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Glutamate neurotransmission is affected in prenatally stressed offspring

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ABSTRACT

Previous studies from our laboratory have shown that male adult offspring of stressed mothers exhibited higher levels of ionotropic and metabotropic glutamate receptors than control rats. These offspring also showed long-lasting astroglial hypertrophy and a reduced dendritic arborization with synaptic loss. Since metabolism of glutamate is dependent on interactions between neurons and surrounding astroglia, our results suggest that glutamate neurotransmitter pathways might be impaired in the brain of prenatally stressed rats. To study the effect of prenatal stress on the metabolism and neurotransmitter function of glutamate, pregnant rats were subjected to restrain stress during the last week of gestation. Brains of the adult offspring were used to assess glutamate metabolism, uptake and release as well as expression of glutamate receptors and transporters. While glutamate metabolism was not affected it was found that prenatal stress (PS) changed the expression of the transporters, thus, producing a higher level of vesicular vGluT-1 in the frontal cortex (FCx) and elevated levels of GLT1 protein and messenger RNA in the hippocampus (HPC) of adult male PS offspring. We also observed increased uptake capacity for glutamate in the FCx of PS male offspring while no such changes were observed in the HPC. The results show that changes mediated by PS on the adult glutamatergic system are brain region specific. Overall, PS produces long-term changes in the glutamatergic system modulating the expression of glutamate transporters and altering synaptic transmission of the adult brain.

1. Introduction

Glutamate (Glu) is the principal excitatory neurotransmitter in the mammalian central nervous system, participating in the integration of brain function and in synaptic plasticity, memory and learning processes. In glutamatergic neurons, de novo synthesis of neurotransmitter Glu has an obligatory requirement of neuron–glia interactions since its precursor glutamine (Gln) can only be synthesized in astrocytes due to the exclusive glial localization of the enzymes pyruvate carboxylase and glutamine synthetase. The former enzyme, pyruvate carboxylase, acts as an anaplerotic pathway for replenishment of tricarboxylic acid (TCA) cycle intermediates which is the prerequisite for production of Gln (Schousboe et al., 2013). This anaplerosis must be coupled to cataplerosis which is not fully understood (Sonnewald, 2014). Subsequent to transfer of Gln from astrocytes to the glutamatergic neurons, these cells convert Gln to Glu in the reaction catalyzed by phosphate activated

Abbreviations: FCx, frontal cortex; HPC, hippocampus; Glu, glutamate; TCA, tricarboxylic acid; PAG, phosphate activated glutaminase; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartate; KA, kainate; GDH, glutamate dehydrogenase; LC-MS, liquid chromatography–mass spectrometry; C, control; PS, prenatal stress; PND, postnatal day; RT-qPCR, real time reverse transcription polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; GPV, glial plasmalemmal vesicles; SYN, synaptosomes; TBOA, threo-β-benzyloxyaspartate; DHT, dihydrokainate; vGluTs, vesicular glutamate transporters; QAR, quantitative receptor autoradiography.

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glutaminase (PAG) in the neuronal mitochondria (Schousboe et al., 2013). When Glu is released to the synaptic cleft, it binds to postsynaptic ionotropic receptors, i.e. those activated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), *N*-methyl-D-aspartate (NMDA) and kainate (KA), and to metabotropic receptors (mGluR 1–8), thus, mediating and modulating synaptic transmission. Subsequently, Glu diffuses in the synaptic cleft and it is internalized by excitatory amino acid transporters (EAATs), primarily localized in surrounding astrocytes (Danbolt, 2001; Gegelashvili and Schousboe, 1997; Zhou and Danbolt, 2013). In the astrocytes, Glu is transformed to Gln through the activity of the enzyme glutamine-synthetase or oxidatively metabolized via conversion to α-ketoglutarate in the glutamate dehydrogenase (GDH) reaction (Schousboe et al., 2013). The trafficking of these amino acids between neurons and glia is named the "glutamine–glutamate cycle" which plays a major role in the maintenance of the Glu pools in cells, in particular the neuronal neurotransmitter pool (Waagepetersen et al., 2005). The significance of this cycle is to secure a rapid removal of Glu from the synaptic space by astrocytic uptake, subsequent replenishment of the neurotransmitter pool in the glutamatergic vesicles and to some extent provision to neurons of a metabolic substrate, i.e., Gln can be a potential fuel (Daikhin and Yudkoff, 2000; Popoli and Pepponi, 2012).

Among glutamate receptors, those activated by NMDA and AMPA play critical roles in associative memory and remodeling of synapses in the hippocampus (HPC) and frontal cortex (FCx). Disruption of these receptors is known to cause cognitive deficits and learning impairments (Lewis, 1997). To date, variations of AMPA and NMDA receptor levels and function have been found in mood disorders (Chen et al., 2010). In addition to this, subunit dysfunction has also been found in neurodegenerative conditions and neuropsychiatric disorders, such as schizophrenia (Coyle et al., 2003). Another major point of regulation for glutamatergic neurotransmission is the EAATs. These transporters show a regional cell type-specific pattern of expression. There are five different types of EAATs: EAAC1, EAAT4 and EAAT5 are predominantly expressed in neuronal extra-synaptic locations while glutamate transporter 1 (GLT-1) and glutamate/aspartate transporter (GLAST) are expressed in glia (Danbolt, 2001). Neuronal transporters appear to contribute less significantly to glutamate uptake than the glial transporters (Gegelashvili et al., 2000). Both, protein levels and kinetics of these transporters are regulated by glutamate receptors, glutamate release and other factors related to glutamatergic neurotransmission (Gegelashvili and Schousboe, 1997). Another well described transporter for Glu is the vesicular glutamate transporter (vGluT). This carrier mediates Glu uptake into synaptic vesicles at the glutamatergic neuronal terminals (Herzog et al., 2006). There are three isoforms of vGluT (1, 2 and 3) that differ in their expression profiles. In the adult brain, vGluT-1 predominates in cerebral cortex, cerebellum and hippocampus and vGlut-2 is present in diencephalon, brainstem and spinal cord. vGluT-3 is the less expressed isoform and is found in non glutamatergic neurons like GABAergic or cholinergic ones. The presence of vGluT in neurons has been taken as a phenotypic trait of glutamatergic neurons with a fundamental role in glutamatergic transmission (Wojcik et al., 2004).

Central nervous system responses to stress are often dependent on the individual's adaptation to her/his environment. In adults, such responses are greatly influenced by previous experiences to stress, especially during the prenatal period (Charil et al., 2010; Finlay and Zigmond, 1997). In this sense, fluctuations in the uterine environment might be transmitted to the fetuses, inducing long term modifications in the structure and function of diverse tissues, increasing the risk of developing diseases in adult life (Cottrell and Seckl, 2009). It was reported that rats exposed to different types of stress during pregnancy produce offspring that show increased vulnerability to anxiety, depression, drug seeking behavior and learning deficits (Maccari and Morley-Fletcher, 2007; Weinstock, 2008). Moreover, phenotypes resembling schizophrenia, hypersensitivity to amphetamine, disrupted social behavior, impaired stress axis regulation and aberrant prefrontal expression of genes involved in synaptic plasticity were also reported (Koenig et al., 2005; Lemaire et al., 2000). The neurochemical basis of many of these disorders has been linked to impairments of the dopaminergic system (Baier et al., 2012), as well as to the serotonergic and noradrenergic systems (Suzuki et al., 2010). In addition, during the last years new evidence has emerged showing that maternal stress leads to malfunction of the glutamatergic system. Yaka et al. (2007) found a synaptic reduction in the GluR1 subunit of the AMPA receptor in the hippocampus of prenatally stressed rats. Similar results were reported in the same area with impaired function of NMDA receptors and reduced long term potentiation (Markham et al., 2010; Son et al., 2006). In prefrontal cortex, prenatal stress seems to affect the modulation of glutamatergic response and NMDA receptor subunits (Fumagalli et al., 2009).

Previous studies from our laboratory have shown that adult offspring of stressed rats exhibited higher levels of ionotropic (NMDA) and metabotropic (mGlu III) glutamate receptors in frontal cortex, striatum and hippocampus compared to control rats (Berger et al., 2002). These animals also show long-lasting astroglial reaction and a reduced dendritic arborization with synaptic loss (Barros et al., 2006a). Since glutamatergic functions are based on a tight relation between neurons and surrounding astroglia, our results suggest that the glutamate neurotransmitter pathway might be impaired in the brain of prenatally stressed offspring. With this hypothesis in mind we addressed a broad analysis of the effect of maternal stress on the glutamatergic synapse of the offspring covering the main steps of the neurotransmitter metabolism. We evaluated glutamate metabolism using [1-^{13}C]glucose injection combined with liquid chromatography–mass spectrometry (LC–MS) analysis of brain extracts, as well as glutamate release and the expression of NMDA and AMPA receptors. We also evaluated the expression and function of the transporters vGluT-1, GLT1 and GLAST in adult male and female offspring of prenatally stressed rats in two brain areas: FCx and HPC. Main findings point out that the expression of the transporters is changed by PS, showing a higher level of vesicular vGluT-1 in the frontal cortex (FCx) and elevated levels of GLT1 protein and messenger RNA in the hippocampus of adult male PS offspring. We provide novel data about the long-lasting consequences of prenatal stress on one of the major neurotransmission systems of the brain.

