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DISTRIBUTION OF MEMBRANE PROGESTERONE RECEPTOR ALPHA IN THE MALE MOUSE AND RAT BRAIN AND ITS REGULATION AFTER TRAUMATIC BRAIN INJURY

D. MEFFRE,[a] F. LABOMBARDA,[a,b] B. DELESPIERRE,[a] A. CHASTRE,[a] A. F. DE NICOLA,[b] D. G. STEIN,[c] M. SCHUMACHER[a] AND R. GUENNOUN[a]*

[a] *UMR 788 INSERM and University Paris-Sud, 94276 Kremlin-Bicêtre, France*

[b] *Laboratory of Neuroendocrine Biochemistry, Instituto de Biologia y Medicina Experimental and Department of Human Biochemistry, University of Buenos Aires, Argentina*

[c] *Department of Emergency Medicine, Brain Research Laboratory, Emory University School of Medicine, Atlanta, USA*

Abstract—Progesterone has been shown to exert pleiotropic actions in the brain of both male and females. In particular, after traumatic brain injury (TBI), progesterone has important neuroprotective effects. In addition to intracellular progesterone receptors, membrane receptors of the hormone such as membrane progesterone receptor (mPR) may also be involved in neuroprotection. Three mPR subtypes (mPRα, mPRβ, and mPRγ) have been described and mPRα is best characterized pharmacologically. In the present study we investigated the distribution, cellular localization and the regulation of mPRα in male mouse and rat brain. We showed by reverse transcription-PCR that mPRα is expressed at similar levels in the male and female mouse brain suggesting that its expression may not be influenced by steroid levels. Treatment of males by estradiol or progesterone did not modify the level of expression of mPRα as shown by Western blot analysis. *In situ* hybridization and immunohistochemistry analysis showed a wide expression of mPRα in particular in the olfactory bulb, striatum, cortex, thalamus, hypothalamus, septum, hippocampus and cerebellum. Double immunofluorescence and confocal microscopy analysis showed that mPRα is expressed by neurons but not by oligodendrocytes and astrocytes. In the rat brain, the distribution of mPRα was similar to that observed in mouse brain; and after TBI, mPRα expression was induced in oligodendrocytes, astrocytes and reactive microglia. The wide neuroanatomical distribution of mPRα suggests that this receptor may play a role beyond neuroendocrine and reproductive functions. However, in the absence of injury its role might be restricted to neurons. The induction of mPRα after TBI in microglia, astrocytes and oligodendrocytes, points to a potential role in mediating the modulatory effects of progesterone in inflammation, ion and water homeostasis and myelin repair in the injured brain.

Key words: membrane progesterone receptor (mPR), neurons, astrocytes, oligodendrocytes, microglia, traumatic brain injury (TBI).

INTRODUCTION

Progesterone has a wide spectrum of actions in the nervous system, although it has generally been thought of as a hormone that influences female reproductive behavior and physiology (Pfaff et al., 1994). Progesterone also modulates various physiological and behavioral functions in both males and females and influences gene sets that modulate social, sexual, and anxiety-related behaviors (Witt et al., 1995; Phelps et al., 1998; Gomez et al., 2002; Schneider et al., 2003; Mong and Pfaff, 2004; Auger and Vanzo, 2006). In addition, progesterone also provides protective and trophic effects (Schumacher et al., 2007; Gibson et al., 2008; Stein, 2008; De Nicola et al., 2009). In the brain, the hormone can modulate synaptogenesis (McEwen and Woolley, 1994; Sakamoto et al., 2001) and stimulate myelination (Ghoumari et al., 2003, 2005; Hussain et al., 2011). The neuroprotective effects of progesterone have been demonstrated in experimental models of neurodegeneration (Vongher and Frye, 1999), brain ischemia (Cervantes et al., 2002; Sayeed et al., 2007; Gibson et al., 2009), and traumatic brain injury (TBI) (Stein, 2001; Stein and Sayeed, 2010). Based on these experimental findings (Schumacher et al., 2007; Brinton et al., 2008; Gibson et al., 2008; Stein, 2008; Stein and Wright, 2010), two phase II trials have already assessed the beneficial effects of progesterone following TBI (Wright et al., 2007; Xiao et al., 2008), and their encouraging outcomes have spurred the launching of two phase III multi-center trials (ProTECT-III, 2011; SyNAPSe, 2011).

Progesterone actions are mediated by its binding to specific receptors; the classical nuclear receptors (PR) which belong to a superfamily of transcription factors (Evans and Hollenberg, 1988). In addition, progesterone

*Corresponding author. Address: UMR 788 INSERM and University Paris-Sud, 80 rue du Général Leclerc, 94276 Bicêtre, France. Tel: +33-1-49-59-18-80; fax: +33-1-45-21-19-40.
E-mail address: rachida.guennoun@inserm.fr (R. Guennoun).

Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase; ANOVA, analysis of variance; AP, antero-posterior; CFC3, Inflammatory proteins C3 complement; DAB, diaminobenzidine; ER, estrogen receptor; GFAP, Glial fibrillary acidic protein; mPR, membrane progesterone receptor; NeuN, neuronal nuclei; Pgrmc1, progesterone receptor membrane component 1; PBS, phosphate buffered saline; PR, progesterone nuclear receptors; SSC, saline-sodium citrate; RT, reverse transcriptase; SEM, standard error of the mean; SDS, sodium dodecyl sulfate; TBI, traumatic brain injury; TBST, TRIS buffer salin -Tween 20.

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has rapid, non-genomic actions initiated at the cell surface by binding to membrane receptors (Falkenstein and Wehling, 2000; Schmidt et al., 2000). While progesterone effects can occur through the classical PR, a role for other progesterone-binding proteins or receptors in the nervous system has long been postulated. Progesterone and its derivatives bind to cell membranes of various rat brain tissues, suggesting non-classical receptor mechanisms of action (Towle and Sze, 1983; Ke and Ramirez, 1990; Tischkau and Ramirez, 1993; Zuloaga et al., 2012). Different non-genomic and rapid effects of progesterone have been observed within the CNS (Ramirez and Dluzen, 1987; Frye, 2001; Balasubramanian et al., 2008a,b; Hwang et al., 2009).

Early speculation regarding the identity of the receptors mediating the rapid effects of progesterone and its metabolites in the brain led to the discovery of steroid binding sites on the $GABA_A$/benzodiazepine receptor chloride channel complex (Lan et al., 1991; Belelli and Lambert, 2005; Hosie et al., 2006). These binding sites have affinity for multiple steroid metabolites, including the progesterone derivative 3α, 5α-tetrahydroprogesterone found in blood and brain tissues and following stress (Rupprecht, 2003; Gunn et al., 2011). A potential membrane receptor of progesterone was first cloned by Wehling's group from porcine liver microsomes (Meyer et al., 1996). Its rat analog is the protein 25-Dx (Selmin et al., 1996) renamed progesterone receptor membrane component 1 (Pgrmc1). Pgrmc1 does not appear to function as a traditional receptor because it requires a binding partner known as serpine mRNA binding protein 1 (Peluso et al., 2005). Pgrmc1 is expressed in the brain (Krebs et al., 2000; Meffre et al., 2005), and in the spinal cord (Labombarda et al., 2003) and its expression is up-regulated after injury (Labombarda et al., 2003; Meffre et al., 2005).

The recent discovery of a family of membrane progesterone receptors (mPRs) offers an alternative mediator of non-genomic progesterone effects in the nervous system. mPR cDNA was first cloned in a fish (Zhu et al., 2003b). Then, other members of this family were identified from several vertebrates including human and mouse (Zhu et al., 2003a). Phylogenetic analysis indicated that these cDNAs comprise three groups: α, β and γ. These proteins have the characteristics of G-protein-coupled receptors with seven transmembrane domains. Using a reverse transcriptase (RT)-PCR, Tang et al. found mRNA of all three mPRs (α, β, and γ) in the human brain (Tang et al., 2005). An mPR with 91% amino acid homology to human mPRα has been cloned from sheep and is expressed in both hypothalamus and pituitary tissues (Ashley et al., 2006). Both mPRα and mPRβ have been detected in the mouse brain (Thomas, 2004). Expression of their mRNAs has been more specifically localized to the medio-basal hypothalamus of both the rat (Liu and Arbogast, 2009) and mouse (Sleiter et al., 2009). mPRα and mPRβ expression in the brain vary with hormonal changes during estrus, with highest mPR expression during proestrus, suggesting receptor up-regulation by estrogen and down-regulation by progesterone (Liu and Arbogast, 2009). However, a recent study showed that the expression of genes encoding mPRα and mPRβ mRNA levels were most robust in the thalamic nuclei and cortex rather than in the hypothalamus (Intlekofer and Petersen, 2011b). The same authors showed that mPRα mRNA was not regulated by estradiol or progesterone treatment (Intlekofer and Petersen, 2011a). A more recent study showed a wide distribution of mPRβ in the female rat brain and the stimulation of its expression by estradiol within the medial septum (Zuloaga et al., 2012).

