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ORIGINAL RESEARCH REPORT

Prenatal restraint stress decreases the expression of alpha-7 nicotinic receptor in the brain of adult rat offspring

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Abstract

Prenatal stress (PS) strongly impacts fetal brain development and function in adulthood. In normal aging and Alzheimer's disease, there is hypothalamic–pituitary–adrenal axis dysfunction and loss of cholinergic neurons and neuronal nicotinic acetylcholine receptors (nAChRs). This study investigated whether prenatal restraint stress affects nAChR expression in the brain of adult offspring. For PS, pregnant dams were placed in a plastic restrainer for 45 min, three times daily during the last week of pregnancy; controls were undisturbed. Male offspring were analyzed at postnatal day (PND) 60 ($n = 4$ rats per group). Western blot (WB) and fluorescence microscopy showed that PS decreased α7-AChR subunit expression (∼50%) in the frontal cortex in the adult offspring. PS decreased significantly the number of α7-AChR-expressing cells in the medial prefrontal cortex (by ∼25%) and in the sensory-motor cortex (by ∼20%) without affecting the total cell number in those areas. No alterations were found in the hippocampus by quantitative polymerase chain reaction (qPCR), or WB analysis, but a detailed fluorescence microscopy analysis showed that PS affected α7-AChR mainly in the CA3 and dentate gyrus subfields: PS decreased α7-AChR subunit expression by ∼25 and ∼30%, respectively. Importantly, PS decreased the number of α7-AChR-expressing cells and the total cell number (by ∼15 and 20%, respectively) in the dentate gyrus. PS differently affected α4-AChR: PS impaired its mRNA expression in the frontal cortex (by ∼50%), without affecting protein levels. These results demonstrate that disturbances during gestation produce long-term alterations in the expression pattern of α7-AChR in rat brain.

Keywords

Aging, cholinergic, cortex, gestational stress, hippocampus, neurodegeneration

History

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Introduction

In the central nervous system, the cholinergic innervations through nicotinic acetylcholine receptors (nAChRs) modulates important biological processes such as learning, memory, plasticity, neuroprotection and neurodegeneration (Gotti et al., 2006; Oddo & LaFerla, 2006). nAChRs are a heterogeneous family of ligand-gated ion channels, in homomeric or heteromeric combinations of α- and β-subunits. The most abundant nAChR subtypes in the brain are the α4β2 and the α7 forms (Gotti & Clementi, 2004; Gotti et al., 2006). Binding of acetylcholine prompts the influx of cations activating membrane depolarization and/or Ca^{2+}-dependent signaling pathways (Feduccia et al., 2012; Gotti & Clementi, 2004). Some nAChRs are located pre-synaptically, where they modulate the release of other neurotransmitters, or in cell bodies and dendrites, where they exert post-synaptic effects, thereby regulating neuronal excitability and neurotransmitter release (Gotti et al., 2006). They are also involved in a wide variety of diseases affecting the nervous system (Barrantes et al., 2000; Gotti & Clementi, 2004).

Cholinergic impairments and cognitive decline related to aging have been widely documented (Terry & Buccafusco, 2003). Synthesis and release of acetylcholine is significantly decreased in senescent mice (Gibson & Peterson, 1981; Gibson et al., 1981). In addition, loss of cholinergic innervation and nAChRs occurs normally in aged rodent and human brains (Casu et al., 2002; Gahring et al., 2005; Nyakas et al., 2011; Perry et al., 2001; Rogers et al., 1998; Schliebs & Arendt, 2011). Similarly, striking loss of cholinergic cells and AChRs in the cerebral cortex and hippocampus (HPC) are neurochemical features of Alzheimer's disease (AD; Francis et al., 1999; Oddo & LaFerla, 2006). Accelerated age-related cognitive decline is associated with an impaired response of

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the hypothalamo-pituitary-adrenal (HPA) axis (Pardon & Rattray, 2008), and dysregulation of this axis has been related to neurodegenerative diseases, including AD (Green et al., 2006). Indeed, susceptibility to stress increases the risk of developing AD in humans (Pardon & Rattray, 2008).

The mechanism by which the pregnant mother transfers stress to the fetus is still a matter of debate (Huizink et al., 2004). It has been proposed that during a stressful pregnancy, a fraction of the increased levels of stress hormones could cross the placenta and reach the fetus, increasing fetal HPA axis activity and affecting brain development (Huizink et al., 2004; Lupien et al., 2009). Unfavorable developmental conditions may cause adaptations that enable fetus survival, but this long-lasting re-programming may have adverse consequences in the adult (Miller & O'Callaghan, 2008). Prenatal stress (PS) in rats induces long-term alterations in the HPA axis in the offspring (Darnaudery & Maccari, 2008; Henry et al., 1994). In concordance, PS or glucocorticoid treatment is associated with long-term impact on the HPA axis in humans (Davis et al., 2011; Entringer et al., 2009; O'Connor et al., 2005; Van Den Bergh et al., 2008). Dysregulation of the HPA axis has been related to depression, anxiety, schizophrenia and neurodegeneration (Green et al., 2006; Hunter, 2012). It has been postulated that some stressors could modulate the HPA axis through acetylcholine via activation of nAChRs (Shytle et al., 2002).

Under normal conditions, glucocorticoids are necessary for adequate brain function, but in excess they modify neurotransmission systems, transcriptional machinery and permanently alter the HPA axis and other endocrine systems, as well as behavior and brain morphology (Lesage et al., 2001; Pardon & Rattray, 2008). PS affects hippocampal neurogenesis in a stress intensity-dependent manner (Coe et al., 2003; Fujioka et al., 2006; Lemaire et al., 2000). An excess of glucocorticoid affects migration of post-mitotic neurons during the development of the cerebral cortex (Fukumoto et al., 2009). In addition, PS accelerates the age-progressive decline of hippocampal cell proliferation (Lemaire et al., 2000).

Based on the above background and our own observations, we hypothesized that PS may alter expression of nAChRs in the brain. Hence, the aim of this study was to analyze whether restraint stress applied in the last week of pregnancy has long-term effects on the expression of the two more abundant nAChR subunits (i.e. α7-AChR and α4-AChR) in two neuropsychiatric-relevant brain regions (i.e. the frontal cortex and the hippocampus) of the rat brain. Employing a combined experimental approach, including molecular and microscopic analyses, we determined that PS does not affect α4-AChR subunit levels although it impairs α7-AChR expression in the cortex and the hippocampus of adult prenatally stressed male rats.

