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Maternal deprivation induces a rapid decline in circulating leptin levels and sexually dimorphic modifications in hypothalamic trophic factors and cell turnover

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ABSTRACT

Pathological outcomes, including metabolic and endocrine disturbances, of maternal deprivation (MD) in Wistar rats depend on gender and the timing of deprivation during development. We analyzed the effect of MD between postnatal days 9 and 10, a critical period in hypothalamic development, on circulating hormones and local production of trophic factors involved in this process, as well as on markers of cell turnover and maturation. Males and females were studied 12 and 24 h after MD and 12 h (MD36) after returning the dam to her pups. Circulating corticosterone levels were increased and glucose and leptin levels decreased throughout the study in both sexes. Hypothalamic mRNA levels of leptin receptor increased significantly at MD24 in both sexes, normalizing in females at MD36, but not in males. In male rats insulin-like growth factor mRNA levels were significantly decreased at MD24 and brain derived neurotrophic factor mRNA levels decreased at MD12 and MD24, with both trophic factors unaffected in females. In males cell proliferation was significantly decreased at MD36, as were the glial structural proteins, glial fibrillary acidic protein and vimentin. In females, nestin levels decreased significantly at MD24. These results indicate that MD differently affects trophic factors and cell-turnover in the hypothalamus of males and females, which may underlie the sex differences seen in the endocrine and metabolic outcome.

Introduction

The intrauterine environment and early neonatal events are important in determining normal physiological functions in later life. Furthermore, the propensity towards pathophysiological processes, including behavioral imbalances, inappropriate stress responses, or the probability of becoming obese or presenting metabolic imbalances, is also modulated by these early events (Renard et al., 2007; Marais et al., 2008; Kappeler et al., 2009). For example, exposing neonatal animals to a single prolonged 24 h period of early (PND9) maternal deprivation (MD) induces short- and long-term behavioral, biochemical, endocrine, cellular (neurons and glia) and immunological alterations, which suggests that this experimental neonatal stress procedure may be of great utility in understanding neurological and probably metabolic disorders with a neurodevelopmental origin (for reviews see Srinivasan and Patel, 2008; Marco et al 2009; De la Fuente et al., 2009; Viveros et al 2009). Maternal deprivation also results in specific metabolic and hormonal alterations, as well as delayed corporal growth (Ellenbroek et al., 2005; Schmidt et al., 2006, Desbonnet et al., 2008) that, equal to what is thought to occur in the development of behavioral deficits, could result from the detrimental effects that the MD-induced increase in corticosterone levels exerts on neurodevelopment, including changes in neurotrophic factors (Mesquita et al., 2007; Marais et al., 2008; Viveros et al 2009). Indeed, in the hippocampus, cortex and striatum this experimental paradigm results in modifications in neurotrophic factors such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Roceri et al., 2004; Choy et al., 2008; Marais et al., 2008; Kikusui et al., 2009), induces synaptic modifications (Yamauchi et al., 2005) and increases neurodegeneration and possibly gliosis (Llorente et al., 2008, López-Gallardo et al., 2008, Viveros et al., 2009); however, the effect of MD on cell-turnover and neuronal development in areas specific to metabolic and hormonal control, such as the hypothalamus, remains to be determined.

Many of the sequelae of MD are sexually dimorphic (Genest et al., 2004; de Jongh et al., 2005; Desbonnet et al., 2008; Llorente et al., 2008, 2009; Viveros et al., 2009; Cirulli et al., 2009; Suárez et al., 2009). This observation is of great interest as many diseases of the central nervous system (CNS) display sexual dimorphism, not only being more prominent in one sex or the other, but also showing sex differences in their response to some treatments (Hurn and Macrae, 2000; Sacco, 2001; Van Den Eeden et al., 2003; Hariz et al., 2003). As brain development is sexually dimorphic, especially in areas that

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are highly susceptible to sex steroids, such as the hypothalamus (MacLusky and Naftolin, 1981), it follows that the rate of cell turnover in specific areas of the brain is different between sexes at certain developmental stages (Nuñez et al., 2001). Hence, as proliferating cells are more susceptible to undergo cell death changes in the cellular environment, including hormone levels, increased stress or any noxious insult at a specific moment during development could result in sexually dimorphic outcomes. Indeed, Oomen and colleagues have recently demonstrated that neurogenesis in the hippocampus is differentially affected by MD in males and females (Oomen et al., 2009).

Changes in nutrition during early development affect body composition and the adult response to further dietary modifications, with males often being more susceptible to many of these manipulations (Cryer and Jones, 1980; Levin 2006; Chen et al., 2009). Maternal stress during gestation and lactation also has a sexually dimorphic impact on the pituitary-adrenal axis (Renard et al., 2007; García-Cáceres et al., in press). Maternal deprivation not only results in dietary restriction, thermal modifications and social stress in the neonate, but also increased stress in the mother that could affect lactation and maternal behaviour (Coutellier et al., 2008). Thus, we hypothesized that during MD circulating and locally produced factors involved in hypothalamic development are modified in a sexually dimorphic manner, differentially affecting cell-turnover in the developing hypothalamus of males and females. To this end, we have analyzed circulating levels of corticosterone, glucose insulin, and leptin, which is involved in development of metabolic circuits (Bouret and Simerly, 2007), at specific time-points throughout 24 h of MD and after return of the mother to her pups. In addition, hypothalamic levels of BDNF, insulin-like growth factor (IGF-1) and its receptor, leptin receptor (ObR) and markers of cell-turnover were analyzed.

Materials and methods

Animals

Adult Wistar rats were purchased from Harlan Interfauna Ibérica S. A. (Barcelona, Spain) and allowed to acclimate for 2 weeks before mating. One male was placed in a cage with two females for 10 days. Rats were maintained at a constant temperature (22 ± 1 °C) and humidity (50 ± 1%) in a reversed 12-h light-dark cycle (lights on at 20:00), and given free access to rat chow (Panlab, Barcelona, Spain) and water. On the day of birth (PND0), litters were culled to eight pups per dam (four males and four females), with no cross-fostering employed. In all experimental groups three different litters were used to reduce the litter effect, with a total of 12 rats in each experimental group. Serum from all rats were run in the hormone analyses unless insufficient sample was obtained for all assays ($n = 11$ or 12). One half of the hypothalami were used for protein analysis ($n = 6$) and one half for mRNA analysis ($n = 6$), with 2 rats from each of the 3 litters used in each analysis.

