Reduced neuroinflammation and enhanced neurogenesis following chronic agomelatine treatment in rats undergoing chronic constant light

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ABSTRACT

Experimental studies have revealed the involvement of neuroinflammation mediated by activated microglia in the pathophysiology of depression, suggesting a novel target for treatment. The atypical antidepressant Agomelatine (Ago) has an advantage compared to the classical antidepressants due to its chronobiotic activity and unique pharmacological profile as a selective agonist at the melatonin receptors and an antagonist at the 5HT2C receptors. We have recently revealed that Ago can exert a potent antidepressant effect in rats exposed to a chronic constant light (CCL). In the present study, we hypothesized that the anti-inflammatory activity of this melatonin analog on activated neuroglia in specific brain structures might contribute to its antidepressant effect in this model. Chronic Ago treatment (40 mg/kg, i.p. for 21 days) was executed during the last 3 weeks of a 6-week period of CCL exposure in rats. The CCL-vehicle-treated rats showed a profound neuroinflammation characterized by microgliosis and astrogliosis in the hippocampus, basolateral amygdala (BL) and partly in the piriform cortex (Pir) confirmed by immunohistochemistry. With the exception of the Pir, the CCL regime was accompanied by neuronal damage, identified by Nissl staining, in the hippocampus and basolateral amygdala and impaired neurogenesis with reduced dendritic complexity of hippocampal neuroprogenitor cells detected by doublecortin-positive cells in the dentate gyrus (DG) subgranular zone compared to the control group. Ago reversed the gliosis in a region-specific manner and partially restored the suppressed DG neurogenesis. Ago failed to produce neuroprotection in CCL exposed rats. The present results suggest that the beneficial effects of Ago represent an important mechanism underlying its antidepressant effect in models characterized by impaired circadian rhythms.

1. Introduction

It is known that most organisms develop a complex circadian system strictly synchronized to the diurnal environmental fluctuations in the light and controlled by neurons of the suprachiasmatic nucleus (SCN) located in the ventral hypothalamus (Reiter et al., 2011a). The accurate and adequate control exerted by this system on peripheral oscillators is crucial for the regulation of cell physiology and thus can prevent or reduce the possibility of functional disturbance. Endogenous biological rhythms can be changed by environmental synchronizers such as light. The pineal gland is the main source of circulating plasma melatonin and its rhythmic secretion modulates the circadian dynamics of many physiological functions (Reiter et al., 2011a). The gland receives neuronal projections from the main biological clock, the SCN, and thereby determines the daily rhythm of hormonal synthesis, secretion, and synchronization of physiological parameters with the light/dark cycle. In the last decades, research has been focused on chronopathology, in terms of potential negative consequences of disturbed circadian biological rhythms for many pathologies from cells to organisms. Experimental data that provided evidence for the chronobiotic efficacy of exogenous melatonin support the hypothesis that regulated by light secretion of this hormone is the main synchronizing signal that

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coordinates internal physiological functions with the light/dark cycle (Reiter et al., 2011b). Exposure to light at night suppresses melatonin release causing a decrease of its synchronizing activity on cells in the periphery. Both removal of the pineal gland or continuous illumination can lead to some pathologies resulting from chronobiological disturbance associated with the inhibition of rhythmic melatonin secretion (Reiter et al., 2011b; Wehr, 1991). Many of these are commonly associated with sleep disorders, elevated blood pressure, sexual dysfunction, insulin resistance, etc. In view of the inherent role of melatonin and its metabolites as potent antioxidants, circumstances showing a melatonin deficiency suggest vulnerability to the damaging effects of free radicals on cell functions (Jou et al., 2010; Paradies et al., 2010). Our previous studies have revealed that melatonin deficiency due to either exposure to continuous light or removal of the pineal gland increases oxidative stress, causes depressive-like behavior accompanied by hypercortisolism as well as disturbed sleep-wake cycle in rats (Tchekalarova et al., 2016, 2018a, 2020a,b,c). Moreover, in agreement with other reports, we have showed that chronic constant light (CCL) regime could instigate various detrimental consequences on physiology and behavior in rats, mainly due to the impaired circadian oscillation in melatonin and corticosterone release in plasma (Fonken et al., 2009; Tapia-Osorio et al., 2013; Tchekalarova et al., 2018).

Literature data, revealing the complex link between signaling pathways associated with the immune system and the molecular clock, support the suggestion that immune cells are closely related and dependent on circadian rhythms (Cartier et al., 2016). Thus, mice exposed to continuous light regime showed exacerbated inflammatory responses which are enhanced after treatment with lipopolysaccharide (Popoli et al., 2009). Due to its selective agonistic binding to melatonin receptors, this melatonin analog has chronobiotic action (Ponol et al., 2013). Furthermore, a reduced survival in conditions of peripheral and central inflammation and strong neuroprotection in limbic structures, including the hippocampus, amygdala, piriform cortex and restoration of the dentate gurus (DG) neurogenesis in condition of melatonin deficit.

2. Materials and methods

2.1. Animals

Twenty-four male Wistar rats (10-12 week-old, weighing 250–300 g) were delivered from the animal facility of the Institute of Neurobiology, Bulgarian Academy of Sciences. They were housed in standard plastic cages (3 per cage) and maintained at 20 ± 2 °C, 50–60% relative humidity in a 12-h light–dark cycle. Water and food pellets were delivered ad libitum. The following groups were used: control rats kept on the 12-h light/dark (LD) regime, lights on 08:00 a.m. and intraperitoneally (i.p.) administered with vehicle (1% hydroxyethyl cellulose, HEC) for three weeks (LD-veh, n = 6); rats housed under the LD regime and i.p. injected with Ago for three weeks (40 mg/kg dissolved in 1% HEC), (gifted by Servier Company, France) (LD-Ago group, n = 6); rats subjected to CCL for six weeks and i.p. injected with HES during the last three weeks of LL regime (LL-veh group, n = 6); rats exposed to CCL for six weeks and i.p. administered with Ago (LL-Ago rats, n = 6). The intensity of the light was 300 lx at the cage level with a light source, a white fluorescent tube. All experimental procedures and protocols were conducted according to the European Communities Council Directive 2010/63/EU and the national rules on animal experiments.