Methods

Animals

Virgin female Wistar rats weighing 250 g were obtained from outbred rats from our own animal facility at the University of Buenos Aires. A maximum of four rats were housed per cage with *ad libitum* access to standard rat chow (Asociación de Cooperativas Argentinas, Buenos Aires, Argentina) and water. Vaginal smears were collected daily for 8 days before mating to determine the stage of the estrous cycle and the day of conception. On the day of proestrus, sexually experienced male Wistar rats weighing 250–300 g were introduced for mating. Vaginal smears were taken on the following morning. The day in which spermatozoa were found in the smear was designated day 1 of pregnancy. A constant light/dark cycle (on at 06:00 h, off at 18:00 h) and temperature of 21–25 °C were maintained. All procedures were in agreement with the standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (CICUAL #121/2013, Facultad de Medicina, Universidad de Buenos Aires, Argentina). Care was taken to minimize the number of rats used.

Prenatal procedures

Pregnant dams ($n = 8$) were randomly assigned to either the control ($n = 4$) or the prenatal stress (PS; $n = 4$) group and were individually housed with *ad libitum* access to standard rat chow and water. Control rats were left undisturbed in the home cage, while PS dams were subjected to a restraint stress procedure, which involved rats being transferred to an experimental room where the stressor was applied. Pregnant females were individually placed into a transparent plastic restrainer, fitted closely to body size, for three 45 min periods per day (09:00, 12:00 and 16:00 h) between days 14 and 21 of the pregnancy. The restrainer had ventilation holes and dimensions appropriate for a pregnant rat of 350 g: internal diameter of 64 mm with an adjustable length of 149–208 mm. This type of stressor was chosen because it has an indirect influence on the fetuses via a direct stress on the mother (Maccari et al., 1995; Ward & Weisz, 1984). It has been reported that pregnant dams exhibit greater corticosterone levels than undisturbed pregnant rats in response to restraint stress (Jensen Pena et al., 2012; Moosavi et al., 2011; Ward & Weisz, 1984; Zagron & Weinstock, 2006). The sessions were performed in a lit environment and no other rats were present in the experimental room during the stress exposure. At the end of the stress session, rats were returned to the animal housing room and were then individually housed with standard rat chow and water *ad libitum*. On the day of parturition (postnatal day, PND 0), litter characteristics were recorded and culled to 10 pups, maintaining similar numbers of males and females wherever possible. Four litters were maintained for each experimental group. Weaning was performed at PND 21. The male and female offspring were housed, by litter, in separate cages after weaning, with no more than five pups per cage, with standard rat chow and water *ad libitum*.

Group assignment

In this study, only male offspring were used. To avoid litter effects, only one rat from each of four litters per group was tested in each experiment. Hence, for this study, ''*n*'' implies that four unique (non-siblings) prenatally stressed or control rats were used separately for each method of analysis

(i.e. quantitative polymerase chain reaction, qPCR; Western blot, WB; or immunofluorescence).

Tissue collection

At PND 60, four males obtained from different litters, belonging to the control or PS group, were rapidly euthanized by decapitation, without anesthesia, between 09:00 h and 10:00 h. As mentioned above, different offspring from different litters were used for WB or qPCR determinations. Both cerebral hemispheres were used in each determination. Brains were removed from the skull for further dissection. Frontal cortex (FCtx; 5.20–1.00 mm antero-posterior [AP] relative to bregma) and the hippocampus (HPC; −1.80 to −6.04 mm AP relative to bregma) were rapidly extracted on ice according to anatomical landmarks corresponding to the Paxinos & Watson (1986) rat brain atlas. Brain tissue samples were weighed and differentially processed for WB or for qPCR analyses as described below.

RNA isolation, cDNA synthesis and qPCR

Total RNA from FCtx and HPC of PND 60 rats from the control or PS groups was isolated with DirectZol RNA Miniprep (Zymo Research, Irvine, CA) following the manufacturer's instructions. Complementary DNA was synthesized by retrotranscription using oligodT and SuperScript™ II Reverse Transcriptase enzyme (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. qPCR reactions were achieved with Kapa SYBR fast qPCR kit (KAPA Biosystems, Wilmington, MA) in triplicate. Data were analyzed using the linear standard curve method. Before each experiment, the calibration curves were validated and samples with Ct values amplified out of the calibrated dynamic range were eliminated. For data normalization, mRNA levels for the reference gene *cyclophilin-a* were measured.

Primers used (5′–3′):

α7-AChR, Chrn7: sense F GGACCGCTTGTGCCTCAT

α7-AChR, Chrn7: antisense R AGGATGCCGATGGTACAG ATG

α4-AChR, Chrn4: sense F GAAGGCGTCCAGTACATTGCA

α4-AChR, Chrn4: antisense R GTCCTCCTTCACCGAGAA GTCA

Cyclophilin-a: sense F CATTCAGTCTTGGCAGTGCAG

Cyclophilin-a: antisense R AAGCATACAGGTCCTGGCAT CT

Western blotting

FCtx and HPC extracts were homogenized in 5 ml/g (per gram of tissue) of ice-cold radioimmunoprecipitation assay buffer (RIPA buffer; 50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate, SDS; 1% protease inhibitors). Following centrifugation of the homogenate for 15 min at 16 000 × *g* at 4 °C, supernatants were recovered and protein concentrations were quantified using the Bradford method. Equal amounts of protein (75 µg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) through 10% acrylamide under reducing conditions and then blotted onto polyvinylidene fluoride (PVNDF) membrane (Immobilon-P; Millipore, Billerica, MA). The blot was blocked in 5% non-fat dry milk in Tris-buffered saline with Tween 20 (TBST; 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 2 h and subsequently incubated overnight at 4 °C with polyclonal anti-α7-AChR (H-302; 1:2000; Santa Cruz Biotechnology Inc., Dallas, TX) or anti-α4-AChR (mAb299; 1:5000; Sigma, St. Louis, MO). After washing in TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h. The bands were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences, Fairfield, CT). Expression of each nAChR subunit was normalized to the β-actin values.

