Behavioural and neuroplastic effects of the new-generation antidepressant agomelatine compared to fluoxetine in glucocorticoid receptor-impaired mice

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Abstract

Major depression is associated with reduced hippocampal volume linked to stress and high glucocorticoid secretion. Glucocorticoid receptor-impaired (GR-i) mice, a transgenic model for affective disorders with hypothalamic–pituitary–adrenal (HPA) axis feedback control deficit, were used to assess the antidepressant-like effects of the mixed melatonin receptor agonist/5-HT2C receptor antagonist, agomelatine, compared to the selective 5-HT reuptake inhibitor (SSRI), fluoxetine, on hippocampal neurogenesis, GR and BDNF expression and antidepressant-responsive behaviour (tail suspension test, TST). GR-i and paired wild-type (WT) mice were given acute or chronic (21 d) treatment with these drugs. Both hippocampal cell proliferation and BDNF mRNA expression were down-regulated in GR-i mice, and these alterations were reversed by chronic agomelatine and fluoxetine treatments, whereas GR mRNA down-regulation was reversed only by agomelatine. Furthermore, chronic agomelatine, but not fluoxetine, increased survival of newly formed cells in the ventral part of the hippocampus without changing their phenotypic differentiation into neurons. In the TST, the enhanced immobility of GR-i mice was reduced to WT level by acute (but not chronic) fluoxetine and chronic (but not acute) agomelatine. These results indicate that agomelatine reversed the neuroplastic changes and helpless behaviour associated with HPA axis alterations in GR-i mice, suggesting neurobiological and behavioural effects mostly similar to those typically seen with classical antidepressants such as fluoxetine, but through clearly distinct mechanisms.

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Introduction

Neuroimaging and post-mortem studies recently showed that major depressive disorder (MDD) is most often associated with structural and functional brain alterations, notably a shrinkage of dendritic spines (Zhou et al. 2004), a reduction of hippocampal volume possibly related to neuronal cell death and neurogenesis deficiency (Rajkowska, 2000; Sheline, 2000), although no clinical studies have up to now supported the latter hypothesis (Czeh & Lucassen, 2007; Reif et al. 2006). Interestingly, such alterations have also been observed in various animal models of stress or depression (Fuchs & Flugge, 1998). In addition, various antidepressant treatments, including selective serotonin reuptake inhibitor (SSRIs), as well as antagonists at hormone receptors responsible for
hypothalamic–pituitary–adrenal axis (HPA) activation, can reverse these changes (Keilhoff et al. 2006; Malberg & Duman, 2003; Mayer et al. 2006).

In rodents, adult neurogenesis, which involves precursor cell proliferation, migration and differentiation, mainly occurs in two brain regions, the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and the subventricular zone in the forebrain. Many factors, including hormones and neurotransmitters, regulate hippocampal neurogenesis (Paizanis et al. 2007). In particular, modifications in the expression of neurotrophins and growth factors like brain-derived neurotrophic factor (BDNF) have been reported to contribute to changes in hippocampal neurogenesis associated with stress-related disorders and antidepressant treatments (Hashimoto et al. 2004; Paizanis et al. 2007).

Compelling data also showed that MDD is often associated with HPA axis dysfunction. HPA axis activity is controlled by a feedback mechanism triggered mainly through the stimulation of brain glucocorticoid receptors (GR) (Dorio et al. 1993; Feldman & Weidenfeld, 1999), and which reduced expression and function have been proposed to contribute to HPA axis alterations in depression (Pariante & Miller, 2001). Accordingly, transgenic mice with impaired brain expression of GR (GR-i) have been generated, as a model presenting many of the neuroendocrine changes associated with depressive disorders, including tonic increase in HPA axis activity and resistance to suppression of corticosterone secretion by dexamethasone (Barden et al. 1997; Froger et al. 2004; Pepin et al. 1992). In addition to having cognitive deficits (Montkowski et al. 1995), GR-i mice are characterized by a reduced CRH expression in the hypothalamus, maladaptive responses of the 5-HT system to stress (Froger et al. 2004), but no changes in ACTH and corticosterone plasma levels at various time-points of the circadian cycle (Barden et al. 1997; Karanth et al. 1997). HPA axis dysregulation in these mice is only apparent under stressful conditions (Barden et al. 1997; Froger et al. 2004) and can be reversed by antidepressant treatment (Barden, 1996; Montkowski et al. 1995).

Our study aimed at further characterizing the depression-like syndrome of these mice by analysing both their hippocampal neurogenesis capacity and their despair-like behaviour in the tail suspension test (TST), in comparison with paired wild-type (WT) controls. In addition, the antidepressant-like activity of the new compound, agomelatine, a potent agonist at melatonin MT₁ and MT₂ receptors (Audinot et al. 2003) and antagonist at the 5-HT₆ receptor (Millan et al. 2003), was also analysed in comparison with the SSRI antidepressant fluoxetine, on hippocampal neurogenesis, BDNF and GR mRNA expression and behaviour in GR-i and WT mice.

