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ORIGINAL ARTICLE

Macrophage migration inhibitory factor is critically involved in basal and fluoxetine-stimulated adult hippocampal cell proliferation and in anxiety, depression, and memory-related behaviors

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Intensive research is devoted to unravel the neurobiological mechanisms mediating adult hippocampal neurogenesis, its regulation by antidepressants, and its behavioral consequences. Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that is expressed in the CNS, where its function is unknown. Here, we show, for the first time, the relevance of MIF expression for adult hippocampal neurogenesis. We identify MIF expression in neurogenic cells (in stem cells, cells undergoing proliferation, and in newly proliferated cells undergoing maturation) in the subgranular zone of the rodent dentate gyrus. A causal function for MIF in cell proliferation was shown using genetic (MIF gene deletion) and pharmacological (treatment with the MIF antagonist Iso-1) approaches. Behaviorally, genetic deletion of MIF resulted in increased anxiety- and depression-like behaviors, as well as of impaired hippocampus-dependent memory. Together, our studies provide evidence supporting a pivotal function for MIF in both basal and antidepressant-stimulated adult hippocampal cell proliferation. Moreover, loss of MIF results in a behavioral phenotype that, to a large extent, corresponds with alterations predicted to arise from reduced hippocampal neurogenesis. These findings underscore MIF as a potentially relevant molecular target for the development of treatments linked to deficits in neurogenesis, as well as to problems related to anxiety, depression, and cognition.
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Introduction

Macrophage migration inhibitory factor (MIF) was originally identified as a T-lymphocytes-derived pro-inflammatory cytokine that inhibits the random migration of macrophages.[1,2] In the immune system, MIF is released in response to inflammatory stimuli including microbial products and glucocorticoid hormones.[3–5] In addition to this pro-inflammatory function, MIF is produced by many other non-immune cells within the body, with high expression levels found in the liver, kidney, anterior pituitary, and brain.[6,7]

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Although the function of MIF in the mature mammalian brain remains unclear, evidence from other organs has identified a function in cellular proliferation. For example, in macrophage cells, MIF acts as an inhibitor of p53 tumor suppressor activity.[8,9] In fibroblasts, MIF was shown to induce cyclin D1 expression and cell cycle progression through activation of the ERK/MAPK cascade.[10] Interestingly, indirect evidence suggests that MIF may also be involved in cellular proliferation in the CNS.[10,11] In the bovine adult brain, MIF expression has been reported in granular cells of the dentate gyrus.[12] *In situ* hybridization has localized MIF in ependymal cells lining the ventricular surfaces and epithelial cells of the choroid plexus, as well as in neurons and astrocytes distributed all over the adult rat brain.[13] Interestingly, both the hippocampus and the ventricles are areas of ongoing cellular proliferation (eventually leading to neurogenesis) in the adult brain.

The subgranular zone (SGZ) of the hippocampal dentate gyrus undergoes limited proliferation and

differentiation of neuronal precursors (neurogenesis) in adulthood.[14] Progenitor cells in the SGZ can give rise to new neurons, which, depending on a number of environmental influences, can migrate into the granule cell layer in which they differentiate into granular neurons[15] and may subsequently become functionally integrated into the hippocampal circuitry.[16–18] Therefore, we aimed here to ascertain the relevance of MIF expression for adult hippocampal neurogenic processes. First, we identified MIF expression in neurogenic niches of the rodent dentate gyrus and characterized the cellular phenotype. To establish a link between MIF and neurogenesis, we used models of altered [both negatively (chronic stress and corticosterone administration) and positively (antidepressant treatment)] adult neurogenesis, whereas the causal function of MIF in basal and antidepressant-stimulated neurogenesis was addressed through pharmacological and knockout genetic approaches. Finally, the behavioral implications of MIF deficiency were also explored.

Materials and methods

Animals

Adult male Wistar rats (3 months), purchased from Charles River (France), were used for the chronic stress experiment. MIF knockout ($MIF^{-/-}$) mice were produced as described earlier[19] and kept under C57BL/6N background [wild-type (WT) controls from this background were obtained from Charles River at 8 weeks of age]. $MIF^{-/-}$ mice were bred and weaned at the Centre Hospitalier Universitaire Vaudois Lausanne and, at approximately 6–8 weeks of age, transported to EPFL for testing. Animals were housed in groups of 3–4 under temperature- and humidity-controlled conditions, and were maintained on a 12 h light/dark cycle with free access to food and water. On delivery to EPFL, animals were allowed to rest for 2 weeks before commencement of testing. Animal experiments were approved through a license issued by the Cantonal Veterinary Authorities (Vaud, Switzerland).

5′Bromodeoxyuridine administration

We have followed two different protocols for 5′bromodeoxyuridine (BrdU; a thymidine analog) administration. For the studies of colocalization between MIF and dividing cells, we injected BrdU (100 mg kg^{-1}, intraperitoneally (i.p.); Sigma-Aldrich, St Louis, MO, USA) dissolved in phosphate buffer (0.1 M) 2 h before killing. For experiments addressed to investigate the survival of the newly proliferated cells, four injections of BrdU (50 mg kg^{-1}, i.p.) were given, one every 12 h, over a period of 2 days, and 15 days before killing.

Tissue preparation for immunohistochemistry

Rats and mice were perfused transcardially under deep chloral hydrate anesthesia, with saline and then 4% paraformaldehyde in sodium phosphate buffer 0.1 M, pH 7.4. After perfusion, the brains were extracted and cryoprotected with 30% sucrose in phosphate buffer. Coronal sections (50 μm) were obtained with a sliding microtome and stored at −20 °C in 30% glycerol; 30% ethylene glycol, 40% PB until used.

Immunohistochemistry for conventional light microscopy

For immunohistochemistry, the tissues were processed using free-floating conditions as follows. Briefly, sections were incubated with 10% methanol, 3% H_2O_2 in phosphate-buffered saline (PBS) for 10 min to block endogenous peroxidase activity. After this, sections were treated for 1 h with 5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in PBS with 0.2% Triton-X 100 (Sigma-Aldrich) and were incubated overnight at room temperature in polyclonal rabbit anti-MIF antibody (1:1000; Zymed, Paris, France), monoclonal mouse anti-ki67 antibody (1:200; Novocastra, Newcastle upon Tyne, UK), monoclonal mouse anti-proliferating cell nuclear antigen antibody (PCNA, 1:200; Sigma-Aldrich), polyclonal goat anti-doublecortin (DCX) antibody (1:200, Santa Cruz, CA, USA), or monoclonal mouse anti-vimentin antibody (1:500, DSHB). After washing, sections were incubated for 30 min with donkey anti-mouse IgG, donkey anti-rabbit IgG or donkey anti-goat IgG biotinilated antibodies (1:250; Jackson ImmunoResearch Laboratories), followed by an avidin–biotin–peroxidase complex (ABC; Vector Laboratories, Peterborough, UK) for 30 min in PBS. Color development was achieved by incubating with 3,3′ diaminobenzidine tetrahydrochloride (Sigma-Aldrich) for 4 min. PBS containing 0.2% Triton-X 100 and 3% normal donkey serum was used for primary and secondary antibodies dilution. All of the sections studied passed through all procedures simultaneously to minimize any difference from immunohistochemical staining itself.

In the case of BrdU immunohistochemistry, endogenous peroxidase activity was inhibited as above, then sections were denatured by incubation for 1 h at 60 °C. After cooling to room temperature, the sections were incubated with 2N HCl for 30 min and then incubated overnight with monoclonal rat anti-BrdU antibody (1:500; Oxford Biotechnology, Oxford, UK) followed by donkey anti-rat IgG biotinilated antibody (as described above).

Double immunohistochemistry for confocal microscopy

To characterize the phenotype of MIF-immunoreactive (IR) elements in the dentate gyrus, we performed double immunohistochemistry using anti-MIF and antibodies against different neuronal, astroglial, microglial, and oligodendroglial markers. We analyzed whether the MIF-IR elements are also IR for different stages of cell proliferation and neurogenesis by using Ki67 and BrdU to mark cell proliferation, vimentin, and anti-glial fibrillar acidic protein (GFAP) for stem

cells, and PSA-NCAM, DCX, and NeuroD for neuronal precursors.

