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ScienceDirect
Pharmacology, Biochemistry and Behavior 85 (2006) 535–544
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Early deprivation increases exploration and locomotion in adult male Wistar offspring

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Received 22 September 2005; received in revised form 19 September 2006; accepted 5 October 2006
Available online 15 November 2006

Abstract

The aim of the study was to investigate the effects of repeated early maternal deprivation (individual separation in warm or cold environment for 4 h/day) during postnatal days 1–15 on emotional responses in novel situations and voluntary alcohol consumption in adult male Wistar rat offspring. Brain monoamine levels and plasma levels of corticosterone were measured at the end of the experiment. Controls were exposed to a brief (3 min) daily handling procedure. As adults, both groups of early deprived rats showed increased nose poking and locomotion in the exploration test compared to controls. Moreover, separated rats kept in room temperature also showed increased locomotion when tested for an extended period of time. There were no differences in alcohol intake, monoamine levels, or corticosterone levels between early deprived animals and controls. In addition, the dams' retrieval behavior of pups was studied, showing that dams of early deprived pups spent more time in the nest with the pups after the 4-h separation period compared to control dams. Our results indicate that early deprived animals show decreased emotionality in novel settings compared to briefly handled controls. Furthermore, the present study demonstrates that methodological issues within the maternal separation paradigm may be influential factors for behavioral changes in adulthood.

Keywords: Alcohol intake; Anxiety; Corticosterone; Early deprivation; Emotionality; Maternal separation; Monoamines; Rat

1. Introduction

Adverse childhood events have been found to be associated with psychiatric symptoms. For example, physical, emotional and sexual abuse in children are proposed to be risk factors for adult depressive and anxiety disorders, and substance abuse (Heim and Nemeroff, 2001; Kendler et al., 2000; Maughan and McCarthy, 1997; Moncrieff et al., 1996; Young et al., 1997). Emerging evidence also suggests that exposure to early life stress is associated with neurobiological changes, which may underlie the increased risk of psychopathology (reviewed in Heim and Nemeroff, 2002, 2001). The aim of the study was to examine emotional behavior, alcohol consumption, brain monoamine and corticosterone levels in rats subjected to early life stress.

In the laboratory rat, the maternal separation (MS) paradigm has been developed to examine early adverse experiences on behavior and neurobiology. Pups repeatedly separated during the postnatal period have shown increased anxiety-related behaviors in adulthood, for example, in the elevated plus maze (Daniels et al., 2004; Huot et al., 2001; Kalinichev et al., 2002; Wigger and Neumann, 1999), two compartment exploratory test (Biagini et al., 1998) and the open field test (Caldji et al., 2000b). Early adverse experience has also been associated with increased voluntary alcohol consumption (Huot et al., 2001; Jaworski et al., 2005; Ploj et al., 2003; Roman et al., 2005), reviewed in Roman and Nylander (2005), and with increased sensitivity to other drugs of abuse, for example, cocaine, morphine and amphetamine (Chretien and Gratton, 2002; Kalinichev et al., 2001; Matthews et al., 1999; Meaney et al., 2002; Zhang et al., 2005).

Moreover, disruption of the hypothalamic–pituitary–adrenal (HPA) axis has been shown in young as well as adult MS animals. For example, MS studies indicate an enhanced secretion of

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doi:10.1016/j.pbb.2006.10.005

adrenocorticotropic hormone (ACTH) and corticosterone in both basal and stressed conditions (Biagini et al., 1998; Daniels et al., 2004; Huot et al., 2001; Kalinichev et al., 2002; Ladd et al., 2005; Liu et al., 2000). MS studies have also shown that a variety of brain neurotransmitter systems are affected by early separations, such as the serotonergic, dopaminergic and noradrenergic systems (Arborelius et al., 2004; Daniels et al., 2004; Gartside et al., 2003; Liu et al., 2000; Matthews et al., 2001; Meaney et al., 2002).

It must, however, be noted that results within the MS paradigm are far from in agreement and might depend on several factors (reviewed in Lehmann and Feldon, 2000; Pryce and Feldon, 2003). We have, for example, recently studied the effects of daily separation of pups on emotional and drug responses, neurochemistry and endocrinology in both male and female adult offspring (Marmendal et al., 2004). In that study, separations were performed for 4 h during postnatal days 1–15 in intact litters kept in incubators. There were mainly no significant alterations in emotionality, alcohol intake, plasma corticosterone and brain opioid peptide levels, either in males or females compared to their respective briefly handled control. A possible explanation for these results might be that the separation of intact litters in incubators did not constitute a severe stressor in the pups. A modification of the separation protocol, which has not been extensively investigated, is by assessing the separation of pups in isolation from both the dam and littermates (i.e. early deprivation (ED), Pryce and Feldon, 2003), a manipulation that has been suggested to possibly be a more severe experience in the pups (Kosten and Kehoe, 2005; Kuhn and Schanberg, 1998; Pryce and Feldon, 2003). Thus, the aim of the present study was to investigate effects of early deprivation on adult animals' emotional behavior (i.e. exploratory and risk assessment behavior, fleeing and freezing responses and spontaneous locomotor activity), voluntary alcohol consumption, brain monoamine levels and endocrine variables. Furthermore, the ambient temperature during separation may be an important factor in MS-studies. To investigate possible effects of ambient temperature in isolated animals, the early deprivation manipulation in the present study occurred either in an incubator or in room temperature, and the control group was briefly handled as in our previous study (Marmendal et al., 2004). No additional stressors were used in adulthood (e.g. in the alcohol consumption test or in conjunction with corticosterone measurements) to further investigate possible disruptions attributable solely to the separation manipulation.

