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Long-term effects of early maternal separation and isolation stress on adulthood behaviour of female rats

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The present study demonstrates the long-term effect of early maternal separation (EMS) and isolation stress on the adult emotionality behaviour of female rats. The maternal separation (MS) of rat pups constituted both separation and isolation from the littermates for three days from post-natal days 5–7 (stress hyporesponsive period, MS(SHRP)) and 16–18 (post-stress hyporesponsive period, MS(PSHRP); 6 h/day) respectively. SHRP is characterized by reduced capacity to secrete stress hormone under stressful situations, which is postulated to be essential for the normal development of hypothalamic–pituitary–adrenal axis. A control group consisted of rat pups never handled or separated from the mother. At post-natal day 61, the rats were exposed to a light/dark test, exploratory activity in a novel environment and passive avoidance test. Both control and MS(PSHRP) groups did not differ in the latency to enter into the dark compartment, number of transitions between light and dark compartments and total motor activities in the preferred dark chamber. However, MS(SHRP) rats exhibited increased activity in the dark chamber in the light/dark test. When exposed to a novel environment, MS(PSHRP) groups exhibited significant decrease in the freezing response when compared to both control and MS(SHRP) groups. Furthermore, following exposure to a passive avoidance test, both MS groups showed decreased latency to enter into the preferred chamber with reduced locomotor activity in the dark compartment, indicating stress-induced decreased attention as a consequence of EMS stress.

Keywords: Anxiety, locomotor activity, maternal separation, stress, passive avoidance.

EARLY post-natal period is a critical phase for brain development, characterized by an enormous capacity for structural and functional reorganization of the neural circuitry in rodents. Any experiences and perturbations during this early period of life are thought to have lasting changes on brain functions and on behaviour during adulthood[1,2]. Aversive experiences during the developmental period can induce time-dependent influence on

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adult behaviour, and can also alter the corticosterone levels[3]. The implication of increased glucocorticoids was further supported by studies in which brief handling can reduce the expression of fear-related behaviour under stressful conditions[4–6]. Similarly, brief handling in neonatal rats caused permanent alterations in hippocampal glucocorticoid receptors, with decreased glucocorticoid responses to stress in adulthood[1,7]. Recent studies on rats in the field have demonstrated that chronic exposure to juvenile stress continuously for three days led to elevated levels of anxiety and poor spatial learning task one month after stress[8]. However, the stress produced by repeated separation from the mother during early post-natal life (early maternal separation stress, EMS) can leave deep scars and result in various mood disorders and cognitive disabilities during adulthood[9]. MS rats as adults exhibit hyper-reactive HPA activity, increased hypothalamic paraventricular nucleus corticotrophic releasing factor and increased median eminence CRF mRNA and protein levels[9], as well as indices of heightened anxiety when compared to the early handled group[10,11].

Studies by Lehmann *et al.*[12] further demonstrated gender-based differences in expression of fear behaviour in rodents. There are no consistent reports yet to show the unconditioned fear and anxiety-like behaviour in rats after maternal separation (MS) along with social isolation stress during early post-natal period. Hence it became more apparent to study systematically the variables involved in this phenomenon and to replicate some of the earlier research in which functional relationships have been reported. The purpose of this study was to investigate further the influence of MS stress along with isolation from littermates during the developmental period, and the response of these rats to novel environmental exposure and to an aversive stimulus during adulthood. Accordingly, the MS stress was designed where the rat pups were being separated from their littermates and also from their dam daily for three days (6 h/day) during the stress-hyporesponsive period (SHRP) and post-stress hyporesponsive period (PSHRP). The MS stress is of shorter periods, tends to minimize the effects of nutritional deficits associated with longer separation and provides a model for poor maternal care in the early days of life. Two months later, the light/dark test, as well as the spontaneous response of MS rats to a novel environment was studied. In addition, the passive avoidance memory test to an aversive stimulus was also studied in the maternally deprived rats.

