Membrane Mineralocorticoid but not Glucocorticoid Receptors of the Dorsal Hippocampus Mediate the Rapid Effects of Corticosterone on Memory Retrieval

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This study was aimed at determining the type of the glucocorticoid membrane receptors (mineralocorticoid receptors (MRs) or glucocorticoid receptors (GRs)) in the dorsal hippocampus (dHPC) involved in the rapid effects of corticosterone or stress on memory retrieval. For that purpose, we synthesized corticosterone–3-O-carboxymethyloxime–bovine serum albumin conjugate (Cort–3CMO–BSA) conjugate (a high MW complex that cannot cross the cell membrane) totally devoid of free corticosterone, stable in physiological conditions. *In a first experiment*, we evidenced that an acute stress (electric footshocks) induced both a dHPC corticosterone rise measured by microdialysis and memory retrieval impairment on delayed alternation task. Both the endocrinal and cognitive effects of stress were blocked by metyrapone (a corticosterone synthesis inhibitor). *In a second experiment*, we showed that bilateral injections of either corticosterone or Cort–3CMO–BSA in dHPC 15 min before memory testing produced impairments similar to those resulting from acute stress. Furthermore, we showed that anisomycin (a protein synthesis inhibitor) failed to block the deleterious effect of Cort–3CMO–BSA on memory. *In a third experiment*, we evidenced that intra-hippocampal injection of RU-28318 (MR antagonist) but not of RU-38486 (GR antagonist) totally blocked the Cort–3CMO–BSA-induced memory retrieval deficit. *In a fourth experiment*, we demonstrated that RU-28318 administered 15 min before stress blocked the stress-induced memory impairments when behavioral testing occurred 15 min but not 60 min after stress. Overall, this study provides strong *in vivo* evidence that the dHPC membrane GRs, mediating the rapid and non-genomic effects of acute stress on memory retrieval, are of MR but not GR type.

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INTRODUCTION

The effects of glucocorticoids (GCs) on memory processes have been attributed to classic steroid mechanisms involving delayed transcriptional regulation (McEwen and Sapolsky, 1995; de Kloet *et al*, 1999; Sapolsky *et al*, 2000; Lupien and Lepage, 2001; McGaugh and Roozendaal, 2002; Donley *et al*, 2005; Joels *et al*, 2006). From a functional point of view, the molecular mechanisms mediating the effects of GCs on memory formation are largely unknown. However, AMPA receptor expression and trafficking have been implicated (Groc *et al*, 2008; Conboy and Sandi, 2010). In addition, recent findings in neuroendocrinology suggest

that steroids can rapidly modulate neuronal activity (within a few minutes) through a non-genomic pathway via an activation of membrane receptors (Borski, 2000; Falkenstein *et al*, 2000; Makara and Haller, 2001; Dallman, 2005; Tasker *et al*, 2006). It has also been shown that GCs increase the release of excitatory amino acids (Venero and Borrell, 1999), reduce unit discharge of hippocampal neuron in rat hippocampus via a rapid non-genomic action (Pfaff *et al*, 1971), and modulate neuronal activity and synaptic excitatory transmission (Chaouloff and Groc, 2011).

However, rapid effects of corticosterone on behavior and cognitive processes are seldom documented (Sajadi *et al*, 2006; Orchinik *et al*, 1991; Sandi *et al*, 1996; Breuner *et al*, 1998). Previous studies from our team provided first clearcut evidence in behaving animals of the involvement of dorsal hippocampal membrane receptors in the mediation of the rapid deleterious effects of stress on memory retrieval. More specifically, we showed that bilateral infusion into the dorsal hippocampus (dHPC) of corticosterone–bovine serum albumin conjugate (Cort–BSA), a high

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MW complex that cannot cross the cell membrane, produced deleterious effects on memory retrieval similar to those resulting from an acute stress administration delivered 5 min before memory testing (Chauveau *et al*, 2010).