Behavioral and neurobiological effects in early separated offspring are proposed, at least in part, to be mediated by the quality of maternal care, which appears to be a critical factor for determining individual differences in stress responsiveness in the rat (Caldji et al., 2000a, 1998; Francis and Meaney, 1999; Liu et al., 1997). Studies have shown that handled and MS offspring receive changed maternal care compared to controls (Huot et al., 2004; Liu et al., 1997; Pryce et al., 2001b; Zimmerberg et al., 2003). In order to investigate one aspect of maternal care behavior, maternal retrieval of pups was observed during the first week after birth, a time period when such behavior is known to be intense in the dams. In our previous study, maternal retrieval behavior was not negatively disrupted in dams of MS litters and these dams spent more time in the nest with pups (Marmendal et al., 2004). Therefore, the final aim in the present study was to once more study the dams' behavioral pattern at the time for dam-litter reunion with a modified separation protocol.

2. Materials and methods

The experimental design of the study is presented in Fig. 1. For more detailed description of the methods carried out, see text.

2.1. Animals

Adult males and virgin Wistar females (Scanbur BK AB, Sollentuna, Sweden) were housed separately in groups of four or five per cage (Macrolon 4; 59×38×20 cm) for 2 weeks to adapt to the novel laboratory conditions before the experiment started. A female in estrous was placed overnight with a male in a breeding cage (Macrolon 3; 42×26×15 cm), and thereafter housed in her original group. At the end of pregnancy, females were housed singly (Macrolon 3) and provided with nesting material. The total of 40 females and their offspring were randomly divided into early deprivation treatment (ED; 20 litters; see Section 2.2 for further description of the separation manipulation) or a brief daily handling procedure (controls; 20 litters). The litters were culled (when possible) to 8 pups per litter (when possible to 4 males and 4 females) on postnatal day 1 (day of birth = day 0). All pups were weighed on postnatal days 1, 5 10, 15, 20, and 25 and in adult age (every 10th day during postnatal days 60–160).

Animals lived in air-conditioned colony rooms (lights off 07:00–19:00 h) at a temperature of 23 °C and a humidity

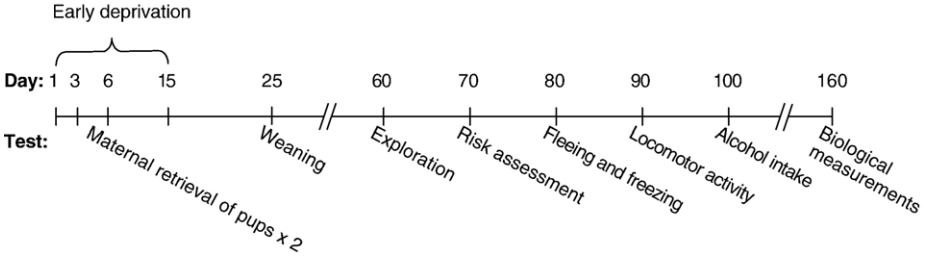

Fig. 1. Time line for the design of the study indicating the different test days and tests used in the experiment. For further explanation of each test, see text.

of 50–60% and they had free access to water and R70 food pellets (Labfor, Lactamin, Vadstena, Sweden). The experiments were approved by the local ethical committee of the Swedish National Board for Laboratory Animals.

2.2. Early deprivation and weaning

All pups in the ED group were separated daily from their littermates and dams for 4 h (10:00–14:00 h) during postnatal days 1–15. The dam was first removed from the nest to a separate cage. All pups were transferred to a room adjacent to the colony room. In addition, the ED group was further divided to two different separation protocols. Two male pups/litter were randomly selected to be included in the study. One of these ED pups were placed individually in a plastic box (18 × 18 × 11 cm high, a cardboard divider allowed the placement of two pups in each box) lined with sawdust, and placed in an incubator set to maintain an ambient temperature of 30 °C (ED_{Inc}). The other ED pup (a sibling) was individually placed in the same manner at room temperature (22 °C; ED_{Room}). The remaining pups in the experimental groups were placed as litters in room temperature during the separation procedure (i.e. separated from the dam) and were not included in the study (except for the Maternal retrieval test). That is, animals were kept in intact litters with their biological dam, two male pups/litter were included in the study (kept in two different temperatures during the separation period) and the rest of the litter were separated from the dam during the separation period but not included in the study. After the pups had been placed, the dam was returned into the maternity cage. At the end of the 4-h separation period, the dam was once again removed from the cage, and the pups were returned to their maternity cage followed by the dam. Regular cage maintenance began on day 7.

Pups who served as controls (one randomly selected male pup/litter) and their siblings (not included in the study, except for the Maternal retrieval test) were identically reared as the ED offspring, and experienced a brief daily handling procedure during postnatal days 1–15 to control for the effects of human handling in the experimental groups. The dam was removed to a separate cage, and the litter was placed in a plastic box (18 × 18 × 11 cm high) at room temperature. Within 3 min, the pups returned to the maternity cage, followed by the dam. On day 25, all offspring were weaned and housed (Macrolon 4; 4 animals/cage) in same treatment group (ED or control animals).

2.3. Behavioral tests

The behavioral tests were conducted sequentially with the same animals and performed during the dark phase of the light/dark cycle. Testing arenas were cleaned with soap and water between each animal tested.

