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Early maternal deprivation immunologically primes hippocampal synapses by redistributing interleukin-1 receptor type I in a sex dependent manner

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ABSTRACT

Challenges experienced in early life cause an enduring phenotypical shift of immune cells towards a sensitised state that may lead to an exacerbated reaction later in life and contribute to increased vulnerability to neurological diseases. Peripheral and central inflammation may affect neuronal function through cytokines such as IL-1. The extent to which an early life challenge induces long-term alteration of immune receptors organization in neurons has not been shown. We investigated whether a single episode of maternal deprivation (MD) on post-natal day (PND) 9 affects: (i) the synapse distribution of IL-1RI together with subunits of NMDA and AMPA receptors; and (ii) the interactions between IL-1RI and the GluN2B subunit of the NMDAR in the long-term, at PND 45. MD increased IL-1RI levels and IL-1RI interactions with GluN2B at the synapse of male hippocampal neurons, without affecting the total number of IL-1RI or NMDAR subunits. Although GluN2B and GluN2A were slightly but not significantly changed at the synapse, their ratio was significantly decreased in the hippocampus of the male rats who had experienced MD; the levels of the GluA1 and GluA2 subunits of the AMPAR were also decreased. These changes were not observed immediately after the MD episode. None of the observed alterations occurred in the hippocampus of the females or in the prefrontal cortex of either sex. These data reveal a long-term, sex-dependent modification in receptor organisation at the hippocampal post-synapses following MD. We suggest that this effect might contribute to priming hippocampal synapses to the action of IL-1 β .

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1. Introduction

Emerging evidence suggests that adverse early-life events may cause an enduring phenotypical shift of the CNS towards an immune-sensitised state (named immune priming). For example, by increasing the expression of receptors for immune molecules on microglia and/or peripheral immune cells, neonatal bacterial infections (Bilbo et al., 2005; Williamson et al., 2011) early life seizures (Somera-Molina et al., 2009), maternal obesity (Bilbo and Tsang,

2010), and pre-natal stress (Vanbesien-Mailliot et al., 2007) promote the adoption of a biochemical setting that persists into adulthood. As a result, adult microglia are ready to sense any subsequent inflammatory trigger and then organise an immediate and “at best” response that leads to an exaggerated production of pro-inflammatory cytokines.

High levels of IL-1 β concur to the pathogenesis of both acute and chronic neurological disorders (Allan et al., 2005; Fogal and Hewett, 2008; Meyer et al., 2011), by disrupting memory processes (Avital et al., 2003; Barrientos et al., 2009), altering synaptic plasticity (Ross et al., 2003; Schneider et al., 1998) and promoting neuronal death (Relton and Rothwell, 1992; Viviani et al., 2003, 2006). Indeed, bacterial neonatal infection in rats leads to HP-dependent memory deficits in the adulthood as the result of an exaggerated IL-1 β production by primed microglia (Williamson et al., 2011). As well, excessive cytokines production (i) increases susceptibility to seizures and neurologic injury in adult animal that have been

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sensitized by seizures early in life (Somera-Molina et al., 2009) and (ii) induces marked changes in anxiety and spatial learning in the adult offspring of obese dams (Bilbo and Tsang, 2010). These observations suggest that the phenotypic shift of CNS immune cells towards a sensitised state acquired early in life set the stage for programming later-life brain behaviour (Bilbo and Schwarz, 2009).

The concept of immune priming, as the acquisition of a setting that emphasizes the immune response, has been exclusively applied to microglia because these are the immunocompetent cells of the CNS. Nevertheless, neuroimmune response is the result of a delicate balance between the production of inflammatory mediators by immune cells and the ability of neurons to sense them through the expression of specific receptors. Interleukin-1 (IL-1) receptor (IL-1RI) is expressed on neurons and involved in various functions ranging from neurotransmission to cell survival. (Allan and Rothwell, 2001; Gadiant and Otten, 1997; McAfoose and Baune, 2009). In particular, a recent hypothesis based on the observation that high levels of IL-1 reached in pathological conditions can induce hyper-excitability of neuronal circuits (Galic et al., 2008; Liu et al., 2013; Rossi et al., 2012), suggest a link between IL-1 and the glutamatergic system as a common mechanism of dysfunction (Fogal and Hewett, 2008).

We have recently described a dynamic and functional interaction between IL-1RI and the NMDA receptor (NMDAR) complex (Gardoni et al., 2011; Viviani et al., 2003). Indeed, both recombinant and glial released IL-1 β potentiates NMDAR functions in primary hippocampal neurons by increasing NMDA-induced intracellular calcium rise (Viviani et al., 2003, 2006). This effect results in the reduction of synaptic spines and exacerbation of neuronal death driven by the overactivation of the GluN2B subunit (Viviani et al., 2003, 2006). Interleukin-1 receptor antagonist (IL-1Ra) and ifenprodil prevent both these effects (Viviani et al., 2003, 2006), confirming the central role of IL-1RI and NMDAR sharing the GluN2B subunit. Moreover, we have found that IL-1RI receptors co-localise with, and bind to the GluN2B subunit of the NMDARs in a specific and highly organised compartment of the glutamatergic synapse: the postsynaptic density (Gardoni et al., 2011). IL-1RI interactions with NMDARs and its localisation at the synaptic membranes is increased in primary hippocampal neurons as a consequence of IL-1 β or NMDA stimulation (Gardoni et al., 2011). These results underline a functional relationship between IL-1RI and the NMDAR through the GluN2B subunit in primary hippocampal neurons, which can be dynamically modulated. It is therefore conceivable that the expression, distribution of IL-1RI and interaction with the NMDAR might contribute to shape the molecular structure of the synapse and define a neuronal “immunophenotype”. We thus hypothesised that, in the same way that early-life adverse events prime microglia, they could also enduringly prime neurons orchestrating IL-1RI expression, distribution and interaction. To investigate this hypothesis, we adopted a model of maternal deprivation (MD). Mother–infant interaction is relevant for brain maturation and vulnerability to disease, involving both the glutamatergic system (Ku et al., 2008; Rodeñas-Ruano et al., 2012) and pro-inflammatory processes (Hennessy et al., 2010).

The aim of this study was to verify whether this specific type of early-life stress can enduringly change the expression and distribution of IL-1RI in the hippocampus and pre-frontal cortex, as well as its interactions with NMDARs. As early-life development is a critical period for functionally shaping the glutamatergic synapse (Bellone and Nicoll, 2007; Gray et al., 2011) these endpoints were evaluated in association with the distribution of the NMDAR and AMPAR subunits. Finally, as sex-dependent alterations in developing brain have been described in neonatal rats exposed to MD (Llorente et al., 2008, 2009; Viveros et al., 2009) we considered male and female rats separately.

