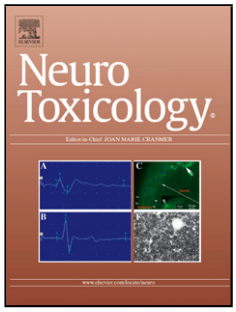

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Reduced maternal behavior caused by gestational stress is predictive of life span changes in risk-taking behavior and gene expression due to altering of the stress/anti-stress balance

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Highlights

- Gestational stress reduces maternal active maternal behavior toward pups.
- Maternal administration of carbetocin prevents stress-induced programming in progeny.
- Increased maternal behavior by carbetocin has long-lasting effect in the offspring.
- The oxytocin system is a potential therapeutic target for stress-related disorders.

Abstract

Exposure of the mother to adverse events during pregnancy is known to induce pathological programming of the HPA axis in the progeny, thereby increasing the vulnerability to neurobehavioral disorders. Maternal care plays a crucial role in the programming of the offspring, and oxytocin plays a key role in mother/pup interaction. Therefore, we investigated whether positive modulation of maternal behavior by activation of the oxytocinergic system could reverse the long-term alterations induced by perinatal stress (PRS; **gestational restraint stress 3 times/day during the last ten days of gestation**) on HPA axis activity, **risk-taking** behavior in the elevated-plus maze, hippocampal mGlu5 receptor and gene expression in Sprague-Dawley rats. Stressed and control unstressed dams were treated during the first *postpartum* week with an oxytocin receptor agonist, carbetocin (1 mg/kg, i.p.). **Remarkably, reduction of maternal behavior was predictive of behavioral disturbances in PRS rats as well as of the impairment of the oxytocin and its receptor gene expression.** *Postpartum* carbetocin corrected the reduction of maternal behavior induced by gestational stress as well as the impaired oxytocinergic system in the PRS progeny, which **was** associated with **reduced risk-taking** behavior. Moreover, *postpartum* carbetocin had an anti-stress effect on HPA axis activity in the adult PRS progeny and increased hippocampal mGlu5 receptor expression in aging. In conclusion, **the activation of the** oxytocinergic system in the early life plays a protective role against the programming effect by adverse experiences and could be considered as a novel and powerful potential therapeutic target for stress-related disorders.

Keywords: maternal behavior; oxytocin receptor agonist; early-life stress; **risk-taking** behavior; transcriptomics; hippocampus.

1. Introduction

Exposure to stress-related events early in life induces a life-long programming effect (Barker, 1995) on stress response, emotional behavior, metabolism, and cerebral plasticity (Maccari et al., 2014, 2017). This may have a considerable impact on the susceptibility to develop age-related disorders (Lesage et al., 2004; Vallée et al., 1999) with evidence for epigenetic transmission across generations (Franklin et al., 2010). In the last decades, studies on programming induced by early life stress (maternal psychosocial stress or *in utero* under-nutrition) have gained a considerable impact. A well characterized animal model of early programming is the perinatal stress (PRS) model in rats (Maccari et al., 1991, 1995, 2014, 2017), in which exposure of pregnant dams to chronic gestational stress programs the progeny to a life-long disruption in the activity and feedback regulation of the hypothalamus-pituitary-adrenal (HPA) axis (Henry et al., 1994; Maccari et al., 1995; Vallée et al., 1999). These alterations are associated with abnormal emotional behavior from infancy on (Laloux et al., 2012; Vallée et al., 1997; Zuena et al., 2008).

A key target of PRS is the hippocampus, a brain region involved in cognition and emotion regulation, where we found a reduced expression of glucocorticoid receptors (Maccari et al., 1991, 1995; Van Waes et al., 2006), which mediate the negative feedback regulation of the HPA axis (Maccari et al., 1991; McEwen et al., 1986; Sapolsky et al., 1986). PRS rats also show a reduced neurogenesis associated with a reduced expression of mGlu2/3 and mGlu5 metabotropic glutamate receptors in the hippocampus (Laloux et al., 2012; Morley-Fletcher et al., 2011; Zuena et al., 2008).

Several lines of evidence suggest that glutamatergic transmission is highly involved in the response to stress (Bagley and Moghaddam, 1997; Moghaddam, 2002; Popoli et al., 2011). An interaction between mGlu receptors and glucocorticoid receptors has been reported in the hippocampus (Nasca et al., 2015a, 2015b). We have consistently shown a defect in glutamate release in the ventral hippocampus of PRS rats (Mairesse et al., 2015; Marrocco et al., 2012, 2014). Because mGlu2 and mGlu3 receptors negatively modulate glutamate release (reviewed by Nicoletti et al., 2011), the reduction in mGlu2/3 protein levels we have seen in the hippocampus of PRS rats might represent an unsuccessful compensatory mechanism aimed at correcting glutamatergic abnormalities in these rats. Interestingly, acute intra-hippocampal injection of a cocktail of mGlu2/3 and $GABA_B$ receptor antagonists, which resulted in a pharmacological enhancement of glutamate release, reversed the alterations in the **risk-taking** behavior in PRS rats, lending credit to the hypothesis that an impairment in glutamate release lies at the core of the pathological phenotype induced by PRS (Marrocco et al., 2012). The

studies conducted over the last decades have conferred a high pharmacological validity to the PRS model suggesting that this animal model can be used for the development of novel therapeutic strategies for stress-related disorders. Studies from our group have shown that both environmental (Morley Fletcher et al., 2003a) and pharmacological (Mairesse et al., 2013, 2015; Marrocco et al., 2014; Morley-Fletcher et al. 2003b, 2004, 2011) interventions at different ages and sensitive periods are able to reverse the phenotype programmed by PRS. A new potential strategy for the treatment of stress-related disorders could be the reinforcement of endogenous anti-stress mechanisms such as the oxytocinergic system. Oxytocin plays a key role in mechanisms of adaptation to stress (Cohen et al., 2010; Zheng et al., 2010) by activating G protein-coupled receptors in the hippocampus, amygdala, and other stress-responsive brain regions.

In addition to its role in the stress/anti-stress balance, oxytocin is also known for its established role in parturition and lactation in all mammalian species (Blanks and Thornton, 2003) and is critically involved in the onset and maintenance of maternal behavior (Bosch and Neuman, 2012; Yoshihara et al., 2017). Central oxytocin infusion in rodents increases the expression of maternal behavior (Pedersen and Prange, 1979; Pedersen et al., 1994), while inhibition of oxytocin receptors produces opposite effects (Pedersen and Boccia, 2003; Pedersen et al., 1985; Rich et al., 2014; van Leengoed et al., 1987). In addition, central infusion of an oxytocin receptor antagonist on *postpartum* day 3 eliminates natural variations in maternal care (Champagne et al., 2001). Remarkably, maternal behavior plays a major protective role on growth, survival, and adaptation of the progeny, considering that at birth the newborn is not fully mature and is completely dependent on its mother for feeding and care (Moriceau and Sullivan, 2005; Tang et al., 2014). Natural variations in maternal behavior have been observed in lactating rodents during the first *postpartum* week (Champagne et al., 2003). Changes in the amount and quality of maternal care have been classified in terms of low and high maternal care (Meaney, 2001) with the low maternal care inducing in the offspring enhanced emotional behavior and a hyper-active HPA axis compared to the high maternal care group (Caldji et al., 2000; Francis et al., 1999). Thus, maternal behavior appears to be a major determinant for both neural development and the HPA axis response to stress of the offspring (Caldji et al., 1998; Champagne and Curley, 2009; Liu et al., 1997). In this line of thought, enhancement of maternal behavior induced by early adoption (Maccari et al., 1995) or early handling (*postpartum* stress of the dam, Koehl et al., 1997) can reverse the hyperactivity and feedback of the HPA axis in PRS animals. Whether enhancement of

maternal behavior *via* the activation of the oxytocinergic system in stressed mothers may intermediate beneficial effects in PRS offspring remains to be investigated.

