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Early Parental Deprivation in the Marmoset Monkey Produces Long-Term Changes in Hippocampal Expression of Genes Involved in Synaptic Plasticity and Implicated in Mood Disorder

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In mood disorder, early stressors including parental separation are vulnerability factors, and hippocampal involvement is prominent. In common marmoset monkeys, daily parental deprivation during infancy produces a prodepressive state of increased basal activity and reactivity in stress systems and mild anhedonia that persists at least to adolescence. Here we examined the expression of eight genes, each implicated in neural plasticity and in the pathophysiology of mood disorder, in the hippocampus of these same adolescent marmosets, relative to their normally reared sibling controls. We also measured hippocampal volume. Early deprivation led to decreases in hippocampal growth-associated protein-43 (GAP-43) mRNA, serotonin 1A receptor (5-HT_{1A}R) mRNA and binding ([^{3}H]WAY100635), and to increased vesicular GABA transporter mRNA. Brain-derived neurotrophic factor (BDNF), synaptophysin, vesicular glutamate transporter 1 (VGluT1), microtubule-associated protein-2, and spinophilin transcripts were unchanged. There were some correlations with *in vivo* biochemical and behavioral indices, including VGluT1 mRNA with reward-seeking behavior, and serotonin 1A receptor mRNA with CSF cortisol. Early deprivation did not affect hippocampal volume. We conclude that early deprivation in a nonhuman primate, in the absence of subsequent stressors, has a long-term effect on the hippocampal expression of genes implicated in synaptic function and plasticity. The reductions in GAP-43 and serotonin 1A receptor expressions are comparable with findings in mood disorder, supporting the possibility that the latter reflect an early developmental contribution to disease vulnerability. Equally, the negative results suggest that other features of mood disorder, such as decreased hippocampal volume and BDNF expression, are related to different aspects of the pathophysiological process.

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INTRODUCTION

Stressful experiences in early life, such as parental separation or abuse, are significant risk factors for mood disorder (Harris *et al*, 1986; Heim and Nemeroff, 2001), likely acting in concert with genetic predisposition (Kendler *et al*, 1993; Jokela *et al*, 2007). Experimental studies, mostly in rodents, have identified a range of putative pathogenic mechanisms that contribute to the relationship between developmental stressors and later depression-like phenotypes (DeBellis *et al*, 1999; Sanchez *et al*, 2001; Pryce *et al*, 2005a; Ansorge *et al*, 2007). These include alterations in the hypothalamo-pituitary-adrenal (HPA) axis (Sapolsky, 2000; Gunnar and Quevedo, 2007), the serotonin (5-HT) system (Gartside *et al*, 2003; Ichise *et al*, 2006), neurotrophins (Duman *et al*, 1997), and synaptic plasticity (Manji *et al*, 2001; Nestler *et al*, 2002; Pittenger and Duman, 2008). The hippocampus is prominently involved in each of these domains (McEwen, 1999; Sapolsky, 2000; Duman and Monteggia, 2006). Supporting the view that similar processes may be relevant clinically, there is an accumulating literature showing a range of broadly comparable changes in the hippocampus of people with mood disorder, either unipolar or bipolar (for review, see Harrison, 2002; Sala *et al*, 2004; Czeh and Lucassen, 2007; Frey *et al*, 2007). These findings include

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smaller hippocampal volume (Campbell *et al*, 2004), fewer serotonin 1A receptors (5-HT_{1A}R) (Drevets *et al*, 1999; Sargent *et al*, 2000; Lopez-Figueroa *et al*, 2004; Hirvonen *et al*, 2008), reductions in synaptic markers (Eastwood and Harrison, 2000; Rosoklija *et al*, 2000; Webster *et al*, 2001; Vawter *et al*, 2002; Tian *et al*, 2007), and decreased expression of mineralocorticoid and glucocorticoid receptors (Lopez *et al*, 1998; Webster *et al*, 2002).

Despite these convergent findings, the molecular links between early life stress and subsequent mood disorder remain poorly understood. One reason has been a paucity of experimental data in the nonhuman primate, requiring extrapolations from the rodent that are compromised by species differences in parenting, endocrine systems, neuroanatomy, and neurodevelopment. Recently, however, a comprehensively assessed primate developmental model of depression vulnerability has been described. In the common marmoset (*Callithrix jacchus*), a small New World monkey, early deprivation (ED) in the form of daily short periods of isolation from the family group during the first month of life causes endocrine stress responses, and leads to biochemical, cardiovascular, and behavioral effects during juvenility and adolescence, indicative of increased basal activity and reactivity in stress systems, and mild anhedonia (Dettling *et al*, 2002a, b, 2007; Pryce *et al*, 2004a, b). These findings as a whole indicate that the ED protocol leads to a 'prodepressive' state that is persistent, extending at least to month 12 of life, which is adolescence in this species.

Here we report an initial examination of the long-term neurobiological effects of the ED manipulation, in the same subjects in which the prodepressive *in vivo* effects were demonstrated. We focused on the hippocampus because of its involvement in stress responses and in the pathophysiology of mood disorder. We measured the expression of eight genes selected in light of reported findings in subjects with mood disorder, and because they serve as markers of processes implicated in the pathophysiological links between stress and depression: synaptophysin, growth-associated protein-43 (GAP-43), brain-derived neurotrophic factor (BDNF), microtubule-associated protein-2 (MAP-2), spinophilin, vesicular glutamate transporter 1 (VGluT1), vesicular GABA transporter (VGAT, also called VIAAT), and the serotonin 1A receptor, as well as estimating hippocampal volume. We also inspected for correlations between these neurobiological parameters measured in adolescence, and the *in vivo* biochemical and behavioral variables that were affected by ED.

MATERIALS AND METHODS

The animals were bred, and all *in vivo* studies conducted, at the Laboratory for Behavioural Neurobiology of the Swiss Federal Institute of Technology, Zurich, under experimental permit in accordance with the Swiss Animal Protection Act (1978). The brains were shipped to Oxford for study under license from the Convention for International Trade in Endangered Species of Wild Fauna and Flora, administered by the Swiss Federal Office for Veterinary Affairs and the UK Department for Environment, Food and Rural Affairs.