These studies were approved by the local ethics committee and complied with the Royal Decree 1201/2005 (BOEn° 252) pertaining to the protection of experimental animals and with the European Communities Council Directive (86/609/EEC).

Maternal deprivation

Maternal deprivation (MD) was performed as previously described (Llorente et al., 2008). Briefly, on the morning of PND9, beginning at 09:00, mothers from the deprived group were removed and placed in a cage beside the home cage in the same room. In order to have baseline measures and to control for possible circadian variations of the variables analyzed, pups of both sexes were sacrificed on the morning of PND9 (Control (Ct) 0, $n = 12$). Both controls and test pups were killed 12 h after the start of MD (Ct12, $n = 12$ and MD12 $n = 12$) and 24 h after MD (Ct24, $n = 12$ and MD24, $n = 12$). On PND10, mothers were returned to the cage of their respective litters and 12 h later another experimental group was sacrificed (Ct36, $n = 12$ and MD36, $n = 16$).

Tissue collection

All rats were sacrificed by rapid decapitation. Trunk blood was collected in tubes containing EDTA (0.5 M) and rapidly placed in ice. The blood was centrifuged (3000 rpm for 15 min) and the plasma collected and stored at −80° until processed. The brain was rapidly removed and the hypothalamus dissected. It was then frozen in liquid nitrogen and stored at −80° until processed.

Blood glucose, plasma insulin, leptin and corticosterone measurements

Blood glucose levels were measured in trunk blood immediately after sacrifice by using an ACCU-CHECK sensor (Roche, Mannheim, Germany).

Plasma insulin and leptin levels were measured with a rat ELISA kit following the manufacture's instructions from Linco Research (St. Charles, MO). The sensitivity of the method for leptin and insulin is 0.04 and 0.2, respectively. The intra-assay variation was 2.2% for leptin and 1.9% for insulin, and the inter-assay variation 3.4% for leptin and 7.6% for insulin. All samples were run in duplicate.

Corticosterone was measured using a solid phase I125 radioimmunoassay (Immunochem Double Antibody Cort, MP Biomedicals, Costa Mesa, CA). The lower detection limit was 3.0 ng/mL and the intra-assay and inter-assay coefficients of variation were 7.3% and 6.9%, respectively.

Protein extraction

Total protein was extracted from individual hypothalami as previously described (5). Total protein concentration was determined by the method of Bradford (Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

Cell death detection ELISA

This photometric enzyme immunoassay, which detects cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) produced after cell death was carried out according to the manufacturer's (Cell Death Detection ELISA, Roche) instructions and as previously described (Lechuga-Sancho et al., 2006). Each sample was measured in duplicate and background measurements subtracted from the mean value of each sample and samples from all experimental groups were run in each assay. Results were normalized by to protein levels in each sample. This assay has a detection limit of approximately 50 dead cells/well with the inter- and intra-assay coefficients of variation being 8.5% and 4.3%, respectively.

Western blotting

Depending on the specific protein to be detected either 15 or 35 μg of protein were resolved on an 8% or 12% SDS-polyacrylamide gel under denaturing conditions. The proteins were then electrotransferred to PVDF membranes (Bio-Rad). Membranes were blocked in T-TBS (Tris–HCl 10 mM ph: 7,5; NaCl 0.1 M; 0.1% Tween 20, Sigma Steinheim, Germany) containing 5% nonfat dried milk for 2 h. Primary antibodies, used at a concentration of 1:1000 unless otherwise stated, were as follows: Anti-proliferating cell nuclear antigen (PCNA) was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-glial fibrillary acidic protein (GFAP) and anti-vimentin from Sigma (St. Louis, MO), anti-nestin (1:500) and anti-insulin-like growth factor-1 (IGF-1) receptor (1:500) were from Chemicon (Temecula, CA), anti-GADPH

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was from AnaSpec (San Jose, CA), anti-Bax (1:1000) and anti-actin (1:1000) was purchased from Neomarkers (Fremont, CA, USA), and anti-Bcl2α (1:500) from Thermo Scientific (Fremont, CA). All primary antibodies were incubated overnight at 4 °C under agitation. The membranes were washed and incubated with the corresponding secondary antibody conjugated with peroxidase (Pierce, Rockford, IL). Bound peroxidase activity was visualized by chemiluminescence (Perkin Elmer Life Science, Boston, MA) and quantified by densitometry using a Gel Logic 1500 Image analysis system (KODAK, Rochester, New York). All results were first normalized to GADPH or actin levels in each lane and then to control values on each blot. All assays were performed a minimum of 2 times combining experimental groups on each gel so that direct comparisons could be made between each group.

Quantitative real-time PCR

RNA extraction from hypothalami was extracted with TRIzol® Reagent (Invitrogen) as previously described (Chomczynski and Sacchi, 1987). Retrotrascriptase polymerase chain reaction (RT-PCR) was performed on total RNA (1 μg) isolated from each individual hypothalamus, with no pooling of samples. A High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) was used according to the manufacturer's protocol on a Peltier thermal Cycler Tetrad2 (BioRad) for RT-PCR. Amplification of the cDNA template was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Expression Assay Mix kits for each detected gene (Applied Biosystems). The commercial reference for each predesigned gene expression assay is as follows: corticotropin-releasing hormone (CRH; Rn01462137_m1), brain-derived neurotrophic factor (BDNF; Rn01484924_m1), insulin-like growth factor I (IGF-I; Rn99999087_m1), IGF-I receptor (IGF-IR; Rn01477918_m1), leptin receptor (ObR; Rn01433250_m1), actin (Rn00667869_m1) and phosphoglycerate kinase 1 (Pgk1; Rn00569117_m1).

Values were normalized to the reference Pgk1 or β-actin. According to manufacturer's guidelines, the $\Delta\Delta C_T$ method was used for relative quantification. Statistics were performed using ΔC_T values.