A drug of choice for the activation of the oxytocinergic system is carbetocin. Carbetocin is a potent and selective oxytocin receptor agonist, which is brain permeant and shows a longer elimination half-life than oxytocin (Dvorská et al., 1992). This drug is currently marketed in different countries for the treatment of *postpartum* bleeding in humans (Chen et al., 2016; Engstrøm et al., 1998; Meshykhi et al., 2016; Onwochei and Monks, 2017; Van der Nelson et al., 2017). Carbetocin is a synthetic peptide that shows no oral bioavailability and therefore cannot be transferred from lactating dams to pups through the milk (Silcox et al., 1993). Thus, carbetocin may exert a direct action on maternal behavior. For this reason, this drug meets the requirements to be used as a pharmacological agent to unveil the role of maternal care in the developmental trajectory of perinatal stress.

Therefore, in the present study, we addressed the question of whether positive modulation of maternal behavior by activation of the oxytocinergic system could prevent PRS-induced alterations in HPA axis activity, but also in **risk-taking** behavior and hippocampal neurochemical parameters and transcriptomics related to the PRS phenotype in their adult life and old age. Specifically, we examined the reprogramming effect of a *postpartum* (1^{st} week) chronic carbetocin administration to lactating dams on maternal behavior and the PRS phenotype in the offspring. We also examined how the interaction between PRS and *postpartum* carbetocin resulted in changes in the gene expression of the stress- and anti-stress balance in the hippocampus.

2. Materials and Methods

2.1. Animals

All experiments followed the rules of the European Communities Council Directive 2010/63/EU. Twenty-five nulliparous female Sprague-Dawley rats, weighing approximately 250 g, were purchased from a commercial breeder (Harlan, France). Animals were housed at constant temperature (22 ± 2 °C) and under a regular 12 h light/dark cycle (lights on at 8.00 a.m.). A vaginal smear using physiological serum (NaCl 0.9%) was performed on the morning following mating with an experienced male (one male per two females). The day on which the smear was sperm positive was considered as embryonic day 0 (E0). After mating, pregnant females (80% of the original purchase, n = 20 females) were individually housed with *ad libitum* access to food (SAFE A04-10, France, 72.4% carbohydrates, 19.3% proteins, 8.4% lipids) and water at a constant temperature (22°C ± 2°C), and under a regular 12 h light/dark cycle (lights on at 8.00 a.m.). On E11, pregnant females were randomly assigned to either the restraint stress or the control group (10 per group). Control females were left undisturbed.

2.2 Perinatal stress protocol

The pregnant females in the restraint stress group were subjected to a restraint stress procedure according to the standard protocol from Maccari's group (Maccari et al., 1995; Morley-Fletcher et al., 2003a). From day 11 of pregnancy until delivery, the dams were subjected to three stress sessions per day (45 min each during the light phase around 9:00 am, 12:00 pm, and 3:00 pm.), during which they were taken to a different room and placed in transparent plastic cylinders with conical end caps and exposed to bright light. Control dams were left undisturbed. At birth, pups were left undisturbed with their mother until weaning 21 days after birth. Only male rats from litters of 10-14 rats with a similar number of males vs. females were used in the present study. Only two male siblings were taken from each litter to remove any litter effect (Chapman and Stern, 1979). The local ethical committee approved the gestational restraint procedure.

2.3. Carbetocin treatment

Either carbetocin (1 mg/kg, SP080756, Polypeptide group, Strasbourg, France) or only its vehicle (saline) was administered i.p. to lactating dams from *postpartum* day 1 to 7 (n =

5/group). The dose and route of administration of carbetocin were selected on the basis of previous reports (Klenerova et al., 2009a, 2009b; Mairesse et al., 2015).

2.4. Analysis of maternal behavior

Control and stressed dams were placed in standard transparent cages on a rack equipped with cameras. The video recording system included 30 small infrared cameras (CMTH with ¼ inch Sony CCD, 3.6 mm lens) attached to a metal structure and placed at about 12 cm distance from the cage wall, allowing whole floor area detection (1 video camera per cage). Two infrared LEDs pointed towards the ceiling to provide diffuse infrared lighting in the room allowing for analysis of behavior. Constant recording (24 h/24 h) was performed by video signals acquired on two 16 channels DVR encoding H.264 format (Avtech, AVC798ZA). The digital video signal was sent by IP to a computer for storage on a hard disk. The Video Viewer Application® (version 0.1.8.4) drove the video recording and replay. From day 1 to day 5 after parturition, maternal active behavior (n = 5 dams per group) was analyzed offline for the 2 h following carbetocin or saline injection. Within each observation period, the behavior of each dam was scored every min (60 observations/h with 2 h of observation per 5 days, i.e. 121 observations/dam/day) for the following behaviors: nursing behavior (active arched back nursing, blanket posture in which the dam lays over the pups, or passive posture in which the dam is lying either on her back or side while nursing the pups), grooming, licking, and carrying pups (Champagne et al., 2003). Data obtained represent the active presence of the dam on the nest expressed in a percentage of the total number of observations. The observer of the maternal behavior was blind to the treatment conditions (PRS and carbetocin treatment).

2.5 Experimental animals

Behavioral and neuroendocrine tests were conducted on the selected male animals at each age point (3 or 16 months of age). TaqMan analyses were conducted at 5 or 22 months of age on these same animals.

All animals were housed in groups of 2-3 per cage, allocated according to their group of maternal treatment. Attention was paid to provide size-adapted cages for aged animals (480 × 375 × 210 mm, surface 1500 cm^2). Behavioral tests were performed between 11:00 a.m. and 3:00 p.m.

2.6. Experimental protocol

In the adult and aged progeny, behavioral, endocrinological, and biochemical measurements were carried out in the temporal sequence indicated in **Fig. 1**.

2.7. ***Risk-taking*** *behavior*

Behavior of both adult and aged control or PRS progeny (n = 5 per group) reared by dams treated with either saline or carbetocin was assessed in the elevated-plus maze (EPM) (Pellow et al., 1985). The test was performed between 1.00 P.M. and 4.00 P.M., lasted for 5 min and began with the placement of the rat in the center of the maze with the head facing a closed arm. The apparatus used consists of four arms (two open without walls and two enclosed by 30 cm high walls), each 50 cm long and 10 cm wide. For aged rats, we used a custom-made EPM apparatus as described by Vallée et al. 1999, with wider closed and open arms (20 × 20 cm). Behavior was recorded on-line with a video camera and manually scored by a trained observer blind to the animals' previous assignment to experimental conditions (PRS and carbetocin treatment) using purpose-specific software (Noldus, The Observer®). The time spent in open and closed arms was measured and the percentage of time spent in open arms was calculated.