The experiments were carried out using female Wistar rats of about 55–60 days old. At the beginning of experiments the body weight of the animal was measured; the average body weight was 200 ± 20 g. Animals had free access to standard pellet food and water, and were maintained on a 12 h light/dark cycle at room temperature and relative humidity at the Central Animal Research Facility (CARF), NIMHANS, Bangalore. Experiments were carried out according to the animal ethics guidelines. Care was taken to minimize the number of animals for the experiments.

Ten adult male and 10 female rats were bred in CARF. The day of delivery was considered as P0. Both mother and rat pups were left undisturbed on the day of birth. MS procedure was carried out at two time-points: MS(SHRP) where the pups were subjected to MS for 6 h from post-natal days 5–7 (P5–P7) and MS(PSHRP) where the pups were subjected to MS for 6 h from post-natal days 16–18 (P16–P18). The MS procedure was carried out from 9.00 a.m. to 3.00 p.m. during the light phase. The separation was performed by placing the rat pups individually in separate polypropylene cages with dimensions $421 \times 290 \times 190$ mm divided into six cubicles equally by a wooden partition. The cages were covered with a stainless-steel mesh lid of size $25 \times 7 \times 14$ mm. After 6 h of MS, the pups were reunited with their dam and littermates. Pups which were not separated from their dams, but were exposed to standard animal facility-rearing conditions served as the control group. Pups were finally weaned on P21 and housed 3–4 per cage (42×26 cm). When all the offspring attained 60 days of age, they were tested for both conditioned and unconditioned fear/anxiety-like behaviour as adults.

Figure 1 represents the design of the experimental protocol for assessing the impact of EMS during SHRP and post-SHRP period on different behaviours during adulthood.

The light/dark transition test is one of the most widely used tests to measure unconditioned anxiety-like behaviour in rodents. The test was based on the natural aversion of rats to brightly illuminated areas and on their spontaneous exploratory behaviour in novel environments. The test was conducted in Coulbourn Habitest rat shuttle cage/passive avoidance cage (Coulbourn Instruments Inc., PA, USA) having dimensions $50.8 \times 25.4 \times 30.5$ cm. The shuttle cage consisted of two compartments of equal size (26×26 cm) separated by a sliding guillotine door (8×8 cm). The light compartment was illuminated with a bright light (400 lux) on the ceiling, whereas the

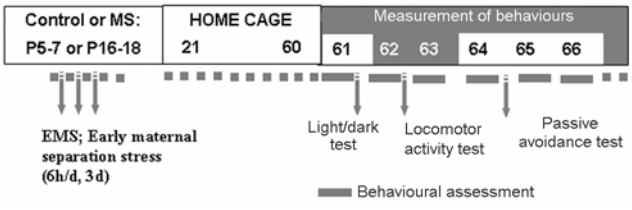

Figure 1. Experimental design for behavioural assessment during adulthood. Maternal separation stress was carried out either during stress-hyporesponsive period (P5–P7; MS(SHRP)) or during post-stress hyporesponsive period (P16–P18; MS(PSHRP)). Control rats did not undergo either handling or separation during neonatal days. Light/dark test was carried out on P61, locomotor activity test on P62; passive avoidance test on P64. P, Post-natal separation.

shock compartment was dark. On the first day, the light/dark test was carried out in all three groups: control (n = 10/group), MS(SHRP) (n = 14/group) and MS(PSHRP) (n = 14/group) by exposing them to the light compartment facing away from the guillotine door. After introduction of a rat, 10 s was allowed for the animal to familiarize with the light compartment. The guillotine door was held open till the end of the experiment. The experiment was conducted using the software Graphic State 3.03 provided with the instrument (Coulbourn Instruments Inc.). The entire experiment was videotaped for offline analysis. The analysis consisted of latency to enter the dark compartment after the guilliton door was open, the number of transitions made by the animal between the two compartments and the total amount of time spent in each chamber separately. The number of transitions indicated the exploratory activity of the rat and the time spent in each compartment was considered as an index of anxiety in rats.