The Early Deprivation Intervention

The marmoset is characterized by monogamous breeding, dizygotic twins, and high levels of caregiving by both parents. In this study, each of nine sets of parents contributed, in random order, one pair of ED twins and one pair of control twins successively, with an interval of 4–6 weeks between termination of the study with the first pair and the birth of the second pair; one set of parents contributed one pair of ED twins only. The total sample was therefore 10 pairs of ED twins and 9 pairs of control twins (Table 1). The ED intervention and its *in vivo* sequelae have been described in detail previously (Dettling *et al*, 2002a, b, 2007; Pryce *et al*, 2004a, b). Briefly, ED infants were separated from their parents for 30–120 min each day, using variable durations as well as times of day, on postnatal days 2–28. The ED was carried out consecutively within each twin pair,

Table 1 Details of animals and *in vivo* data

	Early deprivation ($n=11$)	Controls ($n=9$)	Significant in vivo effects[a]
Sex	7 (M), 4 (F)	5 (M), 4 (F)	
Age (weeks)	48.4 (range: 43–51)	48.2 (range: 47–50)	
Terminal CSF cortisol (μg/dl)	12.2 (1.31)	10.5 (1.5)	ED × family group interaction[b]
Urinary noradrenaline (ng/mg creatinine)[c]	75.9 (6.5)	56.3 (8.1)	ED main effect[d]
Urinary dopamine (ng/mg creatinine)[c]	973 (143)	690 (86)	ED main effect[e]
Social play in infancy (percent time)	4.4 (0.4)	5.6 (1.1)	Trend to ED main effect[f]
Impulsivity in juvenility (from 64 trials)	13.9 (1.7) ($n=9$)	12.3 (3.5) ($n=6$)	ED × session interaction[e]
Reward motivation in adolescence (average rewards)	8.8 (2.4) ($n=8$)	11.9 (1.9) ($n=7$)	ED main effect[d]

Abbreviation: ED, early deprivation.
Values are mean (SEM).
[a]*In vivo* studies were carried out with both sets of twins (ED–ED and Control–Control) from each of 5–9 breeding pairs.
[b]Dettling *et al* (2007).
[c]Males only. Value averaged from samples taken between weeks 9 and 48.
[d]Pryce *et al* (2004a).
[e]Pryce *et al* (2004b).
[f]Dettling *et al* (2002a).

such that one infant remained with the parents at all times. Controls were briefly handled on the back of the carrying parent on days 2–28. After day 28, subjects remained in the home cage with the family group, and there were no further interventions that differed between ED and control subjects. Additional manipulations were: at age 15 weeks, in three families, subjects were surgically operated to implant a radiotransmitter for the measurement of blood pressure; following a 2-week recovery period, endocrine and behavioral measures of these subjects were within the range of other subjects. At age 18–20 weeks, in all families, subjects were studied in six 60-min tests of isolation from the family in a novel physical environment. Otherwise, all behavioral testing, and the collection of physiological samples, were carried out in the home cage.

At age 48 weeks (range: 43–51 weeks), animals were euthanized. For each group, one subject was removed from the home cage to a procedures room, sedated (Saffan, 10 mg/kg, i.m.), and physiological samples were collected. The animal was then deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.), and the brain either removed and frozen immediately (for the molecular studies), or the animal was transcardially perfused (for hippocampal volumetry; see below). At 1–4 days later, the procedure was repeated with the second twin. Two subjects (two control males) were euthanized at 16 and 38 weeks due to injury and excluded from the present study. The frozen brains comprised 11 ED (7 male and 4 female), and 9 controls (5 male and 4 female); the fixed brains comprised 7 ED (4 male and 3 female), and 6 controls (3 male and 3 female, with 3 brains not studied due to poor perfusion). All brains were coded, and all experiments conducted and analyzed blind to group status.

Brain Processing and Sectioning

The brains to be frozen were rinsed with cold saline, immersed in isopentane at -50°C and stored at -80°C. After warming to -20°C, each brain was serially sectioned on a cryostat in the coronal plane at 10 μm, with two adjacent sections mounted onto each slide, and stored at -80°C. Every 20th slide was taken for Nissl staining, and used to identify the anterior and posterior limits of the hippocampus. Within these limits, for each ISH experiment, the hippocampus was sampled at $\sim$2 mm intervals, giving three hippocampal levels (anterior, middle, and posterior), with two sections at each level, per animal.

For brains to be fixed, animals were transcardially perfused with saline for 2 min followed by 4% paraformaldehyde at 4°C for 12 min. The head was removed and fixed in 4% paraformaldehyde at 4°C for 24 h. The brain was then transferred to 10% sucrose solution for 1×24 h at 4°C, and to 30% sucrose solution for 1×24 h, and then stored at -80°C. These brains were cut at 50 μm on a Leica sliding microtome using solid carbon dioxide, and collected in vials containing cryoprotectant (300 ml glycine, 300 ml ethylene glycol, 400 ml distilled water, 5.4 g disodium hydrogen orthophosphate, and 1.57 g sodium dihydrogen orthophosphate per liter). Every fifth section was mounted on 2% gelatine-treated slides, stained with cresyl violet and coverslipped.

In Situ Hybridization with Oligonucleotides

With the exception of BDNF (see below), *in situ* hybridization was performed with olignonucleotide probes. As marmoset cDNA sequences were not available, the proposed target region of each transcript was amplified from marmoset cDNA using RT–PCR with primers designed to the human cDNA sequence. RT–PCR products were then sequenced, and oligonucleotide probes designed that were 100% complementary to the marmoset cDNA sequence (primers and sequences available on request).

For *in situ* hybridization, the frozen sections were thawed, fixed, acetylated, and delipidated (Law *et al*, 2003). Oligonucleotides were 3′ end labeled with [^{35}S]dATP (1250 Ci/mmol; Perkin Elmer, UK) in a 10 : 1 molar ratio using terminal deoxynucleotidyl transferase (Promega, UK). The experimental conditions and film exposure times for each probe were optimized in pilot studies; the definitive experiment for each transcript was performed in a single experimental run. Sections were incubated overnight at 40–42°C with hybridization buffer containing 1.0×10^6 c.p.m. of labeled probe, as described (Law *et al*, 2003). Postincubation washes were carried out in $1 \times$ SSC at 55°C for 3×20 min and 1 h at room temperature. Experimental controls comprised: hybridization with sense-strand probes, hybridization in the presence of 50-fold excess unlabeled probe, and ribonuclease (RNase A 200 μg/ml at 37°C for 20 min) pretreatment. After air drying, slides were apposed to autoradiographic film (Kodak, Rochester, NY, USA) along with ^{14}C microscales (Amersham Pharmacia Biotech, Sweden) for 3 weeks (GAP-43 and serotonin 1A receptor), 2 weeks (synaptophysin, spinophilin and VGAT), or 1 week (VGluT1 and MAP-2).

Riboprobe *In situ* Hybridization For BDNF

BDNF mRNA was detected using a riboprobe, for which a 1.6 kb segment of the human BDNF cDNA (accession no. M61176) was cloned into Bluescript vector (Stratagene) to generate a cDNA template. The specificity of this clone was previously confirmed by northern blotting (Weickert *et al*, 2003). Sense and antisense riboprobes for BDNF were generated from linearized plasmids using a T7 or T3 polymerase (according to insert orientation), an *in vitro* transcription kit as recommended by the manufacturer (Promega) and ^{35}S-UTP (Amersham Pharmacia Biotech), labeled to a specific activity of $\sim 1.6 \times 10^9$ c.p.m./μg and purified by ethanol precipitation. The sequence recognized by this clone is contained within the common BDNF exon (human chromosome 11: 27635676–27637249) and spans the entire protein coding domain. For BDNF *in situ* hybridization, two hippocampal sections per animal were thawed, fixed in 4% formalin in 0.1 M PBS, acetylated, and dehydrated as described (Whitfield *et al*, 1990). Sections were hybridized with 200 μl of hybridization buffer containing 5 ng/ml of radiolabeled probe at 55°C overnight in a humidified chamber. RNase digestions and stringent post-hybridization washes were performed as described (Whitfield *et al*, 1990). After air drying, slides were apposed to film (Kodak Biomax) for 2 weeks along with ^{14}C microscales.

