The stressed brain
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AGOMELATINE REVERSES THE DECREASE IN HIPPOCAMPAL CELL SURVIVAL INDUCED BY CHRONIC MILD STRESS

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ABSTRACT

The antidepressant agomelatine is a MT<sub>1</sub>/MT<sub>2</sub> receptor agonist and 5-HT<sub>2C</sub> antagonist. Its antidepressant activity is proposed to result from the synergy between these sets of receptors. Agomelatine-induced changes in the brain have been reported under basal conditions. Yet, little is known about its effects in the brain exposed to chronic stress as a risk factor for major depressive disorder. Recently, we described agomelatine-induced changes on neuronal activity and adult neurogenesis in the hippocampus of rats subjected to chronic footshock stress. In order to better characterize the actions of agomelatine in the stress-compromised brain, here we investigated its effects on hippocampal neurogenesis in the chronic mild stress (CMS) model. Adult male rats were subjected to various mild stressors for 5 weeks, and treated with agomelatine during the last 3 weeks of the stress period. The sucrose preference test was performed weekly to measure anhedonia, and the marble burying test was carried out at the end of the experiment to assess anxiety-like behavior. In our model, the CMS paradigm did not change sucrose preference; however, it increased marble burying behavior, indicating enhanced anxiety. Interestingly, this stress model differentially affected distinct stages of the neurogenesis process. Whereas CMS did not influence the rate of hippocampal cell proliferation, it significantly decreased the newborn cell survival and doublecortin expression in the dentate gyrus. Importantly, treatment with agomelatine completely normalized stress-affected cell survival and partly reversed reduced doublecortin expression. Taken together, these data show that agomelatine has beneficial effects on hippocampal neurogenesis in the CMS paradigm.
CHAPTER 4

INTRODUCTION

Depression is a devastating illness and a major contributor to the global disease burden (Greden, 2001; Greenberg and Birnbaum, 2005). Treatment of depressive disorder remains a challenge, despite of several classes of antidepressant drugs available. Currently, most of depressed patients are treated with monoaminergic compounds, such as selective serotonin reuptake inhibitors (SSRI), serotonin and noradrenaline reuptake inhibitors (SNRI), and noradrenaline reuptake inhibitors (NRI), which potentiate the brain’s monoaminergic system and elevate the monoamine levels (Wong and Licinio, 2001). However, only about 50% of the depressed patients treated with these medications achieve complete remission (Nestler et al., 2002a; Fagiolini and Kupfer, 2003). Moreover, rather poor tolerability of currently used antidepressants and late onset of their therapeutic effects further increase the risk of unsuccessful treatment (Berton and Nestler, 2006). Thus, there is a need for novel and more efficient medications with fundamentally different mechanism of action.

Search for new targets in treatment of depression has led to the development of the first melatonergic antidepressant, agomelatine (de Bodinat et al., 2010). This novel drug acts as a potent agonist of melatonergic (MT1/MT2) receptors as well as an antagonist of the serotonergic (5-HT2C) receptors (Audinot et al., 2003; Millan et al., 2003). The antidepressant properties of agomelatine have been demonstrated in both animal models (Papp et al., 2003; Bourin et al., 2004; Bertaina-Anglade et al., 2006) and in clinical studies (Kennedy and Emsley, 2006; Montgomery, 2006; Olie and Kasper, 2007; Goodwin et al., 2009). The antidepressant efficacy of agomelatine includes beneficial effect on sleep quality, achieved via synergy between the melatonergic and 5-HT2C receptors (Popoli, 2009). Current literature indicates that the therapeutic actions of agomelatine are mediated through different mechanisms as compared to SSRI and tricyclics (Hanoun et al., 2004; Paizanis et al., 2010). Nevertheless, molecular correlates underlying the antidepressant action of agomelatine merit further investigation.