Materials and methods

Animals

Transgenic mice (strain B6C3F1; line 1.3) (Pepin et al. 1992), heterozygous for the presence of the transgene-expressing rat GR antisense RNA, were mated together. Mice homozygous for the presence of the transgene (GR-i) or its absence (WT) were bred at UMR677 INSERM/UPMC animal facility, and housed under standard conditions [22 ± 1°C, 60% relative humidity, 12-h light/dark cycle (lights on 07:00 hours), food and water available ad libitum]. Behavioural and anatomical studies were performed on 10-wk-old male mice. Genotype was determined by PCR analysis as previously described (Froger et al. 2004).

Procedures involving animals and their care were conducted in accordance with directives of the European Community (Council directive 86/609) and the French Agriculture and Forestry Ministry (Council directive 87-848, October 19, 1987, permissions 75-116 to M.H. and 75-977 to L.L.).

Antidepressant treatments

On the basis of previous data in validated animal models of depression and anxiety (Bourin et al. 2004; Cryan & Lucki, 2000; Millan et al. 2005; Papp et al. 2003; Santarelli et al. 2003), mice received either acutely or chronically for 21 d, at 17:00 hours, i.e. 2 h before the onset of the dark period, agomelatine [50 mg/kg body weight intraperitoneally (i.p.)] dissolved in 1% hydroxy-ethyl-cellulose (HEC), or fluoxetine (10 or 30 mg/kg i.p. for acute administration, and 10 mg/kg i.p. for chronic treatment) dissolved in saline (0.9% NaCl). Control mice received vehicle only under identical conditions.

Immunohistochemistry

Immunoperoxidase staining

In order to quantify hippocampal cell proliferation, animals received a single i.p. injection of 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, France; 75 mg/kg dissolved in saline, 10 mg/ml) 30 min (acute condition) or 16 h (chronic condition) after injection of agomelatine, fluoxetine or their vehicles, and were sacrificed 2 h later, i.e. at 07:30 hours (acute condition) or 11:00 hours (i.e. 18 h after the last injection for chronic treatment). For assessing cell survival and differentiation, BrdU (75 mg/kg i.p.) was injected daily
during the first week of the 21-d antidepressant treatment, and mice were sacrificed 4 wk later (Malberg et al. 2000), i.e. after a 2-wk washout period of antidepressant treatments.

At the selected appropriate time, mice were deeply anaesthetized with pentobarbital (60 mg/kg i.p.), then transcardially perfused with 75 ml of 0.15 M NaCl supplemented with 15 mM NaNO3 followed by 75 ml of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4) at 4 °C. Brains were removed and post-fixed in the same paraformaldehyde-PBS solution overnight at 4 °C. Serial coronal sections (40 μm) were cut throughout the entire hippocampus using a vibratome (VT1000S, Leica, Germany).

Immunohistochemistry for the immature neuron marker doublecortin (DCX) or for BrdU was then performed. Free-floating sections were rinsed in 0.1 M PBS, incubated for 5 min in 3% H2O2/10% methanol in 0.1 M PBS and then rinsed. For BrdU staining only, sections were incubated in 2 M HCl + 0.1% Triton X-100-supplemented 0.1 M PBS (30 min, 37 °C), and then immersed in 0.1 M sodium tetraborate buffer (pH 8.6), for 30 min. Sections were then incubated for 1 h in 0.1 M PBS supplemented with 5% normal rabbit serum (bregma 2.30 to 0.94 mm) for DCX immunohistochemistry. Sections were subsequently transferred to the same but fresh mixture of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4) at 4 °C. Brains were removed and post-fixed in the same paraformaldehyde-PBS solution overnight at 4 °C. Serial coronal sections (40 μm) were cut throughout the entire hippocampus using a vibratome (VT1000S, Leica, Germany).

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**Quantitative analyses**

Cell counts were performed on blind-coded slides examined under 10 × and 20 × objectives (Leica). DG in the dorsal (bregma −0.94 to −2.30 mm) and ventral (bregma −2.30 to −3.80 mm) extensions of the hippocampus (Franklin & Paxinos, 1997) was analysed in BrdU-immunopositive cells in the SGZ defined as a two-cell bodies-wide zone of the hilus along the base of the granule cell layer (GCL), using a computer image analysis system (Mercator, Explora Nova, France) and a mounted video 3CCD camera (Sony, France). Results are presented as the total number of BrdU-immunopositive cells per hippocampus.

For the counting of DCX-positive cell numbers, three sections containing the DG were used per mouse. Sections were taken from one-in-four series from the hippocampus, excluding the most rostral and caudal sections. The selected sections were highly comparable between the different mice regarding their rostrocaudal location. Cells were counted throughout the entire thickness of the section, using 40 × magnification. The average number of immunopositive cells/section was calculated for each mouse. DG volume was measured according to the Cavalieri principle (West & Gundersen, 1990).

