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ORIGINAL INVESTIGATION

Chronic agomelatine treatment corrects behavioral, cellular, and biochemical abnormalities induced by prenatal stress in rats

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Abstract
Rationale and objectives The rat model of prenatal restraint stress (PRS) replicates factors that are implicated in the etiology of anxious/depressive disorders. We used this model to test the therapeutic efficacy of agomelatine, a novel antidepressant that behaves as a mixed MT1/MT2 melatonin receptor agonist/5-HT_{2c} serotonin receptor antagonist.
Results Adult PRS rats showed behavioral, cellular, and biochemical abnormalities that were consistent with an anxious/depressive phenotype. These included an increased immobility in the forced swim test, an anxiety-like behavior in the elevated plus maze, reduced hippocampal levels of phosphorylated cAMP-responsive element binding protein (p-CREB), reduced hippocampal levels of mGlu2/3 and mGlu5 metabotropic glutamate receptors, and reduced neurogenesis in the ventral hippocampus, the specific portion of the hippocampus that encodes memories related to stress and emotions. All of these changes were reversed by a 3- or 6-week treatment with agomelatine (40–50 mg/kg, i.p., once a day). Remarkably, agomelatine had no effect in age-matched control rats, thereby behaving as a "disease-dependent" drug.
Conclusions These data indicate that agomelatine did not act on individual symptoms but corrected all aspects of the pathological epigenetic programming triggered by PRS. Our findings strongly support the antidepressant activity of agomelatine and suggest that the drug impacts mechanisms that lie at the core of anxious/depressive disorders.

Keywords Agomelatine · Prenatal stress · Adult neurogenesis · Ventral hippocampus · Fluoxetine · Phospho-CREB · Metabotropic glutamate receptors

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Introduction

Agomelatine, a mixed MT1/MT2 melatonin receptor agonist/5HT2C serotonin receptor antagonist, was recently approved for the treatment of major depression in Europe, thereby becoming the first antidepressant in the market that does not act primarily on monoaminergic transmission (reviewed by De Bodinat et al. 2010). In preclinical studies, agomelatine was found to modulate the expression of depression-related molecules such as brain-derived neurotrophic factor, basic fibroblast-growth factor-2, and activity-regulated cytoskeleton-associated protein (Calabrese et al. 2010) to enhance adult neurogenesis in the hippocampal dentate gyrus (Soumier et al. 2009; AlAhmed and Herbert 2010; Dagyte et al. 2010, 2011) and to reduce the increase in glutamate release induced by acute stress in the prefrontal/frontal cortex (Tardito et al. 2010). Agomelatine also reverses the neurochemical and behavioral abnormalities in transgenic mice with impaired glucocorticoid receptors (Païzanis et al. 2010) and exerts an antidepressant-like activity in models of learned helplessness (Barden et al. 2005; Bertaina-Anglade et al. 2006), chronic mild stress (Papp et al. 2003), and forced swim test (Bourin et al. 2004). Most of these preclinical models have face validity or pharmacological validity, i.e., they reflect core symptoms of depression or are sensitive to antidepressant drugs (reviewed by Krishnan and Nestler 2008; Nestler and Hyman 2010). We examined the efficacy of agomelatine in the rat model of prenatal restraint stress (PRS), one of the models that replicate putative factors implicated in the etiology of major depression.

According to the developmental hypothesis of mood disorders, stressful events occurring during critical periods of brain development trigger a maladaptive program that alters the mechanisms of resilience to stress across the entire lifespan. Along this line, major depression can be seen as a latent outcome of stressful early life events that become more influential in genetically predisposed individuals (Darnaudéry and Maccari 2008; Weinstock 2008; Lupien et al. 2009; Heim et al. 2009; Shonkoff et al. 2009; Krishnan and Nestler 2008). The rat PRS model is particularly valuable for the study of the pathological consequences of early life stress and for the identification of novel therapeutic strategies in the treatment of depression and anxiety (Darnaudéry and Maccari 2008; Maccari et al. 2003; Maccari and Morley-Fletcher 2007). PRS rats show a long-term impairment in the feedback regulation of the hypothalamus–pituitary–adrenal axis (Maccari et al. 1995), a generalized disorganization of circadian rhythms and wake–sleep cycle (Dugovic et al. 1999; Van Reeth et al. 2000; 2007), an increased depression- and anxiety-like behavior (Vallee et al. 1997; Zuena et al. 2008), and a reduced active coping and social play (Morley-Fletcher et al. 2003a). The value of PRS rats as a model of depression is strengthened by the finding that these animals show a persistent deficit in hippocampal neurogenesis and abnormalities in transcription factors and surface receptors that have been related to the pathophysiology of depression and anxiety (Darnaudery et al. 2006; Koehl et al. 2009; Lemaire et al. 2000; 2006; Schmitz et al. 2002; Zuena et al. 2008).

Here, we report that chronic treatment with agomelatine corrects all biochemical, cellular, and behavioral abnormalities displayed by PRS rats in the adult life. We focused on changes occurring in the hippocampus, a critical region for the interaction between stress and the pathophysiology of mood disorders (López et al. 1999; Sapolsky 2004; Fuchs et al. 2004; Malberg and Schechter 2005; McEwen and Olié 2005; Warner-Schmidt and Duman 2006).

Materials and methods

Animals

Nulliparous female Sprague–Dawley rats, weighing approximately 250 g, were purchased from a commercial breeder (Harlan). The animals were housed at constant temperature (22±2°C) under a 12-h light/dark cycle (lights on at 8.00 a.m.). Tap water and standard food were available ad libitum. During the initial week after their arrival, the female rats were group-housed (four per cage) to synchronize their estrous cycle.

PRS protocol

The animals were subjected to PRS according to our standard protocol (Maccari et al. 1995). From day 11 of pregnancy until delivery, pregnant rats were subjected to three stress sessions per day (45 min each; starting at 09:00, 12:00, and 17:00 h), during which they were placed in transparent plastic cylinders (diameter=7 cm; length=19 cm) and exposed to bright light (650 Lux). Only male offspring from litters containing ten to 14 pups with a comparable number of males and females were used. Rats of 2–3 months of age were used in all experiments. The experiments followed the rules of the European Communities Council Directive 86/609/EEC. The PRS procedure was approved by the local ethical committee. The detailed experimental design is shown in Fig. 1.