In an attempt to unravel the working mechanism of agomelatine, preclinical studies reported a variety of its mediated changes in the brain. In experiments with unchallenged laboratory animals, agomelatine was shown to enhance adult hippocampal neurogenesis, to increase expression of brain-derived neurotrophic factor (BDNF), and to activate several cellular signals, i.e. extracellular signal-regulated kinase 1/2 (ERK1/2), protein kinase B (Akt), and glycogen synthase kinase 3β (GSK3β) (Banasr et al., 2006; Soumier et al., 2009). This information considerably
advanced understanding of agomelatine’s action in the brain. However, since effects of antidepressant drugs might differ in disturbed versus intact systems (Berton and Nestler, 2006), the latter findings under basal conditions are not possible to directly extrapolate to the therapeutic action of agomelatine in depression. This gap may be partially bridged by applying an animal model approach, in order to elucidate effects of agomelatine under conditions that might predispose to mood disorders.

Animal models of chronic stress represent a valuable tool to investigate behavioral, endocrine and neurobiological changes underlying stress-related psychopathologies, such as major depression, and efficacy of antidepressant therapies (Fuchs et al., 2001; Nestler et al., 2002b). The chronic mild stress (CMS) paradigm has been proposed as an animal model of depression (Willner et al., 1992). In laboratory animals, this chronic stress procedure attempts to mimic some of the dysfunctions associated with human depressive disorder. As such, CMS was reported to induce anhedonia, which could be reversed by antidepressant treatment (Willner, 1997; Papp et al., 2003; Bessa et al., 2009). Also, CMS was demonstrated to reduce expression of BDNF and activation of cyclic adenosine monophosphate responsive element binding protein (CREB), both implicated in pathophysiology of depression and working mechanism of antidepressants (Gronli et al., 2006; Castren et al., 2007; Hu et al., 2010). Moreover, CMS was shown to suppress adult hippocampal neurogenesis, suggested to play a role in depressive disorder and action of antidepressant drugs (Jayatissa et al., 2008; Krishnan and Nestler, 2008; Elizalde et al., 2010). Although impairments in hippocampal neurogenesis have not yet provided functional mechanism for the pathophysiology of depression, this phenomenon gained an enormous interest among researchers (Dagyte et al., 2010a). In particular, the notion that hippocampal neurogenesis may be oppositely regulated by stress and antidepressants gave rise to the possibility of using it as a readout in testing the efficacy of novel treatments for major depressive disorder.

Altogether, this encouraged us to apply agomelatine treatment in chronically stressed animals, in order to explore its therapeutic actions and mechanisms behind them. Recently, we characterized agomelatine-induced changes in the brain of rats exposed to chronic footshock stress. In this model, treatment with agomelatine normalized stress-affected neuronal activity and promoted neurogenesis in the hippocampus (Dagyte et al., 2010b). In order to better characterize the action of agomelatine in the stress-compromised brain, here we investigated its effects on hippocampal neurogenesis in the CMS model. We asked whether this stress paradigm induced differential changes in distinct stages of the neurogenesis process (cell proliferation, survival and neuronal differentiation), and whether agomelatine treatment reversed them. In addition, we assessed effects of CMS and agomelatine...
on anhedonia and anxiety-like behavior by using sucrose preference and marble burying tests as respective behavioral paradigms.

**EXPERIMENTAL PROCEDURES**

**Animals and housing**

The experiments were performed using adult male Wistar rats (Harlan, Horst, The Netherlands) weighing 250-300 g at the beginning of the experiment. The animals were housed individually in a climate-controlled room with constant temperature (21±1°C) and a 12 h light / 12 h dark cycle (lights on at 05:00 h). Food and water was available *ad libitum*, except when food deprivation was applied as a stressor. The experiments were approved by the ethical committee for the use of experimental animals of the University of Groningen and carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

**Experimental protocol**

During a 2-week acclimatization period before the start of the experiment, all rats were handled, weighed and trained to consume 1% sucrose solution. Three habituation tests for sucrose were performed, followed by a baseline sucrose preference test (SPT). On the basis of their sucrose preference in the baseline SPT, animals were divided into two matched groups, home-cage control and chronic stress. Both groups were further subdivided into three subgroups, based on the drug treatment (untreated, treated with vehicle or agomelatine). Thus, six groups of rats were used for the present experiment: three groups of control rats, which were either untreated (CTR) or treated with vehicle (CTR Veh) or agomelatine (CTR Ago), and three groups of chronically stressed rats, which were also either untreated (STR) or treated with vehicle (STR Veh) or agomelatine (STR Ago). Each group consisted of 7-8 animals. Stress group rats were exposed to a 5-week chronic mild stress (CMS) procedure, whereas control rats stayed undisturbed in a separate room. Drug treatment started on the third week of the experiment, and continued daily throughout the final three weeks of the 5-week experimental period. At the end of every week, the SPT was applied to all rats. In the last week of the experiment, the marble burying test was performed. At the end of the 5-week experiment, the home-cage control rats were sacrificed concomitantly with the stressed rats which were
euthanized 4-6 h after the last stressor exposure and 18-20 h after the last treatment administration.