Statistical analysis

Data were first analyzed by three-way analysis of variance (ANOVA) with the factors being sex (males and females), MD (maternal non deprived and deprived rats) and time (0, 12, 24 or 36 h). Two-way and one-way ANOVAs were performed when appropriate. Scheffe's *F* test

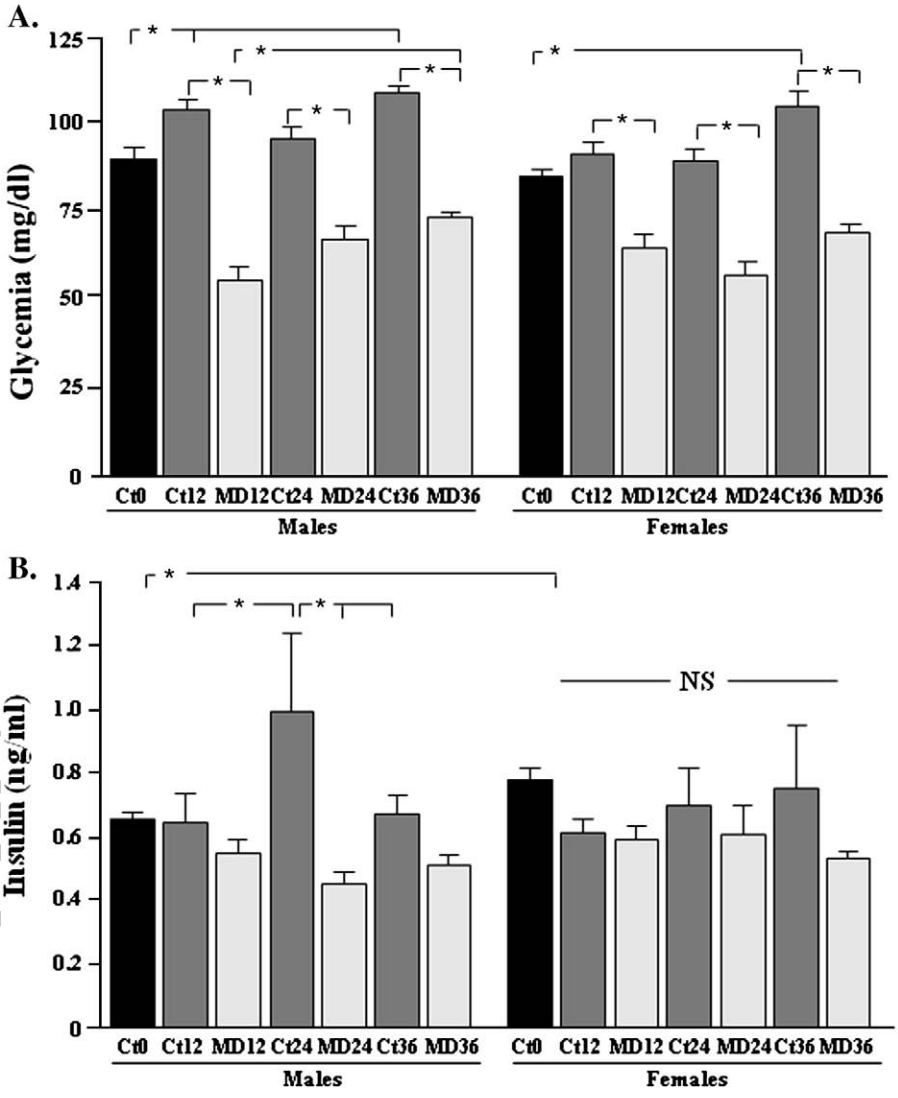

Fig. 1. Circulating glucose (A) and insulin (B) levels in male and female rats that were exposed to maternal deprivation (MD) starting on postnatal day (PND) 9 and sacrificed at 0, 12 and 24 h after the onset of MD, or 12 h (MD36) after being returned to their mother. Ct = control, * = ANOVA $p<0.05$.

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was used for post-hoc comparisons. The level of significance was chosen as $p<0.05$.

Results

Circulating hormone levels and glycemia

There was no effect of sex on glucose levels, however there was a significant effect of time (*DF*: 3, *F*: 13.321, $p<0.0001$) and treatment (*DF*: 1, *F*: 269.173, $p<0.001$) and an interaction between time and treatment (*DF*: 3, *F*: 33.285, $p<0.001$) and sex, time and treatment (*DF*: 3, *F*: 3.739, $p<0.01$). Throughout the study there was a significant decline in glucose levels in those pups that were separated from their mothers and this occurred in both male and females (Fig. 1A).

There was a significant effect of treatment on insulin levels (*DF*: 1, *F*: 7.381, $p<0.01$). In male rats, there was a significant effect of MD on insulin levels (*DF*: 1, *F*: 8.753, $p<0.005$) and this was time-dependent with an interaction between time and treatment (*DF*: 3, *F*: 2.844, $p<0.05$), with insulin levels increasing at 24 h in control male pups and then declining at 36 h. There was no significant effect of time in