To date, studies on mPR expression in the brain were focused on females and regions mainly involved in neuroendocrine functions (Liu and Arbogast, 2009; Sleiter et al., 2009; Intlekofer and Petersen, 2011b,a; Zuloaga et al., 2012). Because of the well-documented and multiple effects of progesterone in the brain, some of which are likely to involve membrane actions, the aims of the present study were to precisely map the regional and cellular distributions of mPRα at both the mRNA and protein levels. We also sought to determine whether mPRα expression is influenced by sex, steroids or TBI. Our results suggest that this receptor may be implicated in different biological effects of progesterone in the brain and particularly in modulating myelination, water homeostasis, and inflammation after TBI.

EXPERIMENTAL PROCEDURES

Animals

Adult (12-week-old) male and female C57BL6 mice and adult male Sprague-Dawley rats (300 g) were obtained from Janvier, France. Animals were housed in a temperature-controlled room on a 12-h light, 12-h dark cycle with food and water *ad libitum*. Experimental protocols were approved by the Direction départementale de la protection des populations du Val-de-Marne, France, authorization number 94-345 to R.G., accredited establishment number 94-043-13). Experiments were performed in accordance with French ethical laws (Act 87-848; Ministère de l'Agriculture et de la Forêt) and the European Communities Council Directives of November 24, 1986 (86/609/EEC) guidelines for the care and use of laboratory animals.

Semi-quantitative RT-PCR and sequencing

RNA extraction. Adult male ($n = 6$) and female ($n = 6$) mice were killed by decapitation. Total brain and spinal cord (positive control) were dissected out, frozen on dry ice and stored at −80 °C until use. Total RNA was extracted using Trizol reagent (Life Technologies, Invitrogen, France) according to the manufacturer's instructions. Frozen samples were directly homogenized in Trizol using a glass–glass homogenizer. The concentration and purity of total RNA were determined by measuring optical density at 260 and 280 nm. All samples were precipitated with ethanol, and then dissolved in distilled water at a concentration of l μg/μl; their quality was verified by gel electrophoresis.

RT. Total RNA was subjected to DNAse 1 (Stratagene, La Jolla, CA, USA) treatment (10 U for 15 min at 37 °C) to remove residual contaminating genomic DNA. cDNA templates for PCR amplification were synthesized from 2 μg of total RNA using a

SuperScript II RNAse H reverse transcriptase kit (Gibco/BRL, Cergy Pontoise, France) for 90 min at 42 °C in the presence of random hexamer primers.

Amplification. An appropriate volume of the cDNA obtained by RT was used in subsequent PCR amplification of mPRα and L19 ribosomal mRNA (endogenous internal standard). Primers for RT-PCR amplification were designed using Oligo Primer Analysis Software version 6.54, Molecular Biology Insights Inc., USA. Primers used for mPRα were 5′-GGCCTCCTTCACCTACCTCT-3′ (sense) and 5′-CAGA CCCGGCTTCTGGCTGT-3′ (antisense); primers for L19 were 5′-CTGAAGGTCAAAGGGAATGTG-3′ (sense) and 5′-GG ACAGAGTCTTGATGATCTC-3′ (antisense). Each PCR contained 100 ng of cDNA template, 1× Taq DNA polymerase buffer, 200 μM of each dNTP, 1 U of Taq DNA polymerase (ATGC), and 0.2 μM of specific primers, in a total volume of 50 μl. The conditions of amplification were 2 min at 94 °C, followed by 30 cycles at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. The PCRs would be expected to yield a product, at 284 bp for mPRα and 194 bp for L19. Aliquots of the amplified products and 100 bp DNA ladder were separated on a 1.2% agarose gel and visualized by ethidium bromide staining. The gels were subsequently quantified using the Biorad Image Analysis System and Molecular Analyst for Macintosh software (Marnes-la-Coquette, France). The relative levels of gene expression were measured by determining the ratio between the products generated from the mPRα genes and the endogenous internal standard (L19) in separate reactions. Validation of semi-quantitative RT-PCR was determined and the optimal number of cycles and cDNA concentration for remaining in the log phase of amplification were chosen (see Labombarda et al., 2010).

Sequencing of PCR products. To confirm the specificity of the RT-PCR amplified mPRα product, it was purified after electrophoresis using the Prepagene Kit (Bio-Rad, Marnes-la-Coquette, France). The identity of the PCR products from the brain, and hence the specificity of the PCR, was confirmed by DNA sequencing (Biofidal, Vaulx en Velin, France).

Antibodies

To detect mPRα protein, a polyclonal antibody (GP47) was generated in rabbits against a synthetic 15 amino acid peptide derived from the C-terminal domain of mouse mPRα (llsqlvrrklhqktk) conjugated to KLH (Eurogentec, Belgium). This antibody has been already characterized and successfully used for mPRα detection in the mouse spinal cord (Labombarda et al., 2010). Specificity of this antibody to detect mPRα was checked by Western blot analysis of the cell lysate and plasma membrane preparations from MDA-MB-231 cells stably transfected with hu-mPR as described previously (Thomas et al., 2007). Plasma membranes of huPR-MDA-MB-231 transfected cells showed a more intense signal compared to untransfected MDA-MB-231 cells. An immunoreactive band around 40 kDa was revealed by the anti-serum, but was not detected in the presence of the blocking peptide or the pre-immune serum. For Western-blot analysis, α-Tubulin antibody (Cell Signaling, Saint Quentin Yvelines, France) was used for normalization. For double immunofluorescence analysis, antibodies for specific markers of cell types were used together with GP47 anti-serum: mouse anti-neuronal nuclei (anti-NeuN, Millipore, Molsheim, France) for neurons, mouse anti-S100 α/β chain (B32.1, Santa Cruz, Heidelberg, Germany) for astrocytes, anti-APC (Ab-7) mouse mAb (CC1, Merck Chemicals Ltd., Nottingham, UK) for oligodendrocytes, and Ox-42 (Santa Cruz, Heidelberg, Germany) for activated microglia. To specifically identify astrocytes, S100 antibody was used rather than GFAP because it is a commonly used astrocytic marker (Wang and Bordey, 2008) which stains the cell body making easy the analysis of double immunolabeling. We recently used it with success to show mPRα expression in astrocytes in the spinal cord (Labombarda et al., 2010).

Western blot analysis

To study the effect of steroid treatments on mPRα expression, adult C57BL6 male mice were used to avoid the influence of the female cycle. Four experimental groups were prepared: (1) controls untreated ($n = 6$); (2) oil ($n = 6$) vehicle injected (sesame oil, s.c.); (3) EB ($n = 6$) estradiol injected (estradiol-17β benzoate, 10 μg/kg/day, s.c., 2 days); (4) Prog ($n = 6$) progesterone injected (4 mg/kg/day, 3 days). The dose and duration of estradiol benzoate treatment were similar to those which have been shown to induce nuclear progesterone receptors (Brown et al., 1987; Greco et al., 2001). The paradigm of progesterone treatment is the one that has previously been shown to prevent neuronal loss after TBI and spinal cord injury (Roof et al., 1994; Thomas et al., 1999) and to modulate motoneurons and glial cell markers after spinal cord injury (Labombarda et al., 2000, 2002; Gonzalez et al., 2004; De Nicola et al., 2009). The progesterone treatment as applied resulted in high levels of progesterone in both the plasma and spinal cord (Labombarda et al., 2003, 2006). Animals were killed 24 h after the last injection.