**Chronic mild stress procedure**

The CMS protocol was adapted from previous studies (Willner et al., 1987; Papp et al., 2003). In the current experiment, the CMS procedure consisted of five different mild stressors, applied randomly every week for 5 weeks. These five stressors were: 16 h of food deprivation, 16 h of soiled cage (500 ml water in 250 g sawdust bedding), 16 h of tilted cage (45°), 12 h of predator sounds (high-pitch medium volume, resembling snake sounds) and 12 h of medium intensity of stroboscopic light flashes. These stressors were applied during the entire dark phase and first 4 h of the light phase, in case of the stressors with 16 h duration. Every experimental week consisted of five days with random stressors, and two days of no-stress.

**Sucrose preference test**

The sucrose preference test (SPT) was conducted as a measure for the anhedonic effect of CMS (Willner et al., 1987). A two bottle preference test was used, where all animals got access to both water and a 1% sucrose solution for 24 hours during a period of no-stress. In order to habituate animals to the sucrose solution, they were trained during the acclimatization period. Three habituation tests were performed, where animals had access to both water and sucrose solution for 24 hours. This training period was followed by the baseline SPT, one day before the start of the CMS protocol. During the 5-week CMS procedure, SPT was performed at the end of each experimental week for 24 hours during a period of no-stress. Bottles with water and sucrose solution were switched regularly to control for the place preference. The final SPT was performed 24 hours before the last treatment administration. Sucrose preference (SP) was calculated as the percentage of sucrose solution ingested relative to the total amount of liquid consumed (SP= \[ \text{sucrose solution, g} / \text{sucrose solution, g + water, g} \] x 100).

**Marble burying test**

The marble burying test was used to measure an anxiety-induced behavioral response to environmental challenge. It was performed in the last week of the experiment, during the light phase of a stress-free period. The method was adapted from previous studies (Ho et al., 2002; Pandey et al., 2009). Briefly, four glass
marbles (2.5 cm in diameter) were placed along a side-wall of each home-cage, and behavior of rats was observed during a 30-min test period. The following parameters were recorded: the number of rats showing burying behavior, and the number of buried marbles (at least two thirds of the surface covered with sawdust). Marble burying behavior reflected an active effort of a rat to hide the unfamiliar object in sawdust bedding, and therefore, it may indicate anxiety-like behavior (Ho et al., 2002).

**Drug treatment**

1% hydroxyethylcellulose (HEC) was used as a vehicle for agomelatine delivery. Agomelatine solution was prepared every day by dissolving agomelatine powder in 1% HEC at a concentration of 40 mg/ml. The choice of agomelatine dose was made on the basis of its activity at this concentration in animal models of depression and anxiety (Papp et al., 2003; Millan et al., 2005), and on neuroplasticity (Banasr et al., 2006; Soumier et al., 2009; Dagyte et al., 2010b). Rats were injected intraperitoneally either with agomelatine (40 mg/kg) or vehicle daily at 15:00 (2 h prior to the dark phase) for 21 days. Injections started on the third week of the 5-week experimental period. Agomelatine and HEC were provided by Servier (France).

**BrdU labeling**

In order to study survival of newly-born cells in the hippocampus during the experiment, rats received an injection of bromodeoxyuridine (BrdU: Sigma, St. Louis, MO, USA), a synthetic analogue of thymidine which is incorporated into newly synthesized DNA of replicating cells during the S phase of the cell cycle (Christie and Cameron, 2006). New cells were labeled 4 days before the start of the experiment with a single intraperitoneal injection (300 mg/kg). This specific time-point of BrdU labeling for studying cell survival was chosen since it was shown that labeled progenitor cells stop proliferating after 4 days (Dayer et al., 2003). Such an injection schedule was similarly applied in our laboratory previously (Van der Borght et al., 2005b; Dagyte et al., 2009; Dagyte et al., 2010b).

