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Voluntary exercise and palatable high-fat diet both improve behavioural profile and stress responses in male rats exposed to early life stress: Role of hippocampus

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Hippocampus;
Glucocorticoid receptor;
Brain-derived neutrophic factor;
5-Hydroxytryptamine receptor 1A (5HT1A) receptor

Summary Childhood trauma induced by adverse early life experience is associated with increased risk of psychological disorders in adulthood. Disruption of normal development has been shown to affect hippocampal morphology and function, influencing adaptations to stress. Here we investigated whether palatable food and/or exercise would ameliorate the behavioural responses following early life stress in rats. Rats were subjected to 15 (S15) or 180 (S180) minutes separation from dams on postnatal days 2–14. After weaning, rats were assigned to either receive chow (C), high-fat diet (HFD), voluntary exercise (running, R), or combined HFD and R for 11 weeks. In addition to anxiety- and depression-like behaviours, response to restraint stress was measured. Glucocorticoid receptor (GR), brain-derived neurotrophic factor (BDNF) and 5-hydroxytryptamine receptor 1A (5HT1A) receptor mRNA in the hippocampus were measured. S180 rats had similar body weight to S15, however their plasma insulin concentrations were double those of S15 rats when consuming HFD; adding exercise reduced plasma insulin. Anxiety-like behaviour in S180 rats, measured using Light Dark test (LDT) and Elevated Plus Maze (EPM) were ameliorated by the provision of HFD, R or HFD + R. A similar effect was observed on depression-like behaviour assessed by forced swim test (FST), with less time being spent immobile. Exposure to early-life stress during development was associated with significant reductions in hippocampal GR, 5HT1A receptor and BDNF mRNA, and these changes were normalized in S180 rats provided with HFD or exercise. Prolonged maternal separation resulted in exacerbated hyperinsulinemia when consuming HFD suggesting that these rats are metabolically disadvantaged. In summary, voluntary exercise alone or in combination with HFD produced beneficial effects on both behaviour and metabolic outcomes in rats exposed to early life stress.

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1. Introduction

Adverse early life experiences during childhood such as childhood trauma, child abuse and neglect have been shown to increase the risk of psychiatric disorders in later life (Bremne and Vermetten, 2001; Heim and Nemeroff, 1999; Sanchez et al., 2001). The effects of early-life stress induced by maternal separation have been studied in several mammalian species since the 1950s (Levine, 1957; Levine and Wiener, 1988; Kuhn and Schanberg, 1998; Gutman and Nemeroff, 2002). Separation of mothers from offspring in their early postnatal period interrupts normal neuronal programming and developing neuroendocrine systems, which subsequently alters offspring behaviour and stress response in adulthood (Kaufman and Rosenblum, 1967; McKinney and Bunney, 1969; Levine and Wiener, 1988; Slotten et al., 2006; Stephanie et al., 2008).

Prolonged maternal separation in early life leads to increased plasma corticosterone and increased anxiety-like behaviour in adulthood (Huot et al., 2001; Millstein and Holmes, 2007). Thus changes in circulating corticosterone following maternal separation likely contribute to dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis, reflected in altered stress responses (Stephanie et al., 2008), subsequently affecting behavioural outcomes. Long-term exposure to corticosterone is detrimental to hippocampal neuron survival and leads to hippocampal atrophy (Sapolsky, 1996). Hippocampal glucocorticoid receptors (GR), which play a pivotal role in the regulation of HPA axis activity, are downregulated under conditions of chronic stress (Kitraki et al., 1999) or in offspring subjected to maternal separation (Meaney et al., 1996; Ladd et al., 2004).

Stress plays an important role in the pathology of depression. Animal studies employing different stressors such as unpredictable mild stressor (Grønli et al., 2006), foot-shock (Rasmusson et al., 2002) and immobilization stress (Smith et al., 1995) demonstrated a significant reduction in hippocampal BNDF mRNA and protein levels. BDNF and its receptor, tropomyosin related kinase receptor B (TrkB) are proposed to be involved in the mechanism of action of antidepressant drugs (Duman and Monteggia, 2006; Castrén and Rantamäki, 2010). The expression of BDNF has been shown to be regulated by corticosterone (Smith et al., 1995; Gourley et al., 2009) and maternal separation (Roceri et al., 2002; Cirulli et al., 2009) in rodents. Thus our current study investigated if interventions such as diet and exercise would alter BDNF mRNA expression following maternal separation in rats.

Serotonin (5HT), a neurotransmitter involved in regulating the HPA axis, also plays a key role in anxiety and depression (Cryan and Leonard, 2000; Stockmeier, 2003) (Rittenhouse et al., 1994; Van de Kar et al., 2001) mainly through subtype 5HT1A and 5HT2A serotonin receptors (Contesse et al., 2000). Activation of these receptors also activates hypothalamic corticotrophin neurons (Mikkelsen et al., 2004) increasing the release of adrenocorticotrophin and circulating corticosterone (Jorgensen et al., 2002). A significant increase in the synthesis and release of 5HT was observed in various brain regions in response to stressors such as cold stress, immobilisation stress, food shock or tail pinch following the activation of HPA activity (for review see Lanfumey et al., 2008). The 5HT1A receptor is one of the primary targets in understanding the mechanisms of antidepressant activity due to its role in controlling the firing of 5HT neurons and the release of 5HT (Cryan and Leonard, 2000).

Previous work from our laboratory that introduced a palatable high-fat diet (HFD) following maternal separation showed that continuous consumption of a palatable HFD ameliorated the behavioural deficits following prolonged maternal separation in male and female rats (Maniam and Morris, 2010b). In a different model of previously unstressed rats, Dallman has consistently reported that 'comfort food' reduced the corticosterone response to an imposed restraint stress (Pecoraro et al., 2004; Dallman et al., 2005; la Fleur et al., 2005). Mechanisms underlying these changes are still not clear, however we observed decreased hippocampal GR mRNA in both pups (Maniam and Morris, 2010b) and dams (Maniam and Morris, 2010a) subjected to prolonged separation, which was reversed by chronic consumption of palatable HFD.

Exercise is not only known to improve metabolic profile but also appears to have a major impact on neurodevelopment and mental health (Cotman and Berchtold, 2002; Pietropaolo et al., 2008). Exercise produces beneficial effects in reducing stress, anxiety and depression-like behaviour (Zheng et al., 2006; Pietropaolo et al., 2008). Studies on the effects of exercise following maternal separation are limited, with only one recent study reporting that exercise improved depression-like behaviour as assessed with forced swim test (FST) (Marais et al., 2009). To explore the possibility of additive effects, we examined the effects of HFD and/or exercise on behavioural and stress responses.