**Immunofluorescent labelling**

Triple immunofluorescent labelling for BrdU, neuron-specific nuclear protein (NeuN), and glial fibrillary acidic protein (GFAP) was performed and analysed by confocal microscopy using rat anti-BrdU (1:100, Abcys), rabbit anti-GFAP (1:300, Dako, France), and mouse anti-NeuN (1:500, Chemicon International, Millipore, France) antibodies. The secondary antibodies used were a biotinylated rabbit anti-rat antibody (1:200; Vector Laboratories) coupled with streptavidin-Alexa Fluor 488 (1:1600; Molecular Probes, USA), an Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:1600; Molecular Probes) and a cy5-conjugated donkey anti-mouse IgG (1:1600; Jackson ImmunoResearch, USA), respectively. Coverslips were finally mounted in Fluoromount-G solution (Clinisciences, France). For phenotype determination, 100 BrdU-positive cells were analysed for the co-expression of BrdU and NeuN (neurons) or BrdU and GFAP (astrocytes) in each mouse, and means ± s.e.m. were calculated from 5–6 mice per group.

**Cell death determination**

Apoptotic cells in adjacent sections were detected using the terminal deoxynucleotidyltransferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) technique adapted from Bauer et al. (2003). After 30-min preincubation in 0.1% sodium citrate/0.1% Triton X-100/PBS at 4 °C, for the blockade of endogenous peroxidases by 3% H2O2/PBS, sections were incubated with TUNEL dilution buffer for 15 min followed by the TdT reaction with biotinylated dUTP in a humid chamber for 1 h at 37 °C (Roche...
Diagnostic, France). Sections were then incubated with the Vector ABC-Elite kit (Abcys), and peroxidase immunostaining was used to detect TUNEL-labelled cells. To obtain a positive control, some sections were treated for 15 min at 37°C with 20 U/ml DNase I (New England Biolabs, France) diluted in DNase buffer prior to the enzymatic reaction. As a negative control, TdT was omitted in the TUNEL reaction mix added to some of the samples. For each mouse, the average number of immunopositive cells/section was calculated.

**Real-time reverse transcription–PCR determinations of GR and BDNF mRNAs**

Mice were killed 30 min after a single injection (acute experiments) or 16 h after the last injection for the 3-wk treatment with agomelatine, fluoxetine or their respective vehicle, and the hippocampus was rapidly dissected and frozen in liquid nitrogen. Total RNA was extracted using the ‘RNaseasy Mini kit’ (Qiagen, France). RNA quality was assessed by capillary electrophoresis using RNA Labchip kits and Agilent Bioanalyzer 2100 (Agilent Technologies, France). Aliquots (900 ng) of each sample were used for reverse transcription (iScript kit; Bio-Rad, USA). For the GR-encoding mRNA, a pair of primers was designed to selectively amplify a 93-bp fragment spanning the 1234–1326 region of the published mouse sequence (NR3C1, NM008173) while for BDNF-encoding mRNA, we used a primer set designed to amplify an amplicon common to all the described splice variants of BDNF transcript (MMBDNFA) and corresponding to the fragments 697–782 of NM008361, 330–410 of NM008360, 197–279 of NM031168, 463–545 of NM010548, 548–624 of NM013693 (Husson et al. 2005). β2-microglobulin was chosen as housekeeping gene and the primer set corresponding to nucleotides 99–185 of the published sequence of the mouse β2-microglobulin mRNA (MMB2MR) was used. Real-time PCR was set up on a myiQ™ apparatus (Bio-Rad) using SYBR Green-containing supermix from Bio-Rad, for 50 cycles of a three-step procedure, including 20 s denaturation at 96°C, 20 s annealing at 62°C, and 20 s extension at 72°C. Semi-quantitative determinations based on the difference in specific ratios (GR or BDNF/β2-microglobulin) between groups were calculated for each experimental series. Specific products were determined as clear single peaks at their melt curves. All sample measurements were performed in duplicate. Gene expression in each sample was normalized to β2-microglobulin expression, i.e.: 2 x (Ct_housekeeping − Ct_gene of interest).

**Behavioural studies**

Mice were tested either 30 min (acute condition) or 16–20 h after the last injection (chronic treatment) of agomelatine, fluoxetine or their respective vehicle. TST was performed using the ID-Tech-Bioseb (France) computerized device following a procedure previously described (Renoir et al. 2008). The total duration of immobility during a 6-min test was determined for each mouse.

**Statistical analyses**

Data (presented as means ± S.E.M.) were analysed using unpaired two-tailed Student’s t test for comparison of two groups with a Welsh’s correction when necessary (Prism 4.0, GraphPad software, USA). A two-way analysis of variance (ANOVA) followed by Bonferroni’s post-hoc test to determine specific group differences, was also used when necessary. In all cases, the significance level was set at p ≤ 0.05.

