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Mechanisms Contributing to the Phase-Dependent Regulation of Neurogenesis by the Novel Antidepressant, Agomelatine, in the Adult Rat Hippocampus

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Agomelatine is a novel antidepressant acting as a melatonergic receptor agonist and serotonergic (5-HT_{2C}) receptor antagonist. In adult rats, chronic agomelatine treatment enhanced cell proliferation and neurogenesis in the ventral hippocampus (VH), a region pertinent to mood disorders. This study compared the effects of agomelatine on cell proliferation, maturation, and survival and investigated the cellular mechanisms underlying these effects. Agomelatine increased the ratio of mature *vs* immature neurons and enhanced neurite outgrowth of granular cells, suggesting an acceleration of maturation. The influence of agomelatine on maturation and survival was accompanied by a selective increase in the levels of BDNF (brain-derived neurotrophic factor) *vs* those of VEGF (vascular endothelial factor) and IGF-1 (insulin-like growth factor 1), which were not affected. Agomelatine also activated several cellular signals (extracellular signal-regulated kinase1/2, protein kinase B, and glycogen synthase kinase 3β) known to be modulated by antidepressants and implicated in the control of proliferation/survival. Furthermore, as agomelatine possesses both melatonergic agonist and serotonergic (5-HT_{2C}) antagonist properties, we determined whether melatonin and 5-HT_{2C} receptor antagonists similarly influence cell proliferation and survival. Only the 5-HT_{2C} receptor antagonists, SB243,213 or S32006, but not melatonin, mimicked the effects of agomelatine on cell proliferation in VH. The promoting effect of agomelatine on survival was not reproduced by the 5-HT_{2C} receptor antagonists or melatonin alone. However, it was blocked by a melatonin antagonist, S22153. These results show that agomelatine treatment facilitates all stages of neurogenesis and suggest that a joint effect of melatonin agonism and 5HT_{2C} antagonism may be involved in promotion by agomelatine of survival in the hippocampus.

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INTRODUCTION

Adult neurogenesis occurs in the dentate gyrus (DG) of the hippocampus of a large number of mammalian species, including humans (Eriksson *et al*, 1998). 'Neurogenesis' refers to a series of developmental steps including proliferation, differentiation and maturation, and it is a prerequisite for the development of fully functional neurons (Song *et al*, 2002). This long and complex process can abort at each one of these critical phases, complicating the study of the functional roles of new neurons and underpinning the importance of understanding the mechanisms involved in each step of cellular generation and survival. Although the implication of altered hippocampal neurogenesis in the pathogenesis of depression remains to be clarified (Kempermann *et al*, 2008), cell proliferation and neurogenesis are generally reduced in animal models of depression and increased by chronic antidepressant treatments (Warner-Schmidt and Duman, 2006). Furthermore, the reduced efficacy of antidepressants for exerting their behavioral effects in animals in which hippocampal neurogenesis has been compromised by irradiation suggests an important role of neurogenesis in the expression of anxiolytic-antidepressant-like properties of antidepressant agents (Santarelli *et al*, 2003; Jiang *et al*, 2005). There are also more recent reports indicating that the implication of neurogenesis in the behavioral effects of antidepressants may depend on the type of drug and its mode of action (Surget *et al*, 2008; Sahay and Hen, 2007). This explains increasing interest in new antidepressants, such as agomelatine (Kennedy, 2007), and in the molecular mechanisms underlying their influence on cellular neuroplasticity and adult neurogenesis. Accumulating evidence supports a role of trophic factors and related signaling cascades in the behavioral and neurogenic effects of antidepressants. This neurotrophic hypothesis of antidepressant actions is based on observations that antidepressants stimulate growth factors such as brain-derived neurotrophic factor (BDNF),

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insulin-like growth factor (IGF-1) and/or vascular endothelial growth factor (VEGF) expression (Schmidt and Duman, 2007; Warner-Schmidt and Duman, 2007). These growth factors generally enhance adult neurogenesis and may exert behavioral antidepressant-like effects (Jin *et al*, 2002; Anderson *et al*, 2002; Khawaja *et al*, 2004; Malberg and Blendy, 2005; Schechter *et al*, 2005; Schmidt and Duman, 2007). Related to the modulation of trophic factors action, antidepressants and mood stabilizers regulate the activity of signal-transduction pathways, such as the extracellular signal-regulated kinase (ERK), protein kinase B (AKT), and glycogen synthase kinase 3β (GSK3β) cascades, which are strongly implicated in synaptic plasticity, response to stress, and induction of mood disorders (Manji *et al*, 2003; Duman *et al*, 2007).

Agomelatine is a novel antidepressant possessing melatonergic receptor agonist (MT1 and MT2) (Audinot *et al*, 2003) and 5-HT_{2C} receptor antagonist properties (Millan *et al*, 2003), which displays robust antidepressant and anxiolytic-like actions in preclinical models (Papp *et al*, 2003; Millan *et al*, 2005) and alleviates various symptoms of major depression in humans (Loo *et al*, 2002; Kennedy and Emsley, 2006; Olié and Kasper, 2007; Dubocovich, 2006). Interestingly, both under basal and stressful conditions, agomelatine enhances hippocampal neurogenesis in rats (Maccari S, unpublished data; Banasr *et al*, 2006), with a distinctive profile in that it (1) selectively increases cell proliferation and neurogenesis in the ventral hippocampus (VH), a region connected with limbic structures, such as the amygdala, the prefrontal cortex and the nucleus accumbens, and strongly implicated in the response to stress (Bannerman *et al*, 2004), and (2) enhances the survival of newly generated cells throughout the entire hippocampus. Furthermore, agomelatine enhances hippocampal cell proliferation only after chronic (21 days), but not acute (4 h) or subchronic (8 days) administration (Banasr *et al*, 2006). This influence of agomelatine on adult neurogenesis in a specific subterritory of the hippocampus provides an insight into the possible functional implication of adult neurogenesis in the control of affective disorders and may also be an instructive model for investigating the cellular mechanisms involved in the effects of antidepressants on cell proliferation and survival, respectively.

Accordingly, using *in vivo* and *in vitro* approaches in combination with confocal analysis and multiple labeling of cells, this study characterized the influence of chronic agomelatine treatment on the phenotypic/morphologic maturation of newly formed granule cells and their survival over time. We also compared the regional influence of agomelatine with the effects of melatonin and those of the selective 5-HT_{2C} receptors antagonists, SB242,084, SB243,213, and S32006, upon hippocampal cell proliferation (bromodeoxyuridine (BrdU) injected the day after the last drug administration) and survival (BrdU injected before the first drug administration). Indeed, we have previously shown that various 5-HT receptor subtypes are involved in the regulation of adult neurogenesis and 5-HT_{2C} receptors have a selective influence depending on the neurogenic zones (Banasr *et al*, 2004). Here, we further specified the agomelatine's action on cell survival by examining the effect of a pretreatment with a melatonin receptor antagonist (S22153). Moreover, using western blots and ELISA techniques, we explored the effects of agomelatine on trophic factors (BDNF, VEGF, and IGF-1 proteins) and intracellular signaling pathways (ERK1/2, AKT, and GSK3β), involved in the control of proliferation and neuronal survival. Because 5-HT_{2C} receptors *per se* have been implicated in the pathogenesis and treatment of anxiety and depression (Millan, 2005), we also examined the effects of the 5-HT_{2C} receptor antagonists on BDNF, VEGF, and IGF-1 levels, as well as cell signaling pathways. Finally, the effects of melatonin on BDNF were also evaluated.