Our previous study did not determine, however, the type of membrane GC receptors (GRs) involved in the rapid effects of Cort-BSA nor that of acute stress on memory retrieval. To our knowledge, only *in vitro* or electrophysiological studies have as yet demonstrated that the rapid effects of corticosterone or stress may be mediated through mineralocorticoid receptors (MRs) activity. Thus, it has been shown that MRs but not GRs are required for non-genomic modulation of hippocampal glutamate transmission by corticosterone (Karst *et al*, 2005). In contrast, in behaving animals, a recent study evidenced that Cort-BSA administered in the medial prefrontal cortex 1 h before memory testing enhances memory consolidation, but impaired working memory, and that these Cort-BSA-induced impairments were blocked by co-administration of a GR but not an MR antagonist (Barseganyan *et al*, 2010). So far, the issue of the type of the membrane-bound steroid receptor involved in the rapid effects of GCs on cognitive processes remains seldom documented in behaving animals, and more particularly as regards the type of hippocampal membrane GRs involved in the rapid effects of corticosterone or stress on memory retrieval. Indeed, several studies have already evidenced the involvement of both types of hippocampus GRs in memory retrieval, but these experiments were not designed to evidence the involvement of MR or GR at the membrane level (Rooszendaal *et al*, 2004; Khaksari *et al*, 2007; Ferguson and Sapolsky, 2007).

Accordingly, as Cort-BSA directly acts on membrane receptors and induced memory retrieval impairments (Chauveau *et al*, 2010), we intended in the study hereby to determine the ability of selective antagonists of the MRs or GRs to block the rapid effects of either acute stress or intra-hippocampus Cort-BSA infusions on memory retrieval. Hence, we synthesized, characterized, and purified a new Cort-BSA complex, that is, Cort-3CMO-BSA (Cort-3-O-carboxymethyloxime-BSA conjugate) totally devoid of free corticosterone that could trigger non-membrane effects. The Cort-3CMO-BSA complex that we developed offers the advantage, as compared to commercial products, of having a higher corticosterone density on carrier BSA.

From a behavioral standpoint, to avoid the use of reinforcements that may interfere either with stress or with the pharmacological treatments, we evaluated memory retrieval processes using non-rewarded alternation behavior, in which the delayed alternation rate is an index of memory performance (Beracochea and Jaffard, 1985; Borde *et al*, 1996; Chauveau *et al*, 2005).

MATERIALS AND METHODS

Experimental Design

Experiment 1 tested the impact of an acute stress delivered 15 min before the test session of the delayed alternation task. In parallel, the time course of the stress-induced corticosterone rise in the dorsal intra-hippocampus was

measured in independent groups of mice. Moreover, to evidence the causal role of the stress-induced hippocampal corticosterone rise on memory retrieval dysfunction, we also evaluated the effects of an intraperitoneal pre-test metyrapone administration (a corticosterone synthesis inhibitor) on both the behavioral and endocrinal measures.

In Experiment 2, corticosterone or Cort-3CMO-BSA was bilaterally administered into the dHPC 15 min before the test session, to determine if these compounds produced memory retrieval deficits similar to those resulting from stress, and the involvement of membrane GRs' activation in the mediation of the rapid deleterious effects of corticosterone on memory retrieval. Moreover, to ensure that the rapid effects of Cort-3CMO-BSA are not mediated by genomic pathway, we administered in independent groups of mice anisomycin (an inhibitor of protein synthesis) 45 min before the infusion of Cort-3CMO-BSA.

In Experiment 3, the GR antagonist RU-38486 (mifepristone) or the MR antagonist RU-28318 was injected into the dHPC 15 min before the injection of Cort-3CMO-BSA. The blockade of either MRs or GRs by selective antagonists should block the effects of intra-hippocampus Cort-3CMO-BSA injection on memory performance, and accordingly will allow determination of the type (MRs or GRs) of the membrane GRs mediating the effects of Cort-3CMO-BSA on memory retrieval.

In Experiment 4, given the results obtained in Experiment 3, the MR antagonist RU-28318 was bilaterally injected into the dHPC 15 min before acute stress delivery; the test session occurred, however, either 15 or 60 min after stress delivery. This experiment allowed us to determine if RU-28318 will block the effects of stress on memory retrieval similarly as for Cort-3CMO-BSA (Experiment 3) and the time course for such an effect.

