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Environmental conditions influence hippocampus-dependent behaviours and brain levels of amyloid precursor protein in rats

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Abstract

Sprague-Dawley rats were reared in enriched (EC; group housing, exposure to stimulating objects, frequent handling) or restricted (RC; individual housing, no exposure to stimulating objects, minimal handling) environments starting on day 23 of life. At six months of age, they underwent behavioural tests to assess 'cognitive' and 'stimulus-response' memory, selective attention, and inflammatory pain processing. Alterations in synapses and cell survival may occur as a result of environment differences; therefore we assessed the brain levels of several proteins implicated in neurite outgrowth, synaptogenesis, and cell survival. Brains were dissected and analysed for amyloid precursor protein (APP) and other synaptic and cytoskeletal proteins using Western blotting. The performance of EC animals in a hidden platform water maze task, and in a test of selective attention (both of which are thought to involve the hippocampus) was superior to that of RC animals. In contrast, performance of RC animals on two stimulus-response tasks, the visible platform water maze test and simple visual discrimination (both of which are thought to be hippocampal independent) was indistinguishable from that of EC animals. Male EC rats displayed a different behavioural response to formalin during the inflammatory phase of nociception – the phase affected by hippocampal processing; a similar trend was observed in females. Female but not male RC rats exhibited elevated plasma corticosterone levels; adrenal weights were unaffected by environmental conditions. Region-specific increases in brain levels of APP, neurofilament-70 (NF-70), and platelet-activating factor receptor (PAF-R) were found in EC rats. These data suggest that enriched animals manifest enhanced functioning of certain hippocampus-mediated behaviours when compared with that of their restricted counterparts; and that brain levels of various synaptic and structural proteins involved in neurite outgrowth, cell survival, and synaptogenesis, are affected by environmental factors.

Introduction

The environmental conditions under which rats are reared are known to affect learning and memory performance (Hebb, 1949). Environmental enrichment or restriction can also affect cerebral size, neuron size, number of glial cells, dendritic branching, spine density, and number of synapses (e.g. Bennett *et al*., 1964; Rosenzweig *et al*., 1971; Volkmar & Greenough, 1972; West & Greenough, 1972; Globus *et al*., 1973; Diamond *et al*., 1975; Katz & Davies, 1984), as well as the turnover of several neurotransmitters (Jones *et al*., 1992; Myhrer *et al*., 1992; Fulford *et al*., 1994; Escorihuela *et al*., 1995). Little is known concerning the mechanisms that underlie these structural and chemical effects.

Stress, and the hormones which are released as a consequence, have been implicated as factors mediating some of the neurological and behavioural consequences of raising animals in different environments (Meaney *et al*., 1991). It has been suggested that enrichment decreases the chronic stress response, ultimately attenuating release of the potentially neurotoxic glucocortoicoid, corticosterone (Devenport et al., 1992). The influence of stress and stress-related mediators on learning and memory is well known (for a review, see Roozendaal 2000). Hippocampal-dependent functions are particularly sensitive to stress and adrenal hormones (Bodnoff *et al*., 1995; de Quervain *et al*., 1998), possibly due to the pronounced concentration of glucocorticoid receptors in the hippocampus (Chao *et al*., 1989; Agarwal *et al*., 1993). Interestingly, hippocampal-dependent forms of learning and memory are also especially affected by environmental conditions (Juraska & Muller, 1984; Nilsson *et al*., 1993; Kempermann *et al*., 1997; Duffy *et al*. 2001).

In this study, we examined the effects of long-term environmental enrichment on behavioural tests believed to measure hippocampal- and striatal-dependent learning and memory processes; selective attention; and inflammatory pain processing. We also determined whether the environmental conditions influenced adrenocortical stress responses by measuring plasma corticosterone levels 90 mins after formalin injection. Weaned male and female Sprague-Dawley rats were exposed to enriched or restricted environmental conditions (EC and RC, respectively) for six months, after which behavioural training and testing ensued. Memory was assessed using a hidden platform version (i.e. a 'cognitive' memory task), and a visible platform version (i.e. a 'stimulus-response' [S-R] task), of the Morris

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water maze; the former is thought to be mediated by the hippocampus and the latter by the dorsal striatum (Packard *et al*., 1989; Packard & McGaugh, 1992; Packard & Teather, 1997, 1998). Subsequently, rats underwent training in another S-R task, simple visual discrimination in a Y-maze. Following acquisition of this task, a test of selective attention was implemented using the 'blocking paradigm'. Disruption of blocking (i.e. the ability to ignore uninformative information) occurs in animals with hippocampal lesions (Rickert *et al*., 1978, 1981; Gallo & Candido, 1995; but also see Garrud *et al*., 1984) or after removal of the cholinergic input to the hippocampus (Baxter *et al*., 1997). Finally, rats were administered a formalin test, a behavioural model of inflammatory pain processing (Dubuisson & Dennis, 1977), to assess whether environmental conditions influence nociceptive responses. It has been suggested that the hippocampus is involved in the processing of pain-related information (McKenna & Melzack, 1992; Wei *et al*. 2000).

To identify possible molecular and cellular bases for the behavioural effects of the EC or RC environments, we measured the levels of several proteins in hippocampus, striatum, cerebellum and frontal cortex using Western blotting. These included synaptic proteins (synaptophysin, APP, synuclein-1 and -2, synapsin-1, and synphillin); cytoskeletal proteins (the light, medium and heavy neurofilaments [NF-L, NF-M, NF-H], glial fibrillary acidic protein), and memory- and stress-related proteins (cyclooxygenase-2, nitric oxide synthase-2, platelet-activating factor receptor [PAF-R]). We elected to evaluate synaptic proteins, such as APP and synapsin-1, as the number of synapses may increase as a result of enrichment (Turner & Greenough, 1985). Moreover, APP has been shown to promote cell survival, neurite outgrowth and synaptogenesis in neurons. The neural cytoskeletal proteins – the heavy, medium, and light neurofilaments – were examined to further previous work that revealed cytoskeletal alterations as a function of environment (i.e. microtubule increase in enriched rats; Jorgensen & Meier, 1979). It has been suggested that a restrictive environment may induce a state of chronic stress (for a review, see Uphouse, 1980), therefore we assessed the levels of NOS-2, which has been shown to be increased in chronically stressed rats (Lopez-Figueroa *et al*., 1998). Finally, both PAF (acting at the PAF-R) and COX-2 have previously been shown to be involved in hippocampal-dependent memory processing (Teather *et al*. 2001, 2002).

Materials and methods

The following experiments were carried out under a protocol approved by the Institutional Animal Care Committee of the Massachusetts Institute of Technology, in accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimise the number of animals used.

Chemicals

All chemicals used in the present experiments were purchased from Sigma (St. Louis, MO, USA), with the exception of Hank's buffered saline solution (Gibco-Life Technologies; Rockville, MD, USA).