On the second day, the same rats were exposed to a novel open chamber to evaluate the anxiety-like behaviour by measuring their locomotor activity. This was conducted in the Coulbourn Habitest modular rat test chamber. The set-up comprised of a drop pan, stainless-steel grid floor for delivery of constant current, a sliding plexiglass door in front and a fixed plexiglass door at the back. A thermal sensor was placed on the top to record the locomotor activity of rats. The fear behaviour of the rats was scored offline using video recordings. The fear behaviour was assessed by measuring freezing responses such as absolute state of immobility with a defensive posture, except that of the respiratory movements. This test was conducted for two consecutive days. The test on the first day enabled us to evaluate the anxiety-like behaviour in a novel environment, while that on the second day served as a measure of anxiety-like behaviour in a familiarized environment. The number of rats used to test anxiety like behaviour was: n = 10/control; 14/MS(SHRP) and 16/MS(PSHRP).

On the fourth experimental day, rats were exposed for evaluating learning and memory. The experiment was performed in a familiar chamber that was used in the light/dark test. The rat was introduced into the light compartment facing away from the guillotine door for all the three steps. The exploratory activity of the rat in the dark compartment was recorded by the thermal sensor placed on top of the dark compartment. The experiment was run using the software Graphic State 3.03. The experimental procedure consisted of habituation, training and testing.

Habituation constituted the first day of the passive avoidance test. It was done to familiarize or habituate the animal with the environment. The duration of this session was 3 min. First, the rat was introduced into the light compartment facing away from the guillotine door. It was then allowed to explore the light compartment for 30 s. At the 31st second, the guillotine door was raised and the rat was allowed to enter the dark compartment. After complete entry into the dark compartment (all four limbs of the rat), the door was shut exactly after 15 s. Subsequently, the exploratory activity of the rat in the dark was measured for 3 s by the thermal sensor. This was called the pre-unconditioned stimulus (pre-US) state.

Training was conducted 24 h post-habituation. The rat was introduced into the light compartment facing away from the guillotine door. Then 30 s after the exploratory activity, the door was raised. The door was shut 15 s past the complete entry of the rat. Following this, the exploratory activity of the rat was recorded for 3 s before delivering the foot shock (pre-US). Succeeding this, a constant current of 0.5 mA was delivered for 1 s. Then the activity of the rat was measured post-US for the next 2 min and the session was terminated. The total duration of the session was 3 min.

In order to test the retention of aversive memory, the rats were exposed once again into the light compartment of the passive avoidance chamber. The testing was conducted 24 h following training. The time taken to enter the preferred dark compartment was manually calculated and the latency was compared with that during preconditioning (habituation). Longer the latency to enter the dark compared to that in habituation trial, better would be the fear memory consolidation in the rat. Similarly, lower the latency to enter the dark chamber compared to that in habituation trial, poorer would be the fear memory.

The light/dark test facilitated evaluating the relative anxiety status of rats. Rats that experienced MS stress both during SHRP, MS(SHRP) and MS(PSHRP), when exposed to a brightly illuminated environment during adulthood, showed reduced time to enter into the dark compartment than control rats (ANOVA: $F_{2,37} = 0.3637$, $P > 0.05$; Figure 2 *a*). Similarly, MS stress did not alter the total number of crossings between light and dark compartments. However, once the rats entered the dark compartments, MS(SHRP) exhibited relative increase in the total number of events compared to that of control

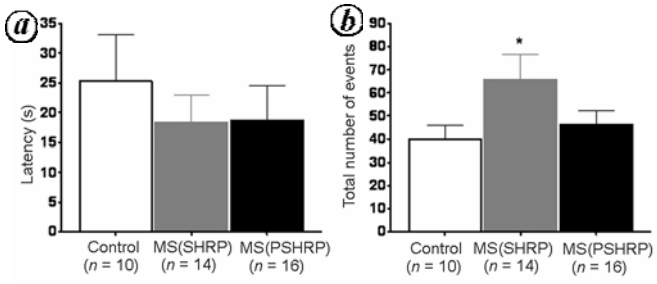

Figure 2. Effects of maternal separation stress on the light/dark test. ***a***, Latency to enter into the dark compartment was used as an index of anxiety-like activity of the rats. No significant differences were observed with respect to latency to enter the dark compartment in the either groups of MS rats. ***b***, MS(SHRP) rats showed statistically significant increase in discrete events in comparison to control rats. Student's t-test, $^*P < 0.03$.