2. Materials and methods

2.1. Animal care

Virgin female Wistar rats weighing 250 g were obtained from outbred rats from the animal facility at the University of Buenos Aires. A maximum of five dams were housed per cage with ad libitum access to standard rat chow (Asociación de Cooperativas Argentinas, Buenos Aires, Argentina) and water. A constant light/dark cycle, with lights on at 06:00 and off at 18:00, and a temperature of 21–25 °C were maintained. Females were individually mated with a sexually experienced male Wistar rat. The day on which vaginal plug was found was designated as the first day of pregnancy. Care was taken to minimize the number of animals used. 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 were approved by the Institutional Animal Care and Use Committee (Facultad de Medicina, Universidad de Buenos Aires).

2.2. Prenatal stress

Pregnant dams (n = 8) were randomly assigned to either the control (C) or the prenatal stress (PS) group and were individually

housed with ad libitum access to standard rat chow and water. A constant light/dark cycle (on at 06:00 h, off at 18:00 h) was maintained at a temperature of 21–25 °C. C rats (n = 4) were left undisturbed in the home cage, while PS dams (n = 4) 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 minutes (min) periods per day (09:00, 12:00 and 16:00 h) between days 14 and 21 of pregnancy. The restrainer had ventilation holes, and dimensions appropriate for a pregnant rat of 350 g: internal diameter 64 mm, and 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 and Weisz, 1984). The sessions were performed in a lit environment. No other subjects were present in the experimental room during the stress exposure. At the end of the stress session, the rats were returned to the animal housing room and were then individually housed with free access to food and water.

On the day of parturition, litter characteristics were recorded and litters were culled to 10 pups, maintaining similar number of males and females, when possible. Weaning was performed at postnatal day (PND) 21. The male and female offspring were housed in separated cages, with no more than 5 rats per cage, and in standard housing conditions. To avoid litter effects, two pups from each litter (a female and a male) were tested for each experiment.

2.3. Liquid chromatography–mass spectrometry (LC–MS)

Male and female offspring of PND 60 were intraperitoneally injected with a [1-^{13}C] glucose solution (99%, pH 7). After 15 min, the animals were decapitated and the head was completely immersed in liquid nitrogen and stored at –80 °C. On the day of experiments the brain was removed from the skull and FCx and HPC were quickly dissected and homogenized with perchloric acid (7%). Homogenates were centrifuged at 4000 *g* for 5 min. The supernatant was saved and the pellet was suspended in distilled water and centrifuged under the same conditions. The two supernatant fractions were mixed and neutralized at pH 6.5–7.5 and freeze-dried. Brain extracts were suspended in water for subsequent analysis. Relevant amino acids were separated and ^{13}C mono-labeling were quantified using the Phenomenex EZ-fast amino acid analysis kit for LC–MS in combination with a Shimadzu LCMS-2010 mass spectrometer coupled to a Shimadzu 10A VP HPLC system. The percentage labeling was calculated as detailed by Walls et al. (2014).

2.4. Glutamate release

Superfusion assay was performed as described in Cabrera and Navarro (1996) with some modifications. Briefly, adult male and female rats of C and PS treatment (n = 4) were killed by decapitation and the FCx and HPC were dissected. Coronal slices 240 μm thick were obtained with a McIlwain tissue chopper. Six to eight slices were incubated in Dubnoff shaker at 37 °C for 10 minutes in Krebs–Ringer bicarbonate-glucose buffer (KRB: NaCl 0.126 M, KCl 2.5 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, Na_2SO_4 1.2 mM, $CaCl_2$ 2.5 mM, glucose 0.1% m/v, ascorbic acid 0.06 mM, pH 7.4 saturated with O_2/CO_2, 95/5%). Thereafter, it was incubated in KRB containing [^{3}H]glutamic acid 0.1% v/v (specific activity: 49.6 Ci/mmol) for 30 min. After the incubation period the tissue was rinsed with fresh KRB, transferred to superfusion chambers and stabilized for 30 minutes with KRB at a flow rate of 0.7 ml/min. Each experiment was started by superfusing fresh buffer KRB in all the chambers. Five successive fractions were collected every 2.5 minutes (basal [^{3}H]-glutamate release fractions). Thereafter, tissue was exposed to 56 mM KCl in KRB and four new fractions were collected every 2.5 minutes (stimulated release fractions). After this collection, superfusion buffer was replaced by regular KRB and three more post stimuli fractions were collected. At the end of the experiments, the tissue was homogenized in 2 ml of perchloric acid (0.2 N) and centrifuged at 4000 *g* for 5 min, 1 ml of the clear supernatant and 1 ml of each fraction were mixed with 3 ml of scintillation fluid (Toluene-PPO-POPOP-Triton X-100 cocktail) and radioactivity was counted on a liquid scintillation counter – β Wallac. Total radioactivity was calculated as the sum of total [^{3}H]Glu collected during superfusion plus the [^{3}H]Glu radioactivity remaining in the tissue. To quantify the effects of stimulation (KCl 56 mM), the radioactivity measured in stimulated fractions was expressed as percent of total [^{3}H]Glu released. Hence, stimulated release is the percentage of increase in [^{3}H]Glu values during stimulation with respect to the percentage of basal release values. The tritiated glutamate may be metabolized which could influence the measurement of its release. Most metabolites except aspartate and GABA will not be released during depolarization and therefore the conclusions regarding stimulated release of excitatory transmitters may only be influenced by release of GABA which is minor compared to that of glutamate.

2.5. Tissue collection

At PND 60, 8 rats from different litters belonging to C or PS group (n = 4) were rapidly euthanized by decapitation. Brains were removed from the skull for further dissection. FCx and HPC were rapidly extracted on ice according to anatomical landmarks corresponding to Paxinos and Watson (1986) rat brain atlas. Brain sections were weighted and differentially processed for quantitative real time reverse transcription polymerase chain reaction (RT-qPCR) analysis or for Western blot.

2.6. mRNA isolation and RT-qPCR

FCx and HPC from PND 60 rats belonging to C or PS groups were immediately homogenized in TRIzolTM reagent (Life Technologies, Carlsbad, CA) to isolate total RNA, according to the manufacturer's instructions. **PolyA (+)** mRNA was purified using the PolyA-Tract mRNA Isolation System (Promega, Madison, WI). Complementary DNA was synthesized using oligodT and SuperScriptTM II Reverse Transcriptase (Life Technologies, Carlsbad, CA). qPCRs were carried out in a **7500** Real-Time PCR System (Applied Biosystems, Life Technologies, Foster City, CA). Quantification of each cDNA was achieved using SYBR Green Master Mix Reagent (Applied Biosystems) in triplicate. Primer sequences **used** are: GLT1 (*Slc1a3*), Fwd: GGTCAATGTAGTGGGCGATTCT, Rev: CTGCGTCTTGGTCATTTCGA; GLAST (*Slc1a6*), Fwd: AATAGTCATTTAATGGCAGGCTCA, Rev: GCG TTAATTTGACGCGGTTAAG; NMDAR1 (*Grin1*), Fwd: GCATCGT AGCTGGGATTTTCC, Rev: TGTGTTCCTACGGGCATCCT and GluR4 (*Grin4*), Fwd: CGTGTGCTGACCCCTGACT, Rev: TCCCGAACTTTGTCTAA TTGCA. Normalization was accomplished employing ***cyclophilin-a*** as housekeeping gene (Fwd: CATTCAGTCTTGGCAGTGCAG, Rev: AAGCATACAGGTCCTGGCATCT). qPCR was analyzed by using the standard curve method. Before each experiment, the calibration curves were validated. Samples with Ct values out of the calibrated dynamic range were eliminated. All procedures followed the manufacturer's instructions. Values shown are relative to the control animals.

2.7. Western blotting

FCx and HPC extracts were homogenized in 5 ml/g (per gram of tissue) of ice-cold RIPA lyses buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycolate, 0.1% SDS, 1% protease inhibitors). Following centrifugation of the homogenate for 15 min at 16,000 *g* at 4°C, the protein was extracted from the supernatant and quantified by the Bradford method (Bradford,

1976). Equal amounts of protein (20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) 10% acrylamide (Laemmli, 1970) under reducing conditions and then blotted onto polyvinylidene fluoride (PVDF) membrane (Immobilon-P; Millipore, Watford, United Kingdom). The blot was blocked with a 5% non-fat dry milk in TBS-T (10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 120 min and subsequently incubated overnight at 4 °C with the appropriate antiserum diluted in blocking solution (rabbit anti GLT-1 1/2500, anti GLAST 1/2000, a kind gift from Dr. Niels Danbolt, University of Oslo, Norway; mouse anti actin 1/5000, Chemicon Millipore MAB1501R). After washing in TBS-T, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody 1/5000 (Danko, P0447 and P0217) in blocking solution for 1 hour (h). The bands were visualized by enhanced chemiluminescence (premix Luminata Crescendo Millipore, WBLUR0500) in a dark box coupled to a digital camera (Leica D-Lux3).

The same procedure was employed to evaluate expression of glutamate receptors of the NMDA and AMPA subtypes. For this purpose 50 μg of tissue proteins was separated in the SDS–PAGE. The primary antibodies used were: rabbit anti NR1ζ (sc-9058, Santa Cruz), diluted 1/100 and rabbit anti GluR4 (8070, Cell Signaling), 1/500. The bands were visualized by enhanced chemiluminescence (ECL plus, Millipore) in a Storm imaging system (GE HealthCare).

The optical density (OD) of the immunopositive bands was measured by the software Imagen Imaje J (Media Cybernetics, Bethesda, Maryland, USA). Each value of the protein expression was normalized to the β-actin values and was express as the relative optical density (ROD).