Tissue preparation for immunofluorescence

At PND 60 rats were deeply anesthetized with a Ketamine/Xylazine mixture (50 and 8 mg/Kg; 35.6 mg/mL and 5.7 mg/mL, respectively, in the injectate; maximum volume injected 0.7 ml; intraperitoneal injection) and perfused through the cardiac left ventricle, initially with 50 mL of cold physiological saline (0.9% NaCl solution containing 0.05% w/v $NaNO_2$ plus 50 I.U. of heparin), followed by perfusion with 500 mL of fixative (4% paraformaldehyde in 100 mM phosphate buffer, PBS, pH 7.4). Brains were then removed, post-fixed for 2 h in 4% paraformaldehyde, cryoprotected by subsequent overnight immersion in 15 and 30% sucrose, and finally stored at −80 °C until processing for immunofluorescence. Eight series of 25-µm-thick coronal sections for FCtx and ten for the HPC were cut on a Leica cryostat (Leica, Wetzlar, Germany). Slices were stored at −20 °C in PBS pH 7.4, with 50% w/v glycerol added as cryoprotector until their use in immunofluorescence studies.

Immunofluorescence

Twenty-five micrometer thick brain sections from both control and prenatally stressed rats were selected according to anatomical landmarks corresponding to the Paxinos & Watson (1986) rat brain atlas (Figure 1). To avoid differences in labeling procedure, all sections from control and prenatally stressed rats were simultaneously processed in the free-floating state. In order to block non-specific binding sites, brain sections were incubated for 1 h at room temperature with 5% v/v normal goat serum in PBS containing 0.15% Triton X-100 (PBST). Sections were incubated for 48 h at 4 °C with primary antibodies to anti-α7-AChR (mAb306, 1/1000, kindly provided by Dr. J. Lindstrom, Perelman School of Medicine, University of Pennsylvania, PA) or anti-α4-AChR (mAb299; 1:500, Sigma). After five rinses in PBST, sections were incubated for 24 h at 4 °C with Texas Red-goat anti-mouse IgG (1/500, kindly provided by Dr. T. Santa Coloma, Institute of Biomedical Research UCA-CONICET, Buenos Aires, Argentina) or Alexa Fluor[555] goat anti-rat IgG (1/500, Molecular Probes, Invitrogen, Carlsbad, CA) for α7-AChR and α4-AChR, respectively. In addition, we used α-Bungarotoxin-Alexa[647] (1/250, Molecular Probes) as double control for α7-AChR labeling. After further washing in PBST, sections were incubated for 20 min with 0.035 mg/ml Hoechst Stain solution (Sigma). Sections were then washed three times in PBST and finally, mounted on

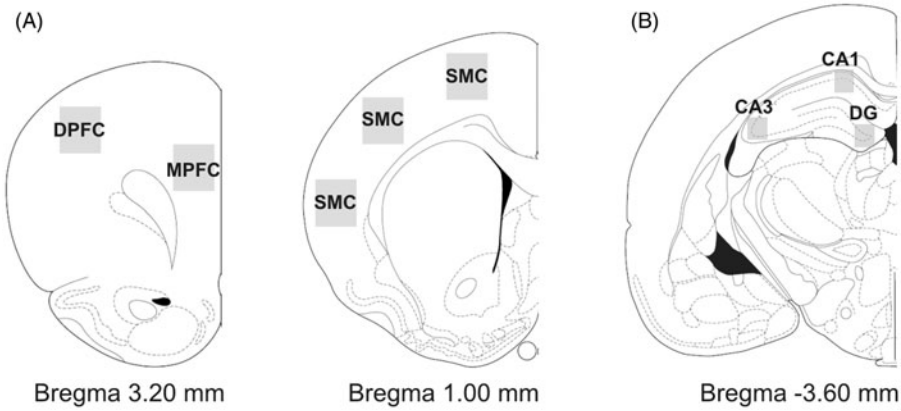

Figure 1. Diagrams of representative coronal sections used for immunofluorescence or cell count analysis of rat brain regions. Samples include 25 µm sections from (A) 3.20 mm to 1.00 mm antero-posterior (AP), (B) −2.80 mm to −4.16 mm AP relative to bregma, according to Paxinos & Watson (1986). DPFC, dorsal prefrontal cortex; MPFC, medial prefrontal cortex; SMC, sensory-motor cortex; CA1, CA3 region of the hippocampus (HPC); DG, dentate gyrus of the HPC. The areas indicated in gray were used for the analysis. The figure is adapted from Paxinos & Watson (1986).

gelatine-coated slides, air dried and coverslipped in 90% glycerol in PBS until observation in a microscope.

Image analysis

In order to ensure objectivity, all measurements were performed on coded slides, under blind conditions, carrying out the measurements on immunolabeled brain sections in both brain hemispheres, from control and prenatally stressed rats with the same standardized observation schedule. A single series of sections from each rat of each group was used for quantification. The brain areas of interest were identified according to Paxinos & Watson (1986) (Figure 1) and the boundaries of the structures in the coronal plane were determined microscopically. Fluorescence images were analyzed with the ImageJ software (National Institutes of Health, Bethesda, MD). For each brain level, fluorescence intensity was measured in the selected area and the non-specific background, measured in a region devoid of α7-AChR, α4-AChR or α-Bungarotoxin-Alexa^{647} label, was subtracted. Neurons immunoreactive with mAb306 (mAb306+, α7-AChR positive neurons) and Hoechst stain positive (total cells), were counted using a computerized image analysis system. Images were captured from slices using a Nikon Eclipse E-600 microscope (Nikon, Melville, NY) with a K2E Apogee CCD camera driven by CCDOPS software (Santa Barbara Instrument Group, Santa Barbara, CA). In order to count mAb306 + and Hoechst-stained cells in FCtx and HPC we analyzed sections from 3.20 to 1.00 mm and from −2.80 to −4.16 mm AP relative to bregma for FCtx and HPC, respectively (Paxinos & Watson, 1986). For the analysis, we delimited the area according to Paxinos & Watson (1986) (Figure 1) in each section, while the number of neurons obtained was normalized to a fixed area (0.5 mm^2). Three brain slices from each rat and brain area were used for the analysis, and an average value was calculated for each brain area per rat. The average section thickness was 25 µm. As measurements were made on every eighth or tenth serial section (i.e. separated by 200–250 µm), it was unlikely that a positive neuron would be counted twice.

Statistical analysis

Fluorescence microscopy and WB data were analyzed using the Student's *t*-test. qPCR data were analyzed with the non-parametric Mann–Whitney *U*-test because data was not normally distributed. All results are presented as the mean ± SEM. The observed differences were considered to be statistically significant when $p < 0.05$. Analysis of data was performed using Origin version 8 OriginLab Graphing Software (OriginLab Corporation, Northampton, MA). *n* values reported in the text or figures represent number of rats (from different litters).

Results

In agreement with previous studies, PS did not interfere with the length of gestation, number, ratio or weight of the pups at birth nor body weight gain of offspring (Barros et al., 2004; Berger et al., 2002; Pallares et al., 2013).