Receptor Autoradiography with [^{3}H]WAY100635

Autoradiographic analysis of serotonin 1A receptor-binding site densities was carried out using [^{3}H]WAY100635 as described (Burnet *et al*, 1997). Briefly, sections were thawed and preincubated at room temperature in 50 mM Tris-HCl buffer (pH7.4) for 30 min, then incubated in 50 mM Tris-HCl containing 3 nM [^{3}H]WAY100635 for 2 h at room temperature. Nonspecific binding was determined by incubation of adjacent sections with 10 μM 5-HT. Slides were washed with the same buffer for 2×4 min at 4°C. Sections were air dried and apposed to Biomax film for 3 weeks.

Image Analysis and Statistical Analysis

Four hippocampal subfields (dentate gyrus, CA3, CA1, and subiculum) were identified, by reference to a marmoset brain atlas (Stephan *et al*, 1980). Pilot studies did not show consistent rostro–caudal differences in hippocampal expression of any of the genes; hence, autoradiographic signals were measured in each subfield, across all hippocampal sections, and the mean value for each subfield was used for the statistical analysis. All analyses were blind to ED or control status.

Data were examined for normality using the Kolmogorov–Smirnov one-sample test. For each gene, comparisons between ED and control animals were made using a two-stage approach. First, by repeated-measures ANOVA (RM-ANOVA) with subfield as within-subjects factor, and group (ED or control) and sex as between-subjects factors. Sex was included because of the prior evidence for sexual dimorphism in hippocampal gene expression (eg Lustig *et al*, 1993; Zhang *et al*, 1999), in animal models of depression and stress, and in the clinical vulnerability to mood disorder (see 'Discussion'). Where the RM-ANOVA revealed a main effect of ED, or an ED-by-subfield interaction, group differences were examined in each subfield separately using one-way ANOVA. Analyses were carried out using SPSS for Windows (v.15.0).

Estimation of Hippocampal Volume

Hippocampal volume was estimated in cresyl violet-stained sections from the perfusion-fixed brains using point counting and Cavalieri's theorem (Walker *et al*, 2002). A grid, 25×25 mm, was thrown over all sections containing the hippocampus from each animal (mean 24 sections, range: 15–32), and the cross-sectional area of the hippocampus, excluding the subiculum, estimated. The sections were spaced 200 μm apart. No correction was made for tissue shrinkage during processing. Left and right hippocampi were measured separately. Data were analyzed using RM-ANOVA with hemisphere as the within-subjects factor and group and sex as between-subjects factors.

Correlations with *In Vivo* Measures

We explored whether the parameters measured here were related to three *in vivo* biochemical and three behavioral indices that were affected by the ED intervention (see Table 1) using Spearman's correlations. As a partial control against multiple testing, we set $\alpha = 0.02$ and followed up each significant correlation by examining whether it persisted after partialling for the effect of ED; we also inspected whether a similar correlation was present in control and ED groups considered separately.

RESULTS

Each transcript was successfully detected (Figures 1 and 2). Specificity of signal was confirmed using the sense probes, cold displacement, and RNase, which produced negligible signals (not shown). The regional distribution of each mRNA, and for [^{3}H]WAY100635 binding, was consistent with findings in the hippocampus of other species (see 'Discussion').

Effect of Early Deprivation on Expression of Presynaptic Genes

For GAP-43 mRNA (Figure 3a), there was an ED-by-subfield interaction ($F(3,48) = 2.97$, $p = 0.041$), with a trend overall effect of ED ($F(1,16) = 3.40$, $p = 0.084$) but no effect of sex or ED-by-sex interaction. GAP-43 mRNA was decreased after ED in the dentate gyrus ($F(1,18) = 4.52$, $p = 0.048$) and CA3 ($F(1,18) = 5.42$, $p = 0.031$), but not in CA1 or subiculum.

Synaptophysin mRNA (Figure 3b) was unaffected by ED and showed no interactions with subfield or sex. There was a trend effect of sex ($p = 0.067$), which reflected higher levels of synaptophysin mRNA in females than males in CA3 ($p = 0.039$) and CA1 ($p = 0.045$).

VGAT mRNA (Figure 3d) showed an ED-by-subfield interaction ($F(3,48) = 2.93$, $p = 0.043$) and a trend overall effect of ED ($F(1,16) = 4.30$, $p = 0.055$), but no effect of sex or ED-by-sex interaction. VGAT mRNA was increased after ED in CA3 ($F(1,18) = 9.28$, $p = 0.007$) and with a trend in dentate gyrus ($F(1,18) = 4.16$, $p = 0.056$), but not in CA1 or subiculum.

VGluT1 mRNA (Figure 3c) and BDNF mRNA (Figure 3e) did not differ after ED or between the sexes, and there were no subfield interactions.

Effect of Early Deprivation on Expression of Postsynaptic Genes

MAP-2 mRNA (Figure 4a) and spinophilin mRNA (Figure 4b) showed no effects of ED, sex, nor an ED-by-subfield interaction.

Effect of Early Deprivation on Expression of Serotonin 1A Receptors

For serotonin 1A receptor mRNA (Figure 4c) there was an ED-by-sex interaction ($F(1,16) = 4.98$, $p = 0.04$) and an ED-by-sex-by-subfield interaction ($F(2,32) = 3.76$, $p = 0.034$). (The subiculum was excluded from the serotonin 1A receptor RM-ANOVA due to missing data). Subsequent analysis showed that, in CA1, serotonin 1A receptor mRNA was decreased following ED ($F(1,16) = 6.41$, $p = 0.022$) with no sex interaction. There was an ED-by-sex interaction in the dentate gyrus ($F(1,16) = 5.10$, $p = 0.038$) and in CA3 ($F(1,6) = 6.74$, $p = 0.019$). In both subfields, serotonin 1A receptor mRNA tended to be decreased after ED in females but increased in males, but the *post hoc* tests in each sex