2.8. Corticosterone response to stress

Reactivity of the HPA axis in response to stress was analyzed in adult control and PRS progeny (n = 5 per group) of dams treated with either saline or carbetocin during the *postpartum* period. The animals were submitted to a 30 min novelty stress exposure during the first half of the light phase of the light/dark cycle (between 9:00 a.m. and 12:00 p.m.). Novelty stress consisted in placing animals in a transparent cylindrical Plexiglas cage (30 cm diameter, 50 cm high) without sawdust and under a bright light (400 lux). Corticosterone levels (ng/ml) were determined on plasma extracted from four blood samples (around 200 µl each) withdrawn from the tail vein of each animal. Samples were collected before stress (T0) and 30, 75 and 120 min afterwards. Plasma corticosterone concentrations were determined using a commercial ELISA kit following manufacturer instructions (DEV9922, Demeditec Diagnostics GmbH, Kiel, Germany). All standards, samples, and controls were run in duplicate concurrently.

2.9. Quantitative mRNA analysis in the hippocampus

The hippocampi (left and right hippocampi were randomized to avoid any lateralization effect) of aged control and PRS rats (n = 5 per group) from dams treated with either saline or

carbetocin during the *postpartum* period were dissected as soon as possible and kept frozen at -80 °C. RNA extraction was performed using the RNeasy Plus mini kit (Qiagen, France). RNA concentration in samples was determined using Nanodrop (ND-1000, Labtech, Germany), and quality verified by RIN (RNA Integrity Number; Bioanalyzer 2100, Agilent Technologies, France). Retrotranscription was performed with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, France). Transcript levels were measured by real-time PCR using TaqMan assays (Applied Biosystems, France). The following TaqMan real-time PCR probes were obtained from Applied Biosystems: glucocorticoid receptor (GR, Nr3c1, Rn00561369_m1); mineralocorticoid receptor (MR, Nr3c2, Rn00565562 oxytocin/neurophysin 1 prepropeptide (oxt, Rn00564446_g1); oxytocin receptor (OxtR, Rn00563503_m1); arginine vasopressin (AVP; Rn00690189_g1). Transcript levels were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Rn001775763_g1) expression. Assay validity was assessed thanks to appropriate negative controls, in which cDNA was omitted. These negative controls were undetermined by software. Acquisition of data (threshold cycle, Ct) was performed by StepOnePlus^{TM} software. A ΔCt (Ct of the considered gene - Ct of the GAPDH gene) and a ΔΔCt (Ct of a particular gene - Ct of the same gene in unstressed rats treated with saline) were calculated.

2.10. Western blot

Ventral hippocampi (left and right hippocampi were randomized to avoid any lateralization effect) of aged control and PRS rats (n = 5 per group) were dissected as soon as possible and immediately stored at -80°C. Immunoblotting analysis was performed on synaptosomes that were isolated by manual homogenization of tissue with a Potter-Elvehjem homogenizer in 10 volumes of HEPES-buffered sucrose (0.32 M sucrose, 4 mM HEPES, pH 7.4). All procedures were performed at 4°C. Homogenates were centrifuged at 1000 g for 10 min and the resulting supernatants were centrifuged at 10,000 g for 15 min. The pellet obtained from the second centrifugation was suspended in 10 volumes of HEPES-buffered sucrose and then spun again at 10,000 g for 15 min. This pellet contained the crude synaptosomal fraction (Marrocco et al., 2012). A Pierce BCA (Thermo Scientific) assay was used to determine protein concentration. Synaptosomal lysates were suspended in Laemmli reducing buffer, and 20 µg of each sample was first separated by electrophoresis on 8% SDS-PAGE gels (Bio-Rad) and then transferred to nitrocellulose membranes (Bio-Rad). The transfer was performed at 4°C in a buffer containing 35 mM Tris, 192 mM glycine, and 20% methanol. After transfer, blots were incubated in a blocking solution containing Tris-buffered saline (TBS), 10% (w/v)

Tween-20, 1% (w/v) non-fat milk and 1% (w/v) bovine serum albumin. We used the rabbit polyclonal anti-mGlu5 receptor (1:1000; catalog AB5675, Millipore) as primary antibody. To ensure that each lane was loaded with an equivalent amount of proteins, the blots were probed with a mouse monoclonal anti-β actin (1:1000; catalog #A5316, Sigma). The primary antibody was incubated overnight at 4°C. HRP-conjugated secondary anti-mouse or anti-rabbit antibodies (purchased from Cell Signaling) were used at a dilution of 1:5000 and were incubated for 1 h at room temperature. Bands were visualized with an enhanced chemiluminescence system (Aurogene). After immunoblotting, images of bands immunoreactive for the target antibody and actin were acquired and the optical density (O.D.) of each band was measured using the ImageJ software. A ratio of target to actin was then determined and these values were compared for statistical significance.

2.11. Statistical Analysis

Data on weight during lactation as well as on **risk-taking** behavior were analyzed by two-way ANOVA (Group: CONT *vs.* PRS; Maternal Treatment: vehicle *vs.* carbetocin), while maternal behavior and corticosterone stress response were analyzed by two-way ANOVA with repeated measures (Group × Maternal Treatment × days or time points). Fisher's LSD test was used to isolate the differences. Significance was set at a p-value of 0.05. However, in mRNA analysis, in order to subdivide data accounting for significant lower-order effects when F was larger than 1, significance was extended to $p < 0.1$ (Snedecor and Cochran, 1967). Correlation analysis of behavioral data of the progeny with maternal behavior or TaqMan analysis in the hippocampus was performed using two-tailed Pearson's correlation analysis.

3. Results

3.1. Postpartum administration of carbetocin reverses the negative effects of gestational stress on maternal behavior and dam's weight gain, whereas it reduces body weight in the progeny.

We measured active maternal behavior (nursing behavior, grooming, licking, carrying pups) in stressed and unstressed dams receiving either saline or carbetocin during the first 5 days *postpartum* (Fig. 2A). In control dams treated with saline, maternal behavior decreased progressively from day 1 to 5 *postpartum*. This decrease was sharper in stressed dams treated with saline, in which a highly significant reduction in active maternal behavior was found at

postpartum days 2 and 3 compared to unstressed dams treated with saline. *Postpartum* carbetocin abrogated the detrimental effect of gestational stress on maternal care (*Group* × *Maternal Treatment effect*, $F(1,16) = 7.538$, $p < 0.05$; * < 0.05 Stressed dam with saline *vs.* Control dam with saline group and # $p < 0.05$ Stressed dam with carbetocin *vs.* Stressed dam with saline group, Fisher's LSD). *Postpartum* carbetocin did not affect maternal behavior in unstressed dams.