Brains were homogenized in an ice-cold lysis buffer (10 mm Tris–Cl (pH 8.0), 150 mm NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 40 μM phenylmethylsulfonyl fluoride, and 1 μM leupeptin). This was followed by incubation for 30 min on ice and then centrifugation at 10,000*g* for 20 min at 4 °C. The supernatant was transferred to new tubes, aliquoted, and stored at −80 °C until electrophoresis was performed. Protein concentrations were determined by the Bradford technique using the Bio-Rad protein assay kit with bovine serum albumin as reference standard (Bradford, 1976). Samples were denatured by adding sample buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.01% bromophenol blue), followed by boiling for 5 min. Twenty micrograms of proteins was separated on 10% SDS-PAGE. Blots were incubated for 1 h at room temperature in 5% non-fat dry milk to block nonspecific binding, and then washed in TBST (4 × 5 min). Blots were then incubated overnight at 4 °C with the anti-mPRα serum GP47 (1/5000). α-Tubulin (Cell Signaling, 1/1000) was used to normalize. After three washes in TBST, membranes were incubated for 1 h with secondary horseradish peroxidase conjugated antibodies (Santa Cruz), and finally washed three times for 15 min with TRIS buffer salin -Tween 20 (TBST). Blots were then treated with enhanced chemiluminescence (Amersham Pharmacia, Velizy-Villacoublay, France) according to the manufacturer's instructions and exposed to X-ray film. The specificity of the GP47 antiserum was confirmed by (1) substituting the antiserum with pre-immune serum; (2) blocking the diluted anti-mPRα-serum GP47 (1/5000) with the peptide (10 μg/ml), and incubating the mixture at room temperature for 2 h (see Labombarda et al., 2010). Signals were acquired directly using Chemidoc Molecular Imager (Bio-Rad, Marnes-la-Coquette, France) and quantified by Quantity-one software (Bio-Rad). Blots were also exposed to X-ray films. Films were scanned and signals quantified using ImageJ software. Data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc tests.

In situ hybridization

Adult male mice ($n = 5$) were anesthetized with ketamine and perfused through the heart with phosphate buffer followed by 1% paraformaldehyde. Brains were dissected out, post-fixed by immersion in the same fixative for 1 h and transferred to a

solution of 15% sucrose in phosphate buffer where they remained overnight. Samples were frozen on powdered dry ice and stored at −80 °C. Serial coronal sections (14 μm) were mounted on 3-aminopropyl triethoxy-silane-subbed slides and stored at −80 °C until use. A specific 40-base synthetic oligonucleotide complementary to mPRα GI2195525 (5′-GATC CTCCTCGGCAGGGAGGGGAGACACTATGATGCGGTG-3′) was chosen. Two scrambled oligonucleotides were used (Scram1: 5′-GCCCTGACGCGTTGGAGTCGAGGTCCAAGG GACTAGGTGA-3′; Scram2: 5′-ACTTCCAGGATCGCTAA GGACTTCCCGTGAAAGGCTCCCG-3′). The sequences were then verified using BLAST search of EMBL and GenBank databases to ensure that there was no homology with other mRNAs. The oligonucleotides were labeled with [^{35}S]dATP (1000 mCi/mmol, Perkin Elmer, France) to a specific activity of 2×10^9 cpm/μg using terminal deoxynucleotidyltransferase (Amersham Pharmacia, Velizy-Villacoublay, France). Adjacent brain sections were incubated in the presence of the ^{35}S-labeled probes according to a previously described protocol (Guennoun et al., 1995). Briefly, 0.25 ng of labeled probe in the hybridization buffer (50% deionized formamide, 10% dextran sulfate, 500 μg/ml denatured salmon sperm DNA, 1% Denhardt's solution, 5% sarcosyl, 250 μg/ml yeast tRNA, 200 mM dithiothreitol, and 20 mM Na_2PO_4 in 2× saline-sodium citrate, SSC) was applied on tissue sections and hybridization proceeded overnight at 40 °C. The following day, sections were rinsed several times using SSC buffer (1× and 0.1×), dehydrated and exposed to X-ray films (Kodak Biomax MR, Sigma–Aldrich, Lyon, France).

Controls for the specificity of the *in situ* hybridization reactions were performed by (1) cold probe competition (the addition of an excess of related or unrelated probes to the hybridization medium before the hybridization of adjacent sections) and (2) the use of scrambled missense oligonucleotides with the same C–G ratio as the antisense probes Scram1 and Scram2.

Immunohistochemical analysis

Adult male mice ($n = 5$) and adult male rats ($n = 5$) were perfused with paraformaldehyde 4%, brains were dissected out, then tissue and sections were prepared as described for *in situ* hybridization. mPRα immunodetection was performed by using an indirect method with avidin–biotin–peroxidase following a standard protocol. Endogenous peroxidase activity was quenched by incubating the slides in 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Slides were then incubated in 3% normal goat serum for 20 min at room temperature to reduce non-specific staining and then incubated overnight at 4 °C with the rabbit anti-mPRα serum GP47 (1/3000). After washing to remove excess primary antibodies, the slides were incubated with biotinylated goat-anti-rabbit IgG (Vector Laboratories) for 2 h at room temperature, washed in phosphate buffered saline (PBS), and incubated with avidin–biotin complex reagent for 30 min at room temperature. The slides were developed using a diaminobenzidine (DAB)–peroxidase substrate for 10 min. Finally, the slides were rinsed in Tris buffer, dehydrated, cleared, and mounted. To show the specificity of the immunostaining, negative controls were run in adjacent sections by substituting the primary antibodies with pre-immune serum and by using pre-adsorption tests as described for Western blot analysis.

Double immunofluorescence staining and confocal microscopy analysis

To identify the type of cells expressing mPRα, double immunofluorescence staining and confocal microscopy analysis were performed using coronal brain slices from male mice ($n = 5$) and from male rat without injury ($n = 5$) and after TBI ($n = 5$). Two successive immunodetections were performed on the same section. mPRα antiserum GP47 at 1/1000, and specific markers of cell types were used: mouse anti-NeuN (1/100) for neurons, mouse anti-S-100 α/β chain (B32.1, 1/50) for astrocytes, anti-APC (Ab-7) mouse mAb (CC1, 1/20) for oligodendrocytes, and Ox-42 (1/50). The Vector M.O.M. immunodetection kit (Clinisciences, Montrouge, France) was used to reduce undesired background staining. One set of slides was first incubated with the rabbit anti-mPRα serum GP47 overnight at 4 °C, after washing the slides were incubated for 2 h at room temperature in the dark with Cy3-goat anti-rabbit IgG (1/200, Chemicon International) washed, and incubated either with monoclonal anti-NeuN, or anti-S-100 α/β chain, or anti-CC1, or anti-Ox-42 for 2 h at room temperature. After washing in PBS, slides were incubated with Alexa Fluor 488-goat anti-mouse secondary antibody (1/200, Molecular Probes, Eugene, OR, USA) for 2 h. The slides were washed in PBS then mounted with fluoromount and observed under the confocal microscopy. Negative controls were also processed using the immune-depleted antibody preparation or normal serum in place of anti-NeuN, or anti-S-100 α/β chain, or anti-CC1, or anti-Ox-42. Double immunofluorescence labeling was visualized using a confocal Zeiss LSM 510 microscope (Carl Zeiss GmbH, Jena, Germany). Images were sequentially acquired with green (488 nm) and red (543 nm) excitations to avoid cross talk between channels. Images were acquired sequentially in a line-scanning mode through an optical section of 1 μm in the *z*-axis and analysis was performed using Bioscan Optimas II software.

TBI

Adult Sprague-Dawley rats (300 g) were housed individually and kept on a reverse light–dark cycle (0.800–20.00 h). Two groups were used ($n = 5$) each; control group without injury to map the mPRα expression in the male rat brain and TBI group to study the impact of injury on mPRα expression. Bilateral contusions of the medial prefrontal cortex were created with a pneumatic impactor device. Briefly, animals were anesthetized with ketamine and xylazine (90 and 10 mg/kg respectively) and placed in a stereotaxic apparatus equipped with a homeothermic blanket system to maintain body core temperature at 37 °C. A midline incision was made in the scalp, the fascia was retracted to expose the cranium, and a craniectomy (6-mm diameter) was made over the midline of the prefrontal cortex with its center 3.0 mm antero-posterior (AP) to bregma. Craniectomies were performed carefully to insure that the dura remained intact in all animals before impact. After removal of the bone, the tip of the impactor (5 mm diameter) was moved to +3.0 mm AP, 0.0 mm (from bregma), and checked for adequate clearance. Trauma was produced by pneumatically activating the piston to impact 2.0 mm dorso-ventral (DV) (from dura) at a velocity of 2.25 m/s with a brain contact time of 0.5 s. Following contusion injury, the wound cavity was cleaned and bleeding stopped before suturing of the scalp. Sham-operated animals underwent only anesthesia, incision and suture of the scalp. Animals were killed 24 h after surgery.