PS effects on α7-AChR mRNA and protein expression in the frontal cortex from adult rat offspring

qPCR

Employing qPCR we found a trend towards downregulation of α7-AChR gene (*Chrn7*) expression in the FCtx of PS offspring (Mann–Whitney test, $p = 0.071$; $n = 4$ rats per group; Figure 2A).

WB

In addition, α7-AChR protein levels, determined by WB, were decreased in the FCtx of PS offspring (Student's *t* test, $p = 0.023$, $n = 4$ rats per group; Figure 2B and C).

Quantitative immunofluorescence

In order to evaluate whether the observed alterations in the expression of α7-AChR in the FCtx were restricted to a specific brain area, we performed immunofluorescence experiments in coronal sections of control and PS rats. In order to evaluate fluorescent intensity (FI, related to protein expression) as well as the relative number of cells expressing α7-AChR, we used another primary antibody (mAb306) to label the α7-AChR. In addition, in the same sections, we employed fluorescent α-bungarotoxin (αBTX) to label the α7-AChR as an antibody control. Total cell number per 0.5 mm^2 was evaluated by Hoechst stain labeling. As shown in Figure 3(A and B), the fluorescent intensity was decreased in PS offspring in the three cortical areas analyzed as compared to undisturbed offspring.

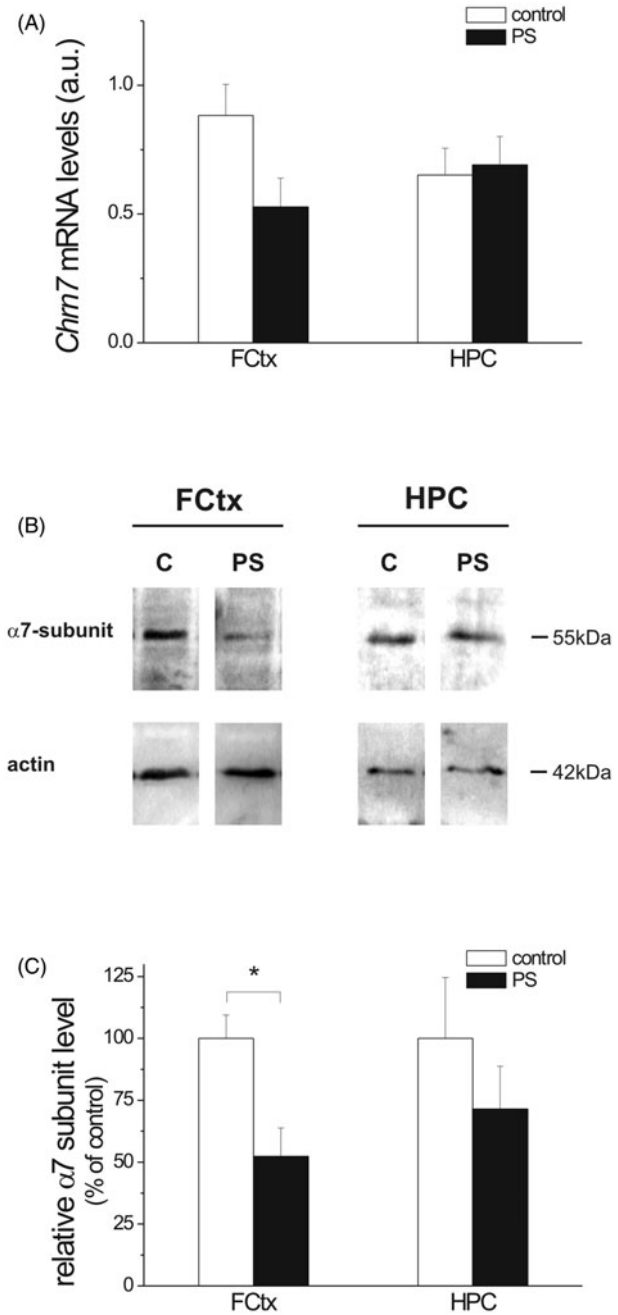

Figure 2. Effect of prenatal stress (PS) on α7-nicotinic acetylcholine receptor (α7-AChR) subunit expression in the frontal cortex (FCtx) and hippocampus (HPC) from adult male rat offspring. (A) α7-AChR (*Chrn7*) mRNA levels in FCtx and HPC in control and PS male offspring at postnatal day (PND) 60. Relative mRNA expression levels were determined by the standard curve method. *Cyclophilin-a* was used as reference gene. Data are expressed as mean ± SEM; Mann–Whitney test, $n = 4$ rats per group. (B) Western blot (WB) analysis from homogenates of FCtx and HPC from control (C) and prenatally stressed rats (PS). A band of ~55 kDa, corresponding to the α7-AChR subunit, was detected with polyclonal antibody H-302 (Santa Cruz Biotechnology). (C) Densitometric quantification and statistical analysis of the results shown in (B). The optical density of each α7-AChR band was normalized to the corresponding actin band (used as load control). Data are expressed as mean ± SEM; *denotes $p < 0.05$; Student's *t*-test; $n = 4$ rats per group.

Dorsal prefrontal cortex

In the dorsal prefrontal cortex (DPFC; Figure 3B, upper three histograms) from PS rats, we observed decreased fluorescent intensity of α7-AChR-labeled cells [FI mAb306: Student's *t* test, $p = 0.047$, $n = 4$ rats per group; FI αBTX: Student's *t* test, $p = 0.027$, $n = 4$ rats per group]. The relative number (per 0.5 mm^2) of α7-AChR-expressing cells (mAb306 + cells) and the relative total number of cells (Hoechst-stained, +, cells) was similar between control and PS rats ($n = 4$ rats per group).

Medial prefrontal cortex

In the medial prefrontal cortex (MPFC; Figure 3B, middle three panels) from PS rats we also observed a decrease in the intensity of fluorescence in α7-AChR-labeled cells [FI mAb306: Student's *t*-test, $p = 0.036$, $n = 4$ rats per group; FI αBTX: Student's *t*-test, $p = 0.025$, $n = 4$ rats per group]. In this cortical area, PS reduced the relative number (per 0.5 mm^2) of α7-AChR-expressing cells [mAb306+/0.5 mm^2: Student's *t*-test, $p = 0.004$, $n = 4$ rats per group] but no significant change was observed in the total number of cells [Hoechst+/0.5 mm^2: n.s., $n = 4$ rats per group].