In general, sections were processed as described above, but the endogenous peroxidase block was omitted. The sections were incubated overnight with rabbit polyclonal anti-MIF antibody (1:1000; Zymed) and one of the following primary IgG antibodies: monoclonal mouse anti-neuronal nuclear antigen (1:100; NeuN, Chemicon, CA, USA), polyclonal rabbit GFAP (1:500; Sigma-Aldrich), monoclonal mouse anti-vimentin (1:500, DSHB), monoclonal mouse RIP (1:500, DSHB), polyclonal goat anti-NeuroD (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal goat anti-DCX (1:200; Santa Cruz Biotechnology), monoclonal mouse anti-ki67 (1:200; Novocastra), and mouse IgM anti-PSA-NCAM (Abcys, Paris, France; 1:1400). After washing, sections were incubated with donkey anti-mouse IgM, donkey anti-mouse IgG, donkey anti-goat IgG, or donkey anti-rabbit IgG secondary antibodies conjugated with Alexa 488 or Alexa 555 (1:200; Molecular Probes, OR, USA) in PBS containing 0.2% Triton-X 100 and 3% normal donkey serum. These general processing of the sections was slightly modified in two cases. The first one was when using the marker for microglia, the biotinylated tomato lectin (1:50, Sigma-Aldrich). After washing, sections were incubated with biotin conjugated with Alexa 488 (1:200, Molecular Probes) in the same solution than the secondary antibodies. The second exception was for the BrdU analyses. In this case, sections were firstly denatured by incubation for 1 h at 60 °C. After cooling to room temperature, the sections were incubated with 2N HCl for 30 min and then incubated overnight with the mix of polyclonal rabbit anti-MIF and monoclonal rat anti-BrdU antibody (1:500; Oxford Biotechnology) followed by donkey anti-rabbit IgG and donkey anti-rat IgG conjugated with Alexa 488 or 555 (as described above).

Sections were mounted on slides and coverslipped using DakoCytomation fluorescent medium (Dako North America, Inc., Carpinteria, CA, USA). Then, they were observed under a confocal microscope (Leica TCS SPE) using a 63X oil objective. Z-series of optical sections (1 μm apart) were obtained using the sequential scanning mode. These stacks were processed with LSM 5 image software. One in 10 series of hippocampal sections from each animal was double labeled as described. Fifty randomly selected IR cells were analyzed in each case to determine the co-expression of MIF and the markers described above (Table 1).

Table 1 Percentage of MIF colocalization with each of the markers used to identify different cell types and proliferation-differentiation states in the SGZ of the hippocampus

Marker	*Mean ± s.e.m.*
MIF/BrdU 2 h	4.0 ± 0.5
MIF/Ki67	3.5 ± 0.9
MIF/GFAP	96.0 ± 1.2
MIF/Vimentin	94.5 ± 1.3
MIF/Nestin	3.5 ± 0.7
MIF/PSA-NCAM	6.5 ± 0.9
MIF/Doublecortin	5.5 ± 1.3
MIF/NeuroD	5.0 ± 0.7
MIF/NeuN	0.0 ± 0.0
MIF/Tomato lectin	0.0 ± 0.0
MIF/RIP	0.0 ± 0.0

Abbreviations: MIF, migration inhibitory factor; SGZ, subgranular zone.

Immunohistochemical quantification

The number of Ki67-, PCNA-, BrdU-, DCX-, PSA-NCAM-, vimentin-, and MIF-IR cells in the dentate gyrus was estimated using a modified version of the fractionator method.[20] Systematic-random series (1:10) of sections covering the entire rostral to caudal extension of this structure were viewed on an Olympus BX40 microscope. Within each section, cells covering 100% of the sample area (that is all dentate gyrus-labeled cells) were counted. Cell somata were identified and counted with a ×40 objective lens. Cells appearing in the upper focal plane were omitted to prevent counting cell caps. The boundaries of the dentate gyrus were determined in parallel series of sections stained with cresyl violet. After cresyl violet staining, the dentate gyrus size in WT and $MIF^{-/-}$ mice was measured by quantifying its extension in every slice from a 1:10 subseries using Image J software (NIH).

Chronic unpredictable stress

During the course of 3 weeks, 24 rats were exposed daily to various stressors that were applied at different times of the day and included bright light (300 l×, 30 min), elevated platform (2 h), predator odor (1 h exposure to 10 μl of trimethylthiazoline, a synthetic compound originally isolated from fox feces), exposure to electric shocks (three 1 s shocks of 0.4 mA), exposure to the context reminder of shocks, acoustic stimulation (78–115 dB noise bursts), forced swimming in tanks of different sizes, exposure to a novel arena, and exposure to elevated plus maze (EPM) (see below for details). Another 24 rats were used as undisturbed controls. Animals were perfused and their brains processed for immunohistochemistry as described above.

Chronic treatment with corticosterone

Brain sections from an earlier published experiment describing an effect of chronic corticosterone treatment in hippocampal neurogenesis[21] were used to assess for the levels of MIF-IR cells in the dentate gyrus. This method of corticosterone administration, the plasma corticosterone levels, and variations in organ weight induced by it have been described in detail earlier.[22] Animals were perfused and their brains processed for immunohistochemistry as described above.

Drug treatments
Mice from each genotype (MIF$^{-/-}$ and WT, $n=8$ per genotype) were chronically administered with the antidepressant fluoxetine [10 mg kg^{-1}, i.p., daily for 14 days; $n=4$ per group] or vehicle (0.9% NaCl; $n=4$ per group). Treatment with Iso-1 (synthesized at the Department of Chemistry, EPFL) [(*S*,*R*)-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester] was also followed in WT animals. Iso-1 is a potent inhibitor of MIF's catalytic and cytokine activity. Iso-1 (7 mg kg^{-1}, i.p. in 5% DMSO in 0.9% NaCl) was injected daily for 14 days. Control mice received vehicle (5% DMSO in 0.9% NaCl).

Corticosterone analyses
Plasma corticosterone levels were analyzed in two cohorts of MIF$^{-/-}$ and WT mice. On the first one (MIF$^{-/-}$ $n=4$, WT $n=5$), basal levels were collected under resting conditions both in the morning and evening by making a small cut close to the end of the tail. On the second one (MIF$^{-/-}$ $n=8$, WT $n=8$), corticosterone response to stress was evaluated by exposing mice to a novel environment (a confined circular box of 25 cm diameter) during 15 min. Blood samples were taken immediately after stress (that is 15 min from the start of stress, min-15) by a small tail incision, and 15 min after the end of the stress procedure (that is 30 min from the start of stress, min-30) by decapitation. Plasma corticosterone was quantified using Correlate-EIA Corticosterone Enzyme Immunoassay Kit (Assay Design, MI, USA) according to the manufacturer's instructions.

Preparation of hippocampal tissue and western blot analyses for corticosteroid receptors
Hippocampal tissue was collected under both basal conditions ($n=10$ per genotype) and 30 min after exposure to stress ($n=4$ per genotype). Each mouse hippocampus was homogenized in ice-cold homogenization buffer [10 mM Hepes/1.0 mM EDTA/2.0 mM EGTA/0.5 mM DTT/0.1 mM PMSF/1% NP-40] containing protease and phosphatase inhibitor cocktail (Roche, Basel, Switzerland). Protein content in whole hippocampal samples was quantified using the *DC* protein assay (Bio-Rad Laboratories AG, Reinach, Switzerland). Equal protein samples were prepared at a concentration of 0.5 μg ml^{-1} in reducing SDS–PAGE buffer (NEB). Proteins were resolved on 10% polyacrylamide gels, and transferred to nitrocellulose membranes. The membrane was then blocked in 5% non-fat milk and rabbit anti-glucocorticoid (1:10 000; Santa Cruz) and anti-mineralocorticoid (1:1000; Santa Cruz) were applied in this blocker over night at 4 °C. Peroxidase-labeled secondary anti-rabbit IgG was applied for 1 h at room temperature followed by washing and band visualized using a chemiluminescence peroxidase substrate (SuperSignal West Dura, Pierce Biotechnology, IL, USA). Immunoreactivity was detected using the Biorad ChemiDoc XRS system. Densitometry analysis on the bands was calculated using Biorad Quantity One (4.2.3) software (Bio-Rad Laboratories AG). Absorbance for each of the synaptic protein antibodies was normalized to within-lane actin absorbance. Average densitometric data are reported for each experimental group as percentage of WT values.