2.2. Tissue preparation

Six weeks after experimental procedures, deeply anesthetized with urethane (1500 mg kg−1) rats from experimental groups exposed to CCL (LL-veh and LL-Ago group) and age-matched control rats (LD-veh and LD-Ago group) were perfused transcardially first with 150 ml ice-cold 0.05 M phosphate buffered saline (PBS), followed by 500 ml cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. After perfusion, the brains were blocked in the coronal plane and cryoprotected in 30% sucrose solution. After 48 h of incubation at 4 °C, serial 40 μm thick sections were cut on a Reichert-Jung freezing microtome at −20 °C and then collected in 24-well flat-bottom plates in a free-floating state in PBS. We stored the tissue sections at −20 °C in a cryoprotectant developed by de Olmos and colleagues and modified by us. It consists of a 30% sucrose solution in PBS with the “antifreeze” additives ethylene glycol (30%). In this way, the tissues stored in this cryoprotectant can later be thawed and treated together.

2.3. Histological procedures

Every sixth section was mounted on chrome-gelatin-coated slides, and Nissl staining with cresyl violet was performed. After staining, the sections were dehydrated in ethanol, cleared in xylene, and embedded in Entellan.

2.4. Immunohistochemical procedures

The immunohistochemical procedure was performed applying the
ABC (avidin–biotin–horseradish peroxidase) method (Hsu et al., 1981). The free-floating sections were washed in PBS/0.3% Triton X-100, treated with 1.2% hydrogen peroxide in absolute methanol for 30 min in order to inactivate endogenous peroxidase. Between each step, the sections were thoroughly rinsed with PBS. Unspecific staining was removed via pre-incubation in 5% normal goat serum (NGS) in PBS for 1 h at room temperature (20 ± 2 °C). The sections were then incubated for 48 h at 4 °C in a humid chamber with primary antibodies. For detection of microglia-specific ionized calcium-binding adaptor molecule 1 (Iba1), which is involved with the membrane ruffling and phagocytosis in activated microglia, a primary rabbit polyclonal antibody (ab153696, Abcam) diluted 1:800 was used. A primary antibody against glial fibrillary acidic protein (GFAP) (rabbit polyclonal; dilution 1:500; Z0334, Dako) was applied for visualization of class-III intermediate filament, a specific marker that distinguishes astrocytes from other glial cells. A microtubule-associated protein expressed by neuronal precursor cells and immature neurons doublecortin (DCX) was detected by means of a primary rabbit polyclonal antibody (working dilution 1:500, ab18723, Abcam). After rinsing in 0.01 M PBS, sections were incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (Dianova, Hamburg, Germany) at a dilution of 1:500 for 2 h at room temperature. The ABC complex (Vector Laboratories, Burlingame, CA, USA) was applied for 2 h at room temperature. Peroxidase activity was visualized using 3,3′ diaminobenzidine as a peroxidase chromogenic substrate for 2–10 min, monitored microscopically. The sections were then mounted on gelatine-coated microscope slides, air-dried, dehydrated in a graded series of ethanol, cleared in xylene, and coveredslipped with Entellan (Merck, Darmstadt, Germany). The immunostained sections were counterstained with 0.1% cresyl violet. For testing of immunoreaction, the omission of the primary antibodies and substitution with normal rabbit serum or PBS was accomplished and no specific immunostaining was found (data not shown). In addition, antigen-antibody preabsorption experiments were performed and preabsorbed antibodies failed to stain brain tissue sections (data not shown).

2.5. Photodocumentation and image analysis

After classical Nissl staining and immunohistochemical reactions for visualizing Iba1, GFAP and DCX in certain brain structures, the slides were scanned at magnification 20x with a base model of Aperio AT2 linear scanning microscope (Leica Biosystems) with software Aperio ImageScope during image acquisition. To quantify cell density, the stained sections were digitalized to produce scans of the respective sections and regions of interest (ROIs). These scans were analyzed by means of ViewPoint Light program (version 1.0.0.9628, PreciPoint, Freising, Germany). The area of the ROI was outlined manually, and the cells of interest were counted by an experienced researcher blinded to the treatment groups.

Analyses were performed on free-floating 40 μm thick coronal sections of dorsal hippocampus at septal (−2.52 mm to −3.24 mm Bregma), septo-temporal (−3.36 mm to −3.96 mm Bregma) and temporal (−4.08 mm to −4.56 mm Bregma) levels and on sections of ventral hippocampus at −4.68 Bregma according to the rat brain stereotaxic atlas (Paxinos and Watson, 2004) and our protocol reported earlier (Tchekalarova et al., 2016, 2017). In each animal, we analyzed 40 μm thick sections from the rostral to the caudal part of the brain in three zones, namely the septal, septo-temporal, and temporal zones. In each animal in each zone (for example, the septal zone) six sections were analyzed and at least three measurements were made on each section in each region of interest (ROI) for analysis. We divide the number of cells by the area and calculate per 1 square millimeter how many cells we have.

In the dorsal hippocampus glial cells were analyzed and counted in the following fields: dCA1, dCA2 (stratum oriens, stratum pyramidale, stratum radiatum, stratum lacunosum moleculare), dCA3a, dCA3b, dCA3c (stratum oriens, stratum pyramidale, stratum lucidum, stratum radiatum).

The enumeration of the positive cells for Iba1 and GFAP was performed in all three distinct layers of the DG and more precisely in the outer molecular layer (MoDG), the middle granular cell layer (GrDG) and the inner polymorphic layer (PoDG, hilus).

Positive glial cells in the three layers of the piriform cortex (Pir) (highly ordered plexiform layer I, the compact cell body layer II and layer III) and in the basolateral nucleus of the amygdala were calculated. In the ventral hippocampus (vHipp), glial cells were examined in the ventral CA1 (vCA1), vCA2 and vCA3 area.

Doublecortin positive cells (DCX+) were counted in the subgranular zone (SGZ) of the dorsal dentate gyrus (dDG).

Nissl-stained sections were analyzed by means of ViewPoint Light program and nerve cells were counted in the pyramidal cell layer (Py) of the following regions: CA1, CA2, CA3a, CA3b, CA3c and also in GrDG, PoDG (hilus), basolateral nucleus of the amygdala (BL), layer II of Pir.