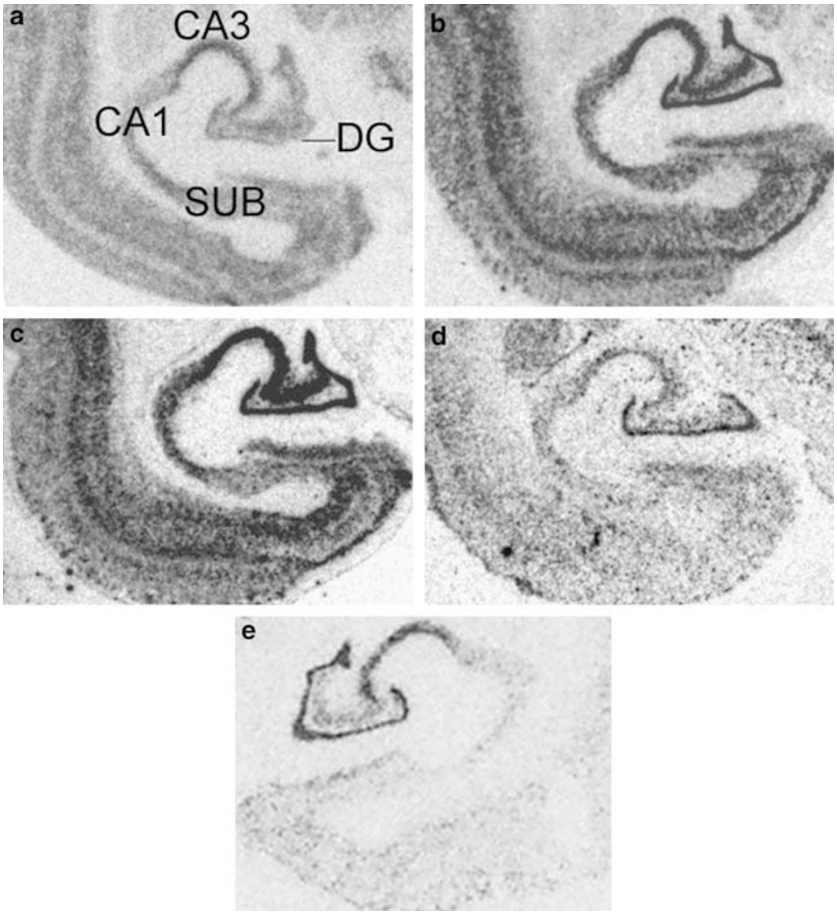

Figure 1 Distribution of five presynaptic protein transcripts in the marmoset hippocampus. (a) Growth-associated protein-43 (GAP-43) mRNA. (b) Synaptophysin mRNA. (c) Vesicular glutamate transporter 1 (VGluT1) mRNA. (d) Vesicular GABA transporter (VGAT) mRNA. (e) Brain-derived neurotrophic factor (BDNF) mRNA. Abbreviations: DG, dentate gyrus; SUB, subiculum.

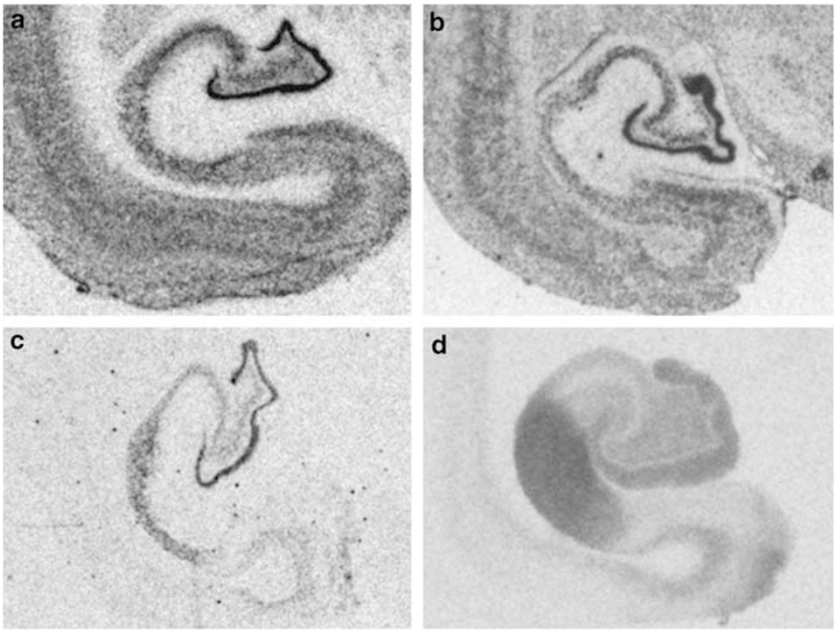

Figure 2 Distribution of four postsynaptically expressed genes in the marmoset hippocampus. (a) Microtubule-associated protein-2 (MAP-2) mRNA. (b) Spinophilin mRNA. (c) Serotonin 1A receptor mRNA. (d) [^{3}H]WAY100635 binding.

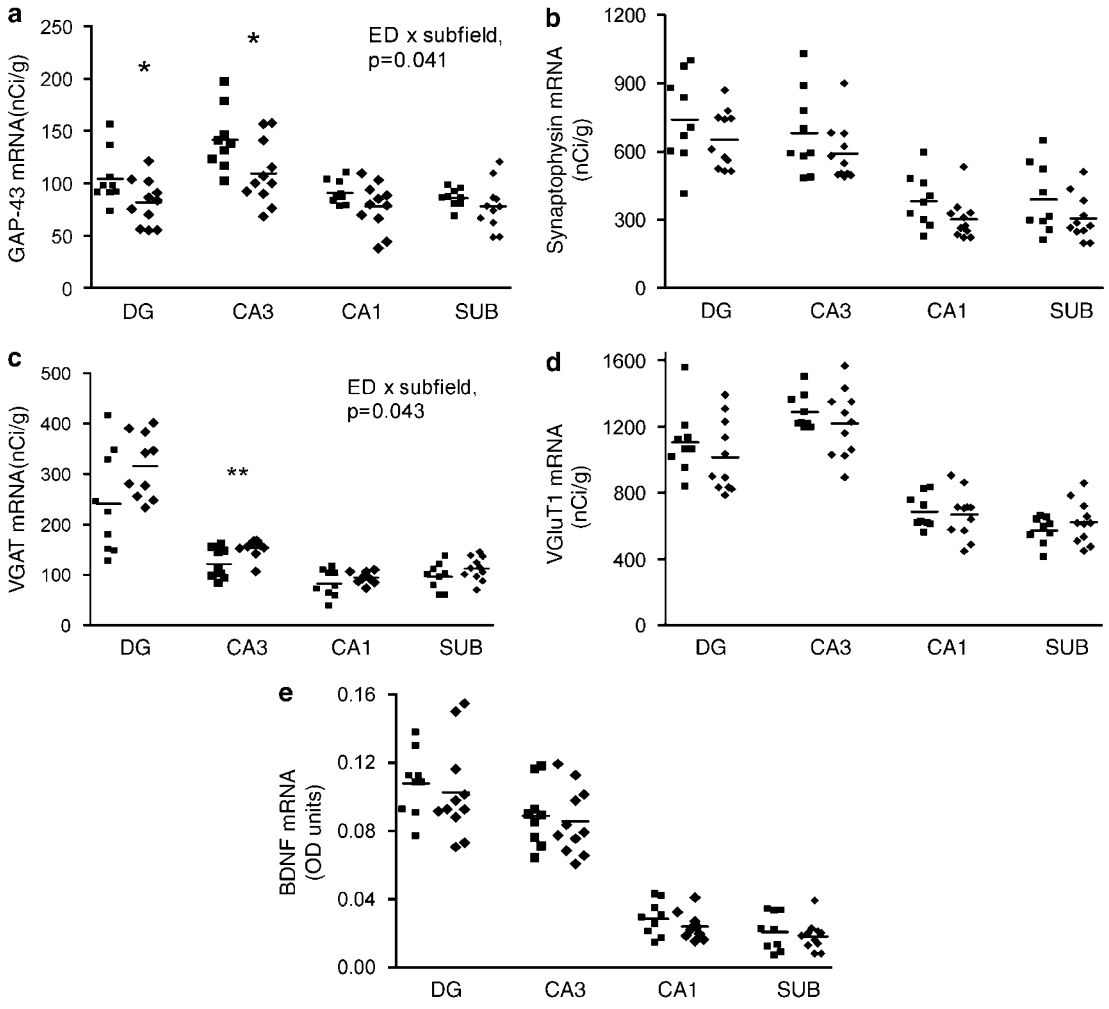

Figure 3 Expression of presynaptic genes in the marmoset hippocampus, in animals subjected to early deprivation (ED, diamonds and controls, squares). (a) Growth-associated protein-43 (GAP-43) mRNA. (b) Synaptophysin mRNA. (c) Vesicular GABA transporter (VGAT) mRNA. (d) Vesicular glutamate transporter I (VGluT1) mRNA. (e) Brain-derived neurotrophic factor (BDNF) mRNA. Abbreviations: DG, dentate gyrus; Sub, subiculum. $^{*}p<0.05$, $^{**}p<0.01$.

were not significant ($p=0.07$–0.16). For example, in CA3, control *vs* ED: females, 66 ± 4 *vs* 52 ± 4 nCi/g ($t=1.63$, $p=0.15$); males, 54 ± 5 *vs* 66 ± 3 ($t=2.03$, $p=0.07$).