General procedures for behavioral testing in MIF$^{-/-}$ mice
Three cohorts of MIF$^{-/-}$ and WT mice were tested at 10–14 weeks of age on a variety of behavioral tests. In two of the cohorts, testing was initiated with the EPM. Then, one of the cohorts was tested in the activity cage (1 day afterward), in the Y-maze test, and in the forced swim test (FST) (1 week inter-test interval). The second cohort was tested in the water maze, in the acoustic fear-conditioning task and in the hot-plate test (1 week inter-test interval). A third cohort of mice was only behaviorally tested in the FST. The behavioral tests are described below. In all the tests (except for the fear conditioning and hot-plate tests), a video camera was used to videotape mice behavior that was automatically recorded and analyzed by the video tracking software (Ethovision, Noldus, Netherlands).

Elevated plus maze
Anxiety-like behavior was assessed using the EPM task, a standardized test for anxiety-like behavior in rodents. This test has been described in detail earlier.[23] Mice ($n=26$–28 per genotype) were placed on the central platform facing a closed arm and allowed to explore the maze for 5 min. The total distance moved, time spent, and number of entries to the open, center, and closed arms were analyzed. Differences in the proportion of time spent in the open arms and close arms are indicative of differences in anxiety-like behavior.

Forced swim test
The mice were individually placed into a glass cylinder (25 cm height, 10 cm diameter) containing 8 cm of water maintained at 25–26 °C. The test lasted 5 min. The time mice spent immobile was measured during the last 4 min of the test period. The floating time, defined as the absence of escape-oriented behaviors, such as swimming or climbing, was scored. Percentage time immobile was calculated.

Spatial learning in the water maze
Mice from each genotype (MIF$^{-/-}$ and WT, $n=10$ per genotype) were tested for their spatial memory. A detailed description of the maze and standard training conditions has been published earlier.[23] Briefly, training was conducted over 3 days (four trials per day with a inter-trial interval of 15 min). The latency to find the platform was measured as an index of the learning and memory abilities of the animals, as no differences in swim speed were found between the two genotypes. One day later (day 4), spatial memory was tested in a probe test and percent time spent in the target and opposite quadrant was measured and

the bias toward the target quadrant served as an indication of spatial memory. After the probe test, a cued version of the water maze task, in which the platform was made visible by attaching a 10 cm flag to the base of the platform, was used to evaluate the motivation and motor abilities of the WT and MIF$^{-/-}$ mice.

Acoustic fear conditioning
Details of the apparatus and training procedure have been described in detail elsewhere.[24] Fear conditioning to the tone was performed on male mice ($n=8$–9/genotype). All trials were video recorded and freezing, which is defined as behavioral immobility, except for respiration movements for at least 2 s, was measured by an experimenter blind to the genotype of the animals. Freezing times were transformed to percentage freezing values.

Hot-plate test
Mice ($n=10$ per genotype) were tested for their analgesic sensitivity using a hot-plate apparatus. The temperature was set at $51\pm0.1\,^{\circ}$C and the cut-off time to stop the trial was 60 s. The parameters recorded were the latencies to front-paw licking, to hind-paw licking, and to jump (all four paws off surface simultaneously).

Statistics
All data for quantitative studies are expressed as mean ± s.e.m. Data was analyzed with Student's two-tailed *t*-test (with Welch's correction, where appropriate) or analysis of variance (one/two way or repeated measures, depending on the experimental design), followed by Bonferroni's or Fischer's LSD *post hoc* analysis, where appropriate. Significance was set at $P<0.05$.

Results

MIF expression in the SGZ of the hippocampus
We observed the presence of MIF-IR cells in several areas of the rat brain including the neocortex (Figures 1e and f), amygdala, hippocampus (Figures 1a–c), thalamic nuclei, and the rostral migratory stream (Figure 1d). Attending to their morphology, MIF-IR cells were small in size and had a multipolar shape (resembling the size and shape of astrocytes) and co-localized with GFAP (Supplementary Figure 2). Most interesting to us, given the reported function of MIF in cell division, was the high level of expression of MIF in the SGZ of the dentate gyrus.

Therefore, we studied the expression of MIF in proliferating cells in the dentate gyrus. In experiments in which BrdU was injected 2 h before killing (to evidence proliferating cells), we observed the expression of MIF (Figure 2a) and Ki67, a proliferative marker (Figure 2b). Here, MIF-IR cells displayed unipolar morphology (Figure 1c), but co-localized with vimentin and GFAP (Figures 2c and d), markers of astrocytes and present in stem cells, as well as with nestin (Figure 2e) that is present in stem cells (type 1 in Kemperman's classification), suggesting significant MIF expression in cytogenic cells. We then undertook a more detailed characterization of the phenotype of these SGZ MIF-IR cells using double immunohistochemistry against MIF and specific markers for cell types at different states of proliferation/differentiation. We did not observe colocalization between MIF and NeuN, the latter a marker of mature neurons (Figure 2g). In addition, we did not find evidence for expression of MIF in neither microglia (using tomato lectin) nor oligodendroglia (using RIP) (Supplementary Figure 1). Some MIF-IR cells co-localized with PSA-NCAM, DCX, or NeuroD, markers for neuroblasts and immature granule neurons in the SGZ of the hippocampus (Figure 2f). The percentage of colocalization between MIF and the different markers analyzed is indicated in Table 1. Overall, these results suggest a potentially functional role for MIF in hippocampal neurogenesis, which we addressed in the following sections.

Correlation between MIF expression and hippocampal neurogenesis
The co-localization of MIF with known markers of proliferative and newly differentiated neurons suggested the possibility that MIF was important for neurogenesis. To functionally link MIF expression in the SGZ of the rat hippocampus to neurogenesis, we studied two models involving impaired neurogenesis: (i) chronic stress and (ii) chronic corticosterone administration (CORT). First, we confirmed that under our experimental conditions, chronic unpredictable stress (CUS) induced a reduction in the number of proliferative cells in the dentate gyrus, as reflected by lower Ki67-IR expression in the stress group than in the control ($P<0.03$; Figure 3a). In parallel, we observed a clear reduction in the amount of MIF-IR cells in the SGZ after CUS ($P<0.002$; Figure 3b). On the other hand, we observed no change in the number of vimentin-IR cells in the SGZ of animals under chronic stress (Figures 3c and d) (6096 ± 342 cells in the control rats vs 5803 ± 67 in stressed rats; $P=0.44$). Vimentin is a marker for precursor cells that we found to co-localize with MIF (Figure 2c); this unaltered presence of vimentin in the MIF indicates that precursor cells are present, but there is a deficit in their proliferation. Second, we analyzed MIF expression in brain slices from an earlier published paper in which a chronic corticosterone treatment was reported to reduce hippocampal neurogenesis[21] Again, we found reduced MIF-IR cells in the corticosterone-treated group as compared with the control group ($P<0.002$) (Figure 3e).