Thus here we hypothesized that voluntary exercise or HFD would ameliorate the behavioural deficits and stress response following prolonged maternal separation in male rats, and that the combination of voluntary exercise and comfort food would have added benefits in regards to the behavioural and stress responses. Given the importance of GR in the stress response and the known effects of both glucocorticoids and BDNF in regulating HPA activity, we explored the underlying mechanisms by measuring the expression of hippocampal GR and BDNF mRNA, in addition to 5HT1A receptor mRNA. Our study is the first to examine the influence of exercise and consumption of HFD following maternal separation on behavioural, physiology and molecular parameters in rats.

2. Materials and methods

2.1. Animal procedures

All animal procedures were approved by the Animal Care and Ethics Committee of the University of New South Wales. Male and Female Sprague-Dawley rats from the Animal Resource Centre (Perth, Australia) were bred in house, maintained under constant temperature (23 °C) and lighting (lights on at 0700–1900 h). Following mating, litters were housed with the dam in polypropylene cages (20 cm × 32 cm × 19 cm) on wood shavings and a metal lid. Rat chow and tap water were provided *ad libitum*. Only pups from litter sizes between 10 and 13 pups were included to minimise alterations in maternal behaviour and pup nutrition.

Litters were assigned either to brief separation of 15 (S15) or prolonged separation of 180 (S180) minutes per day from postnatal days (PND) 2–14 according to our previously

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described protocol (Maniam and Morris, 2010b). Eight litters underwent S15 and nine litters underwent S180 treatment. Across the eight treatment groups, the total number of rats were 109, and ranged from $n = 12–15$ in each group.

In this study, we contrasted S180 with S15 because in the wild the dams leave the pups for periods of approximately 20–25 min (Leon et al., 1978). In this study for both S15 and S180 groups, the separation was commenced at different times between 1300 and 1500 h to reduce the predictability of the intervention. The dam was removed between 1300 and 1500 h and housed singly, then pups were removed from their cage into a small box containing wooden shavings placed on a heating pad at 32 °C in a separate room. At the end of separation, pups were returned to the home cage, followed by the dams. All cages were subjected to weekly cage cleaning during maternal separation time from postnatal days 2–14 for both the S15 and S180 rats.

2.2. Post-weaning diet and exercise intervention

At PND 20, the pups were weaned and housed 3–4 rats per cage to minimize stress. Male offspring from each litter were assigned to each treatment group: standard laboratory chow (11 kJ/g, energy 12% fat, 21% protein, 65% carbohydrate, Gordon's Specialty Stockfeeds, NSW, Australia); highly palatable cafeteria-style diet (HFD); chow and voluntary running wheels (R); or HFD and R combined. Rats were given continuous access to R in their home cage. Each cage was fixed with a running wheel with a rotation counter to calculate the distance run. Both exercised and non-exercised rats were housed 3–4 rats per cage. We undertook video monitoring to ensure all rats accessed running wheels however it is difficult to quantify the actual distance run by each of the rats under these housing conditions. Group housing was chosen to minimise stress. The distance run was reported as the average distance run across the rats in the cage.

The HFD had 32% energy as fat, comprising 3 different types of food of known calories (cakes, biscuits and dim sim) in addition to two types of modified chow; milk chow (15.33 kJ/g with 60% powdered chow, 33% sweetened condensed milk, 7% saturated animal fat) and chocolate/peanut milk chow (17.77 kJ/g with 40% powdered milk, 15% sweetened milk, 13% lard, 12% unsalted peanut, 15% chocolate topping). This diet is a modification of that used in our previous studies (Hansen et al., 2004; Velkoska et al., 2005). Food was given daily at 1700 and 24 h food intake was measured once a week by measuring the food remaining in the cage from the preceding 24 h. Energy intake was calculated from the known energy content of each food and averaged across the rats in the cage.

2.3. Behavioural testing of offspring

All behavioural tests were conducted during the light phase and measurements commenced 2 h after lights on (0900–1200 h).

2.4. Elevated plus maze (EPM)

EPM was conducted at 10 weeks of age during light phase using a plus shaped maze made of opaque perspex with two opposite open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm with 40 cm walls). In a 10-min trial, the time spent in the open and closed arms was scored. Frequency of head dips (the rat dipping its head into the space below the open arm and observing the environment) was also scored as an index of exploratory behaviour.

2.5. Light dark test (LDT)

The light/dark test (LDT) was conducted during light phase at 11 weeks of age. The LDT apparatus comprised bright and dark perspex compartments (24 cm × 24 cm × 27 cm) connected by a small opening (10 cm × 10 cm). The light reading on the floor of the light area was 4000–5000 lx and the dark area was lit between 1 and 10 lx. The rats were gently placed in the light area, facing away from the doorway. The total time spent in light and dark areas were recorded for 5 min.

2.6. Forced swim test (FST)

The forced swim test was conducted at 12 weeks of age during light phase as described previously (Maniam and Morris, 2010a). A training session was conducted by placing individual rats into a vertical plexiglass cylinder (height 40 cm, diameter 18 cm) containing approximately 35 cm of water maintained at 25 °C. The rats were removed after 15 min, towel dried and placed in a heated enclosure before being returned to their home cages. The test was conducted 24 h later, where rats were placed into the cylinder for 5 min and the duration of immobility, swimming and climbing were recorded.

2.7. Restraint stress

Restraint stress was conducted at 14 weeks of age during light phase. Each rat was briefly (30 min) placed in a cloth restrainer and a baseline blood sample was taken by tail clip. The rat remained in the restrainer for 30 min to induce immobilization stress. Serial blood samples were withdrawn at 15, 30, 45 and 60 min following initiation of the stress. Blood was centrifuged at 4 °C, 13 000 rpm and plasma was stored at −20 °C for plasma corticosterone measurement.

2.8. Tissue collection at 15 weeks

At 15 weeks of age, the offspring were anesthetized by ketamine/xylazine (20/80 mg/kg) and decapitated. Cardiac blood was collected and centrifuged (12 000 rpm, 8 min). The plasma was separated and stored at −20 °C for subsequent determination of plasma insulin. Coronal sections were made of the brain at the level of the optic chiasm and the rostral border of the hypothalamus. The hypothalamus was removed and the hippocampus was separated from the cortex and snap frozen in liquid nitrogen and stored at −80 °C for determination of mRNA expression of genes of interest. Epididymal white adipose tissue (WAT), retroperitoneal (Rp) WAT, and visceral WAT were dissected and weighed.

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2.9. Plasma insulin and corticosterone assays

Plasma insulin was measured using commercially available radioimmunoassay kits (Linco, MO, USA) according to the manufacturer's instruction. Plasma corticosterone was analysed using a kit from MP Biomedicals, LLC, Ohio.