Animals

Two pregnant Sprague-Dawley rats were obtained (Charles River, MA, USA) one week prior to giving birth. At postnatal (PN) day 23, pups were removed, and sexes were separated into small groups and allowed to acclimatise for one week. At this time, pups were assigned to either enriched (EC; $n = 9$; 5 males, 4 females) or restricted (RC; $n = 9$; 5 males, 4 females) conditions. Approximately half of each litter was assigned to each condition, and rats were paired according to body weight (at PN day 30). Rats were exposed to these differential rearing conditions for six months. Two RC rats were eliminated from the study due to tumour development (mammary tumour in one female, 5 months of age; testicular tumour in one male, 4.5 months of age). One EC female rat was eliminated (2 months of age) due to insufficient body weight gain and overall unhealthy appearance. No tumours were detected in this rat.

Rearing conditions

All rats were housed in the same rack in plastic cages ($52 \times 32 \times 20$ cm high) with wire lids. Bedding and water were regularly changed, and animals were weighed every 2 weeks, at which time general health assessments were made. Animals had *ad libitum* access to laboratory chow and water until two weeks before the onset of training in an appetitive discrimination task. EC rats were initially housed in groups of 4–5 (same-sex). Group size was decreased as animal size increased (to decrease overcrowding stress). Specifically, after 2 months of enrichment in groups of 4–5, the groups were divided into groups of 2–3. Plastic toys (blocks, balls, cylinders, etc.) placed in the EC cages were rotated between groups weekly; new toys were introduced monthly. EC rats were taken to a 'playroom' (12×6 feet; containing filing cabinets, desks, chairs, boxes and toys) every other day and allowed same-sex contact for 30–45 min. The RC rats were housed individually, without toys, and handled two times a month. To avoid the typical weight gain caused by a sedentary lifestyle (relative to enriched rats), rats were allowed to exercise three times per week in an empty 4×6 ft room. One month prior to behavioural training, RC rats were handled twice weekly in order to alleviate fear and anxiety in subsequent behavioural training procedures. All animals were housed in the same room under standard laboratory conditions on a 12-h light-dark cycle (light from 7:00 to 19:00 h).

Behavioural tests

General water maze apparatus

The water maze was a galvanized circular tank 6 ft (1.83 m) in diameter and 1.5 ft (0.55 m) in height, filled with water (25 ± 2 °C) to a depth of 20 cm and located in a room containing several extramaze cues. Four starting positions (north, south, east and west) were spaced around the perimeter of the tank, dividing the pool into four equal quadrants. The rectangular Plexiglas® escape platform used for the hidden platform task ($11 \times 14 \times 19$ cm) was submerged at a depth of 1 cm. For the visible platform version of the water maze, a white rubber ball (8 cm in diameter), attached to the top of the submerged platform, protruded above the water surface. The platform could be used as a step to mount the ball to escape the water. All water maze procedures were videotaped for further analyses. Behavioural training was carried out between 10:00 and 14:00 h.

Hidden platform water maze task

Rats initially received four training sessions each consisting of four trials (i.e. swims) in the hidden platform water maze task. On each trial the animal was placed into the tank facing the wall at one of four designated start points (N, S, E or W) and allowed to escape onto the hidden platform. The submerged platform was located in the same quadrant on every trial. A different starting point was used on each of the four daily trials, and the order of starting points was quasi-random, but constant for all animals. If an animal did not escape

within 90 s, it was manually guided to the escape platform by the experimenter. After mounting the platform, rats remained on it for 20 s. After each trial, animals were removed from the maze and placed in a holding cage for a 60-s intertrial interval (ITI). The latency to mount the escape platform was recorded and used as a measure of task acquisition.

On the fifth day, animals were given a 60-s probe test to assess their spatial skills. For this test, the platform was removed and the amount of time each animal spent in the four quadrants was measured.

Visible platform water maze task

Rats received two training sessions, each consisting of four trials (i.e. swims). On each trial the animal was placed into the tank facing the wall at one of four designated start points (N, S, E or W), and allowed to escape onto the visibly cued platform. A different starting point was used for each trial, the order of which was quasi-random and the same for all animals. If an animal did not escape within 90 s, it was manually guided to the escape platform by the experimenter. After mounting the platform, rats remained on the platform for 20 s. After each trial, animals were removed from the maze and placed in a holding cage for a 60-s ITI. The latency to mount the escape platform was recorded and used as a measure of task acquisition.

The visible escape platform was placed in a different quadrant on each of the four trials. The locations of the start points were arranged so that the distance to the platform (i.e. proximal or distal) and the location of the platform relative to the start point (i.e. left or right) were counterbalanced across the four trials to control for turning preferences.

Hidden platform water maze retest

As the small sample size did not allow for counterbalancing the order in which the hidden and visible platform tasks were performed (i.e. which task was performed first), and as the order of tasks may influence performance on subsequent tasks, rats were re-trained in the hidden platform task, following training in the visible platform task, to assess whether any initial impairments in cognitive memory performance would be manifest after extensive experience in the water maze. Thus, following training in the visible platform task, animals underwent a second training session in the hidden platform task, with the platform located in the quandrant located diagonal to the original platform location. Animals underwent 4 training trials per day for two days. Animals that had had prior water maze experience reached a retention latency criterion (mean of 10–20 s across 4 trials) within 2 days; in contrast, on initial testing, they required 4 days of training. A second probe trial was carried out on the third day.

Simple visual discrimination task

Training and testing of visual discrimination was performed in a grey Y-maze, consisting of a goal and start box separated by a run area. All walls of the apparatus were 25 cm high, and the maze was placed in a well-lit room. The start box (30 cm long x 15 cm wide) was divided from the rest of the apparatus by a guillotine door. The distance from the door to the choice point (a dividing wall containing two 10×15 cm Plexiglas® doors separated by 7 cm) was 30 cm. The trap doors were equipped with counterweights which permitted gradual closing of the doors to prevent re-entry into the run area. The discriminada were white (stimulus B) and black (stimulus A) cue cards affixed in the doors; the black cue card was always the positive discriminadum. The location (i.e. right or left door) of the positive discriminadum for each training trial was decided according to a pseudorandomised series with the only restrictions being that the positive cue was located in each side an equal number of times per day, and that the positive cue could not be placed in the same door for more than two consecutive trials (to prevent positional preference).

Animals were food restricted for approximately 10 days prior to the onset of the discriminatory training in the modified Y-maze task, to achieve a body weight of 85% of their original weight (at the beginning of food restriction). Animals were habituated to the modified Y-maze training apparatus for three days prior to the onset of training. The first day of habituation consisted of allowing animals to explore the maze apparatus for 15 min with food (FruitLoops™, Kelloggs, IL, USA) scattered around the goal box. The Plexiglas® doors to the goal box contained neutral grey cue cards and were propped open. On the second day of habituation, animals were acclimatized to the trap door by placing the rats in the start box and removing the trap door after a 20-s delay. If an animal did not leave the start box within 60 s, it was gently guided out, and the trap door was closed to prevent re-entry. Fruit loops™ were scattered around the goal box, and the goal box doors were left open with neutral grey cue cards placed in them. On the third day of habituation the trap door was again used, and the goal box doors containing neutral grey cue cards were left ajar. To enter the goal box, animals had to push open the goal box doors. Three days of habituation were required because few rats ate the FruitLoops™ on the first two days, in spite of food restriction. Most rats sampled the food on the third day of habituation, indicating acclimatization to the maze apparatus.

