degree of behavior and sleep changes because any stressful event is a highly potent variable determining the brain function and neuronal impact of the stressor. The stress and anxiety are often viewed as somehow related, and literature suggests that the uncontrollability of stress is a potent initiator of anxiety and that the serotonin systems mediate this process. It has been believed that an understanding of the physiological system and its association with serotonin controlling the centers can provide a sharper knowledge for understanding the stress pathophysiology with physical reality [13]. Although many neurotransmitters are linked to the pathophysiology of stress, research studies have implicated disturbances in the serotonin system and the hypothalamo-pituitary-adrenal (HPA) axis because neurobiological alterations in these systems are most consistently associated with depressive illness [14, 15].

Previous studies have established that extravasation in the blood-brain barrier is mediated by the stimulation of serotonin systems, followed by a secretion of serotonin. Simultaneously, different series of experiments under...
stressful conditions suggest that the prior administration of para-chlorophenylalanine (p-CPA), a serotonin inhibitor, prevent edema development [16, 17] and changes in brain cortical signals [18, 19]. But even through changes in the brain serotonin level are responsible for the alterations of both sleep and behavior following immobilization stress, no report is available that analyzes the effect of the prior administration of p-CPA on these parameters in a model of acute immobilization stress. Therefore with considerations of some established pathophysiological stress markers, in the present study an effort has been made to monitor the effect of the pretreatment of p-CPA on the sleep and behavior of rats subjected under acute immobilization stress.

**Materials and Methods**

**Subjects.** Fifty male Charles Foster albino rats (obtained from the Central Animal House, Institute of Medical Science, Banaras Hindu University, Varanasi, India), weighing 60–80 grams, aged 7–8 weeks, were housed in polypropylene cages (30 cm × 20 cm × 15 cm) on 12L:12D (light from 0700 to 1900) cycle at 24 ± 1°C with food and water ad libitum. The rats were divided into three groups: control group (n = 10); acute immobilization stressed groups (n = 20) and p-CPA pretreated groups with acute immobilization stress (n = 20). The stressed and p-CPA pretreated stressed groups were further divided into two equal groups consisting of 10 rats each for sleep and behavioral recording.

**Immobilization stress model.** The rats were subjected to the acute immobilization stress at room temperature (24 ± 1°C) on specially designed wooden boards for four hours, i.e., from 0800 hours to 1200 hours IST (Indian Standard Time). The limbs of the animals were fixed on the board with adhesive tape, and the whole body of each rat was wrapped with surgical gauze to prevent body movement. The animals were allowed to breathe freely during the immobilization.

**p-CPA pretreatment (n = 20).** The schedule of pretreatment of p-CPA and the dose have been used according to the methods described earlier. p-CPA (Sigma Chemical Co., USA) was injected 100 mg/kg i.p. daily for three consecutive days in rats. Twenty-four hours after the last injection of p-CPA, the animals were subjected to stress. This dose schedule inhibits the serotonin synthesis in the brain and induces a long–lasting depletion in CNS (central nervous system) [19, 20].

**Surgery and electrode implantation.** Under stereotaxic guidance, electrodes for polysomnographic recordings were aseptically and chronically implanted on each rat’s head with the help of pentobarbital (35 mg/kg i.p.) anesthesia. The implantation of electrodes for three electrographic signals, such as EEG (electroencephalogram), EOG (electrooculogram), and EMG (electromyogram) were performed as the method, described earlier [21–23]. The animals were allowed a minimum one-week recovery period from surgery before commencement of the recording of a sleep-wake cycle.

**Polysomnographic recording.** For a recording of polysomnographic data, the animals were placed in a test chamber (35 cm × 25 cm × 30 cm) made of perspex that was located in a constantly illuminated (500–600 lux white light), sound-insulated shielded chamber (300 cm × 180 cm × 240 cm). The recordings of data were played from 1200 hours to 1600 hours IST on the experimental day with the help of an electroencephalograph (Medicare, India) via a signal conditioning box at a chart speed of 7.5 mm/s. The data were recorded with the amplifier settings suggested by Sarbadhikari [21] and the parameter settings given in Table 1.