**Brain collection and immunohistochemistry**

At the end of the experiment rats were anesthetized with sodium pentobarbital and transcardially perfused with heparinized saline followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer. Brains were extracted and post-fixed in the same
solution overnight at 4°C and subsequently cryoprotected by immersion in a 30% buffered sucrose solution for up to 48 h. Coronal serial sections of 30 μm were cut using a cryostat and stored in 0.01 M phosphate-buffered saline with 0.1% sodium azide at 4°C until immunohistochemistry was performed.

In order to assess effects of CMS and agomelatine on different aspects of hippocampal neurogenesis, immunohistochemistry for Ki-67, doublecortin (DCX), and BrdU was performed. Ki-67 is a nuclear protein which is expressed during all phases of the cell cycle, except G0 (Scholzen and Gerdes, 2000), which would therefore give an indication of cell proliferation on the last day of the experiment. DCX protein is expressed in newly-born cells from 1 day to about 4 weeks of age, and might therefore reveal sustained changes in the neurogenesis process and its modulation (Brown et al., 2003; Rao and Shetty, 2004). Finally, since a pool of newborn cells was labeled with an injection of BrdU 4 days prior to the start of the experiment, BrdU immunostaining would indicate changes in survival of new cells in the course of the experiment.

The immunostainings were performed on free-floating sections under continuous mild agitation. BrdU immunohistochemistry required extra steps for DNA denaturation. For this purpose, sections were exposed to 2x saline sodium citrate (2xSSC) containing 50% formamide for 30 min at 65°C, followed by a rinse with 2xSSC, incubation with 2 M HCl for 30 min at 37°C and a washing step with 0.1 M borate buffer. Brain sections for all three immunostainings were preincubated in 3% normal serum and 0.1% TritonX-100, and then incubated with one of the following antibodies for 60 h at 4°C: primary mouse-anti-Ki-67 (1:200; Monosan, Uden, The Netherlands), goat-anti-DCX (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or rat-anti-BrdU (1:800; Serotec, Oxford, UK). Subsequently, sections were rinsed in 0.01 M Tris-buffered saline and incubated for 2 h at room temperature with secondary biotinylated goat-anti-mouse, rabbit-anti-goat or donkey-anti-rat antibody depending on the primary antibody host (1:500 for all secondary antibodies, Jackson ImmunoResearch Suffolk, UK). Then, avidin-biotin complex (1:500, Vector ABC kit, Vector Laboratories, Burlingame, CA, USA) was added for 2 h, after which the staining was visualized with 1 mg/ml diaminobenzidine and 0.003% H2O2. Thereafter, sections were rinsed, mounted on slides, dehydrated and coverslipped for microscopic analysis.

**Quantification**

All analyses were performed by an observer blind to the group assignment. Ki-67 and BrdU positive cells were counted in the subgranular zone (SGZ) and granular
cell layer (GCL) of the dentate gyrus in every twelfth section of the hippocampus under 500 × magnification. Immunopositive cells that were located one cell diameter away from SGZ were also included in the analysis. The number of immunopositive cells in each section was expressed relative to the length of the dentate gyrus in order to correct for variation in size of the hippocampus along the rostro-caudal axis of the brain. The length of the dentate gyrus was measured by delineating the entire SGZ in each hippocampus. DCX immunoreactivity (ir) in cell bodies and dendrites of the newly generated cells was measured by a previously described method (Van der Borght et al., 2007; Dagyte et al., 2009). Measurements were performed with a computerized system (Leica, Cambridge, UK). Images were taken using a Leica charged-coupled device digital camera mounted on a microscope (DMIRB, Leica, Cambridge, UK) at 100 × magnification. In total, 7-8 sections per animal (Bregma – 2.56 to –6.30, according to the rat brain atlas (Paxinos and Watson, 2007) were analyzed by delineating a similarly sized area covering the entire width of the SGZ and GCL in each hippocampus. Optical density (OD) of DCX expression was measured in the dentate gyrus and corrected for non-specific background labeling, which was measured in the corpus callosum. The OD was expressed in arbitrary units corresponding to grey levels using a Quantimet 550 image analysis system (Leica, Cambridge, UK). Simultaneously, a second measure of the DCX immunostaining was taken, complementary to the DCX OD analysis. Within each delineated area covering the SGZ and GCL, the percentage of the total surface that was covered with DCX-positive cell bodies and dendrites was calculated, and presented as area coverage (%). In all three stainings, dorsal and ventral hippocampi were analyzed separately. Since similar changes in the dorsal and ventral parts of hippocampus were detected, these data were pooled and effects in the entire hippocampus were presented.