Animals

The subjects were 6-month-old naive male mice of the C57BL/6 inbred strain obtained from Charles Rivers (L'Arbresle, France). At the time of the experiments, mice weighed between 28 and 32 g. They were housed individually with free access to water on a 12 h light-dark cycle in a temperature-controlled and ventilated room. Tests were conducted during the light phase of the cycle between 0800 and 1200 hours. The number of animals used in each group is given in the Result section.

Surgery and Histology

Mice were anesthetized with a ketamine (1 mg/kg body weight)-xylazine (10 mg/kg body weight) solution and placed on a stereotaxic frame. All stereotaxic coordinates are referenced in μm from the bregma (Paxinos and Franklin, 2001). For pharmacological administration, two stainless-steel guide cannulae (26 G, 8 mm length) were implanted bilaterally 1 mm above the surface of the dHPC ($\text{AP} = -2000$; $L = \pm 1400$; $V = -1000$). Guide cannulae were fixed in place with dental cement and two microscrews attached to the skull. All operated mice were allowed to recover in their home cages in the animal room for at least 7 days before the behavioral experiments began. After completing the behavioral test, all mice were killed to collect

brains. All the brains were coronally sectioned (50 μm thickness). A cresyl violet stain was used to verify the exact probe location in dorsal HPC.

Pharmacological Administration

In Experiment 1, all subjects were habituated for prick effect in the animal room without perforating the skin during 5 consecutive days. On the day of experiment and 30 min before the acute stress delivery, mice received an intraperitoneal injection of metyrapone (Promochem; 35 mg/kg body weight dissolved in a solution of 5% ethanol in saline) in a room different from that used for behavioral testing.

In Experiment 2, a 32-G stainless-steel cannula (9 mm length) attached to a microsyringe with polyethylene catheter tubing was inserted into the guide cannula. The syringes were placed in a constant flow rate pump (0.4 $\mu\text{l}/\text{min}$). Corticosterone (Sigma, France) or Cort-3CMO-BSA was diluted in mock CSF (vehicle) at the concentration of 1 mg/ml, and bilaterally injected (1 μl per side) into the dHPC. The cannulae were left in place for 3 min before removal to allow the diffusion of the drugs from the cannulae tips. Corticosterone or Cort-3CMO-BSA was injected 15 min before behavioral testing. The control group was injected with BSA to verify the absence of any side effects of BSA alone on performance.

In a further step, anisomycin (Sigma) was dissolved in 1 M HCl, diluted in saline, and adjusted to pH 7.5 with NaOH. The concentration of the solution was of 160 $\mu\text{g}/\mu\text{l}$. A measure of 0.8 μl of solution (corresponding to 128 μg) was injected in each hippocampus side. Anisomycin was injected *in situ* in the dHPC 45 min before the Cort-3CMO-BSA. The control group received the anisomycin solution followed 45 min later by BSA.

In Experiment 3, corticosterone receptors antagonists (RU-38486 and RU-28318; Tocris, St Louis, MO) at the concentration of 20 mg/ml or vehicle (mock CSF with the addition of a small amount of absolute ethanol) were injected 15 min before Cort-3CMO-BSA into the dHPC. The antagonists were injected in a volume of 0.40 μl per hippocampus side (0.05 $\mu\text{l}/\text{min}$ during 8 min). Thus, the amounts of antagonists injected in hippocampus were eightfold higher (8 μg) as compared to Cort-3CMO-BSA (1 μg). A small amount of absolute ethanol was first used to dissolve the antagonists and the volumes were then brought up with mock CSF. Behavioral testing occurred 15 min after the Cort-3CMO-BSA injection.

In Experiment 4, the MR antagonist and vehicle solution were prepared similarly to Experiment 3 and injected bilaterally into the dHPC 15 min before stress. Behavioral testing occurred either 15 min ('MR antagonist + stress 15 min' group) or 60 min ('MR antagonist + stress 60 min' group) after the acute stress delivery. They were compared to a non-stressed group receiving the vehicle solution 30 min before the onset of the test session or to a stressed group also receiving the vehicle solution 15 min before stress delivery.

