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Altered gene expression in hippocampus and depressive-like behavior in young adult female mice by early protein malnutrition

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Perinatal development represents a critical period in the life of an individual. A common cause of poor development is that which comes from undernutrition or malnutrition. In particular, protein deprivation during development has been shown to have deep deleterious effects on brain's growth and plasticity. Early-life stress has also been linked with an increased risk to develop different psychopathologies later in life. We have previously shown that perinatal protein malnutrition in mice leads to the appearance of anxiety-related behaviors in the adulthood. We also found evidence that the female offspring was more susceptible to the development of depression-related behaviors. In the present work, we further investigated this behavior together with its molecular bases. We focused our study on the hippocampus, as it is a structure involved in coping with stressful situations. We found an increase in immobility time in the forced swimming test in perinatally malnourished females, and an alteration in the expression of genes related with neuroplasticity, early growth response 1, calcineurin and c-fos. We also found that perinatal malnutrition causes a reduction in the number of neurons in the hippocampus. This reduction, together with altered gene expression, could be related to the increment in immobility time observed in the forced swimming test.

Keywords: Calcineurin, c-fos, depressive-like behavior, early-life stress, Egr1, forced swimming test, hippocampus, malnutrition, mice, perinatal period

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Early perinatal life represents a sensitive and critical period for an individual. The quality of the embryonic environment and postnatal experiences have a great influence on the emotional and cognitive development of the infant and later in life (Andersen 2003; Fox *et al.* 2010; Lupien *et al.* 2009). Indeed, early-life stress, such as extreme poverty, nutritional deficiency, social deprivation or abuse, has long-lasting effects on the brain and has been shown to predict vulnerability or resilience to the onset of psychopathologies including depression, anxiety and autism in the offspring (Danese & McEwen 2012; Kim *et al.* 2013; McCrory *et al.* 2011).

Maternal malnutrition, because of its widespread incidence, remains one of the major early-life adversities (non-genetic factors) affecting the development of newborn's brain (World Health Organization 2012). An increasing number of studies point out that the effects of early-life nutritional inadequacy are persistent and lead to permanent deficits in learning and behavior (Burdge & Lillycrop 2010; Chmurzynska 2010; McGaughy *et al.* 2014). While all nutrients have certain influence on brain maturation, proteins appear to be most critical for the development of neurological functions (Kar *et al.* 2008; Morgane *et al.* 2002). Experimental studies on rodents have shown that protein malnutrition in early stages of life could alter neurogenesis, cell migration, differentiation and plasticity (de Godoy *et al.* 2013; Ranade *et al.* 2008; Rotta *et al.* 2008). It has been shown that both prenatal and postnatal protein malnutrition significantly decrease play behavior in rats (Maria Moreira Camargo & de Sousa Almeida 2005). In addition, reported effects of prenatal and/or postnatal protein malnutrition on the offspring include changes in exploratory behavior and anxiety, altered learning and memory abilities and a different response to aversive stimuli (Alamy & Bengelloun 2012; Belluscio *et al.* 2014; Laus *et al.* 2011; Reyes-Castro *et al.* 2012).

In addition to gene and environment interaction, sex is another important factor to consider in regard to vulnerability to psychopathologies. Sex differences in response to prenatal and early-life stress put males at an increased risk to present neurodevelopmental disorders including autism, attention deficit and Tourette's syndrome, while major depressive disorder affects twice as many females as males (Bale & Epperson 2015; Scott 2011). Although there are no sex differences in affective disorders before puberty, females show an increased risk in adolescence and throughout adulthood. These sex differences are seen in multiple countries and cultures, suggesting there is a biological basis underneath its expression (Altemus 2006). Current research with the use of animal models shows robust neural and behavioral sex differences in response to physiological stimuli such as stress, and to pharmacological agents such as psychoactive drugs and alcohol (Gomez & Luine 2014; Papaioannou *et al.* 2002). In

particular, several evidences show that early-life stress exacerbates emotional dysfunction and depression-like behavior when female but not male rodents are exposed to acute stressful stimuli in adult life (Korosi *et al.* 2012). Recently, we observed that maternal protein restriction during pregnancy and lactation delayed the physical growth and neurodevelopment and negatively affected motivation and exploratory activity of the offspring in a sex-independent manner. However, only female offspring exhibited traits of hopelessness behavior when subjected to the tail suspension test (Belluscio *et al.* 2014).

Given the increased incidence of affective disorders in female mice exposed to early-life adversities, we decided to investigate the molecular basis of the depression-like behavior exhibited by adult female mice that were subjected to perinatal protein malnutrition. We focused our study on the hippocampus because part of the limbic system has a major role in cognition and mood regulation by modulating anxiety states and depression (McEwen *et al.* 2015). We hypothesized that deregulation of hippocampal immediate early gene (IEG) expression in response to acute stressful stimuli is related with the emergence of this emotional trait. We show that, as adults, perinatal protein-restricted female offspring exhibit traits of depression-like behavior. In addition, this offspring displays an altered expression of IEGs, early growth response 1 (Egr1) and c-fos, and calcineurin in response to a traumatic stimulus compared with female offspring born from normal nourished dams.

Materials and methods

Animals and diets

CF-1 mice (crlfcen:CF1) from the colony of the Bioterio Central, Facultad de Ciencias Exactas y Naturales (University of Buenos Aires), were used for all experiments. The diets used in this study were formulated by Research Diets Inc. (New Brunswick, NJ, USA) according to the AIN-93 final report (Reeves *et al.* 1993). Normal protein (NP) diet contains 20% casein, 10% sucrose, 5% alpha cellulose, 7% soybean oil, 3.5% mineral mixture, 1% vitamin mixture, 0.3% L-cystine, 0.25% choline bitartrate and 52.95% starch dextrins. Instead, low-protein (LP) diet contains 8% casein and 64.95% starch dextrins, and was formulated to be isocaloric to the NP diet. Lowering the protein content also lowers the amount of phosphorus in the diet. This was compensated by the addition of potassium phosphate (10.7 g/kg of diet) to the mix.

Female mice (F0) were fed with NP diet for 4 days and then switched to the assigned diet (NP or LP) for 3 days prior mating. Male mice from the F0 were only fed with NP diet for 3 days prior mating. For mating, one male was housed with two nulliparous females for five consecutive days. Ten days after the end of the mating period, female mice were individually housed and kept under the same diet until weaning (P21). Weight was regularly measured during both pregnancy and lactation. On postnatal day 2 (PD2), litters were adjusted to 8–9 pups per litter with a 1:1 male:female ratio when possible. The offspring (F1) was fed with regular laboratory chow after weaning. Their weight was measured on a periodical basis, until animals were transferred to the behavioral testing area 3 weeks before testing. Food consumption was measured on a daily basis during pregnancy, lactation and once a week after weaning in the pups.