2.6. Analysis of the stages of maturation of doublecortin cells

For evaluation of the maturation of DCX+ cells, 8 scanned images of the entire DG were analyzed for each experimental group with ViewPoint Light program. The structural maturation of 70 DCX+ cells was evaluated with a staging system based on the classification of cells in six stages according to Horsey and collaborators (2020). In brief, Stage 1: the DCX+ cell body was located in the SGZ of the dentate gyrus and no visible dendritic processes. Stage 2: the DCX+ cell had 1 or 2 small processes that stayed within the SGZ. Stage 3: the main dendrite of the DCX+ cell expanded into the inner half of the GrDG layer. Stage 4: the principal dendrite reached the outer half of the GrDG. Stage 5: the leading dendrite reached the inner MoDG. Stage 6: the leading dendrite reaches the outer MoDG. The DCX+ cells evaluated for each of the stages were grouped into early (stages 1 and 2), intermediate (stages 3 and 4) and late (stages 5 and 6) stages.

2.7. Statistical analysis

Data are presented as the mean ± SEM. Two-way analysis of variance (ANOVA) was executed followed by a post hoc Bonferroni or Holm Sidak t-test when appropriate in case of main effect of factors Regime (LD and LL) and/or Treatment (vehicle and Ago), respectively. Statistical analysis was performed using SigmaStat®11.0 software. The difference at p < 0.05 indicates a significant difference.

3. Results

3.1. Effect of CCL regime and agomelatine treatment on microglial activation

With the exception of CA2 and CA3c subfield of the dorsal hippocampus (dHipp), six weeks of CCL regimen elevated the number of the Iba1+ cells in all observed brain structures, including the dCA1, dCA3a, dCA3b subfields of dHipp, dDG, vHipp, BL and Pir (Fig. 1A–H; 2A–L; 3A–L). This model of melatonin deficit induced changes in microglia morphology characterized by amoeboid-like shape compared to microglial cells of rats exposed to a diurnal LD period. Interestingly, the chronic Ago treatment per se elevated the Iba1+ cells compared to vehicle-treated and exposed to LD regime rats in some limbic structures such as dHipp (dCA3a (septal), dCA3b (temporal), dPoDG (septo-temporal)), vHipp (vCA1 and vCA2) (Fig. 115; 2M3-5). However, this anti-depressant drug produced a region-dependent suppression of activated microglia in the IL-exposed rats (Fig. 111-3; 2M1-6; 3M1-2).

3.1.1. Dorsal hippocampus (dHipp)

The CCL regime produced region-specific effects in the dHipp with the main regime effect detected for the dCA1 (F1, 65) = 4.011; p = 0.055 septal; F(1, 82) = 21.500; p < 0.001 temporal), dCA3a [F(1, 48) = 21.680; p < 0.001 septal; F(1, 38) = 33.204; p < 0.001 septo-temporal; F(1, 36) =
Fig. 1. (A-H) Immunohistochemical expression of Iba1 protein in the dorsal hippocampus of rats. Immunoreactive microglial cells are observed in the fields (dCA1, dCA3c) of the dorsal hippocampus (dHipp), dorsal polymorphic (dPoDG) and granular (GrDG) layers of the dentate gyrus (DG). Classically ramified microglia was observed in dHipp in both control groups of animals (LD veh and LD Ago). Scale bars: 500 μm (A, C, E, G), 100 μm (B, D, F, H). (I1–I3) Effect of chronic treatment with Agomelatine (Ago) in LD- and LL-exposed rats on microgliosis measured by an anti-Iba1 antibody in the septal, septo-temporal and temporal region of the dorsal molecular layer of the dentate gyrus (dMoDG) (I1), dorsal granular layer of the dentate gyrus (dGrDG) (I2), dorsal polymorphic layer of the dentate gyrus (dPoDG, hilus) (I3). The number of rats in each group (n = 5). Data are presented as means ± S.E.M. *p = 0.02 (I1); **p = 0.035 (I2), ***p = 0.003 (I3), ****p < 0.001 compared to LD-veh group; +p = 0.02 (I2), ++p < 0.001 compared to LD-Ago group; oop = 0.032 (I3), ooop = 0.009 (I1), oop < 0.001 compared to LL-veh group.

5.772; p = 0.022 temporal), dCA3b [F(1, 48) = 13.663; p < 0.001 septal; F(1, 36) = 5.772; p = 0.022 temporal]. Post hoc confirmed a significant increase in the number of activated microglia in all parts of the dCA3a field compared to LD-veh group (septal, septo-temporal and temporal) (p < 0.001) while the dCA1 and dCA3b fields were affected in the septal (p = 0.005 and p = 0.007, respectively) and temporal part (p < 0.001), respectively (Fig. 2M1, 2M4-M5). Two-way ANOVA demonstrated a main Treatment effect for the dCA1 [F(1, 100) = 33.517; p < 0.001 septal; F(1, 65) = 17.057; p < 0.001 septo-temporal; F(1, 82) = 26.476; p < 0.001 temporal], dCA2 [F(1, 48) = 23.312; p < 0.001 septal; F(1, 38) = 5.662; p = 0.023 septo-temporal], dCA3a [F(1, 44) = 12.702; p < 0.001 septal; F(1, 65) = 19.669; p < 0.001 septo-temporal; F(1, 39) = 128.024; p < 0.001 temporal], dCA3b [F(1, 48) = 8.363; p = 0.006 septo-temporal; F(1, 35) = 7.020; p = 0.012 temporal], CA3c [F(1, 54) = 6.273; p = 0.015 septal; F(1, 38) = 23.316; p < 0.001 septo-temporal], respectively, with Regime × Treatment interaction detected in the dCA1 [F(1, 100) = 32.072; p < 0.001 septal; F(1, 65) = 10.354; p = 0.002 septo-temporal; F(1, 82) = 18.505; p < 0.001 temporal], dCA2 [F(1, 48) = 38.455; p = 0.03 septal; F(1, 38) = 38.455; p < 0.001 septo-temporal], dCA3a [F(1, 44) = 73.987; p < 0.001 septal; F(1, 65) = 45.389; p < 0.001 – septo-temporal; F(1, 39) = 128.024; p < 0.001 temporal], dCA3b [F(1, 47) = 7.885; p = 0.007 septal; F(1, 48) = 7.594; p = 0.008 septo-temporal; F(1, 35) = 40.836; p < 0.001 temporal], dCA3c [F(1, 54) = 24.355; p < 0.001 septal; F(1, 45) = 10.433; p = 0.002 septo-temporal]. The Ago treatment in rats exposed to LD regime increased the number of Iba1+ cells in the dCA3 region compared to LD-veh group (septal dCA3a: p < 0.001, temporal dCA3b: p = 0.021) (Fig. 2M4-M5). However, under the LL regime the Ago treatment alleviated microglia activation in the dCA1 (septal and septo-temporal: p < 0.001 compared to LD-veh, LD-Ago and LL-veh group, respectively; temporal: p < 0.001 compared to LL-veh group), the CA3a (septal: p = 0.003 compared to LL-veh group; septo-temporal: p < 0.001 compared to LD-veh, LD-Ago and LL-veh group; temporal: p < 0.001 compared to LL-veh group), dCA3b (septal: p = 0.007 compared to LL-veh group; septo-temporal and temporal: p < 0.001 compared to LD-veh, LD-Ago and LL-veh group; temporal: p < 0.001 compared to LL-veh group), dCA3c (septal: p < 0.001 compared to LD-veh, LD-Ago and LL-veh group; septo-temporal: p < 0.001 compared to LD-veh, LD-Ago and LL-veh group; respectively) and dCA3c (septal: p < 0.001 compared to LD-veh, LD-Ago and LL-veh group; septo-temporal: p < 0.001 compared to LD-veh, LD-Ago and LL-veh group) (Fig. 2M1, 2M4-M6). Although the LL regime did not produce effect on activated microglia in the dCA2 and dCA3c subfield, Ago administration significantly decreased the number of Iba1+ cells in the dCA2 (septal and septo-temporal: p < 0.001 compared to LD-veh, LD-Ago and LL-veh group) (Fig. 2M2, 2M5).
3.1.2. Dorsal dentate gyrus (dDG)