2.8. Fixation and tissue processing

At PND 60, 4 control and 4 stressed rats were deeply anesthetized with xylazine/ketamine hydrochloride solution (Mallinckrodt, 10 and 75 mg/kg, respectively). They were perfused through the cardiac left ventricle, initially with a cold saline solution containing 0.05% w/v $NaNO_2$ plus 50 IU of heparin and subsequently with a cold fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PBS), pH 7.4. Brains were removed, post-fixed in the same cold fixative solution for 4 h, subsequently cryoprotected by overnight immersion in 15 and 30% w/v sucrose and stored at –80 °C until processing for immunohistochemistry. Ten series of coronal brain sections (25 μm thick) for FCx and ten series for HPC were cut on a Leica cryostat. The sections were stored at –20 °C in PBS, pH 7.4, with 50% w/v glycerol added as a cryoprotector until their use in immunocytochemical studies.

2.9. Immunohistochemistry

Brain sections of both C and PS rats (n = 4) were selected according to anatomical landmarks corresponding to the Paxinos and Watson (1986) rat brain atlas: plates 4–13 for FCx and plates 27–45 for the HPC. The sections were simultaneously processed in the free-floating state. To inhibit endogenous peroxidase activity, tissue sections were previously treated with 0.5% v/v H_2O_2 in methanol for 30 min at room temperature. The sections were treated for 1 h with 3% v/v normal goat serum in PBS to block nonspecific binding sites. After two rinses in PBS plus 0.025% v/v Triton X-100 (PBS-X), sections were incubated for overnight at 4 °C with primary antibodies to vGluT1 (1:2000, Synaptic System 135 303) in 5% normal goat serum in PBS. After five rinses in PBS-X, sections were incubated for 1 h at room temperature with biotinylated secondary antibody antirabbit IgG (1/250, Sigma-Aldrich B7389). After further washing in PBS-X, sections were incubated for 1 h with streptavidin–peroxidase complex diluted 1:200. Sections were then washed five times in PBS and twice in 0.1 M acetate buffer, pH 6 (AcB) and development of peroxidase activity was carried out with 0.035% w/v 3,3-diaminobenzidine hydrochloride (DAB) and 0.1% v/v H_2O_2 dissolved in AcB. After the enzymatic reaction step, sections were washed three times in AcB and once in distilled water. Finally, sections were mounted on gelatine coated slides, air dried, and coverslipped using Permount for light microscopic observation.

2.10. Image analysis

To ensure objectivity, all measurements were performed on coded slides, under blind conditions, for every immunolabeled brain sections of both C and PS rats with the same standardized observation schedule. Five serial tissue sections were randomly selected for each area and for each animal. All the observations and images were done under a light microscope (Olympus BX50) with a digital camera (CoolSnap). The images were digitized and processed with the software Imaje Pro plus (version 5.1) to evaluate the OD of the immunolabeled. The OD measures were relativized to the OD values of a control section of the image without immunolabel.

2.11. Glial plasmalemmal vesicle and synaptosome preparation

Glial plasmalemmal vesicles (GPV) and synaptosomes (SYN) were prepared by the method previously described by Nakamura et al. (1993) modified by Dunkley et al. (2008). Briefly, at PND 60 rats were killed by decapitation (n = 4 for each group), their brains quickly removed and the FCx and the HPC were dissected out within a few minutes. Tissue was homogenized with 0.32 M sucrose containing 1 mM EDTA (pH 7.4) (1 ml/1 g) using a Teflon-glass homogenizer, then centrifuged at 1000 *g* for 10 min. Two milliliters of supernatant was layered onto a 4-step Percoll gradient composed by 2 ml of 20, 10, 6 and 2% Percoll (Sigma-Aldrich) in a buffer solution (0.32 M sucrose, 1 mM EDTA, 0.25 mM DTT and 20 mM HEPES, pH 7.4). The Percoll gradients were centrifuged at 33,500 *g* for 5 min. The layer between 2 and 6% Percoll was collected and designated as GPV fraction and the layer between 10 and 20% Percoll was collected and designated as SYN fraction. Both fractions were diluted to 80 ml with sucrose solution and centrifuged at 20,000 *g* for 30 min. The pellets were suspended in reaction medium (125 mM NaCl, 3.5 mM KCl, 1.5 mM $CaCL_2$, 1.2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM HEPES and 10 mM D-glucose, pH 7.4) and used in uptake experiments.

Uptake experiments were carried out using fresh GPV and SYN fractions. The reaction was started by adding 100 μl of sample (about 1 mg/ml) to tubes with 400 μl of reaction medium. The tubes were incubated with 10 nM of L-[3,4-^{3}H]glutamic acid (specific activity: 49.6 Ci/mmol. NET-490, Perkin Elmer) for 3 min at 30 °C (Takarada et al., 2003). It may be argued that the tritiated glutamate can be metabolized but it should be kept in mind that uptake is a prerequisite for metabolism. Hence, measurement of radioactivity will reflect uptake. In other tubes 100 μM of threo-β-benzyloxyaspartate (TBOA, Tocris, Bioscience) or 100 μM of dihydrokainate (DHT, Sigma-Aldrich) was added to the reaction. The incubation was terminated by vacuum-filtration through G/FB Whatman glass fiber-filters and rapid washing, 3 times, with physiological buffer (at 4 °C). Nonspecific uptake was evaluated by parallel incubations of GPV and SYN fraction in the same solutions at 0 °C. The radioactivity on the filters was measured using a liquid scintillation counter – β Wallac. Protein content was estimated using bovine serum albumin as standard (Lowry et al., 1951).

2.12. Statistical analysis

Statistical differences were analyzed using one way ANOVA to detect differences in metabolic determinations (LC–MS) and in the

uptake with inhibitor experiments. For the Western blot, RT-qPCR, immunohistochemistry, glutamate release and glutamate uptake assays results were analyzed by Student's two-tailed t-test. All results are presented as mean ± SEM. The observed differences were considered to be statistically significant when $p < 0.05$. Analysis of data was performed by using InfoStat 2008 and Excel 2008.

3. Results

3.1. Labeling of amino acids in Fcx and HPC of PS offspring

The labeling of Glu, Gln and GABA obtained after injection of [1-^{13}C]glucose was evaluated in FCx and HPC of C and PS offspring, both in males and females (Fig. 1). No statistical differences were found for ^{13}C enrichment (%) regarding mono, double and triple ^{13}C labeled Glu, Gln or GABA between C and PS offspring in neither areas nor gender. Only results for mono labeled metabolites are shown in Fig. 1. Glu metabolism was also examined using [1-^{13}C]glucose and [1,2-^{13}C]acetate injection followed by magnetic resonance spectroscopy analyses of brain extracts from female offspring showing no differences from controls (data not shown).

3.2. Glutamate-stimulated release

Glutamate release was measured by superfusion using FCx and HPC slices obtained from C and PS offspring, both males and females. Levels of [^{3}H]glutamic acid released are presented in Fig. 2. Insets show a representative profile of glutamate released and the arrow indicates the addition of K^{+}. In spite of the apparent increase in FCx of PS offspring ($t = -2.43$; $p = 0.06$), no significant differences were found in any of the situations analyzed.

3.3. Evaluation of glutamate receptors levels

The mRNA expression of the NR1 subunit of the NMDA receptor and the GluR4 subunit of the AMPA receptor were evaluated by RT-PCR in FCx and HPC of male and female PS offspring. No significant differences were found for male rats (Fig. 3A). On the other hand, PS female offspring show a significant increase of NR1 mRNA ($t = -4.71$; $p = 0.002$) (Fig. 3B). The protein expression of NR1 and GluR4 was evaluated by Western Blot in the same subjects (Fig. 4). Although the differences were not statistically significant, NR1 subunit in the FCx of female PS offspring shows an increase (Fig. 4D), matching the increase in mRNA levels of the same area and gender