Agomelatine and fluoxetine treatment

In experiments 1, 2, and 3, the vehicle rats received injections of hydroxyethylcellulose (HEC, 1% suspension in distilled water) in a volume of 2 ml/kg. Agomelatine

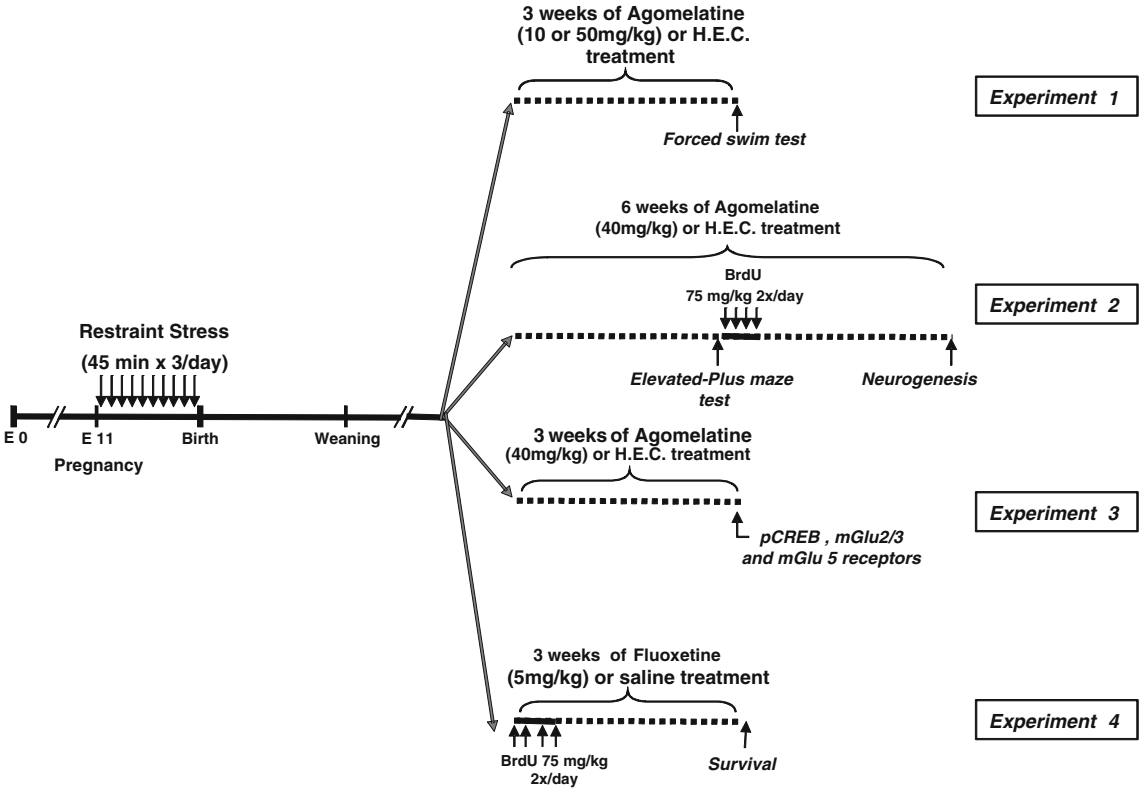


Fig. 1 Experimental design. Four experimental conditions were used in the present report. *Experiment 1* examined the effects of a 3-week agomelatine treatment (10 or 50 mg/kg) on performance in the forced swim test in PRS and control rats. *Experiment 2* examined the effects of chronic agomelatine treatment (40 mg/kg) on performance in the EPM and on hippocampal neurogenesis. Agomelatine treatment was carried out for 6 weeks. After 3 weeks of treatment, an EPM test was performed. Then, the animals were injected two times a day (at 11:00 and 15:00 h) for four consecutive days with BrdU. At 15 h after the last agomelatine injection, the rats were killed for the assessment of neurogenesis. In *Experiment 3*, the animals were treated with 40 mg/kg of agomelatine for 3 weeks and then used for immunoblot analysis of p-CREB and mGlu receptors. In *Experiment 4*, we examined the effect of a 3-week treatment with fluoxetine (5 mg/kg, i.p.) on hippocampal neurogenesis in a separate set of animals. Here, BrdU was administered for 4 days before starting the fluoxetine treatment

(Servier, France) was dissolved in HEC and injected i.p. (2 ml/kg) at doses of 10 or 50 mg/kg in experiment 1 and 40 mg/kg in experiments 2 and 3. This last dose was selected on the basis of previous reports (Van Reeth et al. 1997; Papp et al. 2003; Banasr et al. 2006; Soumier et al. 2009) and on the basis of data from experiment 1. Injections were performed 2 h prior to the onset of the dark phase of the 12-h light/dark cycle, based on the circadian rhythm resynchronization properties and antidepressant activity of agomelatine (Van Reeth et al. 1997; Papp et al. 2003). In experiment 4, the animals were treated with fluoxetine (5 mg/kg, i.p., Sigma) dissolved in saline. The control rats received an equal volume of saline.

Behavioral analysis

In experiments 1 and 2, we examined the effect of a 21-day treatment with agomelatine on depression- and anxiety-like behavior using the forced swim test and the elevated plus maze (EPM) tests, respectively, after 3 weeks of antidepressant treatment. Briefly, one set of animals (n=6 per group) was subjected to an adapted version of the forced swim test (Porsolt et al. 1978) in a cylindrical container (height=59 cm; diameter=25 cm) filled with water at 25°C up to a level of 36 cm. The test was carried out between 13:00 and 16:00 h. Twenty-four hours after a 15-min session (pre-test), the rats were tested for a 5-min session during which immobility latency and duration, climbing, and swimming were measured (Observer 20 Noldus, Wageningen, The Netherlands). Another set of rats (n=7–8 per group) was tested at the EPM as described by Pellow et al. (1985). The EPM test was carried out between 13:00 and 16:00 h, lasted 5 min, and began with the placement of the rat in the center of the maze with the head facing a closed arm. Behavior was automatically analyzed using a video tracking software (View Point, France). The time spent in open and closed arms, respectively, was recorded and the percentage of time spent in open arms was calculated.