**Results**

**Effects of impaired GR function on hippocampal cell proliferation, neurogenesis and cell death**

**Basal conditions**

**Cell proliferation.** In GR-i and WT mice, BrdU-labelled cells located in the SGZ (Fig. 1a) showed irregularly shaped nuclei and diffuse patterns of BrdU staining and were frequently associated in clusters (Fig. 1b) as expected for proliferating neural stem cells (Malberg & Duman, 2003). The total number of DG BrdU-positive cells was significantly lower in naive transgenic GR-i compared to WT mice (about −40%, p < 0.001, Student’s t test), and this difference was noted also in the dorsal (about −35%, p < 0.001, Student’s t test) and ventral (about −50%, p < 0.01, Student’s t test) parts of the hippocampus analysed separately (Fig. 1c). In contrast, DG volume estimate did not reveal any significant difference between GR-i and WT mice (Fig. 1d).

In GR-i and WT mice, age had a significant negative effect on cell proliferation (F<sub>3,32</sub> = 22.84, p < 0.0001), but the significant difference in BrdU labelling between GR-i and WT mice was maintained whatever the age of the animals from 5- to 10-wk-old (F<sub>3,32</sub> = 36.57, p < 0.0001) (data not shown).

**DCX labelling.** Study of growth and maturation of newly born cells in the DG, using DCX labelling (Rao & Shetty, 2004), showed that the number of immature,
DCX-immunopositive neurons was significantly lower (about -30%, p < 0.05, Student’s t test) in GR-i compared to WT naive mice (Fig. 2a–c).

Cell death. TUNEL staining was quite low in basal conditions in GR-i and WT mice (Fig. 3a). As shown in Fig. 3b, no significant difference in TUNEL staining in the hippocampus was observed between control and transgenic mice.

**Effects of acute antidepressant treatments**

Under acute conditions, neither agomelatine (50 mg/kg i.p.) (F\(_{1,8}=0.05, p > 0.05\)) nor fluoxetine (30 mg/kg i.p.) (F\(_{1,11}=2.51, p > 0.05\)) significantly modified the total number of BrdU-labelled cells in the SGZ of the whole hippocampus, in GR-i and WT mice (Fig. 4a, b). Furthermore, regional analysis did not reveal any changes in the number of BrdU-positive cells, in the dorsal (agomelatine: F\(_{1,8}=0.05, p > 0.05\); fluoxetine: F\(_{1,11}=10.57, p > 0.05\)) or the ventral (agomelatine: F\(_{1,8}=0.59, p > 0.05\); fluoxetine: F\(_{1,11}=3.15, p > 0.05\)) parts of the hippocampus after acute treatment with antidepressant vs. vehicle in GR-i and WT mice (Fig. 4a, b).

**Effects of chronic antidepressant treatments**

**Cell proliferation**

A 3-wk treatment with agomelatine (50 mg/kg.d i.p.) differentially promoted cell proliferation in the whole DG (about +20%, p < 0.001, Bonferroni’s post-hoc test) and its ventral part (about +35%, p < 0.01, Bonferroni’s post-hoc test) whereas in GR-i mice, this effect was significant in the whole DG (about +26%, p < 0.001, Bonferroni’s post-hoc test), as well as the dorsal (about +24%, p < 0.05, Bonferroni’s post-hoc test) and ventral (about +34%, p < 0.05, Bonferroni’s post-hoc test) extensions (Fig. 5a). However, no hippocampal volume modifications were associated with these effects (Fig. 5b).
Fig. 2. Immunostaining of DCX in the hippocampus of glucocorticoid receptor-impaired (GR-i) and wild-type (WT) mice under basal conditions. (a) Low-magnification photomicrographs of doublecortin (DCX) immunoreactivity in the dentate gyrus in a WT and a GR-i mouse. Most cell bodies immunopositive for DCX are located in the subgranular zone (SGZ) of the dentate gyrus. (b) High-magnification photomicrograph of DCX-immunopositive cells in the granule cell layer (GCL) with extended ramified dendrites. (c) Numbers of DCX-immunopositive cells/section in GR-i vs. WT mice. Each bar is the mean ± S.E.M. of data obtained in three mice per group. * p < 0.05, Student’s t test.

A 3-wk treatment with fluoxetine (10 mg/kg, i.p.) also induced a significant increase in the number of BrdU-labelled cells in the whole DG (F1,14 = 5.30, p < 0.05) but only in GR-i mice (about +60% over saline-treated transgenic mice, p < 0.001, Bonferroni’s post-hoc test). Regional analysis showed that this effect also reached statistical significance in the dorsal (about +54%, p < 0.05, Bonferroni’s post-hoc test) and ventral (about +64%, p < 0.01, Bonferroni’s post-hoc test) parts of the hippocampus in GR-i but not WT mice (Fig. 5c). On the other hand, fluoxetine treatment did not modify the hippocampal volume in GR-i and WT mice (Fig. 5d).