2. Materials and methods

All of the experiments described were performed in accordance with Spanish Royal Decree 1201/2005 of 21 October 2005 (BOE no. 252) concerning the protection of experimental animals, and in close agreement with the European Community Council Directive of 24 November 1986 (86/609/EEC). The experimental protocol was approved by our local Animal Ethics Committee. Every effort was made to minimise animal suffering and distress.

2.1. Animals

The experimental animals were the offspring of albino Wistar rats purchased from Harlan Laboratories housed in plastic Macro-lon[®] III cages. The parental generation was mated (one male \times two females) in our animal facilities approximately 2 weeks after their arrival. After 10 days, the females were singly housed and animals monitored daily for parturition. On the day of birth (PND 0), litters were sex-balanced and culled to eight pups per dam (four males and four females). Except for the experimental animals (see below), the dams and litters were then left undisturbed until weaning (PND 22), when the animals were separated by sex and housed in groups of four siblings per cage.

All of the animals were kept at a constant temperature (22 \pm 1 $^{\circ}$ C) and humidity (50 \pm 1%) in a reverse 12-h dark-light cycle (lights on at 20.00), with free access to food (commercial diet for rodents A04/A03; SAFE, Augy, France) and water.

2.2. Early maternal deprivation

Early maternal deprivation (MD) was performed as previously described (Llorente et al., 2007). Briefly, on PND 9, the litters underwent 24 h of maternal deprivation: i.e. the dams were removed from their home-cages at 09.00, and the pups were left undisturbed (in the same room) until the next day (PND 10, 09.00) when the dams were returned to their home-cages. Control animals were manipulated in the same way except for the MD episode. Six litters were used to analyse the long-term effects of MD (3 l were assigned to the MD group and another 3 to the control group). These animals (first batch) were sacrificed on PND 45 and were previously evaluated in diverse behavioural tests (Marco et al., 2013). To evaluate the acute effect of MD, an additional batch of animals (6 l:3 l were assigned to the MD group and another 3 to the control group) was sacrificed on PND10.

For western blotting analysis, animals were sacrificed by rapid decapitation; their brains were extracted and the frontal cortex and hippocampi were dissected, frozen in dry ice, and stored at -80° C until use.

For immunohistochemical analyses, half of the animals from the first batch (PND45) were deeply anaesthetised with pentobarbital (100 mg/kg, Normon Veterinary Division) and perfused transcardially with paraformaldehyde 4% (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (PB), pH 7.4. Their brains were post-fixed with paraformaldehyde 4% in PB and cryoprotected in 11% sucrose in 0.1 M phosphate saline buffer (PBS), pH 7.4.

2.3. Materials and antibodies

All of the reagents were purchased from Sigma (Milan, Italy). IL-1R, and IL-1 β antibodies were purchased from Santa Cruz Biotechnology (CA, USA); monoclonal GluN2B and GluA2 antibodies from NeuroMab (Davis, CA, USA); polyclonal GluA1 antibodies from Calbiochem (Merck, Darmstadt, Germany); monoclonal GluN2A antibody from Zymed (San Francisco, CA, USA); monoclonal actin and secondary anti-mouse antibodies from Sigma Aldrich (S. Louis,

MO, USA); and secondary anti-rabbit antibody from Bio-Rad (Hercules, CA, USA).

2.4. Subcellular fractionation

The post-synaptic Triton-insoluble fractions (TIFs) were purified from tissue as previously described (Gardoni et al., 1998, 2001). Briefly, tissues were homogenized in ice-cold sucrose 0.32 M containing Hepes 1 mM, MgCl₂ 1 mM, EDTA 1 mM, NaHCO₃ 1 mM, PMSF 0.1 mM, at pH 7.4 in presence of a complete set of proteases inhibitors (Complete™, Roche Diagnostics, Basel, Switzerland) and phosphatases inhibitors (Sigma–Aldrich). The homogenized tissue was centrifuged at 1,000xg for 10 min. The resulting supernatant (S1) was centrifuged at 13,000g for 15 min to obtain a fraction of mitochondria and synaptosomes (P2 fraction). The pellet was resuspended in buffer containing 75 mM KCl and 1% Triton-X 100 and centrifuged at 100,000g for 1 h. The final pellet was homogenized in a glass-glass potter in 20 mM Hepes. Then, an equal volume of glycerol was added and this fraction, referred as Triton insoluble fraction (TIF), was stored at –80 °C until processing. TIF fraction was used instead of the classical postsynaptic density (PSD) (Gardoni et al., 1998) because the amount of the starting material was very limited. The protein composition of this preparation was, however, carefully tested for the absence of presynaptic markers (Viviani et al., 2006); i.e. synaptophysin and synaptotagmin) as well as for the enrichment in post-synaptic proteins (Gardoni et al., 2011). Similar protein yield was obtained in TIF purified from all experimental groups and the same amount of TIF protein was applied to SDS–PAGE in each lane and electroblotted for all samples.

2.5. Immunoprecipitation and Western blotting

TIFs were immunoprecipitated with antibodies raised against GluN2B and the presence of IL-1RI and GluN2B in the immunocomplex were evaluated by means of western blot as previously reported (Gardoni et al., 2011). Briefly, TIFs (50 µg protein) were incubated overnight at 4 °C in a RIA buffer (200 mM NaCl, 10 mM EDTA, 10 mM Na₂HPO₄, 0.5% NP-40, 0.1% SDS, 10 mM NaF) with GluN2B antibody (1:200), and then for 2 h with protein A-Sepharose beads. Following this incubation, the supernatants were removed, and the beads were washed five times with solubilisation buffer. After the final wash, the beads were resuspended in sample buffer for SDS–PAGE and briefly centrifuged; the supernatants were loaded on 6% SDS–PAGE gels. The presence of GluN2B and IL-1RI in the immunocomplex was evaluated by Western blotting analysis and IL-1RI protein level was normalized for GluN2B immunoreactivity.

Protein content of homogenate and TIF's samples has been quantified by using Bio-Rad (Hercules, CA, USA) protein assay. After measuring protein concentration, all samples have been standardized at 1 µg/ul concentration and 20 µg/sample loaded in each lane. Western blot analysis was performed using antibodies raised against N-Methyl-D-aspartic acid (NMDA) glutamate receptor subunits NR2A and NR2B, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor subunits GluR1, GluR2, interleukin-1 receptor type I (IL1RI) and actin. The specificity of the IL-1RI antibody was tested by pre-absorption with the blocking peptide. Quantification of Western blotting analysis has been performed by means of computer-assisted imaging (Quantity-One® System; Bio-Rad) after normalization on actin levels. Actin was chosen as standard to normalize TIF, since it is highly enriched in the post-synaptic compartment and is directly associated to the GluN2B subunit of the NMDAR (Robison et al., 2005).