Animals were kept in a 12-h light/dark cycle with lights on at 0600 h, and food and water were administered *ad libitum*. Experiments were performed in accordance with local regulations and the National Institutes of Health (NIH) *Guide of the Care and Use of Laboratory Animals* (NIH publication 80-23/96) and were previously approved by the Ethical Committee (CICUAL) of the Facultad de Ciencias Exactas y Naturales, University of Buenos Aires (Protocol 24/12). For each test, only a maximum of three pups per mother were used in order to minimize the litter effect and also to comply with ethical issues. For the tail suspension and open field test, the offspring from 9 NP- and 8 LP-/fed dams were used. For each treatment, three pups from this cohort were left untested and designated to immunohistochemistry analysis. For the forced swimming test (FST) and subsequent mRNA expression analysis, the F0 consisted of 11 NP and 10 LP dams. Three untested animals from this cohort were used for protein extraction and western blot. Male littermates of the F1 were assigned to another set of experiments not related to this manuscript.

Behavioral testing

For behavioral testing, mice were left mainly unhandled, except for routine cage changes. Before every test, subjects were habituated to the testing room (adjacent to the housing area) for 40–50 min. Tests were performed with a constant background noise of 60 dB. Lights were fixed to 100 lx and tests were recorded with a video camera using the ANY-MAZE™ 5.1 VIDEO TRACKING SOFTWARE (Stoelting, Wood Dale, IL, USA). The same cohort of animals was used first for the open field and then for the tail suspension test at 10 and 11 weeks of age, respectively. The FST and subsequent gene expression analysis were performed in a different cohort of animals at 10 weeks of age. All tests were performed between 0900 and 1200 h.

Open field test

The open field test was performed in a 45-cm side square arena with dark walls (30 cm height). Mice were placed next to one of the walls and left to explore the environment for 20 min. The whole session was video-recorded and mice were tracked using the ANY-MAZE.

Tail suspension test

Animals were tested as previously described (Belluscio *et al.* 2014). Briefly, mice were suspended by the tail using cotton adhesive tape to fix them to the apparatus, which consisted of a thin wire supported by two sticks. The whole session (6 min) was video-recorded and immobility time was registered by a blind observer.

Forced swimming test

Animals were placed in a 5-l beaker ($18 \times 26\ cm^2$) containing 3 l of clean water at 25°C for 6 min. Testing sessions were video-recorded using the ANY-MAZE. Immobility was defined as lack of motion of the body with the exception of those movements necessary to keep the head above water (Costa *et al.* 2013), and was recorded by an observer blind to the animal treatment. Only the last 4 min were taken into account for scoring. Animals were killed by cervical dislocation 1 or 24 h after the end of the test, and both hippocampi were dissected, immediately frozen in liquid nitrogen and stored at −80°C until posterior use. Hippocampi from untested animals were used as time 0 controls.

Gene expression analysis

Total RNA was obtained from frozen samples using the RNAzol RT reagent (Sigma), St. Louis, MO, USA according to the manufacturer's instructions. RNA content and purity were measured with a NanoDrop 2000 (Thermo Waltham, MA, USA Scientific). Samples were pretreated with RQ1 DNase (Promega) Madison, WI, USA and cDNA was synthesized using 1 μg of total RNA per 200 U of M-MLV reverse transcriptase (Promega) and oligo(dT) primers. Parallel reverse transcription mixtures with no enzyme were prepared in order to detect any possible DNA contamination. Real-time quantitative reverse transcriptase polymerase chain reaction (PCR) of cDNA samples was performed using the MyiQ2 Two-Color detection system (Bio-Rad, Hercules, CA, USA) and SYBRgreen (Molecular Probes, Eugene, OR, USA) fluorescence chemistry. For the amplification reaction,

Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) was used. Primers were designed to include an exon–exon junction (if possible) using PRIMER-BLAST (NCBI), and were as follows: Gapdh Fw-5′ GGCATTGCTCTCAATGACAA and Rv-5′ GGCCTCTCTTGCTCAGTGTC; Pgk1 Fw-5′ TGGGCAAGGATGTTCTGTTC and Rv-5′ TGCAGTCCCAAAAGCATCAT; Hprt Fw-5′ CAGGCCAGACTTTGTTGGAT and Rv-5′ TTGCGCTCATCTTAGGCTTT; c-fos Fw-5′ TTCGACCATGATGTTCTCGGG and Rv-5′ TTGGCACTAGAGACGGACAG; Egr1 Fw-5′ TTCAATCCTCAAGGGGAGCC and Rv-5′ GAGAAGCGGCCAGTATAGGT; activity-regulated cytoskeleton-associated protein (Arc) Fw-5′ CCTACAGAGCCAGGAGAATGAC and Rv-5′ GAAGAGAGGATGGTGCTGGTG; CalN Fw-5′ GCGATTGATCCCAAGTTGTC and Rv-5′ TGCCCTCCTTCATGAGATGT. For each pair of primers, melting curves were generated to select the appropriate annealing temperature and $MgCl_2$ concentration for the PCR. The PCR amplification protocol was: (1) 95°C for 5 min, (2) 94°C for 5 seconds, (3) 60°C (except for Egr1, for which 61°C was used) for 20 seconds and (4) 72°C for 25 seconds (repeat steps 2–4 40 times). The melting curve was measured from 55 to 95°C with a 0.5°C increment. Serial dilutions of mouse cDNA samples were prepared in order to determine each pair of primer's linear range of amplification. The raw fluorescence data were analyzed using LINREGPCR (Ruijter *et al.* 2009) and N_0 values were normalized against the geometric mean of the N_0 of three reference genes (*Hprt*, *Gapdh* and *Pgk1*).

Western blot

Protein extraction of hippocampi from untested females was carried out using the protocol described in the study of Ericsson *et al.* (2007) with minor modifications. Briefly, homogenization of frozen samples was performed in sodium dodecyl sulfate (SDS) buffer (2% SDS, pH 6.8) with a 1.5 microtube pestle. Samples were subsequently incubated at 70°C for 10 min and centrifuged during 15 min at 12.000 ***g***. Western blot analysis was carried out as previously described (Ogara *et al.* 2014). Protein concentration was estimated using the bicinchoninic acid protein assay kit (Sigma) and 30 μg of protein was loaded on 10% gels. The following antibodies were used: mouse anti-*β*-actin 1:1000 C4 (Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit anti-Egr1 (588, Santa Cruz Biotechnology, 1:500). Anti-mouse and anti-rabbit secondary antibodies conjugated with horseradish peroxidase were purchased from Sigma.