2.10. Molecular analysis

RNA was extracted using Tri-reagent (SIGMA ALDRICH) and treated with DNase I (Invitrogen) to remove any contaminating genomic DNA and stored at −80 °C. Spectrophotometric measurements were done using the Nanodrop Spectrophotometer (Nanodrop Technologies Wilmington, DE, USA) to determine RNA concentration and purity. One microgram of RNA was reverse transcribed to cDNA using Omniscript Reverse Transcription kit (QIAGEN) and stored at −20 °C. Quantitative real-time PCR (qPCR) was performed with 50 ng cDNA using custom designed gene expression assays for Glucocorticoid receptor (GR) (Rn00561369), 5-hydroxytryptamine receptor 1A (5HT1A) (Rn00561409_s1) and brain-derived neutrophic factor (BDNF) (Rn01484928_m1) from Applied Biosystems. GR, 5HT1A and BDNF mRNA levels were compared to housekeeping gene beta actin. There were no statistical differences in beta actin CT values across different groups in hippocampus indicating constant level of expression of the housekeeping genes (data not shown). Analysis was performed using the $^{\Delta\Delta}$CT method (Livak and Schmittgen, 2001).

2.11. Statistical analysis

Data were checked for normality using Wilks *Shapiro* test. Data was expressed as mean ± SEM. Body weight, food intake, plasma insulin, plasma corticosterone, behavioural and mRNA expression data were analysed using 3-way ANOVA followed by post hoc *least significant difference* (LSD). The level of significance was set at $P < 0.05$.

3. Results

3.1. Effect of exercise and HFD on food intake

Average daily food intake from 4 to 10 weeks of age is shown in Fig. 1. Three-way ANOVA revealed no significant interaction between separation × exercise × diet. A main effect of diet ($F(7,16) = 672.4$, $p < 0.001$) and a significant interaction of exercise × diet ($F(7,16) = 13.88$, $p = 0.02$) on food intake were observed. Interestingly, in the chow fed group, energy intake between S180 and S15 rats were similar but the intake was increased by exercise (S180 versus S180R) ($p < 0.05$, Fig. 1). No effect of exercise was observed in the S15 rats consuming chow. As expected, HFD increased energy intake compared to chow across all groups ($p < 0.001$, Fig. 1). Access to running wheels reduced energy intake in S15 rats consuming HFD ($p < 0.05$, Fig. 1) but this difference was absent in S180 rats.

3.2. Wheel running activity

The average distance travelled per day/rat from 4 to 10 weeks of age was not significantly different across all groups.

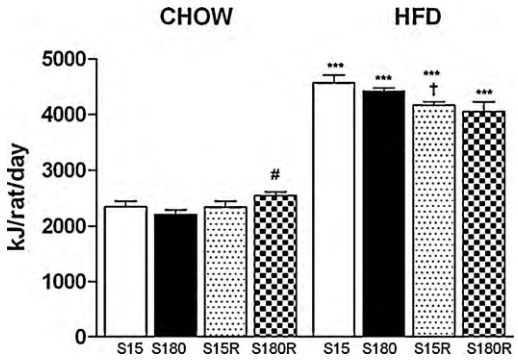

Figure 1 Average energy intake (kJ/day) (4–10 weeks of age) in S180 and S15 rats consuming chow or HFD with or without exercise (R). Data were analysed by three-way ANOVA followed by *post hoc* LSD. Results are expressed as mean ± S.E.M., $n = 12$–15. $^{\dagger}p < 0.05$ compared to S15 consuming the same diet (effect of separation). $^{\#}p < 0.05$ compared to sedentary S180 consuming the same diet (effect of exercise). $^{***}p < 0.001$ compared to chow fed (effect of diet).

However, the distance travelled by all groups of rats gradually decreased from week 6 (time) and reached a plateau at 9–10 weeks of age (Fig. 2).

3.3. Behaviour

3.3.1. Anxiety-like behaviour assessed with EPM

A main effect of maternal separation on the percentage time spent in the open arm of EPM ($F(7,69) = 12.75$, $p = 0.001$) and a significant interaction between diet × exercise ($F(7,69) = 9.99$, $p = 0.002$) were observed. However, there was no significant interaction between separation × diet × exercise. At 10 weeks of age, in those rats consuming chow, S180 rats spent significantly less time in the open arm of the EPM compared to S15 rats, demonstrating increased anxiety-like behaviour ($p < 0.05$, Fig. 3A). Interestingly, this was reduced by exercise, HFD or the combination in S180 rats ($p < 0.05$, Fig. 3A). A similar pattern was observed in percentage entries

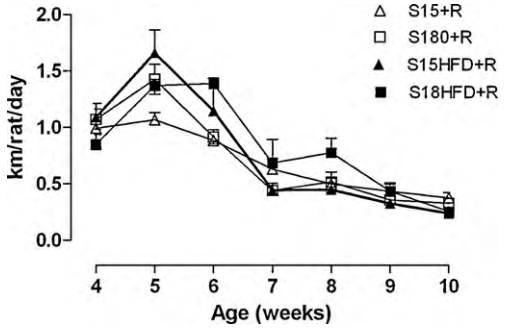

Figure 2 Average distance travelled per day by S180 and S15 rats consuming chow or HFD with exercise (R) from 4 to 10 weeks of age. Rats consuming low fat chow with access to running wheels are represented by open triangle (S15R) and open square (S180R) while those rats consuming HFD with access to running wheels are represented by filled triangle (S15R + HFD) and filled square (S180R+ HFD). Data were analysed by repeated measures with ANOVA followed by *post hoc* LSD. Results are expressed as mean ± S.E.M., $n = 12$–15.