Assessment of hippocampal neurogenesis

As shown in Fig. 1, the animals were injected two times a day (at 11:00 and 15:00 h) for four consecutive days with the thymidine analog bromodeoxyuridine (BrdU, 75 mg/kg/3 ml; i.p.) to label dividing cells. In the groups treated with agomelatine or its vehicle, BrdU was injected after 3 weeks of treatment after the behavioral testing at the EPM. Injections of agomelatine or vehicle were continued for three more weeks (see Fig. 1). In the groups treated with fluoxetine or saline, BrdU was injected in the first 4 days of a 3-week treatment. At 15 h after the last injection of agomelatine or vehicle (n=6 per group) or fluoxetine or saline (n=5 per group), the rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and post-fixed for 24 h in paraformaldehyde. Serial brain sections (40 μm) across the whole hippocampus were made with a vibratome (Leica, France) and processed using a standard immunohistochemical procedure. The dorsal hippocampus (7.2 to 4.4 mm above the interaural line) and the ventral hippocampus (4.3 to 2.7 mm above the interaural line according to Paxinos and Watson 1986) were studied by examining every sixth section of a series or 18 sections per animal. Means for the dorsal and ventral hippocampal regions for each hemisphere were obtained from nine sections per rat.

Immunostaining for BrdU was carried out as described previously (Zuena et al. 2008) using mouse anti-BrdU antibodies (1/500, Boehringer Manneheim, Indianapolis, IN, USA) and diaminobenzidine.

BrdU–NeuN double immunostaining was performed as described previously (Zuena et al. 2008) using rat anti-BrdU (1/500, Immunological Direct, AbCys, France) and mouse anti-NeuN (1/1,000, Chemicon, France) antibodies followed by a TRITC-conjugated donkey anti-rat antibody (Jackson) and an Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes). For BrdU–PSA/NCAM double immunostaining (n=6–8 rats per group), sections were pre-incubated for 30 min in lysine (18.3 mg/ml) and H_2O_2 (1%) and then for 30 min in blocking buffer (10% normal rabbit serum) containing 0.1% Triton X100. The sections were first incubated with mouse anti-PSA-NCAM (IgM, 1/500, AbCys, France) for 72 h at 4°C and then exposed to FITC-conjugated goat anti-mouse IgM (1/200, Molecular Probes, France). After rinsing, the sections were processed for BrdU staining using rat anti-BrdU antibodies (1/100, AbCys, France) for 48 h at 4°C and then exposed for 2 h to an Alexa 594-conjugated donkey anti-rat antibody (1/100, Invitrogen, France).

Double-labeling quantification: the analysis was performed blind using coded sections. The percentage of $BrdU^{\pm}/NeuN^{\pm}$ cells was determined using a confocal microscope (Zeiss, LSM 510, France) equipped with a ×60 oil objective. For each rat, four sections located in representative regions of the dorsal and ventral hippocampus were analyzed, with a minimum of 80 $BrdU^{\pm}$ cells in the entire z axis with a 0.5-μm step in order to exclude false double-labeling. Another series of sections were used for the quantification of $BrdU^{\pm}/PSA/NCAM^{\pm}$ cells, which was performed under similar conditions. Data are expressed as the ratio between double-labeled cells and total $BrdU^{\pm}$ cells.

Immunoblot analysis

In experiment 3, PRS and control rats treated with agomelatine or vehicle for 21 days (Fig. 1) were killed by decapitation and their brains were rapidly removed. The hippocampus was dissected and stored at −80°C. Five animals per group (controls and PRS, vehicle- and agomelatine-treated) were examined in duplicate. Tissue homogenization, sample preparation, and SDS-PAGE were carried out as described previously (Zuena et al. 2008). The following primary antibodies (Upstate Biotechnology, Lake Placid, NY, USA) were used: rabbit anti-CREB (1:500), rabbit anti-pCREB (1:2,000), rabbit anti-mGlu2/3, and anti-mGlu5 receptors (both at 1:1,000). Secondary antibody β-actin directed against rabbit (Sigma, St Louis, MO, USA) was used at a 1:5,000 dilution. Densitometric analysis was carried out using Quantity One Software (Biorad) associated to a GS-800 scanner. The data were normalized by the expression of β-actin.

Statistical analysis

Data, expressed as means ± SEM, were analyzed using the parametric analysis of variance, with group (control vs. PRS) and treatment (vehicle vs. agomelatine) as between-subject variables, followed by Newman–Keuls post hoc comparisons for further examination of differences between the groups. A p-value of 0.05 or less was considered to indicate a significant difference.

Results

Effect of agomelatine treatment on depression- and anxiety-like behavior

In the forced swim test, which is widely used for the evaluation of the efficacy of antidepressants (Porsolt et al.1978), PRS rats showed an increased immobility time as compared to age-matched controls, as expected (Morley-Fletcher et al. 2004, 2003b). A 21-day treatment with agomelatine (50 mg/kg, i.p., once a day) had no effect in

control rats but reduced the immobility time in PRS rats to the same levels observed in control rats (group × treatment interaction, $F(1,20)=4.64$, $p<0.05$) (Fig. 2). No effect was seen with a lower dose of agomelatine (10 mg/kg, once a day) (not shown). Anxiety-like behavior was assessed by measuring the relative time spent in the open and closed arms of the EPM. The PRS rats showed a higher level of anxiety (i.e., they spent less time in the open arms) as compared to control rats. Anxiety was significantly reduced by a 21-day treatment with 50 mg/kg of agomelatine (group × treatment interaction, $F(1,26)=5.22$, $p<0.05$). Agomelatine had no effect on the EPM in control rats (Fig. 3).

Effect of agomelatine treatment on the survival of proliferating progenitor cells in the hippocampal dentate gyrus

Adult PRS rats showed a reduced number of $BrdU^+$ progenitor cells in the hippocampus as compared to age-matched controls (see below). A 21-day treatment with saline did not affect the number of proliferating neuroprogenitors (not shown), indicating that the pharmacological experiments were not biased by chronic mild stress associated with i.p. injections under our conditions. Experiments with agomelatine and fluoxetine (used as a comparator) were not performed at the same time so that each experimental group has its own controls.

BrdU immunostaining in the hippocampal dentate gyrus of control and PRS rats treated with vehicle or agomelatine for 3 weeks prior to and for additional 3 weeks starting from BrdU injection is shown in Fig. 4a–c. We found a prominent effect of PRS on the number of proliferating progenitor cells in the ventral hippocampus of rats treated with vehicle (see also Zuena et al. 2008). Agomelatine had no effect in control rats but reversed the reduction in the number of proliferating progenitor cells in the whole hippocampus of PRS rats (Fig. 4d) (group × treatment interaction, $F(1,26)=5.54$, $p<0.05$). The effect of agomelatine was restricted to the ventral hippocampus (Fig. 4e, f) (dorsal hippocampus**:** group × treatment, $F(1,26)=0.16$; ventral hippocampus: group × treatment, $F(1,26)=6.89$; $p<0.01$).