Statistical analysis

The statistical analysis was performed with GraphPad Prism 4.0. Data are expressed as the mean ± standard error of the mean (SEM) and *n* refers to the number of animals studied. Comparison between male and female was analyzed by Student's *t*-test. Effect of steroid treatment was analyzed by one-way ANOVA followed by Bonferroni post hoc tests. *P* values more than 0.05 were not considered statistically significant.

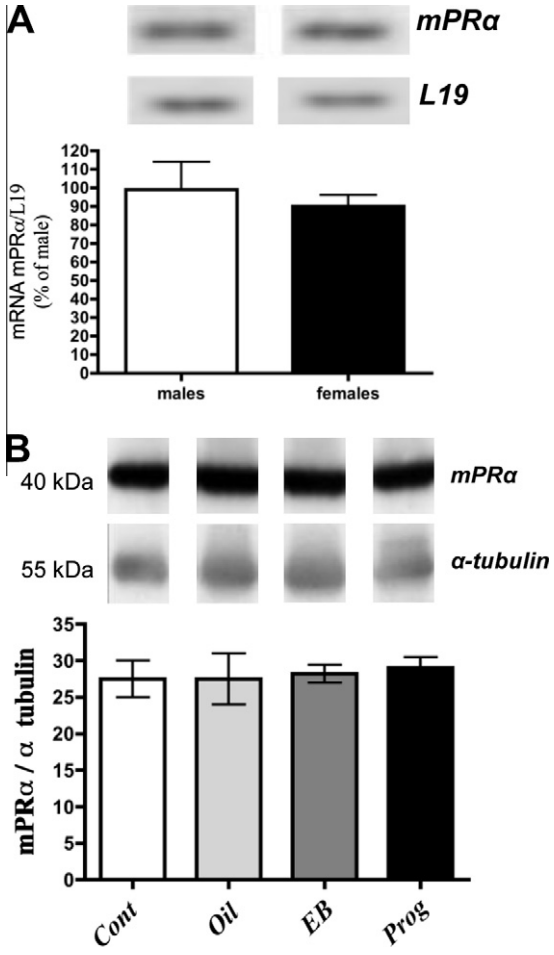

Fig. 1. mRNA analysis of mPRα in male and female mouse brain, and effect of steroid treatment on mPRα expression in males. (A) Semi-quantitative RT-PCR analysis of mPRα in male and female mouse brain. The expression of mPRα is similar in males and females. Values, expressed as mean ± SEM of six mice, were compared by Student's *t*-test. (B) Effects of steroid treatments on mPRα expression: Western blot analysis. A typical Western blot using anti-mPRα serum GP47 (1/5000) and α-Tubulin (1/1000) for normalization and the corresponding quantifications are shown. One strong specific immunoreactive band around 40 kDa was revealed. The intensity of the signal was similar between the untreated and hormone-treated mice. No significant difference in mPRα expression was observed between controls (Cont), oil (vehicle), estradiol benzoate (EB) and progesterone (Prog)-treated mice. Data represent the mean ± SEM of five mice per group. Statistical analysis: one-way ANOVA.

RESULTS

mPRα mRNA is expressed in the adult mouse brain at similar levels in males and females

The presence of mPRα transcripts in the brain was demonstrated by RT-PCR using total RNA isolated from adult male and female mouse brains. As expected, RT-PCR amplification generated a fragment of 284 bp. Sequencing of the amplified products and analysis by the NCBI BLAST program showed that the nucleotide sequence was homologous (100% identity) to the expected fragment in the sequence of mouse mPRα (GI2195525). Similar levels of mPRα transcripts were observed in the brain of males and females as shown by semi-quantitative RT-PCR (Fig. 1A).

mPRα expression in male mouse brain is not modified by estradiol or progesterone treatments

To determine whether brain mPRα protein expression is regulated by estrogen or progesterone, adult male mice were injected s.c. with vehicle alone (oil), estradiol benzoate (EB, 10 μg/kg/day for 2 days) or with progesterone (Prog, 4 mg/kg/day for 3 days). Brains were sampled for protein analysis 24 h after the last injection. Western blot analysis was performed using specific mPRα polyclonal anti-peptide serum, already described and successfully used in the spinal cord (Labombarda et al., 2010). A single immunoreactive band of the expected size for mPRα (40 kDa) was detected in the mouse brain lysates. The intensity of the signal was similar in controls, vehicle, estradiol or progesterone-treated mice (Fig. 1B). No band was detected in controls incubated with the antiserum pre-adsorbed with the immunizing peptide or substituted by the pre-immune serum (not shown). One-way ANOVA showed no significant difference in mPRα expression between controls nontreated and vehicle, estradiol or progesterone-treated mice (Fig. 1B).

mPRα is expressed in several regions throughout the male mouse brain

The regional distribution of mPRα mRNA in the brain was studied by *in situ* hybridization using a specific probe. Strong specific hybridization signals were obtained all over the serial coronal brain sections. Fig. 2 shows examples of the broad expression of this receptor: mPRα mRNA is expressed in the olfactory bulbs (Fig. 2A), cerebral cortex (Fig. 2B–E), striatum (Fig. 2C), septum (Fig. 2C), thalamus (Fig. 2D, E), hypothalamus (Fig. 2D, E), amygdala (Fig. 2D, E), hippocampus (Fig. 2D, E), and cerebellum (Fig. 2F). The specificity of the hybridization signals for mPRα was verified: (1) by performing displacement experiments with an excess of unlabeled competing probes. A 500-fold excess of unlabeled homologous oligonucleotide completely abolished mPRα hybridization signals (Fig. 2C′), whereas a 500-fold excess of unlabeled heterologous oligonucleotide did not modify the quality of the signal; (2) by using two different scrambled oligonucleotides, which gave no specific signal (Fig. 2F′).

Immunohistochemical analysis using the anti-mPRα serum showed a wide regional distribution of mPRα protein, which is consistent with the results obtained by *in situ* hybridization for mPRα mRNA distribution. Fig. 3A shows a representative mPRα immunoreactivity in the granular, mitral and glomerular layers of the olfactory bulb, while Fig. 3B shows the immunolabeled neurons in the cingulate cortex. Specific labeling was evident in the motor cortex (Fig. 3C) as well as in the piriform cortex (not shown). mPRα immunoreactive cells were observed in several nuclei of the hypothalamus: high intensity of staining is evident in the paraventricular nucleus (Fig. 3D), the arcuate nucleus (Fig. 3E) and in