MATERIALS AND METHODS

Animals and Drug Treatments

Seven-week-old male Wistar rats (Charles River, France) were group-housed under standard conditions (12-h light/dark cycle, $20 \pm 2\,^{\circ}C$, food and water *ad libitum*). All procedures were conducted in accordance with the French Agriculture and Forestry Ministry (decree 87848, license 01498). SB242,084, a selective 5-HT_{2C} receptor neutral antagonist (Kennett *et al*, 1997), SB243,213, a selective 5-HT_{2C} receptor inverse agonist (Wood *et al*, 2001), and S32006, a novel 5-HT_{2C} receptor inverse agonist (Dekeyne *et al*, 2008), were injected once a day, at 10 mg/kg i.p. dissolved in sterile water or 1% hydroxyethylcellulose (HEC). Doses of 5-HT_{2C} antagonists were selected on the basis of the well-characterized actions of these drugs in functional studies performed both in our laboratory and elsewhere (Di Matteo *et al*, 1999; Kennett *et al*, 1997; Wood *et al*, 2001; Dekeyne *et al*, 2008). Agomelatine was injected as a suspension in 1% HEC at 40 mg/kg i.p. once a day for 8, 15, or 21 days, and melatonin was injected as a suspension in 1% HEC at 40 mg/kg i.p. once a day for 21 days. The choice of agomelatine and melatonin doses was made on the basis of their activity at this range of dose in animal models of depression and anxiety (Papp *et al*, 2003; Millan *et al*, 2005), and on neurogenesis (Banasr *et al*, 2006). S22153, an MT1/MT2 receptor antagonist (Weibel *et al*, 1999), dissolved in 1% HEC was injected at 10 mg/kg i.p. 15 min before each agomelatine administration (once a day for 15 days). All drugs were provided by IRIS (Institut de Recherches Internationales Servier, France), except melatonin (Sigma-Aldrich), and injected at 17:00. For cell proliferation study, BrdU (200 mg/kg i.p.) was administered 2 h before perfusion. For cell maturation and survival studies, animals received five injections of BrdU (75 mg/kg, 2-h intervals) the first day of treatment and were killed 8, 15, or 21 days later.

Immunohistochemistry and Quantification

Anaesthetized animals with chloral hydrate were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4). Serial coronal sections through the rostro-caudal hippocampal extent were collected and treated as reported (Banasr *et al*, 2006). For BrdU staining, we used a monoclonal mouse anti-BrdU (1 : 200; Dako, France, 48 h, $4\,^{\circ}C$), a biotinylated goat anti-mouse secondary antibody (1 : 200; Dako; 2 h RT) followed by amplification (Elite ABC Kit, Vector) and diaminobenzidine vizualization method. The BrdU-labeled cell quantification was performed as described (Banasr *et al*, 2006).

The BrdU-labeled cells present in the subgranular zone and in the granule cell layer of the DG were examined in the dorsal-rostral (interaural 6.20–3.70 mm) and the ventral-caudal (interaural 3.70–2.28 mm) part of the hippocampus (Paxinos and Watson, 1986).

For multiple labeling, sections were incubated with mouse anti-NeuN (1 : 1000; Chemicon, France), rabbit anti- GFAP (1 : 500; Dako), mouse anti-PSA-NCAM, (1/500, AbCys, France) for 48 h at 4 °C and exposed to respective secondary antibodies: Alexa Fluor 633 goat anti-mouse, Alexa Fluor 546 goat anti-rabbit, and Alexa Fluor 594 goat anti-mouse (1 : 200, Molecular Probes, France; 2 h RT). PSA-NCAM is a marker of neuroplasticity (Gascon *et al*, 2007) and when co-expressed with BrdU, it labels committed neuronal precursors in adult neurogenic niches (Ming and Song, 2005). Sections were then incubated with rat anti-BrdU (1 : 100; Jackson, France) followed by incubation with Alexa Fluor 488 donkey anti-rat antibody (1 : 100; Jackson, France; 2 h RT). The BrdU-positive cells co-labeled with GFAP, NeuN, and PSA-NCAM were visualized with a confocal scanning laser microscope, in z axis with a 0.5 μm step. The percentage of co-expression was determined in the dorsal hippocampus (DH) and VH (25 cells randomly picked in each region per rat).

ELISA and Western Blot Analyses

Animals were killed by decapitation and hippocampi were dissected out and stored at −80 °C until use. Tissue samples were homogenized at 4 °C in Promega lysis buffer (137 mM NaCl; 20 mM Tris-HCl, pH 8.0; 1% NP40; 10% glycerol) for ELISA and in EBM lysis buffer (0.20 mM Tris-HCl, pH 7.5; 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with phosphatase inhibitors (Halt phosphatase inhibitor cocktail from Pierce) for western blots. Both lysis buffers were supplemented with protease inhibitors cocktail (Roche). Samples were then sonicated and lysates were cleared by centrifugation at 4 °C, 15 000 *g*. Protein concentrations were determined according to the Bradford assay using bovine serum albumin as standard. Quantifications of VEGF and IGF-1 proteins were measured with Quantikine M mouse VEGF and IGF-1 enzyme immunoassay kits (R&D Systems) and BDNF protein with a mouse ELISA kit (Emax R immunoAssay System, Promega). ERK, AKT, and GSK3 phosphorylation was studied on samples diluted in loading buffer (Tris-HCl 0.3 M, pH6.8; 4% SDS; 50% glycerol; dithiothreitol 0.5 M). Proteins (50 μg) were resolved by SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. Immunodetection was carried out with mouse anti-phospho-ERK1/2 (1 : 2000; Cell Signaling Technology, CST), rabbit anti-phospho-AKT (1 : 1000; CST), rabbit anti-phospho-GSK3$\alpha\beta$ (1 : 1000; CST), rabbit anti-ERK1/2 (1 : 1000, CST), rabbit anti-AKT (1 : 1000; CST), and rabbit anti-GSK3β (1 : 1000; CST). Antibodies against actin or tubulin (Sigma) were used to control for equal protein loading. Bands were visualized by enhanced chemiluminescence (ECL kit from Pierce) and quantified using Image J software. Immunoreactivity of each sample was then normalized to the amount of actin or tubulin and expressed as a percentage of the value obtained in the vehicle-treated animals.

Hippocampal Primary Culture and Morphometric Analyses

To target the peak of granule cells ontogenesis (Altman and Bayer, 1990), hippocampi were obtained from 4-day-old Wistar rat pups. Pooled hippocampi were mechanically triturated, suspended in Neurobasal-A medium supplemented with 2% heat-inactivated fetal calf serum and plated at 300 000 cells/ml on poly-L-ornithin (10%) precoated coverslips. After 24 h, the entire medium was replaced with Neurobasal-A medium supplemented with 2% B-27 and an antibiotic mixture of penicillin (50 U/ml) and streptomycin (50 μg/ml) (GIBCO, Invitrogen). Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere and daily treated with various concentrations of agomelatine (10^{-8}, 10^{-7}, and 10^{-6} M) or vehicle for 8 days. Agomelatine was diluted in alcohol (10^{-1} M) and then in culture medium up to final concentrations.

Cultures were fixed in 4% PFA, incubated with 3% bovine serum albumin (Sigma-Aldrich) and overnight incubated at 4 °C with mouse anti-nestin (1 : 1000, Chemicon), rabbit anti-GFAP (1 : 500, Dako), mouse anti-MAP-2 (1/250, Sigma-Aldrich), and rabbit anti-Prox-1 (1/2000, Abcys). Prox 1 is a transcription factor allowing the detection of new granule neurons in the DG (Pleasure *et al*, 2000; Brandt *et al*, 2003). Double labeling was performed with Alexa Fluor 488- and 546- goat and rabbit antibodies (1 : 1000, Molecular Probes). Nuclear counterstaining was performed using DAPI (0.001%, Sigma-Aldrich). Labeled cells were counted with a fluorescent microscope in five–six randomly chosen fields on two dishes per experimental condition, from three independent cultures. Total dendritic length and branching point numbers per cell were measured on 100 Prox1-MAP-2 co-labeled cells per dish and experimental condition using Neurite Outgrowth from the Metamorph software.

Statistical Analyses

Data are means ± SEM (5–7 animals per group). For cell proliferation and survival studies, analyses on the number of BrdU-labeled cells in the DH and VH were performed using two-way ANOVA (region × drug treatment). Analysis of cell maturation was performed with a two-way ANOVA (phenotype × drug treatment). For the other studies, statistical analyses were performed with one-way ANOVA followed by Holm-Sidak's test for multiple comparison procedures. The level of statistical significance was set at $p < 0.05$.