MIF$^{-/-}$ mice show lower levels of neurogenesis and exhibit significantly reduced response to fluoxetine-induced cell proliferation than WT mice
The fact that reduced neurogenesis was accompanied by parallel reductions in MIF-IR expression in the dentate gyrus suggested either that MIF was causally

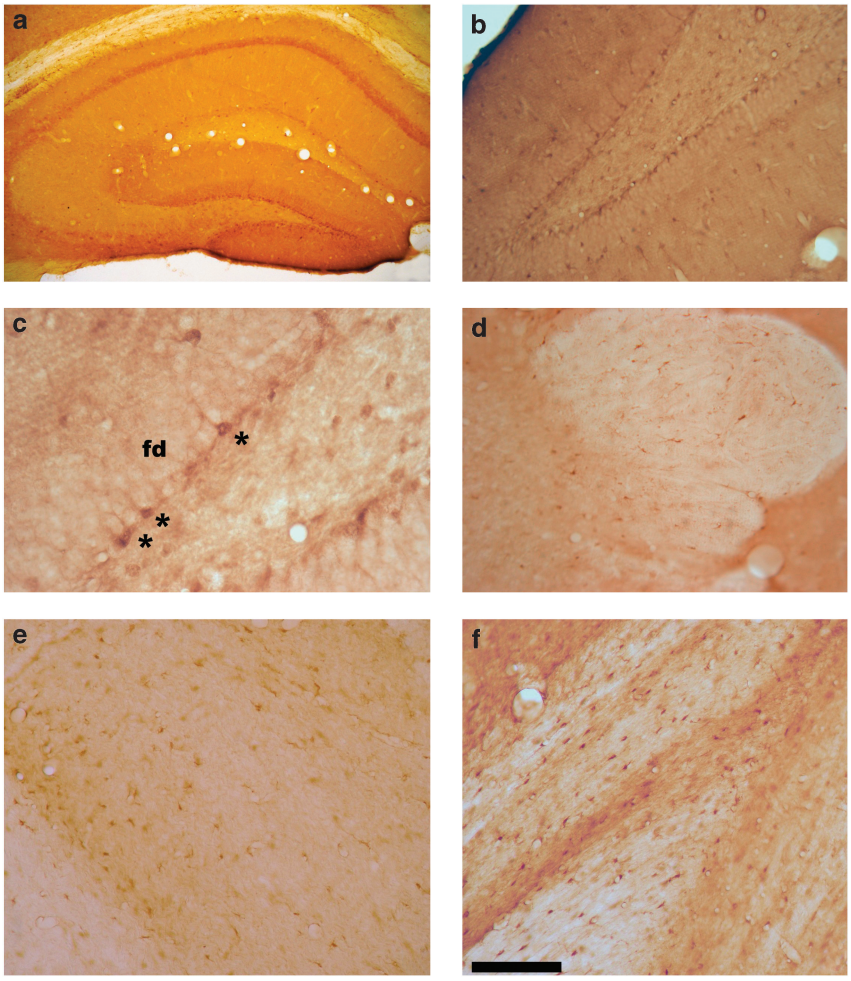

Figure 1 MIF distribution in the brain. (**a**) Panoramic view of the hippocampus. (**b**) Detailed view of the dentate gyrus of the hippocampus. (**c**) A more detailed view shows the presence of MIF-IR cells in the SGZ, a neurogenic area of the adult brain. Asterisks mark cells MIF-IR in the SGZ with the unipolar characteristic morphology; fd, fascia dentata. Examples of expression of MIF in other brain regions: rostral migratory stream (**d**), neo-cortex (**e**), and white matter (**f**). Scale bar: 1 mm (**a**), 200 μm (**b**, **d–f**) and 50 μm (**c**).

implicated in the proliferative deficit or that reduced MIF expression was a consequence of the treatments' effects on neurogenesis. To directly address this causality issue, we compared expression levels in the dentate gyrus of three markers (PCNA, DCX, BrdU) for different neurogenic phases between WT and $MIF^{-/-}$ mice. We found a marked reduction in all three markers of neurogenesis in $MIF^{-/-}$ mice as compared with controls: the difference was already observed for cell proliferation (as indicated by PCNA-IR; $P<0.0001$; Figure 4a), and was maintained for differentiating/immature neurons (as indicated by DCX-IR; $P<0.0001$; Figure 4b) and for cells surviving for 3 weeks [time considered to be required for new cells incorporation into the hippocampal circuitry[25] as their proliferation (as indicated by BrdU-IR; $P<0.0003$; Figure 4c)]. There was no significant change in the level of precursor cells as evidenced by unchanged levels of vimentin in the SGZ in $MIF^{-/-}$ mice (5801 ± 272 cells in the WT group vs 6358 ± 411 in the $MIF^{-/-}$ group; $P=0.27$). Moreover, the overall size of the dentate gyrus was unaffected (data not shown).

The former experiment established the causal implication of MIF on cell proliferation under basal conditions. We questioned, then, whether MIF would also be required for cell proliferation. The SSRI antidepressant fluoxetine has been found to stimulate neurogenesis, particularly cell proliferation.[26,27] Therefore, we examined whether genetic deletion of MIF would interfere with fluoxetine-induced cell proliferation. Fluoxetine (10 mg kg^{-1}, i.p.) was given daily over 14 days. Analysis of variance with genotype (WT and $MIF^{-/-}$) and treatment (vehicle or fluoxetine) as the between group factors revealed significant effects of fluoxetine ($F(1,12)=11.43$; $P=0.005$), genotype ($F(1,12)=68.76$; $P<0.0001$), and an interaction between the two factors ($F(1,12)=9.37$; $P=0.01$) on cell proliferation (as indicated by PCNA-IR expression). *Post hoc* tests indicated again lower cell proliferation in

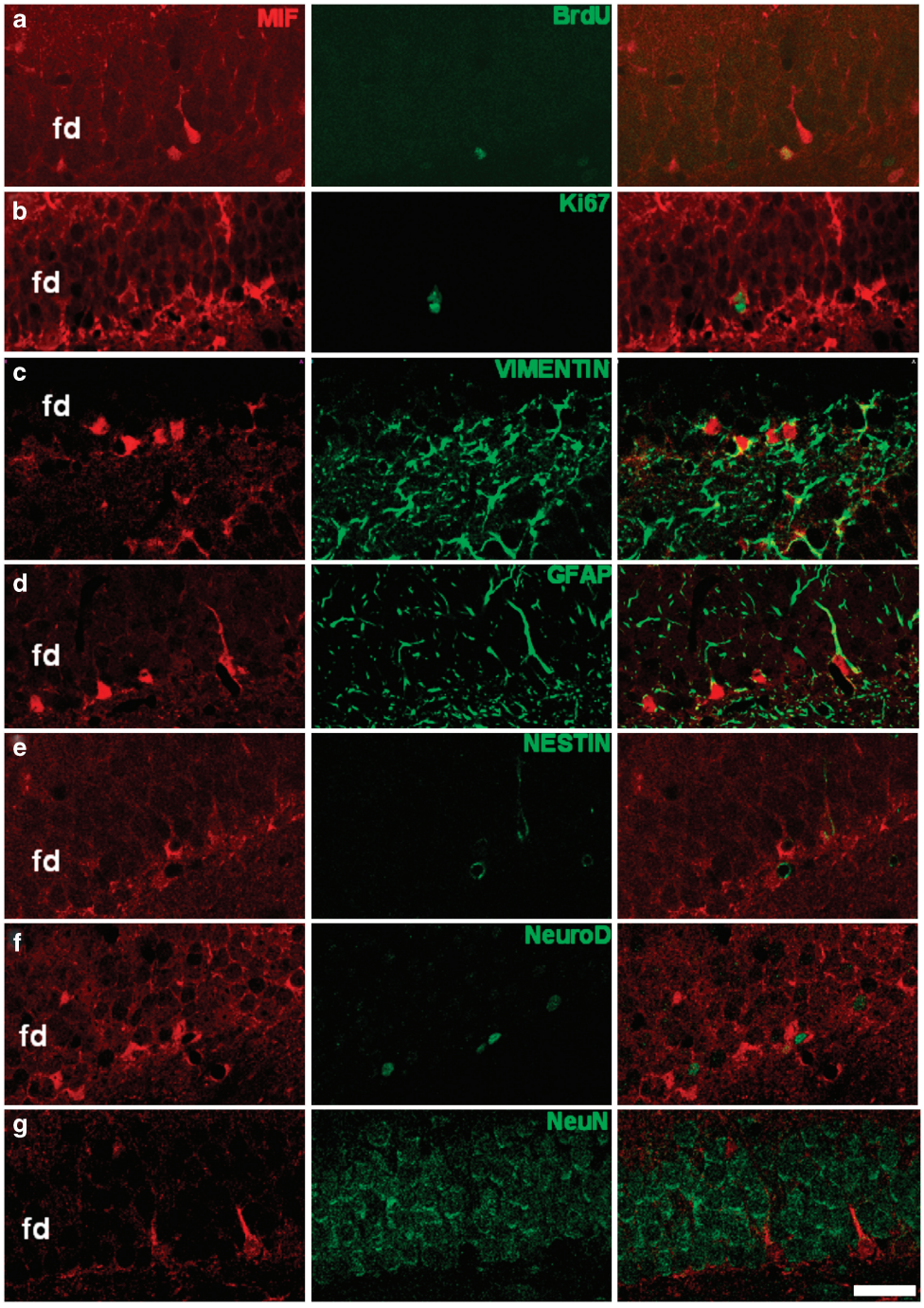

Figure 2 Phenotypical characterization of MIF-IR cells in the hippocampal SGZ. Colocalization of MIF and BrdU (**a**) and MIF and Ki67 (**b**) reveals that MIF-IR cells have the ability to proliferate. Colocalization of MIF and vimentin (**c**) and MIF and GFAP (**d**) reveals that these cells displayed astrocytic-stem cell nature. The observed colocalization between MIF and Nestin (**e**) confirmed the stem-cell nature of these cells (type 1). Some MIF-IR cells displayed colocalization with NeuroD (**f**) revealing the clear immature nature of those cells. (**g**) MIF-IR cells does not colocalize with the neuronal marker NeuN; fd, localization of granule cell layer. Scale bar. 25 μm. Images correspond to focal planes.