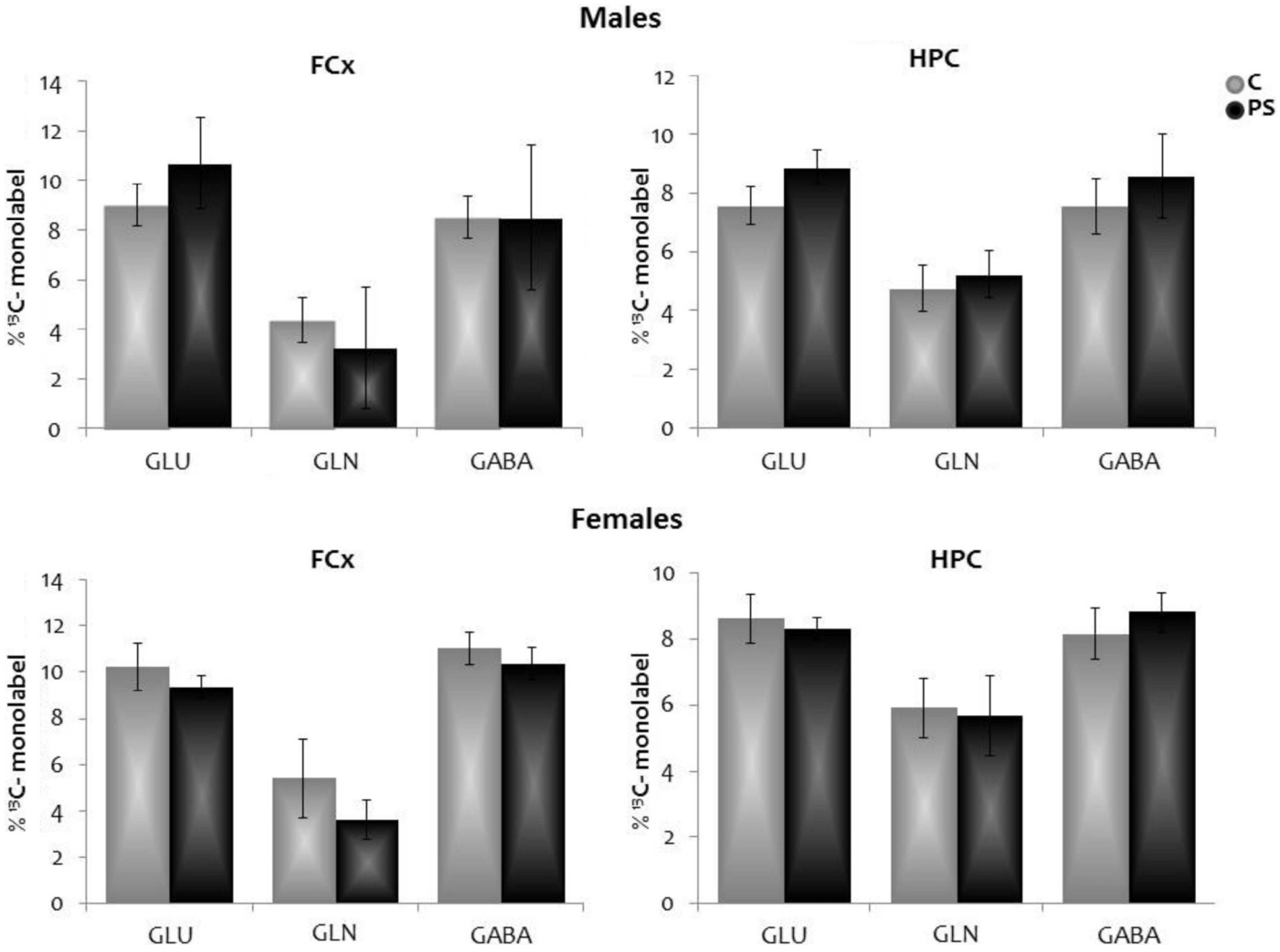

Fig. 1. Percentage of monolabeling (of total ^{13}C label) for glutamate (Glu), glutamine (Gln) and GABA, in FCx and HPC of male and female adult offspring (PND 60). Values are shown as mean ± SEM (n = 4).

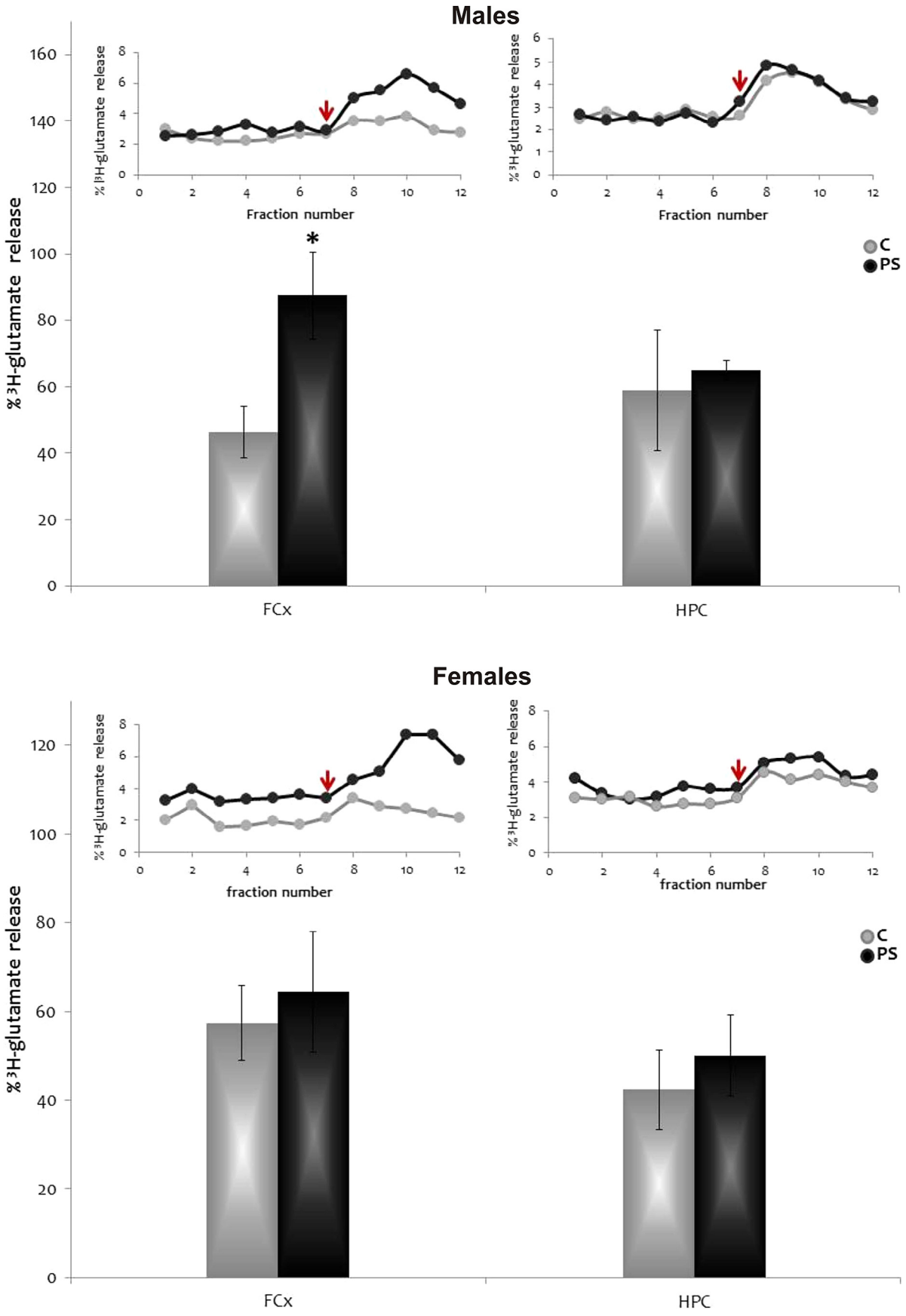

Fig. 2. Release of [^{3}H]glutamate in FCx and HPC slices of C and PS male and female offspring. The insets show a representative profile of [^{3}H]-glutamate release from the slices superfused with Mg^{2+} free Krebs–Ringer bicarbonate-glucose buffer and exposed to K^+ (56 mM). K^+ was superfused for 10 min from fraction 7 (arrow) to fraction 10. The evoked release is the percentage increase after stimulation with respect to the basal release of efflux. Values are means ± SEM (n = 4).

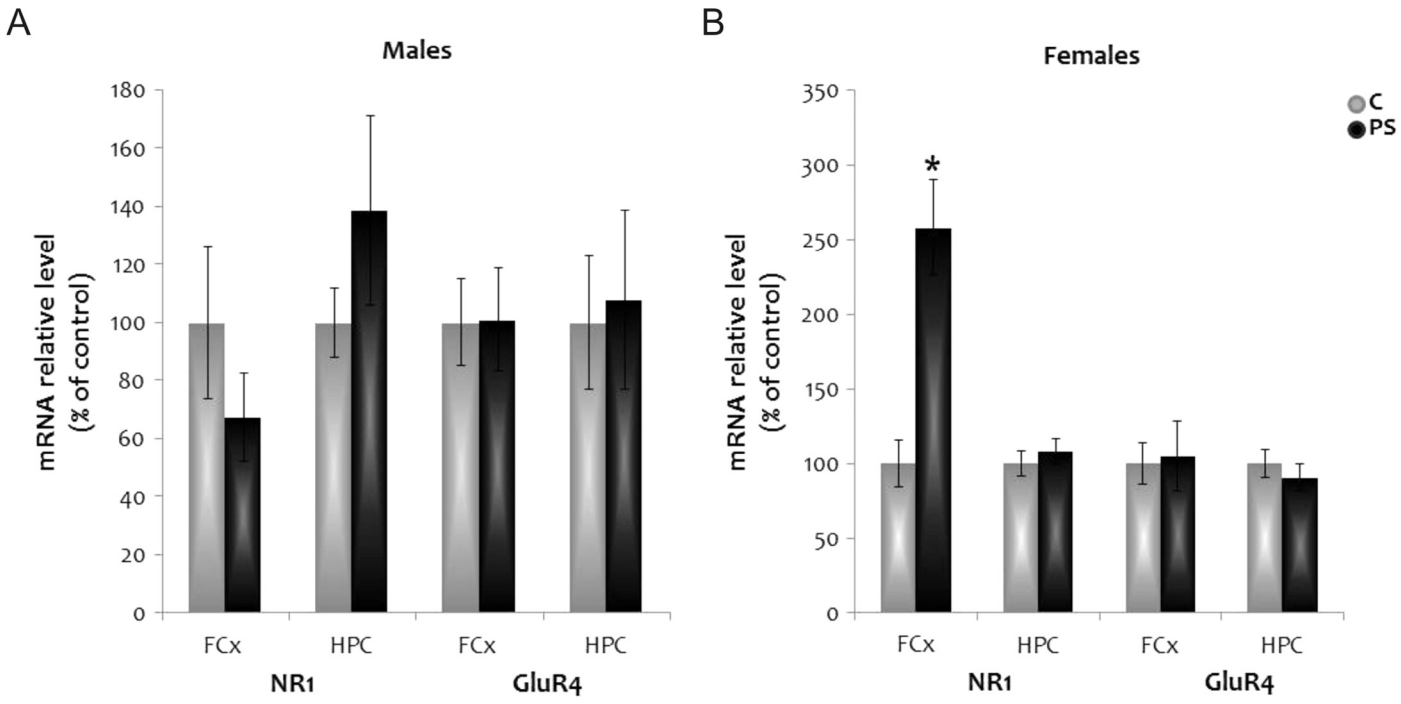

Fig. 3. qRT-PCR analysis for mRNA expression of glutamate receptors subunits in PS and C adult rats. Detection of NR1 and GluR4 subunits was performed in FCx and HPC, for male and female offspring. Histograms show mRNA values normalized to cyclophilin mRNA amount and expressed as percentage of control values. Results are the mean ± SEM ($n = 4$). *Significant difference between C and PS ($p < 0.05$).