RESULTS

Agomelatine Increases Cell Maturation *In Vivo* and *In Vitro*

The degree of maturation of newly formed cells labeled with BrdU *in vivo* was determined at 8 and 15 days of development, using a combination of PSA-NCAM and NeuN labeling to assess different stages of neuronal maturation. At 8 days, under control conditions, the majority of BrdU-labeled cells expressed PSA-NCAM only (Figure 1a). PSA-NCAM-NeuN co-expression was

detected in 10% of BrdU-labeled cells, while almost no new neurons (0.2%) expressing NeuN only were detected. Consistent with the transient expression of PSA-NCAM (Seki, 2002), a 42% decrease in the number of PSA-NCAM-labeled cells excluding NeuN was observed at 15 days, associated with a threefold increase in the number of cells co-expressing PSA-NCAM and NeuN (Figure 1a and b). When compared with the vehicle-treated group at 8 days, agomelatine induced a small but significant decrease in the number of PSA-NCAM cells (9%; $p<0.05$), mirroring the increase in PSA-NCAM-NeuN co-labeled cells (8%; $p<0.05$), and it did not influence NeuN expression (Figure 1a). At 15 days, agomelatine induced also a decrease in the proportion of PSA-NCAM-labeled cells (11%; $p<0.01$) and an increase in the number of NeuN cells (7%; $p<0.05$), while no significant difference was observed in the proportion of cells co-labeled by PSA-NCAM and NeuN (Figure 1b). As illustrated in the pie-chart (Figure 1c) showing the percentages of all BrdU cell types, including unidentified ones, agomelatine produced a shift in differentiation leading to a highly significant increase in the number of mature neurons, which was doubled as compared with control values ($p<0.01$).

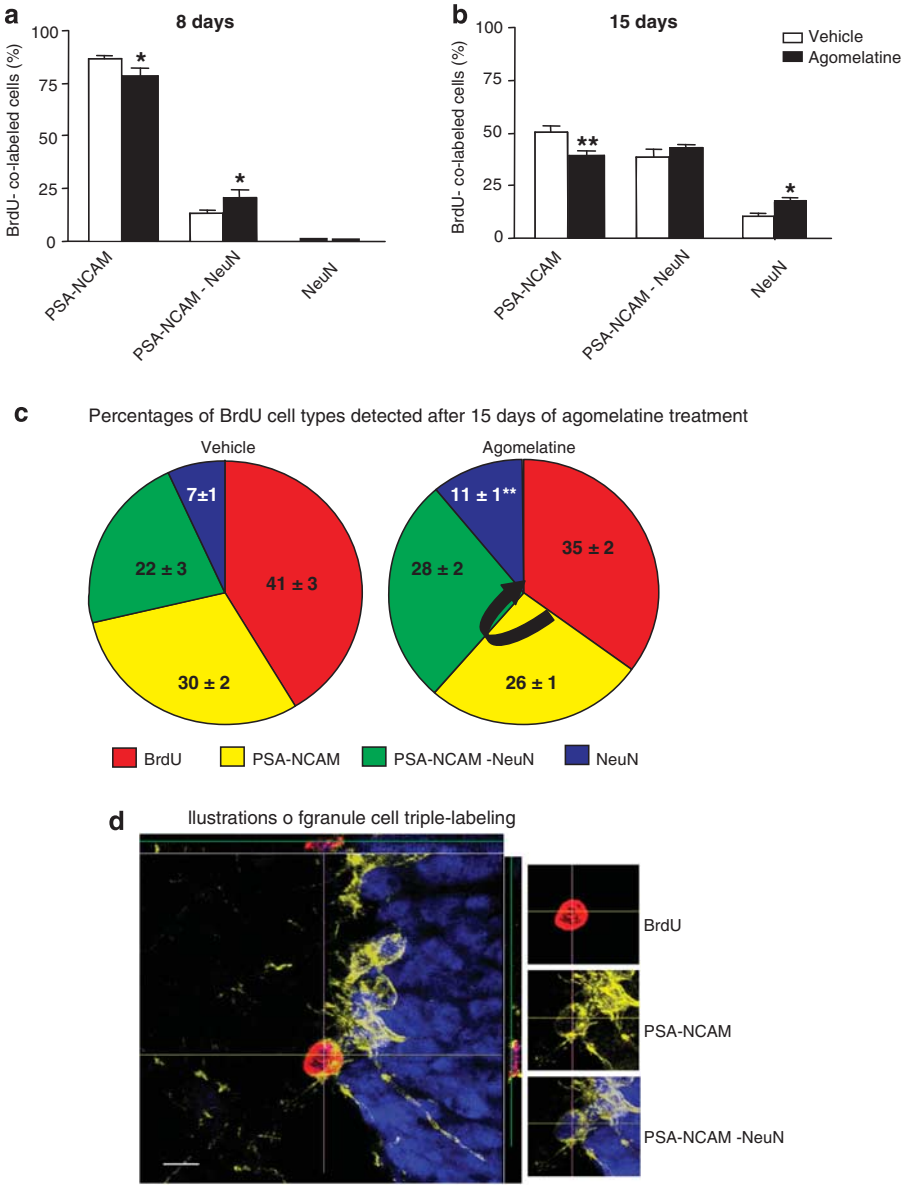

Figure 1 Agomelatine treatment accelerates the phenotypic maturation of newborn granule cells *in vivo*. Ratios of different cell types were estimated at different stages of development using confocal microscopy and multiple labeling of markers selective for immature (PSA-NCAM) and mature neurons (NeuN). Same studies were performed at 8 (a) and 15 days (b) after BrdU injection and compared between agomelatine- *vs* vehicle-treated rats. Bar graphs show decreases in percentage of newborn cells labeled with PSA-NCAM only and corresponding increases in more mature (PSA-NCAM/NeuN) and NeuN only labeled cells, over time. Results are presented as means ± SEM of number of BrdU-co-labeled cells (two-way ANOVA, $*p<0.05$, $**p<0.01$ *vs* vehicle). (c) The pie-chart graphs show the distribution of all BrdU cell types, including unidentified BrdU cells, at 15 days and the shift toward more mature neurons. Results are means ± SEM of percentages expressed relative to BrdU cells for $n=6$–8 rats by group (two-way ANOVA, $*p<0.05$, $**p<0.01$ *vs* vehicle) (d) Illustration of a BrdU cell co-labeled with PSA-NCAM and NeuN (arrow). Scale bar: 10 μ.

To study the effect of agomelatine on morphological maturation, we developed a postnatal hippocampal culture relatively enriched in granule cells as identified by Prox1 labeling (Pleasure *et al*, 2000). At DIV 8, postnatal hippocampal cultures contained a majority of neural progenitor cells identified by Nestin expression (41% of DAPI-labeled cells; Figure 2), and rather similar proportions of astrocytes detected using GFAP (21% of DAPI-labeled cells; Figure 2) and neurons either detected by MAP-2 (18% of DAPI-labeled cells) or Prox1, representing the granule cell population (5% of DAPI-labeled cells; Figure 2). Although the morphologies of GFAP- and Nestin-labeled cells are rather close, we found few double-labeled cells. All Prox1-labeled cells expressed MAP-2 and represented about 20% of this population. At this developmental stage, Prox1-MAP-2 co-labeled cells were characterized by small round-ovoid cell bodies (about 10-μm in diameter) and unipolar primary dendritic trees (Figure 2). Exposure to various concentrations of agomelatine for 8 days did not affect the total number of MAP-2-labeled cells, whereas 10^{-7} M agomelatine significantly increased the number of Prox1-MAP-2-labeled cells (46%; $p<0.05$) (Figure 3a and b). A tendency toward an increase was also observed at 10^{-8} M, whereas there was no effect at the highest dose of 10^{-6} M. Moreover, agomelatine did not affect the number of GFAP-labeled cells (data not shown). Using the Neurite Outgrowth module of Metamorph software, we could trace and quantify the dendritic profile of each Prox1 granule neuron (Figure 3c,d and e). Under control conditions, the mean neurite length of granule neurons was around 110 μm, which was increased by 35 and 38% in cultures treated with 10^{-8} and 10^{-7} M agomelatine, respectively ($p<0.01$, Figure 3c). The number of branching points per granule neuron was also increased at agomelatine concentrations of 10^{-8} M (28%, $p<0.05$) and 10^{-7} M (47%, $p<0.05$) (Figure 3d). Agomelatine (10^{-6} M) had no effect on neurite length or the number of branching points.