**Statistics**

Two-way ANOVA was used to analyze the effects of stress and drug treatment on hippocampal neurogenesis and marble burying. The body weight gain and sucrose preference data were analyzed by repeated measures ANOVA. When significant effects were found, post-hoc analysis was performed using the Fisher LSD test. The level of significance was set at p<0.05.
RESULTS

Body weight gain

Body weight was measured from day -7 to day 35 of the experiment. All rats grew at similar rates during the acclimatization period. However, CMS and agomelatine treatment significantly reduced body weight gain (repeated measures ANOVA, time x stress interaction: $F_{(30,1230)} = 32.85$, $p<0.001$; time x drug interaction: $F_{(60,1230)} = 15.79$, $p<0.001$; figure 4.1).

Sucrose preference test

Preference for 1% sucrose solution over water was measured before the start of the CMS procedure (baseline) and weekly during the 5-week experimental period. Repeated measures ANOVA did not reveal any significant differences in sucrose preference among the groups throughout the experiment ($p>0.05$; figure 4.2).

Marble burying test

The number of buried marbles by each animal was recorded during a 30-min test period. Figure 4.3A depicts the average number of buried marbles in each experi-
mental group. Individual variation associated with this behavioral measure is illustrated in figure 4.3B. The data points correspond to individual animals, and show the number of their buried marbles. Two-way ANOVA revealed a significant stress-induced increase in burying behavior ($F_{(1,41)}=5.18$, $p=0.03$, when the number of animals showing burying behavior was analyzed; $F_{(1,41)}=5.06$, $p=0.03$, when the number of buried marbles across the groups was compared). Agomelatine treatment reduced burying behavior (only 29% of stressed agomelatine-treated rats buried marbles as compared to 63% of stressed non-injected and 50% of stressed vehicle-treated animals), but this effect did not reach statistical significance.

**Hippocampal neurogenesis**

Figure 4.4 shows representative examples of Ki-67 (A), BrdU (B) and DCX (C) immunostainings in the hippocampal dentate gyrus. Changes in different stages of hippocampal neurogenesis after CMS and agomelatine treatment are depicted in Figure 4.5.

Hippocampal cell proliferation was assessed by Ki-67 immunohistochemistry (figure 4.4A). There were no significant differences in the number of proliferating cells among the groups ($p>0.05$; figure 4.5A).

Hippocampal cell survival was assessed by BrdU immunohistochemistry (figure 4.4B). Two-way ANOVA revealed significant stress and drug effects ($F_{(1,41)}=9.77$, $p=0.003$ and $F_{(2,41)}=4.20$, $p=0.02$, respectively; figure 4.5B). Post-hoc Fisher LSD test showed that CMS significantly reduced the number of BrdU-positive cells in both
untreated and vehicle treated animals (CTR vs. STR: p=0.03; CTR Veh vs. STR Veh: p=0.01). Agomelatine reversed this stress-induced effect (STR Veh vs. STR Ago: p=0.02), whereas it did not influence the rate of cell survival in control animals (CTR Veh vs. CTR Ago: p>0.05).

Effects of CMS and agomelatine on newly-born hippocampal neuron maturation were assessed by DCX labeling (figure 4.4C). First, we quantified the OD of DCX expression (figure 4.5C). Two-way ANOVA revealed a significant stress effect and a stress x drug interaction (F(1,41)=12.38, p=0.001 and F(2,41)=11.41, p<0.001, respectively). Post-hoc Fisher LSD test showed that both CMS and agomelatine
Figure 4.4 Representative photomicrographs of Ki-67 (A), BrdU (B) and DCX (C) immunostainings in the hippocampal dentate gyrus. Arrows point at Ki-67 (A), BrdU (B) and DCX (C) labeled cells.