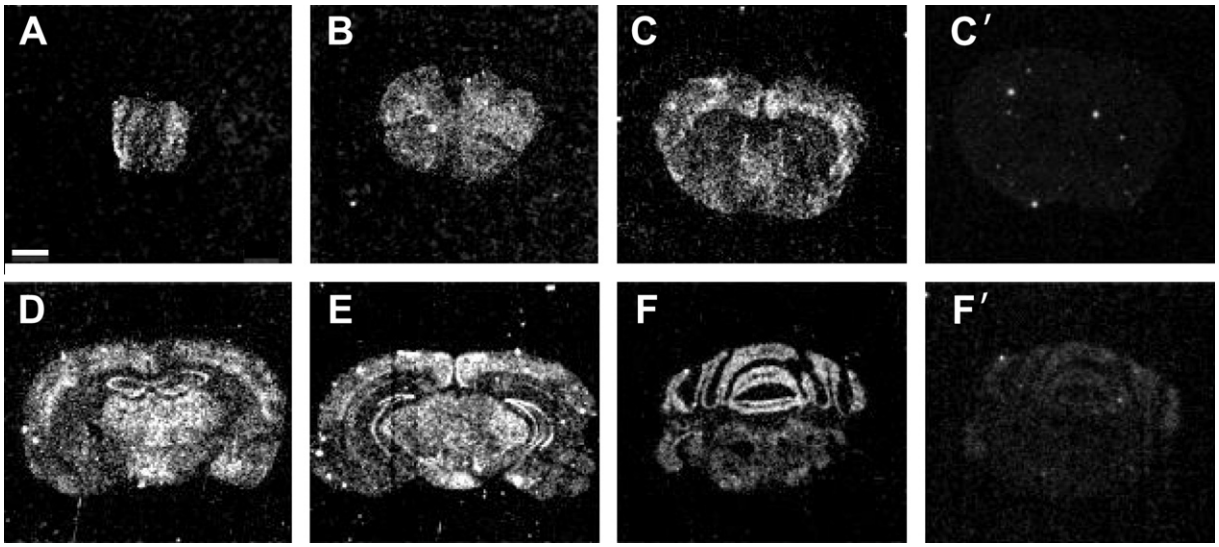

Fig. 2. Regional distribution of mPRα mRNA in the male mouse brain: analysis by *in situ* hybridization. Representative autoradiograms (dark field) after hybridization over coronal brain sections with the mPRα probe (A–F), with an excess of unlabeled probe (C′), or with the scrambled probe, (F′). A broad distribution of mPRα mRNA is observed. Exposure time: 10 days. Scale bar = 0.2 cm.

the ventro-median nucleus (Fig. 3F). Weaker expression was observed in the supra-optic and in the supra-chiasmatic nuclei (not shown). Moderate staining was observed in the tanycytes (Fig. 3E), and in ependymal cells at the level of the third (Fig. 3F), and lateral ventricles (Fig. 3G). Moderate labeling was observed in choroid plexus (Fig. 3I). Septum (Fig. 3G), striatum (Fig. 3H) and habenula (Fig. 3I) also showed positive cells for mPRα. Moderate to high labeling was observed in the hippocampus: Fig. 3J shows immunoreactivity in the neurons of the CA1, CA2 and CA3 regions. In the cerebellum, strong labeling intensity was observed in the Purkinje cell layer (P) while a faint labeling was observed in the granular cell layer (Fig. 3K). No specific labeling was observed on adjacent sections incubated with the antibody pre-adsorbed with the immunization peptide or with the pre-immune serum as shown for the cerebellum in Fig. 3L.

mPRα is expressed by neurons but not in the glia in the male mouse brain

The localization of the mPRα-expressing cells observed by immunohistochemical analysis, as well as their aspect and their size, suggests that the receptor is mainly expressed by neurons. To identify further the types of cells expressing mPRα we performed double immunofluorescence staining (mPRα together with cell-specific markers) followed by confocal microscopy analysis. The cell-specific antibodies used were (i) anti-NeuN for neurons; (ii) CC1 mAb for oligodendrocytes; and (iii) anti-S-100 α/β chain for astrocytes. Results of the double immunofluorescence stains showed that throughout the brain, mPRα was expressed by neurons. Arrows in Fig. 4A show significant and typical examples of mPRα/NeuN co-labeling: the receptor is expressed by neurons in the frontal and pyriform cortex, as well as neurons of different hypothalamic nuclei, hippocampus and striatum. Interestingly, even though the majority of neurons express the receptor, some did not (Fig. 4A, arrowheads). In contrast, mPRα was neither expressed by oligodendrocytes (Fig. 4B, arrowheads) nor by astrocytes (Fig. 4C, arrowheads), as shown by the absence of mPRα/CC1 and mPRα/S100 co-immunostaining, respectively. Consequently, the mPRα-expressing cells observed in Fig. 4B and C (dotted arrows) correspond to another cell type i.e. neurons.

mPRα expression is induced in glia after TBI

mPRα is expressed in several regions throughout the male rat brain. Immunohistochemical analysis showed a wide distribution of mPRα in male rat brain (data not shown) similar to that described for mouse brain (for details see ‘‘mPRα is expressed in several regions throughout the male mouse brain’’ section and Fig. 3). Several mPRα immunoreactive cells were observed in the olfactory bulb, striatum, cortex, thalamus, hypothalamus, septum, hippocampus and cerebellum of the rat brain in the control group.

mPRα expression is induced in glia after TBI. As progesterone has several neuroprotective effects after TBI and some of these effects might be mediated by mPRα, we studied the expression of this receptor in the injured brain. For this, male rats were subjected to bilateral contusions of the medial prefrontal cortex with a pneumatic impactor device, and mPRα expression was studied by double immunofluorescence followed by confocal microscopy analysis 24 h after injury. The analysis was performed in the control group and TBI group in both the core of the lesion and the peri-lesional area. In the control group mPRα immunoreactivity was restricted to $NeuN^+$ cells as shown for the mouse brain in Fig. 4. After TBI, there is an induction of mPRα expression in the glial cells as shown in Figs. 5 and 6.