Immunofluorescence staining

Untested females from the F1 were deeply anesthetized with avertin (0.4 mg avertin/g i.p.) and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde. Brains were postfixed overnight in 4% paraformaldehyde and coronal 60-μm sections were cut using the Integraslice 7550PSDS vibrating microslicer (Campden Instruments, Loughborough, England). Slices were stored in a cryoprotectant solution (25% glycerol, 25% ethylene glycol and 0.1 M of 50% phosphate buffer, pH 7.4) at −20°C. Eight slices spanning the hippocampal region (from Bregma −1.22 mm to Bregma −2.7 mm) of each animal were used for NeuN immunostaining in free floating slides. Sections were rinsed twice in phosphate-buffered saline (PBS) and incubated for 1 h at room temperature in blocking solution (5% goat serum, 0.3% Triton-X-100) and then for 24 h at 4°C in anti-NeuN mouse monoclonal antibody (MAB377, MIllipore, Billerica, MA, USA 1:200) in blocking solution. Slices were then washed twice for 15 min in 0.04% PBS-Tween and then incubated with the secondary antibody for 1 h at room temperature (anti-mouse Alexa 555, Invitrogen, 1:1000) in blocking solution. Sections were rinsed once more in PBS, counterstained with DAPI (4′,6-Diamidino-2-Phenylindole, Dihydrochloride) (Sigma) and then mounted in PBS:glycerol. Absence of non-specific secondary antibody binding was checked using a secondary control without primary antibody in parallel. Images were taken with a confocal microscope (Olympus Fluoview FV1000, Tokyo, Japan) using a ×20 objective and a digital zoom of ×1.8. Two fields of the CA1, one of the CA2, three of the CA3 and three of the dentate gyrus (DG) were selected per slice and two confocal photos were taken per field, 8 μm apart from each other in the *z*- axis. Fields were chosen using the Franklin and Paxinos atlas as a reference (Franklin & Paxinos 2007). NeuN-positive cells were quantified under blind conditions in an area of 0.01486 mm^2 for the CA1, CA2 and CA3 regions, and of 0.00836 mm^2 for the DG. The average number of positive cells was calculated per region for each individual. *N* equals the number of subjects.

Statistical analysis

Statistical tests used throughout the paper are described in the figure legends and text. The Shapiro–Wilks and the Levene tests were used to test the normality and homogeneity of variances, respectively. Studentized residuals were used to detect and remove outliers. Unpaired Student's *t*-test, statistical power, two-way analysis of variance (ANOVA) with orthogonal contrasts and Tukey's posttests were carried out using the INFO-STAT SOFTWARE (Universid ad Nacional de Córdoba, Córdoba, Argentina), while the PRISM 5.01 (GraphPad Software Inc. La Jolla, CA, USA) was used for the repeated-measures (RM) ANOVA with Bonferroni's posttest. A *P*-value of <0.05 was accepted as statistical significance for all tests. For immunofluorescence and western blot experiments, only three animals per diet were used. This number was chosen as it allowed us to detect ~10% differences (in previous experiments) using these techniques with a statistical power of 80%.

Results

Impaired physical development of perinatally protein-malnourished offspring

To assess the impact of dams' malnutrition during pregnancy and lactation on the offspring, the body weight of female mice was measured from weaning until 6 weeks of age (Fig. 1). A two-way ANOVA with RM indicated that there were significant main effects for diet group ($F_{1,135} = 33.16$, $P < 0.0001$) and time ($F_{3,135} = 1181.90$, $P < 0.0001$) but their interaction was not significant ($F_{3,135} = 0.96$, $P = 0.4156$). Bonferroni *post hoc* analysis showed that at weaning the body weights of female LP mice are lower than NP mice ($P < 0.001$) and continues to be significantly lower until being subjected to the behavioral tests ($P < 0.001$). In addition, LP offspring exhibited a delay in both physical and neurological development as shown previously (data not shown; Belluscio *et al.* 2014).

Protein-malnourished female mice display a depression-related behavior

We first performed the tail suspension test to evaluate the effect of perinatal protein malnutrition on behavioral despair, a depression-related behavior. We observed a significant effect of nutritional condition, with LP group spending more time immobile ($t_{45} = 2.294$, $P < 0.05$) comparing with female mice from NP group (Fig. 2a). To further assess this emotional trait, we performed the FST. Similarly, LP mice exhibited higher immobility times ($t_{42} = 2.848$, $P < 0.01$) than NP mice (Fig. 2b). One NP mouse with a very low immobility time was considered an outlier according to the criterion outlined in *Materials and methods* and discarded. The total distance traveled in an open field arena by mice from both groups were similar ($t_{45} = 0.4539$, $P = 0.6521$), indicating that they did not differ significantly in locomotive activity (Fig. 2c). These results suggest that LP female mice exhibit some characteristics of depressive-like behavior.

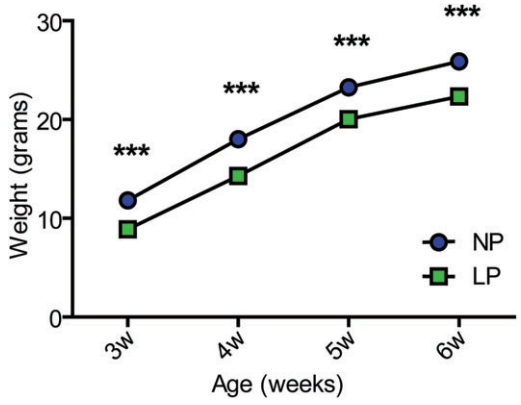

Figure 1: F1 weight after weaning. Mean ± SEM. Female mice from the F1 were weighted once a week after weaning; $n = 22–25$ per treatment. (SEM bars smaller than symbols).Two-way RM ANOVA, Bonferroni, *post hoc* test, $***P < 0.001$.

Altered expression of Egr1 and Arc in female mice subjected to perinatal protein malnutrition

The FST was applied as an acute stress on 10-week-old female mice. Two IEGs underlying experience-induced synaptic plasticity, Egr1 and Arc, were measured in the hippocampus 1 and 24 h after the FST session. The two-way ANOVA showed a main effect for treatment ($F_{1,31} = 15.07$, $P < 0.001$), reflected by a significant decrease in Egr1 mRNA expression in malnourished animals compared with controls (Fig. 3a). Orthogonal contrasts pointed out that Egr1 expression was significantly lower at both basal level ($P < 0.05$) and 1 h after being subjected to the acute stress ($P < 0.01$). No effects for the factor time point ($F_{2,31} = 2.65$, $P = 0.0863$) and no treatment × time point interaction ($F_{2,31} = 0.75$, $P = 0.4799$) were found. Furthermore, immunoblotting showed that these changes are paralleled by protein expression, reflected by diminished levels of Egr1 protein in the hippocampus of LP mice (Fig. 3c,d).

A similar analysis for Arc mRNA expression (Fig. 3b) showed a significant main effect for time ($F_{2,35} = 4.52$, $P < 0.05$), but no differences were observed neither for the diet ($F_{1,35} = 0.15$, $P = 0.6966$) nor for the interaction between time and diet ($F_{2,35} = 0.18$, $P = 0.8351$). Tukey's *post hoc* analysis indicated that Arc mRNA expression was significantly increased 1 h after the mice were exposed to forced swimming in both groups ($P < 0.05$).