The sleep parameters considered for analyses, as discussed by Andersen and Tufik [24], are (a) total sleep time (TST)—sum of all sleep periods during the recording; (b) total time of slow wave sleep (TSWS)—sum of all periods of SWS sleep throughout the recording; (c) total time of rapid eye movement sleep (TREM)—sum of all periods of rapid eye movement sleep throughout the recording; (d) total wake time (TWT)—sum of all periods of waking during the recording; (e) sleep efficiency (EFFIC)—percentage of TST during the recording time; (f) number of slow wave sleep episodes (NSWS)—total number of slow wave sleep episodes in the whole recording period; (g) latency of slow wave sleep (LSWS)—time lag between onset of the recording and the first episode of slow wave sleep; (h) latency of rapid eye movement sleep (LREM)—time lag between onset of the recording and the first episode of rapid eye movement; (i) Stage shift (StS)—number of times the animal changes from one sleep phase to the other.

**Behavioral activity monitoring.** In the present study, the changes in behavior following acute immobilization stress in rats were evaluated by open-field (OF) and elevated plus-maze (EPM) methods [21, 25]. The open field experiments are mainly used to measure the fearfulness and reactivity of the animals. However, the elevated

Table 1. The parameters of amplifier setting for the recording of different electrophysiological signals are given in the table.

<table>
<thead>
<tr>
<th>Signal</th>
<th>Sensitivity in µV/mm</th>
<th>Low-frequency cutoff in Hz</th>
<th>High-frequency cutoff in Hz</th>
<th>50 Hz filter</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEG</td>
<td>10</td>
<td>1</td>
<td>70</td>
<td>In</td>
</tr>
<tr>
<td>EOG</td>
<td>20</td>
<td>0.3</td>
<td>35</td>
<td>In</td>
</tr>
<tr>
<td>EMG</td>
<td>10</td>
<td>5</td>
<td>70</td>
<td>Out</td>
</tr>
</tbody>
</table>
plus maze is used to test their emotionality or anxiety [21, 26].

Open field (OF): The field was a circular arena with the outer diameter being 84 cm. Peripherally there were 16 squares. The inner concentric circle of 56 cm diameter contained 8 squares. The 100 W frosted bulb was placed 1 m above the field, an otherwise dark room during the activity testing. The behavioral parameters of each rat were tested in a wake condition in the OF for 3 min by placing the animal at the center of the apparatus: (i) Immobilization: Each rat had the eyes open, holding its head against the gravity but without any head, body, or limb movements. (ii) Grooming: Rhythmic paw movements over the face and/or head for face washing might include episodes of biting and cleaning of paws. (iii) Rearing: Standing still and upright on its hind limb only. (iv) Ambulation: When all four limbs were in one particular square (central or peripheral) of the open field.

Elevated plus-maze: The maze [21] had two open arms (50 cm × 10 cm) and at right angles to it, two closed arms (50 cm × 10 cm × 40 cm) with the roof uncovered; an open central crossing (10 cm × 10 cm) that was rising to a height of 50 cm. The behavioral parameters of each rat were tested for 5 min in a wake condition in EPM by placing them at the end of an open arm: (i) Transfer latency: Time taken (in seconds) by the animal to move from the outer end of the open arm to either of two closed arms. (ii) Percentage of time in open arms: The percentage of total testing time spent in the open arm. (iii) Percentage of time at central crossing: The percentage of total testing time spent at the crossing of the open and crossed arms. (iv) Number of crossing of the arms: The number of times the animal crosses the center for going one arm to any of the other three arms.

Determination of edema and edematous swelling in the brain. The animals were sacrificed after recordings of behavioral parameters, and the brains were dissected out. The wet and dry weights of each brain were noted and the percentage of water calculated. The dry weight of each brain was determined after repeatedly drying the sample in an oven at 80°C until the weight remained constant. The percentage of edematous swelling was calculated by using following formula [19]:

\[
\% \text{ Water content in experimental animal} = \frac{100 + f}{100 + f} \times 100
\]

where \( f \) = % of swelling caused by edema.

Other parameters. (a) Analysis of plasma corticosterone level: Blood was collected from the femoral vein of anesthetized rats before the removal of brain for determination of edematous swelling. The change, if any, in the level of plasma corticosterone was assayed by the spectrofluorometric method as described earlier [25].

(b) Changes in colonic temperature: The changes in body temperature are an established indication of stressful events. Thus with the help of a thermistor probe (Yellow Spring Co., USA) and a telethermometer (Aplab, India), the core body temperature was recorded before and after the immobilization stress from all stressed and control animals.