For the assessment of neurogenesis, we counted the number of $BrdU^+$ cells co-expressing NeuN, a marker of both immature and mature postmitotic granule cells in the hippocampal dentate gyrus (reviewed by Kempermann et al. 2004). Confocal analysis showed that PRS reduced adult neurogenesis by decreasing the number of proliferating progenitors (Fig. 4j; group × treatment, $F(1,26)=5.49$, $p<0.05$) and indicated that agomelatine treatment reversed the reduction of newly formed neurons (i.e., the number of $BrdU^+/NeuN^+$ cells) induced by PRS (Fig. 4g–j). We also counted the number of $BrdU^+$ cells expressing PSA-NCAM, a marker of type 2 and 3 progenitor cells along the neurogenic pathway (Filippov et al. 2003; Seki and Arai 1999). Figure 5a shows typical cells expressing BrdU and PSA-NCAM in the dentate gyrus, at the internal border of the granule cell layer. PRS reduced the number of $BrdU^+/PSA\text{-}NCAM^+$ cells in the whole hippocampus, and this reduction was reversed by agomelatine treatment (Fig. 5b) (group × treatment interaction; $F(1,16)=24.09$, $p<0.01$). Although the effect of PRS was prevalent in the ventral hippocampus, agomelatine treatment increased the number of $BrdU^+/PSA\text{-}NCAM^+$ cells in both the dorsal and ventral hippocampus of PRS rats (Fig. 5c, d; ($F(1,16)=10.74$, $p<0.01$ and $F(1,16)=17.51$, $p<0.01$, respectively). Agomelatine had

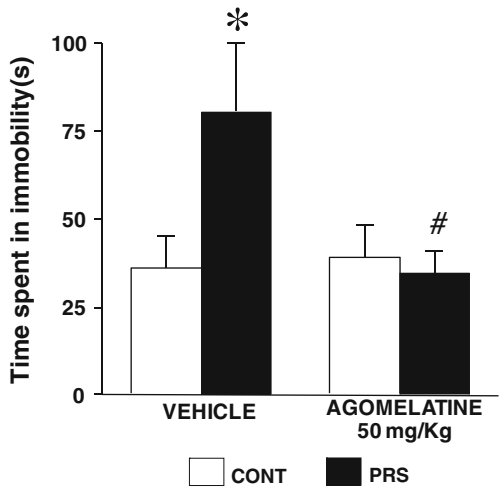


Fig. 2 Agomelatine treatment reduces the immobility time in PRS rats subjected to the forced swim test. Values of immobility time (s) are means ± S.E.M of six animals per group. $p<0.05$ vs. the respective controls (*asterisk*) or vs. PRS rats treated with vehicle (*number sign*). *CONT*, controls

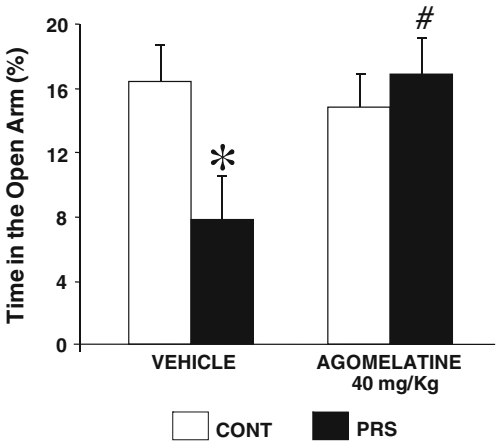


Fig. 3 Agomelatine treatment produces anxiolytic-like effects in PRS rats at the EPM. Values are expressed as means ± S.E.M. of seven to eight animals per group. $p<0.05$ vs. the respective controls (*asterisk*) or vs. PRS rats treated with vehicle (*number sign*). *CONT*, controls

no effect on hippocampal neurogenesis in control rats (Figs. 4j and 5b–d).

We compared the action of agomelatine with that of fluoxetine, a conventional antidepressant that is known to increase the immobility time at the forced swim test in PRS rats (Szymańska et al. 2009). A 3-week treatment with fluoxetine (5 mg/kg, i.p., once a day, starting from the day of BrdU injection) reversed the reduction in the number of $BrdU^+$ in the hippocampus of PRS rats, with no effect in control rats (group × treatment interaction $F(1,18)=7.72$, $p<0.01$; Fig. 6a). The action of fluoxetine was seen in both the dorsal and ventral hippocampus of PRS rats (Fig. 6b; group × treatment interaction $F(1,18)=4.50$, $p<0.05$; Fig. 6c, group effect $F(1,18)=4.78$, $p<0.05$, respectively). Fluoxetine treatment also reversed the reduction in the number of $BrdU^+/NeuN^+$ cells induced by PRS (Fig. 6d–g) (group × treatment interaction $F(1,18)=6.63$, $p<0.01$).

Effect of agomelatine treatment on the hippocampal levels of p-CREB and mGlu2/3 and mGlu5 metabotropic glutamate receptors