decreased the OD of DCX expression in the dentate gyrus (CTR vs. STR: p=0.03; CTR Veh vs. STR Veh: p<0.001; CTR Veh vs. CTR Ago: p<0.001). In stressed animals, agomelatine significantly reversed the decrease in DCX expression (STR Veh vs. STR Ago: p=0.04), but did not restore it to the control level (CTR Veh vs. STR Ago: p=0.004). Additionally, we analyzed the percentage of area covered with DCX-positive cells (figure 4.5D). Two-way ANOVA revealed a significant stress x drug interaction (F_{2,41}=6.89, p=0.003). Post-hoc Fisher LSD test showed that both CMS and agomelatine decreased the percentage of dentate gyrus area covered with DCX-positive cells (CTR vs. STR: p=0.008; CTR Veh vs. STR Veh: p=0.015; CTR Veh vs. CTR Ago: p=0.002). Agomelatine increased DCX area coverage in stressed animals, but this effect did not reach statistical significance (p>0.05).

**DISCUSSION**

The present study investigated action of agomelatine in the rat CMS model. Our data show that this stress paradigm differentially affected distinct stages of the adult neurogenesis process. Whereas CMS did not influence the rate of hippocampal cell proliferation, it significantly decreased the newborn cell survival and double-cortin expression in the dentate gyrus. Notably, treatment with agomelatine completely normalized stress-affected cell survival and partly reversed the reduced doublecortin expression. Altogether, these data show that agomelatine has beneficial effects on hippocampal neurogenesis in the stress-compromised brain.

Exposure to CMS led to a significant decrease in body weight gain. Similar effects on growth were previously reported in male rats subjected to various chronic stress models (Karst and Joels, 2003; Rygula et al., 2005; Dagyte et al., 2009). In the
Figure 4.5 Effects of CMS and agomelatine treatment on adult hippocampal neurogenesis. Neither CMS nor agomelatine influenced hippocampal cell proliferation (A). Whereas CMS decreased hippocampal cell survival, agomelatine completely reversed this stress effect (B). Both CMS and agomelatine treatment reduced optical density of the DCX expression in the dentate gyrus, whereas a combination of stress and drug treatment partly rescued this effect (C). Accordingly, both CMS and agomelatine decreased the percentage of dentate gyrus area covered with the DCX-positive neurons (D).

*p<0.05, **p<0.01, ***p<0.001.
CMS model, reduction in growth may depend on a combination of specific stressors, since both decrease (Bielajew et al., 2002; Ushijima et al., 2006; Jayatissa et al., 2009) and no change in body weight (Grippo et al., 2005; Gronli et al., 2006) were found in this stress paradigm. In our experiment, the food deprivation stressor had obvious effects on growth; however, its induced weight loss was short-lasting and fully recovered on the following day. Thus, the overall reduction in growth in the current CMS model cannot be attributed to a single food deprivation stressor, but rather depends on a synergistic effect of multiple stressors. The 3-week treatment with agomelatine also led to a significant decrease in body weight gain, as observed in our previous study (Dagyte et al., 2010b). This reduction in growth is most likely mediated through agomelatine’s action on melatonergic receptors, as melatonin was reported to decrease body weight in rodents (Wolden-Hanson et al., 2000). In contrast to the growth reduction in rats, agomelatine treatment does not change body weight in humans (Rouillon, 2006; Kasper and Hamon, 2009).