Two-way ANOVA showed a main regime effect for the septo-temporal and temporal dDG as follows: dMoDG [F(1, 102) = 3.822; p = 0.053 septo-temporal; F(1, 98) = 10.268; p = 0.002 temporal], dGrDG [F(1, 89) = 18.29; p < 0.001 septo-temporal; F(1, 76) = 15.972; p < 0.001 temporal] as well as dPoDG [F(1, 49) = 4.477; p = 0.042 septo-temporal; F(1, 53) = 4.347; p = 0.042 temporal]. Six weeks of exposure to CCL did not affect microglia morphology in the septal dDG (p > 0.05 vs LD-veh group) but it elevated the number of Iba1+ cells in the septo-temporal and temporal dDG and this effect was more pronounced in the temporal dDG compared to LD-veh group as follows: the dMoDG (septo-temporal: p = 0.035 and temporal: p < 0.001), the dGrDG (septo-temporal: p = 0.02 and temporal: p < 0.001) and the dPoDG (hilus) (septo-temporal and temporal: p < 0.001) (Fig. 11I-13).

The main treatment effect was also verified for the septo-temporal and temporal dDG as follows: dMoDG [F(1, 102) = 6.143; p = 0.015 septo-temporal; F(1, 98) = 30.274; p < 0.001 temporal], dGrDG [F(1, 76) = 37.929; p < 0.001 temporal], dPoDG [F(1, 49) = 8.634; p = 0.005 septo-temporal] with Regime x Treatment interaction detected in the dMoDG [F(1, 100) = 6.508; p = 0.012 septo-temporal; F(1, 98) = 15.613; p < 0.001 temporal], dGrDG [F(1, 76) = 15.244; p < 0.001 temporal] and dPoDG [F(1, 49) = 24.433; p < 0.001 septo-temporal]. While the Ago treatment in rats exposed to LD regime increased the number of Iba1+ cells in the septo-temporal hilus compared to LD-veh group (p = 0.003), this antidepressant drug attenuated the hyperactive microglia in all temporal parts including the dMoDG and dGrDG (p < 0.001 as well as the hilus (p = 0.032) compared to LL-veh group (Fig. 11I-13). It also produced amelioration of the number of Iba1+ cells in septo-temporal dMoDG (p = 0.009) and the hilus (p < 0.001) compared to LL-veh group (Fig. 11I, 11J).

3.1.3. Ventral hippocampus (vHipp)

The main Regime effect was demonstrated for the vCA1 [F(1, 67) = 5.24; p = 0.045], vCA2 [F(1, 36) = 34.526; p < 0.001] and vCA3 [F(1, 52) = 5.428; p = 0.05]. Hyperactivation of microglia was detected in the vCA1, vCA2 and vCA3 field of the vHipp as a result of six-week exposure to CCL compared to LD-veh group (p < 0.001) (Fig. 2M3). The Treatment effect was also detected for the vCA1 [F(1, 67) = 44.363; p < 0.001], vCA2 [F(1, 36) = 7.253; p = 0.027] and vCA3 [F(1, 52) = 46.877; p < 0.001] with Regime x Treatment interaction for the vCA1 [F(1, 67) = 182.938; p < 0.001], vCA2 [F(1, 36) = 28.347; p < 0.001] and vCA3 [F(1, 52) = 36.478; p < 0.001].

The post hoc test revealed that the chronic Ago treatment suppressed the LL-induced elevation of Iba1+ cells (vCA1 and vCA3: p < 0.001 compared to LD-veh, LD-Ago and LL-veh group; vCA2: p < 0.001 compared to LL-veh group).

3.1.4. The basolateral amygdala (BL) and the piriform cortex (Pir)

Two-way ANOVA demonstrated a main Regime effect for the BL [F(1, 56) = 165.032; p < 0.001- septal; F(1, 61) = 16.538; p < 0.001 septo-temporal; F(1, 63) = 26.055; p < 0.001 temporal], a main Treatment effect [F(1, 61) = 8.720; p = 0.005 septo-temporal; F(1, 63) = 5.652; p = 0.028 temporal] as well as Regime x Treatment effect [F(1,63) = 5.302; p = 0.025 temporal]. The CCL exposure of rats for six weeks remarkably enhanced the activated microglial cells in all regions of the BL (septal and temporal: p < 0.001, septo-temporal: p = 0.003 compared to LD-veh group) (Fig. 3M1). Hyperactivation of microglia was not affected by the Ago treatment in the septal BL (p > 0.05) but was attenuated in the septo-temporal (p = 0.007 compared to LL-veh group) and temporal (p = 0.008 compared to LL-veh group) BL, respectively.