Microdialysis Experiments

At least 7 days before microdialysis experiments, mice were anesthetized with a ketamine (100 mg/kg body

weight)-xylazine (10 mg/kg body weight) mixture and placed on a stereotaxic frame. A single guide microdialysis cannula (CMA/7 Microdialysis probe, CMA Microdialysis, Solna, Sweden) was implanted in the bottom of the parietal cortex at the following coordinates from the bregma (Paxinos and Franklin, 2001): antero-posterior = $-2000 \mu\text{m}$; lateral = $+1400 \mu\text{m}$, and vertical = $-800 \mu\text{m}$. The guide cannula was fixed with dental cement and three micro-screws attached to the skull. All operated mice were allowed to recover in their home cages in the animal room. On the day of the experiment, the microdialysis probe was introduced through the guide cannula and lowered 1 mm below so that the microdialysis membrane was located into the dorsal HPC. At the end of the microdialysis experiment, mice were anesthetized and then transcardially perfused in the left ventricle with saline solution (NaCl 0.9%), followed by formaldehyde (4%). Brains were then post-fixed in a 4% formaldehyde solution for 10 days, and then in a saccharose-formaldehyde solution (30–4% (v/v)) for 2 days. All the brains were coronally sectioned (50 μm thickness). A cresyl violet stain was used to locate the microdialysis probe with utmost accuracy.

In Experiment 1, microdialysis was performed in freely moving animals to determine corticosterone levels in the dHPC after acute stress or metyrapone administration. Probes (CMA/7, membrane length 1 mm; CMA Microdialysis) were perfused continuously with sterile filtered Dulbecco's solution at rate of 0.1 $\mu\text{l}/\text{min}$. At 12 h after probe implantation and extracellular concentration equilibration (2 h at 1 $\mu\text{l}/\text{min}$ flow rate), baseline dialysates (15 min samples) were collected with a flow rate of 1 $\mu\text{l}/\text{min}$ during 2 h. Intraperitoneal metyrapone injections were carried out 30 min before the acute stress delivery. The dialysates were collected during 3 additional hours after stress (flow rate: 1 $\mu\text{l}/\text{min}$; sampling delay: 15 min). The foot-shock delivery system was located in the dialysis cage. Samples were stored at -80°C before analysis. Baseline dialysates were collected for 1 h before intraperitoneal injection of metyrapone or vehicle, and 30 min before acute stress delivery. Free corticosterone levels measured in the dialysates were expressed as the percentage of the averaged baseline values sampled before the injection.

Behavioral Task

Delayed alternation procedure. All tests were performed in a T-maze constructed of gray Plexiglas. Stem and arms were 35 cm long, 10 cm wide, and 25 cm high. Goal arms and the start box were separated from the central alley by horizontal sliding doors. Illumination inside the apparatus (about 50 lx) was provided by a lamp positioned 2 m above the stem. Testing was conducted between 0800 and 1200 hours to avoid circadian variations of corticosterone.

Mice were submitted to the delayed alternation task using a forced-trial procedure. In the acquisition phase, the subjects were forced to enter twice the same goal arm of the maze, as access to the other arm being blocked by the sliding door. The two forced trials were separated by a 30 s interval. The acquisition phase was followed by a test phase implemented 24 h later. During the delay separating the acquisition and test trials, mice were returned to their home cage in the animal room. During the test trial, animals

remained 30 s in the start box. The door was then opened, and animals were free to enter each goal arm within the maze. The correct choice (alternation behavior) is to enter the arm opposite to that entered the day before. In all experiments, animals remained for 30 s in the chosen arm, and were again returned during 30 s to the start box, for a second free choice trial. This additional trial aimed at assessing whether the treatments impaired or not the ability of mice to alternate as well as short-term memory.

In all pharmacological and behavioral experiments, mice were subjected to two forced-trial sessions, the blocked arm of the second session being opposite to the arm blocked at the first session. Each session was separated by at least 1 week (Figure 1).

Acute Stress

The acute stress was administered 15 min before the test trial (first and fourth experiments). Stress was delivered in a room different from the one used for behavioral testing. Mice remained during 1 min in a stress delivery cage. Stressed mice received three successive unavoidable electric footshocks (0.9 mA; 10 ms) in keeping with our previous studies (Celerier *et al*, 2004; Chauveau *et al*, 2010; Tronche *et al*, 2010). Non-stressed mice were placed in the cage in the same conditions, except that they received no footshock. Animals were then placed in their home cage for 15 min and subsequently assigned to the T-maze behavioral procedures.