Our results reveal that exposure to the CMS paradigm did not change the rate of hippocampal cell proliferation. Nevertheless, it significantly decreased the survival of hippocampal cells, born before the stress period. Furthermore, CMS was associated with a reduction in the expression of DCX, a protein present mainly in the newborn immature neurons. It is interesting to note that distinct aspects of the neurogenesis process might be differentially influenced by stressful stimuli. Our data confirm a previously observed discrepancy between stress effects on hippocampal cell proliferation and survival in the CMS model (Lee et al., 2006). A similar dissociation between different stages of neurogenesis was also found in a related paradigm of chronic unpredictable stress (Oomen et al., 2007). These results suggest that the CMS paradigm influences a part, but not the entire process of hippocampal neurogenesis. Such differential effects associated with CMS may hint to the role of this form of stress in regulation of adult neurogenesis in the hippocampus. Sustained glucocorticoid exposure is considered as one of the mediators of stress-induced changes in neurogenesis (Fuchs et al., 2001; de Kloet et al., 2005). A degree of the hypothalamic-pituitary-adrenal (HPA) axis activation in the CMS model varies across different studies. Whereas some researchers report mild to moderate increases in plasma corticosterone concentrations after exposure to CMS (Bielajew et al., 2002; Grippo et al., 2005; Ushijima et al., 2006; Toth et al., 2008), others do not find lasting changes in basal corticosterone levels (Stout et al., 2000; Lee et al., 2006), as also observed in our laboratory (unpublished results). The action of corticosterone is mediated through two types of receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Both of these receptors are barely found on hippocampal progenitors, but their expression increases dynamically while newborn cells progress through different stages of the neurogenesis process to
develop into mature neurons (Cameron et al., 1993; Garcia et al., 2004). Altogether, this points to a limited role of corticosteroids in regulating adult neurogenesis in the CMS model. Furthermore, these observations suggest that a variable sensitivity of the newborn cells to changes in the local glucocorticoid environment might influence stress effects on different aspects of the neurogenesis process (Wong and Herbert, 2004, 2005). As mentioned above, proliferating cells have low GR and MR levels that may contribute to the lack of CMS effects on the rate of cell proliferation. In contrast, the survival of older hippocampal cells, expressing higher GR and MR levels, may be more severely influenced by stress. In addition, many other factors, including various proteins, neurotransmitters and hormones, are suggested to regulate the proliferation and survival of newborn cells, and have differential effects on these two processes (Hagg, 2005; Balu and Lucki, 2009). Growth factors, especially, vascular endothelial growth factor (VEGF) and BDNF, seem to play a crucial role in regulating survival and differentiation of newly generated cells (Cao et al., 2004; Mattson et al., 2004). In line with this notion, reduced neurotrophin expression has been repeatedly reported in the CMS model (Gronli et al., 2006; Bergstrom et al., 2008; Toth et al., 2008; Hu et al., 2010). In sum, adult hippocampal neurogenesis, and cell proliferation in particular, is a highly dynamic process (Dagyte et al., 2009; Ma et al., 2009). Hence, the future research should consider the temporal dynamics of the stress response and time-dependent effects of various stress mediators when designing new studies of this kind (Koolhaas et al., 1997; Buwalda et al., 2001).

Importantly, agomelatine treatment completely reversed the CMS-induced decrease in hippocampal cell survival. This finding confirms the previous observations on actions of agomelatine in the stress-compromised brain (Maccari et al., 2005; Dagyte et al., 2010b) as well as in a transgenic animal model of depression (Paizanis et al., 2010). Moreover, our data show that this antidepressant partly reversed stress-induced reduction in the DCX labeling in the dentate gyrus. Agomelatine treatment in stressed animals significantly increased the OD of DCX expression; however, its effects on the second measure of this analysis, the area covered with DCX-positive cells, did not reach statistical significance. Such discrepancy might indicate that distinct dimensions measured by two methods differ in their magnitude. The OD reveals the intensity of the staining, and thus, reflects the amount of expressed protein. Previously, we showed that changes in the OD of DCX labeling correlate with alterations in the number of immature DCX-positive neurons (Dagyte et al., 2009). We also acknowledged the differential influence of neurogenic regulators, such as stress and antidepressants, on DCX-positive cells (Dagyte et al., 2010b). Earlier studies demonstrated that DCX protein is expressed dynamically by heterogeneous cell populations, ranging from proliferating progenitors to immature yet post-mitotic neurons (Brandt et al., 2003; Kempermann
et al., 2004b; Plumpe et al., 2006). A possibility that stress and antidepressant
treatment differentially regulate the distinct populations of DCX cells was also
confirmed in the CMS model (Jayatissa et al., 2008). According to the proposed
hypothesis, chronic stress might preferentially affect DCX-expressing post-mitotic
neurons, whereas antidepressant drugs might promote the proliferating DCX-
positive progenitors and accelerate their progression towards mature neurons
(Encinas et al., 2006; Jayatissa et al., 2008; Wang et al., 2008; David et al., 2009).
Our DCX data show that CMS reduced both the amount of this protein as well as the
relative number of cell bodies and dendrites that express it. It is feasible that stress
downregulates the expression of DCX by decreasing the number of immature
neurons. On the other hand, agomelatine also reduced both of these measures.
Previous studies revealed that this antidepressant speeds up maturation of newborn
neurons (Soumier et al., 2009), resulting in lower number of DCX-positive cells
(Banasr et al., 2006), and thereby, reduced expression of DCX protein (Dagyte et al.,
2010b). Interestingly, the present data show that agomelatine significantly, albeit
partly, counteracted the stress-induced decrease in DCX reactivity but did not
significantly change the relative number of cells that express this protein. The effect
of agomelatine on stress-altered DCX immunoreactivity was rather mild. Therefore,
it might have not manifested as a significant change in the measurement of area of
coverage, associated with higher individual variation.