2.3.1. Maternal retrieval of pups

All dams were assessed for retrieval behavior of the pups (modified from Hård et al., 1985) when offspring were 3 and 6 days of age. The test was conducted for 15 min immediately after the early deprivation procedure (ED group), and at the corresponding time of the day for the control group (approximately at 14:00 h). For dams to the ED group, the original litter was collected together before the test began (one ED_{Room} and one ED_{Inc} pup and their separated siblings). All control dams and control pups were removed from the maternity cage into separate cages for 5 min and the ED dams were removed before reunion with pups after the separation procedure. Thereafter the pups returned to the maternity cage, on the opposite side of the nest, followed by the dam. The time (s) for retrieval of the first pup to the nest, retrieval of the whole litter and the time (s) the dam spent in the nest with pups was measured. If the dam started to build a new nest upon reunion no measures were recorded. The testing room was illuminated by a 15 W white light bulb.

2.3.2. Exploration

At ~ 60 days of age, animals were tested for exploratory behavior in an illuminated testing room. The test apparatus (modified from File and Wardill, 1975) consisted of a wooden, brown-painted hole-board (80 × 80 × 35 cm high). The floor was divided into 16 squares by lines and each square contained a hole (4 cm in diameter and 2.5 cm deep). The rat was placed in the middle of the arena, and during 2 × 5 min the observer registered the number and cumulative duration (s) of nose pokes (both eyes disappearing in the hole) into the holes and the number of lines crossed (with all four limbs).

2.3.3. Risk assessment

Testing for risk assessment was performed on day 70. The test apparatus (modified from Grewal et al., 1997) comprised an elevated (73 cm above ground level) deep green circular platform (104 cm diameter). A smaller red Perspex circular canopy (70 cm diameter) was supported 10 cm above the platform by a central pillar. The test apparatus was thus divided into an inner, dimly lit covered zone, and an outer, brightly-lit exposed zone. Four lines forming eight 45° sectors divided the platform. The test started by placing the animal under the canopy. During the 10-min testing period the following behaviors were recorded by two observers: the number of stretched attend postures, number of lines crossed with all four limbs and the time spent in the outer exposed zone. A stretched attend posture (SAP) was defined as flexed hind limbs and a flattened lower back position with extended forelimbs when the animal was either standing still or moving slowly.

2.3.4. Fleeing and freezing

Testing for fleeing and freezing, in response to a sudden auditory signal, was performed on postnatal day 80. The test occurred in a circular Plexiglas cage (diameter 45 cm, height 30 cm) illuminated by a 15 W white light bulb (modified from Hård et al., 1983). The floor of the cage was divided by two lines to form four 90° sectors. The rat was placed in the cage and allowed a 5-min adaptation period. A doorbell (95 dB; suspended on the chamber wall) was then turned on for 6 s. During the signal the rat would first attempt to flee and thereafter, typically at the end of the signal, freeze. The number of lines crossed during the signal and the duration (s) of the freezing

response were used as measures of flight distance and freezing, respectively. The observation was terminated when the animal moved some parts of the body after the freezing reaction or if the freezing time exceeded 20 min. Defecation (number of boli deposited) was measured at the end of the test.

2.3.5. Spontaneous locomotor activity

Ten days after the fleeing and freezing test (~ day 90) animals were tested for locomotor activity (60 min), in computerized test chambers made of Plexiglas boxes (70×70×35 cm high; Kungsbacka Mät och Reglerteknik AB). The test box contained two series of invisible infrared photocell beams (high level: 14 cm from box floor; low level: 4 cm from box floor; 9 cm between the photocells; 2.5 cm from the walls) to measure locomotor behavior. The following variables were measured: the lower grid of infrared beams registered forward locomotion (consecutive interruptions of two beams). Rearing was registered by the high-level infrared beams, which registered every interruption of the beams as a rearing count when the rat raised itself onto its haunches. Total peripheral locomotion was recorded by measuring activity counts when the animal was in the periphery of the box (every interruption of the beams 2.5 cm from the walls was counted).

2.3.6. Voluntary alcohol intake

The animals (~ 100 days of age) were gradually familiarized to alcohol by giving them continuous access to a bottle containing an alcohol solution in addition to the water bottle. The alcohol concentration (vol/vol) was gradually increased over a nine-day period (2–4–6%). Thereafter, the animals were housed individually in clear Plexiglas cages (Macrolon 3) and had continuous access to two bottles (plastic 300-ml bottles with ballvalve nipples; Scanbur BK AB, Sweden) containing 6% alcohol solution and tap water for a 3-week period. The voluntary intake of alcohol and water was recorded by weighing the bottles three times a week during the period. The bottles were kept in the same position in the cages during the experiment and cleaned and refilled once a week. Alcohol preference is expressed as proportion of alcohol solution intake relative to total fluid consumption in percent and alcohol intake as gram per kilogram per day (g/kg/day) of absolute alcohol. At the end of the 3-week testing period, all animals were housed in their original groups.

2.4. Biological measurements

2.4.1. Tissue dissection and blood samples

Before decapitation, the alcohol solution was removed to give the animals a 4-week alcohol washout period. Animals (about 160 days of age) were housed individually 1 day prior to decapitation, and decapitated in a separate room between 09:00 and 12:00 h. After decapitation the thymus and adrenal glands were rapidly dissected out and weighed.

At the time of decapitation, blood samples were collected for analysis of plasma corticosterone level on day 160. After centrifugation of the tubes with blood, for 5 min at 375 *g*, the plasma was collected and stored at −80 °C until analyzed. Corticosterone was measured by the use of radioimmunoassay kits supplied by ICN Biomedicals (Costa Mesa, CA).