The main Regime effect was detected for the septo-temporal Pir [F(1, 100) = 17.391; p < 0.001], the main Treatment effect [F(1, 100) = 8.200; p
Fig. 3. Immunohistochemical localization of Iba1 protein in the rat basolateral amygdala (BL) and piriform cortex (Pir). Iba1-immunostained microglial cells are visible in these limbic regions in LD-exposed control rats administered with vehicle (LD veh) (A-C), LD-exposed control rats treated with agomelatine (LD Ago) (D-F), LL-exposed experimental rats administered with vehicle (LL veh) (G-I) and LL-exposed animals treated with agomelatine (LL Ago) (J-L). Note that the activated microglial cells in both the basolateral amygdala and internal polymorphic layer (III) of the piriform cortex are increased in number in rats exposed to chronic constant light (LL veh) The administration of agomelatine to rats exposed to chronic constant light (LL Ago) diminishes the number of Iba1-immunopositive microglial cells in the examined structures. Scale bars = 500 μm (A, D, G, J), 100 μm (B, C, E, F, H, I, K, L). (M1-M2) Effect of chronic treatment with Agomelatine (Ago) in LD- and LL-exposed rats on microgliosis measured by an anti-Iba1 antibody in the septal, septo-temporal and temporal region of the basolateral amygdala (BL) (M1) and the Piriform cortex (Pir) (M2). The number of rats in each group (n = 5). Data are presented as means ± S.E.M. **p < 0.003 (M1), ***p < 0.001 compared to LD-veh group; +++p < 0.001 compared to LD-Ago group; oop = 0.007 (M1), oop = 0.008 (M1), oop < 0.001 compared to LL-veh group.

3.2. Effect of CCL regime and agomelatine treatment on activated astrocytes

Neuroinflammation associated with activated astrocytes was assessed with the astrocyte marker GFAP. With the exception of the dGrDG (septo-temporal) and the dCA3a area (septo-temporal) in the dHipp and the vCA3 area of the vHipp where Ago treatment in LD regime increased astrogliosis compared to vehicle-treated groups, the sections of the two control groups (LD-veh and LD-Ago), processed for GFAP immunostaining, showed normal body shape with low immunoreactivity (Fig. 4A-L, 5A-D). The detected GFAP-immunostained ramified astroglial cells were increased in the whole dDG, area-specifically scattered in the hippocampal layers of the dHipp but not affected in the vHipp as a sequence of six weeks exposure to CCL compared to LD-veh group. In addition, the BL and Pir were also profoundly affected by melatonin deficit (Fig. 6E and F). The intense immunoreactivity for GFAP as well as the cell hypertrophy detected in the dCA1, dCA2 (septo-temporal and temporal), dCA3a (septal, septo-temporal), dCA3b (septo-temporal), dCA3c, dDG (MoDG (septal and septo-temporal), dGrDG and dPoDG), as well as BL was partially or fully suppressed by Ago (Fig. 4M1-M2; 4M4-M6; 5E1-E3; 6I1).

3.2.1. Dorsal hippocampus (dHipp)

Two-way ANOVA revealed the main Regime effect for the dCA1 [F(1, 127) = 52.487; p < 0.001 - septal; F(1, 116) = 67.319; p < 0.001 septo-temporal; F(1, 171) = 60.213; p < 0.001 temporal], the dCA2 [F(1, 71) = 113.730; p < 0.001 septo-temporal and F(1, 58) = 177.410; p < 0.001 temporal], dCA3a [F(1, 86) = 79.829; p < 0.001 septo-temporal and F(1, 92) = 87.427; p < 0.001 temporal], dCA3b [F(1, 62) = 9.432; p = 0.023 septo-temporal], dCA3c [F(1, 65) = 5.62; p = 0.047 septal; F(1, 71) = 225.421; p < 0.001 - septo-temporal and F(1, 84) = 9.812; p = 0.002]. Six-weeks exposure of rats to constant light exerted a profound astrogliosis in the dCA1 area (p < 0.001), dCA2 and dCA3a (septo-temporal and temporal) (p < 0.001), dCA3b (septo-temporal) (p < 0.001), dCA3c (septal: p = 0.027, septo-temporal and temporal: p < 0.001) compared to LD-veh group (Fig. 4M1-M2; 4M4-M6).

The main Treatment effect was shown also for the dCA1 [F(1, 116) = 5.363; p = 0.022 septo-temporal and F(1, 171) = 4.962; p = 0.027 temporal], dCA3a [F(1, 77) = 102.014; p < 0.001 septal; F(1, 80) = 6.530; p = 0.013 septo-temporal], dCA3b [F(1, 47) = 24.073; p < 0.001 septal; F(1, 44) = 6.214; p = 0.017 temporal], dCA3c [F(1, 71) = 31.190; p < 0.001 septo-temporal] with Regime × Treatment interaction for the dCA1 [F(1, 127) = 127.451; p < 0.001 septal and F(1, 116) = 14.631; p = 0.002 temporal], dCA2 [F(1, 71) = 119.277; p < 0.001 septo-temporal], dCA3a...
Two-way ANOVA revealed the main Regime effect for the dMoDG \(\left[F_{(1, 130)} = 10.043; p = 0.009 - \text{septal}; F_{(1, 58)} = 178.245; p < 0.001 \text{-septo-temporal}\right] \), dGrDG \(\left[F_{(1, 67)} = 9.473; p = 0.023 \text{septal}; F_{(1, 58)} = 178.245; p < 0.001 \text{-septo-temporal}; F_{(1, 70)} = 5.114; p = 0.045 \text{-temporal}\right] \), dPoDG \(\left[F_{(1, 90)} = 58.117; p < 0.001 \text{septal}; F_{(1, 72)} = 48.557; p < 0.001 \text{-septo-temporal}; F_{(1, 103)} = 26.646; p < 0.001 \text{-temporal}\right] \). Post hoc test confirmed that the sections of the LL-veh group demonstrated astrogliosis, which is characterized by an intense immunoreactivity for GFAP marker and cell hypertrophy, and was detected in the dMoDG (septal and septo-temporal) \(p < 0.001\), dGrDG (septal, septo-temporal and temporal) \(p < 0.03\) and dPoDG (septal, septo-temporal and temporal) \(p < 0.001\) (Fig. 5E1-E3).