Behavioural training began on day four. At this time discriminada were placed in the doors. Following either a correct or incorrect response, animals were placed in a holding cage for a 120-s ITI. Entry into the door containing the positive cue card was considered a correct response. For each correct response, animals were allowed a small piece of Fruit loop™. An attempt to enter the locked door containing the negative stimulus was considered an incorrect response. When animals performed an incorrect selection, they were immediately removed from the maze and placed in the holding cage prior to the onset of the next trial. Ten training trials were given per day until animals satisfied the criterion of 8 out of 10 correct selections.

Blocking paradigm (compound stimulus training)

After criterion was reached in the discriminatory Y-maze task (i.e. rats trained to associate food reward with stimulus A), the compound training procedure began. At this time, two novel stimuli (a gold bead or two purple beads, stimuli X and Y, respectively) were presented simultaneously with stimuli A and B. Stimulus X was compounded with stimulus A (AX compound paired with food reward) and stimulus Y was compounded with stimulus B (BY compound, or negative compound). In theory (MacKintosh, 1995), A is already an effective reward predictor (from previous training) and learning about stimulus X should be 'blocked' as it provides no new information pertaining to reward. If animals have the ability to block out irrelevant stimuli they will pay little attention to added cues that do not add new information to the task at hand, as selection of the original positive cue will always result in food reward. After the compound cues were added, animals were given 10 training trials for three days, after which the original discriminada (i.e. black and white cue cards) remained and the compounded cues (i.e. beads) were removed. Rats were given 10 training trials for one day to assess retention of the positive stimulus A. This savings test was designed to examine whether the addition of the irrelevant information impaired memory for the relevant stimulus.

Formalin test

Following behavioural testing, rats were allowed access to food *ad libitum* for two weeks, after which time they were run in the formalin test (Dubuisson & Dennis, 1977). Animals were placed in a clear Plexiglas®· formalin test box (30 × 30 × 30 cm), with a mirror positioned at a 45° angle below the floor allowing for unobstructed observation of the animal's paw. Following a 10-min habituation period, animals were removed from the formalin box at which time 50 μL of 1% formalin was injected subcutaneously (s.c.) into the plantar surface of the right hind paw with a 27-gauge needle. The amount of time the rats elevated the injected paw was recorded in five-min intervals during a 70-min period following formalin injection.

The 70-min formalin test produces a biphasic response resulting in an 'early phase' which lasts approximately 5 min (which we will refer to as phase 1), and an 'inflammatory phase' (15–70 min) which may be divided into early and later components, which we will refer to as phase 2. Typically animals elevate their paws following injection (i.e. phase 1) followed by a reduction in paw elevation. Approximately 15–20 min after injection, the dynamic inflammatory phase begins and animals again elevate their paws to varying degrees for the remainder of the testing period. The amount of time animals elevate their paws is used as a behavioural measure of pain.

Harvesting of adrenal glands and blood for corticosterone assay

Immediately following the formalin test, animals were killed with an overdose of sodium pentobarbital (100 mg/kg), and their right-sided adrenal glands were removed and weighed. Trunk blood was collected in polyethylene tubes containing ethylenediamine tetracetic acid (EDTA), and centrifuged at 1200 *g* at 4 °C to remove blood cells and platelets; the supernatant fluid was frozen at –80 °C and used later to determine total (free plus bound) plasma corticosterone levels (ICM Diagnostics, Costa Mesa, CA, USA) as per manufacturers instructions.

Brain dissections

Brains were dissected on a chilled dissection board. A coronal brain matrix (adult rat, 200–500 g; Vibratome, St Loius, MO, USA) was used to produce blocks for dissection. Whole cerebellum, dorsal striatum, and dorsal hippocampus were taken for the study; as much of the frontal cortex was collected as possible using the coordinates and landmarks as illustrated in Paxinos & Watson (1986). The samples were snap-frozen in Eppendorf tubes placed in liquid nitrogen and stored at –80 °C until protein analyses were carried out.

Western blotting

Tissue samples were thawed, lysed, briefly sonicated, and centrifuged at 14000 *g* for 30 min at 4 °C. The lysis buffer (1X) contained Hank's balanced salt solution (HBSS; without MgCl, CaCl, $MgSO_4$, and phenol red), 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/mL aprotinin, 15 μg/mL leupeptin, 2.5 mg/mL Pepstatin A, 1% *n*-dodecyl-*D*-maltoside. Aliquots of the soluble supernatant fluid were used or frozen for later use at –80 °C. The insoluble pellet (crude membrane fraction) was resuspended in a more stringent 3X lysis buffer containing 50 mM Tris-HCL, pH 8.0, 150 mM NaCl, 0.02% sodium azide; 0.1% sodium dodecyl sulfate (SDS), 1% nonidet-40, 0.5% sodium deoxycholate, 0.1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 10 μg/mL aprotinin, 15 μg/mL leupeptin, 2.5 mg/mL Pepstatin A, 50 mM NaF, 1 mM sodium orthovanadate. This suspension was briefly sonicated, and centrifuged at 14000 *g* for 30 min at 4 °C. Protein assays were carried out using the BCA assay (Pierce; Illinois, USA), according to manufacturer's instructions. Soluble and insoluble homogenates (15–40 μg) from the individual brain regions were subjected to electrophoresis on a 4–15% acrylamide gel under reducing conditions. Proteins were transferred to a polyvinylidine difluoride (PVDF) membrane, blocked with Chemiblock (Chemicon, St. Lois, MO, USA) solution for 1 h, and incubated with primary antibody/Chemiblock solution overnight at room temperature on an orbital shaker. Blots were incubated with various antibodies against the proteins of interest. These included APP (1 : 4000; Boehringer Mannheim; Indianapolis, IN, USA), synaptophysin, synuclein-1 (1 : 3000, and 1 : 1000, respectively; Transduction Laboratories, Lexington, KY, USA), synapsin-1, NF-145, NF-70 (1 : 1000, 1 : 2000, 1 : 1000, respectively; Calbiochem, La Jolla, CA, USA), N-200 and glial fibrillary acidic protein (GFAP) (1 : 2000 and 1 : 3000, respectively; Sigma, St. Louis, MO, USA), COX-2, NOS-2, and PAF-R (all 1 : 1000; Santa Cruz; Ann Arbor, MI, USA). Following three rinses in TBS with 1% Triton (TBST), blots were incubated in HRP-conjugated secondary antibodies for 60 min, and then developed using ECL reagent (Santa Cruz; Ann Arbor, MI, USA). Bands were analysed using densitometric software (NIH V.1.61).