Sensory-motor cortex

Similar to the changes observed in the MPFC, in the sensory-motor cortex (SMC; Figure 3B, lower three panels) from PS, we observed a decrease in the intensity of fluorescence in α7-AChR-labeled cells [FI mAb306: Student's *t* test, $p = 0.04$, $n = 4$ rats per group; FI αBTX: Student's *t* test, $p = 0.01$, $n = 4$ rats per group]. In addition, PS reduced the relative number (per 0.5 mm^2) of α7-AChR-expressing cells [mAb306+/0.5 mm^2: Student's *t*-test, $p = 0.02$, $n = 4$ rats per group] but did not alter significantly the total number of cells [Hoechst+/0.5 mm^2: n.s., $n = 4$ rats per group].

PS effects on α7-AChR mRNA and protein expression in the hippocampus (HPC) from adult rat offspring

qPCR

We also analyzed α7-AChR mRNA and protein expression in the HPC from control and PS rats. qPCR did not show changes in α7-AChR mRNA expression in the whole HPC of PS rats ($n = 4$ rats per group; Figure 2A).

WB

Similarly, hippocampal α7-AChR protein levels determined by WB were not affected in PS offspring ($n = 4$ rats per group; Figure 2B and C).

Quantitative immunofluorescence

Measurements on coronal brain sections from control and PS rats showed differential effects of PS on α7-AChR protein expression and the relative number of α7-AChR-expressing cells in specific areas of the HPC.

CA1

Significant changes were not observed in the CA1 area of the HPC of PS rats, either in AChR fluorescence intensity or in the relative number (per 0.5 mm^2) of α7-AChR-expressing cells (Figure 4B, upper three panels; $n = 4$ rats per group).

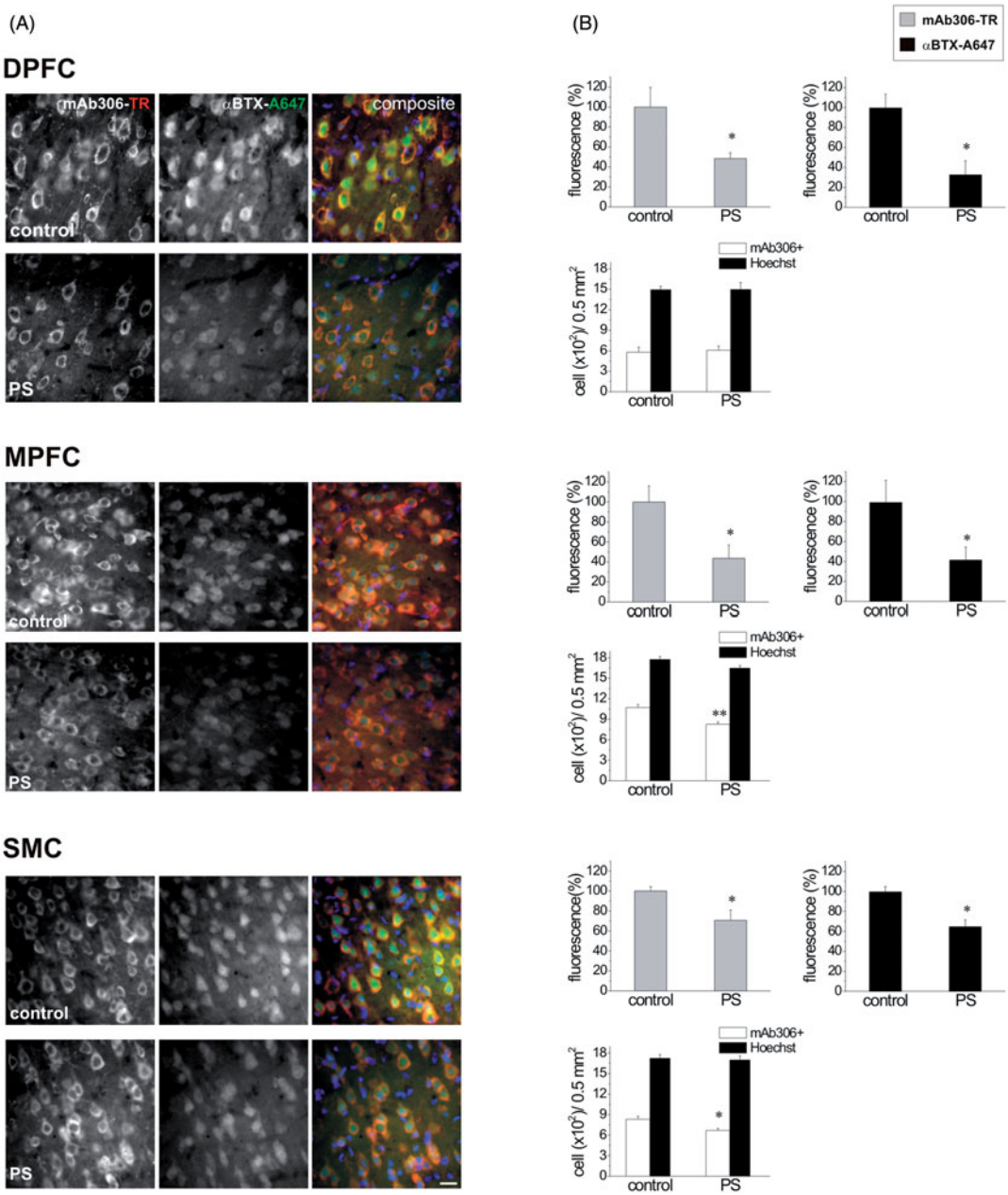

Figure 3. Expression of α7-AChR subunit in the frontal cortex (FCtx) of control and prenatally stressed (PS) male rat offspring. (A) Representative photomicrographs of coronal sections showing α7-AChR positive cells in the indicated brain regions from control and prenatally stressed rats (PS) at postnatal day (PND) 60: dorsal prefrontal cortex (DPFC), medial prefrontal cortex (MPFC), sensory-motor cortex (SMC). α7-AChR positive cells were labeled using monoclonal anti-α7-AChR subunit (mAb306) and Texas Red-goat anti-mouse IgG, *mAb306-TR*, left panel; and α-Bungarotoxin-Alexa^{647}, *αBTX-A647*, middle panel. Total cells were identified by Hoechst stain labeling. A co-localization of the three channels is shown in the right panel, *composite*. Bar, 20 μm. (B) Quantitative analysis of images depicted in A. Fluorescence intensities from mAb306-TR and αBTX-A647 were expressed as % of control. The relative number of α7-AChR expressing cells, mAb306 + and total cells, Hoechst-stained, were indicated by white and black bars, respectively. The number of cells obtained was normalized to a fixed area (0.5 mm^2). Values are reported as mean ± SEM. *, **denote $p < 0.05, < 0.01$, respectively, statistically significant different from control (undisturbed) rats. Student's t test, $n = 4$ rats per group.