(Fig. 3B). No differences were found in any of the other experimental situations analyzed (Fig. 4).

3.4. Measurement of vesicular glutamate transporter level

In order to determine the vesicular transporter expression in C and PS rats we quantified the levels of vGluT-1 protein by immunohistochemistry in FCx and HPC of male and female offspring. PS males show an increase of 36.5% with respect to C subjects in FCx ($t = -3.02$; $p = 0.02$) (Fig. 5E). No variations were observed in males HPC (Fig. 5E) or in the female FCx and HPC (Fig. 5F). To perform a positive control of the localization of the antibody against vGluT-1, we examined the extent of co-localization with synaptophysin antibody (Fig. 5G). The immunofluorescence analysis showed a positive merge of the markers, synaptophysin and vGluT-1, for every evaluated situation.

3.5. Glial glutamate transporters measured in FCx and HPC

The protein and mRNA expression of the main astroglial glutamate transporters (GLT1 and GLAST) was determined within the FCx and HPC in C and PS offspring. The genomic expression of the two glutamate transporters (GLT1 and GLAST) in C and PS rats were assessed by RT-PCR assay (Fig. 6). We observed PCR products for all the samples which suggested the presence of glutamate transporters in the evaluated brain areas. No differences between C and PS were found for both transporters in FCx in males (Fig. 6A). Glutamate transporter GLT1 is overexpressed in PS males HPC by 90% over control value ($t = -3.2$; $p = 0.02$) but no differences were found for GLAST in HPC (Fig. 6A). The expression levels of both transporters were also evaluated in female brain areas and the statistical analysis did not reveal any difference between C and PS rats (Fig. 6B).

Protein levels were detected by Western Blot assays. Statistical analysis of the results indicated that the male PS offspring present an increased level of GLT1 transporter protein, in the HPC of 30% ($t = -3.53$; $p = 0.03$) when compared to control levels (Fig. 7B). No changes were found in male FCx (Fig. 7B) or in female FCx and HPC areas (Fig. 7D). GLAST transporter showed no significant statistical difference between C and PS offspring in any area or gender but a minor increase was detected in GLAST hippocampal levels of PS males ($t = -2.22$; $p = 0.08$) (Fig. 7B).

3.6. Functional evaluation of glial glutamate transporters – glutamate uptake

To evaluate the effect of prenatal stress on the functionality of glutamate transport we measured the uptake of [^{3}H]-glutamate in GPV and SYN fractions from rat frontal cortex and hippocampus in male and female offspring (Fig. 8). In both areas and genders, the level of transport in control samples was higher in GPV than in SYN. In male PS offspring, we observed an increased level of [^{3}H]-glutamate uptake in FCx from GPV ($t = -2.39$; $p = 0.04$) (Fig. 8A). No statistical differences were found in other male experimental situation or in the female offspring.

In order to test the purity of the fractions we assayed control GPV and SYN fractions in the presence of TBOA, a potent blocker of excitatory amino acid transporters and DHK, a selective inhibitor of GLT-1 subtype of glutamate transporter. The figure shows that, as expected, TBOA reduced the uptake activity of GPV fractions by 90% in FCx and 70% in HPC showing that [^{3}H]glutamate uptake is mostly due to the activity of glutamate transporters (Fig. 9). However, DHK reduced the activity of SYN fractions by 80% indicating that most of the uptake is due to GLT1 transporter, most probably reflecting astroglial contamination of the synaptic fractions (see Danbolt, 2001).

4. Discussion

Previous studies from our laboratory have shown that adult offspring of stressed rats exhibited higher levels of ionotropic and

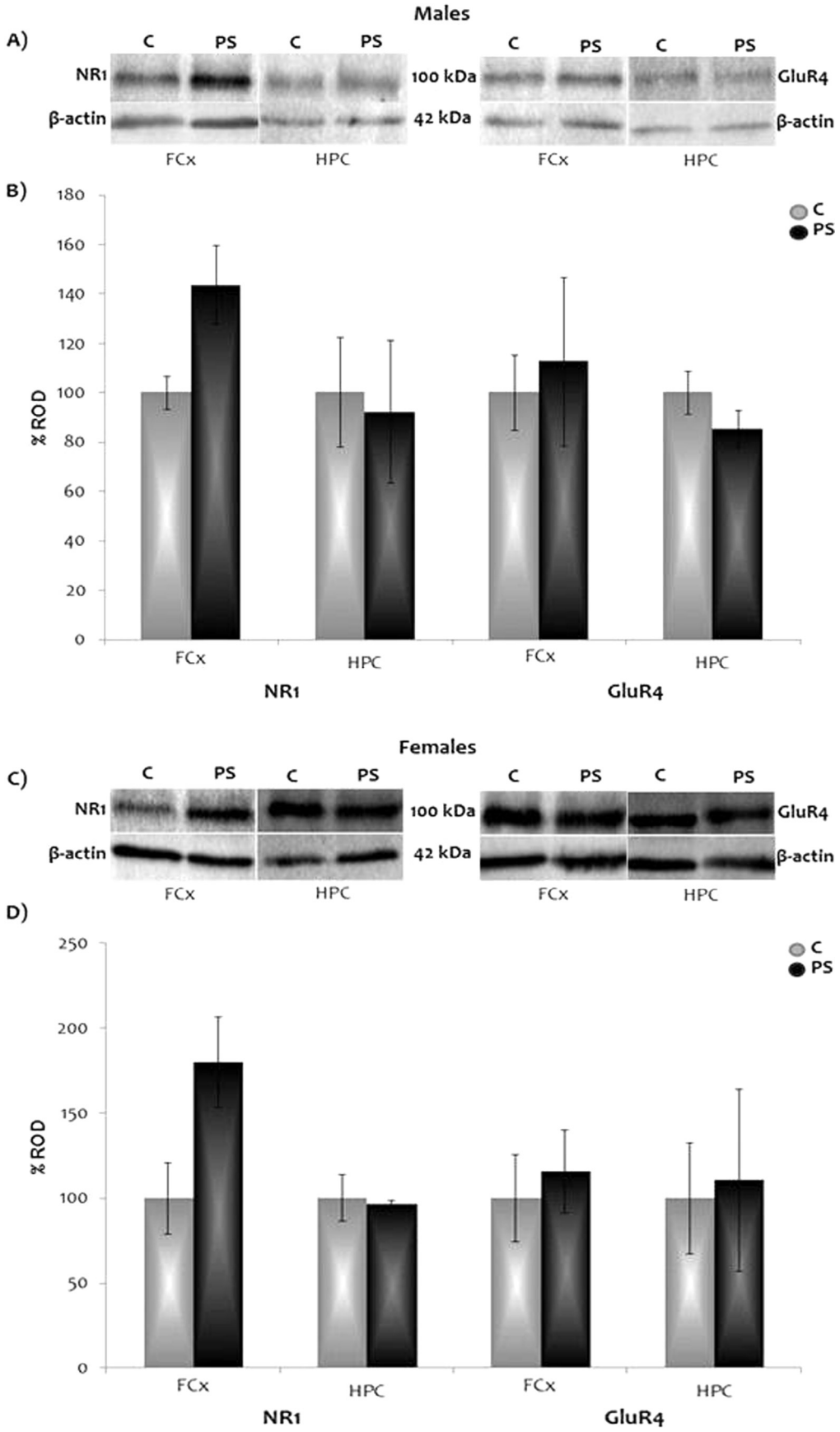

Fig. 4. Protein expression of NR1 and GluR4 glutamate receptors subunits, in the FCx and HPC, of male and female offspring. Representative Western blot images show immunopositive bands for anti-NR1 and anti-GluR4 antibodies and their corresponding β-actin standard bands in males (A) and females (C). Histograms indicate densitometric analysis, reported as the relative optic density (ROD) of control immunolabel, for males (B) and females (D). Values are means ± SEM (n = 4).