$MIF^{-/-}$ than in WT mice (vehicle injected in both cases; $P<0.01$). Moreover, whereas fluoxetine treatment increased cell proliferation in WT mice as compared with vehicle-injected mice ($P<0.01$), it had no effect in $MIF^{-/-}$ mice ($P<0.001$; Figure 4d). The same differences were observed in immature neurons (as indicated by DCX labeling; data not shown).

In this latter experiment, we also measured MIF-IR in the dentate gyrus of WT mice after fluoxetine treatment. Interestingly, a significant increase was

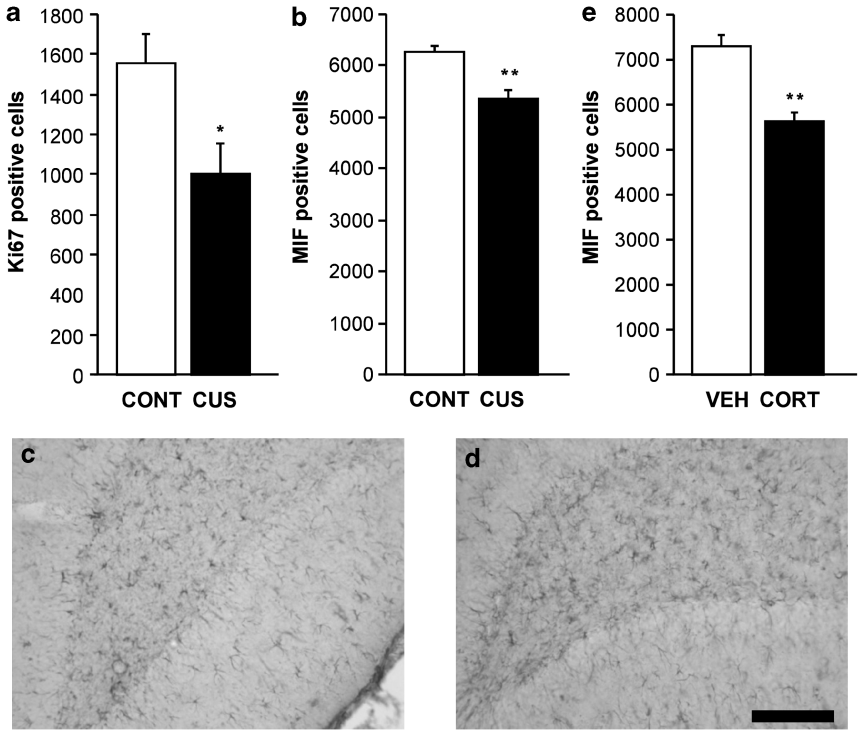

Figure 3 Modulation of MIF in the SGZ by treatments affecting hippocampal cell proliferation (chronic stress, stress hormones administration, and fluoxetine). CUS leads to a reduction in (**a**) the proliferative marker Ki67 and (**b**) a corresponding reduction in expression of MIF-IR cells in the rat SGZ. (**c** and **d**) These changes are not related to changes in hippocampal vimentin expression between (**c**) control and (**d**) CUS. (**e**) Chronic treatment with corticosterone induces a similar reduction in MIF expression in the SGZ zone of the dentate gyrus. All data represents mean ± s.e.m. $*P<0.05$ and $**P<0.01$ compared with corresponding control. Scale bar 100 μm.

found in fluoxetine-treated mice as compared with saline-treated mice ($P<0.05$; Figure 4e), mimicking the enhanced cell proliferation induced by this treatment (Figure 4d).

Finally, pharmacological inhibition of MIF (with 14 days of iso-1 treatment), mimicking post-natal MIF deletion, also modulated hippocampal neurogenesis. Iso-1-treated mice showed a lower number of Ki67-IR cells in the dentate gyrus than vehicle-injected mice ($P=0.04$; Figure 4f). See Supplementary Figure 3 for pilot data indicating that MIF antagonist Iso-1 can also reduce fluoxetine-stimulated cell proliferation.

Plasma glucocorticoid levels and hippocampal corticosteroid receptors in MIF$^{-/-}$ mice

As MIF$^{-/-}$ and WT mice differed in their neurogenic responses, and given that there is a link between MIF and glucocorticoids,[7] and glucocorticoids are known to regulate hippocampal neurogenesis,[28] we set experiments to evaluate plasma corticosterone levels and hippocampal expression of the two types of corticosteroid[29] (mineralocorticoid, and glucocorticoid) receptors both under basal conditions and in response to stress. As shown in Figure 5a, left panel, no significant differences in plasma corticosterone levels between the two genotypes were observed when blood samples were taken under basal conditions at different times during the circadian cycle ($F(1,7)=0.80$; $P=0.40$). Again, when a second cohort of mice was briefly exposed to novelty stress (Figure 5a, right panel), no significant differences in corticosterone responses were observed when plasma samples were taken 15 and 30 min from the beginning of the stress procedure ($F(1,14)=2.34$; $P=0.15$). Similarly, no differences between the two genotypes were found for hippocampal mineralocorticoid or glucocorticoid expression when evaluated under basal conditions (Figure 5b, left panel; *n.s.*) or after exposure to stress (Figure 5b, right panel; *n.s.*).

Characterization of anxiety- and depression-like behaviors in MIF$^{-/-}$ mice

At the behavioral level, hippocampal neurogenesis has been linked to affective behaviors such as anxiety,[30] depression,[31] and to cognitive functions such as learning and memory.[32,33] Therefore, we characterized these behaviors in the MIF$^{-/-}$ mice. Analysis of behavior in the EPM indicated higher indices of anxiety in MIF$^{-/-}$ mice than in controls: mutant mice displayed fewer entries ($P<0.01$; Figure 6a) and spent less time in ($P<0.001$; Figure 6b) the open arms than WT mice. MIF$^{-/-}$ also moved significantly less in all areas of the EPM (Figure 6c). This reduced exploration was further confirmed in the activity cage, with MIF$^{-/-}$ mice moving significantly