Cell survival
A significant effect of genotype was observed on the survival of newly generated cells in the hippocampus. Indeed, in the HEC- and saline-treated groups, the number of labelled cells 4 wk after BrdU injections was lower in GR-i mice compared to WT mice [whole DG: F1,14 = 7.40, p < 0.05; dorsal DG: F1,14 = 4.36, p < 0.05; ventral DG: F1,14 = 10.04, p < 0.01, after HEC treatment (Fig. 6a); whole DG: F1,14 = 11.99, p < 0.05; dorsal DG: F1,14 = 9.76, p < 0.05; ventral DG: F1,14 = 9.94, p < 0.05, after saline treatment (Fig. 6b)].

A 3-wk treatment with agomelatine significantly increased the number of BrdU-positive cells in the ventral DG of GR-i mice (about +55%, p < 0.05, Bonferroni’s post-hoc test) but not WT mice (Fig. 6a). Conversely, no significant changes were noted in its dorsal extension and the whole DG (Fig. 6a) and a 3-wk treatment with fluoxetine (10 mg/kg, i.p.) was ineffective in the dorsal, ventral and whole DG in GR-i and WT mice (Fig. 6b). Furthermore, the treatments did not modify hippocampal DG volumes in GR-i and WT mice (not shown).

Phenotype of surviving cells
To determine the phenotype of BrdU-positive surviving cells, triple immunofluorescent labelling was performed using markers for mature neurons (NeuN) and astrocytes (GFAP) in addition to BrdU (Fig. 6c). Double BrdU- and NeuN- [Fig. 6c(i)] or BrdU- and GFAP- [Fig. 6c(ii)] labelled cells were counted throughout the DG. Ratios of BrdU-NeuN/BrdU-GFAP double-labelled cells were similar in the dorsal and ventral parts of the hippocampus, and therefore corresponding values were pooled for calculations for the whole hippocampus. Most of the surviving DG BrdU-positive cells were found to also express NeuN (of a total of 100 BrdU-immunoreactive cells counted per animal: HEC = 88.0 ± 2.3 and 85.3 ± 1.8;
saline = 91.5 ± 4.7 and 89.5 ± 1.0, in WT and GR-i mice, respectively, means ± S.E.M., n = 5–6), and only a few of them (10–15%) expressed GFAP. Chronic treatment with agomelatine or fluoxetine did not affect the differentiation of newly generated cells towards neuronal or glial phenotype (double BrdU-NeuN immuno-reactive cells: agomelatine = 89.5 ± 1.2 and 89.5 ± 0.8; fluoxetine: 88.1 ± 1.1 and 90.5 ± 1.6, in WT and GR-i mice, respectively, means ± S.E.M., n = 5–6; not significantly different from data above, for vehicle-treated mice).

Hippocampal expression of GR and BDNF mRNAs in naive mice

As expected, hippocampal GR mRNA expression was significantly down-regulated in naive GR-i compared to WT mice (−21.0 ± 7.8%, mean % ± S.E.M., n = 6, p < 0.05, two-tailed Student’s t test). In addition, hippocampal levels of BDNF mRNA were significantly lower in naive GR-i compared to WT mice (−41.0 ± 2.2%, mean % ± S.E.M., n = 6, p < 0.001, two-tailed Student’s t test) (Fig. 7a, b).

Effects of chronic antidepressant treatments

GR mRNA expression

As shown in Fig. 8a, chronic agomelatine administration exerted a significant effect on GR mRNA expression since down-expression of this transcript was reduced from −45% in vehicle (HEC) to only −21% in agomelatine-treated GR-i compared to paired WT mice (Bonferroni’s post-hoc test, p < 0.001) [genotype: F1,20 = 623.1; agomelatine: F1,20 = 82.10; interaction: F1,20 = 82.10]. Conversely, fluoxetine treatment did not significantly modify hippocampal GR mRNA levels in GR-i mice (Fig. 8b) [genotype: F1,20 = 184.1; fluoxetine: F1,20 = 20.24; interaction: F1,20 = 20.24]. Furthermore, neither fluoxetine nor agomelatine significantly modified GR mRNA levels in the hippocampus of WT mice (not shown).