We also measured the effect of gestational/perinatal stress and *postpartum* carbetocin on the dams'- and progeny's body weight at the end of the first week *postpartum*. In the dams (Fig. 2B), systemic carbetocin (1 mg/kg, daily, i.p.) increased body weight gain exclusively in stressed dams (*Maternal treatment effect*, $F(1,16) = 15.76$, $p < 0.05$; # $p < 0.05$ Stressed dam with carbetocin *vs.* Stressed dam with saline group, Fisher's LSD). On the other hand, in the progeny (Fig. 2C), pups from both groups of dams (stressed and unstressed) treated with carbetocin showed reduced body weight. PRS male pups reared by dams treated with saline showed reduced body weight compared to pups reared by unstressed control dams (*Group effect*, $F(1,16) = 6.12$ and *Maternal treatment effect* $F(1,16) = 87.58$, $p < 0.05$); *** < 0.05 PRS-Saline *vs.* CONT-saline and # p < 0.05 PRS-Carbetocin *vs.* PRS-saline and CONT-Carbetocin vs. CONT-saline, Fisher's LSD).**

*3.2. Postpartum administration of carbetocin prevents changes in **risk-taking** behavior, HPA axis response to stress, and hippocampal gene expression induced by PRS in the adult progeny.*

Adult (3 – 5-month-old) offspring of unstressed (control) and stressed dams treated with carbetocin or saline during the first *postpartum* week were tested for risk-taking behavior in **the open arm of** EPM, plasma corticosterone levels in response to novelty stress, and stress and anti-stress gene expression in the hippocampus.

As depicted in Fig. 3A, PRS **reduced risk-taking** behavior measured as spending less time in the open arm of the EPM than did the progeny of unstressed dams treated with saline. Carbetocin administration to lactating dams prevented this behavior in PRS animals (*Group* × *Maternal Treatment effect*, ANOVA: $F(1,16) = 8.793$, $p < 0.01$; * < 0.05 PRS-saline *vs.* CONT-saline and ## $p < 0.01$ PRS-carbetocin *vs.* PRS-saline, Fisher's LSD) with no effect on control animals. As depicted in Fig. 3B, the corticosterone response to novelty stress was greater in PRS offspring of saline-treated dams than in their respective controls. Carbetocin administration to lactating dams normalized corticosterone response in adult PRS rats (main figure: *Maternal Treatment effect*, $F(1,16) = 6.51$, *time effect*, $F(3,48) = 100$, *Group* ×

Maternal treatment effect, $F(1,16) = 6.11$ $p < 0.05$; inset, area under curve (A.U.C.): *Group* × *Maternal Treatment effect*, $F(1,16) = 5.77$; * < 0.05 PRS-Saline *vs.* CONT-saline and # $p < 0.05$ PRS-Carbetocin *vs.* PRS-saline, Fisher's LSD) with no effect in control animals. As shown in Fig. 3C, the expression of stress-related genes (NR3C1, glucocorticoid receptor (GR) gene; NR3C2, mineralocorticoid receptor (MR) gene; and AVP, vasopressin gene) and anti-stress related genes (OXT, oxytocin gene; and OXTR, oxytocin receptor gene) was measured in the hippocampus. Adult PRS rats reared by saline-treated dams showed a significant reduction in MR and OXT mRNA levels, and a significant increase in OXTR and AVP mRNA levels in the hippocampus compared to adult control animals reared by saline-treated dams. Treatment of lactating dams with carbetocin reversed all changes in the expression of stress and anti-stress related genes caused by PRS (GR: not significant; MR: *Group* × *Maternal Treatment* $F(1,16) = 3.990$, $p = 0.06$; AVP: *Group effect* $F(1,16) = 3.595$, $p = 0.07$; OXT: *Maternal Treatment effect* $F(1,16) = 3.037$, $p = 0.08$; OXTR: *Group* × *Maternal Treatment* $F(1,16) = 4.357$, $p = 0.05$; * < 0.05 PRS-Saline *vs.* CONT-saline and # $p < 0.05$ PRS-Carbetocin *vs.* PRS-saline, Fisher's LSD). Carbetocin had no effect on gene expression in the offspring of unstressed dams.

*3.3. Postpartum administration of carbetocin prevents changes in **risk-taking** behavior, mGlu5 receptors, and hippocampal gene expression induced by PRS in the aged progeny.*

The aged (16 – 22-month-old) offspring of dams treated with saline or carbetocin were tested for **risk-taking** behavior in the EPM, mGlu5 receptor levels in the hippocampus, and for stress and anti-stress genes expression in the hippocampus as for adult progeny.

As depicted in Fig. 4A, the 16-month-old PRS offspring of saline-treated dams showed **decreased risk-taking** behavior **in the open arm of** EPM, thereby indicating that the **behavioral** programming caused by PRS lasted throughout the animals' lifespan. Carbetocin administration to lactating dams reversed this profile in PRS (*Group* × *Maternal Treatment effect*, $F(1,16) = 8.677$, $p < 0.01$; * $p < 0.05$ PRS-Saline *vs.* CONT-saline and # $p < 0.05$ PRS-Carbetocin *vs.* PRS-saline, Fisher's LSD). As depicted in Fig. 4B, PRS reduced mGlu5 receptor expression in the hippocampus and carbetocin administration corrected this profile (*Group* × *Maternal Treatment effect*, $F(1,16) = 5.584$, $p < 0.05$; * $p < 0.05$ PRS-Saline *vs.* CONT-saline and # $p < 0.05$ PRS-Carbetocin *vs.* PRS-saline, Fisher's LSD). As shown in Fig. 4C, the expression of stress-related genes and anti-stress related genes was measured in the hippocampus. 22-month-old PRS rats reared by saline-treated dams showed a significant reduction in OXT mRNA levels, and a significant increase in AVP mRNA levels in the

hippocampus compared to aged control animals reared by saline-treated dams. Although not statistically significant, the reduction of MR and the increase of OXTR genes echoed the trend detected in the adult progeny. *Postpartum* carbetocin reversed all changes in the expression of the above-mentioned genes in PRS animals (GR and MR: no significant effect, $F < 1$; AVP: *Group* × *Maternal Treatment effect,* $F(1,16) = 3.973$, $p = 0.06$; OXT: *Group effect,* $F(1,16) = 10.902$, $p < 0.01$ and *Maternal Treatment effect,* $(F1,16) = 5.881$, $p < 0.05$; OXTR: *Group* × *Maternal Treatment effect* $F(1,16) = 3.739$, $p = 0.07$; * $p < 0.05$ PRS-Saline *vs.* CONT-saline and # $p < 0.05$ PRS-Carbetocin *vs.* PRS-saline, Fisher's LSD). As was the case for adult progeny, carbetocin had no effect on the above-mentioned parameters in the aged offspring of unstressed dams.

3.4. Correlation analysis

We analyzed the correlation between **risk-taking behavioral** response in the EPM and gene expression in the hippocampus of the adult and aged progeny. We found that for both the adult (Fig. 5A, left) and aged (Fig. 5A, right) progeny, the **reduced risk-taking** behavior (reduced exploration **of the open arm of** EPM) in PRS rats was correlated with anti-stress related genes. Specifically, a negative correlation was found between OXTR and behavioral response in the EPM for the adult group, while in the aged group we found a positive correlation between OXT gene expression and behavioral response (*Pearson's correlation analysis*: adult progeny, $r = -0.53$ and $p < 0.01$; aged progeny, $r = 0.47$ and $p < 0.05$).