RESULTS

Assessment of stress because of acute immobilization stress

The analysis of results showed that an acute immobilization stress for four hours significantly decreased (\( \Delta T = \))
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Table 4. Analyses of changes in the behavioral activity in OF and EPM apparatuses for p-CPA–untreated stressed and p-CPA–treated stressed rats with respect to the control group of rats. The data are represented as mean (±SE) and compare to the *$P < 0.05$, the **$P < 0.01$ to the respective control group.

<table>
<thead>
<tr>
<th>Behavioral test</th>
<th>Control (n = 10)</th>
<th>Stress (n = 10)</th>
<th>p-CPA pretreated (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open field</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immobilization (s)</td>
<td>71.00 (5.38)</td>
<td>126.40 (7.19)**</td>
<td>118.50 (6.67)**</td>
</tr>
<tr>
<td>Rearing (number)</td>
<td>9.40 (0.83)</td>
<td>1.80 (0.61)**</td>
<td>2.40 (0.45)**</td>
</tr>
<tr>
<td>Grooming (number)</td>
<td>3.60 (0.47)</td>
<td>5.40 (0.51)*</td>
<td>3.20 (0.33)</td>
</tr>
<tr>
<td>Ambulation (squares)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral (number)</td>
<td>67.60 (1.96)</td>
<td>22.80 (2.96)**</td>
<td>29.40 (4.47)**</td>
</tr>
<tr>
<td>Central (number)</td>
<td>3.80 (0.57)</td>
<td>4.60 (0.58)</td>
<td>4.30 (0.45)</td>
</tr>
<tr>
<td>Total (number)</td>
<td>71.40 (1.86)</td>
<td>27.40 (2.48)**</td>
<td>35.70 (5.13)**</td>
</tr>
<tr>
<td>Elevated plus maze</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transfer latency (s)</td>
<td>13.00 (0.82)</td>
<td>20.20 (0.10)*</td>
<td>24.60 (0.33)*</td>
</tr>
<tr>
<td>% Time open arm</td>
<td>5.53 (0.24)</td>
<td>8.33 (0.55)*</td>
<td>9.67 (0.23)*</td>
</tr>
<tr>
<td>% Time center</td>
<td>2.93 (0.69)</td>
<td>4.97 (0.53)*</td>
<td>2.15 (0.35)</td>
</tr>
<tr>
<td>No. arms crossed (number)</td>
<td>2.40 (0.58)</td>
<td>2.55 (0.27)</td>
<td>3.10 (0.67)</td>
</tr>
</tbody>
</table>

1.87 ± 0.12°C) the body temperature ($P < 0.01$), but the plasma corticosterone level was increased significantly ($P < 0.01$) in drug-untreated groups of rats. The p-CPA pretreated group also shows statistically similar results of the drug-untreated group as a significant decrease ($\Delta T = 1.69 \pm 0.07°C$) in body temperature ($P < 0.01$) and the increases in plasma corticosterone levels ($P < 0.01$) in rats were analyzed in comparison to the control group (Table 2). These results indicate that p-CPA pretreatment is not involved in the changes in body temperature or in alterations in plasma corticosterone level following acute immobilization stress.

Sleep parameters

Table 3 shows the results of sleep parameters obtained in four hours of recording following acute immobilization stress. The statistical analysis of results advocate that the p-CPA untreated acute immobilization stress group of rats has significantly increased TST ($P < 0.05$) and TREM ($P < 0.01$), but TWT was significantly decreased ($P < 0.01$) in comparison to the control group. However, statistically significant changes in TST, TREM, and TWT following acute immobilization stress were found reversed in the p-CPA pretreated stressed group, as insignificant changes were recorded in these parameters following immobilization stress in comparison to the control rats. Summarizing the results in terms of sleep efficiency (EFFIC), we observed significantly increased EFFIC ($P < 0.05$) following immobilization stress, while insignificant change in the EFFIC was recorded in the p-CPA pretreated stressed rats in comparison to the control rats. The acute immobilization stress reduced the onset time for slow wave sleep and rapid eye movement sleep in drug-untreated rats, and thus a significant reduction in both LSWS ($P < 0.05$) and LREM ($P < 0.05$) was analyzed in comparison to the control group. Drug (p-CPA) pretreatment does not cause any alteration in these data because insignificant changes were recorded between p-CPA treated and untreated stressed groups. However, no significant differences were found in TSWS, NSWS, or StS between drug-treated stressed rats, drug-untreated stressed rats, and control rats.

Behavioral test

The data of behavioral analysis of control as well as stressed animals for acute immobilization stress are presented in the Table 4.