BDNF mRNA expression

Chronic administration of agomelatine (Fig. 8c) as well as fluoxetine (Fig. 8d) reversed the down-regulation of BDNF mRNA in the hippocampus of GR-i mice,
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Fig. 5. Effects of chronic treatment with agomelatine (a, b) or fluoxetine (c, d) on hippocampal cell proliferation in glucocorticoid receptor-impaired (GR-i) and wild-type (WT) mice. (a, c) BrdU-labelled cells were counted within the entire dentate gyrus (DG) of the hippocampus and its dorsal and ventral parts after 3-wk treatment with agomelatine (50 mg/kg i.p. daily), fluoxetine (10 mg/kg i.p. daily) or their vehicles [hydroxy-ethyl-cellulose (HEC), saline, respectively]. The number of BrdU-labelled cells in each sub-area of the DG is expressed per hippocampus. The proliferative effect of agomelatine was significant in the total DG and its dorsal and ventral parts in GR-i mice, but only in the total and ventral DG in WT mice. The proliferative effect of fluoxetine was significant in the total DG, and its dorsal and ventral parts in GR-i mice. Conversely, fluoxetine did not induce cell proliferation in the DG of WT mice. (b, d) Volume of the DG, in mm³. Volumes were determined by multiplying the sum of the DG area per section by the distance between sections (160 μm). Each bar is the mean ± S.E.M. of 5–7 independent determinations. * p < 0.05, *** p < 0.001, GR-i vs. WT mice, Bonferroni’s post-hoc test. # p < 0.05, ## p < 0.01, ### p < 0.001 agomelatine vs. HEC or fluoxetine vs. saline, Bonferroni’s post-hoc test.

Effects of antidepressant treatments

Acute treatment

Acute agomelatine (50 mg/kg i.p.) had no effect on immobility duration in GR-i and WT mice. In contrast, a two-way ANOVA revealed a significant effect of acute fluoxetine (F<sub>2,44</sub> = 6.84, p < 0.01). In WT mice, 30 mg/kg i.p. fluoxetine led to a significant decrease of immobility time (p < 0.01, Bonferroni’s post-hoc test), whereas, in GR-i mice, fluoxetine at both 10 and 30 mg/kg i.p. induced this effect (p < 0.01 and p < 0.05, respectively, Bonferroni’s post-hoc test) (Fig. 9b).

Chronic treatment

A two-way ANOVA analysis showed a significant effect of chronic agomelatine treatment (50 mg/kg.d

Behaviour in the TST

Basal conditions

Under basal conditions, i.e. without any treatment, naive GR-i mice showed a significantly longer immobility time compared to WT mice (p < 0.001, Student’s t test) during the 6-min observation period of the TST (Fig. 9a).
Fig. 6. Effects of chronic treatment with (a) agomelatine or (b) fluoxetine on hippocampal cell survival and (c) differentiation in glucocorticoid receptor-impaired (GR-i) and wild-type (WT) mice. (a, b) BrdU-labelled cells were counted within the entire dentate gyrus (DG) and its dorsal and ventral parts in mice sacrificed 4 wk after the last injection of BrdU. The number of BrdU-labelled cells in each sub-area of the DG is expressed per hippocampus. (a) Agomelatine exerted a significant effect on cell survival in the ventral part of the DG in GR-i mice. (b) Fluoxetine did not exert a significant effect on cell survival in GR-i or WT mice. Each bar is the mean ± S.E.M. of 4–5 independent determinations. *p < 0.05, **p < 0.01, GR-i vs. WT mice, Bonferroni’s post-hoc test. # p < 0.05, agomelatine vs. hydroxy-ethyl-cellulose (HEC), Bonferroni’s post-hoc test. (c) Confocal microscopy illustration of BrdU (green)-, neuron-specific nuclear protein (NeuN) (blue)-, and glial fibrillary acidic protein (GFAP) (red)-labelled cells in the granule cell layer after 3-wk treatment with agomelatine. Arrows point at cells double-labelled (Merge) for BrdU and NeuN (i, top row of micrographs) or BrdU and GFAP (ii, bottom row of micrographs).

Discussion

Several recent studies have emphasized the role that HPA axis dysfunction may play in the physiopathology of MDD and associated alterations in neuroplasticity and neurogenesis. In this context, the aim of our study was to further investigate the effects of the novel
antidepressant agomelatine (Olie & Kasper, 2007), compared to fluoxetine, in the GR-i transgenic mouse, a murine model of depression with marked alterations in HPA axis function due to GR down-regulation in brain (Froger et al. 2004; Pepin et al. 1992).

Molecular, anatomical and behavioural characteristics of GR-i mice

As previously reported (Froger et al. 2004; Holsboer & Barden, 1996; Pepin et al. 1992), genome incorporation of the transgene encoding GR antisense RNA down-regulated GR expression in GR-i mice. In addition, we also found that BDNF mRNA expression was down-regulated in the hippocampus of GR-i compared to WT mice. This finding is of particular relevance because alterations in neurotrophic factors like hippocampal BDNF have been frequently associated with depression-like symptoms in several animal models (Alfonso et al. 2006), and with MDD in humans (Karege et al. 2002). Neurotrophic factors are known to exert a key role not only in cell differentiation, but also in cell proliferation and survival at adult age (Lee et al. 2002; Sairanen et al. 2005) and the marked reduction of cell proliferation and survival together with the reduced neurogenesis assessed by DCX expression that we observed within the dorsal and ventral parts of the DG might be causally related to the down-regulation of BDNF in GR-i mice. Interestingly, the decreased neurogenesis observed in GR-i mice was not linked to any detectable increase in apoptotic processes.