Intra-Hippocampal Corticosterone Assay

An enzyme immunoassay commercial kit (Correlate-EIA, Assay Designs, Ann Arbor, MI) was used to measure HPC corticosterone concentrations in the microdialysates. The sensitivity of the assay was 18.6 pg/ml. Therefore, baseline sample concentration was more than 10-fold above the sensitivity threshold.

Synthesis of Cort-3CMO-BSA

Chemicals and experimental equipment. Corticosterone (+97.0%) and carboxymethoxylamine hemihydrochloride (+98.0%) were purchased from TCI Europe (Zwijndrecht, Belgium). Anhydrous pyridine (99.8%), dichloromethane,

and methanol (Chromasolv grade) were supplied by Sigma-Aldrich Chemicals (Saint Quentin Fallavier, France). ^1H and ^{13}C NMR spectra were recorded with a Bruker AC-300 FT (^1H : 300 MHz; ^{13}C : 75 MHz). Chemical shifts (δ) and coupling constants (J) are expressed in p.p.m. and Hz, respectively. High-resolution mass spectrum was acquired by the CESAMO (Bordeaux, France) on a QStar Elite mass spectrometer (Applied Biosystems). Thin-layer chromatography (TLC) was performed on SDS TLC plates: thickness 0.25 mm, particle size 15 μm , and pore size 60 Å. Merck silica gel 60 (70–230 mesh and 0.063–0.200 mm) was used for flash chromatography. Spots were revealed with UV as well as KMnO_4 (0.05% in water).

Synthesis of Cort-3CMO. A solution of carboxymethoxylamine hemihydrochloride (82 mg, 0.75 mmol) and anhydrous pyridine (79 mg, 80 μl , 1 mmol) in methanol (5 ml) was added to a solution of corticosterone (173 mg, 0.5 mmol) in methanol (4 ml) at room temperature. The mixture was stirred for 5 h and concentrated under reduced pressure. The residue was purified by flash silica gel chromatography, using dichloromethane/methanol (94:6 (v/v)) as an eluent, to afford the target compound as a white solid (157 mg, 75%, +95 % purity by NMR, R_f = 0.15).

^1H NMR (MeOH- D_4) δ 0.91 (s, 3H, CH_3 -18), 0.91–1.22 (m, 3H, CH_2 -7b, CH-9, CH-14), 1.37 (s, 3H, CH_3 -19), 1.51–1.85 (m, 5H, CH_2 -1b, CH_2 -12b, CH_2 -15, CH_2 -16b), 1.91–2.55 (m, 9H, CH_2 -1a, CH_2 -2b, CH_2 -6, CH_2 -7a, CH-8, CH_2 -12a, CH_2 -16a, CH-17), 3.02 (apparent dt, 1H, J = 17 and 4 Hz, CH_2 -2a), 4.15 (d, 1H, J = 19 Hz, CH_2 -21a), 4.23 (d, 1H, J = 19 Hz, CH_2 -21b), 4.34 (m, 1H, CH-11), 4.56 (s, 2H, O- CH_2 -COOH), and 5.66 (s, 1H, CH-4).

^{13}C NMR (MeOH- D_4) δ 15.0 (CH_3 -18), 18.9 (CH_2 -2), 20.4 (CH_3 -19), 22.0 (CH_2 -16), 24.1 (CH_2 -15), 31.4 (CH_2 -6), 31.9 (CH-8), 33.0 (CH_2 -7), 33.7 (CH_2 -1), 38.5 (C-10), 43.5 (C-13), 47.7 (CH_2 -12), 56.5 (CH-9), 57.7 (CH-14), 59.0 (CH-17), 67.0 (CH-11), 68.6 (CH_2 -21), 69.7 (O- CH_2 -COOH), 114.5 (CH-4), 157.9 (C-3), 161.7 (C-5), 172.8 (COOH), and 210.4 (C-20).