CA3

In contrast, the CA3 area of the HPC from PS rats showed a statistically significant decrease in fluorescence intensity of labeled α7-AChR (Figure 4, middle three panels) [FI mAb306: Student's t test, $p = 0.02$, $n = 4$ rats per group; FI αBTX: Student's t test, $p = 0.014$, $n = 4$ rats per group]. The relative number (per 0.5 mm^2) of α7-AChR-expressing cells as well as the relative number of total cells were similar between control and PS rats (Figure 4, middle three panels; $n = 4$ rats per group).

Dentate gyrus

The dentate gyrus (DG) of the HPC showed a significant decrease in the fluorescent intensity of labeled-α7-AChR in PS offspring [IF mAb306: Student's t-test, $p = 0.022$, $n = 4$ rats per group; FI αBTX: Student's t-test, $p = 0.03$, $n = 4$ rats per group]. PS significantly reduced the relative number (per 0.5 mm^2) of α7-AChR-expressing cells [mAb306+/0.5 mm^2: Student's t test, $p = 0.002$, $n = 4$ rats per group] and the total number of cells [Hoechst+/0.5 mm^2: Student's t test, $p = 0.005$, $n = 4$ rats per group; Figure 4B, lower three panels].

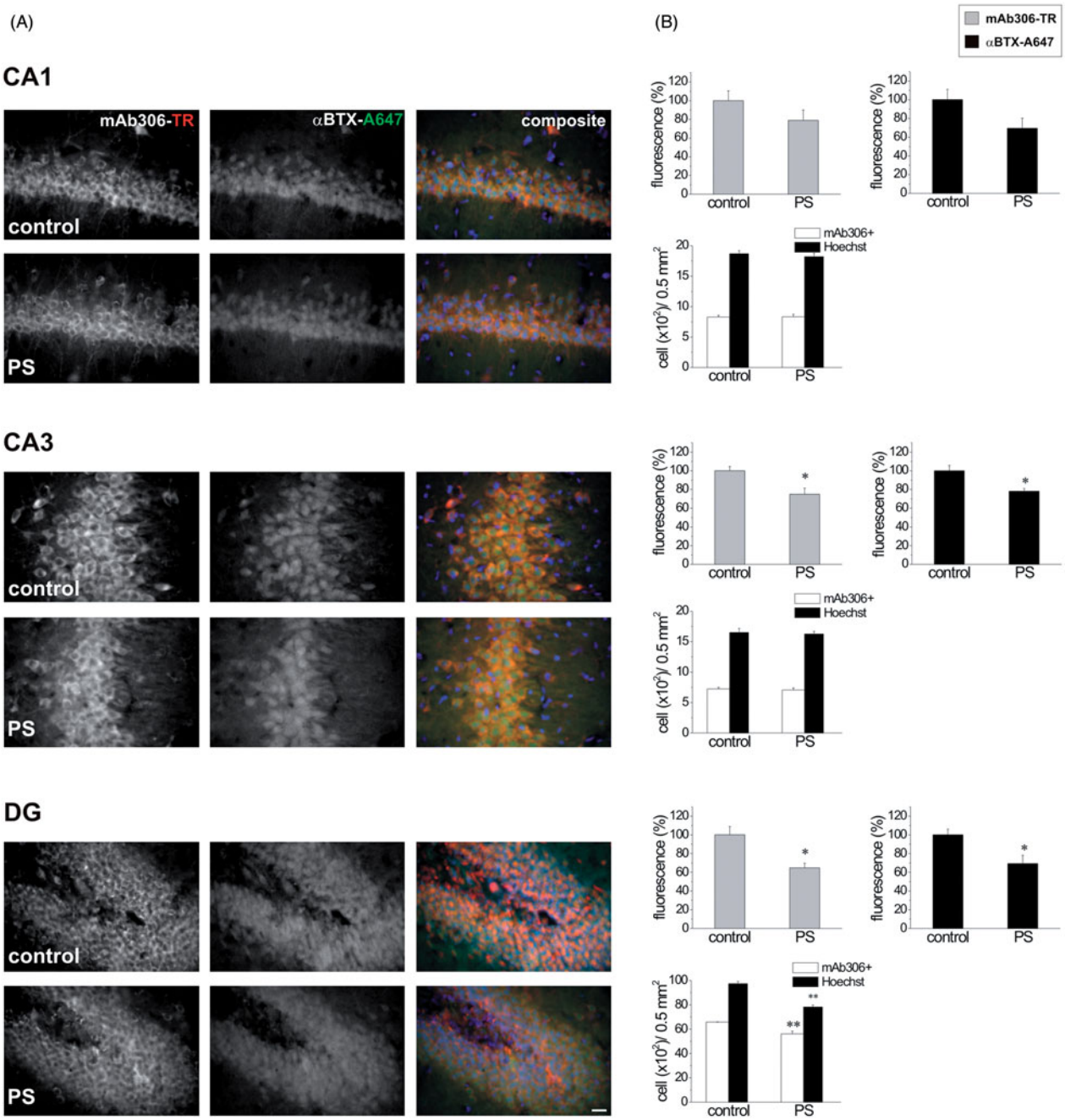

Figure 4. Expression of α7-AChR subunit in the hippocampus (HPC) of control and prenatally stressed (PS) male rat offspring. (A) Representative photomicrographs of coronal sections showing α7-AChR positive cells in the indicated HPC regions from control and prenatally stressed rats (PS) at postnatal day (PND) 60: CA1, CA3 areas of the HPC and dentate gyrus (DG). α7-AChR positive cells were labeled using monoclonal anti-α7-AChR subunit (mAb306) and Texas Red-goat anti-mouse IgG, *mAb306-TR*, left panel; and α-Bungarotoxin-Alexa^{647}, *αBTX-A647*, middle panel. Total cells were identified by Hoechst stain labeling. A co-localization of the three channels is showed in right panel, *composite*. Bar, 20 μm. (B) Quantitative image analysis of conditions described in A. Fluorescence intensities from mAb306-TR and αBTX-A647 were expressed as % of control. The relative number of α7-AChR expressing cells, mAb306 + and total cells, Hoechst-stained, were indicated by white and black bars, respectively. The number of cells obtained was normalized to a fixed area (0.5 mm^2). Values are reported as mean ± SEM. *, **denote $p < 0.05, < 0.01$, respectively, statistically significant different from control (undisturbed) rats. Student's t test, $n = 4$ rats per group.