2.4.2. Brain dissection and monoamine extraction

Immediately after decapitation, the brain was removed and dissected on a chilled petri dish. The following brain areas were taken out for analysis: basal forebrain (medial frontal cortex, nucleus accumbens, olfactory tubercle, septum), dorsal striatum (caudate-putamen), hippocampus, amygdala and remaining cortical tissue. The tissues were weighed and stored at −80 °C until analyzed for dopamine (DA), noradrenaline (NA), serotonin (5-HT), homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA) with high-pressure liquid chromatography with electrochemical detection (HPLC-ED).

The frozen tissues were homogenized in 0.1 M perchloric acid containing Na_2-EDTA (2 mg/ml), glutathione (0.5 mg/ml) and D,L-alpha-methyl-DOPA (100 ng/ml) using Branson Sonifier 250. The samples were centrifuged for 10 min (10 000 *g*, 4 °C) and the supernatants were taken for analysis. The HPLC-ED system consisted of a Gynkotek P580 pump, a CMA 200 autosampler and a stainless steel column (4.6×150 mm) packed with Nucleosil RP18 5u (Jones Chromatography). The mobile phase (pH=2.74) was made up of K_2HPO_4 (0.012 M) and citric acid (0.04 M), containing Na-octyl-sulphate (63 mg/l), Na_2-EDTA (20 mg/l) and methanol (8%), the flow rate being 0.8 ml/min. HPLC-ED employed Antec Decade electrochemical detector including VT-03Hy-Ref. Currents were monitored using Chromelion PC1 software.

2.5. Statistics

Half of the litters were assessed with respect to the separation manipulation (two male pups were selected from each litter to further study and separated either in incubator or in room temperature, that is, ED_{Inc} or ED_{Room} group respectively) and the remaining litters served as controls (one male pup from each litter were selected to further study). Due to possible litter effects among siblings in the ED_{Inc} and ED_{Room} group, one-way ANOVA was not used. The between groups comparisons (i.e. ED_{Inc} vs. controls, ED_{Room} vs. controls and dams to ED litters vs. control dams) were analyzed with Student's unpaired

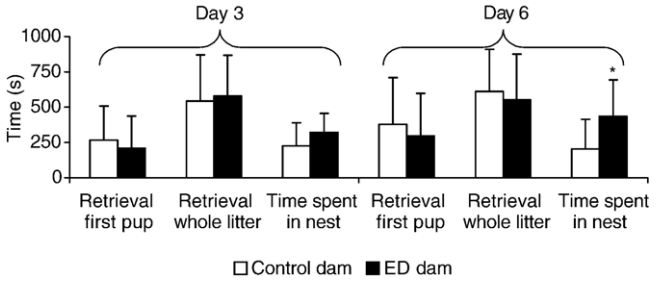

Fig. 2. Mean±SD values for measured parameters in the test of maternal retrieval of pups in control and experimental dams (ED dam; $n=12–19$/group). The test was conducted for 15 min on days 3 and 6, after the separation procedure, and at the corresponding time of the day for the control group. *$p<0.05$ vs. Control (Student's unpaired *t*-test).

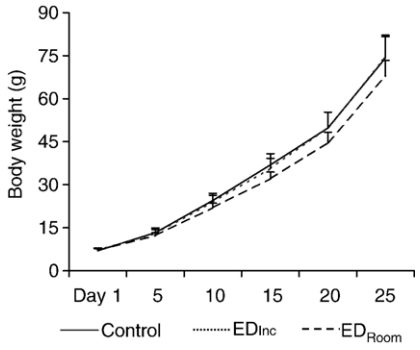

Fig. 3. Mean±SD values for body weight (g) on day 1, 5, 10, 15, 20 and 25 in controls, early deprived animals kept in an incubator (ED_{Inc}) and early deprived animals kept in room temperature (ED_{Room}; $n=16–17$/group).

t-test (two-tailed). Body weight measurements during early development were subjected to a 2 (group: ED_{Inc}/ED_{Room} vs. controls)×6 (age: day 1, 5, 10, 15, 20 vs. 25) ANOVA, and during adulthood to a 2 (group: ED_{Inc}/ED_{Room} vs. controls)×7 (age: day 60, 70, 80, 90, 110, 120 vs. 160) ANOVA, with repeated measurement on the second factor. Since Mauchly's Test of Sphericity indicated non-homogeneity of variance in the repeated measurements ANOVA, Greenhouse-Geisser epsilon corrected degrees of freedom was used to calculate the significance of the *F*-ratio. Moreover, since the two-way ANOVA was used twice for the data from the control group (in comparison to the ED_{Inc} and ED_{Room} groups), the level of significance (5%) was adjusted to 2.5% for each analysis. All other comparisons between the control and the two ED groups were, due to multiple comparisons, made with adjustment of the alpha level by the Bonferroni–Hochberg procedure (Hochberg, 1988). Data are presented as mean±SD.

3. Results

3.1. Maternal retrieval of pups

Data for two control dams and one dam of ED litters on day 3 were excluded in the statistical analyses due to new nest building, and on day 6 four of the dams of ED litters were excluded for the same reason. Furthermore, only data for litters containing eight pups were included in the statistical analyses for the parameters "time for retrieval of whole litter" and "time spent in the nest" (2–3 control litters and 4 ED litters were therefore excluded on both day 3 and 6). Analysis showed no significant differences between control and ED dams in time for retrieval of the first pup or of the whole litter to the nest on day 3 and 6 (Fig. 2). Comparison between the two groups of dams on day 6 showed that the dams of ED litters spent significantly more time in the nest with pups compared to control dams ($t=2.62$; $p<0.05$).