2.6. Immunohistochemistry

Immunohistochemistry was performed as previously described (Llorente et al., 2008). Coronal brain sections (30 µm thick) were cut in a LEICA CM3050 cryostat, collected onto alternate gelatin-coated slides (four slices per slide), air dried and stored at –30 °C until use. For the immunohistochemistry of IL-1RI or IL-1β, the slides were incubated overnight at 4 °C with the primary anti-IL-1RI (1:200) or anti-IL-1β antibody (1:200). After several washes in PBS containing 0.25% Triton X-100, the slides were incubated with a FITC-conjugated secondary antibody (1:500), washed and mounted in an anti-fading water-based medium (Vectashield, VECTOR Laboratories, Burlingame, CA) containing DNAbinding fluorescent dye DAPI (2 µg/mL; Sigma–Aldrich) to assure visual observation of the nuclear morphology. Omission of the primary antibody was used as a control. The fluorescence analysis was made using an Axio Imager A1 microscope (Zeiss, Germany).

2.7. Quantitative evaluation of IL-1β-, IL-1RI- and DAPI-positive cells

The evaluation was made using a 10x objective in the medial hippocampus (bregma –3.30 to –10.00 mm) and the pre-frontal cortex (bregma 4.20 to 4.70) (Paxinos et al., 1998). Four slides per region and per animal were randomly selected, and three tissue sections per slide were analysed. In the hippocampal formation, we focused on areas CA1 and CA3, and the dentate gyrus (DG). For both CA subfields, we analyzed the zone corresponding to the stratum pyramidale (SP), and the stratum oriens (SO) together with the zone corresponding to strata radiatum, lacunosum, and moleculare (SRLM). For DG we analyzed the granular cell layer and the polymorphic layer. In the prefrontal cortex we analyzed all the cortical layers.

The number of IL-1β- IL-1RI- and DAPI-positive cells was estimated in a total of five counting frames (width 0.215 and length 0.26 mm) per slide and for each analysed area and region (a total of 72 tissue sections per region and per experimental group) and all of the counts were made on coded sections. Cell nuclei from immunoreactive cells that came into focus were counted. The data are expressed as the ratio between the number of IL-1β- and IL-1RI-positive cells the number of DAPI-positive nuclei in the same frame, in order to avoid local variations between animals.

2.8. IL-1β assay

IL-1β was measured by a commercially available ELISA (Quantikine, R&D Systems, Abingdon, UK) and using the soluble S1 fraction obtained during the subcellular fractionation procedure of all the investigated tissues.

2.9. Statistical analysis

Males and females were independently analysed, “n” refers to the number of rats used. Statistical analysis between C and MD groups was performed within each sex using an unpaired Student's *t*-test. A significance level of 95% (*p* < 0.05) was considered statistically significant.

3. Results

Initially all of the measurements in the male and female rats were made on PND 45: i.e. 35 days after the single episode of maternal deprivation (PND 9–10), to evaluate whether MD contributes to later changes of the neuronal immunophenotype. This time point was chosen to perform all the biochemical assessments on the basis of the observation that MD induces brain and behavioural

abnormalities in adolescent and adult rats (Ellenbroek and Riva, 2003; Llorente et al., 2009).

The distribution of IL-1RI and the GluN2A and GluN2B subunits of NMDARs was initially investigated by means of Western blotting using the post-synaptic TIFs purified from control and MD rat hippocampi (Hp). The amount of IL-1RI was significantly higher in the males who had experienced MD (Fig. 1A; $n = 6$, $p < 0.01$ vs. control). The levels of the GluN2A and GluN2B subunits did not significantly change in MD males, although GluN2A shows a decreasing trend while GluN2B slightly increase (Fig. 1A). This effect was male specific as no significant changes were observed in the females (Fig. 1B). As the subunit composition of synaptic NMDARs can

Table 1

Ratio of GluN2A/GluN2B subunits of the NMDAR as in TIF of Hp and PFC of males and females rat, control and MD. GluN2A/GluN2B ratios have been calculated on values normalized on actin. Data are expressed as % of control and expressed as means \pm SEM ($n = 6$ for each experimental group). * $p < 0.05$ vs. control; Student's t -test.

TIFs	GluN2A/GluN2B (% of control)		GluN2A/GluN2B (% of control)	
	Males	Females	Control	MD
Hp	100 \pm 3.9	54.8 \pm 5.8**	100 \pm 10.3	103 \pm 10.3
PFC	100 \pm 7.7	100 \pm 6.6	100 \pm 12.9	129 \pm 19.3