PS and α4-AChR mRNA and protein expression in the FCtx and HPC from adult rat offspring

We also analyzed α4-AChR gene (*Chrn4*) and protein expression in the FCtx and HPC from control and PS rats.

qPCR

PS dramatically downregulated α4-AChR mRNA expression in the FCtx (Mann–Whitney test, $p = 0.008$, $n = 4$; Figure 5A). No significant difference was found for α4-AChR mRNA expression in the HPC ($n = 4$ rats per group; Figure 5A).

WB

α4-AChR protein levels in the FCtx or HPC determined by WB were not affected in PS offspring ($n = 4$ rats per group; Figure 5B and C).

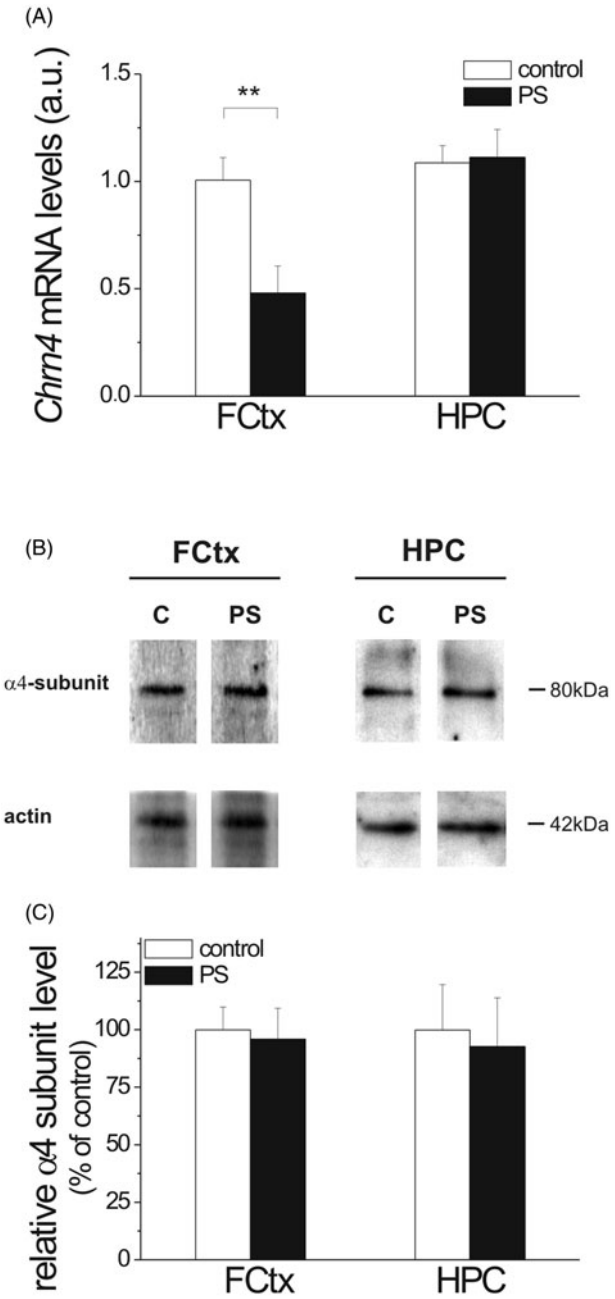

Figure 5. PS effects on the α4-AChR subunit expression in the rat frontal cortex (FCtx) from adult male rat offspring. (A) α4-AChR (*Chrn4*) mRNA levels in frontal cortex (FCtx) and Hippocampus (HPC) in control and prenatally stressed (PS) offspring at PND60. Relative mRNA expression levels were determined by the standard curve method. *Cyclophilin-a* was used as reference gene. Data are expressed as mean ± SEM; **denote $p < 0.01$. Mann–Whitney test, $n = 4$. (B) Western blot (WB) analysis from homogenates of FCtx and HPC from control (C) and prenatally stressed rats (PS). A band of ~80 KDa corresponding to the α4-AChR subunit was detected with monoclonal antibody mAb299 (Sigma). (C) Densitometric quantification and statistical analysis of the results shown in B. The optical density of each α4-AChR band was normalized to the corresponding actin band (used as load control). Data are expressed as mean ± SEM; $n = 4$ rats per group.

Quantitative immunofluorescence

In agreement with WB experiments, we did not find differences in the expression of α4-AChR in the DPFC, MPFC or SMC between undisturbed or PS rats (data not shown).

Discussion

In this study, we analyzed the consequences of PS on gene and protein expression of nAChRs in the brain of the adult male offspring. Our results demonstrate that prenatally exposure to maternal restraint stress reduced protein expression of α7-AChR in the FCtx and HPC in adulthood. In addition, only α4-AChR mRNA expression in the FCtx was affected in prenatally stressed rats. We demonstrated that PS has long lasting consequences in the pattern of nAChR expression in the brain of prenatally stressed rat offspring.

Exposure to different stressful events during the last week of pregnancy in rats interferes with correct progeny development, producing delays in motor development, impaired adaptation to stressful conditions, altered sexual behavior, learning deficits, anomalies in neuronal development and brain morphology and changes in cerebral asymmetry that persist through adulthood (Baier et al., 2012; Darnaudery & Maccari, 2008; Fride & Weinstock, 1989; Huizink et al., 2004; Weinstock, 2001, 2008).

As a consequence of maternal stress, it has been postulated that increased levels of stress hormones during pregnancy could cross the placenta and reach the fetus, promoting an increased fetal HPA axis activity and affecting brain development (Huizink et al., 2004; Lupien et al., 2009). Glucocorticoids act on glucocorticoid receptors which are expressed throughout the brain. These receptors can function as transcription factors and regulate gene expression. Thus, glucocorticoids may potentially have long-lasting effects over the function of various brain regions (Lupien et al., 2009). In addition, glucocorticoids may have non-genomic actions evidently mediated by binding to membrane proteins such as ligand-gated ion channels (Shi et al., 2002). The α7-AChR gene contains a corticosteroid-response element, the genomic target of glucocorticoid receptors (Hunter, 2012; Leonard et al., 2002). Moreover, it has been observed that glucocorticoids and chronic or prenatal stress affects α7-AChR gene expression (Carrasco-Serrano & Criado, 2004; Hunter et al., 2010; Neeley et al., 2011).