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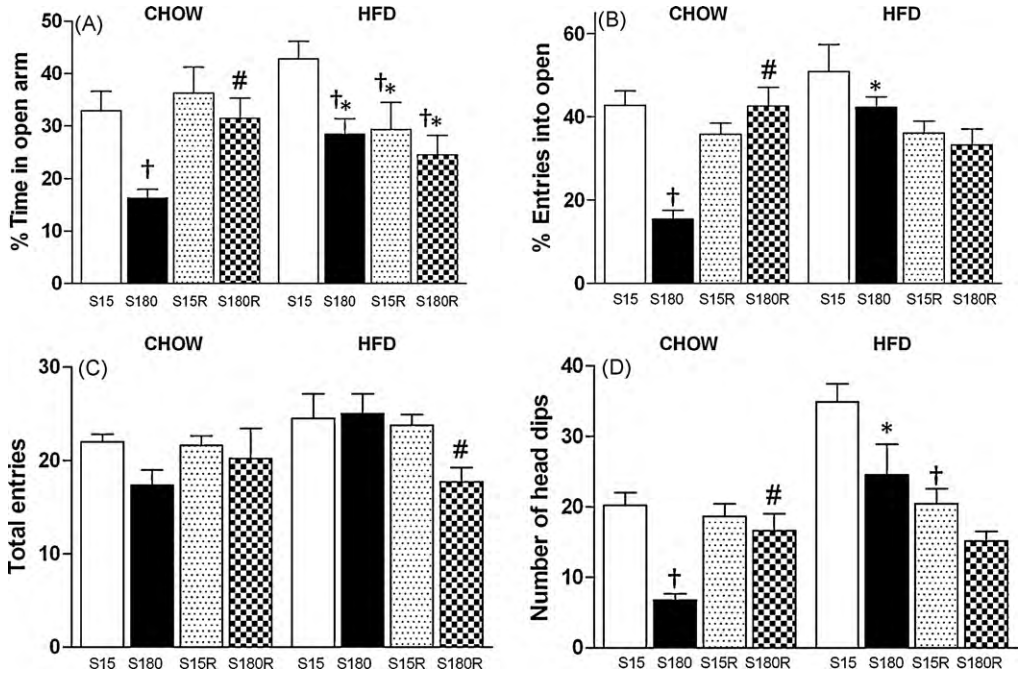

Figure 3 Percentage time spent in the open arm of EPM (A), percentage entries into open arm (B), total entries into both arms (C) and number of head dips (D) in S15 and S180 rats consuming chow or HFD with or without exercise (R). Data were analysed by three-way ANOVA followed by *post hoc* LSD. Results are expressed as mean ± S.E.M., $n = 9–11$. $^{\dagger}p < 0.05$ compared to S15 consuming the same diet (effect of separation). $^{\#}p < 0.05$ compared to sedentary S180 consuming the same diet (effect of exercise). $^{*}p < 0.05$ compared to chow fed (effect of diet).

made into the open arm of EPM, which is also an index of anxiety-like behaviour (Fig. 3B) and in this parameter, main effects of separation ($F(7,69) = 9.97$, $p = 0.002$) and diet ($F(7,69) = 6.86$, $p = 0.011$) with significant interaction between exercise × separation × diet ($F(7,69) = 7.98$, $p = 0.006$) were observed. In terms of locomotor activity, measured by total entries made into both closed and open EPM arms, the S180 + R consuming HFD made significantly less total entries compared to S180 rats consuming the same diet ($p < 0.05$, Fig. 3C). S180 rats on chow also had decreased exploratory behaviour, with 60% less frequency of head dips compared to S15 rats ($p < 0.05$, Fig. 3D). This was increased in S180 + R and S180 rats fed HFD ($p < 0.05$, Fig. 3D). Surprisingly, the combination of HFD and exercise reduced the frequency of head dips in S15 rats ($p < 0.05$, Fig. 3D). A similar trend was observed in S180 rats (S180R + HFD versus S180HFD) but this failed to reach significance.

3.3.2. Anxiety-like behaviour with light dark test

Anxiety-like behaviour was measured with LDT at 11 weeks of age. Three-way ANOVA revealed no significant interaction between separation × exercise × diet. Compared to the EPM, less marked effects on the percentage time spent in the light area across groups were observed. There was no significant separation × diet × exercise interaction. However, *t*-test revealed a significant difference between S15 and S180 rats consuming chow, with S180 rats spending less time in the light area compared to S15 rats ($t(17)$, p = 0.031, Fig. 4A). When number of entries to the light area was examined, a main effect of separation ($F(7,77) = 8.113$,

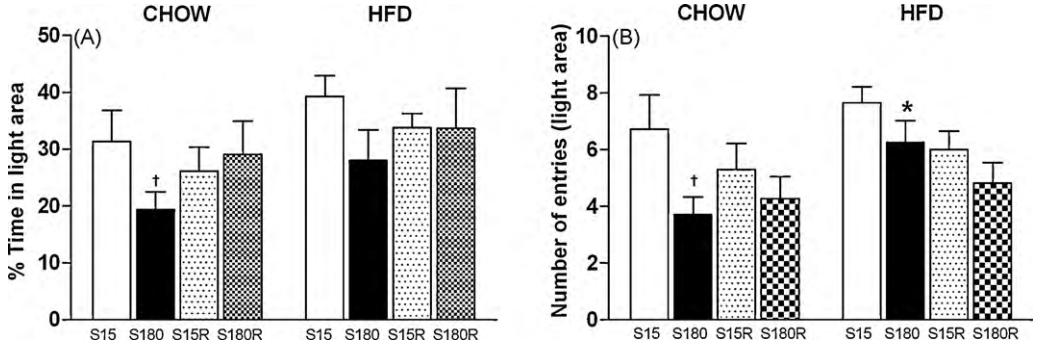

Figure 4 Percentage time in light area of LDT (A) and number of entries into light area (B) in S180 and S15 rats consuming chow or HFD with or without exercise (R). Data were analysed by three-way ANOVA followed by *post hoc* LSD. Results are expressed as mean ± S.E.M., $n = 9–12$. $^{\dagger}p < 0.05$ compared to S15 (effect of separation). $^{*}p < 0.05$ compared to chow fed (Effect of diet).

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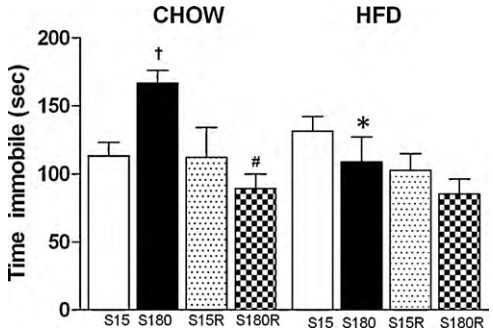

Figure 5 Time spent immobile in the forced swim test in S180 and S15 rats consuming chow or HFD with or without exercise (R). Data were analysed by three-way ANOVA followed by *post hoc* LSD. Results are expressed as mean ± S.E.M., $n = 10–11$. $^{\dagger}p < 0.05$ compared to S15 (effect of separation). $^{\#}p < 0.05$ compared to sedentary S180 consuming the same diet (effect of exercise). $^{*}p < 0.05$ compared to chow fed (effect of diet).

$p = 0.006$) and a main effect of diet ($F(7,77) = 4.109$, $p = 0.046$) were observed. Thus S180 rats made significantly less entries to the light area compared to S15 rats ($p < 0.05$, Fig. 4B) and this was reversed by access to HFD but not exercise ($p < 0.05$, Fig. 4B).