Malnourished mice exhibit a lower induction of c-fos after acute stress

We next assessed the expression of c-fos after exposure of mice to forced swimming (Fig. 3e). Expression of the IEG c-fos is commonly used as a marker for neuronal activity as it is often expressed when neurons become activated (Herrera & Robertson 1996). The two-way ANOVA showed a significant effect of diet ($F_{1,30} = 4.19$, $P < 0.05$) and time ($F_{2,30} = 43.78$, $P < 0.0001$) but not in their interaction ($F_{2,30} = 3.11$, $P = 0.0591$). Tukey's *post hoc* analysis indicated

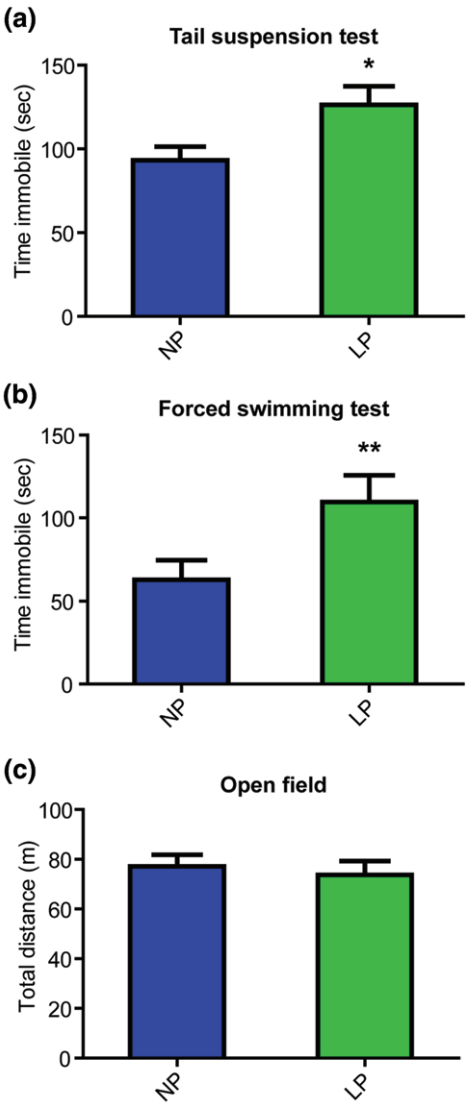

Figure 2: Depressive-like behavior and locomotion in the F1. Mean + SEM. Female mice of the F1 were tested for: (a) tail suspension test ($n = 21–26$ per treatment), (b) FST ($n = 26–19$ per treatment) and (c) open field test ($n = 21–26$ per treatment). Student's *t*-test, $*P < 0.05$ and $**P < 0.01$.

that c-fos mRNA expression was increased 1 h after the acute stress in both NP and LP groups ($P < 0.05$). However, the c-fos mRNA level attained in the NP group was higher than the LP group ($P < 0.05$).

Lower levels of calcineurin in hippocampus of malnourished mice after acute stress

Calcineurin is a serine/threonine protein phosphatase that regulates neurotransmission, neuronal structure and plasticity, and neuronal excitability in mood disorders. Increasing evidence has suggested that calcineurin is involved in the regulation of depressive-like behavior

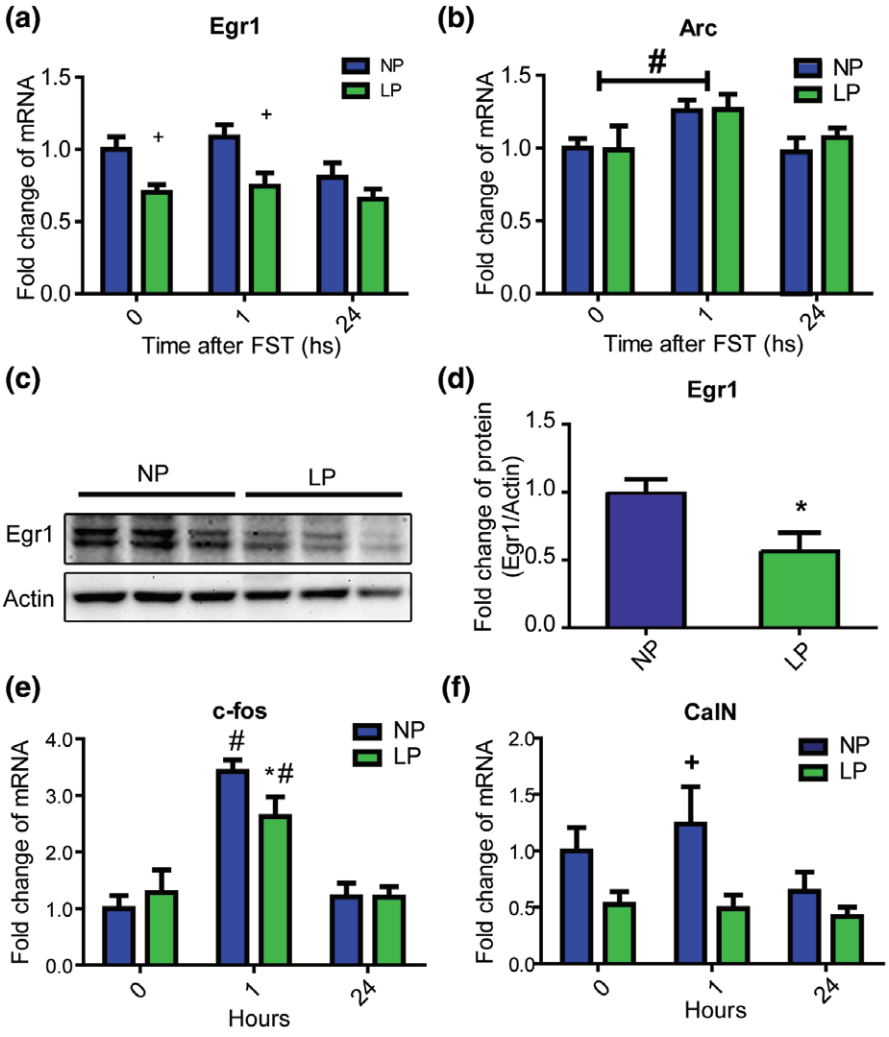

Figure 3: Hippocampal expression of Egr1, Arc, c-fos and CaIN genes after the FST. Mean + SEM. Real-time reverse transcriptase PCR for Egr1 (a), Arc (b), c-fos (e) and CaIN (f) mRNAs. Fold change relative to the geometric mean of three housekeeping genes (*Hprt1*, *Gapdh* and *Pgk1*) and then to NP at time 0; $n = 5–7$ per combination of time and treatment. Two-way ANOVA, $*P < 0.05$ for differences between diet, $\#P < 0.05$ for differences with the respective time 0 using Tukey's *post hoc* test and $+P < 0.05$ orthogonal contrast between diets. Egr1 protein levels in the hippocampus of female mice not subjected to the FST measured by western blot (c) and its quantification (d); $n = 3$ per treatment. Student's *t*-test, $*P < 0.05$.

(Crozatier *et al.* 2007; Zhu *et al.* 2011). Then, we evaluated the expression of the catalytic subunit of calcineurin A in female mice subjected to perinatal protein malnutrition at different times after the FST. We observed a significant effect for the diet factor ($F_{1,33} = 5.93$, $P < 0.01$) (Fig. 3f). No effects for the factor time point ($F_{2,33} = 1.56$, $P = 0.2256$) and no treatment × time point interaction ($F_{2,33} = 0.54$, $P = 0.5904$) were found. Orthogonal contrasts indicated that the levels of calcineurin A mRNA in LP mice were significantly lower 1 h after being subjected to the acute stress than their NP counterparts ($P < 0.05$). Although not significant, a similar tendency was observed in mice at basal conditions. These results show that perinatal protein malnutrition led to diminished levels of calcineurin A and increased depressive-like behavior.