The density of [^{3}H]WAY100635-binding sites showed an ED-by-subfield interaction ($F(3,48)=3.01$, $p=0.039$) but no interaction with sex. [^{3}H]WAY100635 binding was decreased by ED in CA1 ($F(1,16)=4.70$, $p=0.046$) with no change in dentate gyrus or subiculum (Figure 4d). In CA3, there was an ED-by-sex interaction ($F(1,16)=5.62$, $p=0.031$); *post hoc* tests showed increased CA3 [^{3}H]WAY100635 binding in males after ED (controls *vs* ED: 16.2 ± 1.0 *vs* 19.4 ± 1.0 nCi/g, $t=2.30$, $p=0.043$) with no alteration in females (controls *vs* ED: 16.4 ± 2.0 *vs* 18.9 ± 0.6 nCi/g, $t=1.21$, $p=0.27$).

Effect of Early Deprivation on Hippocampal Volume

For hippocampal volume, there was an effect of sex ($F(1,19)=5.59$, $p=0.042$), with the hippocampus being larger in females (54.3 ± 2.5 mm^3) than males (46.2 ± 2.3 mm^3). However, there was no effect of ED, no ED-by-sex interaction, and no left–right difference (Table 2).

Correlations with *In Vivo* Measures

Several of the exploratory correlations between the *in vivo* measures and adolescent gene expression met our criteria for significance. (a) Social play in infancy, which is decreased by ED (Dettling *et al*, 2002a), correlated inversely with [^{3}H]WAY100635 binding in CA3 (Figure 5a; $r=-0.606$, $n=20$, $p=0.005$; partialling for ED: $r=-0.566$, d.f. $=17$, $p=0.011$), and with a similar trend in ED and control groups (respectively, $r=-0.790$, $n=11$, $p=0.004$; $r=-0.594$, $n=9$, $p=0.09$). (b) In adolescence, ED marmosets exhibited reduced motivation for palatable reward in terms of fewer operant responses on a progressive ratio reinforcement schedule (Pryce *et al*, 2004a), and this measure of mild anhedonia correlated with VGluT1 mRNA in CA1 (Figure 5b; $r=0.707$, $n=15$, $p=0.003$; partialling for ED: $r=0.655$, d.f. $=12$, $p=0.011$); the correlation was present in ED ($r=0.719$, $n=8$, $p=0.045$) and control ($r=0.893$, $n=7$, $p=0.007$) groups. (c) Urinary noradrenaline is raised by ED, from late infancy to adolescence (Pryce *et al*, 2004a), and average levels correlated negatively with synaptophysin mRNA (Figure 5c; $r=-0.733$, $n=11$, $p=0.01$), with a similar finding in ED ($r=-0.899$, $n=6$,

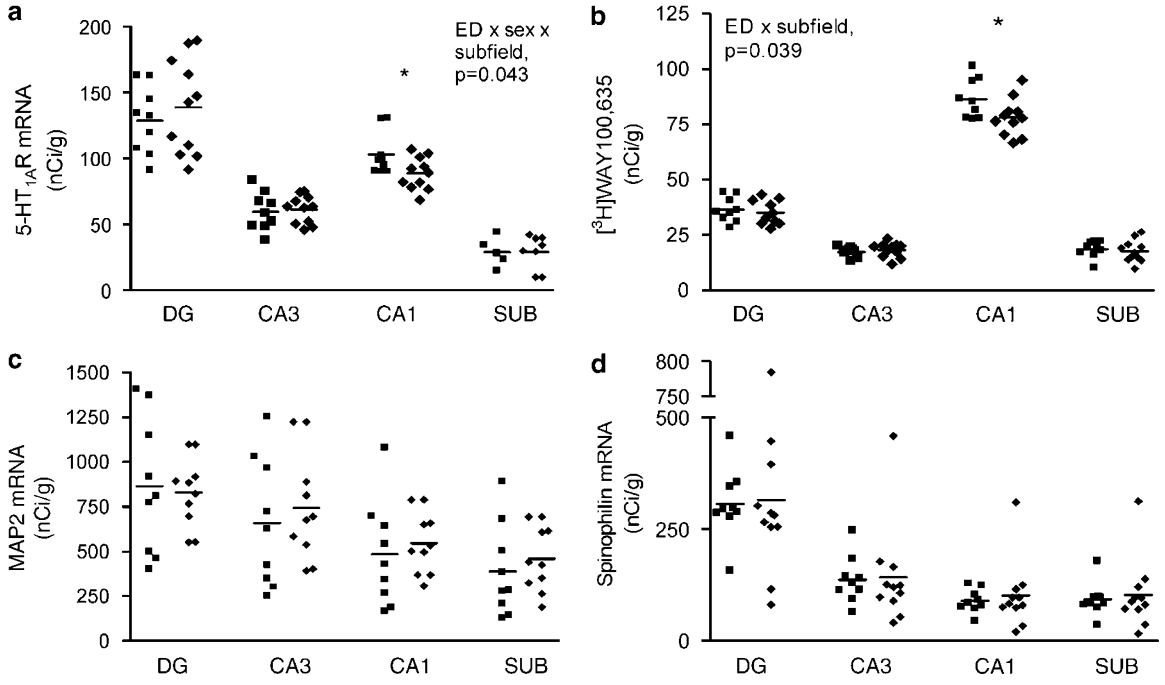

Figure 4 Expression of postsynaptic genes in the marmoset hippocampus, in animals subjected to early deprivation (ED, diamonds and controls, squares). (a) Serotonin 1A receptor mRNA. In addition to the decrease in CA1 in the ED group, there are ED-by-sex interactions in DG and CA3 (see text). (b) [^{3}H]WAY100635 binding. In addition to the decrease in CA1 in the ED group, there is an ED-by-sex interaction in CA3 (see text). (c) Microtubule-associated protein-2 (MAP-2) mRNA. (d) Spinophilin mRNA. One animal in the ED group had spinophilin mRNA values >2 SD greater than all others; there remains no difference between ED and control groups when it is omitted. Abbreviations: DG, dentate gyrus; Sub, subiculum. *$p<0.05$.

Table 2 Hippocampal volumes following early deprivation

	Controls ($n=6$)	ED animals ($n=7$)
Left hippocampus (mm^3)	49.3 (3.1)	51.7 (2.1)
Right hippocampus (mm^3)	46.6 (3.5)	51.8 (3.3)

Abbreviation: ED, early deprivation.