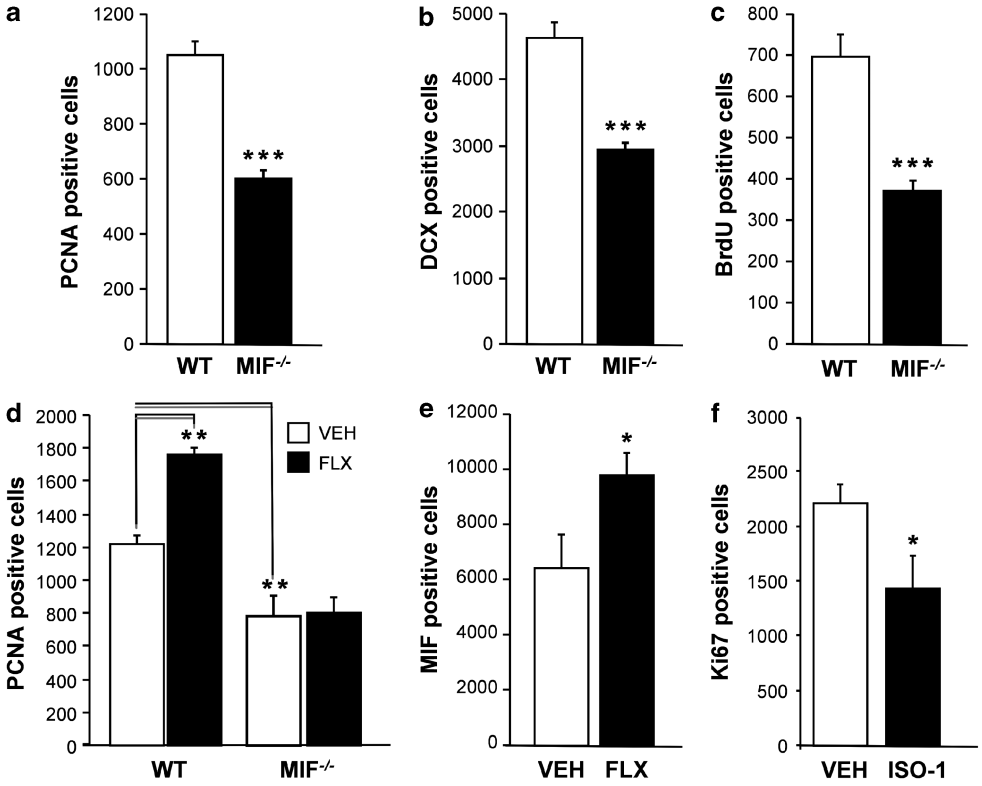

Figure 4 Hippocampal cell proliferation and neurogenesis in MIF$^{-/-}$ mice. Reduced expression of (**a**) PCNA (proliferation marker), (**b**) DCX (neurogenesis marker), and (**c**) BrdU (cell survival and integration marker) IR cells in the SGZ of the dentate gyrus of MIF$^{-/-}$ mice compared with WT controls. (**d**) 14-day treatment with fluoxetine (10 mg kg^{-1}) increases the number of PCNA IR cells in the dentate gyrus of WT, but MIF$^{-/-}$ mice. (**e**) Chronic fluoxetine treatment (10 mg kg^{-1} for 14 days) induces increase in the number of MIF-IR cells in the SGZ of the dentate gyrus. (**f**) Hippocampal Ki67 positive cell number is reduced after Iso-1 treatment (7 mg kg^{-1} for 14 days). All data represents mean ± s.e.m. *$P<0.05$, **$P<0.01$, and ***$P<0.001$ compared with corresponding control or as indicated.

shorter distance than WT controls ($P<0.01$; data not shown).

Depression-like behavior was evaluated in the FST. MIF$^{-/-}$ mice spent significantly more time immobile than WT mice ($P<0.05$; Figure 6d), which is suggestive of higher depression-like behavior in the mutant mice. However, in a pilot study performed in behaviorally naive mice, depression-like behavior (that is floating) in the FST was equivalent for MIF$^{-/-}$ and WT mice (Supplementary Figure 4).

Performance of MIF$^{-/-}$ mice in hippocampus-dependent and -independent learning tasks

Spatial learning and memory abilities of MIF$^{-/-}$ and WT mice were tested in the hippocampus-dependent water maze task.[34,35] A repeated measures analysis of variance indicated (i) a significant effect of time ($F(11,198)=6.60$; $P<0.001$), with animals showing reduced escape latencies over training trials (Figure 7a); (ii) a significant overall effect of genotype ($F(1,18)=6.34$; $P=0.02$), with MIF$^{-/-}$mice performing worse than WT controls; and (iii) a significant interaction between time and genotype ($F(11,198)=2.45$; $P=0.007$). *Post hoc* analyses indicated that MIF$^{-/-}$ mice performance was particularly impaired on the first trial of day 3 (Trial 9; $P<0.05$), suggesting a deficit for the mutant mice in the long-term memory. Further evidence of the long-term memory dysfunction in these mice was found in the probe test, conducted 24 h after the last trial on day 3. Whereas WT mice showed a clear preference for the target vs the opposite quadrant ($P<0.0001$), MIF$^{-/-}$ mice failed to show this preference (*n.s.*) (Figure 7b), performing, in fact, at random (that is their time in the target quadrant did not differ from 25%, chance level). To check whether the cognitive deficit observed in the MIF$^{-/-}$ mice was not related to a motor deficit or altered motivation, we performed a further training session in which the platform was made visible (cued) to the mice. Here, no differences were found between MIF$^{-/-}$ and WT animals in their latencies to find the platform (*n.s.*; Figure 7a, visible), suggesting that the learning and memory deficits apparent in the knockout mice are not related to visual and/or motivational deficits. Further evidence that it is long-term memory, but not short-term memory that is affected in MIF$^{-/-}$ mice, was obtained in the Y-maze, in which animals did not differ in short- or working-memory measures (data not shown).

Then, WT and MIF$^{-/-}$ mice were tested in the hippocampus-independent, but amygdala-dependent acoustic fear-conditioning task (Figure 7c). When

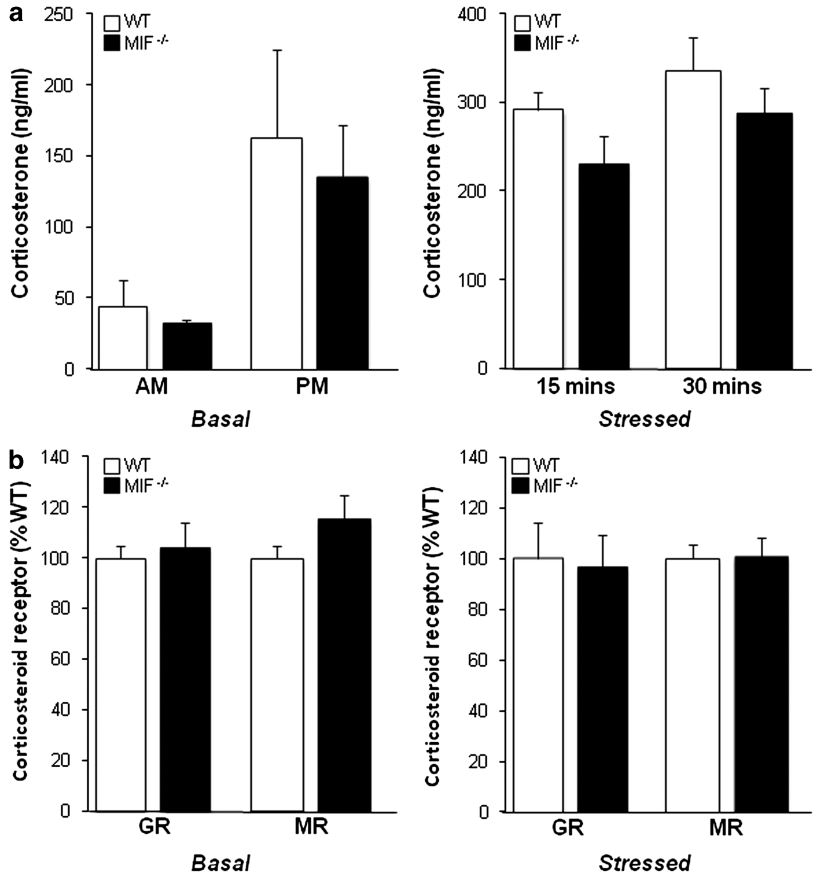

Figure 5 Corticosterone hormone and receptor levels in basal and stressed mice. (**a**) Basal (morning and evening; left panel, $n=4$) and post-stress (15 min or 30 min; right panel, $n=8$) corticosterone levels did not differ in MIF$^{-/-}$ compared with WT mice. (**b**) Basal (left panel, $n=10$) and post-stress (right panel, $n=8$) levels of hippocampal corticosteroid (that is mineralocorticoid and glucocorticoid) receptors were similar in both genotypes. All data represents mean ± s.e.m.