HR-MS (ESI $^+$): m/z 442.2200 for $[\text{M} + \text{Na}]^+$ (calcd for $[\text{C}_{23}\text{H}_{33}\text{NO}_6\text{Na}]^+$, 442.2206).

Synthesis of Cort-3CMO-BSA conjugate. Cort-3CMO was coupled to BSA by the activated ester method. Briefly, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (21 mg, 110 μmol , 1.1 equiv.) and *N*-hydroxysuccinimide (12.7 mg, 110 μmol , 1.1 equiv.) were added to a solution of Cort-3CMO (42 mg, 100 μmol , 50 equiv.) in anhydrous DMF (2 ml) at 0 °C. The mixture was stirred for 2 h and was then added to an ice-cooled solution of BSA (134 mg, 2 μmol) in 0.2 M borate-boric buffer, pH 8.7 (5 ml). The resulting mixture was allowed to warm at room temperature for 6 h, and then dialyzed against phosphate buffer saline (0.01 M, pH 7.4, 0.9% NaCl) and distilled water. The Cort-3CMO-BSA conjugate was lyophilized and stored at –20 °C. The corticosterone density on BSA was analyzed by matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS). The molecular ratio of covalently linked corticosterone was about 30 per molecule of BSA.

Stability of Cort-3CMO-BSA conjugate. To verify the stability of Cort-3CMO-BSA once administered *in vivo*, we diluted Cort-3CMO-BSA in mouse CSF at the concentration

Figure 1 Memory testing procedure: animals are first submitted to an acquisition phase in which they are forced to enter twice the same arm of the maze (two forced trials); then, after a 24-h delay interval, they are submitted to the test phase in which they can freely enter either arm of the maze. An alternation is scored when the mouse enters the arm opposite to the one visited in the acquisition phase. Treatments (stress, and drug infusions in the dHPC) are delivered before the test phase, according to the experimental schedule described in the Materials and methods section.

of 1 mg/ml. Mouse CSF was sampled on 12 mice according to the technique described by Liu and Duff (2008). We analyzed the solution after 15, 60, and 120 min at a thermostated temperature of 37 °C. The solution was analyzed using ELISA technique (Arbor assays), allowing determination of only free corticosterone but not of conjugated forms. In each solution, free corticosterone concentrations were inferior to the limit of detection of the technique (16.9 pg/ml). Thus, we can assume that after 2 h in CSF at 37 °C, the concentration of free corticosterone was inferior to 0.0169 p.p.m. In consequence, the Cort-3CMO-BSA complex appears to be stable once administered *in vivo*.

Statistical Analysis

Statistical analyses were performed using the Statview 5.0 software. The data were analyzed using one- or two-way

factorial analyses of variance (ANOVAs), followed, when adequate, by *post hoc* comparisons (Scheffe's test). Data were expressed as means $\pm$ SEM. Comparisons of retrieval performances with chance level were calculated with one-sample Student's *t*-test (with hypothesized mean = chance level = 50%). Microdialysis data were analyzed using one- or two-way repeated-measure ANOVA as appropriate, followed, when adequate, by *post hoc* testing (Bonferroni's test).

RESULTS

First Experiment: Effects of Acute Stress and Metyrapone on Delayed Alternation Rates and Hippocampal Corticosterone Concentrations

Effects of acute stress delivered 15 min before test session. Data are represented in Figure 2a. The data revealed a

Figure 2 (a) Effects of stress on delayed alternation performance. Stress, delivered 15 min before test session, significantly reduced alternation rates: ****p* < 0.001; chance level: 50%. (b) Effects of stress and metyrapone (inhibitor of corticosterone synthesis) on delayed alternation rates. Metyrapone (35 mg/kg) injected intraperitoneally 30 min before stress delivery blocked the deleterious effects of stress on alternation rates; **p* < 0.05; ***p* < 0.01; chance level: 50%. (c) Time-course evolution of corticosterone in the dorsal hippocampus measured by microdialysis in stressed animals pretreated or not with metyrapone (35 mg/kg). The peak of corticosterone is observed at about 60 min and return to baseline at about 90 min after stress delivery. Results are expressed in relative concentration; comparison to baseline: **p* < 0.05; ***p* < 0.01; ****p* < 0.001. Metyrapone-treated mice exhibit no difference after stress delivery as compared to baseline for each time.