We analyzed the correlation between behavioral responses of the progeny with the active maternal behavior at day 3 (Fig. 5B). We found that for both the adult (Fig. 5B, left) and aged progeny (Fig. 5B, right), the **reduced risk-taking** behavior in PRS rats was correlated with low maternal behavior (positive correlation; *Pearson's correlation analysis*: adult progeny, $r = 0.68$ and $p < 0.01$; aged progeny, $r = 0.58$ and $p < 0.01$). To extend the analysis to the effect of maternal behavior on the programming of the PRS phenotype, we analyzed the correlation between gene expressions in the progeny with the active maternal behavior at day 3 (Fig. 5C). In the adult progeny, a negative correlation was found between gene expression and maternal behavior, with a higher expression of OXTRs correlated with reduced maternal behavior (Fig. 5C, left; *Pearson's correlation analysis*: OXTR, $r = -0.47$ and $p < 0.05$; OXT, $r = 0.26$ $p > 0.1$, n.s. **not shown**). On the other hand, a positive correlation was found between gene expression in the aged progeny and maternal behavior with a lower expression of OXT genes correlated with reduced maternal behavior (Fig. 5C, right, *Pearson's correlation analysis*: OXT, $r = 0.57$ and $p < 0.01$; OXTR, $r = -0.21$, $p > 0.1$, n.s. **not shown**).

4. Discussion

We confirmed that PRS has a dramatic and persistent impact on behavior, stress response, and hippocampal gene expression in both adult and aged rats, as previously shown (Bale, 2015; Brunton and Russel, 2011; Darnaudéry and Maccari, 2008; Monteleone et al., 2014; Reynaert et al., 2016; Vallée et al., 1999). Moreover, we showed that PRS impaired the expression of genes involved in the regulation of the stress status in the hippocampus, such as oxytocin and oxytocin receptors. So far, the effects of gestational stress on oxytocin and oxytocin receptor mRNA levels have been mostly investigated in brain areas such as the hypothalamus and the amygdala (Champagne and Meaney, 2006; Hillerer et al., 2011; Murgatroyd et al., 2015). Here, we have shown that repeated administration of carbetocin in the early *postpartum* period **increased risk-taking** behavior in both adult and aged PRS rats. In addition, carbetocin administration to dams induced an anti-stress effect by correcting the abnormal phenotype caused by PRS in the adult progeny. Remarkably, carbetocin treatment of the mothers did not increase body weight in PRS rats at PND7, indicating that the rescuing effect on the PRS phenotype was not due to increased feeding resulting from the well-known pro-lactating effect of oxytocin. Carbetocin treatment to the dams normalized the activity of the HPA axis in the adult PRS progeny, and normalized the expression of mGlu5 receptors in the ventral hippocampus of aged PRS rats. mGlu5 receptors are involved in the regulation of emotional behavior (Ballard et al., 2005; Inta et al., 2013) and hippocampal neurogenesis (Di Giorgi Gerevini et al., 2004; Morley Fletcher et al., 2011; Zuena et al., 2008). The reduction of mGlu5 in aged PRS rats strengthens the hypothesis that glutamatergic neurotransmission is abnormal in PRS animals (Marrocco et al., 2012; Zuena et al., 2008) throughout the lifespan. The prevention of mGlu5 abnormalities in PRS rats from dams treated with carbetocin supports the evidence that by acting on glutamate neurotransmission, oxytocin receptors might be specifically targeted in the treatment of stress-related disorders. Indeed, we previously demonstrated that presynaptic oxytocin receptors localized in glutamatergic nerve terminals of the ventral hippocampus are critically involved in the rescuing effect of an adult carbetocin treatment on PRS-induced abnormalities of the HPA axis and behavior (Mairesse et al., 2015).

Postpartum administration of carbetocin to the mother fully reversed potentially long-lasting changes in the expression of stress and anti-stress genes in the hippocampus, which reflect the developmental programming induced by PRS, in adult (with the exception of the GR gene)

and aged PRS rats. Remarkably, PRS induced a reduction of OXT mRNA levels associated with a compensatory increase of OXTR gene levels in the progeny throughout the entire lifespan. These effects were prevented by early treatment with carbetocin, a drug that behaves as an OTXR agonist. Thus, an impairment of maternal care in early postnatal life causes profound alterations in the anti-stress oxytocinergic system. This suggests that the oxytocinergic system may lie at the core of the pathogenesis of stress-related disorders, which might be prevented by increased maternal behavior after birth (e.g., by administering an OXTR agonist to the lactating dam). In agreement with our findings, de Souza et al. (2013) have shown that cross fostering reverses the reduction in the hypothalamic oxytocin *tonus* caused by prenatal stress.

It was also interesting to find that carbetocin treatment could reverse the increase in vasopressin (AVP) mRNA levels found in the hippocampus of PRS rats. So far, changes in the AVP system in response to PRS had been reported in the hypothalamus (de Souza et al., 2013; Lukas et al., 2010; Zhang et al., 2012), but never in the hippocampus. It is possible that a balance between OXT and AVP in the hippocampus regulates the formation of contextual memories related to stressful events, thereby influencing the overall process of stress coping. Remarkably, the increased expression of the AVP gene in the hippocampus of PRS rats is consistent with regulatory role of AVP in enhancing HPA axis reactivity to stress (Zelena et al., 2015), which is characteristic of the PRS model (Henry et al., 1994; Maccari et al., 1991; 1995; Vallée et al., 1999). Interestingly, *postpartum* carbetocin administration reversed the same molecular and behavioral parameters in the hippocampus, as does adult chronic carbetocin treatment, i.e. it led to a correction of the HPA axis negative feedback mechanisms, stress and anti-stress gene expression, and synaptic glutamate release (Mairesse et al., 2015). The fact that *postpartum* carbetocin administration had the same effect as adult carbetocin treatment indicates a short-term effect of carbetocin when administered in adulthood and a reprogramming (long-term) effect lasting until an advanced age when administered in early development.

Changes in OXT and OXTR genes were correlated with changes in **risk-taking** behavior **in the open arm of** EPM induced by PRS. A negative correlation between OXTR gene expression and behavior for the adult progeny as well as a positive correlation between OXT gene expression and behavior for the aged progeny were observed. Taken together, these results indicate a hypofunctioning of the oxytocinergic system throughout the lifespan in the PRS rat model. These data suggest an accelerated aging of the oxytocinergic system, and we have already reported that the stress response is likewise affected (Vallée et al., 1999). Our

findings on oxytocinergic system are in agreement with the work of Neumann's group, which evidenced an age-related decrease of the oxytocinergic system in the hypothalamus of rodent models (Keck et al., 2000).