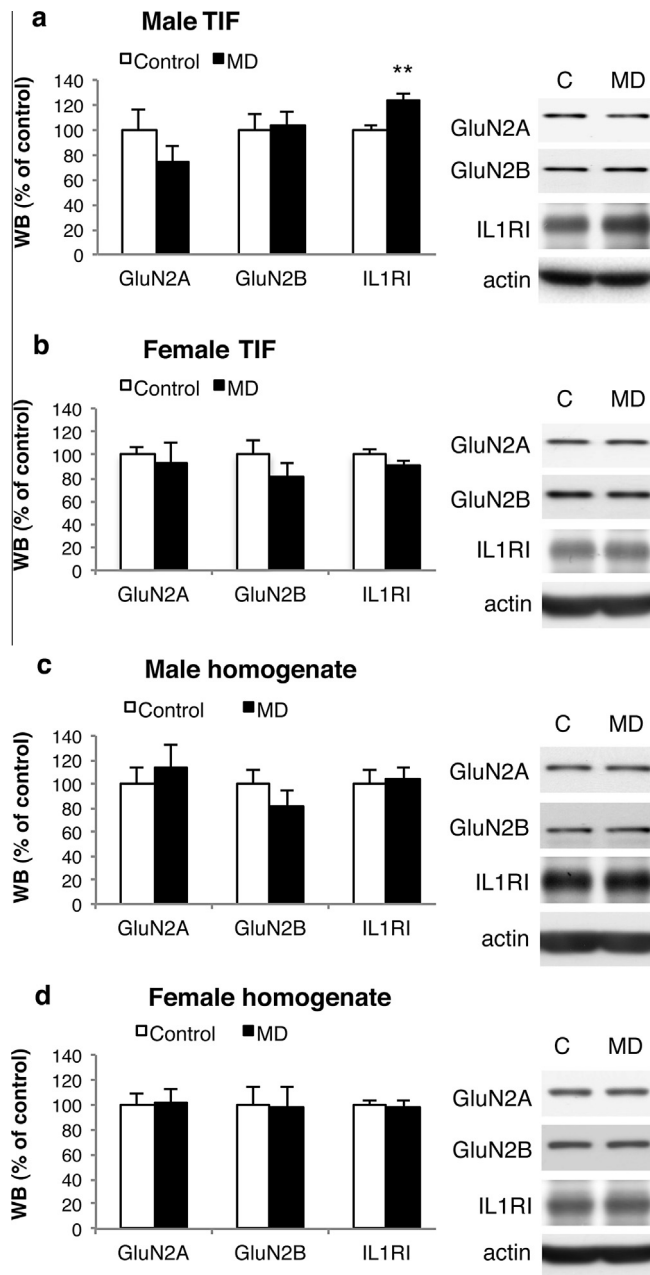


Fig. 1. Effect of maternal deprivation (MD) on IL-1RI, GluN2A and GluN2B levels in the hippocampus of 45-day-old male and female rats. Quantification (left) and representative Western blots of the proteins (right) in male (a) and female TIFs (b), and male (c) and female total homogenates (d) of control (C) and MD rats. The specificity of the IL-1RI antibody was tested by pre-absorption with the blocking peptide (data not shown). The data were normalised for actin immunoreactivity. Mean values \pm SE ($n = 6$). ** $p < 0.01$ vs. control; Student's t -test.

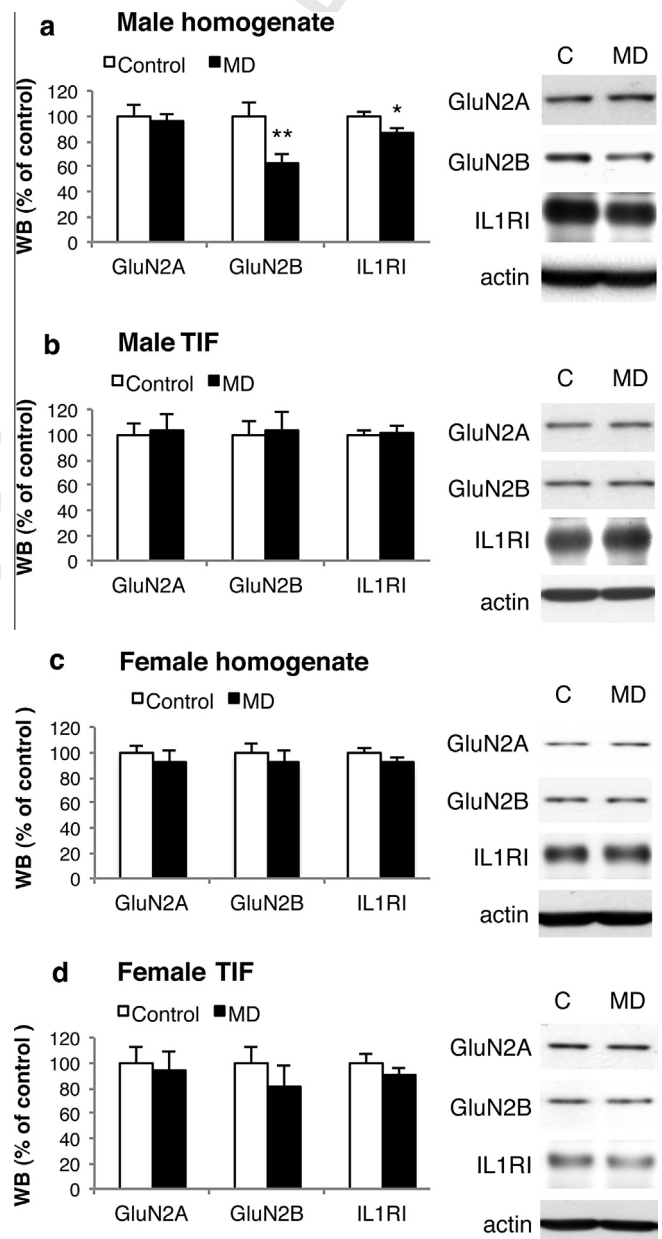


Fig. 2. Effect of maternal deprivation (MD) on IL-1RI, GluN2A and GluN2B levels in the pre-frontal cortex of 45-day-old male and female rats. Quantification (left) and representative Western blots of the proteins (right) in male (a) and female total homogenates (c), and male (b) and female TIFs (d) of control (C) and MD rats. The data were normalised for actin immunoreactivity. Mean values \pm SE ($n = 6$). ** $p < 0.01$ vs. control; Student's t -test.

quickly change at neonatal synapses, switching from the predominance of GluN2B- to GluN2A-containing receptors (Bellone and Nicoll, 2007; Paoletti et al., 2013), we also quantified the levels of the GluN2B to GluN2A subunits in the TIFs obtained from control and MD rats as GluN2A/GluN2B ratio (Table 1). This allowed to evaluate whether MD interferes with the expected shift and, if so, in which brain areas. On PND 45, the GluN2A/GluN2B ratio was reduced in the Hp of the MD males (Table 1; $n = 6, p < 0.01$ vs. control). No significant changes were observed in females (Table 1).

In order to clarify whether the increase in IL-1RI was due to receptor redistribution rather than a generalised increase in expression, IL-1RI, GluN2A and GluN2B levels were evaluated in total Hp homogenates. There was no difference in total protein expression in the control and MD groups of either sex (Fig. 1C, D).

Pre-frontal cortex (PFC) is another brain area involved in cognition and reactions to stress that is rich in IL-1RI (Plata-Salaman et al., 2000) and undergoes important maturational changes (Crews et al., 2007; Marco et al., 2011). PFC samples from male

and female rats were therefore processed for TIF and Western blotting, and assessed for IL-1RI, GluN2B and GluN2A expression. Unlike the Hp samples, the PFC homogenates of the male rats who had experienced MD had reduced amounts of IL-1RI (Fig. 2A; $n = 6, p < 0.05$ vs. control) and GluN2B (Fig. 2A; $n = 6, p < 0.01$ vs. control), whereas the levels of GluN2A were the same as those found in the controls (Fig. 2A), although none of the considered proteins was altered at the post-synapse. As shown in Fig. 2B, the TIF levels of IL-1RI, GluN2B and GluN2A were not changed after deprivation. Neither the total expression of IL-1RI, GluN2B and GluN2A (Fig. 2C) nor their distribution to the post-synaptic fraction (Fig. 2D) changed in the PFC of the females who had experienced MD. Furthermore, no modification of the GluN2A/GluN2B ratio was observed in both the male and female PFC (Table 1).