significant between-group difference ($F(1, 58) = 12.7$; $p = 0.0007$). More precisely, non-stressed mice ($N = 30$) exhibited alternation rates ($86.6 \pm 6.3\%$) significantly above those observed in stressed ones ($N = 30$; $46.6 \pm 9.2\%$). In contrast, both groups exhibited similar short-term alternation rates evaluated 30 s after the delayed test session ($90.0 \pm 5.5\%$ vs $80.0 \pm 7.4\%$ for non-stressed and stressed groups respectively; $F(1, 58) = 1.16$; $p = 0.28$).

Effects of metyrapone administered 30 min before acute stress on delayed alternation rate. Data are presented in Figure 2b. The data revealed a significant between-group difference ($F(3, 67) = 3.5$; $p = 0.018$). More specifically, whereas vehicle ($N = 18$; $77.7 \pm 10.0\%$), metyrapone ($N = 17$; $76.4 \pm 10.6\%$), and metyrapone + stress ($N = 18$; $88.8 \pm 7.6\%$) groups exhibited similar performances (NS in all comparisons), the stressed group ($N = 18$) displayed significantly lower performances ($44.4 \pm 12\%$) as compared to vehicle and metyrapone groups ($p < 0.05$ in both comparisons) and as compared to the metyrapone + stress group ($p < 0.01$).

In contrast, all groups exhibited similar short-term alternation rates evaluated 30 s after the 24-h delayed test session ($83.3 \pm 9.0\%$, $76.4 \pm 10.0\%$, $77.7 \pm 10.0\%$, and $88.8 \pm 7.6\%$ for vehicle, metyrapone, vehicle + stress, and metyrapone + stress groups, respectively; $F(1, 58) = 1.16$; $p = 0.28$).

Stress-induced intra-hippocampus corticosterone rise. Figure 2c represents corticosterone levels in the dHPC. Results are expressed in percentage of variation of baseline. Two-way repeated-measure ANOVAs performed on corticosterone kinetic evidenced a significant interaction between Treatments $\times$ Time ($F(15, 180) = 3.275$; $p \leq 0.001$). Bonferroni's *t*-test did not reveal any differences between the groups (vehicle: $N = 8$; metyrapone: $N = 8$) in the pre-stress period for each factor.

Vehicle As compared to the last pre-stress sample ($108.66 \pm 10.98\%$; 'time = 0'), stress induced a progressive and significant increase in corticosterone levels from 15 min ($131.32 \pm 8.23\%$; $t = 2.99$; $p \leq 0.05$) to 90 min ($179.75 \pm 21.32\%$; $t = 2.992$; $p \leq 0.05$). Furthermore, the highest difference was observed 60 min after stress administration (200.24 ± 31.63 ; $t = 4.062$; $p \leq 0.001$).

Metyrapone As compared to the last pre-stress sample ($102.86 \pm 21.04\%$; 'time = 0'), the stress-induced increase in corticosterone levels observed in vehicle-treated mice was not observed in metyrapone-treated ones. As a consequence, the progressive and significant increase in corticosterone levels in vehicle-treated mice was observed 15–90 min after stress delivery, as compared to metyrapone-treated mice (15 min post-stress delay: $131.32 \pm 8.23\%$ and $68 \pm 3.5\%$, respectively; $t = 3.96$; $p = 0.03$; 90 min post-stress delay: $179.75 \pm 21.32\%$ vs $122 \pm 20.5\%$; $t = 2.96$; $p < 0.05$). Finally, no significant difference was observed between vehicle- and metyrapone-treated mice from the 105 min post-stress delay point ($118.50 \pm 25.79\%$ and $105.75 \pm 12.19\%$ respectively; $t = 0.476$; NS) up to the 150 min point ($93.89 \pm 14.25\%$ and $124.49 \pm 14.99\%$, respectively; $t = 1.143$; NS).