Reduced neuronal density in hippocampus of protein-malnourished female mice

To evaluate the impact of early-life malnutrition on hippocampus structure later in adulthood, we analyzed the neuronal density of its different regions (Fig. 4). A significant decrease in NeuN-positive cells was found in LP mice when compared with NP mice in the following areas: CA1 ($t_4 = 2.400$, $P < 0.05$), CA3 ($t_4 = 2.476$, $P < 0.05$) and DG ($t_4 = 5.112$, $P < 0.01$). No differences were observed in CA2 between both groups ($t_4 = 1.267$, $P = 0.1369$).

Discussion

The main goal of this paper is to explore the molecular basis of the depressive-like behavior observed in female mice subjected to perinatal protein malnutrition. Exposure to perinatal stress has three major effects on adult behavior: learning impairments, enhanced sensitivity to drugs of abuse and increases in anxiety- and depression-related behaviors (Lupien *et al.* 2009). In this study, differences indicating altered levels of despair behavior in the perinatal protein-malnourished animals were observed after nutritional recovery. Behavioral performance in the tail suspension test and FST shows longer immobility periods in LP mice than

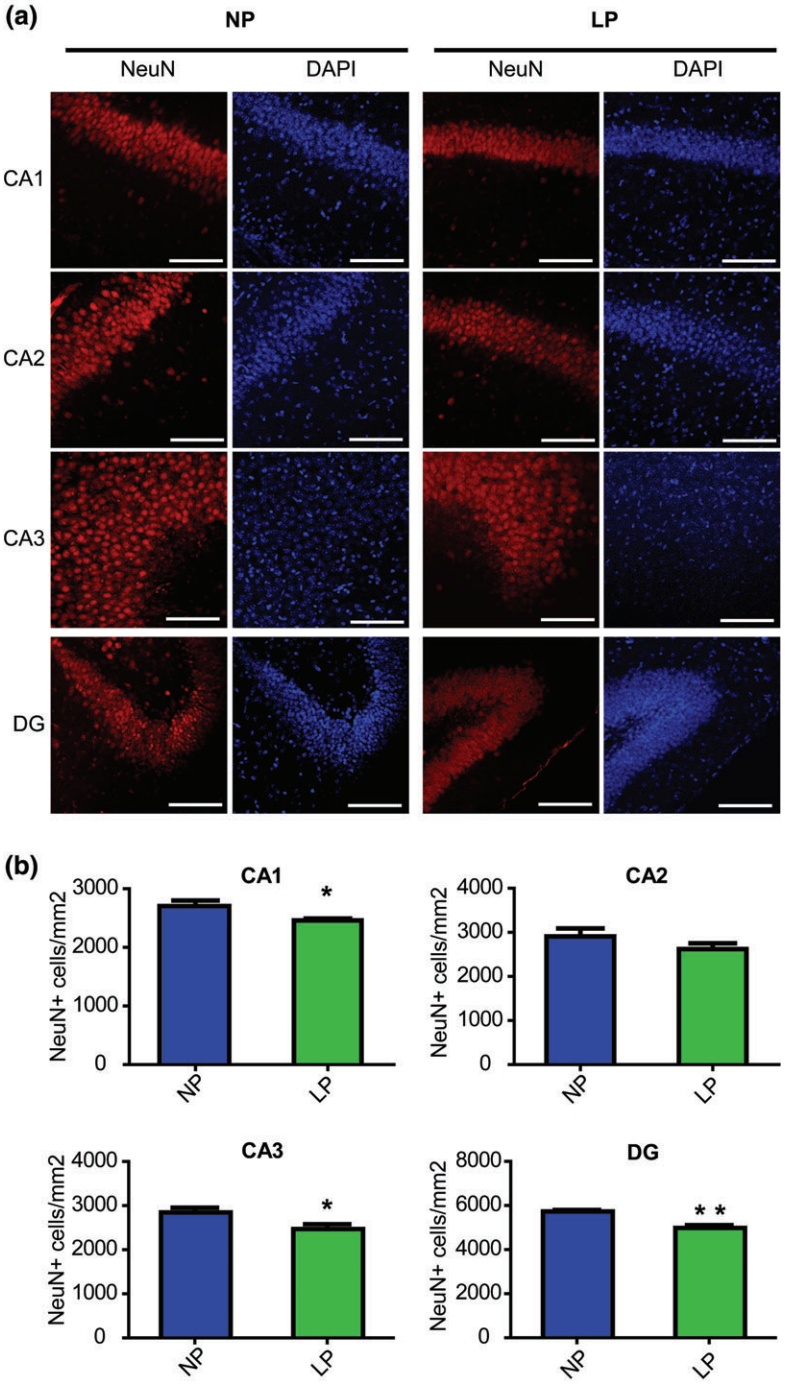

Figure 4: NeuN-positive cells density in the CA1, CA2, CA3 and DG regions of the hippocampus. (a) NeuN immunofluorescence, representative images. Scale bar: 100 μm. Images were taken with a ×20 objective and a digital zoom of ×1.8. (b) NeuN+ cells/mm^2; $n=3$ animals per treatment. Mean + SEM. Student's *t*-test, $*P<0.05$ and $**P<0.01$.

in NP mice. Similar results were observed in rats subjected to protein restriction (de Godoy *et al.* 2013). These results show the negative and long-lasting effects of reduced protein intake during pregnancy and lactation in the female offspring, later in adulthood. Low maternal care has been extensively associated with an altered response to stress in the offspring (Curley & Champagne 2015). We have previously shown that protein restriction during pregnancy and lactation leads to impaired maternal behavior and to the emergence of anxiety-like behaviors in both male and female mice, while depressive-like behavior was only found in female mice (Belluscio *et al.* 2014). This could be linked to the observation that dams spend more time licking and grooming male pups potentially making female pups more vulnerable to stress (Hao *et al.* 2011). On the other hand, it was also shown that this sex bias is lost in some models of prenatal stress (Power

& Moore 1986). Ultimately, differences in depressive-like behavior are a result of the interaction between sex and dietary factors, with maternal care being a potential intermediate of these interactions.

Psychologically stressful events are known to have a long-lasting impact on behavior. The consolidation of such, largely adaptive, behavioral responses to stressful events involves changes in gene expression in limbic brain regions such as the hippocampus and amygdala (Reul 2014). In particular, the expression of IEGs such as Egr1, c-fos, and Arc is rapidly and selectively upregulated in subsets of neurons in specific brain regions, in response to various acute stressors (Bilang-Bleuel *et al.* 2002; Carter *et al.* 2015). Expression of these IEGs has therefore been widely used as a molecular marker for neuronal populations that undergo plastic changes underlying formation of memories in response to stressful events. In hippocampus, rapid transcription of Egr1 directs the response to behavioral tasks. In addition, Egr1 regulates Arc expression which is involved in synaptic plasticity playing an important role in modulating dendritic spine density and remodeling. In turn, c-fos expression is also rapidly induced after an acute challenge and inhibition of this induction has been associated with the appearance of depression traits.