IL-1RI expression in male and female Hp and PFC was also evaluated by means of immunohistochemistry (Fig 3). IL-1RI showed more intense immunoreactivity in the pyramidal cell layer of CA1 and CA3, and the granular cell layer of the DG than in the

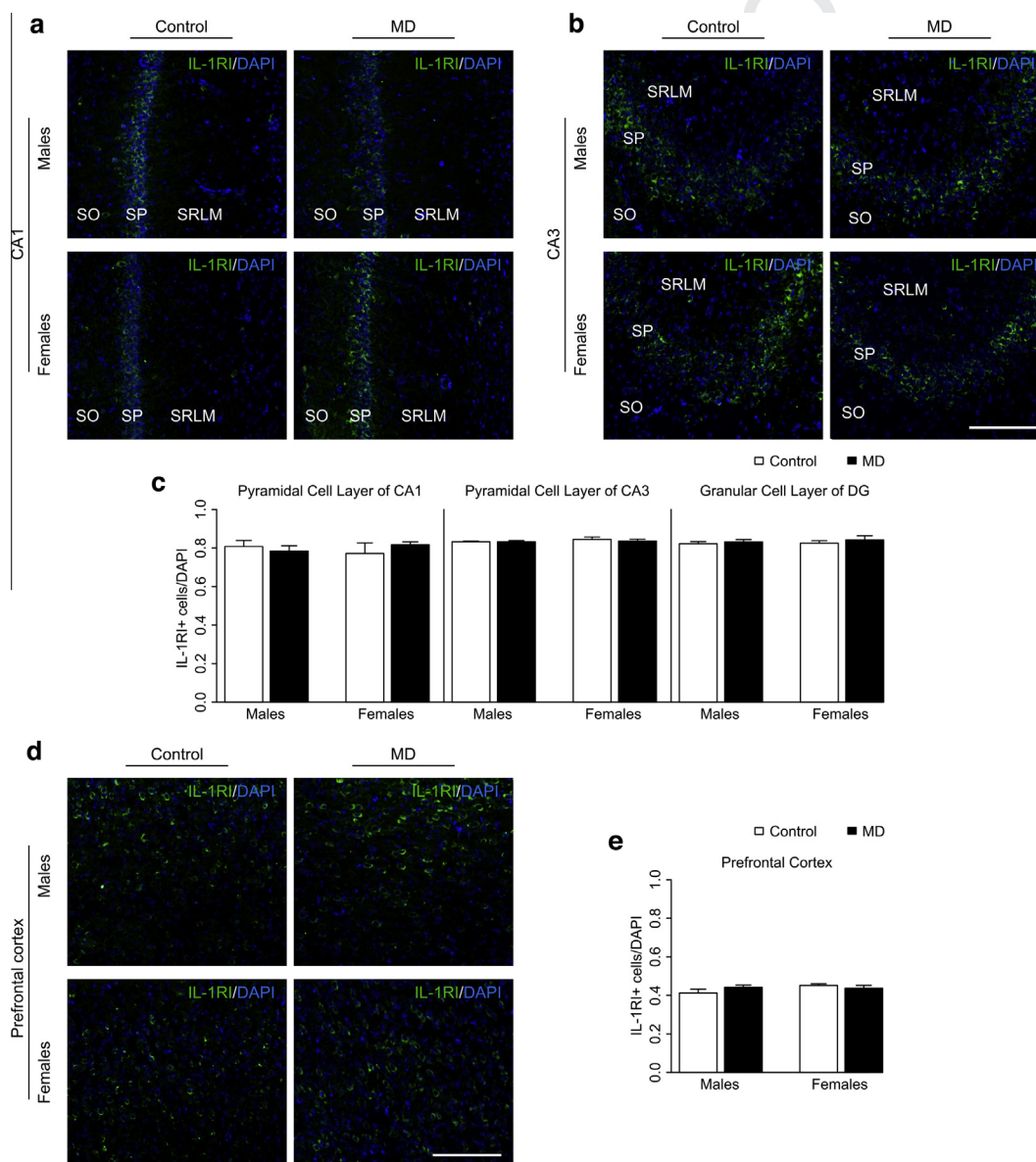


Fig. 3. Effects of maternal deprivation (MD) on the number of IL-1RI positive cells (IL-1RI + cells/DAPI) in 45-day-old male and female rats. Representative microphotographs of IL-1RI + cells/DAPI in the hippocampus (CA1 and CA3; a and b) and pre-frontal cortex (d). Scale bar 150 μ m. SO: stratum oriens; SP: stratum pyramidale; SRLM: stratum radiatum-lacunosum moleculare. The cells were quantified in areas CA1 and CA3, and the dentate gyrus of the hippocampal formation (c), and in the pre-frontal cortex (e). Mean values \pm SEM ($n = 6$).

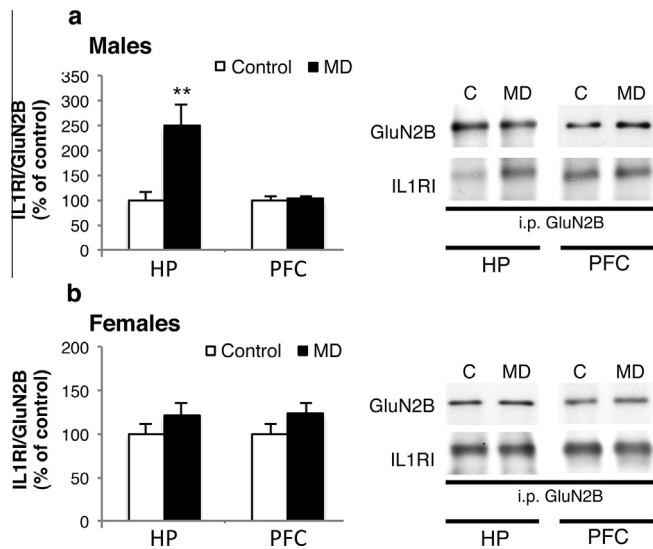


Fig. 4. Effect of maternal deprivation (MD) on IL-1RI co-immunoprecipitation with the GluN2B subunit of the NMDAR in male and female hippocampus and pre-frontal cortex. TIFs (50 μ g proteins) purified from male (a) and female (b) hippocampus and pre-frontal cortex were immunoprecipitated (i.p.) with an antibody specific for GluN2B. The presence of GluN2B and IL-1RI in the immunocomplex was evaluated by Western blotting in the control (C) and MD animals. IL-1RI was normalized for GluN2B immunoreactivity (left panel). Mean values \pm SE ($n = 6$). ** $p < 0.01$ vs. control; Student's t -test.

pre-frontal cortex. No change in the IL-1RI + cells/DAPI ratio in the MD rats of either sex were evident (Fig. 3), supporting the observation that the IL-1RI levels in the hippocampal TIFs of male MD rats is due to changes in the receptor's subcellular distribution.