Agomelatine Increases Cell Survival *In Vivo*

As antidepressant effects on neurogenesis depend on the duration of treatment, we first determined the time course of cell survival following 8, 15, and 21 days of agomelatine administration (Figure 4). Under control condition, the mean number of BrdU cells/DG decreased significantly between 8 and 15 days post-BrdU injections (two-way ANOVA, time × region, $p<0.05$). When compared with the vehicle-treated (control) group, agomelatine had no effect at 8 days in the DH or VH, but it produced significant increases in the number of BrdU-labeled cells in both regions after 15 or 21 days administration. We thus used the 15 or 21 days administration for the next experiments on cell survival.

We then compared the effects of 21 days administration of agomelatine, melatonin, and 5-HT_{2C} receptor antagonists on cell survival. Only agomelatine produced significant increases in both the DH and VH (23 and 33%, respectively, $p<0.05$), whereas SB243,213, S32006, SB242,084, and melatonin had no effect (Figure 5c and d). These changes led to similar increases in newly formed neurons or astrocytes, as shown by the confocal microscopy analyses revealing no effect of agomelatine on the respective percentages of BrdU-NeuN- *vs* BrdU-GFAP-labeled cells neither in the DH nor in the VH (Table 1). As melatonin has been implicated in hippocampal cell survival (Kong *et al*, 2008; Quiros *et al*, 2008; Manda *et al*, 2009; Ramirez-Rodriguez *et al*, 2009), we tried to show a possible implication of melatonin agonist properties in the effect of agomelatine by adding a melatonin inhibitor together with agomelatine. We found that the daily pretreatment with the

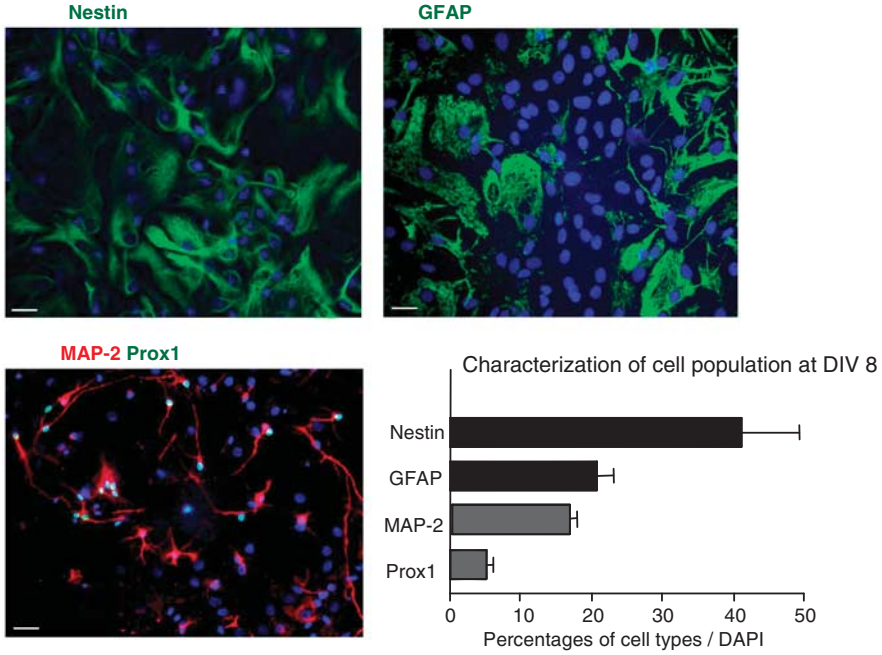

Figure 2 Cellular characterization of postnatal (P4) hippocampal cultures at 8 days *in vitro* (DIV 8). Neural progenitor cells, astrocytes, and neurons were detected with Nestin, GFAP, and MAP-2 antibodies, respectively. Granular cells were identified with Prox1 expression. Results are expressed as a percentage of DAPI-labeled cells (blue) in three independent experiments. Scale bar: 50 μ.

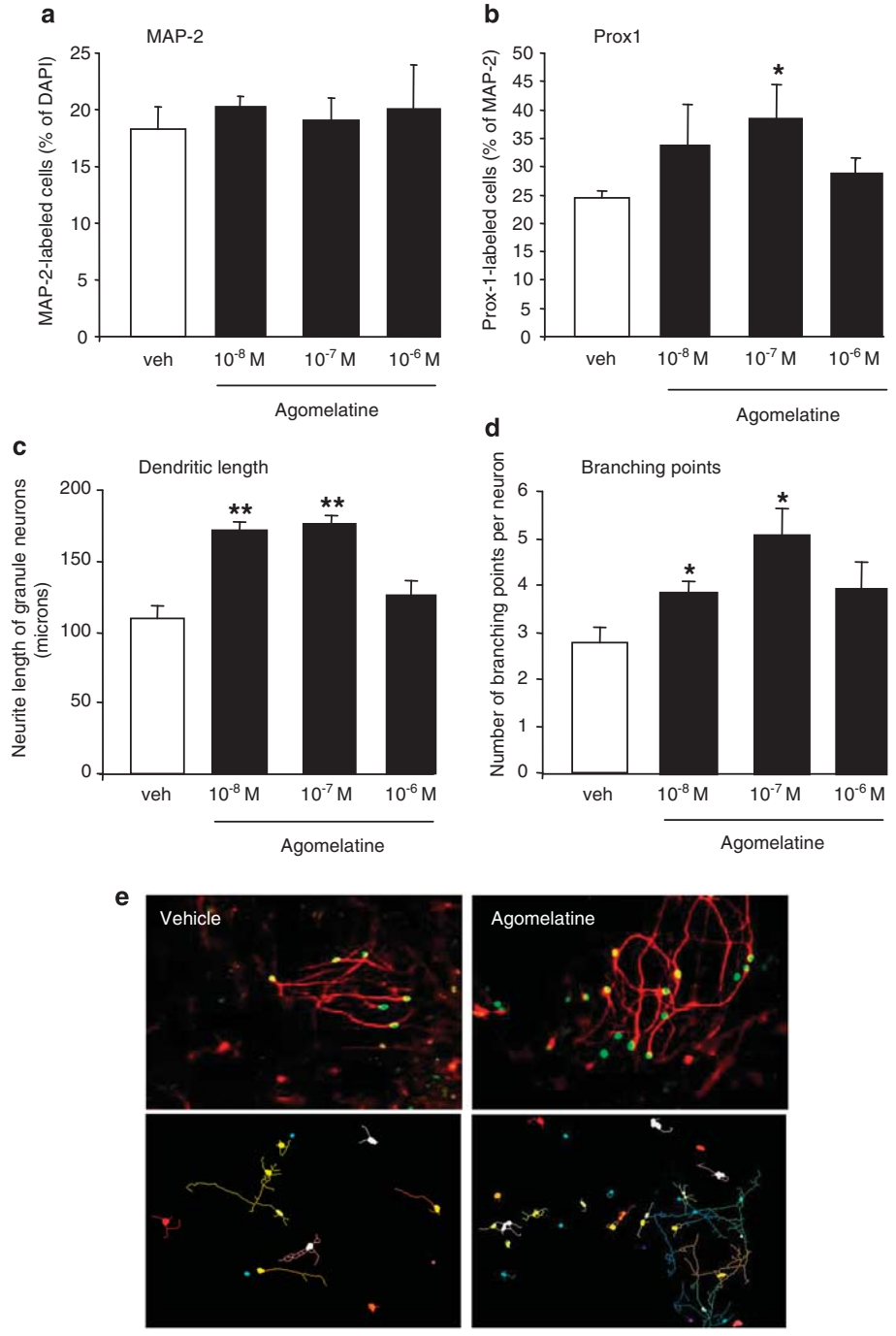

Figure 3 Agomelatine treatment increases the morphologic maturation of granule cells *in vitro*. Quantifications of MAP-2 (a) and Prox1-labeled (b) cells in postnatal hippocampal cultures at DIV 8 treated with various concentrations of agomelatine (10^{-8}, 10^{-7}, and 10^{-6} M) or vehicle. Agomelatine treatment did not affect the number of MAP-2 cells (a) and increased the percentage of Prox1 cells (b), their dendritic length (c), and number of branching points (d). Results are means ± SEM of the percentage of DAPI (a) or MAP-2-labeled cells (b). Metamorph software was used to measure dendritic length and branching points per granule neuron expressed as means ± SEM (c, d). Representative examples of Prox1-MAP2 cells in vehicle- and agomelatine (10^{-7} M)-treated cultures, analyzed with Metamorph software (e). Cells were analyzed in three independent experiments for each condition (ANOVA $*p<0.05$; $**p<0.01$ *vs* vehicle). Scale bar: 50 μ.