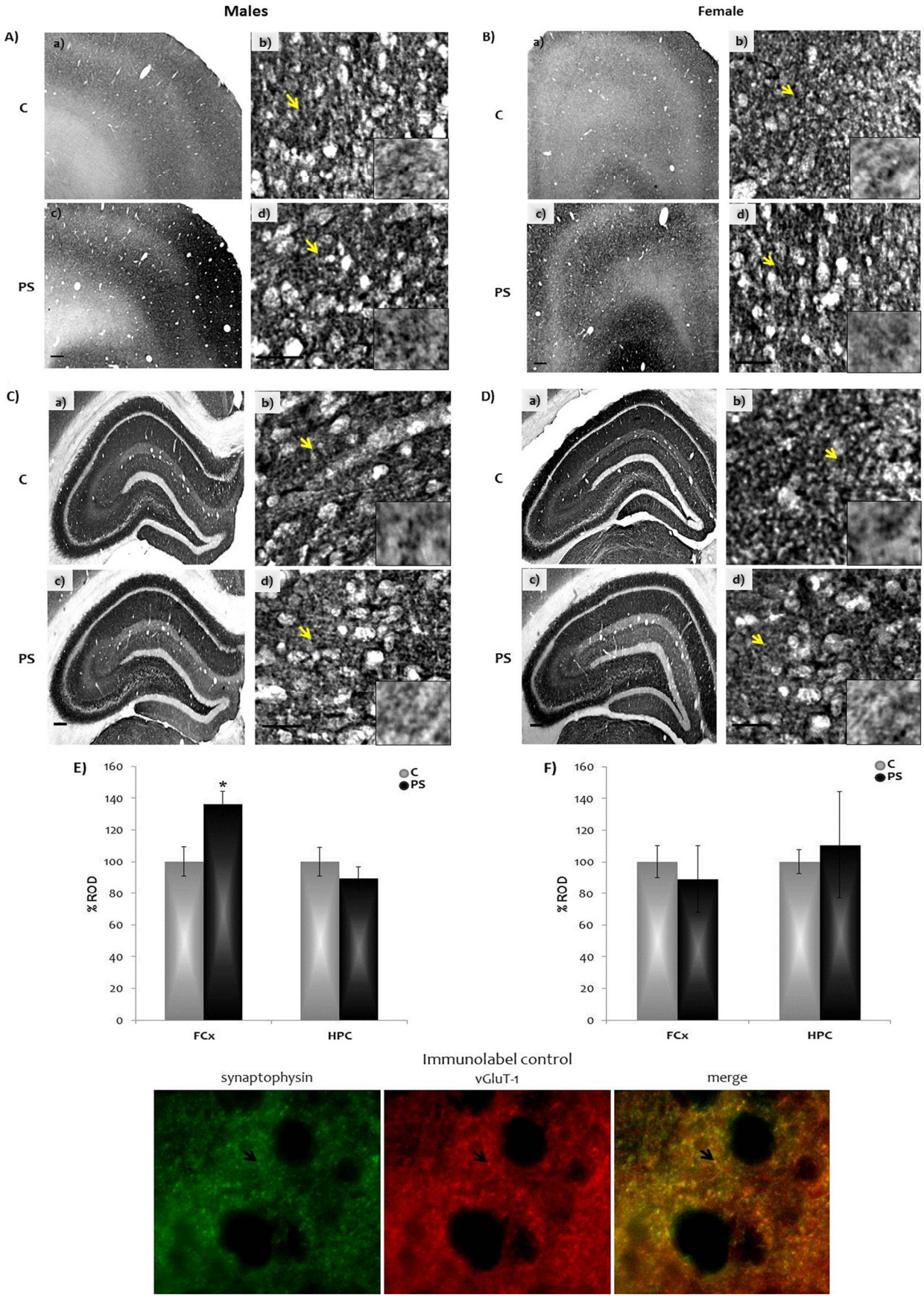

Fig. 5. Evaluation of vGluT-1 immunolabel in C and PS adult offspring. Pictures show representative images of male (A and C) and female (B and D) brain areas. (A and B) FCx images at two different magnifications: (a and c) 4×; (b and d) 40×. (C and D) HPC images at two different magnifications: (a and c) 4×; (b and d) 40×. Scale bar: 200 μm for 4× magnification and 20 μm for 40×. Insets show images at 100× magnification. (E and F) Relative optical density (ROD) of vGlut-1 in males (E) and females (F) for both brain areas. Values are means ± SEM ($n = 4$) expressed as % of the C values. *Significant difference between C and PS ($p < 0.05$). (G) Immunolabel control image shows the fluorescent staining for anti- vGluT-1 (red), anti-synaptophysin (green) antibodies. Merged images show the overlapping signals for synaptophysin and vGluT-1. Arrows point to the immunolabel of vGluT-1.

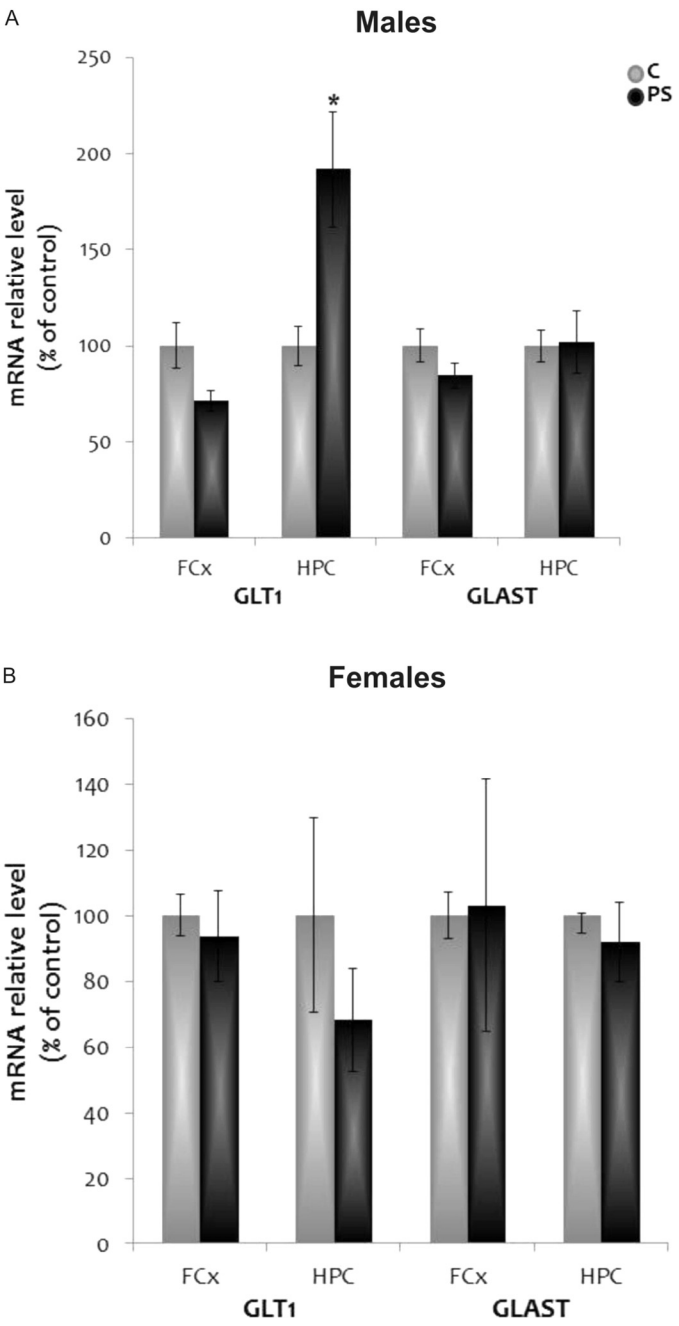

Fig. 6. RT-PCR analysis for mRNA expression of glutamate transporters GLT1 and GLAST in FCx and HPC of PS and C adult male (A) and female (B) offspring. The mRNA levels were normalized to cyclophilin mRNA and expressed as % of the control. Values are means ± SEM (n = 4). *Significant difference between C and PS ($p < 0.05$).

metabotropic glutamate receptors than control rats. These animals also show long-lasting astroglial reaction and a reduced dendritic arborization with synaptic loss. Since metabolism of glutamate is linked to a cycle between neurons and surrounding astroglia, we hypothesized that glutamate metabolism might be impaired in the brain of a prenatally stressed rats. To confirm our assumption we addressed a thorough study of the main steps in the glutamate cycle that is completed between the neuron and the astrocyte, in the prefrontal cortex and hippocampus of the prenatally stress offspring. The main findings of this study show that immobilization of the pregnant dam during the third week of pregnancy results in an up-regulation of the mRNA and protein levels of GLT1 in the hippocampus simultaneously with an increase in the uptake of glutamate and elevated levels of vGluT-1 protein in the prefrontal cortex of adult male offspring.

Glutamate release, binding to receptors and transport take place between three key neural elements: the astrocyte, the presynaptic neuron and the postsynaptic neuron, i.e. the tripartite synapse (Parpura et al., 2012). In the presynaptic neuron glutamate is packaged into synaptic vesicles by the family of vesicular glutamate transporters (vGluTs). Once released into the synaptic cleft, glutamate may occupy and activate the receptors located on both neurons and astrocytes. The removal of glutamate from the synapse is facilitated by a family of plasma membrane excitatory amino acid transporters (EAATs) generally localized to astrocytes (Anderson and Swanson, 2000).

The glial–neuronal metabolic relationship mediated by Glu–Gln interconversion appears to be essential for glutamatergic neurotransmission (Gruetter et al., 2001). Most of the Glu is present in neurons (Ottersen et al., 1992) whereas astrocytes synthesize most of the glutamine (references in Schousboe et al., 2013). More pyruvate derived from glucose is metabolized in the neuronal TCA cycle (Hassel et al., 1995; Qu et al., 2000) and in agreement with this the ^{13}C labeling was higher in glutamate than in glutamine in the present experiment. In our study no significant alterations were found in the ^{13}C labeling of metabolites from [1-^{13}C]glucose in PS rats suggesting that the prenatal insult does not affect glutamate homeostasis and the glutamate–glutamine cycle activity in between neurons and astrocytes. The observed changes in plasma membrane glutamate transport may compensate and adjust glutamate homeostasis to control levels.