3.3.3. Depression-like behaviour assessed with FST

Three-way ANOVA revealed a significant interaction between separation, diet and exercise on time spent immobile ($F(7,80) = 5.64$, $p = 0.02$). Time spent immobile as an index of depression-like behaviour was measured and a main effect of exercise ($F(7,80) = 11.91$, $p = 0.001$) was observed with no effect of maternal separation or diet. The interaction between separation × diet ($p = 0.068$) or separation × exercise ($p = 0.067$) almost reached significance. At 12 weeks of age, S180 rats spent significantly more time being immobile compared to S15 rats, indicating increased depression-like behaviour ($p < 0.05$, Fig. 5). Those S180 rats having access to running wheels or HFD or the combination spent significantly less time immobile ($p < 0.05$, Fig. 5).

3.3.4. Stress response

The response to restraint stress was measured at 13 weeks of age. A significant interaction between separation × exercise × diet for basal plasma corticosterone ($F(7,58) = 4.079$, $p = 0.048$) was observed. S180 rats had significantly higher basal plasma corticosterone compared to S15 rats ($p < 0.05$, Fig. 6A) and access to continuous running wheels reduced basal plasma corticosterone in S180 rats consuming chow ($p < 0.05$, Fig. 6A). However, HFD and the combination of HFD and exercise did not significantly alter basal plasma corticosterone in S180 rats.

In the area under the curve of plasma corticosterone concentration in response to stress, ANOVA showed a significant interaction between separation, exercise and diet ($F(7,58) = 4.28$, $p = 0.043$). The S180 rats consuming chow had significantly elevated plasma corticosterone, as evidenced by a greater area under the curve compared to S15 rats consuming the same diet ($p < 0.05$, Fig. 6B). Interestingly, exercising S180 rats and S180 rats fed HFD had overall lower corticosterone responses to stress ($p < 0.05$, Fig. 6B).

3.4. Plasma hormone at death

3.4.1. Insulin

Three-way ANOVA revealed a significant interaction between separation, diet and exercise on plasma insulin concentrations ($F(7,75) = 5.64$, $p = 0.02$). At 14 weeks of age, as expected, main effects of diet ($F(7,75) = 73.17$, $p < 0.001$) and exercise ($F(7,75) = 24.73$, $p < 0.001$) on plasma insulin concentrations were observed. Plasma insulin concentrations in rats consuming chow were not significantly different across separation groups but HFD increased plasma insulin concentrations more than twice compared to chow fed rats. Interestingly, the effect of separation on plasma insulin concentrations approached significance ($F(7,75) = 3.45$, $p = 0.067$). Significant interactions between separation × exercise ($F(7,75) = 6.90$, $p = 0.010$) and separation × diet ($F(7,75) = 4.06$, $p = 0.047$) on plasma insulin concentrations were present. In those rats consuming HFD, plasma insulin in S180 was two times those of S15 and almost 4 times more plasma insulin compared to chow fed rats

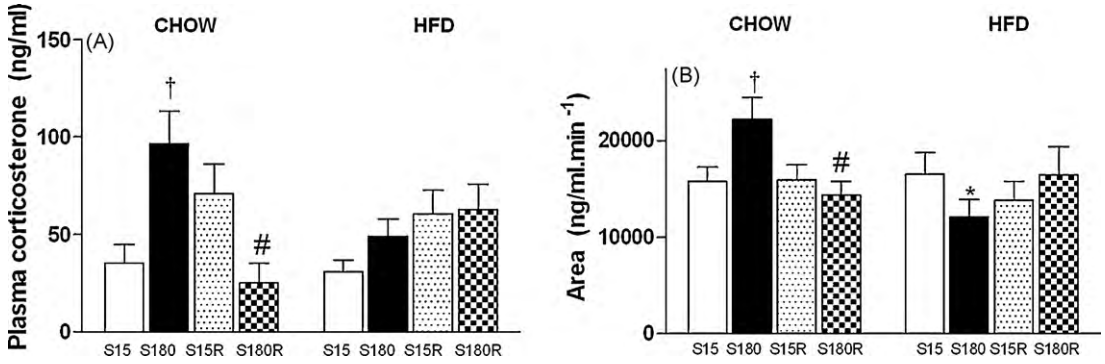

Figure 6 Basal plasma corticosterone concentrations (A) and area under the corticosterone response curve following restraint stress (B) in S180 and S15 rats consuming chow or HFD with or without exercise (R). Data were analysed by three-way ANOVA with followed by *post hoc* LSD. Results are expressed as mean ± S.E.M., $n = 8–10$. $^{\dagger}p < 0.05$ compared to S15 consuming the same diet (effect of separation). $^{\#}p < 0.05$ compared to sedentary S180 consuming the same diet (effect of exercise). $^{*}p < 0.05$ compared to chow fed (effect of diet).

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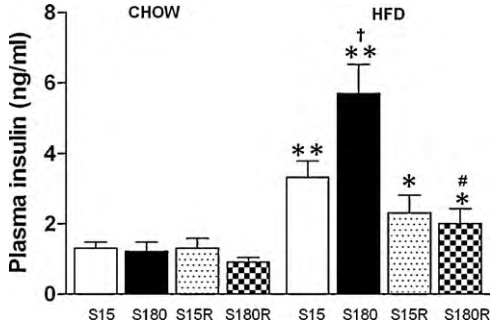

Figure 7 Plasma insulin concentrations at death in S180 and S15 rats consuming chow or HFD with or without exercise (R) (14 weeks of age). Data were analysed by three-way ANOVA followed by *post hoc* LSD. Results are expressed as mean ± S.E.M., $n = 10–12$. $^{\dagger}p < 0.05$ compared to S15 consuming the same diet (effect of separation). $^{\#}p < 0.05$ compared to sedentary S180 consuming the same diet (effect of exercise). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to chow fed (effect of diet).

($p < 0.01$, Fig. 7). Rats consuming HFD with access to running wheels had plasma insulin levels less than half of S180 rats consuming HFD ($p < 0.05$, Fig. 7).

3.4.2. Body weight, BMI and fat mass

A main effect of maternal separation ($F(7,101) = 5.03$, $p = 0.027$), diet ($F(7,101) = 159.67$, $p < 0.001$) and a significant exercise × diet interaction ($F(7,101) = 6.09$, $p = 0.015$) on body weight were observed. At the end of the study, in those rats consuming chow, neither separation nor exercise altered body weight across groups. As expected, HFD increased body weight by approximately 30% compared to chow fed rats across groups ($p < 0.05$, Table 1). The effect of separation was evident in those rats consuming HFD and having access to running wheels with S180 rats being approximately 7% lighter than S15 rats ($p < 0.05$, Table 1).