Table 1
Mean±SD values for the number of stretched attend postures (SAP), the number of lines crossed and the time spent in the outer exposed zone of the arena (s) in controls ($n=18$), early deprived animals kept in an incubator (ED_{Inc}; $n=18$) and early deprived animals kept in room temperature (ED_{Room}; $n=20$)

	No. of SAP	No. of crossings	Time in exposed zone (seconds)
Control	10.2±3.6	65.4±20.1	7.1±10.6
ED_{Inc}	12.2±5.4	63.6±20.3	7.3±13.7
ED_{Room}	15.3±5.0**	78.8±23.4	17.7±22.0

The test was performed on day 70.
**$p<0.01$ vs. Control (Student's unpaired *t*-test).

3.2. Postnatal development

3.2.1. Body weight

For the statistical analyses of body weights, only litters containing eight pups were included (three litters with 6–7 pups were excluded in both ED and control group). A two-way ANOVA for ED_{Inc} vs. control animals revealed a significant main effect for age (F [1.27, 39.45]=2411.50; $p<0.001$), no significant main effect for group or a group×age interaction (Fig. 3). The same analysis for ED_{Room} vs. control animals revealed a significant main effect for age (F [1.35, 41.75]=

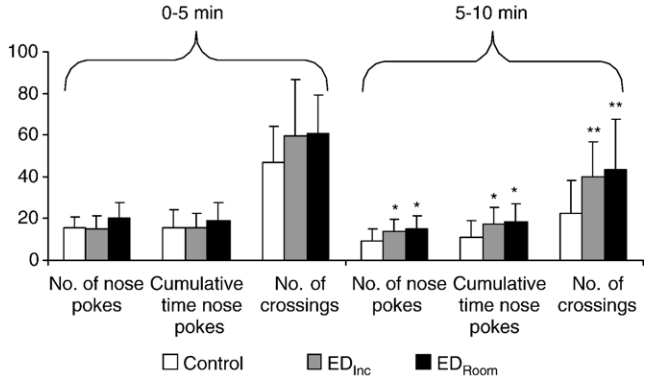

Fig. 4. Mean±SD values for number and cumulative time (s) of nose pokes and lines crossed in controls, early deprived animals kept in an incubator (ED_{Inc}) and early deprived animals kept in room temperature (ED_{Room}; $n=16–20$/group). The test was performed for 2×5 min and the animals were 60 days of age. *$p<0.05$ and **$p<0.01$ vs. Control (Student's unpaired *t*-test).

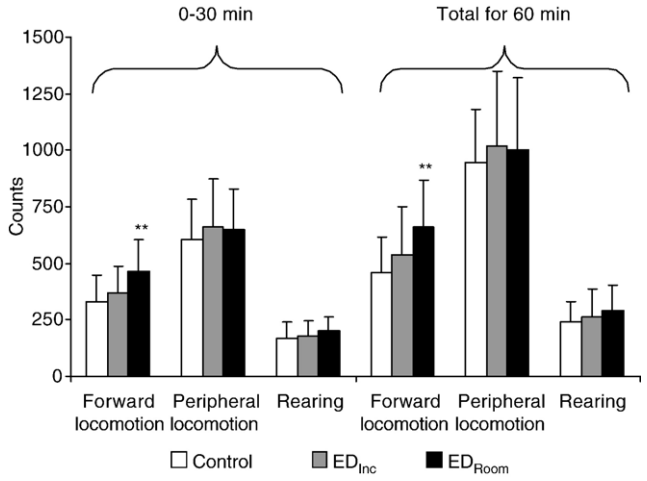

Fig. 5. Mean±SD values for counts in forward and peripheral locomotion and rearing (1 h) in controls, early deprived animals kept in an incubator (ED_{Inc}) and early deprived animals kept in room temperature (ED_{Room}; $n=18–20$/group). Animals were 90 days of age at the test occasion. **$p<0.01$ vs. Control (Student's unpaired *t*-test).

Table 2
Mean±SD values for alcohol preference (% of total fluid intake), alcohol intake (g/kg/day of absolute alcohol), water intake (ml/kg/day) and total fluid intake (ml/kg/day) during 3 weeks in controls (n=18), early deprived animals kept in an incubator (ED_{Inc}; n=18) and early deprived animals kept in room temperature (ED_{Room}; n=20)

	Alcohol preference	Alcohol intake	Water intake	Total fluid intake
Week 1				
Control	25.3±32.2	0.8±1.0	42.5±20.3	57.7±9.9
ED_{Inc}	17.0±13.8	0.6±0.7	47.7±12.3	59.4±17.3
ED_{Room}	17.5±13.9	0.5±0.4	45.6±11.0	55.4±8.5
Week 2				
Control	28.6±28.8	0.9±0.9	44.2±21.7	61.3±10.3
ED_{Inc}	19.5±15.1	0.5±0.4	47.8±17.2	58.6±14.2
MS_{Room}	25.2±22.7	0.8±0.7	46.9±19.1	62.2±13.1
Week 3				
Control	28.2±31.6	0.8±0.9	41.7±21.9	57.0±11.5
ED_{Inc}	21.7±19.3	0.6±0.7	45.5±18.1	58.0±17.5
ED_{Room}	24.5±21.4	0.7±0.6	41.8±14.4	55.3±10.9

Animals were about 100 days of age at start of the test.
There were no significant differences between controls and the ED groups (Student's unpaired t-test).

2774.15; $p<0.001$), for group (F [1, 31]=11.50; $p<0.01$) and a group×age interaction (F [1.35, 41.75]=8.09; $p<0.01$), indicating that ED_{Room} animals had a lower body weight, and the rate of weight gain was slower compared to controls.