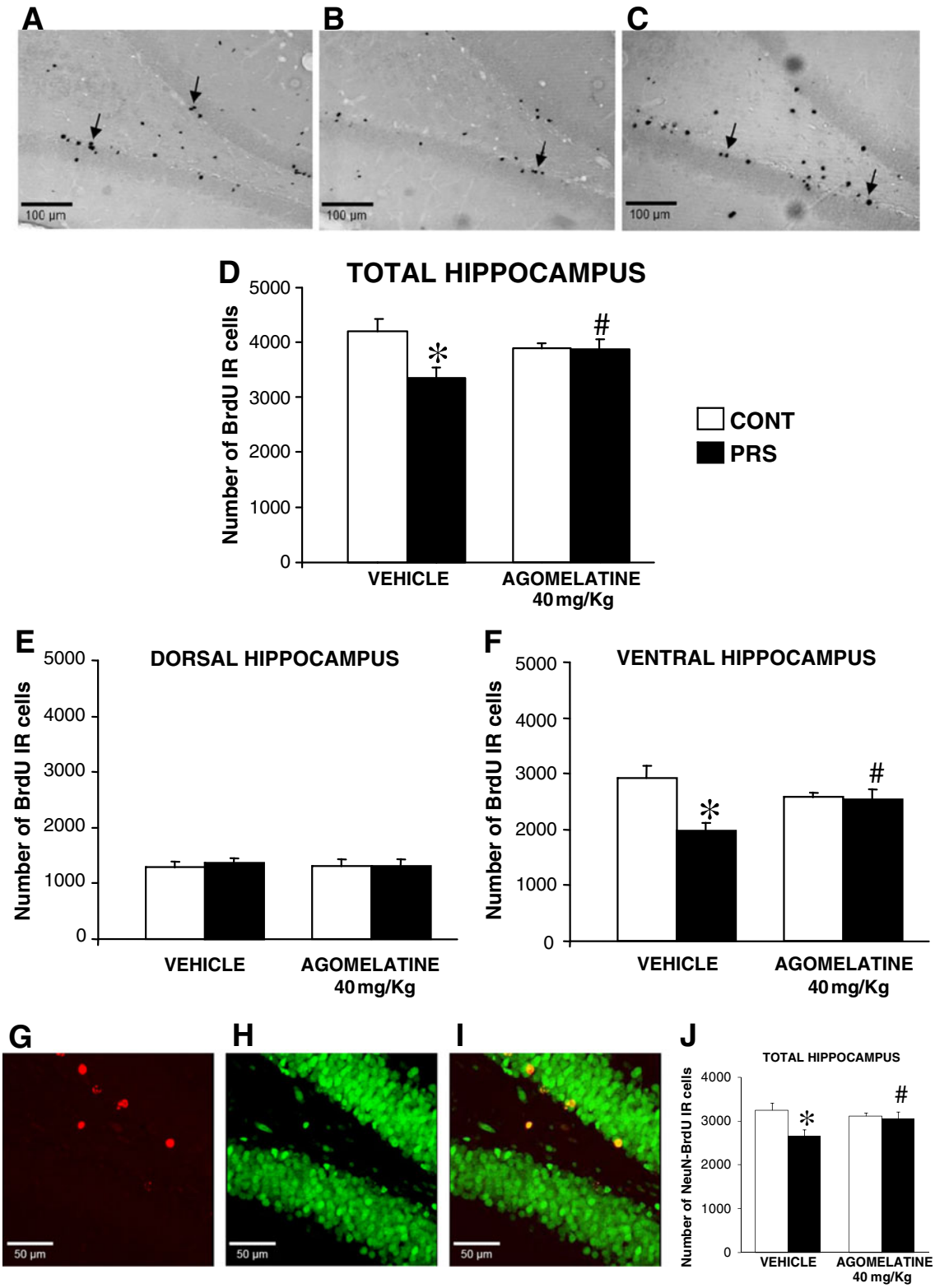
The hippocampal levels of Ser133-phosphorylated CREB (p-CREB), mGlu2/3 receptors, and mGlu5 receptors were measured by immunoblotting. p-CREB was detected as a single band at about 43 kDa (Fig. 7a). mGlu2/3 receptors were detected as a band at about 100 kDa, corresponding to receptor monomers, and a higher molecular size band, corresponding to receptor dimmers (Fig. 7b). mGlu5 receptors were detected as a major band at about 140 kDa, corresponding to receptor monomers; the dimeric band was nearly undetectable under the given conditions (Fig. 7c). The PRS rats treated with vehicle for 21 days showed a reduced expression of p-CREB, mGlu2/3 receptors, and mGlu5 receptors in the hippocampus, as compared to control rats treated with vehicle (see also Zuena et al. 2008). Chronic agomelatine treatment (here, 40 mg/kg, i.p., once a day for 21 days) reversed the reduction in p-CREB, mGlu2/3 receptors, and mGlu5 receptors in PRS rats (Fig. 7a, group × treatment interaction, $F(1,15)=5.23$, $p<0.05$; Fig. 7b, group × treatment interaction, $F(1,16)=15$, $p<0.005$; Fig. 7c, group × treatment interaction ($F(1,16)=36.22$, $p<0.01$). Agomelatine had no effect in control rats (Fig. 7a–c).

Discussion

Our data show for the first time the efficacy of agomelatine in a model that replicates the developmental factors involved in the etiology of anxious/depressive disorders (reviewed by Krishnan and Nestler 2008; Nestler and Hyman 2010) and allows to examine the outcome of early life stress in the adult. Agomelatine treatment corrected *all* abnormalities displayed by PRS rats, suggesting that the drug impacts all mechanisms that lie at the core of the maladaptive programming induced by PRS. We wish to highlight that agomelatine had no effect on any of the parameters that we have tested in control rats. This suggests that agomelatine, at least in PRS rats, acts as an etiopathogenetic drug and its action is specific to the pathological state (i.e., agomelatine behaves as a "disease-dependent" drug).

As expected (Zuena et al. 2008), PRS rats showed a deficit in adult neurogenesis, which was more prominent in the ventral portion of the hippocampus. A recent review (Fanselow and Dong 2010) highlights the distinct functions performed by the ventral (or temporal) and dorsal (or septal) portions of the hippocampus. The ventral hippocampus (corresponding to the anterior hippocampus in primates) is mainly related to stress, emotions, and affect, whereas the dorsal hippocampus (the posterior hippocampus in primates) performs primarily cognitive functions (Henke 1990; Moser et al. 1995). Lesions of the most ventral quarters of the hippocampus affect anxiety-like behavior at the EPM, increasing the entry in the open (unprotected) arms of the maze (Kjelstrup et al. 2002). Thus, the reduced neurogenesis in the ventral hippocampus found in PRS rats is consistent with the abnormal behavior of these animals at the EPM. Agomelatine treatment preferentially enhanced neurogenesis in the ventral hippocampus of PRS rats as it does in transgenic mice with defective glucocorticoid receptors (Païzanis et al. 2010). Other antidepressant drugs also affect the neurogenesis in the ventral hippocampus in different animal models of depression (Bisgaard et al. 2007; Jayatissa et al. 2006; Marais et al. 2009; Surget et al. 2008). Remarkably, agomelatine and fluoxetine treatment did not affect the hippocampal neurogenesis in control rats under the given experimental conditions. This contrasts with other reports showing an effect of agomelatine (Banasr et al. 2006;

Fig. 4 Agomelatine treatment reverses the reduction of neuroprogenitor cells in the hippocampus of PRS rats. Immunohistochemistry for $BrdU^+$ cells in the subgranular zone of the dentate gyrus of control rats treated with vehicle, PRS rats treated with vehicle, and PRS rats treated with agomelatine are shown in **a**, **b**, and **c**, respectively. Counts of $BrdU^+$ cells are shown in **d**–f) for the total, dorsal, and ventral hippocampus. Data represent the sum of $BrdU^+$ cells in the subgranular zone and the granule cell layer of the two hippocampi of each animal. The total number of $BrdU^+$ cells per dentate gyrus was estimated by multiplying the number of cells counted by six since every sixth section was sampled. Values are means ± S.E.M. of six to eight rats per group. Representative confocal images of $BrdU^+$ cells (*red*), $NeuN^+$ cells (*green*), and $BrdU^+/NeuN^+$ cells (*yellow*) (**g**–**i**). The effect of PRS and/or agomelatine treatment on the number of $BrdU^+/NeuN^+$ cells is shown in **j**, where values are means ± S.E.M. of six rats per group. $p<0.05$ vs. the respective controls (asterisk) or vs. PRS rats treated with vehicle (*number sign*). *CONT*, controls