Accelerated age-related cognitive decline is related to an impaired response of the HPA axis (Pardon & Rattray, 2008) and prenatally stressed rats have an increased responsiveness of the HPA axis to stress events (Darnaudery & Maccari, 2008; Henry et al., 1994). Recent studies on humans have shown that PS or glucocorticoid treatments are associated with long-term impact on the HPA axis in human offspring (Davis et al., 2011; Entringer et al., 2009; O'connor et al., 2005; Van Den Bergh et al., 2008). Dysregulation of the HPA axis has been related to neurodegenerative diseases such as AD (Green et al., 2006) and in addition, it has been shown that stress increases the susceptibility to develop AD pathology (Buynitsky & Mostofsky, 2009; Pardon & Rattray, 2008). Evidence from studies performed in rats and monkeys (Coe et al., 2003; Lemaire et al., 2000) demonstrated that PS affects hippocampal neurogenesis, and in rats, PS accelerates the normal-age progressive decline in hippocampal cell proliferation (Lemaire et al., 2000). Also, an excess of glucocorticoid affects migration of post-mitotic neurons during the development of the cerebral cortex (Fukumoto et al., 2009).

Stress contributes to a number of brain disorders and various studies have shown the relationship between stress and AChRs (Hunter, 2012). For example, prenatal restraint stress enhanced hippocampal acetylcholine release in adult rats (Day et al., 1998). Also, chronic restraint stress (across 21 days) increased the level of α7-AChR mRNA but decreased α7-AChR protein level in the HPC of adult Sprague–Dawley rats (Hunter et al., 2010). Recently, Schulz et al. (2013) applying an unpredictable variable stress protocol to pregnant Sprague–Dawley rats found that PS increased the levels of α4β2-AChR subtype in the HPC of male and female offspring, reporting minor changes on the hippocampal α7-AChR subtype. It is important to highlight that several PS protocols exist in the literature which vary in type of stressor, daily frequency, length of application and week of gestation (Baier et al., 2012; Huizink et al., 2004). These factors, as well as the animal strain used, may contribute to the differences reported on the effects of PS (Charil et al., 2010; Neeley et al., 2011).

Employing the Wistar strain, this report demonstrated that prenatal restraint stress impaired protein expression of α7-AChR, and caused a trend towards downregulation in the expression of the corresponding gene, in the FCtx of the brain of the adult offspring. In addition, we found that in the MPFC and SMC areas of the FCtx of prenatally stressed rats, the relative number of α7-AChR-expressing cells was lower than in control rats. Of interest here, is that we recently showed, that chronic corticosterone treatment affects cell proliferation and cell differentiation through the modulation of α7-AChR expression and function in a neuronal cell line exhibiting a cholinergic phenotype (Baier et al., 2014). We can therefore hypothesize that these mechanisms could be operative in the developing brain of a fetus exposed to stress hormones: hence, higher corticosterone levels may interact with, and/or modulate the expression and functionality of nAChRs, finally affecting the characteristics or the fate of the affected neurons in the developing brain.

In the HPC, we did not find alterations in the expression of α7-AChR mRNA but changes were observed in α7-AChR protein levels in the CA3 and dentate gyrus areas, suggesting that in this brain territory, PS affected the post-translational regulation of α7-AChR. Interestingly, we found that in the dentate gyrus of rats exposed to PS, the relative number of α7-AChR-expressing cells and the relative total cell number were negatively affected. Learning and memory deficits, as well as hippocampal neurodegeneration and cholinergic system dysfunction, were more severe in APP transgenic mice (Tg2576, APP) lacking α7-AChR (A7KO–APP) (Hernandez et al., 2010).

Neurogenesis in the adult dentate gyrus is necessary for proper hippocampal function (Campbell et al., 2010). Corticosterone modulates adult neurogenesis in the HPC (Aztiria et al., 2007; Cameron & Gould, 1994) and increased levels of this glucocorticoid seem to inhibit neuronal differentiation in this cerebral area (Wong & Herbert, 2006). Moreover, PS affects the neurogenesis as well as the number of granule cells in the dentate gyrus of prenatally stressed rats (Lemaire et al., 2000). Campbell et al. (2010) showed that the α7-AChR is essential for correct hippocampal circuitry formation and function, adult neurogenesis and the integration of newer neurons in the dentate gyrus. Thus, lower expression levels of α7-AChR in the dentate gyrus due to PS could affect the downstream signaling pathway associated with this protein and could be responsible, at least in part, for the alterations described above.

AChRs containing the α4-AChR subunit seem to be differentially modulated by PS. PS decreased α4-AChR gene expression in the FCtx, but α4-AChR protein expression was not altered in this area. These data indicate that there might be a post-translational mechanism in the FCtx involved in or responsible for supporting normal levels of α4-AChR. This observation could be the result of a native compensatory mechanism for maintaining adequate AChR signaling (Putz et al., 2008).

Accumulating evidence suggests that the α7AChR plays a critical role in the pathophysiology of neuropsychiatric disorders, including schizophrenia and AD (Gotti & Clementi, 2004). Schizophrenic patients show alterations in HPA axis function (Corcoran et al., 2003) and post-mortem analysis shows a significant decrease in the level of α7AChR in the FCtx and HPC (Guan et al., 1999; Hunter, 2012; Martin & Freedman, 2007; Toyohara & Hashimoto, 2010). The hypothesis that schizophrenia has a neurodevelopmental origin is now the most widely accepted explanation for the pathophysiology of this disease (Koenig et al., 2002; Lewis & Levitt, 2002). At present, most models that explain the cause of schizophrenia propose interactive effects between multiple susceptibility genes and environmental factors (Lewis & Levitt, 2002; Schaaf, 2014). Some recent articles connected early life insults with AD-neuropathology in animal models. Stressing rats by maternal separation increases beta-secretase (BACE1) expression and amyloid-β levels in the adult offspring (Martisova et al., 2012). Furthermore, exercise during pregnancy protects against AD-like pathology and improved brain plasticity in TgCRND8 mice (Herring et al., 2012). Also, maternal stress during the first week of gestation in APPswe/PS1dE9 mice affects behavioral phenotype and AD-related neuropathology (Sierksma et al., 2013); and prenatally dexamethasone treatment sensitizes rats to subsequent injury by chlorpyrifos, enhancing the long-term deficits on ACh function (Slotkin et al., 2013).

In this study, we describe that rats exposed to PS show abnormalities in adulthood that concern the pattern of AChR expression in FCtx and the HPC. Considering that loss of cholinergic neurons and neuronal AChR are characteristic of some neurological diseases, we can hypothesize that prenatally stressed rats can be more susceptible to, or to develop early in its lifespan, disorders involving loss of AChR.

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Declaration of interest

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