melatonin antagonist, S 22153, just before agomelatine administration abolished the agomelatine effect on cell survival at 15 days post-BrdU injection. Indeed, the mean number ± SEM of BrdU cells of treated rats in both subregions (DH: 777 ± 62; VH: 547 ± 24) was not any more different from control (DH: 767 ± 45; VH: 487 ± 47).

Agomelatine Increases Cell Proliferation *In Vivo*

The effects of agomelatine, melatonin, and 5-HT2C antagonists were also compared with cell proliferation after 21 days of treatment. We corroborated our previous study (Banasr *et al*, 2006), showing that agomelatine induced a

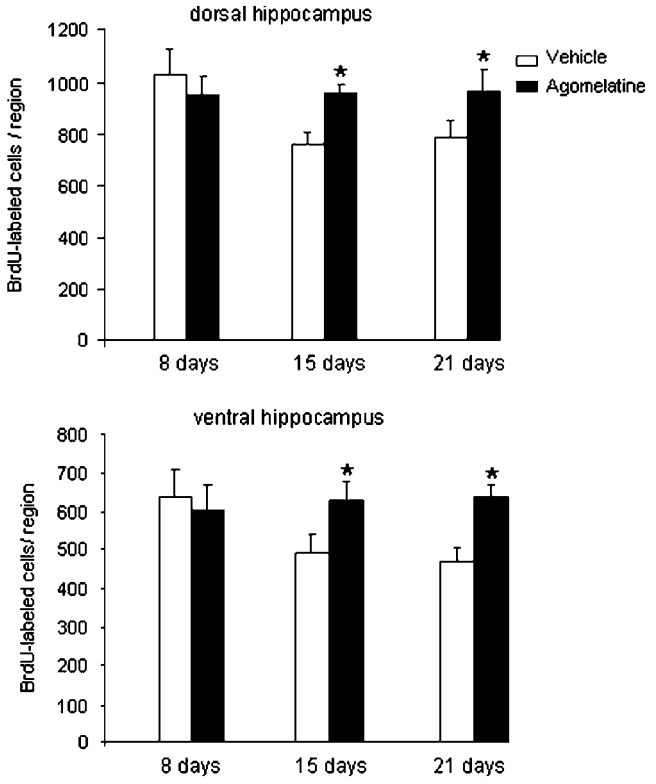

Figure 4 Time course analysis of cell survival. Hippocampal BrdU-labeled cells were quantified after 8, 15, or 21 days of agomelatine treatment (40 mg/kg i.p.). BrdU was injected the first day of treatment. Results are means ± SEM of the number of BrdU-labeled cells per region quantified in the dorsal and ventral GCL for $n = 7$–8 rats per group (two-way ANOVA $*p < 0.05$ *vs* vehicle).

39% increase ($p < 0.05$) in the percentage of BrdU-labeled cells in the VH, whereas the DH was not affected (Figure 5a and b). The 5-HT_{2C} receptor antagonists (inverse agonists), S32006 and SB242,313, also produced significant increases in cell proliferation in the VH (30%, $p < 0.05$ and 42%, $p < 0.01$, respectively) without affecting the DH (Figure 5a and b). In contrast, SB242,084 (a neutral antagonist) was ineffective (Figure 5a and b). Melatonin induced no significant change in cell proliferation in either the VH or the DH (Figure 5a and b).

Agomelatine has Selective Effect on BDNF Level and Stimulates ERK1/2, AKT, and GSK3β Cell Signaling Pathways

Changes in hippocampal BDNF, VEGF, and IGF-1 levels were examined after chronic (21 days) agomelatine treatment. ELISA analyses performed in hippocampal tissue extracts 16 h after the last drug administration showed that agomelatine treatment induced significant increases in the whole hippocampal BDNF level (28%; $p = 0.02$; agomelatine group: 32.4 ± 1.3; control group: 25.3 ± 0.8 pg/mg prot.) but produced no change in VEGF or IGF-1 levels. Quantifications of trophic factors were performed separately on the DH and VH to search for a possible regional effect that could have been masked in the global analysis However, no regional change was observed for VEGF or IGF1, while agomelatine induced significant increases in BDNF level both in the DH and VH (37 and 19% respectively, $p < 0.05$) (Figure 6).

The effects of chronic agomelatine administration on the level of phosphorylation of ERK1/2, AKT, and GSK3β in hippocampal tissue, 16 h after the last drug injection were also examined. Although no change was observed in total protein levels in any case, a twofold increase in ratios of P-ERK1/2 to ERK1/2 ($p < 0.01$), and 30 and 57% increases for ratios of P-AKT to AKT and of P-GSK3β to GSK3β, respectively ($p < 0.05$) were detetected in agomelatine-treated rats (Figure 7).

Effects of 5-HT2C Receptor Antagonists on Trophic Factors and Cell Signaling Pathways

To test a potential implication of 5-HT_{2C} receptors in agomelatine-induced changes in neurogenesis by trophic factors, we also measured BDNF, VEGF, and IGF-1 levels in hippocampal tissue after chronic (21 days) administration of SB242,084 or SB243,213, but did not find any significant effect (Table 2).

With regard to cell signaling pathways (Figure 8), these treatments induced significant increases in the level of phosphorylation of ERK1/2 (33 and 34%, respectively; $p < 0.05$). By contrast, decreases in the level of phosphorylation of AKT and GSK3β were detected in hippocampal tissue obtained from rats treated with SB243,213 (21 and 29%, respectively; $p < 0.05$).

Effect of Melatonin on BDNF Level

We also examined the consequences of 21 days treatment with melatonin on hippocampal level of BDNF. Compared with agomelatine treatment, melatonin induced a smaller increase at the limit of significance (17%; $p = 0.05$) in hippocampal BDNF level (melatonin group: 29.6 ± 0.5; control group: 25.3 ± 0.8 pg/mg prot.).

DISCUSSION

The present experiments show that agomelatine influences various phases of adult hippocampal neurogenesis, including cell proliferation, maturation, and survival, with distinctive patterns of action. Our data suggest a role for 5-HT_{2C} receptor blockade in the regulation of cell proliferation in the VH, whereas control of cell survival throughout the whole hippocampus depends upon the joint action of agomelatine at both types of receptors. Furthermore, the results also suggest that recruitment of BDNF and the ERK and AKT–GSK3β signaling pathways participate in the induction of hippocampal neurogenesis by agomelatine.