3.3. Adult behavior (days 60–100)

3.3.1. Exploration

In the second period of testing, ED_{Inc} showed significantly more nose pokes (t=2.34; $p<0.05$) with a longer cumulative duration (s; t=2.47; $p<0.05$) and more crossings (t=3.07; $p<0.01$) compared to controls in the exploration test (Fig. 4). The same pattern was observed between ED_{Room} and control animals: during the second 5-min period ED_{Room} animals exhibited significantly more nose pokes (t=2.64; $p<0.05$), with a longer cumulative duration (s; t=2.71; $p<0.05$), and more crossings (t=2.94; $p<0.01$), relative to controls. No significant differences among the groups were observed in the first 5-min period of testing.

3.3.2. Risk assessment

ED_{Room} animals performed significantly more number of SAPs compared to controls (t=3.50; $p<0.01$; Table 1). There were no significant differences between ED_{Inc} and control animals.

3.3.3. Fleeing and freezing

There were no significant differences between controls and ED_{Inc} or ED_{Room} animals in fleeing (Control: 4.0±2.7, ED_{Inc}: 5.0±2.8 and ED_{Room}: 5.2±3.1 number of lines crossed) and freezing responses (Control: 52.6±69.0, ED_{Inc}: 106.9±184.4 and ED_{Room}: 120.5±234.9 s), or in number of fecal boli deposited (Control: 2.3±2.8, ED_{Inc}: 1.7±1.9 and ED_{Room}: 1.0±2.2; n=17–20/group).

Table 3
Mean±SD values (ng/g tissue) for monoamines and their metabolites in different brain areas on day 160 in controls, early deprived animals kept in an incubator (ED_{Inc}) and early deprived animals kept in room temperature (ED_{Room}; n=10–12/group)

	Monoamines			Metabolites		
	NA	DA	5-HT	DOPAC	HVA	5-HIAA
Basal forebrain						
Control	733.9±195.4	3299.8±590.3	1076.5±240.4	600.5±127.7	117.7±39.3	662.5±57.3
ED_{Inc}	716.3±138.2	3161.5±541.6	1011.6±120.5	604.9±127.0	142.5±64.6	656.7±80.6
ED_{Room}	664.5±174.6	3318.9±924.9	978.7±140.3	603.5±153.7	99.5±22.1	619.0±100.0
Dorsal striatum						
Control	165.0±94.4	6924.8±1033.5	495.0±68.2	1177.6±233.1	165.7±51.2	416.2±66.5
ED_{Inc}	151.7±42.7	6314.3±686.7	480.0±78.3	1061.1±137.4	141.1±31.2	385.2±38.3
ED_{Room}	152.0±36.4	6685.2±514.2	483.7±67.7	1160.4±248.9	155.1±38.9	396.3±67.5
Hippocampus						
Control	418.6±54.0	21.1±8.5	512.4±72.2	9.5±2.8	7.7±2.3	351.5±57.7
ED_{Inc}	410.2±33.0	20.1±11.5	511.6±64.9	8.4±2.3	8.2±2.0	347.1±36.9
ED_{Room}	398.2±31.0	15.6±3.8	499.5±96.4	7.2±2.1	7.7±3.1	332.5±74.3
Amygdala						
Control	484.8±48.1	840.7±229.4	878.8±67.1	104.3±28.6	21.6±5.0	426.0±58.4
ED_{Inc}	472.4±34.0	823.1±295.1	888.2±60.6	95.7±35.6	19.8±7.0	418.0±51.3
ED_{Room}	475.3±66.8	995.4±286.5	905.6±114.3	120.3±40.9	26.4±17.1	417.2±64.7
Remaining cortical tissue						
Control	260.0±28.6	210.9±140.8	371.6±56.5	36.2±14.0	38.7±11.4	149.2±31.5
ED_{Inc}	277.8±26.7	135.1±38.5	397.9±66.0	30.8±6.2	34.8±9.2	160.4±19.5
ED_{Room}	263.6±26.2	154.2±139.8	390.2±59.7	31.6±16.8	36.0±10.5	157.0±23.7

NA = noradrenaline; DA = dopamine; 5-HT = serotonin; DOPAC = 3,4-dihydroxyphenylacetic acid; HVA = homovanillic acid; 5-HIAA = 5-hydroxyindoleacetic acid.
There were no significant differences between controls and ED groups (Student's unpaired t-test).

3.3.4. Spontaneous locomotor activity

ED_{Room} animals showed significantly increased total forward locomotion (i.e. 60 min) compared to controls ($t=3.45$; $p<0.01$, Fig. 5). The increased locomotion in ED_{Room} animals was already present for the first 30 min of the test ($t=3.09$; $p<0.01$), and even for the first 15 min of the test (control: 200±100, ED_{Room}: 295±129; $t=2.54$; $p<0.02$). There were no significant differences in spontaneous locomotor activity between ED_{Inc} and control animals.

3.3.5. Voluntary alcohol intake

As seen in Table 2, there were no significant differences in any of the fluid parameters (i.e. alcohol preference, alcohol intake, water intake and total fluid intake) between the ED_{Inc} or ED_{Room} and control animals during the three-week alcohol consumption period.