The main Treatment effect was shown also for the dMoDG \(F_{(1, 130)} = 4.827; p = 0.03 \text{septal}; F_{(1, 58)} = 21.546; p < 0.001 \text{-septo-temporal}\) and dGrDG \(F_{(1, 67)} = 13.084; p < 0.001 \text{septal}; F_{(1, 58)} = 21.546; p < 0.001 \text{-septo-temporal}\) and dPoDG \(F_{(1, 103)} = 11.535; p < 0.001 \text{temporal}\) with Regime × Treatment interaction for the dMoDG \(F_{(1, 130)} = 11.999; p < 0.001 \text{septal}; F_{(1, 58)} = 23.366; p < 0.001 \text{septo-temporal}; F_{(1, 58)} = 23.366; p < 0.001 \text{-septo-temporal}\) and PoDG \(F_{(1, 90)} = 18.000; p < 0.001 \text{septal}; F_{(1, 72)} = 13.362; p < 0.001 \text{-septo-temporal}\). The Ago treatment in LD regime increased GFAP immunoreactivity only in the septo-temporal dGrDG \(p = 0.032 \text{compared to LD-veh group}\) (Fig. 5E2). The same treatment in LL regime partially restored to control levels the GFAP immunoreactivity in the dMoDG (septal) \(p < 0.001\) compared to LD-veh, LD-Ago, dMoDG (septal and septo-temporal) \(p = 0.015\) compared to LL-veh group (Fig. 5E1-E3).

The effective suppression of this antidepressant drug was detected in the septal dMoDG and dGrDG \(p < 0.001 \text{compared to LL-veh group}\) as well as temporal dGrDG \(p = 0.028 \text{compared to LL-veh group}\) (Fig. 5E1, E2).

### 3.2.2. Dorsal dentate gyrus (dDG)

Two-way ANOVA demonstrated a main Treatment effect \(F_{(1, 48)} = 20.197; p = 0.001\) (Fig. 5E1-E3). However, no significant differences were found between the regimes for the treatment effects. Post hoc analysis revealed that the treatment with Ago in LL regime partially suppressed the GFAP immunoreactivity in the dMoDG (septal) \(p < 0.001\), dGrDG (septal, septo-temporal and temporal) \(p < 0.03\) and dPoDG (septal, septo-temporal and temporal) \(p < 0.001\) (Fig. 5E1-E3).}

### 3.2.3. Ventral hippocampus

Two-way ANOVA demonstrated a main Treatment effect \(F_{(1, 48)} = 26.646; p < 0.001 \text{-temporal}\) (Fig. 5E1-E3). Post hoc test confirmed that the sections of the LL-veh group demonstrated astrogliosis, which is characterized by an intense immunoreactivity for GFAP marker and cell hypertrophy, and was detected in the dMoDG (septal and septo-temporal) \(p < 0.001\), dGrDG (septal, septo-temporal and temporal) \(p < 0.03\) and dPoDG (septal, septo-temporal and temporal) \(p < 0.001\) (Fig. 5E1-E3).
Fig. 5. Immunohistochemical expression of a glial fibrillary acidic protein (GFAP) in the dorsal polymorphic (dPoDG) and granular (GrDG) layers of the dentate gyrus (DG). Representative images of GFAP immunoreactivity in the dDG (A) of LD-exposed vehicle-treated control rats (LD veh), (B) LD-exposed control rats treated with agomelatine (LD Ago), (C) LL-exposed vehicle-treated rats (LL veh) and (D) LL-exposed experimental rats treated with agomelatine (LL Ago). Note that the exposure of animals to chronic constant light causes a profound cell hypertrophy and astrogliosis in PoDG of the dorsal hippocampus (C). The treatment with Ago in LL regime partially suppressed the GFAP immunoreactivity in LL-Ago treated rats (D). Scale bars = 100 μm. (E1-E3) Effect of chronic treatment with Agomelatine (Ago) in LD- and LL-exposed rats on astrogliosis measured by an anti-GFAP antibody in the septal, septo-temporal and temporal region of the dorsal molecular layer of the dentate gyrus (DG) (dMoDG) (E1), dorsal granular layer of the DG (dGrDG) (E2), dorsal polymorphic layer of the DG (dPoDG, hilus) (E3). The number of rats in each group (n = 5). Data are presented as means ± S.E.M. *p = 0.03 (E2); **p = 0.032 (E2), *p = 0.012 (E3); *p = 0.019 (E3); *p = 0.025 (E3), ***p < 0.001 compared to LD-veh group; +p = 0.012 (E3); +p = 0.019 (E3); +p = 0.025 (E3), ++p < 0.001 compared to LD-Ago group; op = 0.028 (E2), ooop < 0.001 compared to LL-veh group.
12.773; p < 0.001] as well as Regime × Treatment interaction [F(1, 48) = 6.638; p = 0.013] for the vCA3. Unlike the activated microglia detected in the whole vHipp of LL-exposed rats, in this structure astrocytes were not affected by the CCR regime (Fig. 4 M3). The chronic Ago treatment elevated the GFAP+ cells in LD-exposed rats (vCA3 area: p < 0.001 compared to LD-veh group) while decreased number of GFAP+ cells was detected in LL-rats treated with Ago (p < 0.001 compared to LD-Ago group).

3.2.4. The basolateral amygdala (BL) and the piriform cortex (Pir)

The main Regime effect was verified for the BL [F(1, 66) = 124.055; p < 0.001 septal; F(1, 56) = 80.545; p < 0.001 septo-temporal; F(1, 66) = 14.410; p < 0.001 temporal]. The exposure of rats to CCL regime for six weeks significantly elevated the number of GFAP+ cells in all parts of the BL (p < 0.001 compared to LD-veh group) (Fig. 6I1). The main Treatment effect was also detected for the BL [F(1, 66) = 27.024; p < 0.001 septal; F(1, 56) = 30.840; p < 0.001 septo-temporal] with Regime × Treatment interaction [F(1, 66) = 14.509; p < 0.001 septo-temporal; F(1, 121) = 32.63; p < 0.001 temporal]. While the astrogliosis was partially affected by the Ago treatment in the septal BL (p < 0.001 compared to LD-veh, LD-Ago and LL-veh group), it was significantly attenuated in the septo-temporal (p < 0.001) as well as in the temporal BL (p = 0.015) compared to LL-veh group, respectively. Similar to the BL, the CCL regimen enhanced inflammatory response associated with activated astrocytes in the Pir (p < 0.001 compared to LD-veh group) (Fig. 6I2). The chronic Ago treatment was unable to reverse the CCL-induced increase of the number of GFAP+ cells (p < 0.001 compared to LD-veh and LD-Ago group, respectively).