Data analyses

The statistical significance of differences in various measures associated with exposure to the EC or RC environments was analysed by unpaired Student's *t*-tests or analysis of variance (ANOVA) with repeated measures where appropriate. Scheffe's posthoc analyses were then carried out when significant differences were demonstrated. Data were initially analysed using sex as a factor; if no gender effect was found, male and female data were pooled.

Results

Effect of environment on adrenal and body weights and corticosterone levels

Environment (EC vs. RC) failed to affect body weight significantly (RC rats weighed 2–4% more than EC rats on average; data not shown). This suggests that allowing RC rats to exercise prevents the body weight differences commonly found in restricted/enriched studies. As anticipated, males weighed significantly more than females ($F_{1,13} = 48.356$, $P < 0.0001$; ANOVA). Environment also failed to affect wet adrenal weight (data not shown). Adrenals of

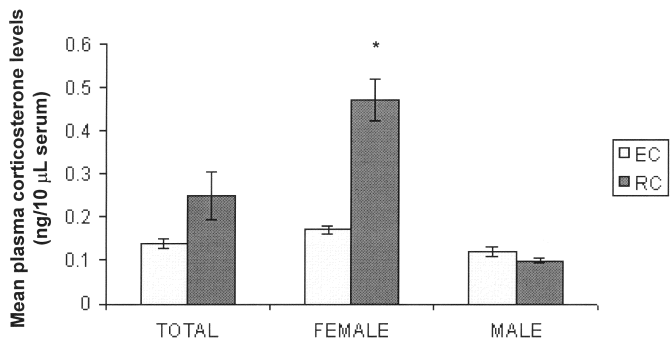

FIG. 1. Effect of 6 months of environmental enrichment or restriction on plasma corticosterone levels. Samples were harvested 90 min after injection of formalin into the hindpaw. Values are means ± SEMs of 4–5 animals per group. *$P < 0.05$ vs. restricted group of the same sex (posthoc *t*-test).

females were significantly larger than those of males, for both environments ($F_{1,13} = 26.35$, $P < 0.001$; ANOVA).

Main effects of environment ($F_{1,13} = 9.645$, $P < 0.01$) and sex ($F_{1,13} = 13.3$, $P < 0.005$) on plasma corticosterone levels, as well as a sex-by–environment interaction ($F_{1,1} = 6.68$, $P < 0.05$) were revealed by ANOVA analysis. (Fig. 1). Post-hoc analysis indicated that female rats had significantly higher corticosterone levels (90 min after formalin) compared to males ($P < 0.01$); RC females had significantly higher corticosterone levels compared to EC females ($P < 0.001$), suggesting that female RC rats have an enhanced stress response relative to EC females.

Effect of environment on performance on a hidden platform water maze task

Both EC and RC rats improved over the course of training in the hidden platform water maze task (Fig. 2A), as evidenced by a significant trial block effect ($F_{1,3} = 11.284$, $P < 0.001$; ANOVA). Animals raised in a restricted environment exhibited higher mean retention latencies than enriched rats, during acquisition of a hidden platform water maze task ($F_{1,13} = 15.483$, $P < 0.002$). Post-hoc analysis revealed that EC rats performed significantly better than RC rats on all four days of hidden platform training ($P < 0.05$)

Both RC and EC rats used a spatial strategy when searching for the escape platform during the probe trial, as they spent more time in the quadrant that formerly contained the platform ($F_{1,3} = 17.5$, $P < 0.001$; ANOVA). However, enriched rats spent significantly longer than restricted rats in the correct quadrant ($F_{1,13} = 9.266$, $P < 0.01$; Fig. 2B), indicating that EC rats had superior spatial memory retention. The number of times EC rats swam through the quadrant where the platform had been located (i.e. platform crossings) was also significantly higher (data not shown).

Effect of environment on performance on a visible platform water maze task

Both EC and RC rats were better able to locate the visible platform on the second than the first training day (Fig. 3), as a significant trial block effect was detected ($F_{1,1} = 14.195$, $P < 0.001$). EC and RC rats exhibited acquisition of the visible platform water maze task, indicating that environment failed to affect performance of this S-R task.

It should be noted that on the initial trials (i.e. trials 1 and 2 on the first day of training), EC rats displayed a ‘spatial hangover’ or perseveration effect in that they searched in the quadrant that had

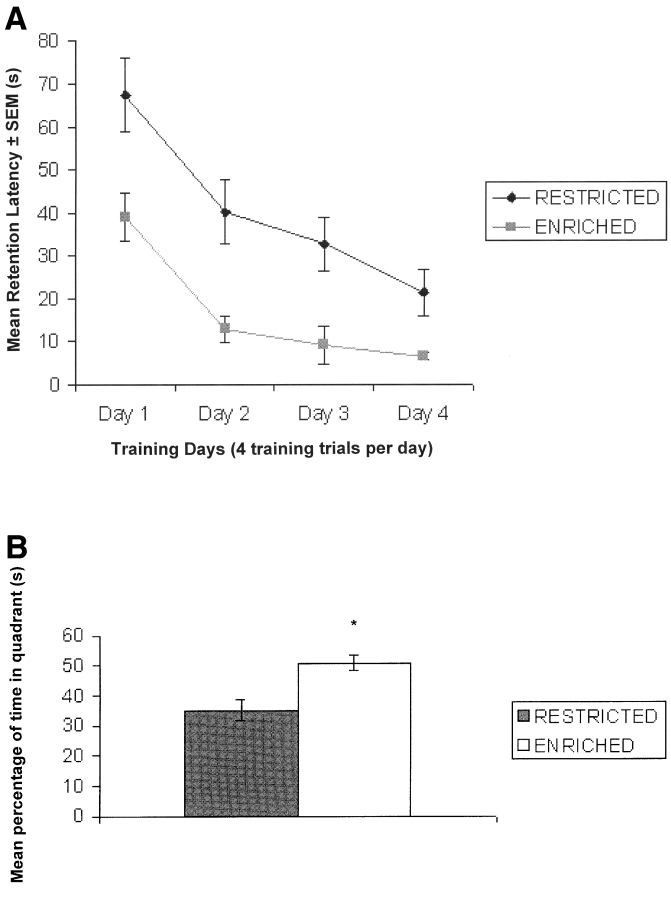

FIG. 2. Effect of 6 months of environmental enrichment or restriction on (A) acquisition of the hidden platform water maze task, and (B) time spent searching the quadrant that previously held the platform during the 60 s probe test. RC rats differed from EC rats on each day of task acquisition and spent less time in the quadrant that had previously contained the platform. Data are represented as means (s) ± SEMs (retention latencies or mean percentage time for A and B, respectively) of 7–8 animals (male and female data were combined as no effect of sex was found). *$P < 0.05$ vs. restricted group (Fischer’s PLSD posthoc).