Open field (OF). When analyzed statistically, after 4 h of immobilization stress on an open field, the mobility in the p-CPA untreated group of animals was found significantly reduced ($P < 0.01$). The rearing behavior was also significantly reduced ($P < 0.01$), but the reverse was observed in grooming ($P < 0.05$). A significant difference in ambulation behavior was found because there was less ambulation in peripheral ($P < 0.01$) and total squares ($P < 0.01$) of the p-CPA untreated stress group of rats compared to the control group. However, no significant difference was found in ambulation on the central squares in this group of rats. Conversely, only grooming behavior was observed with insignificant changes in comparison to control rats in p-CPA pretreated stressed rats. But the other parameters show similar variations as drug-untreated stressed rats.

Elevated plus maze (EPM). Following four hours of acute immobilization stress, the transfer latency ($P < 0.05$), percentage time on open arms ($P < 0.05$), and the percentage time on center ($P < 0.05$) were significantly increased in p-CPA untreated stressed rats compared to the control group because these rats had taken more time to enter the closed arm and spent more time on the open arm and the central square. For the number of arms crossed, both the stressed and control groups showed no significant difference. A pretreatment of p-CPA following acute immobilization stress reverses the change in percentage time on the center as insignificant changes were analyzed in compari-
son to the control rats. However, the data of other parameters were found statistically the same as those of the drug-untreated group of rats.

**Edema and edematous swelling in brain**

The result indicates that four hours of immobilization stress increases the brain water content by 3.28%, which corresponds to a 15.22% increase in volume swelling in the drug-untreated group of rats, but the p-CPA pretreated groups of rats show relatively less increase in water content (1.07%).

**DISCUSSION**

Immobilization stress is a well-known method to produce psychological stress. It is very effective in eliciting typical nonspecific stress manifestations accompanied with a discharge of medullary catecholamines, and if prolonged, it produces hypothermia in rats [9]. The results from previous studies have also established a close association between the brain edema and the breakdown of blood-brain barrier in stressful conditions. In unison, pharmacological studies clearly indicated that an increased level of serotonin in stress is a major contributing factor for increased blood-brain barrier permeability either directly or through some indirect mechanism [17], and if the synthesis of the serotonin is blocked with the help of a serotonin synthesis inhibitor such as p-CPA, it prevents the edema formation in the brain [19], which is strikingly similar to the present findings.

In the present manuscript, the experiments were carried out on a model approach as described by Sharma et al. [20] to analyze the changes in behavior and sleep following acute immobilization stress. The dose dependency of the p-CPA has not been examined in this particular manuscript. However, there is certainly a dose-dependent relationship between the p-CPA pretreatment and the serotonin action. Literature suggests that either a single injection of p-CPA (400 mg/kg body weight) [8] or pretreatment with 100 mg/kg body weight [20, 27] for three consecutive days causes no apparent variation in behavior or electrophysiological record. That is why there was no need to add a separate group of animals for drug treated and stress. Aside from that, it has been shown that p-CPA selectively decreases the concentration of serotonin in brain without altering any other neurotransmitters, and the drug in itself has no pharmacological action on the brain [8].

It has also been confirmed that the corticosterone secretion in prolonged immobilization stress suppresses the repetitive sleep, which results in insignificant change in slow wave sleep episodes [3]. The significant increase in TST and EFFIC was analyzed following acute immobilization stress due to the result of a significant increase in TREM at the cost of a significant decrease in TWT. The comparative sleep analyses from the drug-untreated immobilization stress and p-CPA pretreated immobilization stress indicate that most of the changes produced by the immobilization stress have been reversed to the control values. The reversal of the data of TST, TREM, TWT, and EFFIC strongly suggest the powerful participation of serotonin in alterations in these parameters because of acute immobilization stress. The outcomes of the present study also propose that aside from the blockage of serotonin secretion, p-CPA also participates in the suppression of CRH in locus coeruleus either directly or indirectly. This may be one of the possible grounds for the reason that most of the changes recorded in sleep-wake cycles in acute immobilization-stressed rats were reversed by the pretreatment of p-CPA, but no alterations in the level of the plasma corticosterone level in the p-CPA-pretreated stressed group were recorded in comparison with the drug-untreated immobilization stressed group. In the rat hypothalamus, it is reported that the concentration of serotonin is greater in the LHA than in other regions. Further, through different studies it has been shown that various stressful stimuli cause significant increases in serotonergic activity in the brain [12]. Jouvet [8], in his review article on brain amines and sleep, mentioned that an increase in serotonin level will increase the REM sleep. The literature also suggests that immobilization stress stimulates the limbic areas of the brain and pathways projecting from these areas to the hypothalamus, and it also stimulates corticotropin-releasing hormone (CRH) secretion into the pituitary-adrenal axis. However, CRH not only stimulates pituitary to increase the corticotropin secretion, but it also increases the sympathetic outflow to adrenals resulting in an increased output of both cortical and medullary hormones [28]. The increased rapid eye movement sleep, induced by immobilization stress, is thus mediated by the endogenous CRH because the brain CRH acts as a neurotransmitter in the locus coeruleus under stressful conditions [2] and because of the increased serotonin level in the brain [8].