3.3. Effect of CCL regime and agomelatine treatment on neuronal damage

Neuronal morphology was evaluated by cresyl violet staining (Fig. 7A–L; 8A–D; 9A–D). Two-way ANOVA analysis of morphological changes as a sequence of melatonin deficit demonstrated the main Regime effect for dCA1 [F(1, 199) = 15.325; p < 0.001 septal; F(1, 141) = 14.509; p < 0.001 septo-temporal; F(1, 121) = 32.63; p < 0.001 temporal], dCA2 [F(1, 63) = 40.920; p < 0.001], dCA3a [F(1, 122) = 7.613; p = 0.007 septal; F(1, 95) = 28.765; p < 0.001 septo-temporal], dCA3b [F(1, 83) = 11.120; p < 0.001 temporal], dCA3c [F(1, 85) = 28.765; p < 0.001 septo-temporal; F(1, 83) = 36.127; p < 0.001 temporal], dGrDG [F(1, 106)
Fig. 7. Representative photomicrographs of Nissl-stained coronal sections of the dorsal hippocampal formation in (A–C) LD-exposed control rats administered with vehicle (LD-veh), (D–F) LD-exposed control rats treated with agomelatine (LD-Ago), (G–I) LL-exposed experimental rats administered with vehicle (LL-veh) and (J–L) LL-exposed animals treated with agomelatine (LL-Ago). Scale bars: 500 μm (A, D, G, J), 100 μm (B, C, E, F, H, I, K, L). (M1–M6) Effect of chronic treatment with Ago in LD- and LL-exposed rats on neuronal loss measured by Nissl staining in the dorsal subfields dCA1 (M1), dCA2 (M2), dCA3a (M4), dCA3b (M5), dCA3c (M6) of the dorsal hippocampus in septo-temporal, septal-temporal, temporal zone and in the ventral subfields vCA1, vCA2, vCA3 of the ventral hippocampus (vHipp) (M3). The number of rats in each group (n = 5). Data are presented as means ± S.E.M. *p < 0.025 (M1), *p = 0.014 (M2), *p = 0.016 (M3), **p < 0.001 compared to LD-Ago.***p < 0.001 compared to LD-veh group; +p = 0.025 (M1), +p = 0.013 (M4), +++p < 0.001 compared to LD-Ago.

3.4. Effects of CCL regime and agomelatine treatment on neurogenesis

Exposure to chronic light as well as Ago-related impact on the hippocampal neurogenesis was explored by quantitative analysis of DCX-expressing cells (immature neurons) in the DG SGZ. Representative photomicrographs depicting proliferation of NPCs via counting DCX+ cell immunoreactivity across LD-veh, LD-Ago, LL-veh and LL-Ago group are shown in Fig. 10A–D. Most of the DCX+ cells were located in SGZ, fewer in the inner one-third region of the dentate granule cell layer and the minimum amount of immunostained cells with longer dendrite was located in the inner molecular layer (stage 5) or in the outer molecular layer (stage 6) (Fig. 10E–I).

Examination of DCX+ cells demonstrated a significant effect of the Regime [F(1, 54) = 13.150, p < 0.001] on neurogenesis in the septo-temporal part of the dDG while a main Ago effect was evident in the septal [F(1, 62) = 12.134, p < 0.001] and temporal [F(1, 39) = 13.678, p < 0.001] part of the SGZ of DG. Post hoc test demonstrated that the total number of DCX+ cells was diminished in the septo-temporal part of the SGZ in the LL-veh group compared to the LD-veh group (p = 0.025) while the Ago treatment produced a strong enhancement of DCX+ cell number both in LD- (septal: p = 0.031 and temporal: p = 0.014 compared to the LD-veh group) and LL-treated group (septal: p = 0.008 and temporal: p = 0.011 compared to the LL-veh group), respectively (Fig. 10F).

Further, DCX+ cells were divided into three separate stages depending on the degree of their structural maturation i.e. according to the location and the rate of growth of apical dendritic processes in the neuronal morphology was shown in the Pir in this model of melatonin deficit (Fig. 9E1–E2). The chronic Ago treatment did not produce neuroprotective effect in regions with detected neuronal damage in the LL group whereas it showed a neuroprotective activity only in the temporal BL (p < 0.001 compared to LL-veh group) (Fig. 9E1).
SGZ. Exposure to chronic light caused an increased number of early stage DCX+ cells (stages 1 + 2) compared to LD exposure [Main Regime effect: F(1, 43) = 55.100, p < 0.001]. More detected NPCs without or too short dendritic processes in the SGZ were from LL-veh and LL-Ago group compared to LD-veh and LD-Ago group, respectively (p < 0.001). Two-way ANOVA revealed a main Regime [F(1, 47) = 129.986, p < 0.001] and Treatment effect [F(1, 47) = 10.596, p = 0.002].

While Ago treatment in the LD exposed rats increased the number of DCX+ cells in intermediate stage (3 + 4) (p < 0.001 compared to LD-veh group), CCL exposure led to a lower number of intermediate stage DCX+ cells than the LD-rats (p < 0.001 LL-veh and LL-Ago compared to LD-veh and LD-Ago group, respectively). Interestingly, the CCL exposure attenuated the proportion of late stage DCX+ cells compared to the LD regime [F(1, 14) = 87.641, p < 0.001; p < 0.001 LL-veh compared to LD-veh group] while Ago treatment partially enhanced the number of DCX+ cells in the late stage [Regime × Treatment interaction: F(1, 14) = 13.557, p = 0.004; p = 0.003 LL-Ago compared to LD-veh and LD-Ago group, p = 0.008 LL-Ago compared to LL-veh group] (Fig. 10G).

4. Discussion
In the present study, we report that CCL exposure in rats affected the late phase of the inflammatory response characterized by changes in microglial and astrocytic morphology in stress-related brain regions such as the hippocampus, BL and Pir. This model also induces neuronal damage in these structure and deficient neurogenesis in the SGZ, specifically in the intermediate and late stages which is detected in the septo-ventral portion of the dHipp. Chronic treatment with Ago
attenuates the inflammatory response and partially recovered blocked neurogenesis in the late neuroblast stage in rats exposed to CCL. However, this antidepressant drug is unable to exert neuroprotection in the damaged regions.