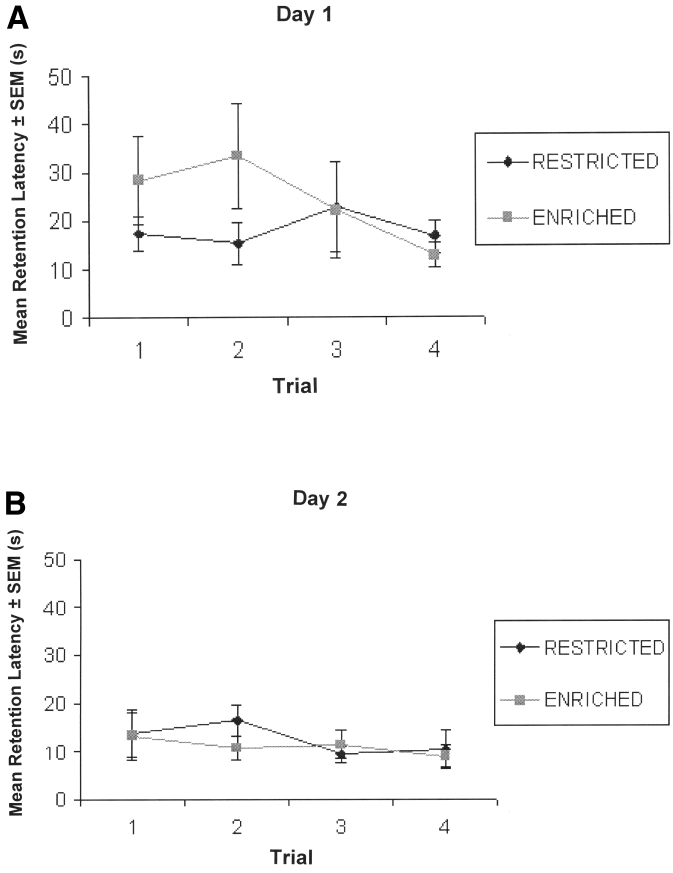

FIG. 3. Effect of 6 months of environmental enrichment or restriction on acquisition of the visible platform water maze task. Data are represented as training trial means (s) ± SEMs (retention latencies) of 7–8 animals (male and female data were combined as no effect of sex was found) for 2 days of training. Most EC rats perform poorly on the initial 2 trials of day 1 as they continued to approach the original location of the hidden platform. However, they readily acquired the visible platform task after these initial attempts proved to be unsuccessful.

previously contained the hidden platform from previous training (Fig. 3), attesting to the EC rats' superior cognitive memory performance from prior experience with the hidden platform task. By trial 3 of day 1, however, EC rats readily mastered the S-R rules required for visible platform acquisition.

Effect of environment on performance on a hidden-platform water maze task retest

As EC and RC rats acquired the visible platform task so readily, we assessed acquisition of another hidden platform task with the platform located in a different location. Prior water maze experience benefited both EC and RC rats, as both readily acquired the second hidden platform task within two days of training (Fig. 4A). Both RC and EC rats improved across the two training days as shown by the significant trial block effect ($F_{1,1} = 14.195$, $P < 0.001$; ANOVA). Animals raised in an enriched environment exhibited significantly lower retention latencies ($F_{1,13} = 7.076$, $P < 0.02$).

The results of a 60-s probe test confirmed that EC rats spent more time in the quadrant that previously held the hidden platform than did their RC counterparts ($F_{1,13} = 8.51$, $P < 0.01$; Fig. 4B), indicating that EC rats had superior spatial memory for the platform location, even after extensive experience in the water maze.

Effect of environment on performance on a visual discrimination task

Environment failed to affect acquisition of an appetitive visual discrimination task, when the mean numbers of days to criterion were compared (Fig. 5A; $P = 0.1922$). The numbers of errors (summation of the total number of errors to criterion) committed by RC and EC rats did not differ (data not shown).

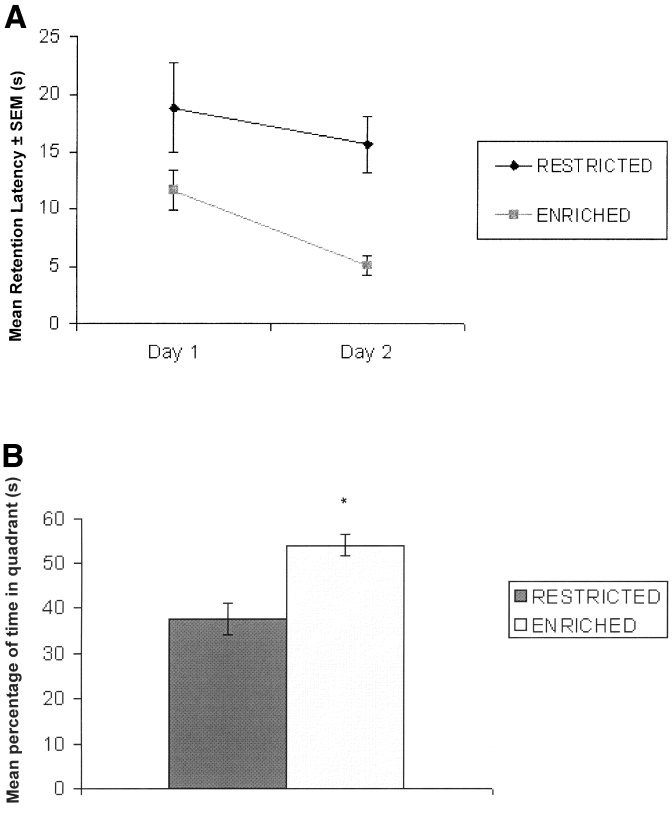

FIG. 4. Effect of 6 months of environmental enrichment or restriction on (A) acquisition of a second hidden platform water maze task, and (B) time spent searching the quadrant that previously held the platform during the 60 s probe test. Data are represented as means (s) ± SEMs (retention latencies or mean percentage time for A and B, respectively) of 7–8 animals (male and female data were combined as no effect of sex was found). *$P < 0.05$ vs. restricted group (Fischer's PLSD posthoc).