In the BMI measurements, a main effect of diet ($F(7,101) = 144.29$, $p < 0.001$) was observed while the effect of separation almost reached significance ($F(7,101) = 3.09$, $p = 0.081$). The interaction between diet and exercise also approached significance ($F(7,101) = 3.92$, $p = 0.050$). Similar to body weight, neither separation nor exercise affected BMI measurements in those rats consuming chow. However, as expected HFD increased BMI across groups compared to chow fed rats ($p < 0.05$, Table 1). In line with reduced body weight, S180R rats consuming HFD had significantly lower BMI compared to S15R rats consuming the same diet. On the other hand, exercise did not significantly alter BMI in S180 rats consuming HFD however statistic analysis reveal that the exercise effect almost reached significance ($p = 0.067$, Table 1).

Main effects of separation ($F(7,101) = 12.73$, $p = 0.001$), exercise ($F(7,101) = 3.95$, $p = 0.049$) and diet ($F(7,101) = 292.52$, $p < 0.001$) on total WAT mass were observed. In addition, a significant interaction between exercise and diet ($F(7,101) = 5.97$, $p = 0.016$) was observed with no significant separation × exercise × diet interaction. In those rats consuming chow, separation did not alter total WAT mass across groups. Surprisingly, S180 rats consuming HFD had approximately 20% less total WAT compared to S15 rats consuming the same diet ($p < 0.05$, Table 1). The exercise

Table 1 Effect of separation, exercise and diet on body weight, BMI and fat mass.

	CHOW				HFD			
	S15	S180	S15R	S180R	S15	S180	S15R	S180R
Body weight (g)	428.7 ± 11.2	416.6 ± 8.5	443.9 ± 12.3	437.7 ± 8.6	562.3 ± 15.9***	541.8 ± 17.7***	546.9 ± 10.3***	508.9 ± 10.0‡,***
BMI (kg/m^2)	0.429 ± 0.01	0.432 ± 0.01	0.444 ± 0.01	0.438 ± 0.01	0.562 ± 0.02***	0.542 ± 0.02***	0.547 ± 0.01***	0.509 ± 0.01‡,***
Rp+Visceral WAT (g)	6.97 ± 0.40	6.34 ± 0.53	7.40 ± 0.72	6.51 ± 0.49	18.41 ± 1.56***	10.26 ± 0.78†,***	13.63 ± 1.26†,***	15.66 ± 1.22$^{\#,***}$
% Rp+Visceral/body weight	1.62 ± 0.08	1.46 ± 0.09	1.64 ± 0.11	1.48 ± 0.09	4.40 ± 0.25***	3.34 ± 0.19†,***	3.88 ± ± 0.13***	3.04 ± 0.19***
Total WAT (g)	14.08 ± 0.70	13.33 ± 0.89	13.63 ± 1.26	11.52 ± 0.86	41.24 ± 3.02***	32.47 ± 2.72†,***	36.19 ± 1.31***	29.68 ± 2.30***
% Total WAT/body weight	3.28 ± 0.14	2.97 ± 0.17	3.02 ± 0.19	2.61 ± 0.15	7.25 ± 0.35***	5.90 ± 0.34†,***	6.60 ± 0.19***	5.77 ± 0.35***

Data were analysed by three-way ANOVA followed by *post hoc* LSD. Results are expressed as mean ± S.E.M., $n = 12–15$.
† $p < 0.05$ compared to S15 rats consuming the same diet.
‡ $p < 0.05$ compared to S15R consuming the same diet
‡‡ $p < 0.01$ compared to S15R consuming the same diet.
$^{\#}$ $p < 0.05$ compared to S180.
*** $p < 0.001$ compared to chow fed.

effect on total WAT mass in S15 rats consuming HFD approached significance ($p = 0.078$, Table 1). In terms of percentage total WAT over body weight, main effects of separation ($F(7,101) = 16.97$, $p < 0.001$) and diet ($F(7,101) = 370.32$, $p < 0.001$) were observed. In addition, significant interactions between separation × diet ($F(7,101) = 4.24$, $p = 0.042$) and exercise × diet ($F(7,101) = 4.01$, $p = 0.048$) were present. S180 rats consuming HFD had less percentage total WAT over body weight compared to S15 rats consuming the same diet ($p < 0.05$, Table 1). A similar pattern was observed in the abdominal fat mass and percentage abdominal fat mass.

3.5. Hippocampal GR, BDNF and 5HT1A receptor mRNA

3.5.1. GR mRNA

Significant interactions between separation × diet ($F(7,53) = 4.495$, $p = 0.039$) and diet × exercise ($F(7,53) = 6.872$, $p = 0.011$) with no significant interaction between exercise, separation and diet were observed. Hippocampal GR mRNA was significantly decreased by approximately 30% in S180 rats consuming chow compared to S15 rats on the same diet, and its expression was returned to levels equivalent to S15 rats in S180R ($p = 0.012$, Fig. 8) and S180 rats fed HFD ($p = 0.029$, Fig. 8).

3.5.2. 5HT1A mRNA

Similar to GR mRNA expression, for 5HT1A mRNA, interaction between exercise, separation and diet was not significant but there appeared to be significant interactions between diet × exercise ($F(7,53) = 6.040$, $p = 0.017$) and separation × exercise ($F(7,53) = 6.014$, $p = 0.017$). S180 rats had approximately 30% lower hippocampal 5HT1A mRNA expression compared to S15 rats consuming chow ($p = 0.032$, Fig. 9). This was returned to S15 levels in S180 rats who exercised ($p = 0.007$, Fig. 9) and S180 rats on HFD ($p < 0.05$, Fig. 9). Surprisingly, no significant differences of this expression were observed between S180 rats fed chow and S180 + R consuming HFD (Fig. 9).

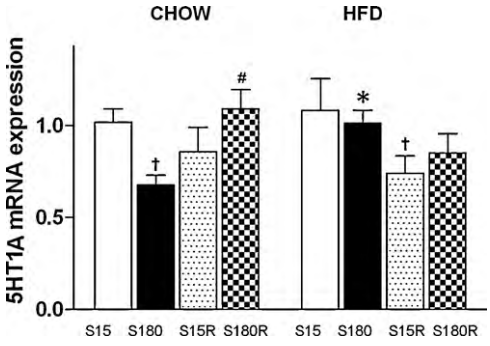

Figure 9 Hippocampal 5HT1A receptor mRNA expression in S180 and S15 rats consuming chow or HFD with or without exercise (R). Data were analysed by three-way ANOVA followed by *post hoc* LSD. Results are expressed as mean ± S.E.M., $n = 7–9$. $^{\dagger}p < 0.05$ compared to S15 consuming the same diet (effect of separation). $^{\#}p < 0.05$ compared to sedentary S180 consuming the same diet (effect of exercise). $^{*}p < 0.05$ compared to chow fed (effect of diet).