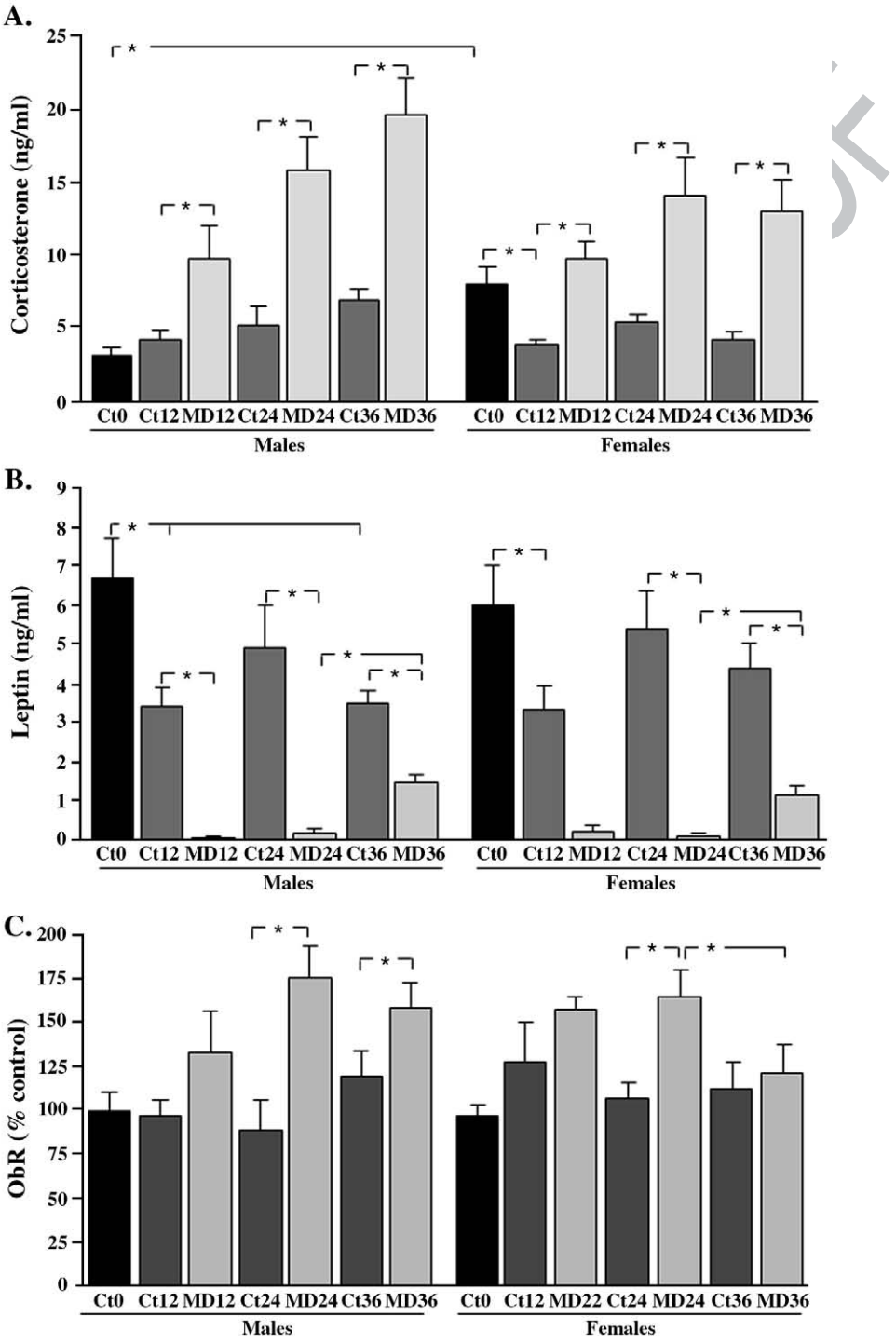

Fig. 2. Circulating corticosterone (A) and leptin (B) levels and hypothalamic mRNA levels for the leptin receptor (C) in male and female rats that were exposed to maternal deprivation (MD) starting on postnatal day (PND) 9 and sacrificed at 0, 12 and 24 h after the onset of MD, or 12 h (MD36) after being returned to their mother. ObR = leptin receptor, Ct = control, * = ANOVA $p<0.05$.

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MD males and MD24 males had significantly lower insulin levels 24 h after separation from their mothers than their controls and basal insulin levels were significantly higher in female pups than in males (*DF*: 13, *F*: 1.867, $p<0.05$). However, in females there was no effect of either time or treatment (Fig. 1B).

There was a significant effect of both time (*DF*: 3, *F*: 22.757, $p<0.0001$) and treatment (*DF*: 1, *F*: 138.615, $p<0.0001$), with an interaction between sex and time (*DF*: 1, *F*: 11.464, $p<0.0001$) and time and treatment (*DF*: 3, *F*: 20.166, $p<0.0001$) on circulating corticosterone levels (Fig. 2A). At baseline, females had significantly higher corticosterone levels compared to males. MD resulted in significantly elevated levels compared to controls at all time-points throughout the study in both sexes.

Circulating leptin levels were affect by both time (*DF*: 3, *F*: 37.067, $p<0.0001$) and treatment (*DF*: 1, *F*: 66.080; $p<0.0001$) with an interaction between these two factors (*DF*: 3, *F*: 9.374; $p<0.0001$). There was no effect of sex. In both male and female control rats, there was a significant change in leptin levels throughout time, decreasing