Soumier et al. 2009) and fluoxetine (Malberg et al. 2000) on neurogenesis in "normal" rats. The potential explanations for this discrepancy include the different strains of rats (Sprague–Dawley rats here vs. Wistar rats in other reports) and, more importantly, the different housing conditions. The PRS and control rats used here spent their whole life in the same animal house, whereas the adult rats used by Banasr et al. (2006) and Soumier et al. (2009) were

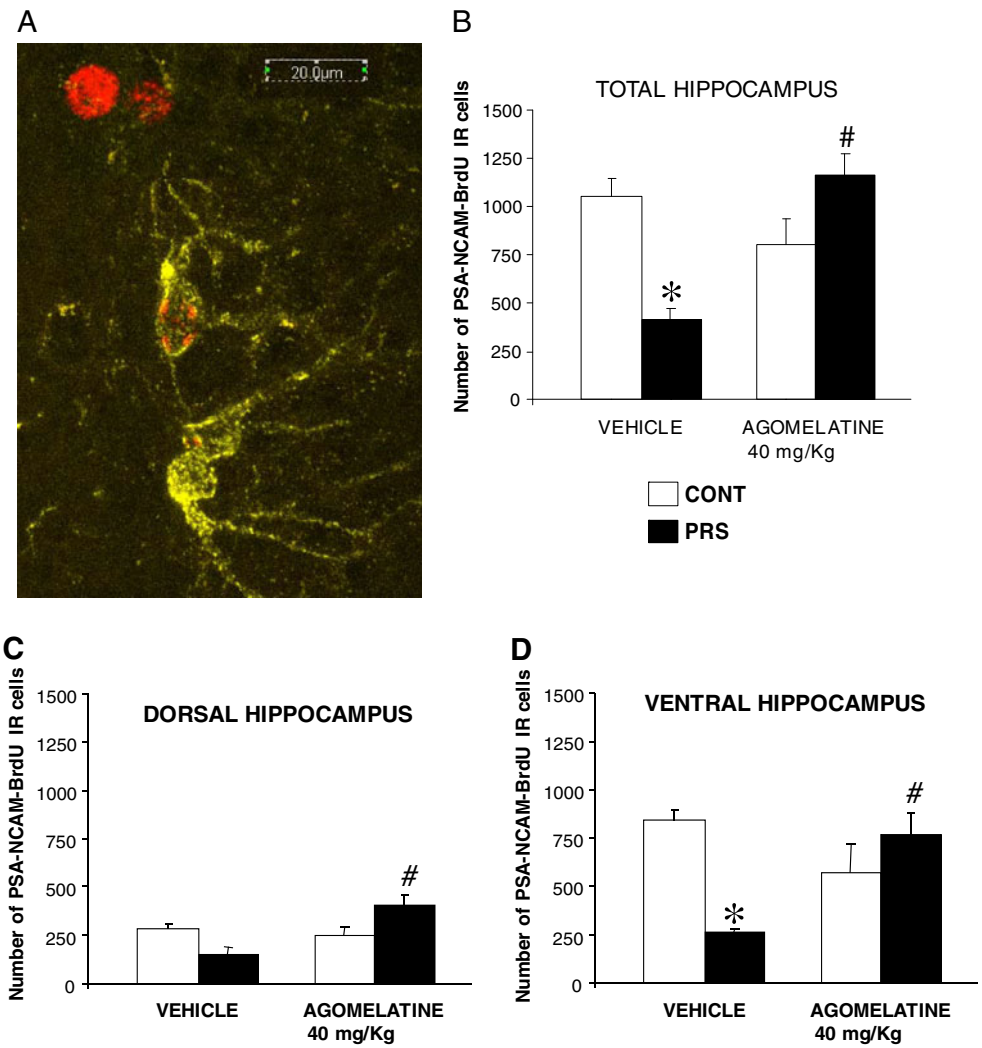


Fig. 5 Agomelatine reverses the reduction in hippocampal neurogenesis induced by PRS in adult rats. Double fluorescence immunostaining of PSA-NCAM (*green*) and BrdU (*red*) is shown in **a**, where representative cells are observed at the border between the granule cell layer and the hilus of the dentate gyrus. Counts of PSA-NCAM$^+$/BrdU$^+$ cells are shown in **b**–**d** for the total, dorsal, and ventral hippocampus of control and PRS rats treated for 6 weeks with vehicle or agomelatine. Values are means ± S.E.M. of six to eight rats per group. Representative confocal images of BrdU$^+$ cells (*red*), NeuN$^+$ cells (*green*), and BrdU$^+$/NeuN$^+$ cells (*yellow*) are shown in **g**–**i**. The effect of PRS and/or agomelatine treatment on the number of BrdU$^+$/NeuN$^+$ cells is shown in **l**, where values are means ± S.E.M. of six rats per group. $p<0.05$ vs. the respective controls (*asterisk*) or vs. PRS rats treated with vehicle (*number sign*). *CONT*, controls

obtained from an external source. Thus, our control rats never experienced the stress associated with changes in the housing conditions. To what extent the increase in hippocampal neurogenesis contributes to the antidepressant-like effect of agomelatine in PRS rats is unclear. In general, the role of a defective hippocampal neurogenesis in the pathophysiology of depression is debated. A decreased number of progenitor cells in the hippocampal dentate gyrus is seen in animal models of depression—including chronic stress and chronic high corticosterone injections (Brummelte and Galea 2010; Czéh et al. 2001; Koo et al. 2010; Warner-Schmidt and Duman 2006; Zhang et al. 2010) as well as in the hippocampus of elderly depressed patients (Lucassen et al. 2010b). However, there is also evidence that a reduced neurogenesis in the dentate gyrus does not associate with a depressive phenotype in response to chronic stress (Vollmayr et al. 2003; Jayatissa et al. 2009; 2010). It is a general belief that hippocampal neurogenesis is enhanced by antidepressant drugs (Santarelli et al. 2003; reviewed by Malberg and Schechter 2005; Tanti and Belzung 2010). However, there are exceptions (Marlatt et al. 2010) and, in addition, the action of antidepressants on neurogenesis might be critically influenced by age, gender, and stress context (Oitzl et al. 2000; Navailles et al. 2008; Lucassen et al. 2009; 2010a; Oomen et al. 2009, 2010). In a mouse model of depression induced by chronic corticosterone treatment, only some "therapeutic effects" of fluoxetine are neurogenesis-dependent (David et al. 2009), and there is also evidence that antidepressants retain their "therapeutic" efficacy when neurogenesis is blocked by a cytostatic drug (Bessa et al. 2009).