Supporting previous reports, the present work also suggests that acute immobilization stress produces hypothermia in rats [9], which is supposed to be independent of serotonin level in the brain. The decrease in the appearance of the slow wave sleep and rapid eye movement sleep in the drug-untreated and the drug-treated groups following acute immobilization stress suggest that a decrease in the LSWS and LREM might have occurred because of the more regulatory mechanism of the brain under hyperthermia produced resulting from acute immobilization stress. Similar to the previous findings [29, 30], the results of the present study indicated that TST is a function of thermoregulatory drive because hypothalamic temperature has been shown to have a profound effect on sleep in a variety of mammalian species [31]. Graf et al. [30] have suggested that two possible mechanisms mediate this influence. First, hypnogenic systems exist in the basal forebrain, and hypothalamic warming may directly influence
the neurons involved. Second, hypothalamic temperature is a major feedback signal in the thermoregulatory system in mammals, and the activity of the thermoregulatory network might have a direct influence in the state of sleep-wakefulness. Sakaguchi et al. [32] favored the latter influence of hypothalamic temperatures on TST. They showed that TST was closely correlated with the thermoregulatory error signal defined as the difference between the hypothalamic temperature threshold for the heat production response and the actual hypothalamic temperature.

The present report demonstrates that acute immobilization stress induces a decrease in mobilization in OF and transfer latency in EPM. Along with these changes, a decrease in rearing and ambulation behavior was also observed, while grooming was found to have significantly increased. These changes in behavioral parameters revealed that immobilization stress altered the excitability of the nervous system similarly, as reported by Sarbadhikari and his co-workers [21]. As a result of this, the subjects were showing augmented mobility in OF ambulation testing. Similar to the present finding, Abraham and Gogate [33] showed that high stress increases grooming behavior. Later on, in EPM, rats were found spending more time on open arms and center rather on closed arms. This behavior may represent the change in emotionality in the rats following immobilization stress, which resulted in animals showing fear of stress and trying to escape. It has been reported that stress-induced release of CRH affects the central nervous system directly and exhibits neurotropic action, which is important for mobilizing behavior response to stress. CRH produces a dose-dependent locomotor activation in rats. After a higher dose, more-bizarre behavioral effects have been observed, including elevated walking, repetitive locomotion, and pawing rapidly against the cage [33].

The p-CPA pretreatment in rats does not demonstrate a great alteration in behavior in them following acute immobilization stress because significant alterations in behavior were noted only in two parameters, such as grooming in the open field and percentage time on the center of the EPM. The p-CPA pretreatment changed these behaviors of rats and reversed them at the level of control rats. Because the behavior of an animal is very complex to analyze and involves various types of biochemicals and different types of feedback and/or feedforward mechanisms regulate the behavior of the subject. However, the reversal of grooming behavior on OF and percentage time on center focused some light on the involvements of serotonin in alterations of behavior following acute immobilization stress.

Although the behavioral abnormalities and alteration in the sleep-wake cycle as observed in acute immobilization-stressed-animals are highly dependent on alterations in brain neurochemicals, it is difficult to indicate only one specific neurotransmitter responsible for the change in a particular parameter. However, it has been a possibility that acute immobilization stress induced extravasations in the blood-brain barrier permeability that may allow circulating neurohormones, especially serotonin to enter the brain leading to changes in sleep-wake cycles and behavioral abnormalities as suggested by [17]. Thus to extract more information regarding the involvements of different types of neurochemicals and their antagonists, in alterations in each parameter of the sleep-wake cycle and behavior of the subjects, an extensive, detailed, and systematic experimental investigation is needed.

REFERENCES
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