Given the specific and dynamic association between IL-1RI and GluN2B, (Gardoni et al., 2011; Viviani et al., 2006) we used co-immunoprecipitation experiments to investigate whether MD could modulate their direct interaction at synapses. TIFs from Hp and PFC of all rats were immunoprecipitated with an antibody specific for GluN2B. Each sample was then evaluated for the presence of IL-1RI. Figure 4A shows an increased co-precipitation of IL-1RI together with GluN2B in the Hp ($n = 6$, $p < 0.01$ vs. control), but not in the PFC, TIFs of the MD males. Once again no change was evident in the Hp or PFC TIFs of the MD females (Fig. 4B).

Due to the impact of MD on IL-1 β signalling system in hippocampal neurons, we used an ELISA to measure the amount of IL-1 β in the plasma, Hp and PFC of the control and MD rats. Regardless of whether they had experienced neonatal MD or not, IL-1 β was virtually undetectable in the Hp and PFC on PND 45, and there was no significant between-group difference in plasma concentrations (male control and MD rats 19.3 ± 1.83 and 20.1 ± 2.5 pg/mL, $n = 6$, $p > 0.05$; female control and MD rats 20.4 ± 1.87 and 22.4 ± 2.44 pg/mL, $n = 6$, $p > 0.05$). Also there was no difference in the immunohistochemistry for IL-1 β of the Hp (CA1, CA3 and the DG) or PFC between the-groups (Fig. 5), thus excluding the possibility of an increase in IL-1 β levels in discrete portions of the selected brain areas.

Our findings show that MD drastically changes the biochemical structure of male Hp post-synapses by enhancing the relationship between IL-1RI and NMDARs and favouring the predominance of the GluN2B subunit over GluN2A. As NMDAR subunit expression and activity contribute to changes in AMPAR levels at the synapse (Gray et al., 2011), we evaluated GluA1 and GluA2 levels in the Hp and PFC TIFs of the four groups. Once again, the only substantial difference was that the Hp of the male MD rats had significantly lower GluA1 (Fig. 6A; $n = 6$, $p < 0.01$ vs. control) and GluA2 levels (Fig. 6A; $n = 6$, $p < 0.01$ vs. control) than the controls.

We finally investigated whether the changes observed within male Hp represent the result of the acute deprivation. Thus, Hp obtained by PND10 male rats shortly after MD and by matched controls were processed for TIFs and analyzed by Western blot to evaluate (i) IL-1RI enrichment, (ii) levels of GluA1 and GluA2, (iii) GluN2A/GluN2B ratio and (iiii) IL-1RI/GluN2B co-precipitation. IL-1 β levels were also evaluated by ELISA. The increased IL-1RI, as well as the reduction of GluA1 and GluA2 levels and the GluN2A/GluN2B unbalance at the post-synapse of MD male rats appear to be a long-term consequence of the early events, since no significant changes between controls (C) and MD were observed for IL-1RI (C: 100 ± 6.32 ; MD: 94.4 ± 5.18 , $n = 6$, $p > 0.05$), GluA1 (C: 100 ± 22.5 , MD: 91 ± 8.85 , $n = 6$, $p > 0.05$), GluA2 (100 ± 18.78 , MD: 92 ± 9.84 , $n = 6$, $p > 0.05$) and GluN2A/GluN2B ratio (C: 100 ± 10.1 , MD: 87.8 ± 9.5 , $n = 6$, $p > 0.05$) at PND10. On the other hand, IL-1RI/GluN2B co-precipitation slightly, although not significantly, increased in Hp TIF of PND10 males subjected to MD (C: 100 ± 25.3 ; MD: 128 ± 16.2 ; $n = 5$, $p > 0.05$). For what concerns IL-1 β this cytokine was detectable in controls (10.11 ± 1.575 pg/100 μ g prot, $n = 6$) while MD blunted the response (3.88 ± 1.36 pg/100 μ g prot, $n = 6$, $p < 0.05$ vs. control). Due to the sex specificity of MD induced effects, IL-1 β was then measured also in female Hp. IL-1 β was undetectable in females both in C and MD.

4. Discussion

The major finding of this study is that a single episode of MD experienced early in life (PND 9), contributes to enhancing the distribution of IL-1RI and increasing its interactions with the GluN2B subunit of the NMDARs at neuronal post-synapses, thus leading to long-lasting alterations in the IL-1RI/NMDAR setting that extends to adolescence (PND 45). The changes occur in synapses characterised by an unbalanced shift of the NMDAR GluN2B and GluN2A subunits, and impoverishment of the AMPA component. They are also highly specific in terms of anatomy and gender as they exclusively affect the hippocampus of male rats. These findings may reflect a novel mechanism by means of which early-life adverse events determines alterations in molecular players of neuronal response to pro-inflammatory cytokines and cellular plasticity.

The observation that early-life infections can permanently alter stress reactivity, disease susceptibility and vulnerability to cognitive and neuropsychiatric disorders (Hornig et al., 1999; Rantakallio et al., 1997; Shi et al., 2003) initially suggested that the immune response contributes to this process. It has so far been demonstrated that perinatal exposure to both immune (i.e. *Escherichia coli* (Bilbo et al., 2005), Lipopolysaccharide – LPS (Galic et al., 2008)) and non-immune triggers (i.e. convulsants (Somera-Molina et al., 2009), pre-natal stress (Vanbesien-Mailliot et al., 2007), a high-fat diet (Bilbo and Tsang, 2010)) promotes the adoption of an enduring biochemical setting by microglia and/or peripheral immune cells that favours an exaggerated pro-inflammatory response upon activation with a subsequent challenge (Bilbo et al., 2005; Bilbo and Tsang, 2010; Galic et al., 2008; Somera-Molina et al., 2009). The observation that excessive expression of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α critically modulate cognitive functions (McAfoose and Baune, 2009), neuronal survival (Fogal and Hewett, 2008) and has been related to the psychiatric disorders (Capuron and Dantzer, 2003; Meyer et al., 2011), further strengthens the hypothesis of a key role for the immune system in early-life programming of later life behaviour (Bilbo and Schwarz, 2009).