The neurobiological effects achieved in the CMS model granted valuable
information on the action of agomelatine in the stress-compromised brain.
Strikingly, however, CMS did not induce anhedonia as measured by preference for
1% sucrose solution over water. In fact, rats showed a very high preference for
sucrose which did not diminish during a 5-week stress period. Both control and
stressed animals drank an excessive amount of sweetened solution as compared to
their daily water intake. Such elevated sucrose consumption indicates that rats
liked/wanted it highly. Notably, different neural systems underlie the conditions of
liking and wanting of pleasurable stimuli, suggesting a possible dissociation between
these processes (Berridge, 2009). Although usually a brain likes the rewards that it
wants, yet, sometimes it may just want them (Berridge et al., 2009). In view of this,
the excess intake of sucrose solution may indicate a strong wanting component of
reward, not necessarily accompanied by a liking element. The measurement of
sucrose consumption over water, thus, might not adequately reflect the hedonic
behavior. Consequently, data on sucrose intake and preference in the CMS studies
should be interpreted with caution. In some cases, for instance, the so called
anhedonic state is a result of increased sucrose consumption by control animals,
without a change in sucrose intake by their stressed counterparts (Gronli et al.,
2006). Altogether, there is a great deal of controversy in the methods used for
determination of anhedonia, and in their measured effects in CMS studies. Despite numerous reports on stress-induced changes in either sucrose preference or intake (Papp et al., 2003; Bekris et al., 2005; Grippo et al., 2005; Koo and Duman, 2008; Toth et al., 2008; Bessa et al., 2009; Jayatissa et al., 2009; Elizalde et al., 2010; Hu et al., 2010), literature also reveals many contrasting results (Matthews et al., 1995; Forbes et al., 1996; Nielsen et al., 2000; Haidkind et al., 2003; Bergstrom et al., 2008; Wu and Wang, 2010). Such controversy is suggested to stem from differences in experimental designs and animal strains used; however, a solid explanation for discrepant findings is still lacking (Nielsen et al., 2000; Willner, 2005; Wu and Wang, 2010).

Interestingly, our CMS paradigm induced changes in marble burying behavior. Both the number of rats showing this behavior as well as the number of their buried marbles was increased in the groups subjected to CMS. The marble burying test is suggested to measure anxiety-like and impulsive behavior, and is proposed to detect potential antidepressant drugs; yet, the functional interpretation of marble burying remains uncertain (Gyertyan, 1995; Borsini et al., 2002; Millan et al., 2002). Nevertheless, our findings reveal that exposure to CMS influenced the animals' response to a novel object in the cage. Increased burying behavior might indicate stress-heightened anxiety (Farley et al., 2010). Although agomelatine had no statistically significant effects in the marble burying test, it did show a tendency to reduce the burying behavior. This observation may reflect the anxiolytic action of agomelatine, previously demonstrated in various animal models (Millan et al., 2005; Papp et al., 2006), including the marble burying test (unpublished results).

In summary, the present study used the CMS model in order to elucidate effects of stress and agomelatine treatment on adult hippocampal neurogenesis. Interestingly, this stress paradigm differentially affected distinct aspects of the neurogenesis process: whereas CMS did not alter the rate of cell proliferation, it decreased the newborn cell survival and DCX expression in the dentate gyrus. Importantly, treatment with agomelatine interfered with these stress-associated changes in the brain. This antidepressant completely normalized stress-affected cell survival and partly reversed reduced DCX expression. Taken together, these findings reveal the ability of agomelatine to counteract stress-induced changes in hippocampal neurogenesis, and confirm its previously reported actions in the stress-compromised brain.
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