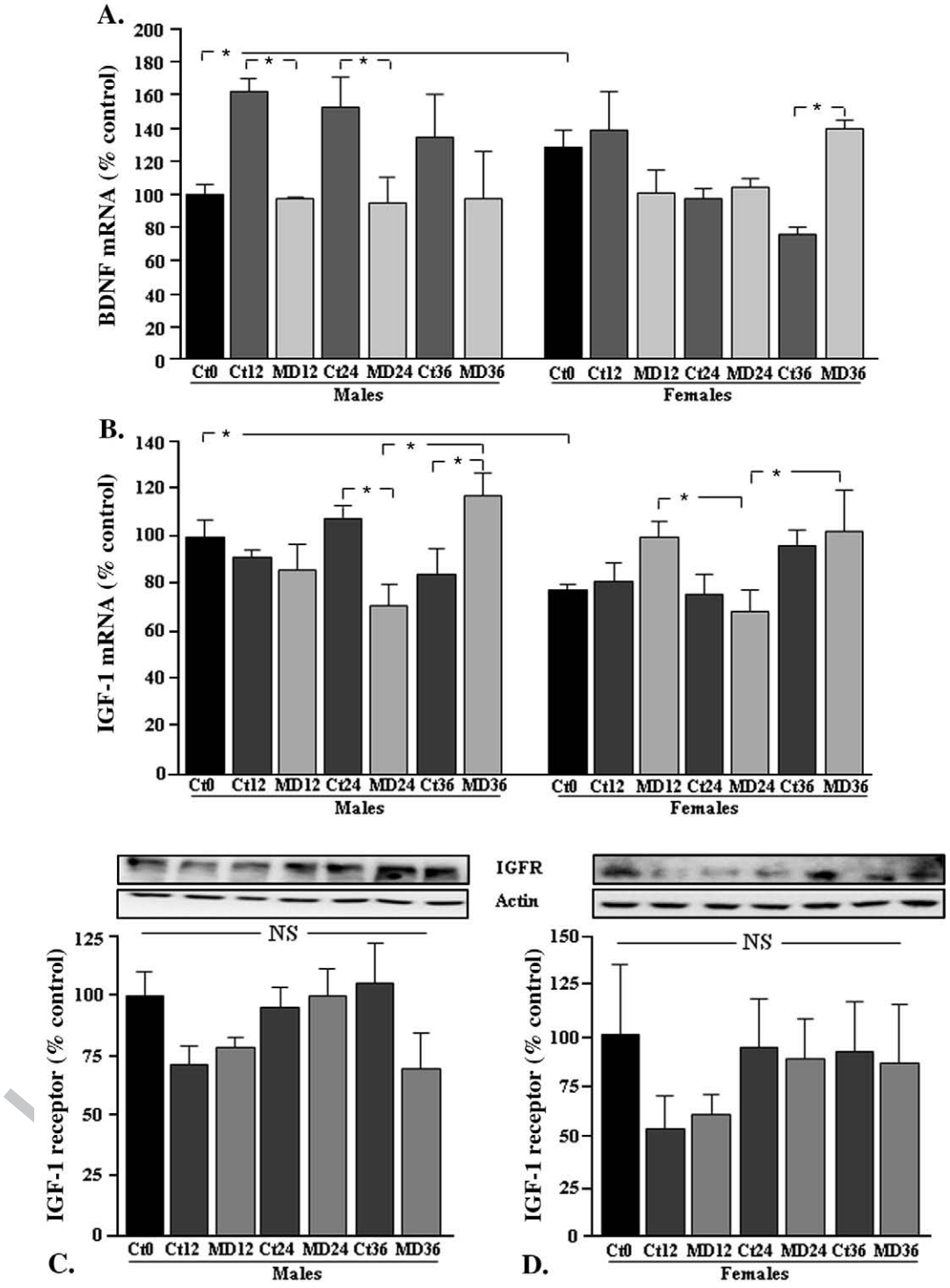

Fig. 3. Hypothalamic mRNA levels of (A) brain-derived neurotrophic factor (BDNF) and (B) insulin-like growth factor (IGF-1) and (C) IGF-1 receptor protein levels (IGFR) in male and female rats that were exposed to maternal deprivation (MD) starting on postnatal day (PND) 9 and sacrificed at 0, 12 and 24 h after the onset of MD, or 12 h (MD36) after being returned to their mother. Ct = control, * = ANOVA $p<0.05$.

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at 12 h in control animals and increasing at 24 h, and then decreasing again at 36 h, with this last decrease only significant in males (*DF*: 13, *F*: 16.629, $p<0.0001$). In both males and females, leptin levels were significantly reduced in MD pups compared to controls at all time-points of the study. In both sexes there was a significant increase in leptin levels after being replaced with their mother for 12 h (Fig. 2B).

Hypothalamic trophic factors and receptors

Hypothalamic ObR mRNA levels were significantly affected by time (*DF*: 3, *F*: 4.643, $p<0.005$ and treatment (*DF*: 1, *F*: 18.376, $p<0.0001$), but not by sex. In addition, there was a significant interaction between time and treatment (*DF*: 3, *F*: 3.974, $p<0.02$). Twenty-four hours after separation from their mother, both male and female pups had a significant increase in hypothalamic ObR mRNA levels (Fig. 2C; *DF*: 13, *F*: 4.222, $p<0.003$). After being returned to their mothers, this increase persisted in male pups, but returned to control levels in female pups.

Hypothalamic BDNF mRNA levels were affected by treatment (*DF*: 1, *F*: 4.528, $p<0.05$) with a significant interaction between sex and treatment (*DF*, 1, *F*: 5.199, $p<0.03$). At baseline, males had significantly lower BDNF mRNA levels than females. Male MD pups had significantly lower BDNF mRNA levels compared to their controls at 12 and 24 h, while MD female rats had significantly increased levels at 36 h (Fig. 3A; *DF* 13, *F*: 2.889, $p<0.02$).

There was a significant effect of sex (*DF*: 1, *F*: 4.833, $p<0.05$) and an interaction between sex and time (*DF*: 3, *F*: 4.012, $p<0.01$) on hypothalamic IGF-1 mRNA levels (Fig. 3B). In male rats there was a significant interaction between time and treatment (*DF*: 3, *F*: 4.125, $p<0.01$) with MD males having significantly decreased IGF-1 mRNA levels at 24 h and significantly increased levels at 36 h compared to their controls (Fig. 3B; *DF*: 6:, *F*: 2.642, $p<0.03$). In female rats there was a significant effect of time on IGF-1 mRNA levels (*DF*: 3, *F*: 3.671, $p<0.02$). At baseline, male pups had significantly higher IGF-1 mRNA levels than females. Whereas in control females there was no significant change in IGF-1 mRNA levels, in MD females these levels significantly decreased between 12 and 24 h of separation and increased again at 36 h (*DF*: 13, *F*:2.637, $p<0.005$).

Protein levels for IGF-1 receptor protein were not affected by MD or time in either males (Fig. 3C) or female pups (Fig. 3D).

Hypothalamic cell turnover

To determine the effect of MD on hypothalamic cell turnover, overall cell death and proliferation were analyzed. No significant effect of MD, sex or time was found on cell death during the study period as determined by ELISA (Fig. 4A). This was coincident with no significant change in the levels of the pro-apoptotic protein BAX or in the anti-apoptotic protein Bcl-2 α in any of the experimental groups (data not shown).