3.4. Biological measurements

3.4.1. Adult body weight, thymus gland, adrenal glands and corticosterone levels

In adult animals, analysis of body weight in ED_{Inc} and control animals revealed a significant main effect for age (F [3.15, 107.21]=852.88; $p<0.001$), and no significant main effect for group or a group×age interaction between control and ED_{Inc} animals (data not shown). The same pattern was seen in ED_{Room} and control animals: a significant main effect for age (F [2.24, 80.79]=1034.18; $p<0.001$) was found, and no significant main effect for group or a group×age interaction (data not shown; $n=18$–20/group). At the time for decapitation, no significant differences in weight of the thymus gland (mg/g body weight) were found between ED_{Inc} (0.69±0.08; $n=18$) and control animals (0.74±0.15; $n=18$), or between ED_{Room} (0.74±0.15; $n=20$) and control animals. Nor were there any significant differences in the weight of the adrenal glands (mg/g body weight) between ED_{Inc} (0.12±0.02; $n=17$) and control animals (0.13±0.02; $n=17$), or between ED_{Room} (0.13±0.03; $n=20$) and control animals. No significant differences in plasma corticosterone levels (ng/ml) were found between ED_{Inc} (201±57; $n=18$) and control (236±88; $n=17$) animals, or between ED_{Room} (208±44; $n=20$) and control animals on day 160.

3.4.2. Brain monoamine levels

Biochemical analyses showed no significant differences between controls and ED_{Inc} or ED_{Room} groups in levels of brain monoamines and their metabolites (Table 3).

4. Discussion

The major finding in the present study was that early deprived pups showed significantly enhanced locomotion and exploratory behavior in a novel testing arena as adults (regardless of ambient temperature during the separation procedure) compared to briefly handled controls. In addition, the enhanced activity level in separated animals was even more pronounced in the group of animals that were kept in room temperature when isolated as pups. As adults, the ED_{Room} animals showed more SAPs when testing their risk assessment behavior. In addition, they showed an increased spontaneous forward locomotion when activity was measured for an extensive period of time (i.e. 60 min). Although no differences between ED and control animals were revealed in fleeing and freezing responses, alcohol intake or in the biological measurements, the reduced anxiety-like behaviors in both ED groups are noteworthy in the exploration and risk assessment tests and the locomotor activity.

Despite wide variations in use of protocols within the MS paradigm, the MS manipulation has been proposed to increase anxiety-related behaviors in adulthood (e.g. Caldji et al., 2000b; Daniels et al., 2004; Huot et al., 2001; Kalinichev et al., 2002; Wigger and Neumann, 1999). The present finding of reduced anxiety-related behaviors in early deprived animals is, however, in agreement with other MS studies (Kaneko et al., 1994; McIntosh et al., 1999; Ploj et al., 2002; Suárez et al., 2004). It should be noted that the control group in the present study was a briefly handled group (i.e. controls animals were briefly handled during the days when the ED manipulation was assessed). Although this group is not commonly used in the field, this group could be argued to be expected to yield results in favor of the hypotheses within the MS field (when assuming that the briefly handled group resembles the commonly used animal facility reared; AFR or handled group; H). In addition, the separation manipulation in the present study was assessed with isolated pups, which may be considered as a more severe stress experience than separation in litters. In this kind of experimental setting increased emotional behavior and increased basal corticosterone levels has been reported in ED animals in relation to briefly handled controls (Biagini et al., 1998). The increased emotionality in ED animals relative to briefly handled control was, however, not found in a study by Zimmerberg and Shartrand (1992). Similarities between these studies are, e.g. treatment of the control group, gender and isolation of pups in incubators during the MS-procedure. However, methodological procedures also differ between these studies, e.g. duration, timing and number of separations, testing apparatus and testing time.

When using the briefly handled control group and separating pups as litters, MS animals have also shown increased emotionality (Matthews et al., 1996b; von Hoersten et al., 1993). These findings are, however, contrasted by several reports where no significant changes among these groups were found (Biagini et al., 1998; Kaneko et al., 1994; Marmendal et al., 2004; Matthews et al., 1996a,b, 1999; von Hoersten et al., 1993), or even decreased anxiety in MS animals (Kaneko et al., 1994). Although the ED-manipulation could be seen as a more severe separation treatment than MS, results for ED in relation to the standard H and/or AFR groups on emotional responses and basal stress hormones have shown to not differ among the groups in adulthood (McIntosh et al., 1999; Pryce et al., 2001a, 2003). However, a depression-like state was reported in ED relative to H animals has been reported (reference to submitted manuscript in Rüedi-Bettschen et al., 2004b). Possible explanations to the diverse outcomes could be the use of different models for measuring aspects of anxiety-like/emotional behavior, as it is thought to be multidimensional and represented

by, for example, locomotor activity, anxiety, exploration, risk assessment and arousal (Archer, 1973; Ohl et al., 2001). These components are argued to be poorly defined and different models may cover different aspects of anxiety-like behavior (Archer, 1973; File, 1992; Ramos et al., 1997; Walsh and Cummins, 1976; Yilmazer-Hanke et al., 2004). For example, the open field is thought to induce moderate anxiety in the rat when exposed to the novel environment with no possibilities to escape. The anxiety is reduced if using the two-compartment exploratory test, where the rat has a safe enclosure, and therefore is proposed to predominantly reflect exploratory behavior (Dulawa et al., 1999). The strain of rat seem also be a factor to take into account in MS-studies (Ellenbroek and Cools, 2000; Neumann et al., 2005), and even sub-lines of the same rat strain may represent a source of variability in stress reactivity (Paré and Kluczynski, 1997), which must take into account when comparing results (e.g. between the present study and Marmendal et al., 2004 as different vendors were used). In addition, animals in the present study were repeatedly used in the behavioral tests, which may have influenced the behavioral outcome. Although this is an important concern to take into account, it could be argued that if only looking at the results for the initial behavioral test (i.e. exploration on day 60), both ED groups clearly show increased nose poking and crossings (Fig. 4) compared to controls. In an attempt to reduce the impact of repetitive testing with the same animals, the behavioral tests in the present study were spaced approximately 10 days apart. Furthermore, the behavioral tests employed might be considered as relatively mild stressors for the animals. Looking closer at the behavioral tests in the present study, the experimental groups' reduced emotionality was more pronounced in tests prominently measuring exploration, while not affecting fearfulness, e.g. in the freezing test. Exploration and fearfulness are aspects of emotionality, and it has been argued that they not always are negatively related (Archer, 1973).