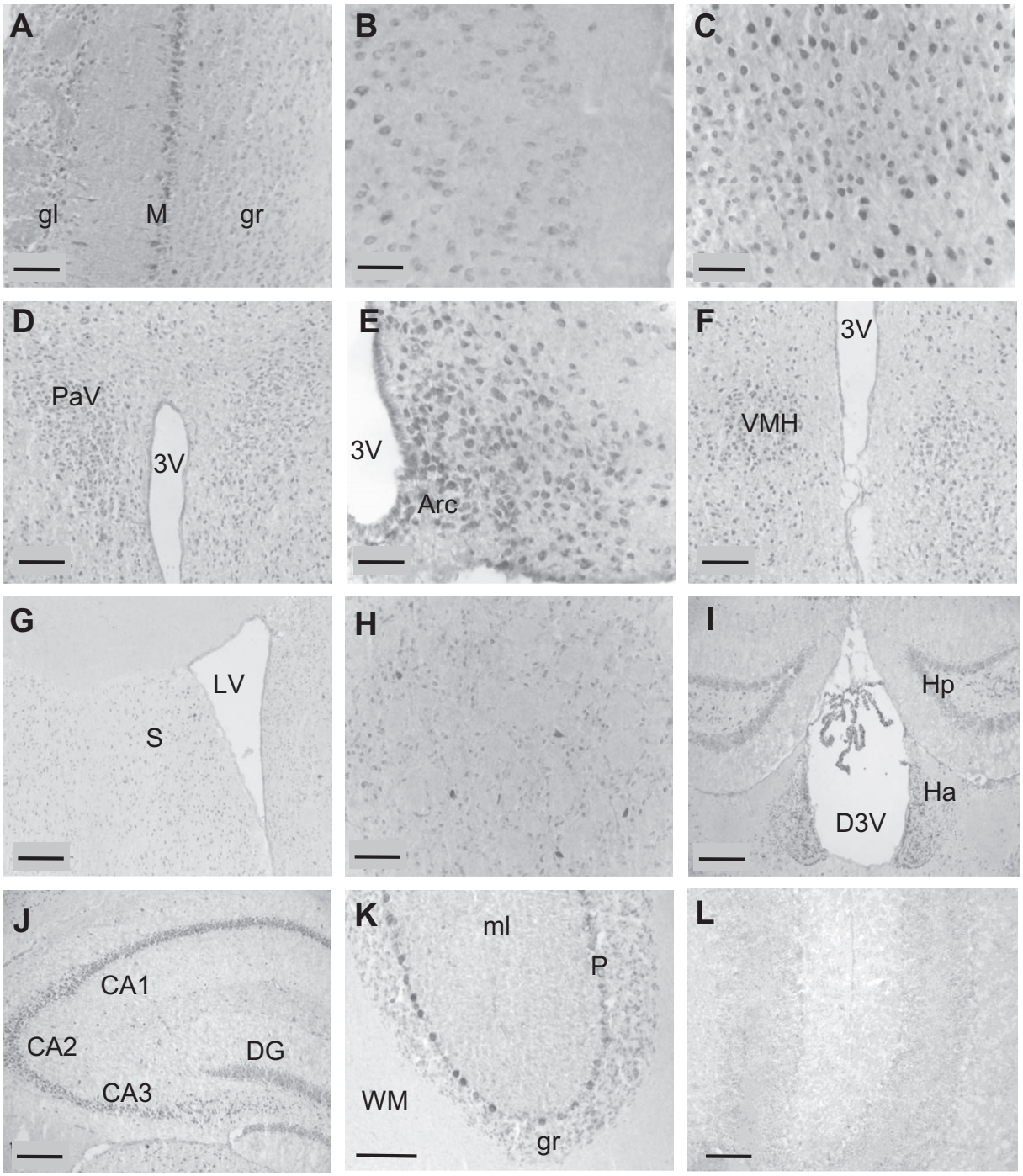

Fig. 3. mPRα distribution in the mouse brain: immunohistological analysis. mPRα is expressed in a variety of structures throughout the brain. Representative images of mPRα immunoreactivity in selected brain regions showing varying levels of labeling intensity and density are shown. (A) Olfactory bulb, (B) cingulate cortex, (C) motor cortex, (D) paraventricular nucleus (PaV), (E) arcuate nucleus (Arc), (F) ventromedial nucleus (VMH), (G) septum, (H) striatum, (I) habenula, (J) hippocampus, (K) cerebellum. mPRα immunodetection was performed using GP47 antibody (1/3000). Biotinylated secondary antibody and DAB–peroxidase detection were used. In control sections, no specific labeling was observed (L) after pre-adsorption with the immunizing peptide (10 μg/ml). CA1–CA3, fields 1–3 of Ammon's horn; DG, dentate gyrus; gl, glomerular cell layer; gr, granular cell layer; Ha, habenula; Hp, hippocampus; LV, lateral ventricle; M, mitral cell layer; ml, molecular cell layer; P, Purkinje cells; S, septum; WM, white matter; 3V, third ventricle; 4V, fourth ventricle. Scale bar = 100 μm (A, D, F, K, L), 50 μm (B, E), 350 μm (C), and 200 μm (G–J).

In the core of the lesion in the prefrontal cortex, the density of NeuN$^+$ cells was low due to the contusion. Several cells were mPRα$^+$, however there were nearly no cells co-labeled with NeuN and mPRα antibodies (Fig. 5A, arrows). This observation contrasts with what was observed in the intact cortex where the majority of NeuN$^+$ cells showed mPRα immunostaining. This indicated that there are other cell types which express

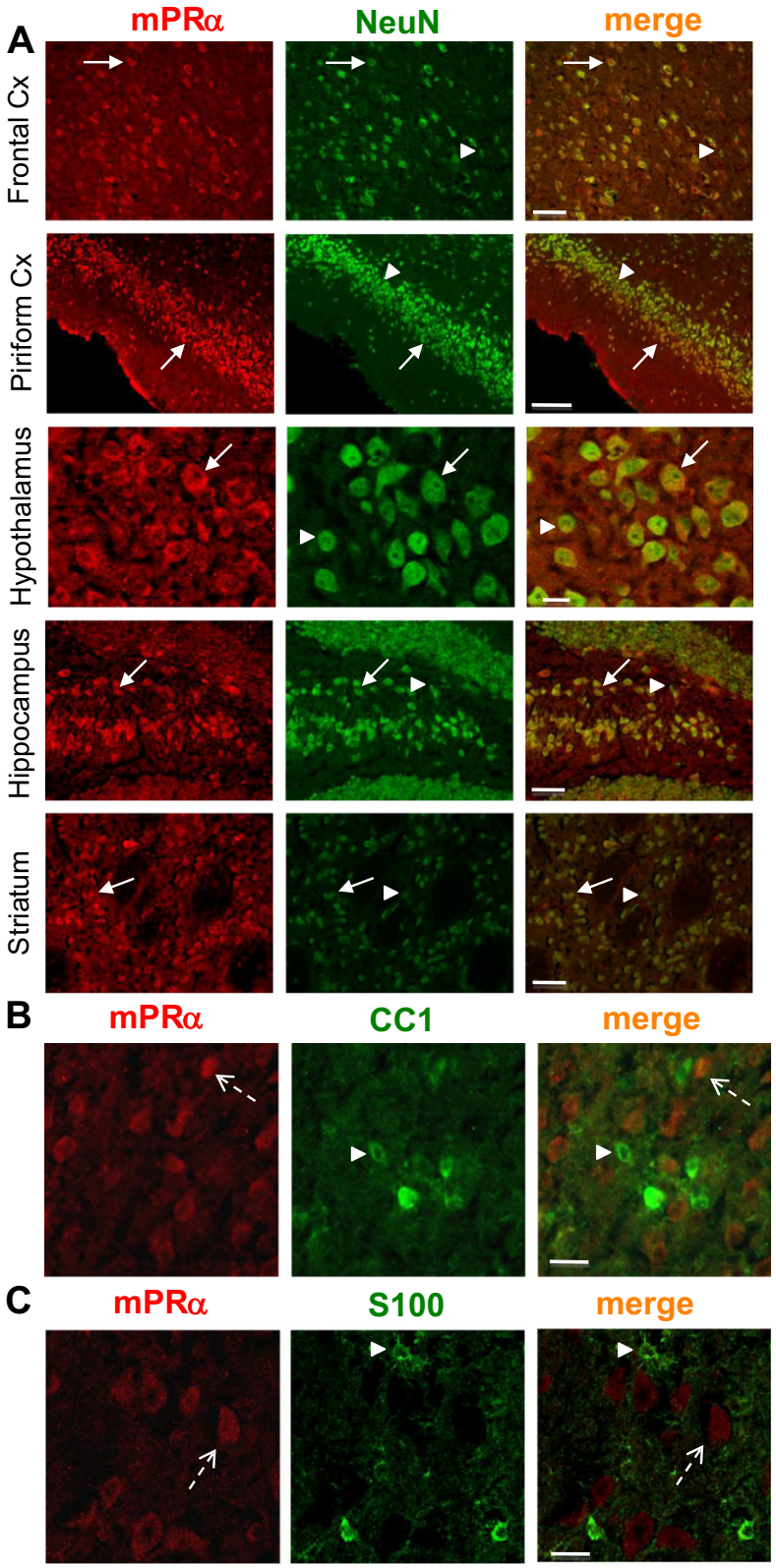

Fig. 4. mPRα is expressed in neurons throughout the mouse brain: analysis by double immunofluorescence and confocal microscopy. Immunostaining with the rabbit polyclonal anti-mPRα GP47 (red) and mouse monoclonal antibody (green) NeuN in (A), CC1 in (B), and S100 in (C), to specifically detect neurons, oligodendrocytes and astrocytes respectively. In A–C, arrows show cells that express mPRα and cell-type marker; arrowheads show cells that express cell type-markers which lack mPRα expression; dotted arrows show cells that express mPRα and which are not co-labeled with the cell type-marker. Images indicate that mPRα is only expressed by neurons (mPRα/NeuN colocalization). Scale bar = 50 μm for all the panels except for the hypothalamus panel (A) and 100 μm (B, C).