$p=0.015$) and control ($r=-0.700$, $n=5$, $p=0.18$) groups. (d) Finally, terminal (ie adolescent) CSF cortisol level correlated with serotonin 1A receptor mRNA, especially in the dentate gyrus (Figure 5d; $r=0.530$, $n=20$, $p=0.02$). The correlation survived partialling for the effect of ED ($r=0.503$; d.f.$=16$; $p=0.033$), sex ($r=0.519$; d.f.$=16$; $p=0.027$), or both ($r=0.496$; d.f.$=15$; $p=0.043$). The correlation was present in the ED group ($r=0.818$, $n=11$, $p=0.002$) but not in the controls ($r=0.095$, $n=8$, $p=0.82$).

Hippocampal volume did not correlate with any of the *in vivo* measures, and neither impulsivity nor urinary dopamine correlated with expression of any of the genes.

DISCUSSION

As noted in the Introduction, early life stressors in rodents, such as maternal separation, produce long-term biochemical, endocrine, and behavioral alterations. These complement, and may help explain, the epidemiological findings that early life adversity confers an increased risk of mood and emotional disorders later in life in humans. The hippocampus is centrally implicated in the response to early life adversity, and in mood disorder. Here, we studied a recently validated nonhuman primate model of depression vulnerability to explore the hypothesis that ED would produce long-term alterations in the expression of genes implicated in hippocampal synaptic plasticity and functioning, and in the pathophysiology of mood disorder. Our hypothesis was supported by some findings (eg decreased GAP-43 and serotonin 1A receptor expression) but not others (eg unchanged BDNF mRNA and hippocampal volume).

Expression Profiles of the Genes in the Marmoset Hippocampus

Each transcript showed a regional distribution within the marmoset hippocampus that was as anticipated from *in situ* hybridization studies in other primates and in rodents. The profile for [^{3}H]WAY100635 with enhancement in CA1 was also as expected (Burnet *et al*, 1997). Here we note two points of interest.

In the rodent, GAP-43 mRNA is expressed by neurons in the hippocampus proper but is barely detectable in the dentate gyrus (McNamara *et al*, 1996). In contrast, in the macaque (Higo *et al*, 1998) and human (Eastwood and Harrison, 1998), GAP-43 mRNA is strongly expressed by granule cells of the dentate gyrus; indeed, it is more abundant therein than in other hippocampal subfields. The New World marmoset appears in this respect to lie between the rodent and these higher Old World primate species, with signal for dentate gyrus GAP-43 mRNA being robust, but less than that seen over CA3.

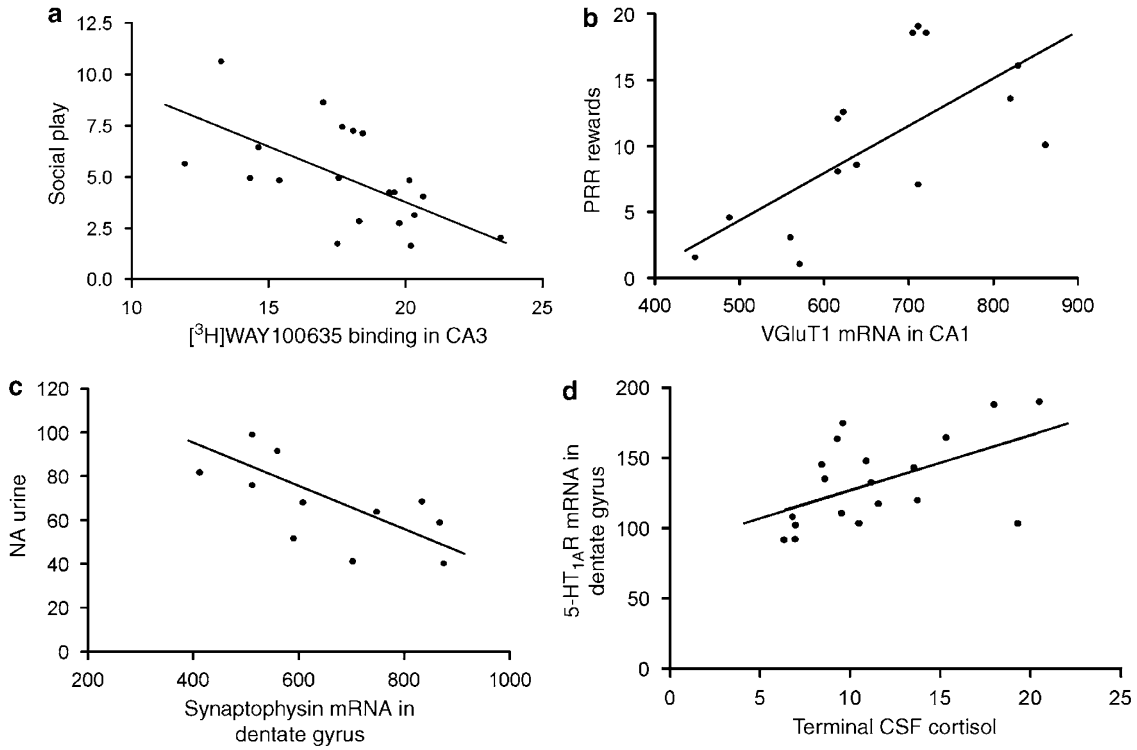

Figure 5 Correlations between gene expression and *in vivo* measures. (a) [^{3}H]WAY100635 binding in CA3 correlates inversely with social play during infancy ($r=-0.606$, $p=0.005$, $n=20$). (b) Vesicular glutamate transporter 1 (VGluT1) mRNA in CA1 correlates with the number of progressive ratio reinforcements, a measure of motivation for reward ($r=0.707$, $p=0.003$, $n=15$). (c) Dentate gyrus synaptophysin mRNA correlates inversely with urinary noradrenaline, measured in males only ($r=-0.733$, $p=0.01$, $n=11$). (d) Dentate gyrus serotonin 1A receptor (5-HT$_{1A}$R) mRNA correlates with CSF cortisol ($r=0.530$, $p=0.02$, $n=20$).

The VGAT mRNA dentate gyrus signal was shown in emulsion-dipped sections to arise from expression by hilar neurons adjacent to the granule cell layer (data not shown); similarly, the punctate VGAT mRNA signal in the other subfields arose from scattered medium-sized cells rather than from the pyramidal neurons. This pattern is the same as that observed in the rat hippocampus (McIntire *et al*, 1997) and is consistent with VGAT mRNA being expressed by GABAergic neurons, in line with its role as the vesicular GABA transporter.