Glutamate is removed from the extracellular space by the family of Na^+-dependent high-affinity transporters (Shigeri et al., 2004). Among these transporters, GLT-1 and GLAST have the most predominant role in clearing the amino acid (Robinson, 1998) and they act in a brain area dependent way (Huang and Bergles, 2004). Several studies have established that the expression and maintenance of glutamate transporters depend on the contact between neurons and astrocytes as well as on neuronally released factors. Moreover, transporters may be regulated independently of changes in the genomic expression upon activation or inactivation of the transporters proteins and redistribution at the plasma membrane (Gegelashvili et al., 1997, 2000; Sheldon and Robinson, 2007; Swanson et al., 1997; Yang et al., 2010). We evaluated the effect of prenatal stress on GLT1 and GLAST at FCx and HPC and we find an over-expression of GLT1 mRNA and protein in HPC. The functional expression of the transporters was measured through the uptake of [^3H]glutamate in a synaptosomal and a gliosomal fraction. The gliosomal fraction of frontal cortex showed an increase in the prenatally stressed offspring. However, in order to characterize the type of transporter present in each fraction, we employed TBOA and DHK, two specific transporter inhibitors. This experiment allowed us to draw two major conclusions: (a) most of the uptake in the GVP fraction was due to GLT1 since 80% of the activity was abolished by DHK and (b) synaptosomal uptake levels are to a large extent the result of a glial contamination, since DHK blocked synaptosomal uptake (Danbolt, 2001).

Glutamate vesicular transporters (vGluT) are responsible for packing the neurotransmitter into the synaptic vesicles and along with glial transporters primarily mediate the uptake that regulates the system (Raudensky and Yamamoto, 2007). In a large number of diseases associated with excitotoxicity an increase in vGluT expression was reported. Kim et al. (2005) found an up-regulation of vGluT-1 protein in hypoxic rats and it has been demonstrated that stressed animals also exhibit a higher level of expression of the vesicular transporter (Raudensky and Yamamoto, 2007). Increased levels of vesicular transporters may induce an increase in Glu release, at least in the long-term control of quantal size, and this could lead

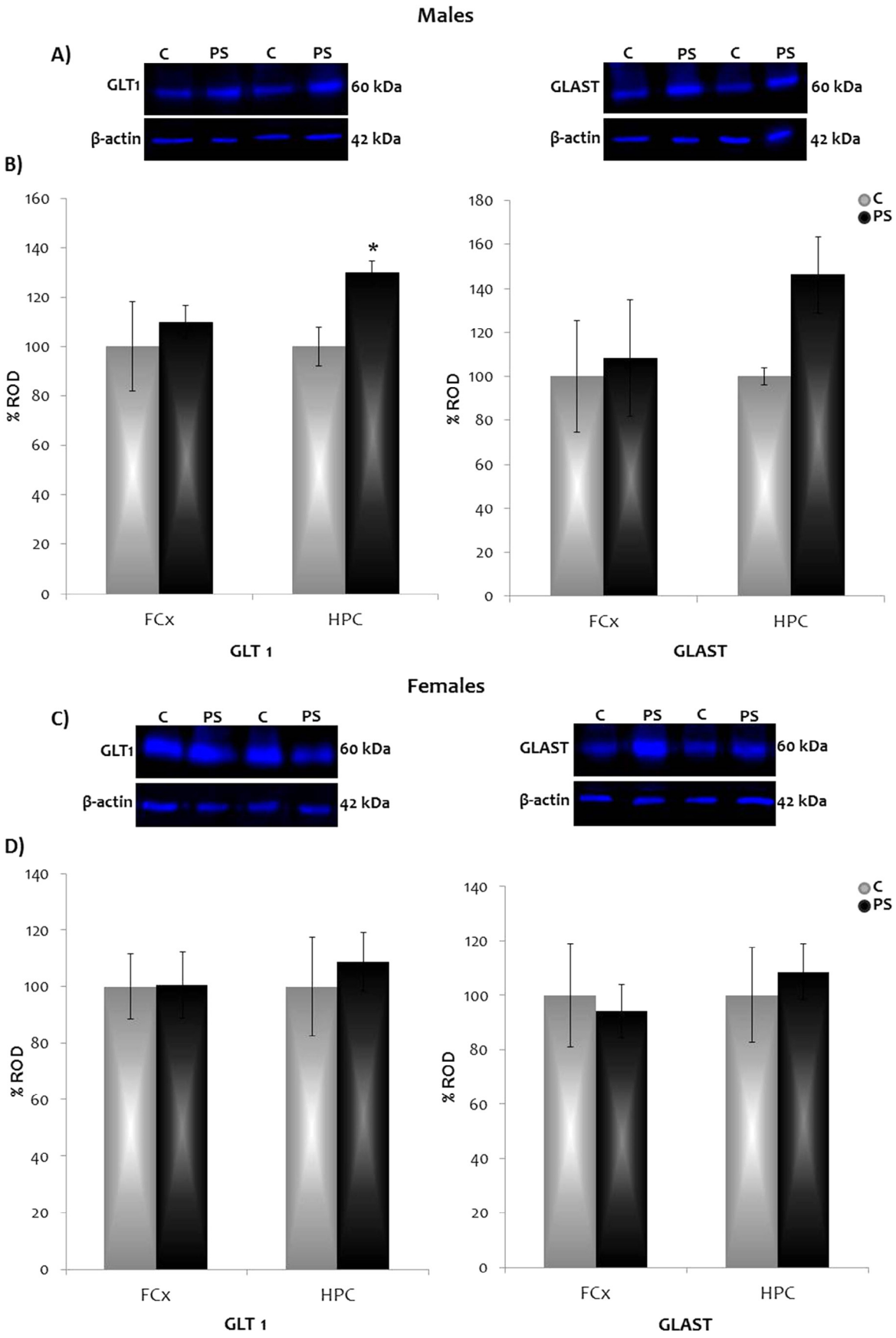

Fig. 7. Protein expression of transporters GLT1 and GLAST, in male and female adult PS and C offspring in two brain areas, FCx and HPC. (A and C) Representative western blot images showing immunopositive bands for anti-GLT1 and anti-GLAST antibodies and their corresponding β-actin standard bands in males and females respectively. (B and D) Relative optic density (ROD) expressed as percentage of control immunolabel, for males (B) and females (D). Values are means ± SEM (n = 4). *Significant difference between C and PS ($p < 0.05$).

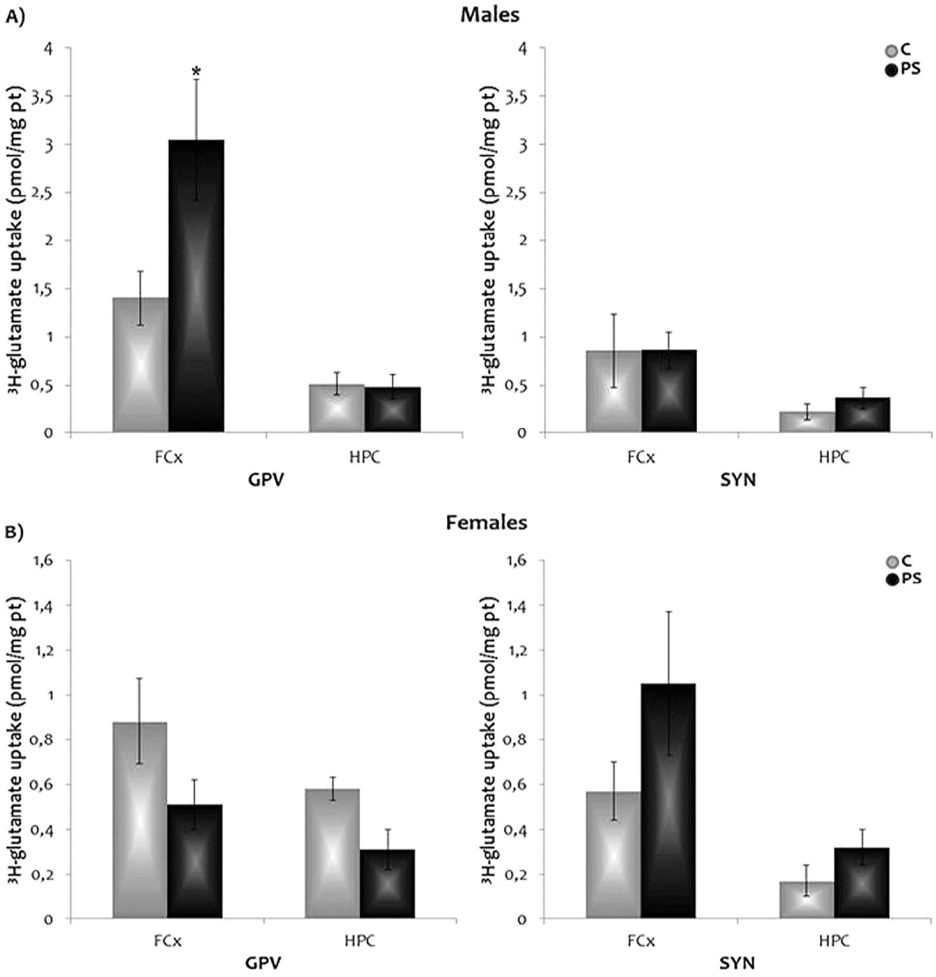

Fig. 8. Uptake of [^{3}H]-glutamate in GVP and SYN fractions isolated from rat FCx and HPC. (A) [^{3}H]-glutamate uptake for adult C and PS male offspring. (B) [^{3}H]-glutamate uptake for adult C and PS female offspring. Values are means ± SEM (n = 6). *Significant difference between C and PS ($p < 0.05$).