Neuroimmune responses are the result of a delicate balance between the production of inflammatory mediators by immune cells and the ability of neurons to sense them through the expression of specific receptors. Although the impact of early-life challenge in shaping the response of immune cells start to emerge (Bilbo and

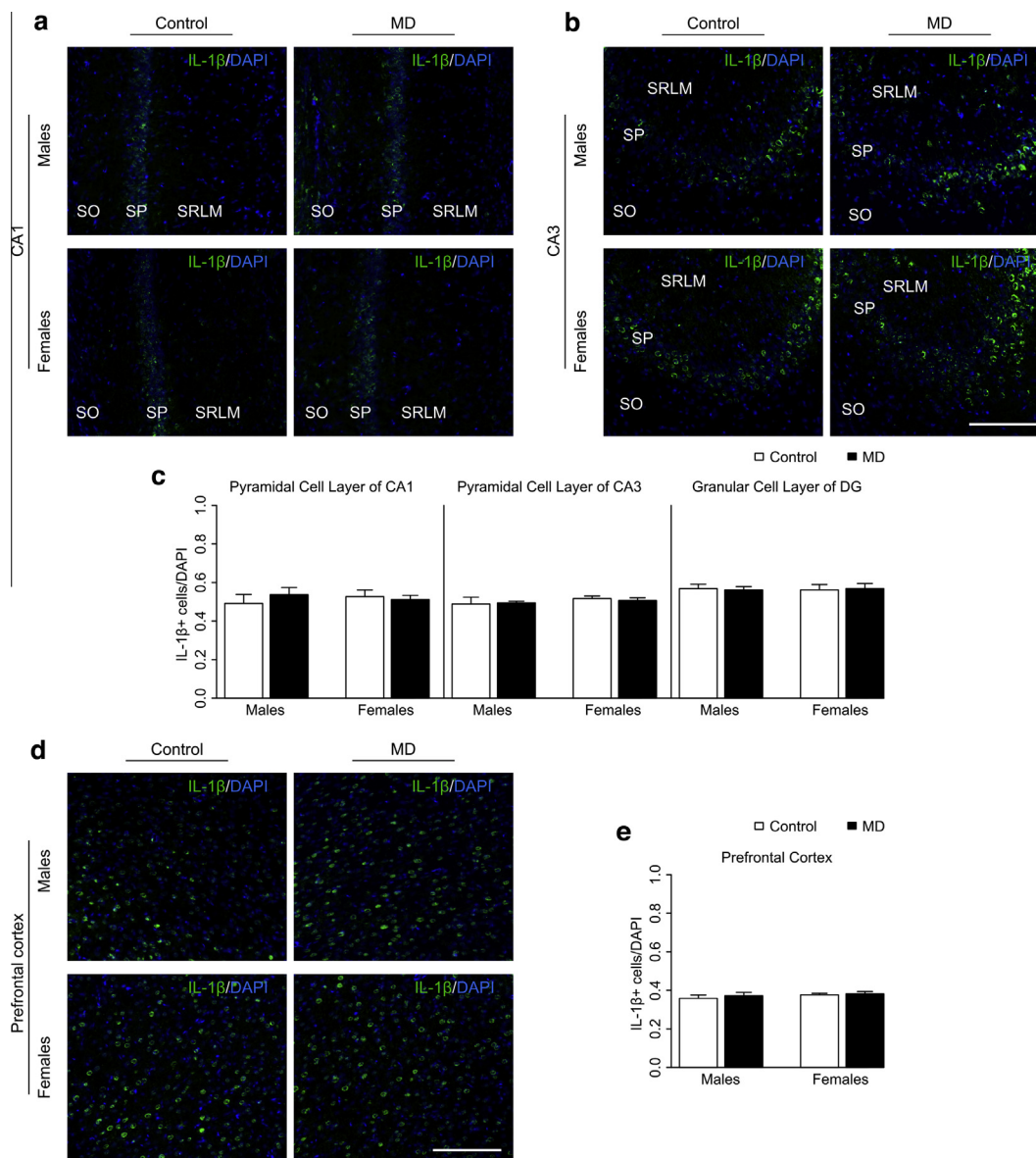


Fig. 5. Effects of maternal deprivation (MD) on the number of IL-1 β positive cells (IL-1 β + cells/DAPI) in 45-day-old male and female rats. Representative microphotographs of IL-1 β + cells/DAPI in the hippocampus (CA1 and CA3; a and b) and pre-frontal cortex (d) of 45-day-old male and female rats. Scale bar 150 μ m. SO: stratum oriens; SP: stratum pyramidale; SRLM: stratum radiatum-lacunosum moleculare. The cells were quantified in areas CA1 and CA3, and the dentate gyrus of the hippocampal formation (c), and in the pre-frontal cortex (e). Mean values \pm SEM ($n = 6$).

Schwarz, 2009; Bilbo et al., 2012), the extent to which this specific challenge induces long-term alteration in the organization of immune receptors in neurons has been completely neglected. We explored this possibility in a model of early-life stress mimicked by maternal separation. In particular, a single 24-h period of MD in rats on post-natal day (PND) 9–10 results in (i) behavioural changes in adolescence and adulthood which resemble those found in the affective disorders, including depressive-like responses (Llorente et al., 2007), enhanced impulsivity (Marco et al., 2007), disruption in pre-pulse inhibition (Ellenbroek and Cools, 2002; Ellenbroek et al., 2005) and cognitive deficits (Llorente et al., 2011); as well as in (ii) developmental alterations in glia and neurochemical changes suggestive of an altered plasticity (Ellenbroek and Riva, 2003; Marco et al., 2013; Viveros et al., 2009).

Our observation that MD contributes to enduring changes in the distribution of the IL-1 receptor in hippocampal neurons of young animals provides the first evidence that the IL-1RI/NMDAR

relationship is modulated *in vivo* and opens up a new perspective. Microglia are not solely responsible for an altered immune response within the CNS (Frank et al., 2007), but neurons could also participate by dynamically modulating the distribution of a receptor central to the neuroinflammatory response. The enrichment of IL-1RI at the dendritic spines of hippocampal neurons of MD rats suggests a different susceptibility of this compartment to IL-1 in respect to control animals.