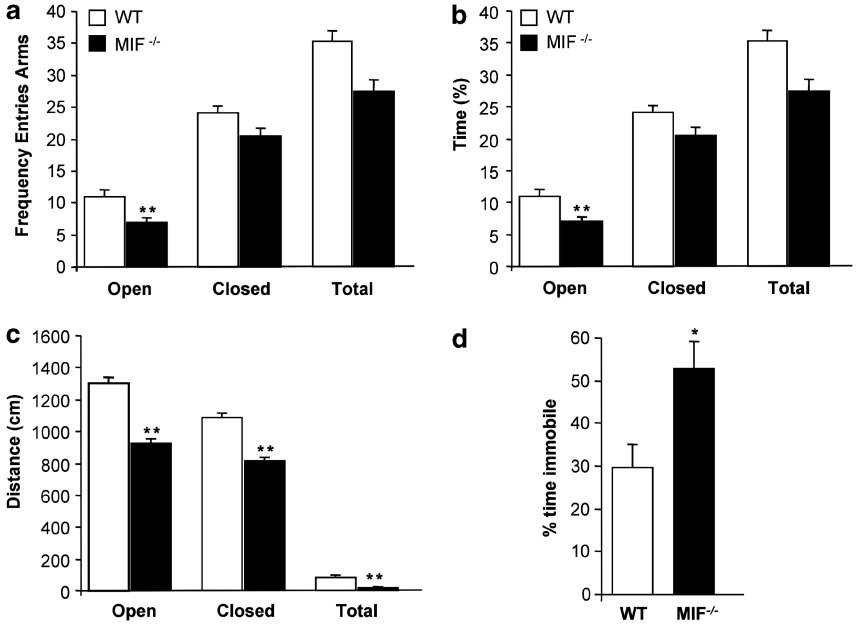

Figure 6 Increased anxiety- and depressive-like symptoms in MIF$^{-/-}$ mice. (**a**) Behavioral measurements in the EPM are represented as frequency of entries in the different arms, (**b**) percent time spent in the different EPM areas and (**c**) distance (cm) moved in the different arms. All data are group means (± s.e.m., $N=28$–30 per group). (**d**) Percentage time floating in MIF$^{-/-}$mice ($n=5$) is significantly longer than WT controls ($n=6$) in the FST, data are group means over the last 4 min of the test. $^{*}P<0.05$, $^{**}P<0.01$ vs WT.

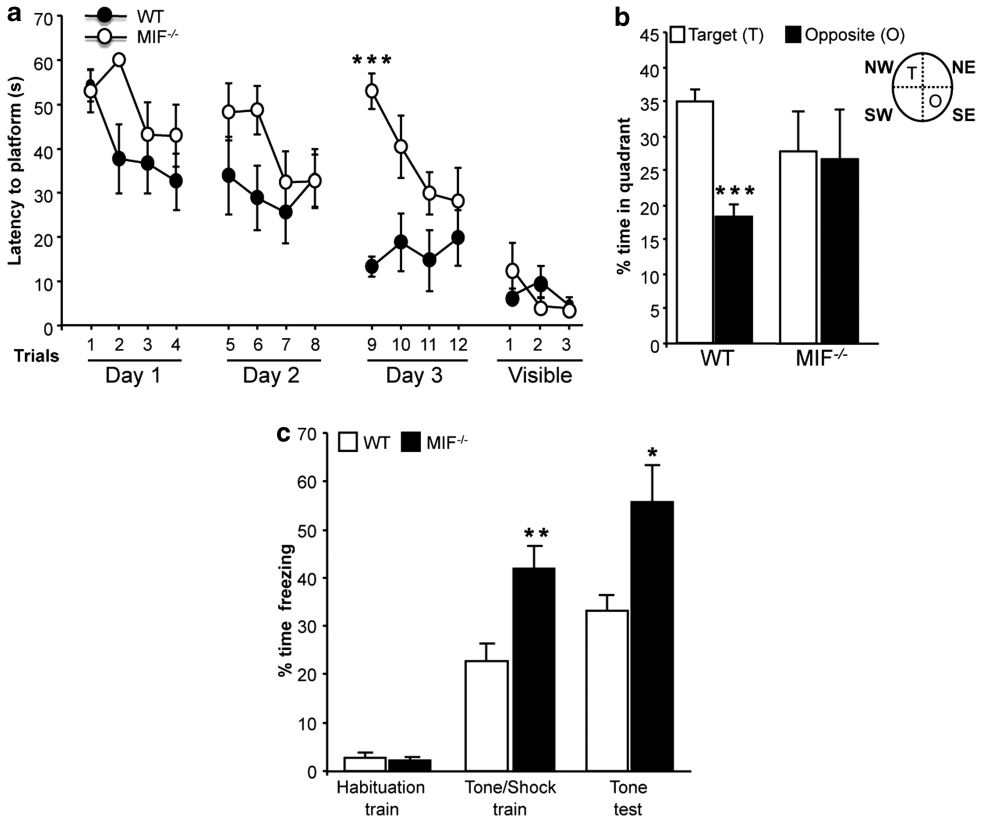

Figure 7 Impaired hippocampal-dependent spatial learning and memory, but intact amygdala-dependent fear conditioning in MIF$^{-/-}$ mice. (**a**) Male mice WT and MIF$^{-/-}$ mice ($n=10$ per genotype) were trained in water maze for 3 acquisition days, followed by a probe test and a visible platform test. MIF$^{-/-}$ mice were profoundly impaired in acquisition of spatial reference compared with WT mice. (**b**) Memory was tested by performance in the probe test conducted 24 h after Trial 12. WT mice showed a target compared with opposite quadrant preference that was absent in MIF$^{-/-}$ mice. $^{*}P<0.05$, $^{***}P<0.0001$. (**c**) In the acoustic fear-conditioning test, MIF$^{-/-}$ mice ($n=7$) showed significantly more freezing both during the training period and recall (test) period compared with WT controls ($n=8$). $^{*}P<0.05$, $^{**}P<0.01$. All data are mean ± s.e.m.

mice were placed in the conditioning apparatus, both genotypes showed minimal freezing (WT: 2.64 ± 1.06%; MIF$^{-/-}$: 2.11 ± 0.75%). On presentation of the shock-tone combination during the training session, MIF$^{-/-}$ mice froze more during the post-shock time than WT mice ($P<0.01$). During the test given 24 h later, MIF$^{-/-}$ also showed significantly higher freezing than WT mice ($P<0.05$). Our results in the hot plate indicated that a differential nociceptive sensitivity is unlikely to explain the observed differences in the acoustic fear-conditioning test: latencies to retract the paws in the hot-plate test were equivalent for both genotypes (WT: 23.07 ± 0.87 s; MIF$^{-/-}$: 20.80 ± 1.42 s; *n.s.*). Altogether, these data indicates that genetic deletion of MIF has profound effects on anxiety- and depression-like behavior as well as on learning and memory function.

Discussion

In this study, we have identified the expression of MIF in astrocytes and, most notably, in neurogenic cells in the SGZ of the rodent dentate gyrus and characterized its presence in stem cells and in both cells undergoing proliferation and in recently proliferated cells undergoing maturation, but not in mature neurons, microglia, or oligodendrocytes. We found that, in the SGZ, MIF expression is modulated in parallel to cell proliferation by treatments that are well established for their modulatory actions on adult hippocampal neurogenesis: stress, glucocorticoid, and fluoxetine treatments. To assess for the causal function of MIF in SGZ cell proliferation, we used genetic and pharmacological approaches. Both, genetic deletion of MIF and chronic treatment with the MIF antagonist Iso-1 resulted in reduced cell proliferation and MIF deletion also abolished the enhanced proliferation induced by chronic fluoxetine treatment. The behavioral phenotype of mice with genetic deletion of MIF was indicative of increased anxiety- and depression-like behaviors, as well as of impaired hippocampus-dependent memory.

Our findings are in agreement with an earlier study by Ogata *et al.*[13] with regards to the identity of post-mitotic cells expressing MIF (that is astrocytes, but not mature neurons, microglia, or oligodendrocytes)

and the first to identify MIF expression in hippocampal neurogenic cells. Although, in their paper, Ogata *et al*.[13] found mRNA expression in neurons and astrocytes, in agreement with our data, protein expression—as assessed by immunohistochemistry—was restricted to astrocytes.

Our work reveals a new critical function for MIF in adult hippocampal cell proliferation. This fits with former studies that had reported MIF expression in neurogenic areas in the CNS[36] and identified proliferative functions for MIF in non-brain tissues.[37] Strikingly, our data from the gene knockout and antagonist treatment approaches indicate a function for MIF in hippocampal cell proliferation under basal (that is antidepressant-independent) conditions for a subset, but not the entire set of progenitor cells. This contrasts with the effects of other molecular mechanisms, such as cyclin D2, found to be required for virtually the entire basal hippocampal neurogenesis observed in mice.[38] Importantly, our study also suggests that MIF is required for the stimulation of hippocampal cell proliferation by antidepressants (at least by the SSRI fluoxetine), highlighting MIF as a potential important molecular target to develop novel neurogenesis-related antidepressant treatments.