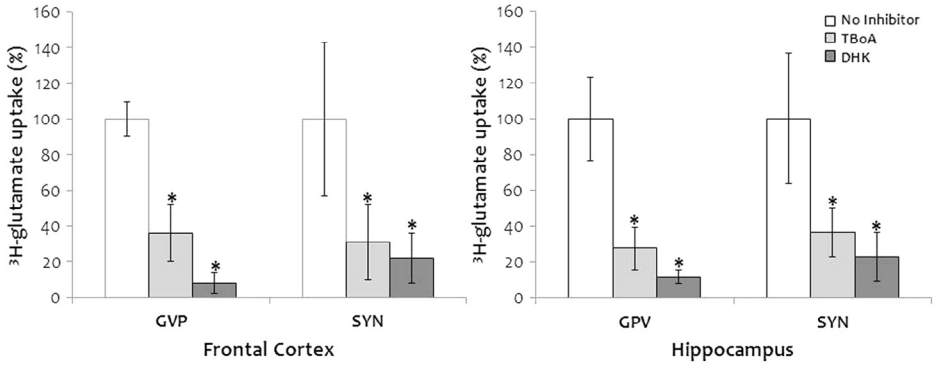

Fig. 9. Representative histogram (only PS male offspring are shown) of the percentage of [^{3}H]-glutamate taken up by GVP and SYN fractions, in the presence of two transporter inhibitors: TBOA and DHK. The analysis was conducted in two brain areas: frontal cortex and hippocampus. Values are mean ± SEM (n = 6) expressed as % of the fraction without inhibitor. *Significant difference between TBoA or DHK vs. No Inhibitor in the same fraction, $p < 0.05$.

to variability in synaptic efficacy and excitotoxic neurodegeneration (Liu, 2003; Moechars et al., 2006; Reimer et al., 1998; Wilson et al., 2005). In our hands, vGluT-1 expression was found to increase in FCx of PS male offspring. In any case, and in agreement with other authors, the enhancement of v-GlutT-1 expression may contribute to the dendritic remodeling and neurodegeneration observed in the stress models (Musazzi et al., 2011). In agreement with our results, Marrocco et al. (2012) found that the reduced glutamate release in PS was not due to an impaired glutamate synthesis in presynaptic terminals. These authors proposed that PS causes a long-lasting dysfunction in the intrinsic machinery controlling exocytotic glutamate release in the ventral hippocampus. It is interesting to point out that these authors detected alterations in the vesicle-associated proteins in the hippocampus. Albeit not studied in the frontal cortex, our observations of an increase in the vGluT protein in the frontal cortex of the PS offspring might well be associated

with alteration of the SNARE complex and/or proteins such as synaptophysin, synapsins, munc-18 and Rab3A, that regulate the trafficking of synaptic vesicles. In fact, in one of our previous reports we showed that PS exerts a reduction in the synaptophysin immunoreactivity in frontal cortex of adult offspring (Barros et al., 2006a).

Prenatal restraint stress in rats is a documented model known to induce neurobiological and behavioral alterations in the offspring (Maccari and Morley-Fletcher, 2007) and it causes neuropsychiatric disorders such as depression, obsessive compulsive disorder and schizophrenia among others (Joels and Baram, 2009). A well-established outcome of stress is the impairment of glutamatergic neurotransmission in different brain areas (Musazzi et al., 2011). Zhang et al. (2013) demonstrated that prenatal restraint stress induced attenuation of EAAT2 (GLT 1) and EAAT3 as well as pGluR1 variation in the hippocampus, striatum and frontal cortex of 1 month old rat offspring and this treatment also induced a depression-like behavior. Martisova et al. (2012) reported an elevated GLT1 expression in the hippocampus of maternal separated adult offspring as a consequence of elevated levels of glutamate released. On the other hand, Matute et al. (2005), in agreement with our results, suggested that the increase in glutamate uptake in the prefrontal cortex reduces transmission efficacy in a similar fashion as was observed in animal models of schizophrenia and schizophrenic patients. Taken together, these results allow us to hypothesize that the variations seen in the PS rats might be a compensatory neuroprotective process against glutamatergic hyperactivity and excitotoxicity. The specificity of the changes in the hippocampus could reflect impaired glutamatergic function in the area which could be the location for memory and learning deficits of PS rat models (Schulz et al., 2011; Son et al., 2006; Yaka et al., 2007). Modifications of the system similar to these have been found in other models, such as transporter knockout and knockdown (Regan et al., 2007; Ueda et al., 2002; Zhang et al., 2009). Even though GLT1 mRNA and protein levels were not altered in FCx of PS offspring, an increase in GPV uptake of glutamate was observed. The insult suffered during development may have caused a hyperfunction of the GLT1 transporter that ultimately could decrease glutamatergic signaling in the prefrontal cortex and this modification has been proposed to contribute to schizophrenia (Tsai and Coyle, 2002). These types of impairments might be the missing link in an already proposed relation between prenatal insults and schizotypal traits (Koenig et al., 2005; Lahti et al., 2009; Moore and Susser, 2011). Moreover, the impairment of the astrocytic uptake of glutamate exerted by PS might lead to a reduced concentration of glutamate in the microenvironment of the synaptic cleft. These changes in glutamate labeling in the synaptic cleft might only be a local change that is lost when the tissue is homogenized.

Since changes in the presynaptic neuron and in the astrocytes were found, it was decided to evaluate the postsynaptic side of the system. Using Western blot we measured the protein levels of NMDA subunit NR1 and AMPA subunit GluR4 in adult rats male and female, C and PS, in both areas. No significant differences were detected between C and PS in any of the evaluated situations. In parallel studies mRNA levels for the same proteins were evaluated and the results showed a significantly increased level of NR1 mRNA in FCx of female offspring. Previous results from our laboratory showed that PS exerted an increase in NMDA receptor expression both in medial frontal cortex and hippocampus of adult male rats (Berger et al., 2002). In this study, PS offspring at PND 90 were employed to detect receptors by quantitative autoradiography. Quantitative receptor autoradiography (QAR) allows visualizing membrane receptors in discrete areas, while in the present study receptor levels were quantified by Western blot in whole brain area homogenates using specific antibodies. Discrepancy with present results might be related to differences in technique and age of the animals. Interestingly, the literature also shows inconsistencies over this issue. Some authors have shown no changes in GluR subunits, or in protein or mRNA levels, in PS rat brain areas (Fumagalli et al., 2009; Neeley et al., 2011) while Yaka et al. (2007) detected a decrease in NR1 in the CA1 of male PS rats and Matrisciano et al. (2012) found similar changes in the expression of mGlu 2 and 3. In any case, all these results support the hypothesis that glutamatergic receptor function and expression are being modulated by prenatal insults. The results also point out that the variations in the system depend on the PS paradigm applied, the brain area to be studied and the kind of animal model employed.

Alterations in glutamate neurotransmission are believed to play a role in the pathophysiology of several neuropsychiatric disorders such as depression, anxiety and drug addiction (Chen et al., 2010; McEwen, 2012; Ongur et al., 2008). Male PS offspring shows a prominent anxious-like phenotype (Zuena et al., 2008). In our model, male PS rats show an anxiogenic behavior in the elevated plus maze (Barros et al., 2006b), an alteration that in view of the present results might be related to impairment of the glutamate neurotransmission in the frontal cortex.

In this study none of the changes observed in males offspring were detected in the females. Few studies report prenatal stress consequences in both sexes and the majority agree that learning deficits, LTP and dendritic density reductions, among others are seen mainly in males while females are more susceptible to anxiety, depression and response of hypothalamic–pituitary–adrenal axis (Weinstock, 2001, 2008). Moreover, Bowman et al. (2004) reported gender differences in PS offspring in the hippocampal and prefrontal cortex concentration of noradrenaline, serotonin and dopamine. Gender associated variations in the hippocampus structure and reaction to glucocorticoids (Liu et al., 2006) have also been found. It is also well established that estrogens can increase spines, glutamate receptor binding and LTP in the hippocampus (Woolley et al., 1997). This could explain the gender specific response to PS and the female capability to overcome insults received during brain development.

5. Conclusion

The present study was undertaken to explore the metabolism of the glutamatergic synapse in the prenatally stressed adult rats and to test the hypothesis that prenatal insults promote variations in the system that modify its function. While glutamate metabolism and release were not affected it was found that PS changed the expression of the transporters, thus, producing a higher level of vesicular vGluT-1 in the FCx and elevated levels of GLT1 protein and messenger RNA in the HPC of adult male PS offspring. We also observed increased uptake capacity for glutamate in the FCx of PS male offspring while no such changes were observed in the HPC. Moreover, the results show that changes mediated by PS on the adult glutamatergic system are brain region specific. The changes reported in this study, might be precluding a deficit in the synaptic strength as a result of the exacerbated uptake of neurotransmitter and a concomitant attempt to compensate this by an elevated Glu release. However, in view of our previous results showing increase in neuronal ionotropic and metabotropic receptors that are known to modulate the glial transporters, our present results provide new insight into the crucial crosstalk between neuron and glia, that seems to be impaired after an early insult. Future studies might dilucidate if the astroglial reaction following PS is cause or consequence of the concomitant glutamate metabolism impairment observed in this study.

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