Cell proliferation, as indicated by relative levels of PCNA, were significantly affected by time (*DF*: 3, *F*: 2.892, $p<0.05$), with an interaction between MD and time (*DF*: 3, *F*: 3.801, $p<0.02$) in male rats. In control male rats there was a significant decline in PCNA levels at 12 h that returned to control levels by 24 h. This temporal change was not seen in MD males, but at the 36 hr study point MD males had significantly lower PCNA levels than their controls (Fig. 4B; *DF* 6, *F*: 3.800, $p<0.005$). In control female rats PCNA levels increased

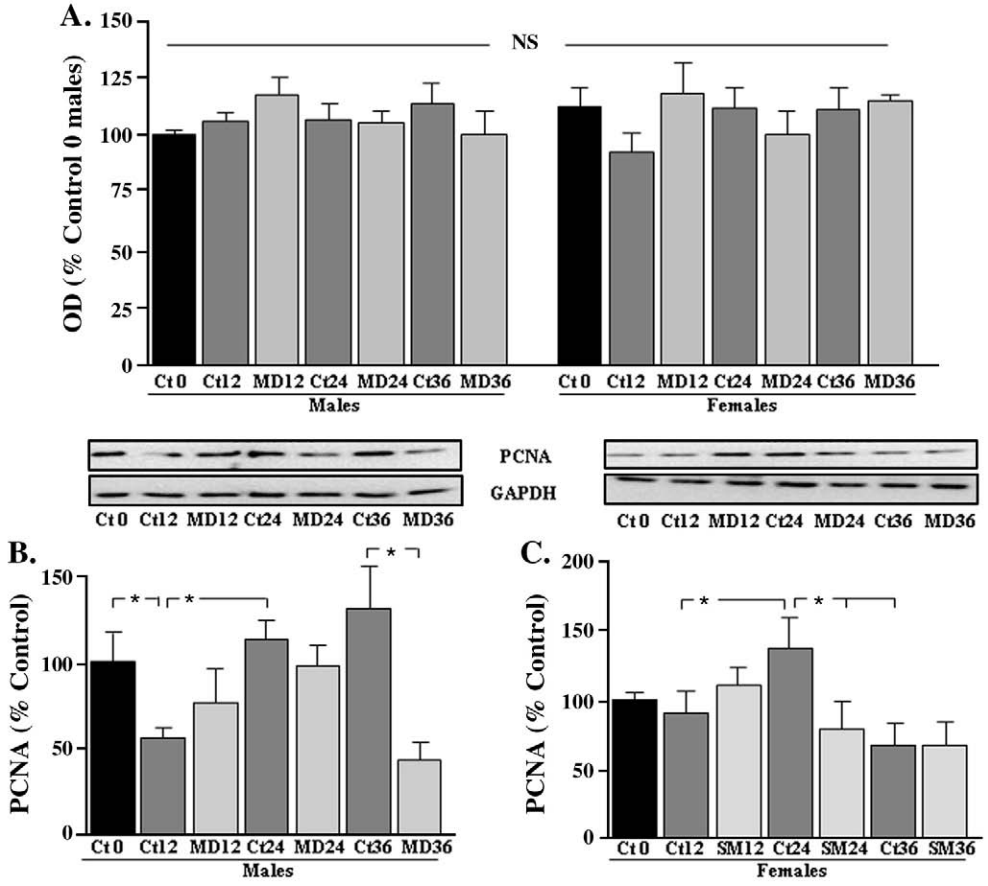

Fig. 4. Relative levels of cell death (A) and proliferation (B and C) in the hypothalamus of male and female rats that were exposed to maternal deprivation (MD) starting on postnatal day (PND) 9 and sacrificed at 0, 12 and 24 h after the onset of MD, or 12 h (MD36) after being returned to their mother. OD = optical density, PCNA = proliferating cell nuclear antigen, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, Ct = control, * = ANOVA $p<0.05$.

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significantly at 24 h, declining again at 36 h. This resulted in MD female pups having significantly lower PCNA levels at 24 h compared to their controls (Fig. 4C; *DF* 6, *F*: 2.522, $p<0.04$).

Levels of nestin, an intermediate filament expressed in proliferating and immature cells, were significantly affected by time in the hypothalamus of male (*DF*: 3, *F*: 8.532, $p<0.0005$) and female (*DF*: 3, *F*: 15.941, $p<0.0001$) rats. In female rats there was also an effect of MD (*DF*: 1, *F*: 5.472, $p<0.03$). Nestin levels changed throughout time in both control and MD males, decreasing at 12 h and increasing again at 24 h, with a decline again at 36 h but this did not reach statistical significance in control males (Fig. 5A); *DF*: 6, *F*: 4.912, $p<0.001$). In females, nestin levels also decreased significantly at 12 h in both control and MD animals, rising again at 24 h and declining significantly at 36 h. At 24 h, the rise in nestin levels was significantly lower in MD females than in control females (Fig. 5B; *DF*: 6, *F*: 9.871, $p<0.0001$).

Levels of the glial cell filament, GFAP, was significantly affected by MD (*DF*: 1, *F*: 11.177, $p<0.002$), with an interaction between time and MD (*DF*: 3, *F*: 4.709, $p<0.007$) in male rats. In control males GFAP levels increased gradually throughout time, while in MD males there was no change in GFAP levels, resulting in them having significantly lower levels compared to their controls (Fig. 5C, *DF*: 6, *F*: 6.022, $p<0.0002$). There were no significant differences in GFAP levels between the female groups (Fig. 5D).

In males there was a significant effect of time (*DF*: 3, *F*: 10.240; $p<0.0002$) on vimentin levels, with a significant decrease at 12 h in both control and MD males that increased at 24 h (Fig. 5E). In addition, MD36 males had significantly lower vimentin levels than their