In the present study, we also showed for the first time an association of the reduction of active maternal behavior in the PRS rat model with behavioral and biochemical outcomes in the offspring. Our finding that early *postpartum* carbetocin administration to the dam enhances maternal behavior and prevents all the pathological outcomes of PRS throughout the entire lifespan of the progeny proves that the defect in maternal care induced by gestational stress programs the development of the offspring. Interestingly, enhancement of maternal behavior by early adoption is able to correct the outcomes induced by PRS (Maccari et al., 1995). The increased maternal care induced by early postnatal carbetocin administration that we report here might be a consequence of the anti-stress effect resulting from the activation of oxytocin receptors in the CNS of the dams (Cohen et al., 2010; Jurek et al., 2015; Windle et al., 1997; 2004; Zheng et al., 2010). This is consistent with data obtained by Champagne et al. (2001), where central infusion of an oxytocin receptor antagonist reduced maternal behavior in high licking dams. Accordingly, high licking dams (high maternal behavior) also displayed a reduced OXTR expression (hypothalamus and amygdala) and a low maternal behavior when exposed to stress (Champagne and Meaney, 2006). This suggests a fundamental interrelationship between the oxytocinergic system and maternal behavior.

Correlation analysis demonstrated that reduction of maternal behavior was predictive of **behavioral** disturbances in PRS rats. We found that the reduced exploration of the EPM (**reduced risk-taking** behavior in PRS rats) was positively correlated with low maternal behavior. This correlation was observed for both adult and aged rats. In sum, these findings indicate that the PRS-induced modifications were functionally related to the reduction in maternal care, because they were corrected by carbetocin treatment to the dam, which enhanced maternal behavior. Maternal behavior was also predictive of changes in gene expression of the oxytocin anti-stress system. In particular, we observed a negative correlation between OXTR genes in the adult progeny and low levels of maternal behavior as well as a positive correlation between low levels of OXT gene expression in the aged progeny and low maternal behavior. These findings support the crucial role of maternal behavior in programming the developmental trajectory of the progeny by shaping the oxytocinergic system. This is in agreement with the seminal finding that naturally occurring variations in maternal behavior (dams exhibiting high or low maternal care) influence GR expression in the hippocampus by an epigenetic mechanism based on gene promoter methylation (Champagne

and Curley, 2009; Weaver et al., 2004). We demonstrate here that impaired maternal behavior is a key determinant for the developmental programming caused by perinatal stress, and that an early postnatal activation of the anti-stress oxytocinergic system might represent a key strategy to restrain the life-long adverse consequences of perinatal stress. Interestingly, a relationship between OXT gene expression and social behaviors has been shown, indicating an epigenetic role for OXTRs (Kumsta et al., 2013) and, in humans, epigenetic variations that decrease expression of the OXTR gene may play a critical role in the vulnerability to *postpartum* depression (Bell et al., 2015; Kimmel et al., 2016).

5. Conclusions

Our findings provide new insights into the complex and multifaceted interaction between the stress and anti-stress oxytocinergic system, indicating that this balance is involved in the late expression of the pathological programming induced by early life stress. The evidence that the improvement in maternal behavior caused by *postpartum* carbetocin treatment to the dams was able to prevent PRS outcomes both in adulthood and at an advanced age, suggests that all these alterations are components of unique developmental programming which is critically controlled by maternal behavior.

Finally, our findings raise the interesting possibility that carbetocin/oxytocin treatment to lactating mothers with increased risk in developing a *postpartum* depression could pave the way to new therapeutic strategies in the treatment of stress-related disorders.

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Conflict of interest

The authors declare no conflict of interest.

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Legends

Fig. 1. Timeline of the experimental procedures.

After the PRS procedure dams were treated for 1 week with carbetocin or saline and maternal behavior was assessed during the first five days. Analysis of **risk-taking behavior in the open arm of elevated-plus maze** and endocrine and biochemical parameters was performed in two separate sets of rats in the progeny at the adult (3 – 5-month-old) and aged (16 – 22-month-old) stage, and were carried out in the temporal sequence reported in the grey boxes. Abbreviations: E = embryonic day; PND = postnatal day.

Fig. 2. *Postpartum* carbetocin administration to lactating dams corrected the early defect in maternal care caused by gestational stress and modified body weight in the progeny.

A. Maternal behavior measured in the first five days *postpartum*. B. Dams' weight gain during the first postpartum week. Values are means ± SEM of 5 females per group. * $p < 0.05$ Stressed dams with saline *vs.* the unstressed control group and # $p < 0.05$ Stressed dams with carbetocin *vs.* Stressed dams treated with saline. C. Body weight of the progeny at PND7. Values are means ± SEM of 5 animals per group. * $p < 0.05$ PRS-Saline *vs.* CONT-Saline; # $p < 0.05$ PRS-Carbetocin *vs.* the PRS Saline group and **CONT-Carbetocin vs. the CONT-saline group**.

Fig. 3. *Postpartum* carbetocin administration to dams prevented alterations in behavior, HPA axis response to stress, and changes in hippocampal gene expressions in the perinatally stressed adult progeny.

A. **Risk-taking** behavior **in the open arm** of EPM in adult offspring of stressed and unstressed dams treated with saline or carbetocin. B. Corticosterone response to novelty stress. In the inset: A.U.C. of corticosterone response. C. Quantitative mRNA analyses of stress-related and anti-stress related genes in the hippocampus. All values are means ± SEM of 5 animals per group. * $p < 0.05$ PRS-Saline *vs.* CONT-Saline; # $p < 0.05$ and ## $p < 0.01$ PRS-Carbetocin *vs.* the PRS Saline group.

Fig. 4. *Postpartum* carbetocin administration to dams prevented alterations in behavior, mGlu5 receptors, and changes in hippocampal gene expression in the perinatally stressed aged progeny.

A. **Risk-taking** behavior **in the open arm of** EPM in the aged progeny of stressed and unstressed dams treated with saline or carbetocin. B. Immunoblots with mGlu5 receptor

antibody in the ventral hippocampus. Results are expressed as the ratio of the optical density (O.D.) of the receptor antibody and the β-actin band, the empty band corresponding to the position of the molecular weight marker. C. Quantitative mRNA analyses of stress-related and anti-stress related genes in the hippocampus. All values are means ± SEM of 5 animals per group. * $p < 0.05$ PRS-Saline *vs.* CONT-Saline; # $p < 0.05$ PRS-Carbetocin *vs.* the PRS-Saline group.

Fig. 5. Correlation analyses.

A. Correlation analyses between behavior in the EPM and anti-stress genes expression in adult and aged progeny. **B.** Correlation analyses between **risk-taking** behavior in the adult and aged progeny and maternal behavior at day 3. **C.** Correlation analyses between anti-stress gene expression in the adult and aged progeny and maternal behavior at day 3.

Figure 1.