Agomelatine Selectively Increases Cell Proliferation in VH: Possible Mediation By 5-HT_{2c} Receptors

The selective agomelatine-induced increase in cell proliferation in the VH is of particular interest with regard to the anatomical and functional differences between the VH (temporal pole) and DH (septal pole) (Moser and Moser, 1998; Bannerman *et al*, 2004). The projections of the VH to the prefrontal cortex and its strong connection with the amygdala support the view that

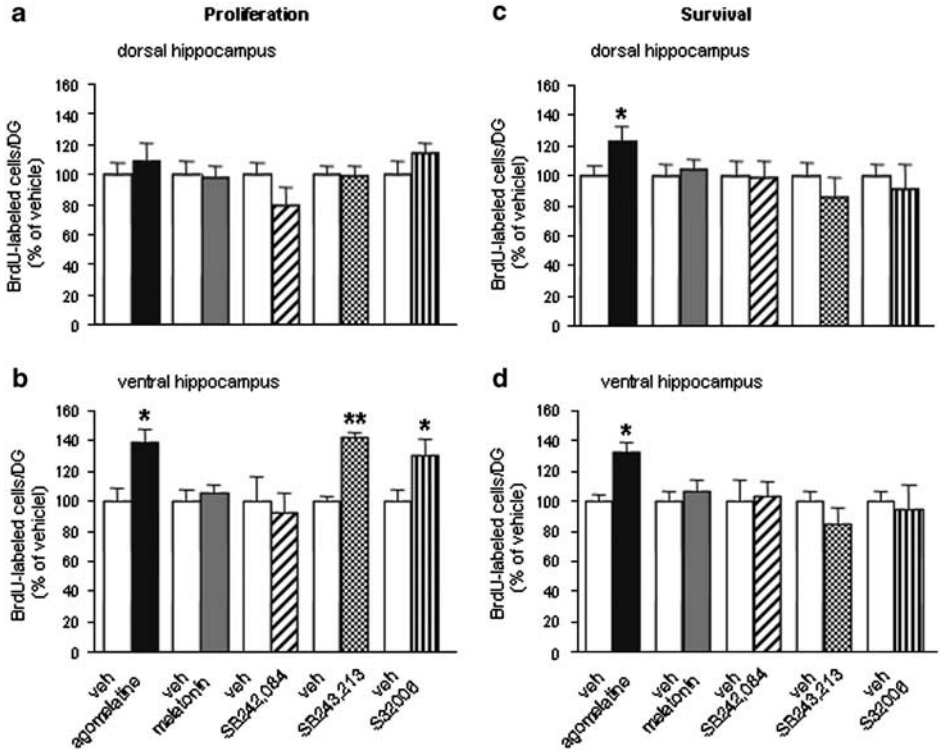

Figure 5 Comparative effects of agomelatine, melatonin, and 5-HT_{2C} receptor antagonists on cell proliferation and survival after 21 days treatment. BrdU was injected either at the end (proliferation) or beginning (survival) of drug treatments. BrdU-labeled cells were quantified in the SGZ (proliferation), or GCL (survival) both in the dorsal (a, c) and ventral (b, d) hippocampus. Rats were treated once daily with agomelatine or melatonin (40 mg/kg i.p., each) or various 5-HT_{2C} receptor antagonists (10 mg/kg i.p.). Treatment with agomelatine, SB243,213, and S32006 selectively increased cell proliferation in the ventral hippocampus, whereas an increase in cell survival was observed only after agomelatine treatment in the ventral and dorsal hippocampus. Results are means ± SEM of the number of BrdU-labeled cells expressed as a percentage of respective controls, for $n = 6$–8 rats per group (two-way ANOVA $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* vehicle).

Table 1 Percentages of BrdU Cells Co-expressing NeuN or GFAP

	BrdU unidentified		BrdU/NeuN		BrdU/GFAP	
	Dorsal	**Ventral**	**Dorsal**	**Ventral**	**Dorsal**	**Ventral**
Vehicle	32 ± 3	34 ± 4	65 ± 3	63 ± 4	3 ± 2	3 ± 1
Agomelatine	37 ± 4	37 ± 5	62 ± 4	61 ± 6	3 ± 1	2 ± 1

Daily administrations of agomelatine or vehicle given for 21 days and starting with BrdU injection did not change the ratio neuron (NeuN)/glia (GFAP). Results are means ± SEM of BrdU-labeled cells expressed in percentages, quantified in the GCL of the dorsal and ventral hippocampus (25 cells per rat, each) for five rats per group.

the VH is particularly involved in 'emotional circuitry' and more specialized for the control of anxiety and depression-related functions, whereas the DH is more implicated in cognitive functions (Bannerman *et al*, 2004; Engin and Treit, 2007). Although this regional dissociation regarding the implication of new neurons in learning and memory processes is not so simple (Snyder *et al*, 2008), several studies showed selective decreases in neurogenesis in the VH following various stress exposures, which elicit depressive-like behavior in adult rat (Kim *et al*, 2005; Jayatissa *et al*, 2006; Lagace *et al*, 2006). Moreover, prenatal stress induced a selective reduction in neurogenesis in the VH associated with anxious behavior and reversed by agomelatine treatment (Maccari S, unpublished data). These results further suggest that this regional stimulatory effect of agomelatine on cell proliferation is not mediated by melatonin receptors, but rather by the blockade of 5-HT_{2C} receptors. Indeed, two different 5-HT_{2C} receptor antagonists, SB243,213 and S32006 (Wood *et al*, 2001; Dekeyne *et al*, 2008), mimicked agomelatine's effect on cell proliferation in the VH only. In line with these observations, it has also been demonstrated that 5-HT_{2C} receptor-binding sites are more densely expressed in the VH compared with DH (Holmes *et al*, 1995). Interestingly, the anxiolytic properties of agomelatine principally reflect acute blockade of 5-HT_{2C} receptors (Millan *et al*, 2005), and 5-HT_{2C} receptors located in the VH are specifically involved in the response to anxiety (Alves *et al*, 2004). Altogether, these data reinforce the hypothesis of a functional dissociation in neurogenesis between hippocampal subregions that may be related to how hippocampal circuit dynamics underlie affective disorders (Meltzer *et al*, 2005; Airan *et al*, 2007).

Intriguingly, in contrast to SB243,213 and S32006, another antagonist, SB242,084 administered at a functionally equivalent dose, failed to induce proliferation in the VH. One factor which may account for this difference is that SB242,084 consistently behaves as a neutral antagonist at unedited, wild-type 5-HT_{2C} receptors, whereas SB243,203 and S32006 are essentially inverse agonists (Kennett *et al*,

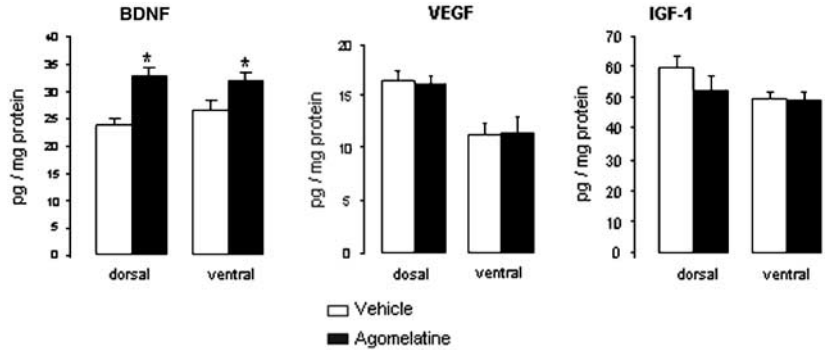

Figure 6 Effects of agomelatine treatment on trophic factor levels in the hippocampus. Levels of VEGF, IGF-1, and BDNF protein were measured in the dorsal and ventral hippocampus 16 h after the last vehicle or agomelatine treatment (21 days, 40 mg/kg i.p.). Hippocampal extracts were analyzed by ELISA assays. Results are means ± SEM pg/mg total protein for six rats per group (ANOVA $*p<0.05$ *vs* vehicle).