We wish to highlight that the scope of this article *is not* to demonstrate that the antidepressant activity of agomelatine is related to an enhanced hippocampal neurogenesis. What is important here is that chronic agomelatine treatment corrects all aspects of the pathological programming triggered by PRS, which includes a defective neurogenesis in the ventral hippocampus. Newly formed neurons in the hippocampal dentate gyrus are involved in the temporal encoding of new memories acting as "pattern integrators"

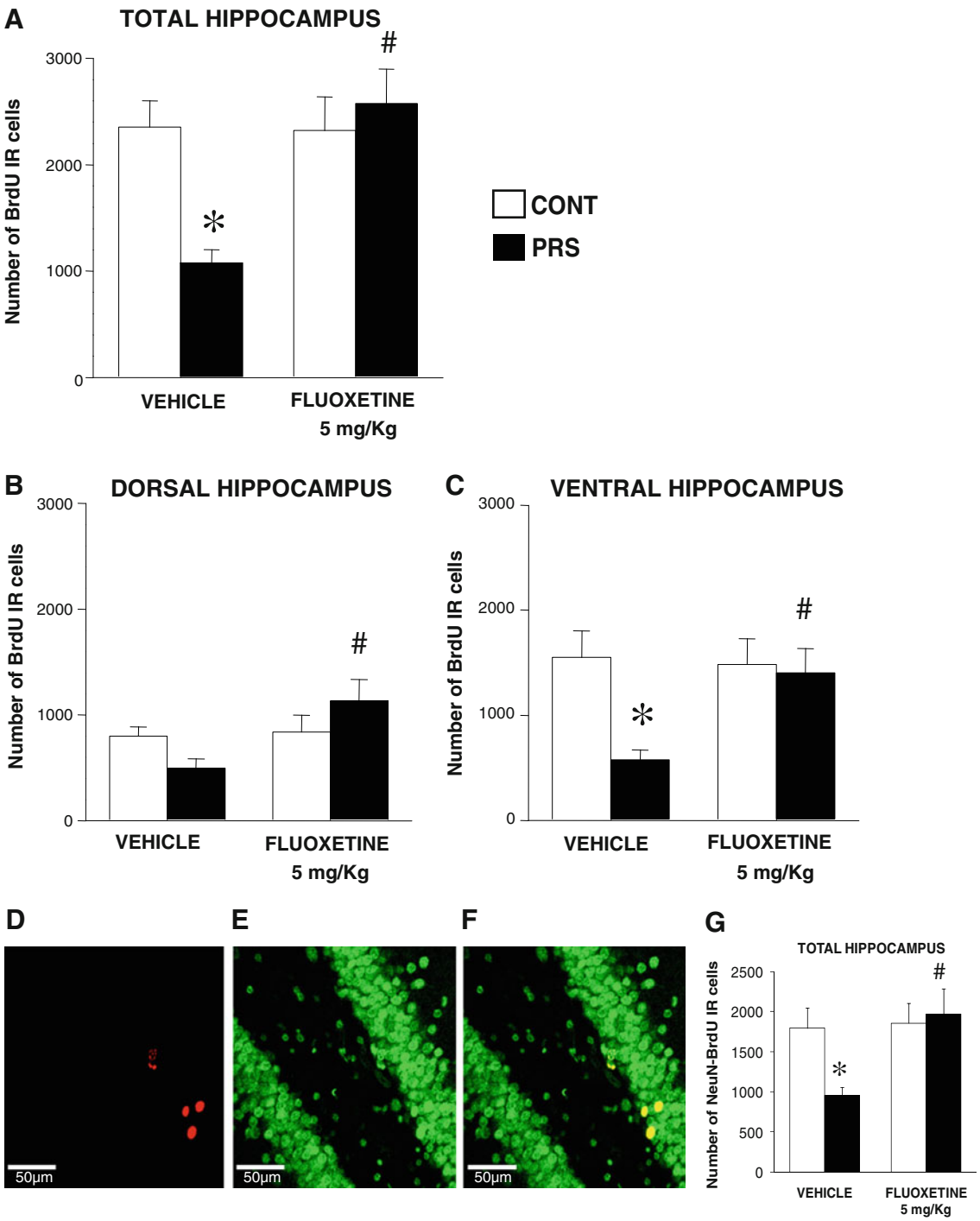


Fig. 6 A 21-day treatment with fluoxetine reverses the reduction of hippocampal neuroprogenitor cells induced by PRS in adult rats. Counts of $BrdU^+$ cells in the whole hippocampus, dorsal hippocampus, and ventral hippocampus are shown in **a**, **b**, and **c**, respectively. Values are means ± S.E.M. of six rats per group. Representative confocal images of $BrdU^+$ cells (*red*), $NeuN^+$ cells (*green*), and $BrdU^+/NeuN^+$ cells (*yellow*) are shown in **d**–**f**. The effect of PRS and/or fluoxetine treatment on the number of $BrdU^+/NeuN^+$ cells is shown in **g**, where values are means ± S.E.M. of five rats per group. $p<0.05$ vs. the respective controls (*asterisk*) or vs. PRS rats treated with vehicle (*number sign*). *CONT*, controls

(reviewed by Aimone et al. 2010). In response to agomelatine, the ventral hippocampus of PRS rats may re-acquire the ability to link stress-related events that occur simultaneously or close in time and to separate recent and remote stress-related memories, a mechanism that may critically affect their resilience to stress.