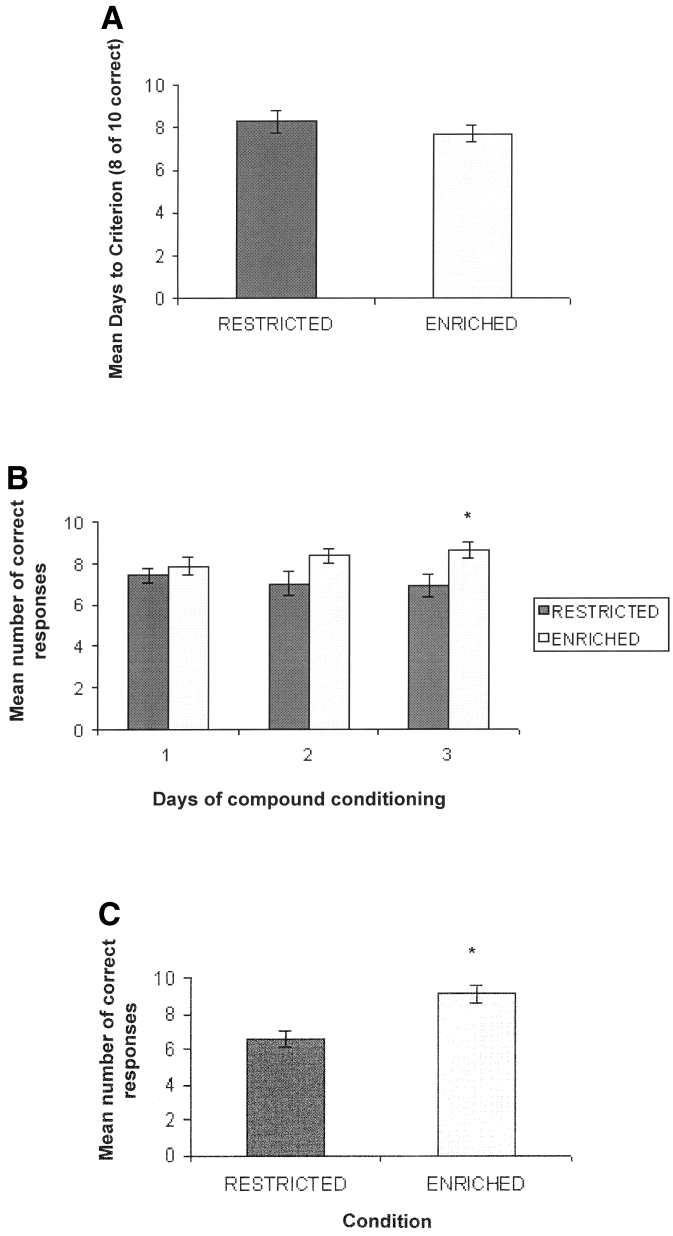

FIG. 5. Effect of 6 months of environmental enrichment or restriction on (A) acquisition of the visual discrimination task in the modified Y-maze, on (B) compound conditioning (i.e. introduction of irrelevant cue to the already trained relevant cue discrimination), and on (C) savings of original relevant cue learning after compound conditioning. Data are represented as means (s) ± SEMs (mean days to criterion of 8 out of 10 correct or mean number of correct responses out of 10) of 7–8 animals (male and female data were combined as no effect of sex was found). *$P < 0.05$ vs. restricted group (Fischer's PLSD posthoc).

Effect of environment on performance on a blocking test

Addition of an irrelevant cue to the previously learned cue in the discrimination task significantly impaired RC but not EC rat performance ($F = 4.944$, $P < 0.05$; ANOVA). Post-hoc analysis indicated a significant difference between the RC and EC groups in the number of correct choices on days 2 and 3 of compound conditioning (Fig. 5B) suggesting that RC rats were confused by the addition of the compound cues. A savings test for the original positive discriminada (i.e. black cue card) indicated that EC rats retained more savings for the positive stimulus (i.e. relevant cue) ($P < 0.05$; Fig. 5C). These data suggest that RC rats are more distracted by the addition of the irrelevant cues, and that memory for the relevant information is impeded as a result.

Effect of environment on inflammatory nociception-related behaviour

In general, nociceptive responses of female rats were greater than those of males, as illustrated by a significant main effect of sex ($F_{1,13} = 5.45$, $P < 0.05$; ANOVA). Hence, individual *t*-tests were carried out to compare the behavioural responses of male and female EC and RC groups during each phase of the formalin test. No effects of environment were observed during phase 1 (initial 10 min postformalin injection) (Fig. 6A), however, in the initial 50 min of phase 2 (10–60 min postinjection), EC males displayed increased nociceptive responses, as evidenced by increased paw elevation times ($P < 0.05$; Fig. 6B). Similar behaviour was observed in female rats, however, this trend did not attain statistical significance ($P = 0.11$). During the final 10 min of phase 2 (60–70 min postformalin injection), RC males displayed greater paw elevation times then their EC counterparts ($P < 0.05$; Fig. 6C). Female rats displayed a similar but not significant tendency ($P = 0.085$).

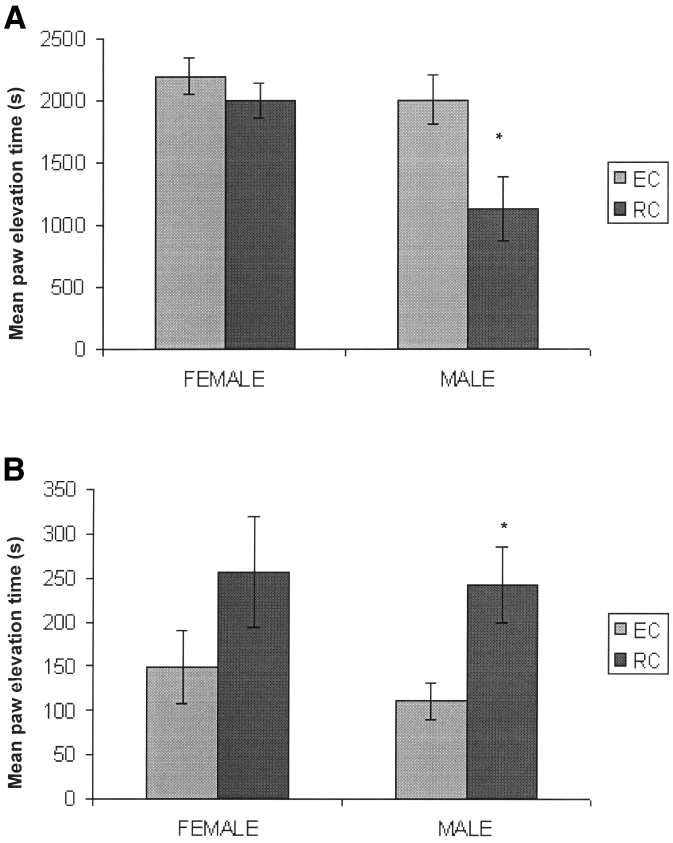

FIG. 6. Effect of 6 months of environmental enrichment or restriction on (A) nocieptive responses during the initial 50 min (10–60 min postformalin injection) of the phase 2 of the formalin test, and on (B) nocieptive responses during the last 10 min (60–70 min postformalin injection) of the phase 2 of the formalin test. Data are represented as means (s) ± SEMs of 4–5 animals per group. *$P < 0.05$ vs. restricted group of the same sex (posthoc *t*-test).