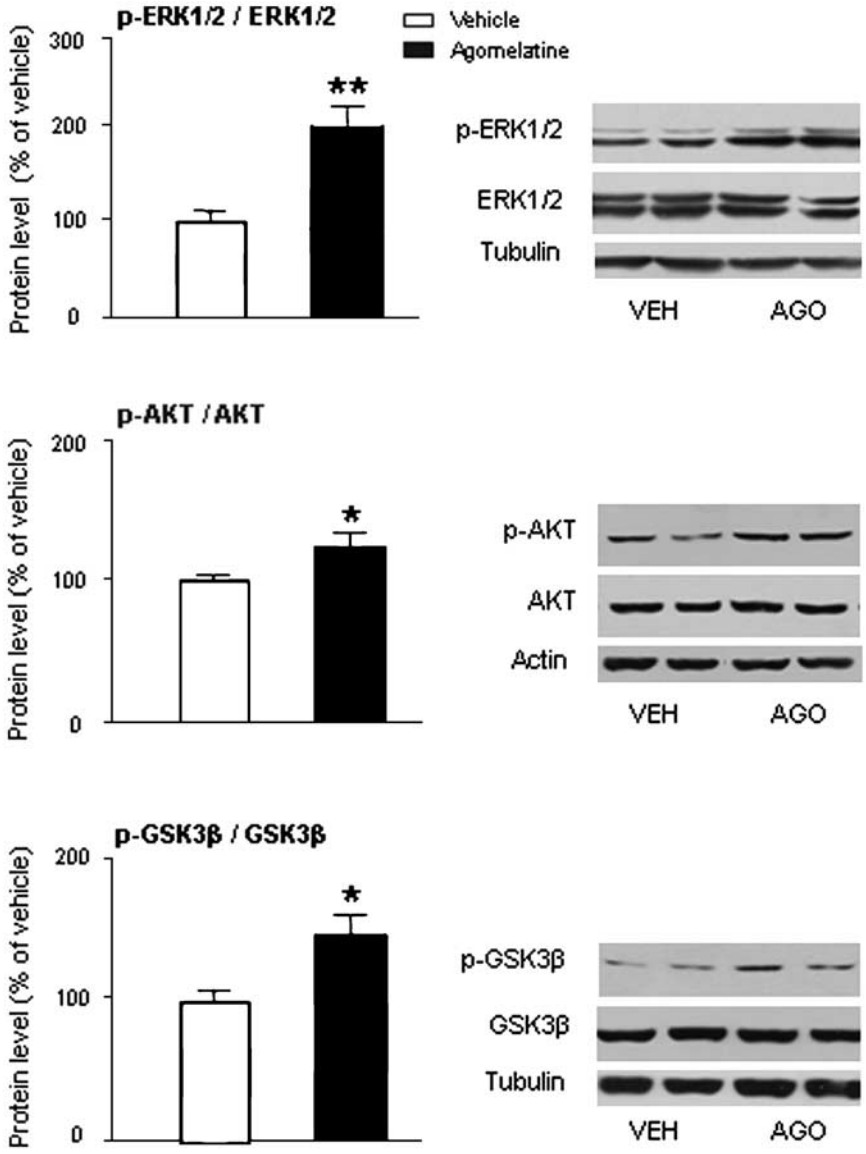

Figure 7 Effects of agomelatine on the ratio of levels of phosphorylated to total ERK1/2, AKT, and GSK3β protein. Hippocampal tissue was taken 16 h after the last vehicle or agomelatine treatment (21 days, 40 mg/kg i.p). Data are calculated as optical density (OD), expressed relative to the corresponding level of actin (AKT) or tubulin (ERK1/2 and GSK3β), normalized to the corresponding total ERK1/2, AKT, or GSK3β level. Results are presented as mean ± SEM percentage relative to vehicle levels (one-way ANOVA, $*p<0.05$; $**p<0.01$).

Table 2 Effects of 5-HT_{2C} Antagonists on Hippocampal Levels of Trophic Factors

(pg/mg prot.)	BDNF	VEGF	IGF-I
Vehicle	21.7 ± 1.0	17.6 ± 0.8	91.3 ± 4.1
SB242,084	19,8 ± 1.4	17.3 ± 0.8	96.4 ± 3.4
SB243,213	17.7 ± 1.1	16.5 ± 0.5	88.1 ± 3.2

Daily administrations of 5-HT2C antagonists or vehicle given for 21 days did not change the levels of trophic factors measured in the whole hippocampus. Results are means ± SEM for 6 rats per group.

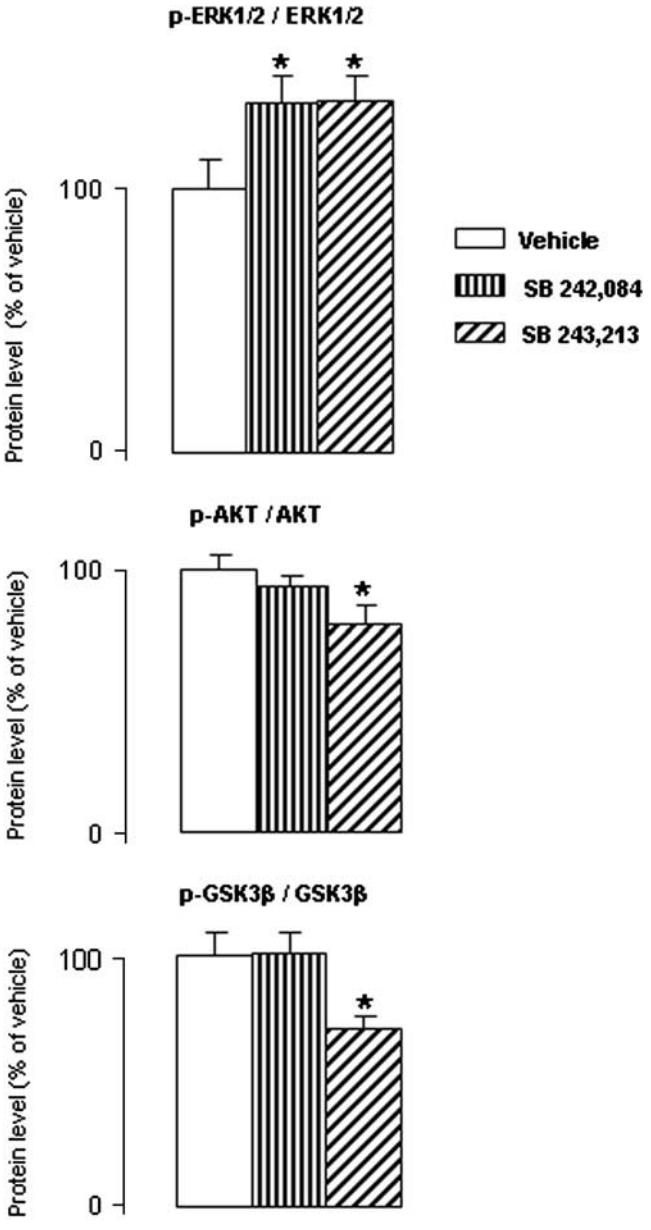

Figure 8 Effects of 5-HT_{2C} receptor antagonists on the ratio of levels of phosphorylated to total ERK1/2, AKT, and GSK3β protein. Hippocampal tissue was taken 16 h after the last vehicle, SB242084 or SB243213 treatment (21 days, 10 mg/kg i.p). Data are calculated as optical density (OD), expressed relative to the corresponding level of actin (AKT) or tubulin (ERK1/2 and GSK3β), normalized to the corresponding total ERK1/2, AKT, or GSK3β level. Results are presented as mean ± SEM percentage relative to vehicle levels (one-way ANOVA, *$p < 0.05$).

1997; Wood *et al*, 2001; Berg *et al*, 2006; Dekeyne *et al*, 2008; Chanrion *et al*, 2008; Millan MJ unpublished observations). The notion that inverse agonism is required for enhancing proliferation in the VH is supported by evidence that certain cerebral populations of 5-HT_{2C} receptors are constitutively active (Berg *et al*, 2005), including sites tonically inhibitory, through GABAergic interneurones, to ascending dopaminergic or noradrenergic systems that are known to increase cell proliferation (Invernizzi *et al*, 2007; Hoglinger *et al*, 2004; Kulkarni *et al*, 2002; Millan *et al*, 2008; Aloyo *et al*, 2009). However, the interpretation of these data is also complicated by the fact that constitutive activity at 5-HT_{2C} sites is regulated by mRNA editing that differs between brain regions; extensively edited 5-HT_{2C} receptor isoforms being constitutively silent (Burns *et al*, 1997; Sanders-Bush *et al*, 2003). Notably, the cell population and receptor isoform controlling VH proliferation remain to be identified. Moreover, the possible neutral antagonist *vs* inverse agonist actions of agomelatine are still under exploration and they will anyway be modified by its concomitant stimulation of melatonin receptors.