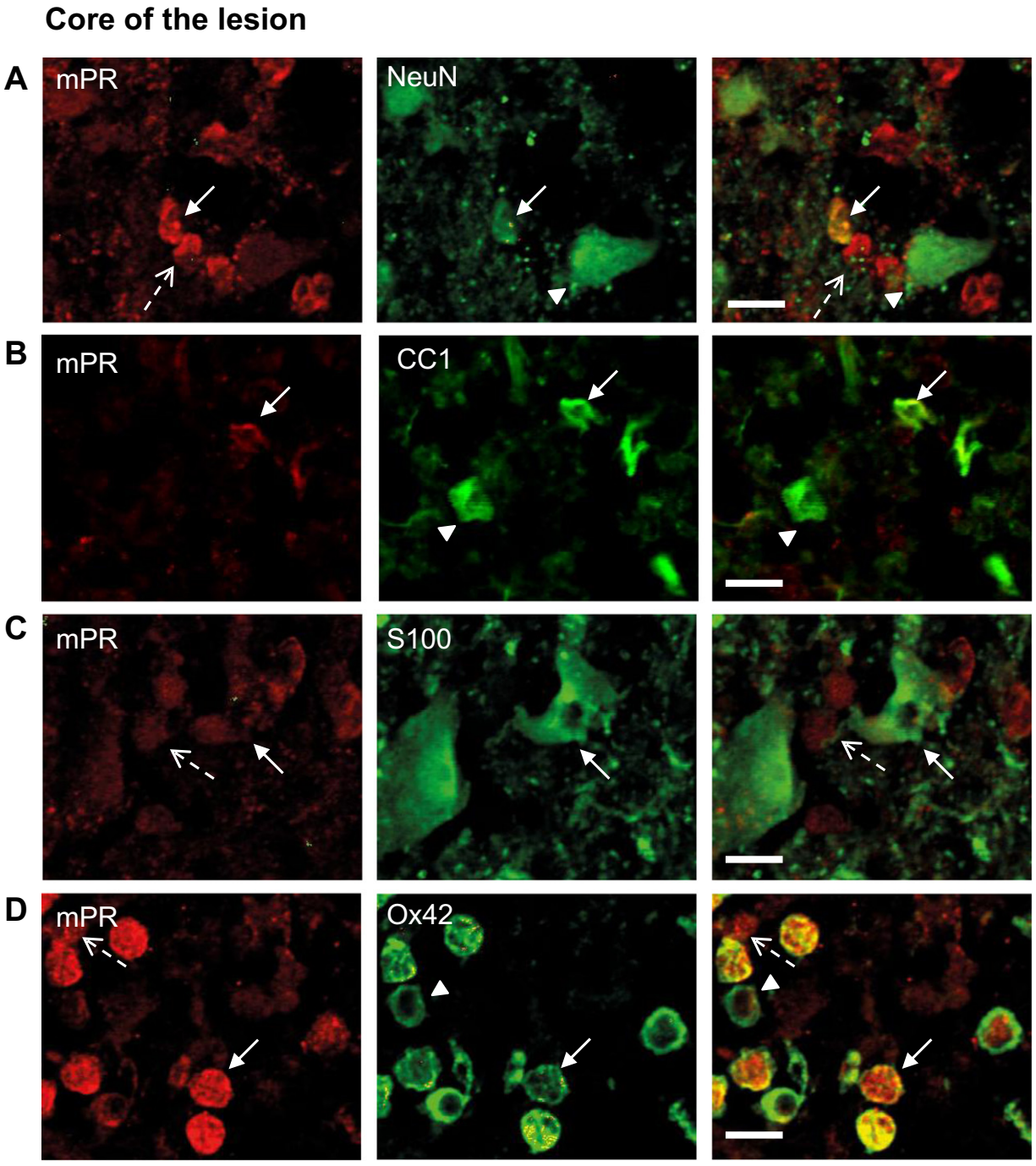

Fig. 5. Effects of traumatic brain injury on mPRα expression in the core of the lesion: analysis by double immunofluorescence and confocal microscopy. Immunostaining with the rabbit polyclonal anti-mPRα GP47 (red) and mouse monoclonal antibody (green) NeuN (A), CC1 (B), S100 (C) and Ox-42 (D) to specifically detect neurons, oligodendrocytes, astrocytes and activated microglia respectively. Arrows show cells that express mPRα and cell type marker; arrowheads show cells that express cell type markers which lack mPRα expression; dotted arrows show cells that express mPRα and which are not co-labeled with the cell type-marker. After TBI, mPRα expression was decreased in neurons (A) and induced in oligodendrocytes (B), astrocytes (C) and reactive microglia (D). Scale bar = 10 μm.

mPRα after injury (Fig. 5A, dotted arrows). Some $CC1^+$ cells were co-stained with mPRα (Fig. 5B, arrows). Several cells $S100^+$ were faintly stained with mPRα (Fig. 5C, arrows). Interestingly, mPRα expression was highly induced in the reactive microglial cells, as shown by the large number of mPRα/Ox-42 co-labeled cells (Fig. 5D, arrows). Fig. 6 summarizes the results observed in the peri-lesional area in the cortex under the lesion site. In contrast to what was observed in the core of the lesion, a large mPRα/NeuN colocalization was observed (Fig. 6A, arrows), as for the non-injured rats (Fig. 4A). The intensity of mPRα signal in neurons was similar to that observed in the intact cortex of the non-injured rats. In addition, mPRα expression was

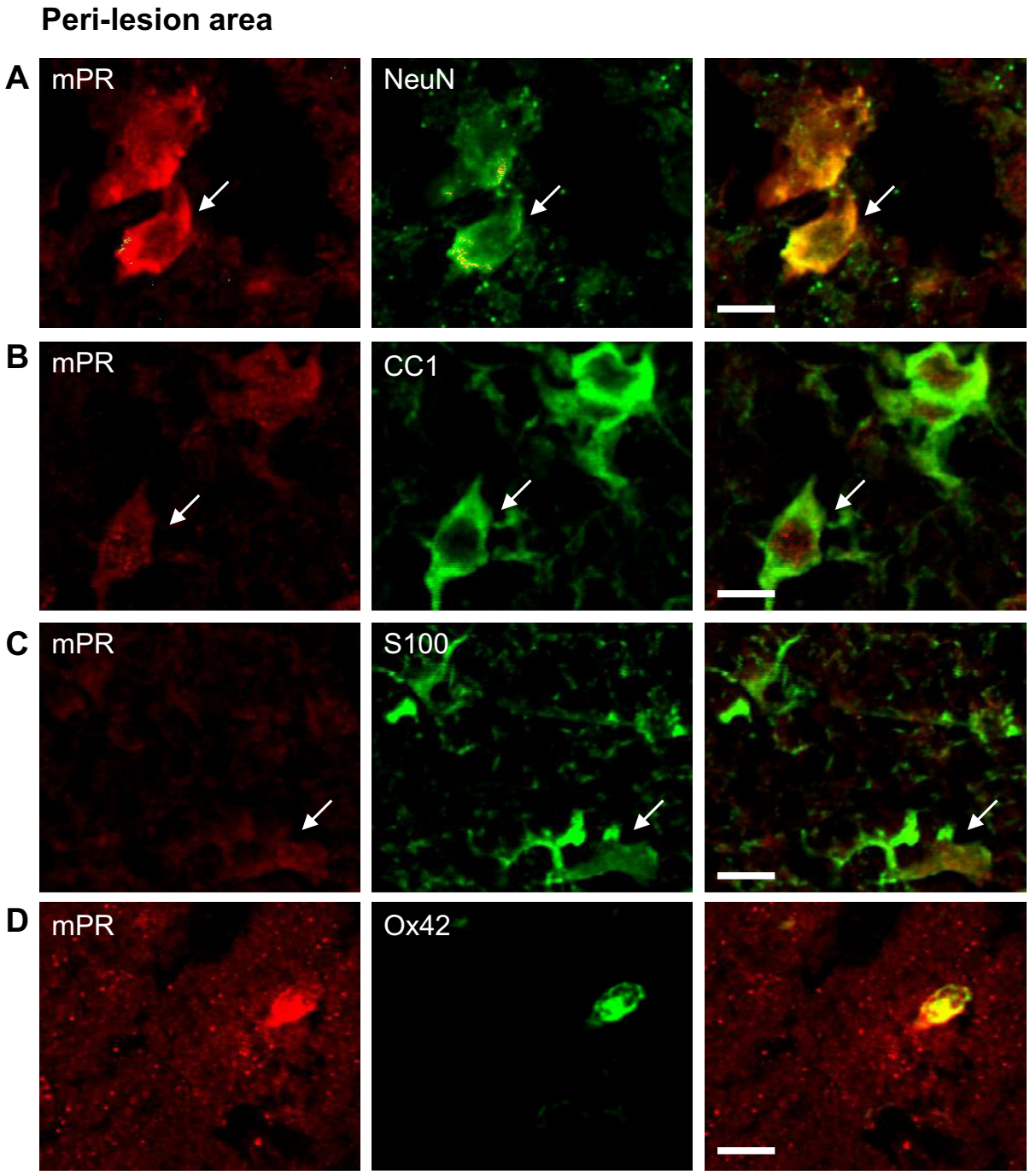

Fig. 6. Effects of traumatic brain injury on mPRα expression in the peri-lesion area: analysis by double immunofluorescence and confocal microscopy. Immunostaining with the rabbit polyclonal anti-mPRα GP47 (red) and mouse monoclonal antibody (green) NeuN (A), CC1 (B), S100 (C) and Ox-42 (D) to specifically detect neurons, oligodendrocytes, astrocytes and activated microglia respectively. Arrows show cells that express mPRα and cell type marker; arrowheads show cells that express cell type markers which lack mPRα expression; dotted arrows show cells that express mPRα and which are not co-labeled with the cell type-marker. After TBI, mPRα expression was induced in oligodendrocytes (B), astrocytes (C) and reactive microglia (D). Scale bar = 10 μm.

induced in oligodendrocytes: almost all $CC1^+$ cells co-express mPRα (Fig. 6B, arrows). In contrast, only faint mPRα labeling was observed in astrocytes, as shown in Fig. 6C. Few activated microglial cells $Ox\text{-}42^+$ were observed in the peri-lesion area and they express mPRα (Fig. 6D).