We observed two important differences in expression of these IEGs. First, Egr1 mRNA expression level was significantly lower in mice from LP group than in mice from NP group. Moreover, this difference remained after mice being subjected to forced swimming stress. Second, although the expression of c-fos was induced in both NP and LP groups after being exposed to the acute stress, the expression levels reached by NP mice were greater than in LP mice. In both cases, mRNA levels returned to basal levels 24 h after performing FST. Changes in IEG expression were unlikely to be caused by novelty instead of stress-induced activity as LP mice habituated equally to NP mice to a novel non-stressful arena.

Several evidences showed that induction of IEGs, especially Egr1 and c-fos, in hippocampus is important to long-term memory formation in response to a stressful event, allowing adaptation and optimization of behavioral responses when similar circumstances are encountered again. Expression of c-fos in the DG was increased after rats' exposure to forced swimming stress (Gutièrrez-Mecinas *et al.* 2011). During development, Egr1 is increasingly expressed postnatally in hippocampus and remains stable in the CA1 throughout adulthood. The expression of Egr1 appears to be tightly linked to NMDA receptors activity. This observation led to the suggestion that high basal levels of Egr1 are maintained by synaptic activity in response to physiological stimuli. Integrity of the ERK/MAPK signaling pathway is also required for proper Egr1 expression (Veyrac *et al.* 2014). Similarly, induction of c-fos in the hippocampus requires the activation of ERK/MAPK pathway via NMDA receptors (Mifsud *et al.* 2011). Hence, the lower basal level of Egr1 and the minor stress-dependent induction of c-fos observed in hippocampus of LP mice could reflect a disturbed functionality of this pathway caused by early malnutrition. In this scenario, the attenuated hippocampal IEG expression in perinatally malnourished mice could explain, at least partially, the behavioral disorders detected in adulthood.

We have observed a pronounced effect for diet on the calcineurin A expression reflected by a decrease in its mRNA in malnourished mice compared with controls that were statistically significant 1 h after mice were exposed to acute stress. Calcineurin or protein phosphatase 2B is a calcium-regulated serine/threonine protein phosphatase enriched in the brain that consists of two subunits: the catalytic A subunit and regulatory B subunit (Klee *et al.* 1998). When activated by calcium and calmodulin, calcineurin dephosphorylates the pre-synaptic protein synapsin I and participates in neurotransmission, neuronal structure and neuronal excitability (Mansuy 2003). Calcineurin functions in concert with another serine/threonine protein phosphatase, the protein phosphatase-1, as well as related protein kinases such as the Ca^{2+}/calmodulin-dependent protein kinase or the protein kinases A and C to control the activity of specific targets such as ion channels, neurotransmitter receptors, signaling enzymes, transcription and translation factors. Contribution of calcineurin with complex brain functions such as learning and memory has already been extensively shown (de la Fuente *et al.* 2014; Xia & Storm 2005). More recently, calcineurin has been shown to be associated with depressive disorders. Local downregulation of calcineurin in the amygdala induced depressive-like behavior, reflected by increased immobility in the FST and tail suspension tests (Bahi *et al.* 2009; Mineur *et al.* 2014). Chronic psychosocial stress, which is associated with the onset of depression, significantly reduced basal calcineurin levels in the DG (Gerges *et al.* 2003). In addition, depressive-like behaviors were induced by pharmacological calcineurin inhibition in mice hippocampus (Crozatier *et al.* 2007). Deficient calcineurin expression was recently found to be associated with schizophrenia in humans and schizophrenia-like symptoms in a knockout mouse model (Miyakawa *et al.* 2003; Takase *et al.* 2012).

These findings suggest that calcineurin plays a critical role in the pathophysiology of affective disorders and could be a link between malnutrition at early life and depressive-like behavior at adulthood. Although little is known about the neurobiological mechanisms that underlie the mood-regulating effects of calcineurin, recent findings suggest that metabotropic glutamatergic receptor 2/3-dependent pathways participate in the development of depression and the behavioral responses to antidepressant treatment (Zhu *et al.* 2011).

The hippocampus is strongly modulated by life experiences, hormonal and environmental conditions and is one of the several limbic structures that have been extensively studied in individuals with depression. Magnetic resonance imaging studies of these subjects have consistently shown a reduction in hippocampal volume. Moreover, the frequency of depressive episodes and how long the depression remains untreated correlate with the magnitude of reduction in hippocampal volume (MacQueen *et al.* 2003; Ruocco *et al.* 2012). Altered hippocampal function, in turn, may influence the activity of neural circuitry in the prefrontal cortex, amygdala and nucleus accumbens, structures that receive inputs from the hippocampus and are associated with emotionality. Moreover, optimal function of the hippocampal formation is critical for modulation of the hypothalamic–pituitary axis and regulation of the stress response, dysregulation of which is

observed in almost half of all depressed individuals (Sahay & Hen 2007). Consistent with our results, the hippocampus has been shown to be particularly susceptible to early-life environment alterations. This susceptibility is largely explained by the protracted development of this structure. In rodents, hippocampal growth takes place mainly during late gestation and the first 2 weeks after birth, with the addition of new neurons throughout life. We showed that protein malnutrition during embryonic and lactation periods reduced the number of neurons in CA1, CA3 and DG regions of mice adult hippocampus. These results are coincident with those observed by other authors having shown that nutrient deficiency during early life results in long-lasting structural abnormalities in the hippocampus of the offspring, including decreased number of neurons, spines, synapses and dendritic arborization (Alamy & Bengelloun 2012; Lukoyanov & Andrade 2000; Matos *et al.* 2011).

Conclusion

In summary, we show that female mice that were subjected to protein malnutrition during pre- and post-natal development have a diminished expression of IEGs and calcineurin in hippocampus when confronted with an acute stress in the adulthood. Additionally, these mice exhibit a fewer number of neurons in the hippocampus, especially in CA3 and DG, regions that are critical for stress response. Stress induces a number of biological responses which enables the organism to adapt to the challenge and increase its likelihood of survival. Therefore, the inability to cope with stressful situations could underlie the increased vulnerability of early-life malnourished mice to depression. Epidemiological studies have shown that depressive symptoms are elevated in adolescents who experienced significant malnutrition early in life (Galler *et al.* 2010; Waber *et al.* 2011). As childhood malnutrition and undernutrition unfortunately remains a significant problem, appropriate animal models will help to understand the effect of malnutrition and undernutrition in humans and especially on brain development in children.

References

Alamy, M. & Bengelloun, W.A. (2012) Malnutrition and brain development: an analysis of the effects of inadequate diet during different stages of life in rat. *Neurosci Biobehav Rev* **36**, 1463–1480.

Altemus, M. (2006) Sex differences in depression and anxiety disorders: potential biological determinants. *Horm Behav* **50**, 534–538.