Agomelatine Promotes Maturation and Survival in the Hippocampus: BDNF Involvement?

How antidepressant influences the different phases of neurogenesis, and particularly maturation, has been poorly investigated, although it is a crucial step for the future integration and function of newly formed cells. A recent study showed that fluoxetine, known to enhance the proliferation of early progenitors cells in the adult brain (Encinas *et al*, 2006), also increases the maturation and survival of newborn neurons at 21 days post-BrdU (Wang *et al*, 2008). Here, we found that agomelatine appeared to induce an early acceleration of cell maturation at 8 days of development. This observation would benefit from direct comparisons with other classes of antidepressants, as it suggests that agomelatine precociously influences the immature neurons at a stage when they are synaptically silent but still respond to neurotransmitters, hormones, and trophic factors (Overstreet-Wadiche and Westbrook, 2006; Laplagne *et al*, 2006). Similarly, agomelatine increased the proportion of mature *vs* immature neurons at 15 days of development, when the cells start to develop dendritic arborization, to extend axon terminals for establishing synapses with their targets, and to be integrated into hippocampal circuitry (Overstreet-Wadiche and Westbrook, 2006). As dentate granule cells born during early postnatal and adult periods have very similar behavior (Laplagne *et al*, 2006), we used postnatal hippocampal cultures to evaluate the effects of agomelatine on the dendritic development of granule cells, which has an important role in the functional integration of newly formed neurons into hippocampal networks (Overstreet-Wadiche and Westbrook, 2006). Likely resulting from changes in proliferation, maturation, and survival, agomelatine selectively increases the number of granule cells expressing Prox1 and promotes their dendritic extension and arborization. In addition, *in vivo* agomelatine treatment also increased the survival of newborn cells at 15 and 21 days. This study further suggests that the early acceleration of maturation by agomelatine can induce an increase in the survival of newborn granule cells

at a critical period of development. Interestingly, results from our previous study show that 8 days of agomelatine treatment does not affect cell proliferation (Banasr *et al*, 2006), reinforcing the view of distinct regulation of proliferation *vs* maturation or survival (Lee *et al*, 2006; Plumpe *et al*, 2006; Olson *et al*, 2006; Hernandez-Rabaza *et al*, 2006).

Consistent with this view, although the agomelatine-induced stimulation of cell proliferation may preferentially involve 5-HT_{2C} receptor blockade, the increase in cell maturation and survival may be because of a joint action on melatonergic and 5-HT_{2C} receptors. Indeed, the agomelatine-induced increase in cell survival was suppressed by a pretreatment with a melatonin receptor antagonist, whereas neither the 5-HT_{2C} receptor antagonists nor melatonin alone mimicked this effect. The implication of melatonin agonist properties of agomelatine in this process is consistent with recent data showing a melatonin-induced increase of new granule cell maturation and survival in mice (Ramirez-Rodriguez *et al*, 2009). This neurotrophic role of melatonin was also suggested by *in vitro* data on viability and differentiation of neural stem cells (NSCs), which was associated to an increase in BDNF expression (Kong *et al*, 2008), even if in our experimental conditions melatonin alone does not modify cell survival. Indeed, we also found an increase in hippocampal BDNF level in agomelatine- and melatonin-treated rats, but for melatonin the increase is less pronounced. This difference in the influence of agomelatine and melatonine on BDNF levels may explain the lack of melatonin effects on cell survival in our experimental conditions and supports the need of a joint action between melatonergic agonist and 5-HT_{2C} antagonist properties for agomelatine effects on cell survival. Indeed, BDNF has long been involved in maturation and survival (Sairanen *et al*, 2005; Bergami *et al*, 2008), although it has also been recently associated with the regulation of proliferation of hippocampal progenitors (Li *et al*, 2008). Our results are consistent with the general protective action of melatonin on neurons, potentially mediated by BDNF (Quiros *et al*, 2008; Manda *et al*, 2009; Imbesi *et al*, 2008) . By contrast, melatonin does not modulate cell proliferation in mice (Ramirez-Rodriguez *et al*, 2009), and the contribution of 5-HT_{2C} receptors in the agomelatine-induced increases in survival by BDNF level remains to be clarified, bearing in mind that a previous study performed under other conditions found that a chronic treatment for 2 weeks with S32006 elevated mRNA encoding hippocampal BDNF (Dekeyne *et al*, 2008). Here, the 5-HT_{2C} receptor antagonists had no effect on BDNF, reinforcing the hypothesis that under the present experimental conditions, 5-HT_{2C} receptors are preferentially involved in the regulation of cell proliferation rather than survival.

We also showed that agomelatine increases the phosphorylation of ERK1/2, AKT, and GSK3β known to transduce the effects of antidepressant agent and mood stabilizers on proliferation of hippocampal neural progenitors (Jiang *et al*, 2005; Wexler *et al*, 2008; Silva *et al*, 2008). The enhanced survival of new granule cells may also be related to the activation of MAPK (ERK1/2) and PI-3K signaling pathways, as previously shown *in vitro* (Almeida *et al*, 2005), and hippocampal NSCs can be protected from apoptosis by antidepressant-induced activation of BDNF and the MAPK pathway (Peng *et al*, 2008). Furthermore, inhibition of GSK3β by phosphorylation following lithium-induced AKT activation has been shown to exert anti-apoptotic effects (Jope and Bijur, 2002). These results are consistent with the neurotrophic-neuroprotective effects of mood stabilizers and antidepressants thought, at least partially, to be mediated by inhibition of GSK3β (Manji *et al*, 2003; Silva *et al*, 2008). Furthermore, indirect (upstream) regulation of GSK3 constitutes a new target for the control of affective disorders and highlights the role of neuroplasticity in their induction and treatment (Mathew *et al*, 2008; Jope and Roh, 2006; O'Brien and Klein, 2007; McClung and Nestler, 2008). Indeed, pharmacological inhibition of GSK3β activity was shown to produce antidepressant-like effects in rodents (Gould *et al*, 2004).

Melatonin has also been shown to activate the prosurvival AKT pathway and inhibit GSK3β activity in various brain regions (Lee *et al*, 2006; Tajes Orduña *et al*, 2009), which is consistent with its neuroprotective action and increases the ERK pathway (Kilic *et al*, 2005). By contrast, despite increases in ERK phosphorylation by SB243,213, we detected a significant negative regulation of GSK3β, reinforcing the view that blockade of 5-HT_{2C} receptors does not necessarily favor cell survival, and may preferentially enhance cell proliferation as suggested by the activation of ERK. It should be noted that this influence of 5-HT_{2C} receptor blockade on ERK may not be direct, as Werry *et al* (2008) showed that activation of heterologously expressed 5-HT_{2C} receptors in CHO cells recruits ERK. Although this difference between the current and previous work reflects the 5-HT_{2C} receptor isoform, constitutive activity, the duration of exposure to drugs, the choice or ligand used and tissue differences that remains to be elucidated (Werry *et al*, 2008; Millan *et al*, 2008), our study clearly demonstrated a stimulating effect of the two 5-HT_{2C} antagonists on ERK pathway, *in vivo*, on the rat hippocampus.

In conclusion, among the different phases of hippocampal neurogenesis stimulated by agomelatine, the rapid and early increase in maturation at a critical period of neuronal development likely influences the functional integration of newborn cells into hippocampal circuitry, an effect that may be related to the rapid clinical efficacy of agomelatine (Kasper and Lemoine, 2008). Although the links between hippocampal neurogenesis and psychiatric disorders are far to be elucidated (Vollmayr *et al*, 2007; Sahay and Hen, 2007; Fuchs, 2007; Eisch *et al*, 2008; Kempermann *et al*, 2008), a better understanding of the regulation of neurogenesis by antidepressants and how they influence distinct phases of progenitor cell development may yield insights into the physiological mechanisms that underlie antidepressant behavioral efficacy.

DISCLOSURE/CONFLICT OF INTEREST

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research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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