Effect of Early Deprivation on Expression of Synaptic Protein Genes

Hippocampal plasticity in rodents is affected by maternal care, rearing conditions and by other developmental stressors (Liu *et al*, 2000; Andersen and Teicher, 2004; Bogoch *et al*, 2007). One manifestation is a decreased expression of genes that serve as markers of presynaptic terminal density, activity, and/or plasticity, such as synaptophysin and GAP-43 (Andersen and Teicher, 2004; Bredy *et al*, 2004; Reines *et al*, 2008). The same approach has been taken to assess whether similar processes occur in the hippocampus of subjects with mood disorder, and the data show reductions in expression of several presynaptic proteins (Eastwood and Harrison, 2000; Fatemi *et al*, 2001; Webster *et al*, 2001; Vawter *et al*, 2002; Tian *et al*, 2007). Here, we found that ED decreases hippocampal expression of GAP-43, suggestive of a long-term effect on hippocampal synaptic plasticity, at least as indicated by this marker (Benowitz and Routtenberg, 1997). In contrast, synaptophysin expression did not change following ED. Synaptophysin is usually considered a marker of presynaptic terminal density rather than plasticity (Harrison and Eastwood, 2001), and as such our data suggest that overall hippocampal synaptic density is not markedly altered by ED. However, dentate gyrus synaptophysin expression was inversely correlated with level of urinary noradrenaline, which is increased in ED marmosets (Pryce *et al*, 2004a) as well as in depressed children with a history of early life adversity (DeBellis *et al*, 1999), and is indicative of sympathoadrenal hyperactivity. This suggests that presynaptic terminal density may be important for the hippocampus in regulating autonomic functions (Fuchs *et al*, 1995).

VGluT1 and VGAT are vesicular transporters for glutamate and GABA, respectively. Effects on both transmitters have been reported after other developmental interventions and stressors in rats (eg Hsu *et al*, 2003; Pickering *et al*, 2006) and hippocampal VGluT1 expression is modulated by antidepressants (Tordera *et al*, 2005). In mood disorder, there are emerging data to support both glutamatergic (Choudary *et al*, 2005; Yildiz-Yesiloglu and Ankerst, 2006; Hashimoto *et al*, 2007) and GABAergic (Brambilla *et al*, 2003; Taylor *et al*, 2003) dysfunction. Following ED, we found that VGAT mRNA was increased whereas VGluT1 mRNA was unchanged. Importantly, there is a quantitative relationship between the expression of these transporters and the release of their transmitter (De Gois *et al*, 2005;

Wilson *et al*, 2005); moreover, vesicular loading by VGAT is directly coupled to GABA synthesis (Jin *et al*, 2003). Hence, the elevation of VGAT expression following ED suggests an enhancement of hippocampal GABA signaling, a possibility that requires direct investigation. GABA signaling is altered in rodents following developmental stressors (Hsu *et al*, 2003; Caldji *et al*, 2000) and in subjects with mood disorder (Brambilla *et al*, 2003; Taylor *et al*, 2003; Frey *et al*, 2007), including altered hippocampal expression of GABAergic genes (Sequeira *et al*, 2007). However, these prior findings tend to implicate impaired GABA functioning. As such, the increased VGAT expression observed here may be an adaptive response to the effects of ED on other determinants of GABA signaling. If so, then extrapolating to humans, perhaps enhanced GABA functioning (including increased VGAT) is protective, and that depression occurs when this compensatory process fails. To test this hypothesis, it would be of interest to assess GABA function in healthy subjects at risk of mood disorder by virtue of their exposure to early life stressors, and to follow them longitudinally and study the effect of a first depressive episode. Unchanged expression of VGluT1 suggests that major alterations in glutamate release do not occur after ED but, in the context of enhanced VGAT expression, implies that the balance between inhibitory and excitatory hippocampal transmission is affected. Also, the correlation with responses on the test of motivation for palatable reward that revealed mild anhedonia in ED adolescents provides an indication that hippocampal VGluT1 expression may be functionally relevant (ie with regard to anhedonia) even in the absence of an overall effect of ED. In rodent models of anhedonia induced by chronic stress in adulthood, the hippocampus is certainly a major region of altered activity and gene expression (Holderbach *et al*, 2007; Bergstrom *et al*, 2007). In mood disorder, hippocampal VGluT1 mRNA is unaltered (McCullumsmith and Meador-Woodruff, 2003) and VGAT expression has not been reported.

In addition to studying these presynaptic genes, we utilized molecular markers of dendrites and dendritic spines, namely MAP-2 and spinophilin respectively, to assess the postsynaptic component of synaptic plasticity. In mood disorder, there are preliminary data showing a reduction in spinophilin expression (Law *et al*, 2004) and for morphological alterations in hippocampal dendrites and spine densities (Rosoklija *et al*, 2000). However, no differences in expression of either MAP-2 or spinophilin were seen here, and as such we found no evidence to support an alteration in postsynaptic plasticity after ED. Undoubtedly many other genes are involved in dendritic plasticity, and there is much more to the process than simply changes in gene expression. Nevertheless, the discrepancy following ED between the changes seen presynaptically, and the lack of change postsynaptically, is notable; it was also seen in a microarray study of the hippocampal response to prenatal stress in rodents (Bogoch *et al*, 2007).

Effect of Early Deprivation on Serotonin 1A Receptors

Serotonin is involved in neurodevelopment (Whitaker-Azmitia, 2001), in stress responses (Chaouloff *et al*, 1999), and in the pathophysiology of mood disorder (Deakin, 1998; Mann, 1999), with the serotonin 1A receptor being important in all these situations (Cowen *et al*, 1994; Sibille and Hen, 2001; Strobel *et al*, 2003). Three neuroimaging studies of mood disorder show decreased hippocampal serotonin 1A receptor binding (Drevets *et al*, 1999; Sargent *et al*, 2000; Hirvonen *et al*, 2008), and hippocampal serotonin 1A receptor mRNA is also decreased (Lopez-Figueroa *et al*, 2004). Reduced hippocampal serotonin 1A receptor binding has also been seen in long-tailed macaques with behavioral depression (Shively *et al*, 2006), and serotonin 1A receptor expression and/or function are decreased by some experimental stressors in rodents (Lopez *et al*, 1998; Van Riel *et al*, 2004; Griffin *et al*, 2005). Moreover, a polymorphism in the serotonin 1A receptor gene has been associated with depression-related personality traits and with lower postsynaptic serotonin 1A receptor expression (Strobel *et al*, 2003; Czesak *et al*, 2006).

Although hippocampal serotonin 1A receptor reductions have not always been replicated in mood disorder (Stockmeier, 2003; Parsey *et al*, 2006), or following maternal separation in rodents (Neumaier *et al*, 2002), the evidence as a whole led us to predict that ED would result in lowered hippocampal serotonin 1A receptor expression. This is what was observed, in CA1, for both serotonin 1A receptor mRNA and receptor binding. Our results suggest that a diminished serotonin 1A receptor availability, particularly in CA1, may be an 'at risk' marker for mood disorder, consistent with the fact that hippocampal serotonin 1A receptor binding remains decreased after recovery from depression (Sargent *et al*, 2000; Bhagwagar *et al*, 2004), and that it may arise as a result of early adverse experiences.

Apart from CA1, interpretation of the serotonin 1A receptor findings is complicated by the interactions between ED and sex. In CA3 and dentate gyrus, only females showed decreases in serotonin 1A receptor mRNA and [^{3}H]WAY100635 binding, whereas males tended to show increases. Although the *post hoc* statistical tests for males and females separately were not conclusive (reflecting the small sample size), these findings are in line with other results suggestive of sexual dimorphisms, affecting relationships between serotonin 1A receptor function, stress, and mood disorder. For example, neonatal handling affects hippocampal serotonin 1A receptors more in female than male rats (Stamatakis *et al*, 2006), and females have a greater vulnerability to mood disorder clinically (Kendler *et al*, 2006) and in animal models of depression (Faraday, 2002; Barna *et al*, 2003). If our finding extrapolates to humans, it might indicate that women are more prone to serotonin 1A receptor downregulation in response to environmental stressors, and that this may play a role in their greater vulnerability to mood disorder.