and MS(PSHRP) rats. Statistical analysis with Student's *t*-test further revealed significant differences between control and MS(SHRP) groups, indicating increased exploratory activity in the dark compartment ($t_{22} = 1.876$, $P < 0.0370$; Figure 2 *b*).

The spontaneous exploratory behaviour of rodents in response to a novel environment and light was also considered as an index of innate aversive behaviour of the rat. Statistical analysis with one-way analysis of variance test suggested significant impact of MS stress on spontaneous exploratory behaviour of the rats in a novel environment (ANOVA: $F_{2,37} = 5.900$, $P < 0.006$) by the MS(PSHRP) group of rats than that of the control and MS(SHRP) group (Figure 3). Further with Student's *t*-test suggesting a significant decrease in the freezing behaviour of the MS(PSHRP) rats ($t_{24} = 2.022$, $P < 0.05$), thus it is evident that MS stress had reverse effects on the rat behaviour of the MS(PSHRP) group.

In the passive avoidance test, the latencies to enter the dark compartment during habituation and testing were compared between groups. Here the rats were trained by giving minimum intensity of foot shock, so that control rats may not exhibit prolonged time to enter into the dark compartment. Although all these groups of rats received mild foot shock (0.5 mA), control rats displayed little or no evidence of fear behaviour throughout the testing period ($t_9 = 0.6108$, $P > 0.05$). However, MS(SHRP) and MS(PSHRP) rats displayed much less fear behaviour, in terms of decreased latency to enter into the preferred dark chamber. The entire data were obtained after 24 h of an electric foot shock.

Statistical analysis with one-way ANOVA suggested no overall effect on the total spontaneous behaviour of the rats ($F_{5,74} = 2.061$, $P < 0.08$). Further Student's *t*-test analysis suggested that MS stress during SHRP period showed reduced latency to enter into the dark compartment after training ($t_{13} = 2.743$, $P < 0.0168$; Figure 4 *a*). Similarly, the MS(PSHRP) group also showed significantly reduced latency to enter the dark compartment during testing ($t_{15} = 4.118$, $P < 0.0009$; Figure 4 *a*) which in turn implies that MS did have severe impact on the aversive memory when compared to control animals.

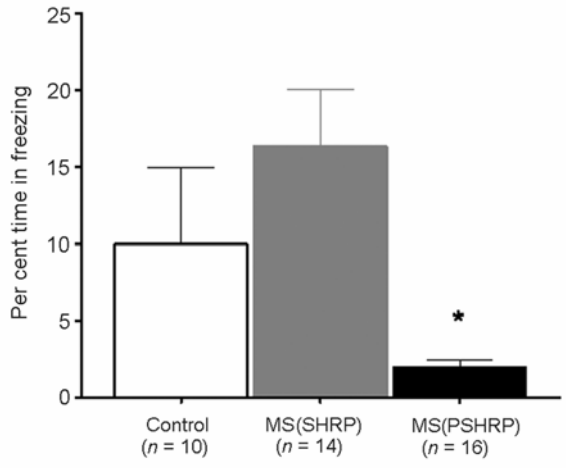

Figure 3. Exposure to a novel environment showed statistically significant decrease in time spent freezing among MS(PSHRP) group of rats in comparison to control. Student's *t*-test was performed. *$P < 0.05$.

Discrete movements or events were recorded as a measure of spontaneous exploratory activity inside the dark chamber. One-way ANOVA suggested an overall impact on the spontaneous activity (ANOVA: $F_{5,75} = 2.466$, $P < 0.04$; Figure 4 *b*). Further, to compare the different stages of testing, Student's *t*-test was applied. A significant decrease in activity inside the dark chamber was observed in the maternally separated groups during post-SHRP period compared to that of control rats ($t_{15} = 2.365$, $P < 0.0319$) (Figure 4 *b*).