Andersen, S.L. (2003) Trajectories of brain development: point of vulnerability or window of opportunity? *Neurosci Biobehav Rev* **27**, 3–18.

Bahi, A., Mineur, Y.S. & Picciotto, M.R. (2009) Blockade of protein phosphatase 2B activity in the amygdala increases anxiety- and depression-like behaviors in mice. *Biol Psychiatry* **66**, 1139–1146.

Bale, T.L. & Epperson, C.N. (2015) Sex differences and stress across the lifespan. *Nat Neurosci* **18**, 35–42.

Belluscio, L.M., Berardino, B.G., Ferroni, N.M., Ceruti, J.M. & Cánepa, E.T. (2014) Early protein malnutrition negatively impacts physical growth and neurological reflexes and evokes anxiety and depressive-like behaviors. *Physiol Behav* **129**, 237–254.

Bilang-Bleuel, A., Rech, J., De Carli, S., Holsboer, F. & Reul, J.M.H.M. (2002) Forced swimming evokes a biphasic response in CREB phosphorylation in extrahypothalamic limbic and neocortical brain structures in the rat. *Eur J Neurosci* **15**, 1048–1060.

Burdge, G.C. & Lillycrop, K.A. (2010) Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. *Annu Rev Nutr* **30**, 315–339.

Carter, S.D., Mifsud, K.R. & Reul, J.M.H.M. (2015) Distinct epigenetic and gene expression changes in rat hippocampal neurons after Morris water maze training. *Front Behav Neurosci* **9**, 156.

Chmurzynska, A. (2010) Fetal programming: link between early nutrition, DNA methylation, and complex diseases. *Nutr Rev* **68**, 87–98.

Costa, A.P.R., Vieira, C., Bohner, L.O.L., Silva, C.F., Santos, E.C.D.S., De Lima, T.C.M. & Lino-de-Oliveira, C. (2013) A proposal for refining the forced swim test in Swiss mice. *Prog Neuropsychopharmacol Biol Psychiatry* **45**, 150–155.

Crozatier, C., Farley, S., Mansuy, I.M., Dumas, S., Giros, B. & Tzavara, E.T. (2007) Calcineurin (protein phosphatase 2B) is involved in the mechanisms of action of antidepressants. *Neuroscience* **144**, 1470–1476.

Curley, J.P. & Champagne, F.A. (2015) Influence of maternal care on the developing brain: mechanisms, temporal dynamics and sensitive periods. *Front Neuroendocrinol* **40**, 52–66.

Danese, A. & McEwen, B.S. (2012) Adverse childhood experiences, allostasis, allostatic load, and age-related disease. *Physiol Behav* **106**, 29–39.

Ericsson, C., Peredo, I. & Nistér, M. (2007) Optimized protein extraction from cryopreserved brain tissue samples. *Acta Oncol* **46**, 10–20.

Fox, S.E., Levitt, P. & Nelson, C.A. (2010) How the timing and quality of early experiences influence the development of brain architecture. *Child Dev* **81**, 28–40.

Franklin, K.B.J. & Paxinos, G. (2007) *The Mouse Brain in Stereotaxic Coordinates*, 3rd edn. Academic Press, London.

de la Fuente, V., Federman, N., Fustiñana, M.S., Zalcman, G. & Romano, A. (2014) Calcineurin phosphatase as a negative regulator of fear memory in hippocampus: control on nuclear factor-κB signaling in consolidation and reconsolidation. *Hippocampus* **24**, 1549–1561.

Galler, J.R., Bryce, C.P., Waber, D., Hock, R.S., Exner, N., Eaglesfield, D., Fitzmaurice, G. & Harrison, R. (2010) Early childhood malnutrition predicts depressive symptoms at ages 11–17. *J Child Psychol Psychiatry* **51**, 789–798.

Gerges, N.Z., Aleisa, A.M., Schwarz, L.A. & Alkadhi, K.A. (2003) Chronic psychosocial stress decreases calcineurin in the dentate gyrus: a possible mechanism for preservation of early LTP. *Neuroscience* **117**, 869–874.

de Godoy, M.A., de Souza, A.S., Lobo, M.A., Sampaio, O.V.K., Moraes, L., Baldanza, M.R., Magri, T.P.R., Wernerck de Castro, J.P.S., Tavares do Carmo, M.D.G., Soares-Mota, M., Rocha, M.S., Mendez-Otero, R. & Santiago, M.F. (2013) Effects of protein restriction during gestation and lactation on cell proliferation in the hippocampus and subventricular zone: functional implications. Protein restriction alters hippocampal/SVZ cell proliferation. *Brain Res* **1496**, 10–27.

Gomez, J.L. & Luine, V.N. (2014) Female rats exposed to stress and alcohol show impaired memory and increased depressive-like behaviors. *Physiol Behav* **123**, 47–54.

Gutièrrez-Mecinas, M., Trollope, A.F., Collins, A., Morfett, H., Hesketh, S.A., Kersanté, F. & Reul, J.M.H.M. (2011) Long-lasting behavioral responses to stress involve a direct interaction of glucocorticoid receptors with ERK1/2-MSK1-Elk-1 signaling. *Proc Natl Acad Sci U S A* **108**, 13806–13811.

Hao, Y., Huang, W., Nielsen, D.A. & Kosten, T.A. (2011) Litter gender composition and sex affect maternal behavior and DNA methylation levels of the Oprm1 gene in rat offspring. *Front Psychiatry* **2**, 21.

Herrera, D.G. & Robertson, H.A. (1996) Activation of c-fos in the brain. *Prog Neurobiol* **50**, 83–107.

Kar, B.R., Rao, S.L. & Chandramouli, B.A. (2008) Cognitive development in children with chronic protein energy malnutrition. *Behav Brain Funct* **4**, 31.

Kim, P., Evans, G. & Angstadt, M. (2013) Effects of childhood poverty and chronic stress on emotion regulatory brain function in adulthood. *Proc Natl Acad Sci U S A* **110**, 18442–18447.

Klee, C.B., Ren, H. & Wang, X. (1998) Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *J Biol Chem* **273**, 13367–13370.

Korosi, A., Naninck, E.F.G., Oomen, C.A., Schouten, M., Krugers, H., Fitzsimons, C. & Lucassen, P.J. (2012) Early-life stress mediated modulation of adult neurogenesis and behavior. *Behav Brain Res* **227**, 400–409.

Laus, M.F., Vales, L.D.M.F., Costa, T.M.B. & Almeida, S.S. (2011) Early postnatal protein-calorie malnutrition and cognition: a review of human and animal studies. *Int J Environ Res Public Health* **8**, 590–612.

Lukoyanov, N.V. & Andrade, J.P. (2000) Behavioral effects of protein deprivation and rehabilitation in adult rats: relevance to morphological alterations in the hippocampal formation. *Behav Brain Res* **112**, 85–97.

Lupien, S.J., McEwen, B.S., Gunnar, M.R. & Heim, C. (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat Rev Neurosci* **10**, 434–445.