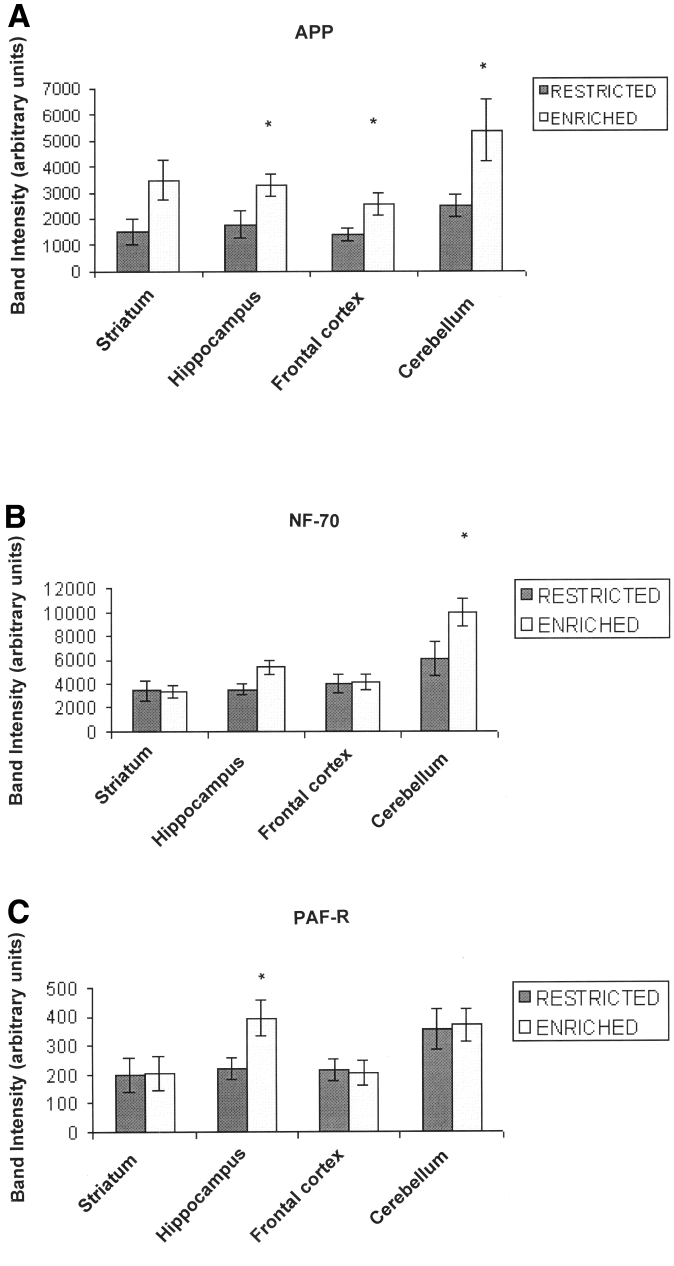

FIG. 7. Effect of 6 months of environmental enrichment or restriction on regional expression of (A)APP (B)NF-70, and (C)PAF-R. Data are represented as means of band intensity (arbitrary units) ± SEMs of 7–8 animals (male and female data were combined as no effect of sex was found). *$P < 0.05$ vs. restricted group (Fischer's PLSD posthoc). Regions were processed and analysed separately; the arbitrary units for one structure should not be compared with another in terms of protein concentration.

Effect of environment on protein levels

EC rats had significantly higher levels of APP than RC rats in the cerebellum, striatum, frontal cortex and hippocampus ($P < 0.05$; ANOVA) (Fig. 7A). No significant differences were detected for any of the other synaptic proteins studied (synphillin-1, synaptophysin, synapsin-1, or synuclein-1; data not shown). EC rats also had significantly higher levels of NF-70 in cerebellum and hippocampus ($P < 0.05$; ANOVA), but not in frontal cortex or striatum (Fig. 7B). EC rats had higher PAF-R levels in hippocampus ($P < 0.05$; ANOVA), but not in other brain structures (Fig. 7C). No detectable differences were observed in cyclooxygenase-2, glial fibrillary acidic acid, or nitric oxide synthase-2 in any of the brain regions analysed (data not shown).

Discussion

Effect of environment on memory and attentional mechanisms

These data show that rats reared in an enriched environment display a pattern of enhanced behavioural function in tasks believed to be dependent on the hippocampus, but not in tasks dependent on the striatum. Specifically, acquisition of two hidden platform water maze tasks was significantly better in EC rats than in RC rats. EC rats also showed enhanced retention of spatial location in subsequent probe tests. In contrast, EC and RC rats acquired the visible platform water maze task at similar rates. It is unlikely that this enhanced EC rat performance is caused by differences in sensorimotor activity, motivation, or emotion, inasmuch as performance of the visible platform task would also be affected by such nonmnemonic factors. Furthermore, performance of an appetitive visual discriminatory S-R task was not affected by environment, as previously described in mice (Jones *et al*., 1992) and rats (Mogensen, 1991). Hippocampal-dependent but not hippocampal-independent fear conditioning has also been found to be affected by environment (Duffy *et al*. 2001). These differences in the responses of different types or aspects of memory to environmental exposure provide further evidence for the existence of multiple memory systems, as suggested by pharmacological (e.g. Packard & Teather, 1997, 1998) and lesion (e.g. Packard *et al*., 1989; Packard & McGaugh, 1992; McDonald & White, 1994, 1994) studies.

These data also show that environmental conditions affect blocking performance, an outcome often attributed to attentional processes (Holland & Gallagher, 1993; Rickert *et al*., 1978; Solomon, 1977). Mackintosh (1995) proposed that animals learn to attend to relatively good predictors of reinforcement (e.g. for a food reward) and to disregard redundant information. As the food reward is already well predicted when the compound cue is introduced, any attention paid to the added cue will rapidly diminish, inasmuch as it provides no new information (Gallagher & Holland, 1994). Our results show that RC, but not EC, rats pay attention to irrelevant information at the expense of relevant information.

It has been suggested that attention is an important element in consolidation and retrieval of cognitive (spatial) forms of memory, but not in procedural (stimulus-response) forms (Arkhipov, 1999). The former but not the latter type of memory is affected by environmental enrichment, hence the enhanced selective attention displayed by the enriched rats in the present study may underlie the enhanced cognitive memory displayed by these rats. It is of interest to note that the hippocampus is involved in attention (for review, see Dutar *et al*., 1985), and that social isolation is associated with attentional deficits (Heidbreder *et al*., 2000). Moreover, the noradrenergic innervation from the locus coeruleus to the hippocampus is involved in visual selective attention (Mohammed *et al*., 1986), and noradrenaline depletion has been shown to attenuate the environment-induced changes in memory (Mohammed *et al*., 1986; Benloucif *et al*., 1995).

Effect of environment on nociception

Enriched and restricted animals displayed different nociceptive responses during phase 2 (i.e. the inflammatory phase of nociception), but not phase 1, of the formalin test. Enriched male rats displayed increased nociceptive behaviour compared with that of restricted males during the initial 50 min of phase 2 (i.e. 10–60 min postformalin injection). In contrast, during the final 10 min (i.e. 60–70 min postinjection), male RC rats displayed greater nociceptive responses than EC rats. A similar but not significant trend was observed in female rats. As females displayed greater reactivity to pain than males did, a 'ceiling effect' may have prevented the detection of significant differences in female rats. Previous experiments have consistently shown that male and female rats react differently to aversive stimulation (e.g. Bodnar *et al*., 1988; Heinsbroek *et al*., 1991; Kepler *et al*., 1991) and to pain processing (Aloisi *et al*., 1996).