Our results demonstrate signs of microgliosis and astrogliosis detected by the markers Iba1 and GFAP, respectively. These signaling markers of late phase of inflammatory response are accompanied by neuronal damage in different limbic regions, including the dHipp as well as BL and the Pir. However, while the vHipp shows an increment in the Iba1 but not in GFAP staining, other structures such as the CA2 and CA3c subfield of dHipp have intact microglia but activated astrocytes and neuronal loss. The inflammatory signaling pathway in brain, including pro-inflammatory glial activation plays a crucial role for development of depressive state and could be an underlying mechanism of antidepressant activity of many drugs. The positive link between depression and microglial activation is detected both in patients (Nakatomi et al., 2014) and experimental rats (Iwata et al., 2016; Lee et al., 2018). Recently, we have reported that exposure to chronic lighting conditions of rats can produce depressive-like responses with diurnal variability and impaired melatonin release in plasma (Tchekalarova et al., 2018a) which data are in agreement with other studies (Fonken et al., 2009; Tapia-Osorio et al., 2013). Depression is characterized by brain inflammation and suppressed processes of neurogenesis (Eisch and Petrik, 2012; Lee et al., 2018). Neuroinflammatory response associated with augmented microglia is demonstrated in a chronic mild stress (CMS) model of depression specifically in the dHipp but not in vHipp (Yirmiya et al., 2015), thus suggesting model-dependent vulnerability of different limbic structures to activation of microglia. Moreover, high plasma corticosteroid level can induce enhancement of M1 pro-inflammatory microglial phenotype in the brain (Nair and Bonneau, 2006). The hyperactive hypothalamic-pituitary-adrenal (HPA) axis is considered a hallmark of major depressive disorder (Varghese and Brown, 2001). Patients with depression are frequently reported to have hypercortisolemia related to disrupted feedback regulation (Murphy, 1991; Pariente and Lightman, 2008). We also have found that two rat models of melatonin deficit (pinealctomy and exposure to CCL) can exhibit depressive responses associated with abnormal HPA axis (Tchekalarova et al., 2016, 2018a, 2018a) which is consistent with previous reports in other animal models of depression characterized by a sustained elevation of plasma corticosterone (Skupio et al., 2015). Moreover, treatment with Ago can exert antidepressant activity via restoration of feedback inhibitory control of HPA axis and neuroprotection in limbic structures (Tchekalarova et al., 2016).

As our previous and other literature data suggest Ago exerts a potent anti-inflammatory, neuroprotective and stimulatory effect on neurogenesis in different models with depressive-like symptomatic (Tchekalarova et al., 2016, 2018a, 2018a; Morley-Fletcher et al., 2011; Soumier et al., 2009). Therefore, we hypothesized that this antidepressant drug will affect positively an activated microglia and astrocytes as well as neuronal damage with concomitant beneficial influence on neurogenesis in the SGZ of DG in rats exposed to CCL. Recently, we have demonstrated that Ago exerts a chronobiologic antidepressant effect under CCL regime via correction of disturbed diurnal behavioral responses and impaired circadian rhythms of plasma melatonin release (Tchekalarova et al., 2018a). In the present study, with few exceptions such as in septo-temporal GrDG and septal BL, the treatment with Ago produces a
In the present study, the melatonin analog Ago was unable to attenuate morphological features of cellular damage in the CCL rat model of melatonin deficit. This result is surprising given the strong neuroprotective activity of Ago in the limbic structures following epileptic seizures (Tchekalarova et al., 2017). At the same time, the present data are in line with our previous findings that Ago could produce an antidepressant and anti-inflammatory effect without affecting the neuronal damage in association with behavioral changes in the streptozotocin-induced Alzheimer’s disease model.

Recently, we have reported that the circadian rhythm of MT1 and MT2 receptor expression in the hippocampus is not altered under CCL condition while Ago treatment elevates the MT1 receptors in the same structure at 10:00 a.m. in the LL group (Tchekalarova et al., 2020a). The limitation of our work is that we did not study the role of serotonin 5-HT2C receptors in the effects of Ago in this model of melatonin deficit. However, as in the previous studies (Ilijeva et al., 2019; Tchekalarova et al., 2017) the treatment with Ago was performed in the afternoon before the subjective dark phase when its chronobiotic capacity might be suggested to be preferably mediated via MT receptors. We can hypothetize that the anti-inflammatory effect of this antidepressant drug is mediated via MT1 receptors which are expressed in the pyramidal neurons of the hippocampal CA1-4 region (Savaskan et al., 2002) and are increased by Ago treatment in the CCL model.

Doublecortin is a reliable marker of neurogenesis within a progenitor late period and the immature postmitotic neurons (Couillard-Despres et al., 2005; Glesser et al., 1999). Our findings for a reduction in the number of DCX+ cells in the septo-temporal SGZ of DG under LL regime both in the periphery and brain (Molteni et al., 2013). These findings are in support of the idea that the antidepressant effect of Ago in a model of melatonin deficit is closely related to its anti-neurogenic capacity during the late phase against activated microglia.

In the present study, the melatonin analog Ago was unable to attenuate morphological features of cellular damage in the CCL rat model of melatonin deficit. This result is surprising given the strong neuroprotective activity of Ago in the limbic structures following epileptic seizures (Tchekalarova et al., 2017). At the same time, the present data are in line with our previous findings that Ago could produce an antidepressant and anti-inflammatory effect without affecting the neuronal damage in association with behavioral changes brain structures in the streptozotocin-induced Alzheimer’s disease model.
effect of this antidepressant. Soumier et al. (2009) have reported that the underlying mechanism of Ago enhancing effect on neurogenesis in intact rats detected both in dHipp and vHipp is associated with an increase in the levels of brain-derived neurotrophic factor (BDNF). Recently, we have shown that while CCL exposure disrupts sleep-wake cycle via impaired BDNF expression in the hippocampus during the light phase, the chronic Ago treatment exerts chronotropic activity through correction of sleep architecture and BDNF expression in the hippocampus (Tchekalarova et al., 2020a). Furthermore, the above-mentioned effects of this melatonin analog are mediated via simultaneous activation of melanin receptors and antagonism on 5HT 1A receptors (Tchekalarova et al., 2020a). Furthermore, the above-mentioned effects of this antidepressant, induces regional changes in hippocampal neurogenesis. Biol. Psychiat. 59, 1087–1096. https://doi.org/10.1016/j.biopsych.2005.11.025, Carlson, D.E., Chiu, W.C., 2008. The absence of circadian cues during recovery from sepsis modifies pituitary-adrenocortical function and impacts survival. Shock 29, 127–132. https://doi.org/10.1097/01.shk.0000301142.52629.67.

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