The dorsal hippocampus has been functionally associated with learning and memory whereas its ventral part is mostly concerned with fear-related behaviours and anxiety (Bannerman et al. 2004). Interestingly, GR-i mice have been shown to display numerous abnormalities regarding spatial learning and short-term memory (Rousse et al. 1997), anxiety (Montkowski et al. 1995) and responses to stress (Froger et al. 2004; Linthorst et al. 2000). All these alterations might theoretically be linked to the deficits observed in hippocampal cell proliferation and survival, probably causally correlated with the decreased BDNF expression.

Along with a deficit in hippocampal neurogenesis, GR-i mice exhibited a significant increase in immobility time in TST, indicating clear-cut despair-like behaviour similar to that reported in other animal models of affective disorders (Cryan et al. 2005). Interestingly, these data differed from those obtained in the forced swim test (FST; Barden et al. 2005), where GR-i mice appeared to be less immobile than paired WT controls. However, it should be emphasized that the latter test was shown to be less informative in mice than in rats (Borsini & Meli, 1988), and also to reflect some adaptive strategy unrelated to despair behaviour (Lucki, 2001; Thierry et al. 1984). Furthermore, TST and FST probably activate distinct neurochemical pathways underlying animal performance (Bai et al. 2001) and this might also account for the differences observed here between GR-i and WT mice.

Effects of antidepressant treatments

When given acutely, neither agomelatine nor fluoxetine enhanced cell proliferation in the hippocampal SGZ of GR-i and WT mice. These results are consistent with previous studies showing that chronic administration of antidepressants is necessary to increase cell proliferation and neurogenesis in the hippocampus (Banasr et al. 2006; Malberg et al. 2000; Santarelli et al. 2003).

However, fluoxetine at the acute dose of 30 mg/kg i.p. was effective in shortening immobility in the TST in GR-i and WT mice, in agreement with previous observations on other mouse strains (Cryan et al. 2005). In contrast to fluoxetine, acute agomelatine did not affect immobility in the TST as also reported for the FST in Swiss male mice (Bourin et al. 2004).

Under chronic treatment conditions, agomelatine enhanced cell proliferation only in the ventral part of the hippocampus in WT mice. However, it should be noted that the number of proliferating cells in this region was slightly lower in WT mice injected with HEC,
the agomelatine vehicle, than in naive mice. Whether HEC, by inducing an unspecific stress, could reduce cell proliferation and whether agomelatine could reverse this effect is an intriguing point to be considered. However, the regional effect of agomelatine, that matches previous data in Wistar rats (Banasr et al. 2006), further emphasizes the critical implication of the ventral hippocampus in stress and response to antidepressants. In contrast, chronic fluoxetine was found to affect cell proliferation in WT mice in neither the dorsal nor the ventral part of the hippocampus. These data were unexpected because earlier studies

<table>
<thead>
<tr>
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<th>Hydroxy-ethyl-cellulose</th>
<th>Agomelatine</th>
<th>Saline</th>
<th>Fluoxetine</th>
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<tbody>
<tr>
<td>GR</td>
<td>104.50 ± 20.37</td>
<td>117.80 ± 3.70</td>
<td>101.80 ± 13.62</td>
<td>99.19 ± 46.64</td>
</tr>
<tr>
<td>BDNF</td>
<td>102.00 ± 11.37</td>
<td>108.40 ± 1.89</td>
<td>102.10 ± 15.01</td>
<td>99.51 ± 11.79</td>
</tr>
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Mean GR and BDNF mRNA/β₂-microglobulin mRNA ratios ± S.E.M. of three independent determinations.

Fig. 8. Real-time RT–PCR quantification of glucocorticoid receptor (GR) and BDNF mRNA in the hippocampus of GR-impaired (GR-i) and wild-type (WT) mice – effects of chronic agomelatine and fluoxetine treatments. Effects of (a) agomelatine and (b) fluoxetine treatments on GR mRNA levels. Bars represent mean GR mRNA/β₂-microglobulin mRNA ratios ± S.E.M. of six independent determinations expressed as percent of WT mice. Chronic agomelatine – but not fluoxetine – administration significantly reduced GR mRNA down-expression in GR-i mice compared to paired WT mice. *** p < 0.001 agomelatine vs. hydroxy-ethyl-cellulose (HEC) in GR-i mice, Bonferroni's post-hoc test. Effects of (c) agomelatine and (d) fluoxetine treatments on BDNF mRNA levels. Bars represent mean BDNF mRNA/β₂-microglobulin mRNA ratios ± S.E.M. of six independent determinations expressed as percent of WT mice. * p < 0.05, *** p < 0.001 agomelatine or fluoxetine vs. respective vehicle in GR-i mice, Bonferroni's post-hoc test. Chronic administration of agomelatine (p < 0.05, Bonferroni's post-hoc test) as well as fluoxetine (p < 0.001, Bonferroni's post-hoc test) reversed the down-regulation of BDNF mRNA in GR-i mice. Range of Ct values were respectively 20.21–23.59 for GR, 20.55–24.90 for BDNF and 18.83–21.73 for β₂-microglobulin.
have shown a promoting effect of chronic fluoxetine on cell proliferation in WT rodents (Kodama et al. 2004; Malberg et al. 2000; Santarelli et al. 2003). However, other reports pointed out that chronic treatment with this SSRI exerted only a minor effect on hippocampal cell proliferation, and was even ineffective in unstressed BALB/CJ mice (Alonso et al. 2004; Holick et al. 2008). These findings suggest that, at least in WT mice, fluoxetine-induced cell proliferation might be strain dependent.