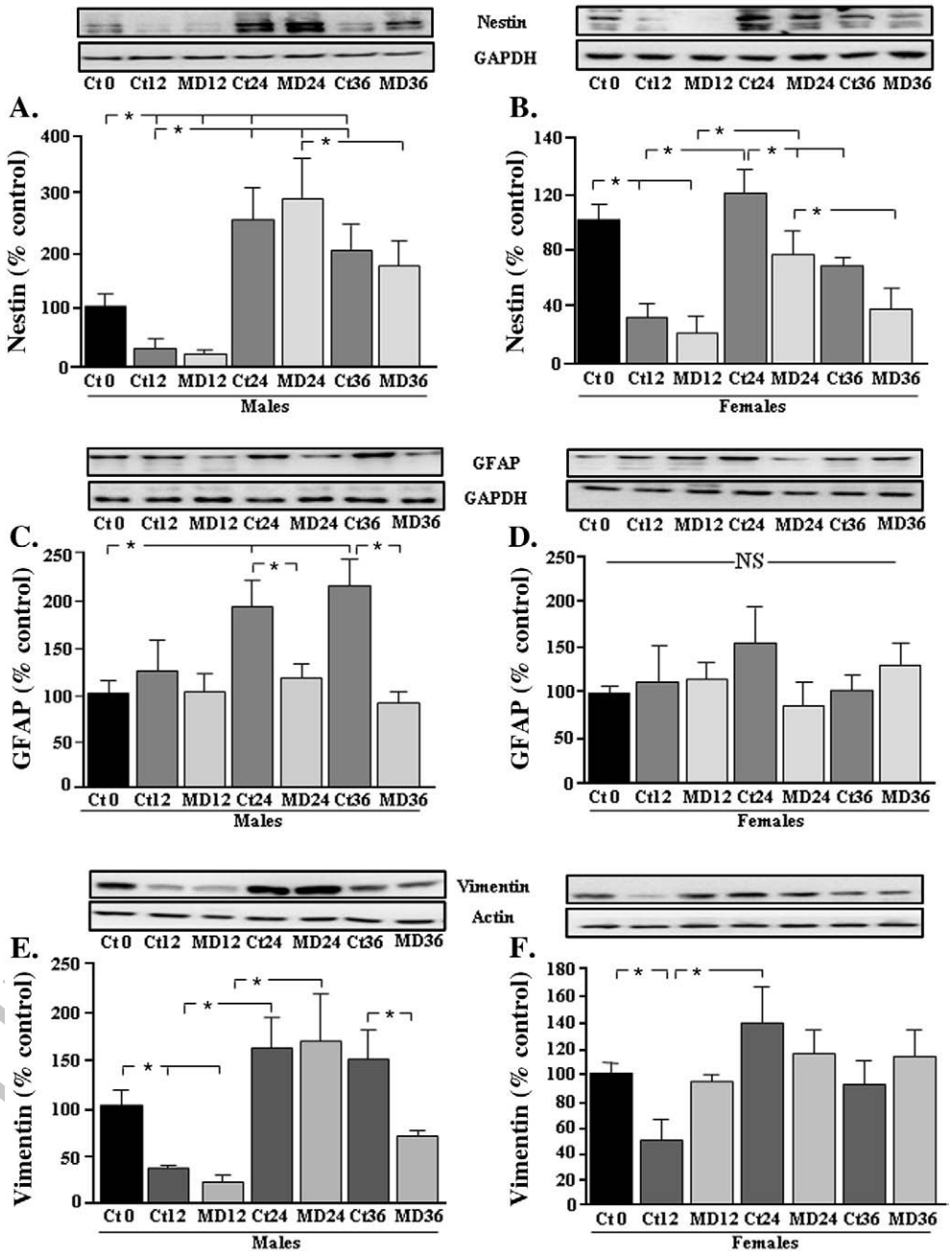

Fig. 5. Relative levels of nestin (A and B), glial fibrillary acidic protein (GFAP; C and D) and vimentin (E and F) in the hypothalamus of male and female rats that were exposed to maternal deprivation (MD) starting on postnatal day (PND) 9 and sacrificed at 0, 12 and 24 h after the onset of MD, or 12 h (MD36) after being returned to their mother. GAPDH = glyceraldehyde 3-phosphate dehydrogenase, Ct = control, * = ANOVA $p<0.05$.

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controls. Vimentin levels were significantly affected by time in female animals (*DF*: 3, *F*: 3.143, $p<0.05$).There was a significant decrease at 12 h in control animals that rose again by 24 h (Fig. 5F; *DF*: 6, *F* 2.478, $p<0.05$).

Discussion

The mammalian hypothalamus is sexually dimorphic with both the early hormonal environment and genetics playing important roles in this phenomenon (Sakuma, 2009; Carrer and Cambiasso, 2002). As hypothalamic neurons from male rats mature earlier than those from females, even before the critical period of steroid imprinting (Carrer and Cambiasso, 2002), it follows that hypothalamic development could be differently affected in males and females by environmental changes. Here we show that neonatal male and female rats have different hypothalamic levels of BDNF and IGF-1, two important neurotrophic factors, and that MD modulates them in a sexually dimorphic manner. Thus, the differential changes in these trophic factors could induce the sex differences in hypothalamic cell proliferation and maturation in response to MD, which in turn is involved in the sexually dimorphic endocrine outcomes.

At PND9, basal circulating corticosterone levels were higher in females than in males, in agreement with previous reports in neonatal animals and adults (Genest et al., 2004; Llorente et al., 2008; Mitsushima et al., 2008). Maternal deprivation significantly increased circulating corticosterone in both sexes throughout the study, similar to that reported at different ages and times following MD (Schmidt et al., 2006; Llorente et al., 2008; Ooman et al., 2009; Kikusui et al., 2009). The persistence in elevated corticosterone levels even 12 h after the dam was returned to her pups could be partially related to the mother's behaviour (Coutellier et al., 2008; Moles et al., 2008; Ooman et al., 2009). However, our previous data showing that corticosterone levels are significantly increased in maternally deprived animals at PND13 (Viveros et al 2009) suggest that the alterations in corticosterone levels are long lasting. Moreover, there is evidence indicating that the hypothalamic-pituitary-adrenal axis of these animals may be persistently and differentially altered until adulthood (De la Fuente et al., 2009; Viveros et al., 2009).

Changes in nutritional factors such as glucose are suggested to precede and participate in the rise in corticosterone levels in MD (Schmidt et al., 2006, 2008). Here glycemia declined in response to MD in both sexes and did not normalize by the end of the study, similar to the maintenance of elevated corticosterone levels. Although maternal care increases when the dam is returned to her pups, with males receiving more care than females (Ooman et al., 2009), the pups may not immediately return to normal feeding or a normal stress status. Furthermore, the difference in maternal care could be involved in some of the sexually dimorphic outcomes.

Although there were no differences in basal glycemia, males had significantly lower insulin levels than females, in accordance with a previous report of sex differences at PND12 rats (Vital et al., 2006). There was a very high variation in insulin levels in control animals that is most likely the result of random nursing. Insulin levels tended to decrease with MD, but this was only significant in males at MD24, which again may be due to the high variability in control rats.