MacQueen, G.M., Campbell, S., McEwen, B.S., Macdonald, K., Amano, S., Joffe, R.T., Nahmias, C. & Young, L.T. (2003) Course of illness, hippocampal function, and hippocampal volume in major depression. *Proc Natl Acad Sci U S A* **100**, 1387–1392.

Mansuy, I.M. (2003) Calcineurin in memory and bidirectional plasticity. *Biochem Biophys Res Commun* **311**, 1195–1208.

Maria Moreira Camargo, L. & de Sousa Almeida, S. (2005) Early postnatal protein malnutrition changes the development of social play in rats. *Physiol Behav* **85**, 246–251.

Matos, R.J.B., Orozco-Solís, R., Lopes de Souza, S., Manhães-de-Castro, R. & Bolaños-Jiménez, F. (2011) Nutrient restriction during early life reduces cell proliferation in the hippocampus at adulthood but does not impair the neuronal differentiation process of the new generated cells. *Neuroscience* **196**, 16–24.

McCrory, E., De Brito, S.A. & Viding, E. (2011) The impact of childhood maltreatment: a review of neurobiological and genetic factors. *Front Psychiatry* **2**, 48.

McEwen, B.S., Bowles, N.P., Gray, J.D., Hill, M.N., Hunter, R.G., Karatsoreos, I.N. & Nasca, C. (2015) Review mechanisms of stress in the brain. *Nat Neurosci* **18**, 1353–1363.

McGaughy, J.A., Amaral, A.C., Rushmore, R.J., Mokler, D.J., Morgane, P.J., Rosene, D.L. & Galler, J.R. (2014) Prenatal malnutrition leads to deficits in attentional set shifting and decreases metabolic activity in prefrontal subregions that control executive function. *Dev Neurosci* **36**, 532–541.

Mifsud, K.R., Gutièrrez-Mecinas, M., Trollope, A.F., Collins, A., Saunderson, E.A. & Reul, J.M.H.M. (2011) Epigenetic mechanisms in stress and adaptation. *Brain Behav Immun* **25**, 1305–1315.

Mineur, Y.S., Taylor, S.R. & Picciotto, M.R. (2014) Calcineurin down-regulation in the amygdala is sufficient to induce anxiety-like and depression-like behaviors in C57BL/6J male mice. *Biol Psychiatry* **75**, 991–998.

Miyakawa, T., Leiter, L.M., Gerber, D.J., Gainetdinov, R.R., Sotnikova, T.D., Zeng, H., Caron, M.G. & Tonegawa, S. (2003) Conditional calcineurin knockout mice exhibit multiple abnormal behaviors related to schizophrenia. *Proc Natl Acad Sci U S A* **100**, 8987–8992.

Morgane, P.J., Mokler, D.J. & Galler, J.R. (2002) Effects of prenatal protein malnutrition on the hippocampal formation. *Neurosci Biobehav Rev* **26**, 471–483.

Ogara, M.F., Belluscio, L.M., de la Fuente, V., Berardino, B.G., Sonzogni, S.V., Byk, L., Marazita, M. & Cánepa, E.T. (2014) CDK5-mediated phosphorylation of p19INK4d avoids DNA damage-induced neurodegeneration in mouse hippocampus and prevents loss of cognitive functions. *Biochim Biophys Acta* **1843**, 1309–1324.

Papaioannou, A., Gerozissis, K., Prokopiou, A., Bolaris, S. & Stylianopoulou, F. (2002) Sex differences in the effects of neonatal handling on the animal's response to stress and the vulnerability for depressive behaviour. *Behav Brain Res* **129**, 131–139.

Power, K.L. & Moore, C.L. (1986) Prenatal stress eliminates differential maternal attention to male offspring in Norway rats. *Physiol Behav* **38**, 667–671.

Ranade, S.C., Rose, A., Rao, M., Gallego, J., Gressens, P. & Mani, S. (2008) Different types of nutritional deficiencies affect different domains of spatial memory function checked in a radial arm maze. *Neuroscience* **152**, 859–866.

Reeves, P.G., Nielsen, F.H. & Fahey, G.C. (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**, 1939–1951.

Reul, J.M.H.M. (2014) Making memories of stressful events: a journey along epigenetic, gene transcription, and signaling pathways. *Front Psychiatry* **5**, 1–11.

Reyes-Castro, L.A., Rodriguez, J.S., Charco, R., Bautista, C.J., Larrea, F., Nathanielsz, P.W. & Zambrano, E. (2012) Maternal protein restriction in the rat during pregnancy and/or lactation alters cognitive and anxiety behaviors of female offspring. *Int J Dev Neurosci* **30**, 39–45.

Rotta, L.N., Leszczinski, D.N., Brusque, A.M., Pereira, P., Brum, L.F.S., Nogueira, C.W., Frizzo, M.E.S., Perry, M.L.S. & Souza, D.O. (2008) Effects of undernutrition on glutamatergic parameters in the cerebral cortex of young rats. *Physiol Behav* **94**, 580–585.

Ruijter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., Van Den Hoff, M.J.B. & Moorman, A.F.M. (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res* **37**, e45.

Ruocco, A.C., Amirthavasagam, S. & Zakzanis, K.K. (2012) Amygdala and hippocampal volume reductions as candidate endophenotypes for borderline personality disorder: a meta-analysis of magnetic resonance imaging studies. *Psychiatry Res* **201**, 245–252.

Sahay, A. & Hen, R. (2007) Adult hippocampal neurogenesis in depression. *Nat Neurosci* **10**, 1110–1115.

Scott, K.M. (2011) Sex differences in the disability associated with mental disorders. *Curr Opin Psychiatry* **24**, 331–335.

Takase, K., Yamamoto, Y. & Yagami, T. (2012) Maternal deprivation in the middle of a stress hyporesponsive period decreases hippocampal calcineurin expression and causes abnormal social and cognitive behaviours in adult male Wistar rats: relevance to negative symptoms of schizophrenia. *Behav Brain Res* **232**, 306–315.

Veyrac, A., Besnard, A., Caboche, J., Davis, S. & Laroche, S. (2014) The transcription factor Zif268/Egr1, brain plasticity, and memory. In Zafar, U.K. & Muly, E.C. (eds), *Progress in Molecular Biology and Translational Science*. Academic Press, London, pp. 89–129.

Waber, D.P., Eaglesfield, D., Fitzmaurice, G.M., Bryce, C., Harrison, R.H. & Galler, J.R. (2011) Cognitive impairment as a mediator in the developmental pathway from infant malnutrition to adolescent depressive symptoms in Barbadian youth. *J Dev Behav Pediatr* **32**, 225–232.

World Health Organization. (2012) Global burden of disease. URL http://www.who.int/healthinfo/global_burden_disease/gbd/en/ (Date of last access 2 January 2016).

Xia, Z. & Storm, D.R. (2005) The role of calmodulin as a signal integrator for synaptic plasticity. *Nat Rev Neurosci* **6**, 267–276.

Zhu, W.L., Shi, H.S., Wang, S.J., Wu, P., Ding, Z.B. & Lu, L. (2011) Hippocampal CA3 calcineurin activity participates in depressive-like behavior in rats. *J Neurochem* **117**, 1075–1086.

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