3.5.3. BDNF mRNA

Significant interactions between exercise, diet and separation ($F(7,53) = 4.182$, $p = 0.046$); diet × separation ($F(7,53) = 4.194$, $p = 0.046$); and exercise × diet ($F(7,53) = 5.26$, $p = 0.026$) were observed. The overall pattern of change was similar to that observed with 5HT1A mRNA, with reduced hippocampal BDNF mRNA in S180 rats, and reversal by exercise in S180 + R rats or HFD consumption ($p < 0.05$, Fig. 10). However, S180 + R consuming HFD had no significant differences in BDNF mRNA expression compared to S180 rats fed chow (Fig. 10).

4. Discussion

This study demonstrated that prolonged, 3 h daily, separation for the first 2 weeks of life resulted in increased anxiety and depression-like behaviour and elevated restraint stress response in adult rats. Prolonged separation was associated with reduced hippocampal mRNA expression of BDNF, GR and 5HT1A. The novel finding of this study is that continuous long-

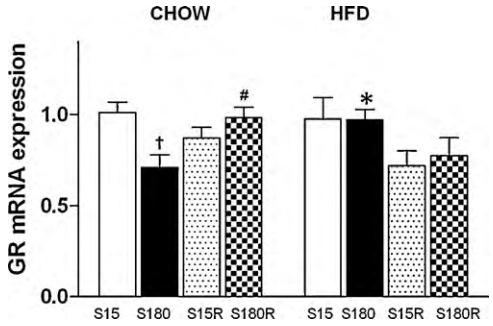

Figure 8 Hippocampal GR mRNA expression in S180 and S15 rats consuming chow or HFD with or without exercise (R). Data were analysed by three-way ANOVA with followed by *post hoc* LSD. Results are expressed as mean ± S.E.M., $n = 7–9$. $^{\dagger}p < 0.05$ compared to S15 consuming the same diet (effect of separation). $^{\#}p < 0.05$ compared to sedentary S180 consuming the same diet (effect of exercise). $^{*}p < 0.05$ compared to chow fed (effect of diet).

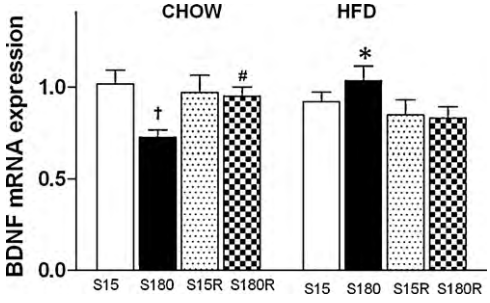

Figure 10 Hippocampal BDNF mRNA expression in S180 and S15 rats consuming chow or HFD with or without exercise (R). Data were analysed by three-way ANOVA followed by *post hoc* LSD. Results are expressed as mean ± S.E.M., $n = 7–9$. $^{\dagger}p < 0.05$ compared to S15 consuming the same diet (effect of separation). $^{\#}p < 0.05$ compared to sedentary S180 consuming the same diet (effect of exercise). $p < 0.05$ compared to chow fed (effect of diet).

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term access to HFD or voluntary exercise produced similar beneficial effects in ameliorating the behavioural deficits induced by prolonged maternal separation. This was in line with increased hippocampal GR, BDNF and decreased 5HT1A mRNA expression. Interestingly, the combination of exercise and HFD did not produce added benefits on behavioural and stress responses, and this intervention did not normalize the decreased GR, BDNF and 5HT1A mRNA observed in S180 rats. One interpretation of this result is that other mechanisms contribute to the alleviation of behavioural deficits induced by combined HFD and exercise, and this possibility warrants further attention.

Our study supports data showing that prolonged separation of 3 h daily for the first 2 weeks of life results in increased anxiety (EPM and LDT) and depression-like behaviour (FST) in addition to increased corticosterone response to stress (Slotten et al., 2006; Stephanie et al., 2008). The current study demonstrated that a shorter period of access to HFD (11 weeks) versus 16 weeks in our previous study (Maniam and Morris, 2010b) produced similar beneficial effects on behavioural responses. Here, we extended our investigation to study an alternate, healthier intervention, voluntary running, in addition to HFD, or the combination, following maternal separation. Depression-like behaviour assessed with FST showed that HFD decreased immobility time in S180 rats supporting the therapeutic value of HFD which was previously shown to increase hedonic behaviour in rats exposed to early life stress (Maniam and Morris, 2010b). In addition, access to exercise produced a similar effect. In another maternal separation model, voluntary exercise reduced depression-like behaviour only in those rats exposed to subsequent chronic stress at adulthood (Marais et al., 2009). Moreover, access to running was shorter (6 weeks, 5 days/week), and plasma corticosterone response to stress was not measured. Thus our study is the first to comprehensively assess behavioural, physiological and molecular changes following continuous chronic access to running wheels or HFD or their combination from weaning for 11 weeks following maternal separation.

The average distance rats ran during the experimental window averaged less than 1 km/day, in line with other data in this strain (Johnson and Mitchell, 2003). Despite the absence of wheel activity differences across groups, access to running wheels appeared to have no benefit for S15 rats but produced beneficial effects in those rats exposed to prolonged maternal separation in terms of physiological and behavioural responses and the molecular changes in brain markers which regulate the stress response.

A novel finding of this study is the markedly reduced plasma corticosterone responses to restraint stress induced by exercise or HFD in S180 rats. Rats with combined exercise and HFD had a trend of reduced plasma corticosterone but this did not reach significance. We further investigated the underlying mechanism(s) contributing to the HFD and exercise related improvement in behaviour by targeting mediators in the hippocampus that regulate HPA axis activity known to be involved in anxiety- and depression-like behaviours. GR, which is abundantly found in the hippocampus, plays a pivotal role in the negative feedback of HPA axis activity. The diet intervention we instituted normalized hippocampal GR mRNA to that of control S15 rats. Voluntary exercise had similar effects. The increase in hippocampal GR mRNA induced by diet or exercise intervention was in line with the decreased plasma corticosterone in response to restraint stress in S180 rats. Continuous access to voluntary running or HFD during development improved HPA axis sensitivity in 180 rats, which may explain the improved behavioural profiles. Other studies on acute stress models showed that voluntary exercise improved HPA axis sensitivity with increased adrenal sensitivity to adrenocorticotrophin after restraint stress (Fediuc et al., 2006) and footshock or cage switch (Dishman et al., 1998). In regards to dietary effects, in another study increased expression of GR mRNA in the CA1 region of the hippocampus was observed in dietary obesity and these rats demonstrated less anxiety-like behaviour compared to chow fed rats, with increased activity in the open field test (Michel et al., 2003). However, in our study, surprisingly the expression of GR mRNA was not altered in those rats given both HFD and exercise, even though they were less anxious and had less depression-like behaviour compared to S180 rats. This finding suggests that upregulation of hippocampal GR mRNA by HFD or exercise alone may not be the sole contributor to the observed behaviours. To this end we investigated other mediators in the hippocampus that are involved in the regulation of stress and HPA axis activity.