Conversely, the experiments involving chronic stress or chronic corticosterone treatment showed that MIF is an accompanying factor to the inhibitory effects of these treatments on cell proliferation[39–42] further supporting a function for MIF in basal (that is non-antidepressant-induced) cell proliferation. However, these experiments do not allow discerning what comes first as a consequence of stress and glucocorticoid treatments: whether MIF is first targeted and, hence, hippocampal cell proliferation is inhibited or the converse, MIF expression is reduced because of an inhibitory effect in the dividing cells. Further experiments with the $MIF^{-/-}$ mice are required to directly address the causal implication of MIF in stress-related reduction in hippocampal cell proliferation. Given the described modulatory actions of glucocorticoids in hippocampal neurogenesis,[43] we evaluated glucocorticoid responses and glucocorticoid regulatory mechanisms in $MIF^{-/-}$ mice. Importantly, no differences were found between MIF deficient and WT mice in plasma corticosterone responses or in hippocampal mineralocorticoid and glucocorticoid receptors neither under basal conditions nor after exposure to stress. These observations strongly suggest that alterations in glucocorticoid-related levels are not the cause for the reductions in neurogenesis observed in the $MIF^{-/-}$ mice.

Altogether, our immunohistochemical studies identify an interesting link between MIF expression in the SGZ of the dentate gyrus and changes in neurogenesis induced by antidepressant and stress-related treatments. This raises the question of the potential involvement of MIF in behaviors that have been reported to be related (even when discrepancies in the literature still exist) to the interaction between these treatments and hippocampal neurogenesis.[27,30,31,44,45] To address this issue, we tested $MIF^{-/-}$ mice for their anxiety- and depression-like behaviors, as well as for their hippocampus-dependent and -independent learning.

Our behavioral results collectively indicate that MIF deficiency is associated with a phenotype characterized by increased anxiety- (as evaluated in the EPM) and depression-like (as evaluated in the FST) behaviors, as well as by impaired hippocampus-dependent (as evaluated in the water maze) memory function. Studies based on strategies aimed at reducing neurogenesis in mice, such as focal hippocampal irradiation, initially proposed that a decrease in neurogenesis *per se* does not induce behavioral symptoms associated with depression,[27,46–48] but rather, that neurogenesis is required for some of the behavioral effects of the antidepressant drugs;[27,49] but see also Refs 50,51 for evidence indicating that antidepressant effects on depression-like behaviors are not dependent on neurogenesis. More recent studies with alternative approaches have supported a role for neurogenesis in anxiety.[52–54] One of the most conclusive evidence was obtained with transgenic mice in which hippocampal neurogenesis was specifically impaired (through the over-expression of the pro-apoptotic protein Bax in neuronal precursors). These mice exhibited a striking increase in anxiety-related behaviors (as evaluated among other tests in the EPM), whereas depression-like behaviors (as evaluated among other tests in the FST) were not affected.[30] Recently, an important modulatory function has been proposed for anxiety on hippocampal cell proliferation involving both amygdala-dependent and -independent mechanisms.[55] Our present data further supports a key function for hippocampal neurogenesis (and hence of MIF as an important molecular pathway regulating cell proliferation) in anxiety-like behavior.

However, given the lack of clear evidence in the existing literature linking a deficit in neurogenesis with depression-like behavior under basal (that is not antidepressant-stimulated) conditions, we should be cautious about the conclusions that can be drawn from the FST data. Among the possible explanations that can be put forward are (i) that there is a link between MIF expression and depression-like behavior in the FST that is independent of hippocampal neurogenesis; (ii) that the genetic deletion of MIF might have modified other biological factors, which are critical for the expression of this depression-like behavior; (iii) that the depression-like behavior observed in $MIF^{-/-}$ mice is the emerging result of having exposed these mice with their heightened anxiety levels to a series of experimental conditions before being tested in the FST. Although our data does not allow us to assert or to exclude any of these possibilities, a pilot study performed in behaviorally naive mice, and showing no differences in the FST between the two genotypes, gave support to the latter. In addition, this hypothesis is in line with substantial experimental and clinical evidence supporting a key

function for high anxiety trait in the emergence of depression.[56]

Our learning results also support a strong link between MIF expression and hippocampus-dependent learning. $MIF^{-/-}$ mice were impaired in their formation of a long-term memory for the platform location in the water maze. It is noteworthy that within each training session, these mice were able to learn and that their deficit was circumscribed to the maintenance of the memory into a long-term storage (as indicated by their recall failure on the first trial of training session 3 and in the probe trial). Specificity of this deficit for a hippocampus-dependent memory was supported by the lack of deficit found in the $MIF^{-/-}$ mice in a hippocampus-independent (amygdala-dependent) learning task, the acoustic fear conditioning. In fact, the $MIF^{-/-}$ mice showed higher fear responses than WT mice both during training and testing, which might be a reflection of their more anxious phenotype instead of a reflection of enhanced fear memory (note that no differences between the two genotypes are observed when the ratio between freezing at testing and freezing at training is considered). In this connection, it is important to mention that a recent study has shown an upregulation of the MIF receptor, CD74, after high-frequency long-term potentiation induction.[57] Although, to our knowledge, it is not as yet known whether CD74 is expressed in neural precursors, recent work has revealed its expression in microglia and neurons.[58] Long-term potentiation can induce proliferation of progenitor cells in the dentate gyrus.[59,60] In light of the proposed function here for MIF in adult neurogenesis, these two events may be linked. However, again a note of caution should be added to remind that other factors (developmental, others) might also account for the learning deficits observed in our $MIF^{-/-}$ mice.

Although hotly debated, a function for neurogenesis in hippocampus-dependent learning and memory has been proposed. Several lines of evidence support this hypothesis: (i) strong correlation between the rate of neurogenesis and spatial learning in the water maze among mice inbred strains[61] and in old rats from an outbreed strain.[62] (ii) manipulations in which inhibiting hippocampal neurogenesis resulted in hippocampus-dependent learning deficits;[32,45,63] and (iii) observations that learning itself can enhance the survival rate of new hippocampal neurons,[64–66] and induce apoptosis of more immature cells and proliferation of neural precursors.[67] However, other studies found the opposite (that is negative) correlation between the rate of hippocampal cell proliferation and spatial learning[68] or failed to find deficits in hippocampus-dependent learning after ablation of hippocampal neurogenesis.[46,69,70] Recent evidence using both local X-irradiation and genetic approaches has revealed a key function for hippocampal neurogenesis in spatial pattern separation, a key cognitive function of the dentate gyrus.[71] Our study adds to the positive evidence accumulated in this debate establishing a link between hippocampal neurogenesis and the formation of long-term spatial memories and goes beyond by highlighting MIF as a key factor in these mnemonic processes.

A recent study[72] has identified MIF as a possible susceptibility gene for autism spectrum disorder. The authors found genetic associations between known functional polymorphisms in the promoter for MIF and autism spectrum disorder-related behaviors. Moreover, probands with autism spectrum disorder were reported to exhibit higher plasma MIF levels than their unaffected siblings, and these levels correlated with the severity of multiple autism spectrum disorder symptoms. These findings raise the intriguing possibility that some of our current findings might have implications for the understanding of the neurobiological mechanisms implicated in autism.

In conclusion, we provide evidence supporting a pivotal function for MIF in both basal and antidepressant-stimulated adult hippocampal cell proliferation (and hence, eventually neurogenesis). We also showed that, in addition to affecting cell proliferation in the dentate gyrus, loss of MIF results in a behavioral phenotype (increased anxiety- and depression-like behaviors, and impaired hippocampus-dependent spatial learning) that to a large extent corresponds with alterations that would be predicted from reduced hippocampal neurogenesis. Altogether, these findings underscore MIF as a potentially relevant molecular target for the development of treatments linked to deficits in neurogenesis, as well as to problems related to anxiety, depression, and cognition.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)