During development, the subunit composition of synaptic NMDARs changes from the predominance of GluN2B- to GluN2A-containing receptors (Bellone and Nicoll, 2007), and it is thought that this shift in the GluN2A/GluN2B ratio tightly regulates AMPAR recruitment to form mature synapses (Gray et al., 2011). In addition to inducing the different compartmentalisation of IL-1RI, MD contributes to re-organising the excitatory glutamatergic synapses in the male hippocampus. We did not observe the physiological GluN2B/GluN2A switch in the spines of our MD rats, and we found

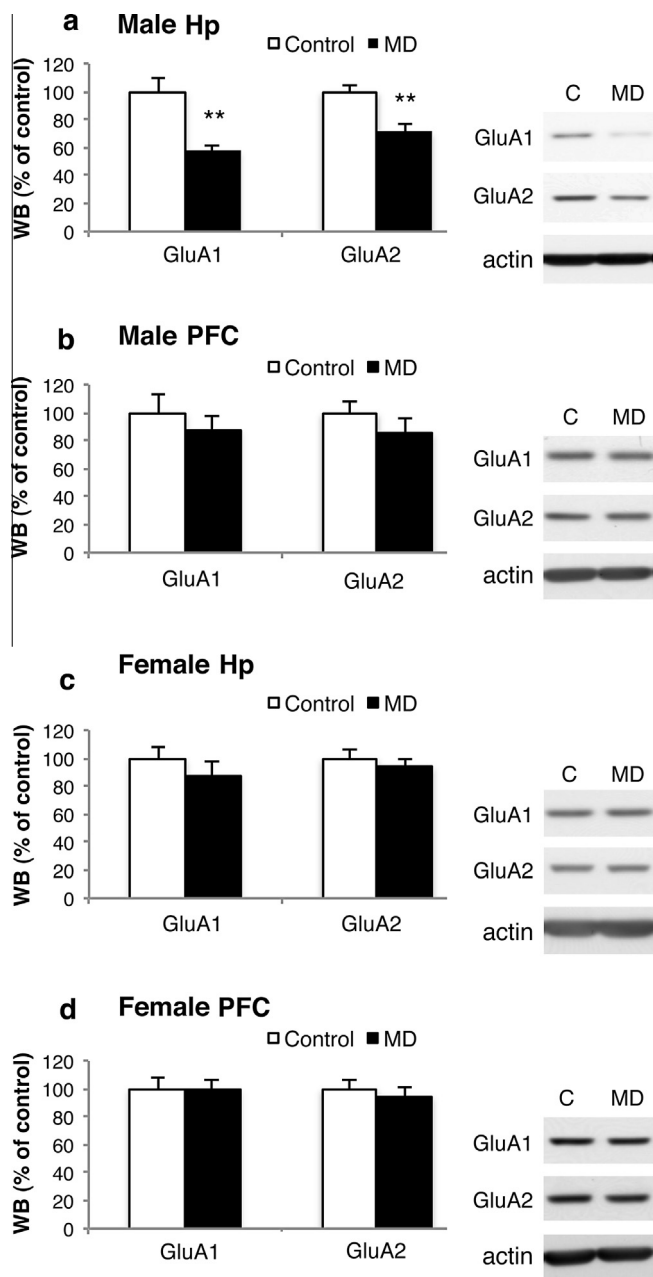


Fig. 6. Effect of maternal deprivation (MD) on the synaptic levels of GluA1 and GluA2 in the hippocampus and pre-frontal cortex of 45-day-old male and female rats. Quantification (left) and representative Western blots of the proteins in the TIFs of the male hippocampus (a) and pre-frontal cortex (b), and the female hippocampus (c) and pre-frontal cortex (d). The data were normalised for actin immunoreactivity. Mean values \pm SE ($n = 6$). ** $p < 0.01$ vs. control; Student's t -test.

a reduced number of AMPA receptor subunits. These results show that MD interferes with the normal maturational programme of male rat hippocampus and are in accordance with electrophysiological data showing that MD rats exhibits enhanced sensitivity to GluN2B-selective antagonists and slower excitatory postsynaptic currents decay kinetics (Rodenas-Ruano et al., 2012).

All the changes induced by 24 h MD and observed at PND 45 were not evident immediately after MD at PND10, suggesting that the reorganization of the hippocampal synapse may represent the long-term consequence of developmental abnormalities. Nevertheless, the slight increase of IL-1RI/GluN2B co-precipitation and reduction of GluN2A, although not significant but consistent with what observed in male Hp at PND45, may suggest that male Hp are anyway setting for those changes that will be observed on PND45.

In this scenario, the observation that MD increases the interaction between IL-1RI and the GluN2B subunit of the NMDAR, might have functional relevance for hippocampal neuronal excitability and synaptic plasticity later-in life. We have previously demonstrated that IL-1 enhances NMDAR activity in primary hippocampal neurons, and therefore their sensitivity to glutamate (Viviani et al., 2003). *In vitro*, this results in a reduction in number of spines (Viviani et al., 2006) followed by neuronal death (Viviani et al., 2003, 2006). The effect is preceded by the enrichment with both the GluN2B subunit (Viviani et al., 2006) and the IL-1RI (Viviani and Gardoni unpublished observation) at the post-synaptic spine and the contribution of IL-1RI is fundamental as suggested by the protective role of the IL-1 receptor antagonist (Viviani et al., 2003, 2006). Beside this, IL-1 β can also exert appreciable influences on various forms of synaptic plasticity (Coogan et al., 1999; Ross et al., 2003; Schneider et al., 1998), which are critically modulated by both the NMDA and AMPA receptors. It is worth noting that previous results have shown that, in males, maternal separation (at PND9) impairs emotional LTP-reinforcement in adolescent animals (Gruss et al., 2008). Although it may be inferred that the reorganization of the hippocampal synapse in terms of re-distribution of both IL-1RI and glutamatergic receptors in MD animals could affect its functionality, additional studies are necessary to understand how this setting prompted by MD can impact behavioural deficits and pathological disorders in adolescence and adulthood. Beside the different organization of the glutamatergic synapse, another important point emerges from our data: the sex dependence of the effect. Sex-dependent alterations in developing brain and adolescent behaviour have been described in neonatal rats experiencing MD (Llorente et al., 2008, 2009), with male hippocampus showing greater susceptibility than female hippocampus (Llorente et al., 2009). Accordingly, MD-induced spine re-organisation of the distribution of IL-1RI and glutamatergic receptors is restricted to male hippocampus at PND45. It is also interesting to note a sex-dymorphic neuroimmune response within the hippocampal formation at PND10 in both control and MD rats. We observed that IL-1 β is elevated only in control males while the response is blunted by MD. These results are in agreement with previous observation showing that IL-1 β levels are naturally elevated in the neonatal brain, peaking around P2 and declining thereafter into adulthood (Giulian et al., 1988). Interestingly, IL1 β was undetectable in the Hp of both control and MD animals on PND 45. It is thus conceivable that IL-1 β might be necessary during development and MD might interfere by reducing IL-1 β in a critical period for Hp development. Further studies are required to elucidate whether this unbalance might play a role in the reorganization of IL-1RI at the adolescent hippocampal synapse.

In conclusion, the present data show a dynamic regulation of IL-1RI *in vivo* in a model of early-life stress that leads to long-term receptor enrichment at synapses and increased interactions with NMDARs in male hippocampus. Compelling data show that this mechanism is part of a complex re-organisation of the excitatory glutamatergic synapses. The notion explored here is that MD-induced redistribution of IL-1RI in neurons might provide a novel molecular basis contributing to the critical role of the immune response in the early-life programming of later-life brain.

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