In the current study we observed that hippocampal BDNF mRNA was reduced in S180 rats in accordance with earlier reports (McEwen, 2000; Molteni et al., 2002; Mirescu et al., 2004; Greisen et al., 2005). Provision of HFD normalized BDNF mRNA levels only in S180 rats while no effect of HFD was observed in S15 rats. Thus diet had minimal effect in control rats, but was able to 'rescue' the reduced BDNF in separated rats. Previous studies reported that an energy rich diet high in fat and mixed with sugar resulted in decreased hippocampal BDNF levels which was in line with reduced cognitive performance (Molteni et al., 2002; Kanoski et al., 2007). The 11 weeks of voluntary exercise also increased hippocampal BDNF mRNA, in agreement with a recent finding in a similar model (Marais et al., 2009). Voluntary exercise-induced BDNF increases were also shown to modulate depression-like behaviour induced by acute stress (Russo-Neustadt et al., 2001) and chronic unpredictable stress (Zheng et al., 2006). A recent study demonstrated that chronic antidepressant treatment in rats reduced immobility time in the FST and increased hippocampal BDNF mRNA (Larsen et al., 2010). Interestingly, in our study, adding exercise to S180 rats consuming HFD did not increase BDNF mRNA, a similar pattern to that of GR mRNA expression.

Serotonin has also been implicated in the pathogenesis of depression (Stockmeier, 2003), and increased 5HT signaling, which activates the transcription factor CREB, was suggested to mediate the expression of BDNF (Mattson et al., 2004). In a voluntary running model, increased CREB mRNA and synapsin I mRNA, which is involved in 5HT release, was observed in rats that were exercising (Vaynman et al., 2003). In the current study, the decreased 5-HT1A receptor mRNA in S180 rats coincided with behavioural deficits and increased stress response. These data are line with other studies where 3-h separated pups had a lower density of 5HT1A receptors, compared to 15 min controls (Vicentic et al., 2006) while selective activation of 5HT1A receptors attenuated anxiety-like behavior in rats bred for high anxiety/depressive phenotypes (Brunelli et al., 2009). The HFD or exercise in S180 rats reversed the reduction in 5HT1A receptor mRNA expres-

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sion and this coincided with improved behavioural profiles. However, the combination of HFD and exercise produced no changes in 5HT1A receptor expression which was similar pattern to that of GR and BDNF mRNA expression. Thus the combined intervention confers no added benefit on behavioural responses, and may be mediated by another mechanism or through other brain regions, and elucidation of these possibilities requires further investigation.

Few studies have looked at the impact of early life stress induced by maternal separation on food intake and subsequent weight gain. In our study, maternal separation had no significant impact on adult body weight in S180 rats compared to S15 controls across diet groups. In another study, 6 h of separation from the mother resulted in lighter pups at 3 weeks of age compared to controls, but this difference disappeared with age (Iwasaki et al., 2000). Several other reports showed that pups subjected to maternal separation have decreased body weight (Tönjes et al., 1986; McIntosh et al., 1999). These findings and our data suggest that prolonged maternal separation does not produce an obese phenotype and we have shown that this is evident in both diets. Interestingly, in our hands the combination of exercise and HFD resulted in S180 rats being lighter compared to S15 controls under the same diet. Although body weight was not altered by prolonged separation in our study, we observed decreased fat mass in S180 rats consuming HFD, which was in line with significantly lower energy intake for the entire experimental period compared to S15 controls on the same diet. Adding exercise to S180 rats on HFD did not alter fat mass but in the control rats, exercise reduced abdominal fat mass (S15 HFD versus S15 HFD + R). This finding suggests that prolonged separation may have altered fat metabolism in those consuming HFD and thus masked the effect of exercise on fat mass. This further suggests that metabolic changes are caused by prolonged separation, an important consideration for future studies.

Our plasma insulin data also point to metabolic consequences of early life stress. Amongst rats consuming HFD the markedly elevated plasma insulin in S180 compared to S15 rats suggest that early life stress may lead to hyperinsulinaemia later in life if consuming HFD. This needs further investigation by examining fasting insulin concentrations, and insulin sensitivity. Prolonged maternal separation may have resulted in altered programming of insulin secretion, which only becomes evident when rats are consuming HFD. To our knowledge there are no data exploring the influence of early life stress on insulin secretion. Schizophrenic patients showed impaired insulin sensitivity (Steiner et al., 2010) and increased circulating insulin-related peptide (Guest et al., 2010). Rats subjected to single prolonged separation have behavioural deficits resembling specific symptoms present in schizophrenia (Llorente et al., 2009) thus suggesting a possible link between early life stress and insulin sensitivity which requires further investigation. Besides beneficial effects on behavioural and stress response, the combined intervention of HFD and exercise markedly reduced the elevated insulin observed in S180 rats. Thus our finding further underlines the beneficial effect of exercise in reducing the risk of developing metabolic disorders (Zachwieja et al., 1997; Yan et al., 2007).

Overall, this study is the first to demonstrate improved behavioural and stress responses in prolonged separated rats following either continuous access to a palatable, high-fat mixed diet or to exercise. The combined intervention had no added benefits. These changes were partly mediated through molecular changes in the hippocampal GR mRNA, 5HT1A receptor mRNA and BDNF mRNA as the combination of exercise and HFD improved behavioural and metabolic profiles but the hippocampal markers measured in this study were not altered compared to S180 rats, thus other mechanisms likely contribute. In the present study, we also failed to see an obese phenotype in S180 rats. Early, prolonged maternal separation led to decreased fat mass and markedly elevated plasma insulin on exposure to HFD, suggesting these rats may be metabolically disadvantaged. In conclusion, voluntary exercise, alone or in combination with HFD, produced beneficial effects on both the behaviour and metabolic outcomes following early life stress induced by maternal separation.

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Conflict of interest

All authors declare that they have no conflict of interest.

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