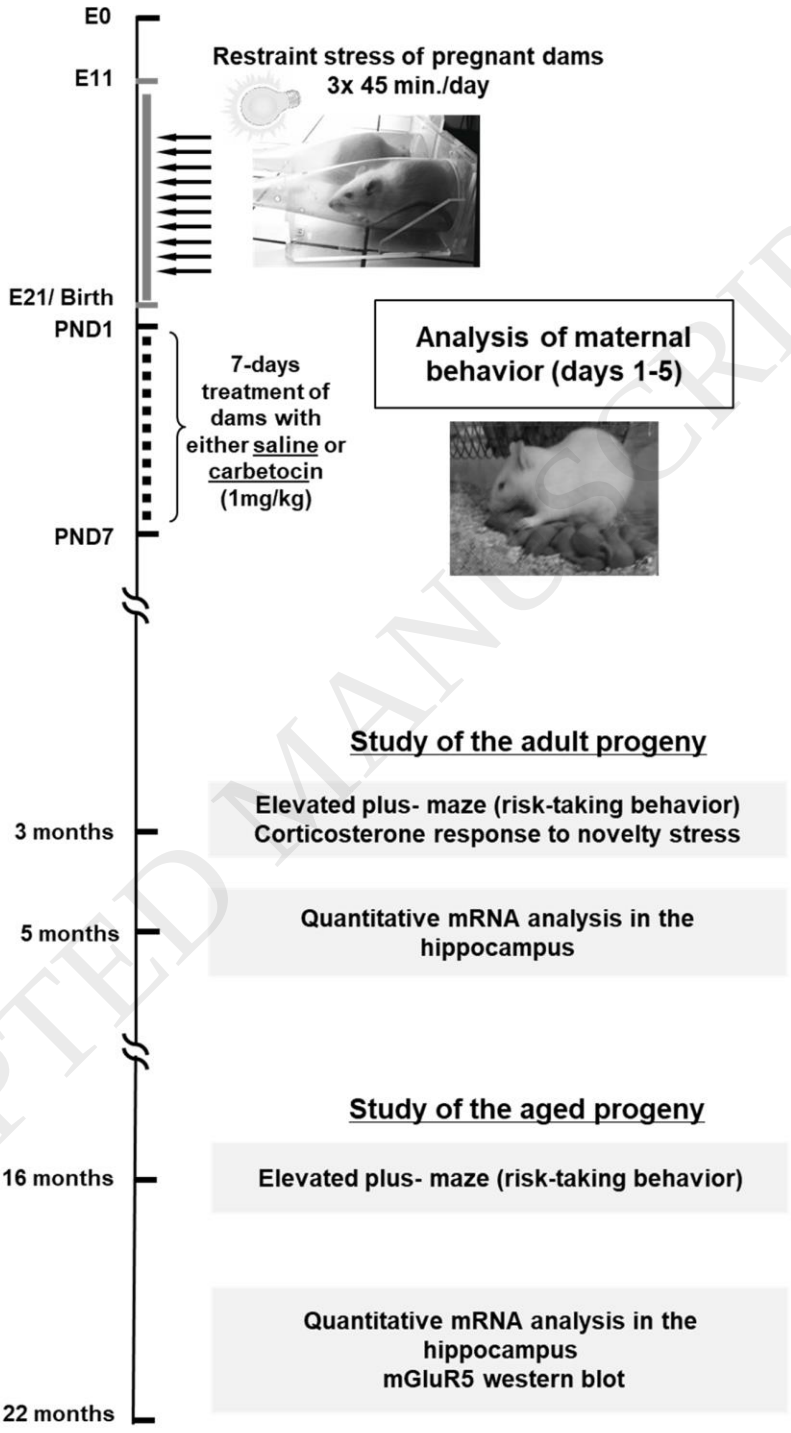
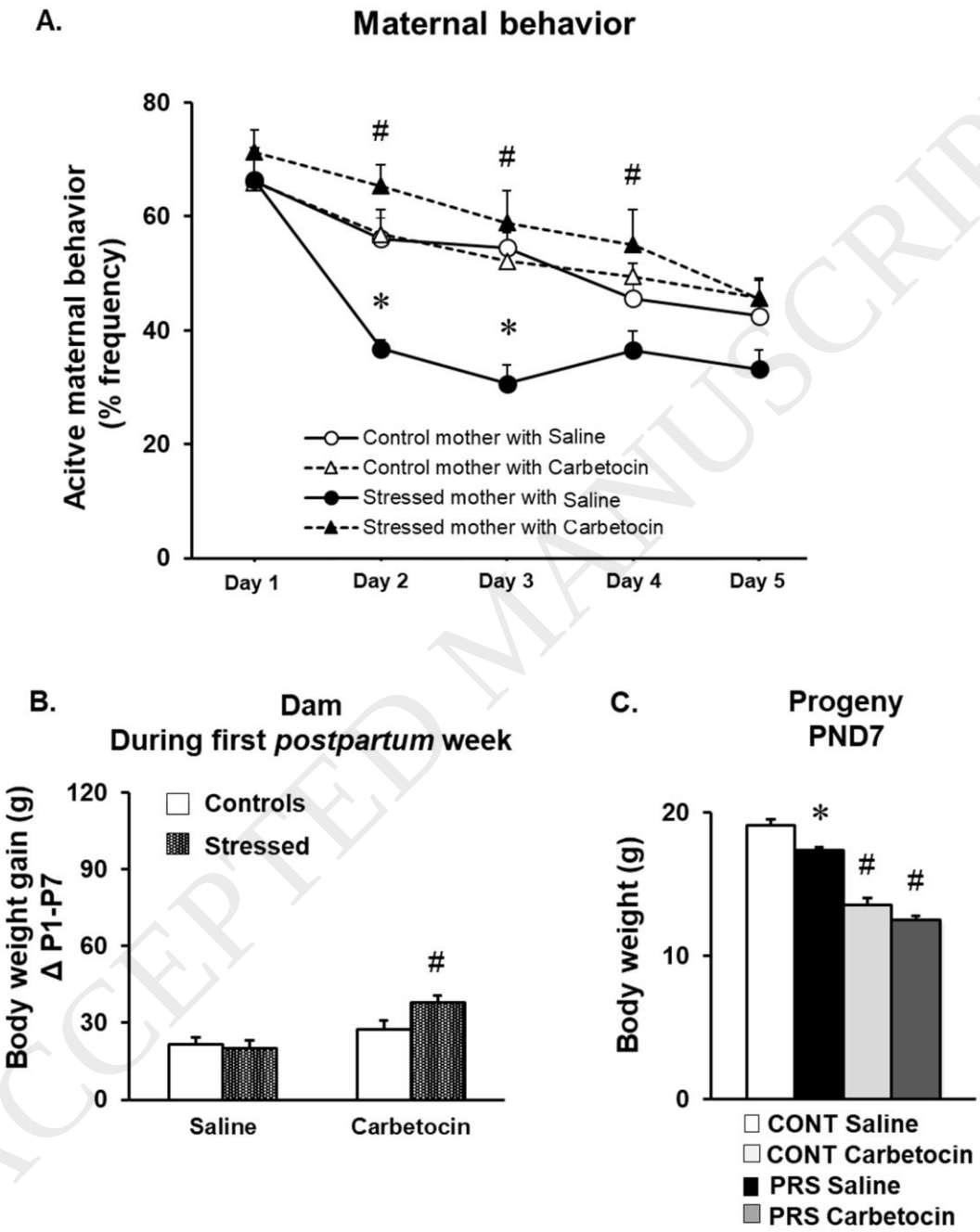


Figure 2.