Agomelatine also reversed the reduction in the levels of p-CREB, mGlu2/3 receptors, and mGlu5 receptors found in

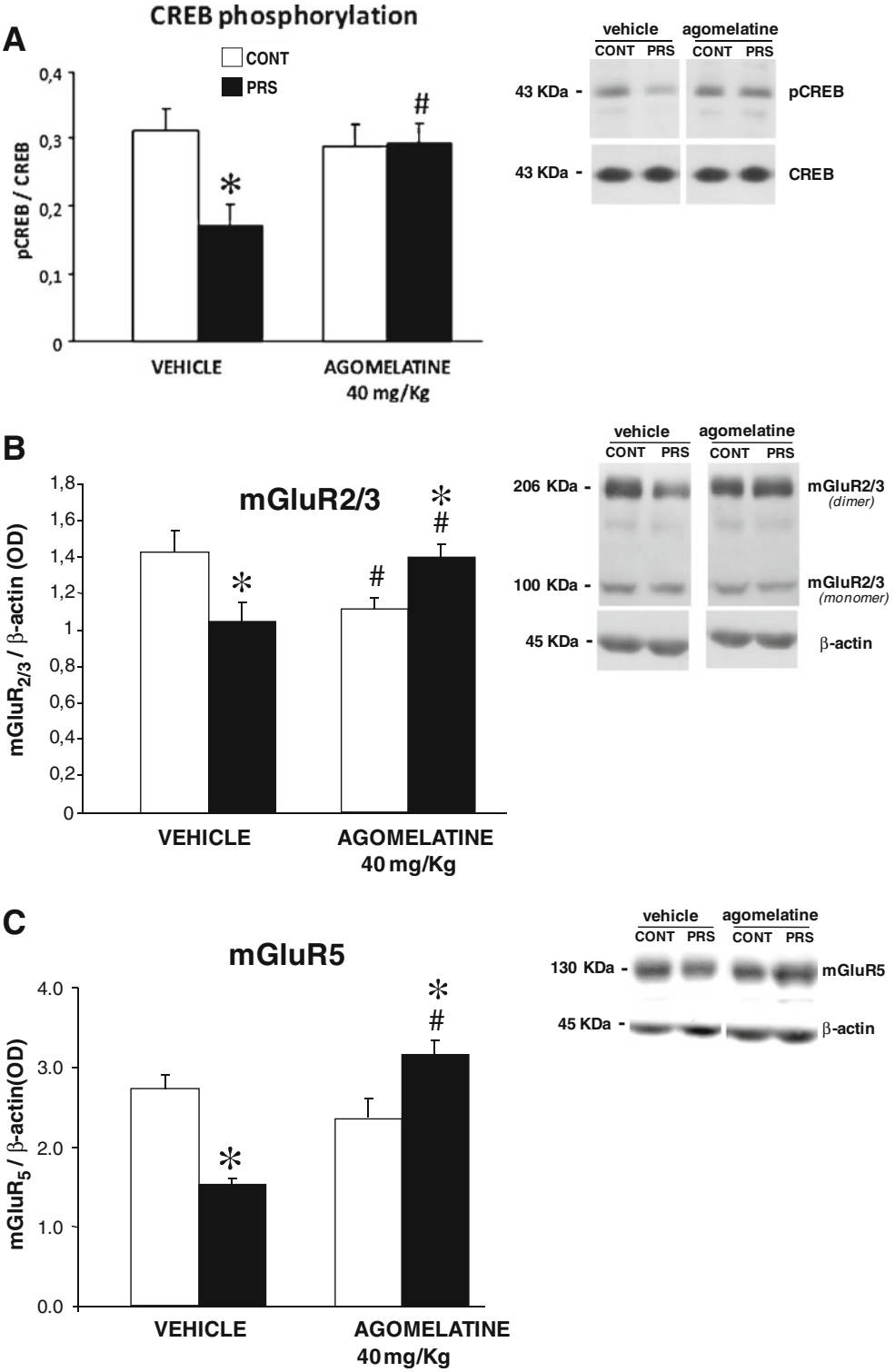


Fig. 7 Agomelatine reverses the reduction in p-CREB, mGlu2/3, and mGlu5 receptor levels in the hippocampus of PRS rats. Representative immunoblots of p-CREB, mGlu2/3, and mGlu5 receptors are shown in **a**, **b**, and **c**, respectively. Densitometric values (normalized by levels of β-actin) are means ± S.E.M. of five individual determinations. $p<0.05$ vs. the respective controls (*asterisk*) or vs. PRS rats treated with vehicle (*number sign*). *CONT*, controls

the hippocampus of PRS rats (see also Zuena et al. 2008). CREB phosphorylation, a process that enhances the transcriptional activity of CREB, is induced by chronic antidepressant treatment in heterologous expression systems (Abdel-Razaq et al. 2007) as well as in the rat hippocampus and cerebral cortex (Tiraboschi et al. 2004). In addition, p-CREB levels in peripheral blood T lymphocytes positively correlate with the response of depressed patients to antidepressant medication (Koch et al. 2002). Reductions in hippocampal levels of mGlu2/3 receptors have been found not only in PRS rats (Zuena et al. 2008; see present data) but also in Flinders Sensitive Line (FSL)

rats (Matrisciano et al. 2008), which are considered as a genetic model of depression (Overstreet et al. 2005). Chronic treatment with the antidepressant, imipramine, upregulates the expression and function of mGlu2/3 receptors in the rat hippocampus (Matrisciano et al. 2002), and the pharmacological activation of mGlu2/3 receptors shortens the latency of antidepressant medication in FSL rats (Matrisciano et al. 2007, 2008). mGlu5 receptors are critically involved in the regulation of synaptic plasticity in the hippocampus (reviewed by Nicoletti et al. 2010), and a reduced expression of these receptors in the hippocampus might contribute to the pathophysiology of cognitive dysfunction and to the low resilience to stress in PRS rats (Darnaudéry and Maccari 2008). Interestingly, CREB, mGlu3 receptors, and mGlu5 receptors are potentially linked to changes in neurogenesis in PRS rats. CREB represents a convergence point of multiple intracellular signaling pathways that regulate hippocampal neurogenesis, although its precise role is unclear (reviewed by Gass and Riva 2007; Dworkin and Mantamadiotis 2010). The activation of mGlu3 and mGlu5 receptors enhances the proliferation of neuroprogenitors in brain niches of adult neurogenesis, including the subgranular zone of the hippocampal dentate gyrus (Di Giorgi-Gerevini et al. 2005; Melchiorri et al. 2007). Thus, cellular and biochemical abnormalities corrected by agomelatine in the hippocampus of PRS rats might be interconnected in the context of an epigenetic programming that alters the resilience to stress and leads to an anxious/depressive phenotype.

In conclusion, our data demonstrate that chronic agomelatine treatment corrects the enduring pathological changes in neuroplasticity and behavior that develop in response to PRS. Whether the drug is effective in other models of early life stress (e.g., models of early postnatal stress) remains to be examined. Our data support the antidepressant action of agomelatine and, at the same time, supports the predictive value of the PRS model, lending credit to the hypothesis that depression and anxiety develop as late consequences of a pathological programming triggered early in life.

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