Other possible contributing factors to the diverse results in MS/ED studies might be, for example, timing and number of separations, use of normal or reversed light/dark cycle, choice of rat strain and the age of the animals when tested as adults (Ellenbroek and Cools, 2000; Lehmann and Feldon, 2000; Pryce and Feldon, 2003). For example, assessing biological measures (e.g. corticosterone and monoamines) during the dark phase and in a non-stressful condition in the present investigation may have affected the results, as it has been shown that these factors may contribute significantly in experiments (Liu et al., 2000; Retana-Marquez et al., 2003). However, it has also been suggested that separations performed during the dark phase and at room temperature are a relevant MS-protocol for investigating impaired affective systems (Rüedi-Bettschen et al., 2005). In addition, in the present study animals were individually housed 1 day prior to decapitation, a factor that may have interfered with biological measurements. When increased alcohol intake has been reported in MS animals compared to handled or facility reared groups (Huot et al., 2001; Jaworski et al., 2005; Ploj et al., 2003; Roman et al., 2005), methodological factors differ between the studies which may have contributed to the results. For example, the use of sweetened alcohol fluid (Huot et al., 2001; Jaworski et al., 2005) and restraint stress during the alcohol testing period (Ploj et al., 2003), as well as a strain of alcohol-preferring rats (Roman et al., 2005).

According to the different MS protocols used in the literature, perhaps none of our protocols may be considered as an extreme form of deprivation in our laboratory conditions, and therefore did not cause enhanced anxiety in the separated animals. For example, it has been proposed that separations must exceed 6 h to constitute a severe experience in the rat pup (Kuhn and Schanberg, 1998). A potential protective factor for ED pups in the present study may have been an increased maternal care in ED dams. It has been proposed that increased maternal care behaviors, such as arched back nursing and licking/grooming, may provide a mechanism for resistance to later adversities in the offspring (Francis et al., 1996; Meaney, 2001). There is a possibility that dams of ED litters in the present study performed maternal care that was counteracting the effect of the deprivation episode, which in turn may have reduced behavioral fearfulness in separated animals later in adulthood. This may have occurred by increased maternal care in dams of ED litters, for example, due to receiving relatively cold pups to the home cage after the separation procedure, which has been reported in other studies (Rüedi-Bettschen et al., 2004a; Stern and Johnson, 1990). However, it has to be noted that maternal behavior in the present study was studied for a rather short time period and possible permanent MS induced alterations cannot be concluded.

It has been reported that single longer separations (12–24 h), or when the pup is repeatedly individually isolated for shorter periods (2–3 h), results in decreased weaning weights (reviewed in Lehmann and Feldon, 2000). The present study showed that isolated pups in room temperature displayed growth retardation during early ages and no differences among the groups were seen in adult ages. Lower body weight in early ages in MS animals have also been found by others, although separated animals sometimes still weighed less in adult ages (McIntosh et al., 1999; Zimmerberg and Shartrand, 1992). It is possible that body weight retardation in early ages in ED_{Room} animals in the present study were due to more energy spent on heat production while separated in room temperature (Jans et al., 1985; Stone et al., 1976). Early deprivation in room temperature clearly affected the pups, also in their behavioral outcome in adult ages. However, in the present study it is not possible to determine in what degree the results for the ED_{Room} group derives from cold stress only or a combination of cold stress and isolation.

In summary, the main finding in the present study was that ED animals showed decreased emotionality compared to brief handled controls. This was predominantly shown in measurements reflecting exploratory behavior. In addition, our studies have shown that methodological issues within the MS paradigm may be important influential factors for physical growth and behavioral changes in adulthood. When comparing our different separation protocols used (alternating housing conditions and ambient temperature during separation, Marmendal et al., 2004), while holding other factors constant, for example, duration, timing and number of separations, reversed light/dark

cycle, control group, animal husbandry procedures and some of the tests, it seems as these methodological issues may have influenced the outcome of our results. Although different sublines of Wistar rats were used in these studies, no significant behavioral differences were reported when holding maternally separated pups in intact litters in warmth during the separation procedure, while increased ambulation and exploration were seen in the present study (more pronounced in the ED_{Room} group). These results may reflect that our present separation protocol induces behavioral changes that may reflect an enhanced ability to cope with stressors. However, to further investigate the behavioral profile of early deprived animals (especially the ED_{Room} group), additional tests could be used, for example, other tests of anxiety, learning/memory, possible impulsive behavior and sensitivity to other drugs of abuse. Furthermore, the use of animals with a vulnerable genetic disposition and the use of additional stressors in adulthood (either acute or chronic) could be another way to further investigate possible interactive effects in maternally separated animals. This would constitute a proposed model for vulnerability to psychiatric illness in humans (Heim and Nemeroff, 2001).

Acknowledgements

We are indebted to Mrs. Birgit Linder for excellent technical assistance. This study was supported by grants from the Alcohol Research Council of the Swedish Alcohol Retailing Monopoly (00/4:3), Adlerbertska forskningsstiftelsen, Rådman och fru Ernst Collianders stiftelse för välgörande ändamål, Stiftelsen Goljes Minne, Helge Ax:son Johnsons Stiftelse and Neranders fond psykiatri.

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