Salzmann and colleagues (2004) report decreased circulating leptin levels 24 h after MD (PND10) and suggest that this decline participates in the stress response both during MD and afterwards. Furthermore, as the rapid decline in leptin cannot be rescued by glucose administration it may not be due solely to lack of nutrition (Schmitz et al., 2006). We found leptin to be almost undetectable 12 h after the onset of MD and to remain low even 12 h after the mother's return to the home cage. Moreover, leptin remains decreased in MD rats at PND13 (Viveros et al., 2009). As one of the main sources of leptin in the stomach during neonatal life is suggested to be maternal milk (Oliver et al., 2002), leptin variations in control rats could follow feeding, with the lack of nursing involved in the decline in leptin levels in MD rats. Although leptin levels are known to follow a circadian rhythm (Froy, 2007), to our knowledge PND9–10 is the earliest age where this has been reported in rats.

Leptin is fundamental for the normal development of specific hypothalamic circuits (Pinto et al., 2004; Bouret et al., 2004) and changes in this hormone during development can result in metabolic disturbance later in life (McMillen et al., 2006). The surge in circulating leptin between postnatal days 6 and 12, peaking at PND10, is not thought to be involved in appetite regulation (Ahima et al., 1998), but most likely has developmental implications. Indeed, blockage of this leptin peak modulates food intake behaviour and energy expenditure in the adult animal (Djiane and Attig, 2008). Thus, the dramatic decline in leptin at PND9–10 could be involved in the later metabolic disturbances, including decreased leptin levels, induced by MD (Matsumoto et al., 2006; Viveros et al., 2009). The decline in leptin could also participate in the up-regulation of hypothalamic ObR mRNA levels in response to MD. In females, however, ObR mRNA levels normalized at MD36 even though leptin remained significantly decreased. In both sexes leptin levels increased significantly between MD24 and MD36, which may have been sufficient to normalize ObR levels in females, but not in males. Indeed, females are reported to be more sensitive to the hypothalamic effects of leptin (Clegg et al., 2003).

Leptin also induces BDNF levels in the hypothalamus (Komori et al., 2006). This neurotrophic factor is important for neuronal survival, differentiation and proliferation (Pencea et al., 2001; Tapia-Arancibia et al., 2004; Coupé et al., 2009) and is also involved in nutritional regulation and stimulates hypothalamic ObR levels (Byerly et al., 2009). However, the decline in leptin during MD was not always associated with a decrease in BDNF. Although hypothalamic BDNF mRNA levels decreased at 12 and 24 h of separation in male rats, normalizing 12 h after being returned to their mothers, there was no change in females during MD. Moreover, hypothalamic BDNF increased 12 h after the termination of MD in females. . In males the decline in BDNF levels recovered rapidly after resuming maternal care; however, the long-term effects in the adult hypothalamus remain to be elucidated as Roceri et al., (2002) report a significant decline in BDNF in the hippocampus of adult MD rats. In addition to leptin, other factors, such as stress and glucocorticoids modulate BDNF (Smith et al., 1995; Givalois et al., 2004) and the time dependent variations in BDNF observed in control rats could be the consequence of normal circadian variations of these factors.

Insulin-like growth factor-1 is important for hypothalamic development, with its local production being high during early postnatal life (D'Ercole and Ye, 2008). We found males to have higher levels than females at PND9, with MD decreasing IGF-1 in males at 24 h and increasing it at 36 h, with no effect in females. However, in both sexes IGF-1 mRNA levels rose significantly between the termination of MD (24 hr) and 12 h after being replaced with their mother. However, there was no change in IGF receptor levels. It is possible that specific nuclei respond differently to this experimental paradigm and these more subtle changes in receptor levels cannot be detected by the methodology employed here. Indeed, specific hypothalamic nuclei are known to have sexually dimorphic responses to IGF-1 (Daftary and Gore, 2003).

Cell division in different tissues, including the brain, (Guzman-Marin et al., 2007), is influenced by circadian rhythms and disruption of this pattern may have pathological outcomes (Lévi et al., 2007). In control rats markers of cell proliferation and structural proteins expressed in immature cells, such as nestin and vimentin, changed throughout the 36-h study period, as did BDNF. Maternal deprivation decreased levels of the cell proliferation marker, PCNA, at MD24 in females and MD36 in males. This temporal difference could be because the degree of cell turnover in some hypothalamic regions is clearly sexually dimorphic (Sakuma, 2009). However, the temporal

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changes in nestin levels were not affected by MD in either sex, suggesting no affect on the rate of neuron maturation. In contrast, the temporal rise in the astrocyte structural protein, GFAP, observed in control males was annulled by MD. There was no difference in GFAP between any of the groups of females. In the adult hypothalamus GFAP levels are higher in males than in females, which is at least partially due to the neonatal sex steroid environment (Chowen et al., 1995). Thus, this increase in levels in males could be related to the neonatal testosterone surge (Forest, 1979). Maternal deprivation modulates adult GFAP levels in the hippocampus, cerebellum and cortex (Llorente et al., 2008; López-Gallardo et al., 2008; Llorente et al., 2009, Viveros et al 2009, Musholt et al., 2009), with some of these effects being sexually dimorphic (Llorente et al., 2008, 2009; Viveros et al., 2009). Whether GFAP levels in the hypothalamus of adult rats are modulated by MD remains to be determined.

In summary, here we demonstrate that early MD has sexually dimorphic effects on the developing hypothalamus. Not only are the basal levels of trophic factors such as IGF-1 and BDNF different between the sexes, but they are modulated in a sex-specific manner during the separation period. The sex differences in trophic factors are associated with differences in cell proliferation and cell specific markers, indicating that maternal deprivation differentially affects cell development in the hypothalamus of male and female rats, which could underlie, at least in part, the differences observed between the sexes in the pathological effects in the adult animal. In contrast, both males and females have a significant increase in circulating corticosterone and a dramatic decrease in leptin levels in response to MD, which may be involved with some of the later endocrine outcomes, but are most likely not involved in the different responses between the sexes.

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