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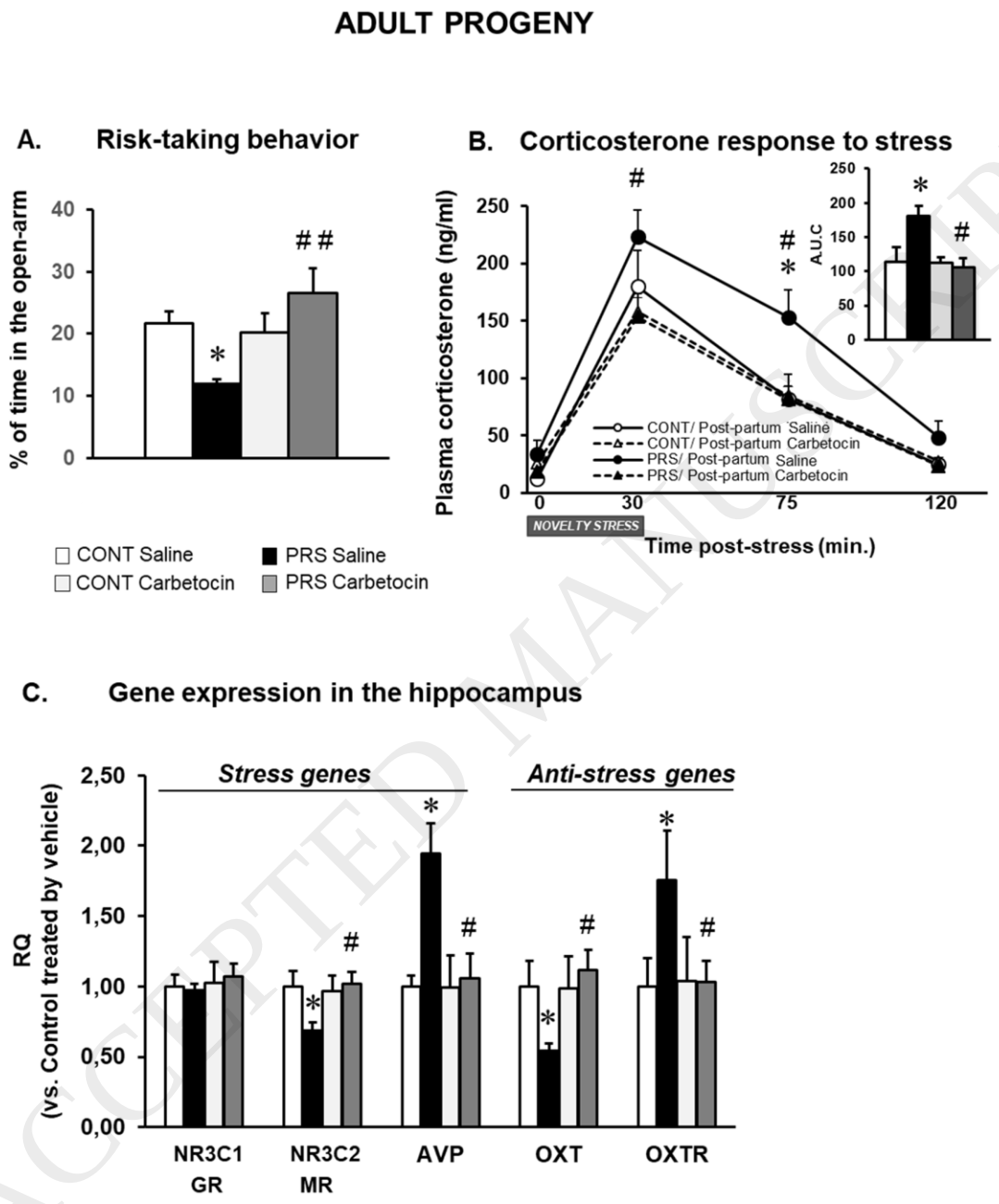

Figure 4.

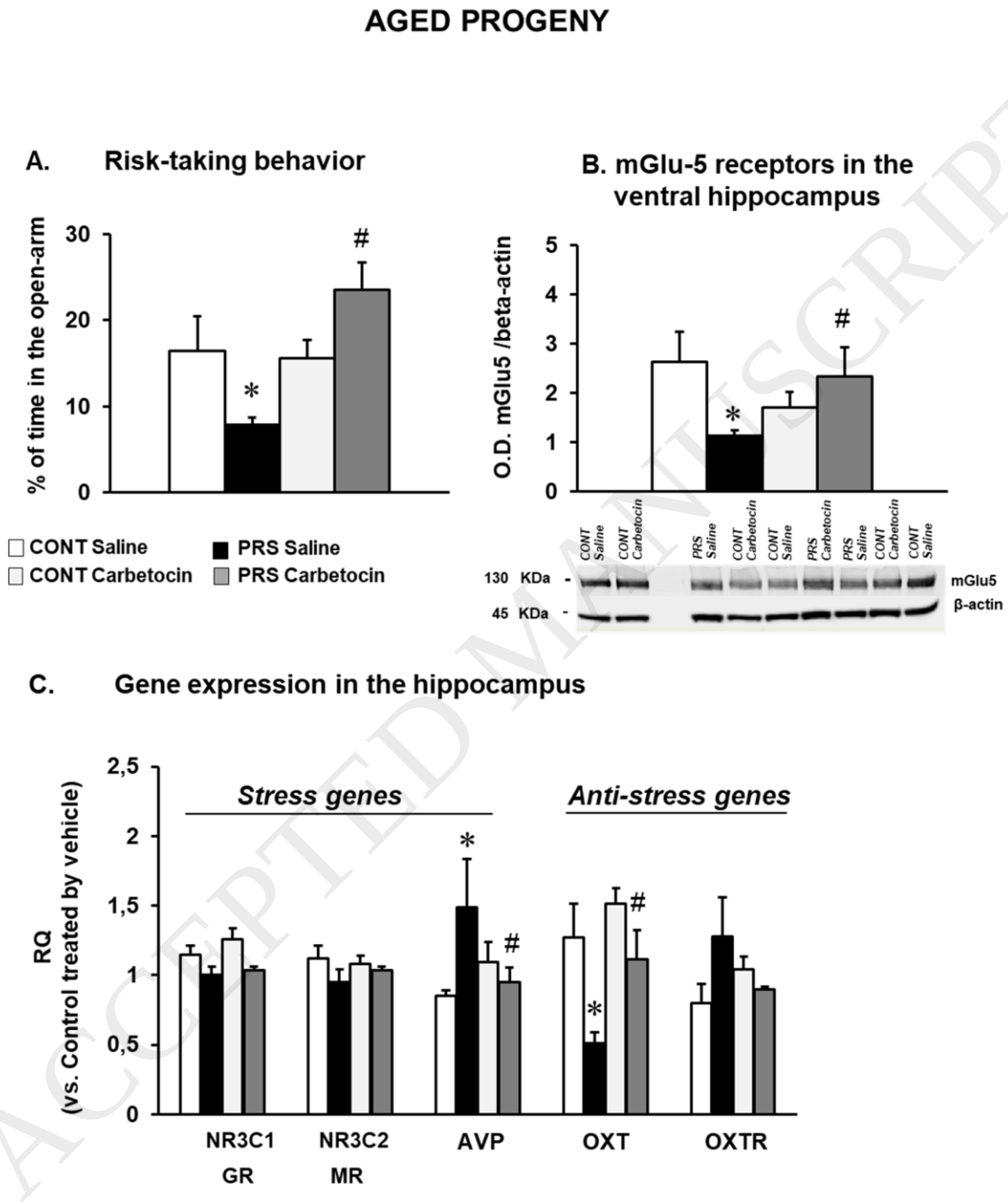

Figure 5.