A possible explanation for the finding that EC animals displayed increased reactivity during the initial part of phase 2 of formalin-induced nociception, and stopped exhibiting nociception-generated behaviour sooner than RC rats is that the EC animals underwent more rapid central processing of this information. This processing might have included the hippocampus as this structure is involved in phase 2, but not phase 1, of the formalin test (McKenna & Melzack, 1992). The involvement of the hippocampus in pain processing is also supported by results from physiological (Dutar *et al*., 1985), pharmacological (Soulairac *et al*., 1967; Dutar *et al*., 1985; McKenna & Melzack, 1992), and behavioural (Corkin, 1984; Wei *et al*., 2000) studies. Furthermore, hippocampal activity is changed by persistent pain and these effects are different in males and females (Aloisi *et al*., 1996).

Effect of environment on physiological stress measures

Female rats had larger adrenal glands and higher plasma corticosterone levels than males, and, unlike males, exhibited significant elevations in corticosterone after exposure to a restricted environment. However, males and females exhibited similar memory and attentional responses, suggesting that the increased stress-induced HPA-activity in restricted females was most likely not responsible for the cognitive deficits displayed by RC rats. These data should not be interpreted as evidence against the 'reduced corticosterone hypothesis' to explain the alterations in behaviour that occur due to environmental enrichment (Devenport *et al*., 1992), as it is possible that HPA-activity differences due to restriction-evoked stress occurred at an earlier stage of development in RC and EC rats. Indeed, stress early in life has been shown to result in long-term memory deficits and selective loss of hippocampal neurons (Brunson *et al*. 2001).

Effect of environment on protein expression

These data show that rats living in enriched or restricted environments for six months display regional differences in brain levels of certain proteins involved in synaptic plasticity and neuronal architecture; levels of APP, NF-70 and PAF-R protein are increased in enriched rats.

Increases in APP, a transmembrane glycoprotein implicated in Alzheimer's disease, are observed in hippocampus, cerebellum, striatum, and frontal cortex. In neuronal cells, APP or its soluble

fragment (APPs) promote cell survival, neurite outgrowth, and synaptogenesis, and can modulate plasticity. APP is involved in memory processing in chicks (Milevsnic *et al*. 2000) and rodents (Doyle *et al*., 1990; Huber *et al*., 1993), and APP knockout mice show impaired cognition (Muller *et al*., 1994; Zheng *et al*., 1994). Moreover, APP levels may be greater in rats that learn more readily than others (Huber *et al*., 1997). Whether the increased levels of APP that we observed in enriched rats are involved in their performance on hippocampal-dependent behaviours can only be conjectured. However, EC rats are reported to have more synapses per neuron (Greenough *et al*., 1985; Turner & Greenough, 1985), suggesting that APP is affecting synaptogenesis in EC rats and perhaps ultimately enhancing plasticity and learning and memory mechanisms.

To our knowledge, this study is the first to demonstrate that enrichment affects the levels of a synaptic protein. No significant alterations in the expression of other synaptic proteins (synapsin-1, synaptophysin, synphillin-1, synuclein-1) were observed, suggesting that the effect on APP expression is selective. However, it should be noted that the Western blot procedures that we used to quantify proteins may be insufficiently sensitive to detect smaller differences in some proteins. Thus, we cannot conclude that differences in the expression of other proteins does not occur due to enrichment; perhaps such differences may be detectable using more sensitive techniques, such as immunohistochemistry.

We observed a significant increase in levels of one neurofilament protein, NF-70, but not in two others, NF-145 and NF-200, within the cerebellum and hippocampus of EC rats. These proteins help maintain the structural integrity of neurons (Nixon & Sihag, 1991) and participate in axonal transport (Grafstein & Forman, 1980). Our findings suggest that the neurocytoskeletal profile of EC rats may differ from that of RC rats, particularly when previous work is taken into account showing that EC rats have increased levels of microtubules in occipital cortex (Jorgensen & Meier, 1979). Although we only found a significant alteration in NF-70; this is the most abundant neurofilament, and we cannot rule out that other (less-abundant) neurofilamaents are not altered by environmental conditions. More sensitive techniques such as immunohistochemistry, at the light or even electron microscope levels, may have to be carried out to fully assess potential cytoskeletal alterations caused by environment.

We also found increased levels of the platelet-activating factor receptor in hippocampus of enriched rats. PAF is thought to be a critical mediator of hippocampus-dependent memory processing (Jerusalinsky *et al*., 1994; Izquierdo *et al*., 1995; Packard *et al*., 1996; Teather *et al*., 2001, 2002) perhaps acting by enhancing the release of glutamate (Clark *et al*., 1992) or acetylcholine (Bussolino *et al*., 1988). The increased levels of the PAF-R may be involved in the enhanced glutamate and acetylcholine efflux previously demonstrated in enriched rats (Myhrer *et al*., 1992; Wongwitdecha & Marsden, 1996).

General conclusions

Exposing developing rats chronically to a complex, enriched environment was associated with enhanced spatial or cognitive memory skills, but not with reinforcement systems as measured by S-R or procedural memory tasks (which is thought to be mediated by the striatum rather than the hippocampus). As cognitive (or spatial) memory is mediated by the hippocampus, whereas S-R (or procedural) memory is mediated by the striatum, these findings indicate that environmental effects can influence hippocampal processing. In addition to performance differences on different types of memory tasks, enriched rats also have a greater capacity to selectively attend to relevant information. This ability may predispose enriched rats to have better memory skills in certain types of tasks that require attention, such as cognitive memory. Selective attention has been shown to involve the hippocampus, again suggesting that enriched rats have superior performance in hippocampal-dependent tasks. Enhanced hippocampal functioning may explain the altered nociception in enriched rats during late phase of the formalin test as it has been proposed that the hippocampus is involved in processing pain-related information.

Enrichment – which has previously been shown to increase the numbers of neurons and synapses – also increased brain levels of APP, a transmembrane protein whose soluble metabolites stimulate neurite outgrowth; the light neurofilament protein (NF-70); and the receptor for PAF, which is also known to participate in hippocampal memory functions. Whether these protein changes underlie the alterations in hippocampus-mediated behaviours seen in enriched rats remains to be determined. Previous work suggests a role for many of these proteins in plasticity and behaviour, thus warranting further study into this area.

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Abbreviations

APP, Amyloid precursor protein; EC, enriched conditions; ITI, intertrial interval; PAF, platelet-activating factor; PAF-R, platelet-activating factor receptor; PN, postnatal; RC, restricted conditions; S-R, stimulus-response.

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