In contrast to that noted in WT mice, the neurogenic effects of agomelatine and fluoxetine were highly significant in GR-i mice as administration of either drug up-regulated cell proliferation in the SGZ of the DG in these transgenic animals when administered chronically. Similar data have been obtained in other rodent models of depression with predictive validity. In particular, chronic treatment with agomelatine was found to significantly reverse the deficit in hippocampal cell proliferation, up to control levels, in prenatally
expression has been described through a positive influence of fluoxetine on GR expression in GR-i mice. Alternatively of chronic agomelatine, but not fluoxetine, two antidepressant drugs under study was the effectiveness of GR-mediated negative feedback control of HPA axis in GR-i mice only, in agreement with previous data in Sprague-Dawley rats (Maccari et al. 2005). In contrast, chronic fluoxetine did not increase hippocampal cell survival in GR-i and WT mice, also in accord with data in rats (Malberg et al. 2000).

Another difference that appeared between the two antidepressant drugs under study was the effectiveness of chronic agomelatine, but not fluoxetine, in increasing GR mRNA expression in GR-i mice. Although a positive influence of fluoxetine on GR expression has been described in vitro (Fariante et al. 2003), SSRIs appeared to have variable effects in vivo (Bjartmar et al. 2000; Seckl & Fink, 1992). Whether, chronic agomelatine but not fluoxetine would restore the effectiveness of GR-mediated negative feedback control of HPA axis in GR-i mice is a relevant question to be addressed in future studies.

In contrast to the differences emphasized above, both agomelatine and fluoxetine, under chronic treatment conditions, were found to reverse the down-regulation of hippocampal BDNF mRNA in GR-i mice, in line with previous data in other rodent models (Castren et al. 2007; Duman & Monteggia, 2006; Vinet et al. 2004). In humans, post-mortem studies also showed that the expression of BDNF was significantly higher in patients treated with antidepressant (Klempin & Kempermann, 2007). According to Castren et al. (2007), chromatin remodelling by antidepressant treatment might be causally related to BDNF up-regulation. Indeed, HPA axis activation in mice, as that occurring after stress or in GR-i mice, produced a long-lasting methylation of histone-3 subunits correlated with suppression of BDNF gene transcription (Tsankova et al. 2006). Chronic antidepressant treatment may counteract the reduction in BDNF mRNA expression by inducing the acetylation of the same histone subunit. Interestingly, an antidepressant treatment that induced histone acetylation in mice previously subjected to social stress was without effect in non-stressed controls (Tsankova et al. 2006), in line with our findings that BDNF mRNA was up-regulated in GR-i mice but not WT mice after chronic treatment with agomelatine or fluoxetine. The possibility therefore exists that increased BDNF signalling is required for the induction of the behavioural response to antidepressant drugs, specifically in animals with depression-like symptoms (Castren et al. 2007; Saarelainen et al. 2003).

In conclusion, our data showed that chronic agomelatine and fluoxetine treatments were able to increase hippocampal cell proliferation and BDNF mRNA levels in GR-i mice, a validated model of depression (Froger et al. 2004; Pepin et al. 1992). However, whether these effects are causally related to behavioural improvement is still a question to be addressed. If, in GR-i mice, the decreased immobility in the TST after chronic agomelatine seemed to occur concomitantly with the promotion of neurogenesis, this was not the case with fluoxetine. With this SSRI, behavioural improvement was observed only under acute treatment conditions whereas induction of cell proliferation occurred under chronic treatment conditions. These data are consistent with those recently published about the occurrence of possible mismatch between the effects of SSRI on neurogenesis on the one hand and behaviour on the other (Holick et al. 2008). Nevertheless, our data provided further support to the idea that increase in hippocampal neurogenesis involves mechanisms, which include the regulation of growth factors such as BDNF, that could be involved in the therapeutic effects of antidepressants.

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Statement of Interest
None.
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