Stressful experiences during early life have an impact on the physiological as well as behavioural responses to a natural calamity in adulthood. There are several evidences to substantiate this on adulthood behaviour[13–15]. We have hypothesized that MS stress along with isolation stress might have stronger impact on the adulthood emotionality of rat behaviour. In support of this, we have demonstrated that rats that experienced MS and isolation stress during SHRP exhibited a significantly higher exploratory activity in the preferred dark chamber than the rats reared under standard housing conditions. When these maternally separated rats were exposed to a novel environment, they exhibited significant decrease in the freezing behaviour and hence increased exploratory activities when compared to the control group. This is in agreement with a similar study[16] which has demonstrated higher level of activity by rats subjected to MS in a novel environment. Yet another study[17] also supports our findings by demonstrating attenuated freezing in the novel open field area, in female rats exposed to MS during SHRP (MS of 6 h per day from post-natal days 2–14). We have also observed that the MS(PSHRP) rats showed significant decrease in freezing behaviour in a novel environment, but no differences were seen in the light/dark test. However, after exposure to an aversive experience, these rats exhibited significantly decreased latency to enter into the dark chamber with lesser activity. This indicates that EMS along with isolation stress was stronger when compared to late MS[18]. There also appears to be altered adrenocorticotropic hormone response to stress and the neuronal stress markers such as high c-fos and CRH mRNA in the early maternally separated rats. This could be one of the reasons underlying the decreased anxiety-like behaviour exhibited by the PSHRP maternally separated groups.

However, following passive avoidance training for the electric foot shock, both MS(SHRP) and MS(PSHRP) rats took lesser time to enter into the dark compartment during the testing session compared to that during

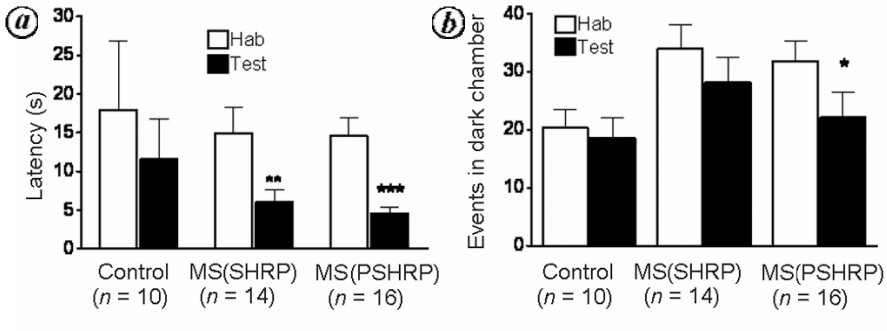

Figure 4. Passive avoidance test showing decreased latency to enter into the preferred chamber. Data showed decreased latency to enter into the dark compartment and also decreased events in the dark compartment by both MS(SHRP) and MS(PSHRP) rats. Hab, Habituation; Test, Retention test, $*P < 0.03$; $**P < 0.01$; $***P < 0.0009$.

habituation. The decreased latency to enter into the dark compartment was associated with a decrease in the total number of discrete events. The impairment in the aversive memory with an inability to remember to avoid the aversive compartment in a passive avoidance chamber, suggests stress-induced decreased attention as a consequence of MS. The present findings expand on the work by others, suggesting significant EMS caused impairment in learning and memory, as tested by spatial alteration and foot-shock sensitivity tests[19]. Similarly, rats subjected to MS of 3 h/day from post-natal day 1 to day 21 suffer from cognitive impairment that has been demonstrated using Morris water maze and novel object recognition test[20]. In conclusion, it appears reasonable to assume a close association between maternal care during early life and adulthood behaviour, as evidenced with impaired aversive memory deficits induced by EMS during and after SHRP.

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