If hippocampal serotonin 1A receptor reduction is part of the molecular mechanisms that mediate between early life stress and an enhanced risk of mood disorder, one co-candidate is HPA axis dysfunction. Hippocampal serotonin 1A receptor expression is regulated by corticosteroids, with elevated levels of the latter typically reducing expression of the former (Burnet *et al*, 1992; Chalmers *et al*, 1993; Le Corre *et al*, 1997; Meijer *et al*, 1997; Neumaier *et al*, 2000; but see Montgomery *et al*, 2001), in part by transcriptional regulation (Meijer *et al*, 2000). However, we found no inverse correlations between cortisol and serotonin 1A

receptor mRNA or binding; indeed the only correlation we did observe was positive (Figure 5d). Of note, ED induces marked acute increases in cortisol in infants (Dettling *et al*, 2002a), but in juvenility and adolescence the elevations are modest and are not seen in morning samples but only in afternoon samples (Pryce *et al*, 2004b; Dettling *et al*, 2007). This suggests that any contribution of ED-induced hypercortisolism to altered serotonin 1A receptor levels occurred in infancy; perhaps the acute increases in cortisol in response to ED influenced the long-term set-point of serotonin 1A receptor expression (see Ansorge *et al*, 2007). Further study is required to establish the interplay between HPA axis alterations and serotonin 1A receptor reductions, and the relationship of both to the effects of ED.

There was an inverse correlation between social play in infancy and CA3 serotonin 1A receptor binding in adolescence, which suggests that the reduced social play observed in ED infants might have been related to increased 5-HT function, as reported for peri-adolescent rats (Homberg *et al*, 2007).

Effect of Early Deprivation on BDNF Expression

The prominent BDNF hypothesis of mood disorder postulates that a deficiency of BDNF is important in its pathophysiology, and that BDNF normalization is a key part of the therapeutic mechanism of antidepressants (Duman *et al*, 1997; Hashimoto *et al*, 2004). The theory has a firm preclinical basis, in that there is much evidence for a critical role of BDNF in neurodevelopment and plasticity (Huang and Reichardt, 2001), and for hippocampal BDNF being reduced after a range of stressors (Duman and Monteggia, 2006), including in adult rats that had been subjected to brief maternal deprivation (Roceri *et al*, 2002). The roles of BDNF in human hippocampal function are also not in doubt (Egan *et al*, 2003). In depressed subjects, several studies show reduced serum BDNF levels (see Duman and Monteggia, 2006), and two found decreased hippocampal BDNF expression in suicide victims, many of whom were depressed (Dwivedi *et al*, 2003; Karege *et al*, 2005), whereas another reported higher hippocampal BDNF immunoreactivity in depressed subjects on antidepressants at death compared to those who were not (Chen *et al*, 2001).

Given these findings, we predicted that BDNF expression would be lower following ED, reflecting the intervention itself and its prodepressive consequences. However, we found no changes in BDNF mRNA. Notably, contrary to Roceri *et al* (2002), rodent studies of repeated maternal separation find hippocampal BDNF expression in adulthood to be unchanged (Roceri *et al*, 2004) or increased (Greisen *et al*, 2005), or to differ between splice variants (Nair *et al*, 2006). These inconsistent findings likely reflect the complex temporal and molecular regulation of BDNF expression (Pruunsild *et al*, 2007), and other factors such as the timing and duration of the intervention, and the interval between the intervention and measurement (Pryce *et al*, 2005a; Lippmann *et al*, 2007). Any of these variables might explain our negative BDNF result. With regard to mood disorder, the present finding raises the possibility that reduced BDNF in that situation is independent of the early stressor component of pathogenesis. For example, it might be part of a genetic predisposition, or not emerge until a significant depressive episode occurs. In any event, there is also a negative study of hippocampal BDNF expression in mood disorder (see Knable *et al*, 2004), and criticism of the 'deficient BDNF hypothesis' itself (Groves, 2007).

Effect of Early Deprivation on Hippocampal Volume

We had anticipated that the ED intervention might lead to hippocampal atrophy, given that human imaging studies have reported smaller hippocampi not only in mood disorder (Campbell *et al*, 2004) but also in people who had experienced severe early life stress, regardless of their later psychiatric diagnosis (Vythilingam *et al*, 2002; Geuze *et al*, 2005). However, not all such studies have been positive (Cohen *et al*, 2006; Lenze *et al*, 2008) and we did not find any effect of ED on hippocampal volume. This finding supports the view that hippocampal atrophy in mood disorder is not related to the early developmental component of pathogenesis, but is a correlate or consequence of the disorder (Czeh and Lucassen, 2007). This interpretation is consistent with the fact that reduced hippocampal volume is best established in those with chronic or recurrent depressive episodes (Sheline *et al*, 1996; Campbell *et al*, 2004). Although the ED animals exhibited decreased motivation for reward, suggestive of mild anhedonia (Pryce *et al*, 2004b), they did not exhibit a true depression-like phenotype. In the ED paradigm, stress is confined to a specific period and thereafter subjects remain undisturbed in the family group: it would be of interest to assess whether hippocampal volume would be reduced if ED were combined with a subsequent stressor applied in adolescence. Finally, there may be a genetic predisposition to smaller hippocampal volume in mood disorder (Frodl *et al*, 2007), which would not be captured here.

CONCLUSIONS

The present study shows that early life stress in a nonhuman primate produces long-term effects on hippocampal gene expression, suggestive of alterations in synaptic functioning and plasticity. The correlations seen between gene expression and the *in vivo* behavioral and biochemical effects of ED speak to the potential functional significance of the molecular alterations. The congruence between the ED effect and observations in mood disorder, especially the reduction in serotonin 1A receptors, suggests that hippocampal involvement in the disorder begins prior to the first illness episode, and may reflect at least partly the role that early developmental adversity plays in its origins. Conversely, the lack of effect of ED on other indices, such as hippocampal volume and BDNF mRNA, suggests that their involvement in mood disorder arises from different aspects of the pathogenic process, or may be secondary to the effects of illness or its treatment.

Clearly, many further issues await investigation in this marmoset model of the long-term effects of early life stress. First, to measure the encoded proteins; this was not possible here due to tissue limitations. Second, to ascertain the role that HPA axis alterations, notably glucocorticoid and mineralocorticoid receptors, play in the response to ED (Pryce *et al*, 2005b). Third, to examine other limbic regions

such as the medial prefrontal cortex, wherein comparable changes related to stress (Cohen *et al*, 2006; Ichise *et al*, 2006; Burton *et al*, 2007) and mood disorder (Manji *et al*, 2001; Harrison, 2002; Choudary *et al*, 2005) also occur. Finally, expression profiling (Sabatini *et al*, 2007) and study of the cellular changes that may follow ED, such as altered hippocampal neurogenesis (Sahay and Hen, 2007), will help paint a more detailed picture.

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DISCLOSURE/CONFLICT OF INTEREST

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