DISCUSSION

We report here a detailed study of regional and cellular pattern of expression of mPRα in the male mouse and rat brain. The level of mPRα expression in brain is high, even in males and in the absence of any estrogen

stimulation. Our results show that this expression is not modified by estradiol or progesterone treatment as was shown by Intlekofer and Petersen in the neuroendocrine regions of female rat brain (Intlekofer and Petersen, 2011a). However, as the effects exerted by steroids depend on the dose, time, duration of treatment, sex, reproductive status and other physiological or experimental conditions a possible fine regulation of mPRα by steroids is not excluded. Indeed, mPRβ expression in the female rat brain has been shown to be regulated by estradiol in the medial septum but not in the medial preoptic area, horizontal diagonal band or oculomotor nucleus (Zuloaga et al., 2012), indicating that estrogens can regulate mPR within discrete brain regions. Therefore, additional studies using different doses and duration of treatment combined with immunohistochemistry analysis or Western blot analysis of different brain subregions are needed to show a possible dose-dependent, or region-specific regulations of mPRα by steroids.

We showed an extensive expression and anatomical distribution of the mPRα receptor in the mouse and rat brain, suggesting a diversity of functioning. The wide distribution of mPRα in the brain suggests that this receptor may mediate several progesterone actions including neuroendocrine regulation and reproductive function in the hypothalamus and also neuroprotection, cognition, motor and sensory functions, aggression and anxiety in other brain regions. Progesterone has been indeed shown to regulate these different functions (Gomez et al., 2002; Schumacher et al., 2007; Brinton et al., 2008; de Sousa et al., 2010); however the mechanisms of action and the receptors involved are understudied. It is possible that some of these progesterone actions may be mediated in part by mPRα. Some regions, like hypothalamus and cortex, appear to express PR, mPRα and Pgrmc1. The overlapping distribution of the different receptors in specific brain regions suggests that there may be a synergism or antagonism between the progesterone receptors and/or the genes they regulate. Like the other progesterone binding proteins PR and Pgrmc1, mPRα is expressed by neurons, suggesting the possibility that the same neuron may express the three receptors.

The wide expression of mPRα in the brain is very similar to the previously described distribution pattern of the 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD), enzyme that converts pregnenolone to progesterone. Thus, *in situ* hybridization analysis revealed that 3β-HSD is expressed throughout the brain (Guennoun et al., 1995). Notably, 3β-HSD expression also appeared to be relatively stable, since TBI resulted in an increase in progesterone (Meffre et al., 2007b) without affecting 3β-HSD expression (Meffre et al., 2007a). The same observations have been made in the spinal cord, where we showed a large distribution of both 3β-HSD (Coirini et al., 2002) and mPRα (Labombarda et al., 2010), and an increase of progesterone levels after injury without a change in 3β-HSD expression (Labombarda et al., 2006). Together with the present observation of the large and steady expression of mPRα, these findings point to an important role for progesterone as a local autocrine/paracrine messenger within the nervous system, with the involvement of membrane receptor signaling. This new concept has important implications for understanding steroid actions in health and disease.

The effects of progesterone in TBI have been extensively studied in rats (Stein, 2001; Stein and Sayeed, 2010). In particular, progesterone has been shown to attenuate cerebral edema, improve spatial learning performance, reduce sensory neglect, and inhibit the increase of some inflammatory cytokines and inflammation-related factors, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, Inflammatory proteins C3 complement (CFC3), Glial fibrillary acidic protein (GFAP) and nuclear factor (NF)-κB (Shear et al., 2002; He et al., 2004; Pettus et al., 2005; Stein, 2008; Sayeed and Stein, 2009; Cekic and Stein, 2010; Stein and Sayeed, 2010; Stein and Wright, 2010). We showed that the distribution of mPRα in rat brain is similar to that observed in mouse brain. At the cellular level, in basal conditions, mPRα is expressed only by neurons. After TBI, mPRα expression is decreased in neurons in the core of the lesion, but is unaltered in the peri-lesion area, indicating that only direct primary injury affects mPR expression in the neurons. This also suggests that progesterone effects on neurons in the core of the lesion might mainly be mediated via Pgrmc1 whose expression has been shown to be increased in the neurons in the lesion site (Meffre et al., 2005).

TBI affects not only neurons but also endothelial cells, astrocytes, oligodendrocytes and microglia. Neuroprotection strategies should inhibit neuron death and optimize glial functions. In addition to direct neuroprotective effects on neurons, progesterone has been shown to act synergistically on glial cells after TBI (Feeser and Loria, 2011). Several studies showed the importance of glial cells as targets for the neuroprotective and anti-inflammatory actions of progesterone (Stein, 2008; De Nicola et al., 2009). Indeed, progesterone downregulates the expression of pro-inflammatory cytokines by microglia and astroglia and thereby reduces brain inflammation. Progesterone also attenuates brain edema in part by regulating the expression of aquaporin 4 in astrocytes (Guo et al., 2006). In addition, progesterone may act on glial cells by regulating the synthesis of other steroids which may affect both neurons and glia. Progesterone has multiple mechanisms of actions (Schumacher et al., 2007). After TBI, we observed the expression of mPRα not only in neurons, but in glia as well. Indeed, mPRα expression was induced in oligodendrocytes, astrocytes and activated microglia in the core of the lesion and in the peri-lesioned area. These observations suggest that progesterone after binding to mPRα may have direct membrane effects in these different glial cell types to regulate several parameters such as cell survival, edema formation, inflammation and myelin repair. By acting on microglia and astroglia, progesterone may reduce reactive gliosis and the release of pro-inflammatory cytokines. Actions on astrocytes are also

important to modulate the expression of aquaporin 4 and to control the function of the neurovascular unit and to reduce brain edema. The ideal drug should be able to block the multiple cellular events that lead to brain damage after TBI. Different experimental studies have shown that progesterone is an attractive potential neuroprotective candidate after TBI (De Nicola et al., 2009; Stein and Sayeed, 2010). Our present results suggest that mPRα might play a pivotal role in mediating progesterone effects in neurons, glia and microglia after TBI.

The nervous system has been shown to adapt the expression of steroid receptors after injury. Indeed, the expression of androgen receptors and estrogen receptor (ER)-α is induced in glial cells after injury (Garcia-Ovejero et al., 2002). ER-X, a potential membrane receptor of estrogens, which is not expressed in the brain of adult mice, is re-expressed after cerebral ischemia (Toran-Allerand et al., 2002). We have also shown that the expression of Pgrmc1 changes after TBI and spinal cord injury (Labombarda et al., 2003; Meffre et al., 2005; Guennoun et al., 2008). These findings suggest that the expression and the mechanisms of action of steroids may differ according to the physiopathological context.

CONCLUSION

We showed a widespread expression of mPRα in many brain regions of male mouse and rat, suggesting a role of mPRα in the regulation of different functions including reproduction, neuroprotection, cognition and behavior. In the basal conditions mPRα is expressed in neurons. After TBI, mPRα is induced in glia suggesting its potential role in mediating the beneficial effects of progesterone in inflammation, ion and water homeostasis and myelin repair in the injured brain.

COMPETING INTERESTS

D.M., F.L., B.D., A.C., A.F.D.N., M.S. and R.G. have nothing to disclose.

D.G.S. is entitled to royalties from products of BHR Pharmaceuticals Ltd. related to the use of progesterone in TBI and stroke, and may receive research funding from BHR Pharmaceuticals, which is developing products related to this research. In addition, he serves as a consultant to BHR Pharmaceuticals and receives compensation for these services. The terms of this arrangement have been reviewed and approved by Emory University, which receives the largest share of fees in accordance with its conflict of interest policies.

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