Second Experiment: Effects of Intra-Hippocampus Corticosterone or Cort-3CMO-BSA Injections on Delayed Alternation Rates

Effects of intra-hippocampus corticosterone injection. Data are presented in Figure 3a. The data revealed a significant between-group difference ($F(2, 57) = 4.3$; $p = 0.018$). More precisely, whereas non-operated ($N = 20$; $75.0 \pm 9.9\%$) and sham-operated + vehicle ($N = 20$; $85.0 \pm 8.2\%$) groups exhibited similar performances (NS), the corticosterone-injected group exhibited significantly lower performances ($N = 20$; $44.4 \pm 12\%$) as compared to non-operated ($p < 0.05$) and sham-operated + vehicle ($p < 0.01$) groups.

In contrast, all groups exhibited similar short-term alternation rates evaluated 30 s after the 24-h delayed test session ($85.0 \pm 8.1\%$, $75.0 \pm 9.9\%$, and $80.0 \pm 9.1\%$ for, respectively, non-operated, sham-operated + vehicle, and corticosterone groups; $F(2, 57) < 1.0$).

Effects of intra-hippocampus Cort-3CMO-BSA injection. Data are displayed in Figure 3b. The data revealed a significant between-group difference ($F(2, 33) = 5.31$; $p = 0.008$). More pointedly, whereas non-operated ($72.9 \pm 4.7\%$) and BSA-injected ($71.1 \pm 5.1\%$) groups exhibited similar performances (NS), the Cort-3CMO-BSA-injected group exhibited significantly lower performances ($40.1 \pm 5.4\%$) as compared to the two other groups ($p < 0.01$ in both comparisons).

In contrast, all groups exhibited similar short-term alternation rates evaluated 30 s after the delayed test session ($80.8 \pm 5.1\%$, $70.0 \pm 8.2\%$, and $75 \pm 4.1\%$ for, respectively, non-operated, sham-operated + vehicle, and corticosterone groups; $F(2, 57) < 1.0$).

Effects of anisomycin administered before Cort-3CMO-BSA. Data are displayed in Figure 3c. The data revealed a significant between-group difference ($F(1, 22) = 5.90$; $p = 0.023$). More pointedly, whereas anisomycin + BSA-injected mice ($N = 12$) exhibited a high level of alternation performance ($79.2 \pm 11.4\%$), anisomycin + Cort-3CMO-BSA-treated mice ($N = 12$) showed a significant decrease in alternation performance ($41.6 \pm 10.3\%$; $p < 0.05$). In contrast, all groups exhibited similar short-term alternation rates evaluated 30 s after the delayed test session ($75.0 \pm 9.7\%$ and $87.5 \pm 6.5\%$ for, respectively, anisomycin + BSA and anisomycin + Cort-3CMO-BSA, respectively; $F(1, 22) = 1.13$; $p = 0.29$).

Third Experiment: Effects of Intra-Hippocampus MR or GR Antagonists Administered Alone or with Cort-3CMO-BSA on Delayed Alternation Rates

Effects of intra-hippocampus MR or GR antagonists on delayed alternation rates. Data are represented in Figure 4a. The data revealed a nonsignificant between-group difference ($F(3, 44) < 1.0$). Performance of non-operated group ($N = 14$; $85.7 \pm 9.7\%$) were similar to that of vehicle-injected mice ($N = 14$; $75 \pm 13.0\%$) and to groups receiving either MR ($N = 10$; $80 \pm 8.1\%$) or GR antagonists ($N = 10$; $75 \pm 8.3\%$).

All groups exhibited similar short-term alternation rates evaluated 30 s after the 24-h delayed test session

Figure 3 (a) Effect of corticosterone on delayed alternation performance. Corticosterone (1 mg/ml) was bilaterally injected in the dorsal hippocampus 15 min before the test session. Control groups were either non-operated or received the vehicle solution. Corticosterone reduced alternation rates significantly as compared to control groups; * $p < 0.05$; ** $p < 0.01$. (b) Effect of Cort-3CMO-BSA (corticosterone-3-O-carboxymethyloxime-BSA conjugate) on delayed alternation performance. Cort-3CMO-BSA (1 mg/ml) was bilaterally injected in the dorsal hippocampus 15 min before the test session. Control groups were either non-operated animals or animals receiving BSA only to verify the absence of any side effect of BSA alone on performance. Cort-3CMO-BSA reduced alternation rates significantly as compared to control groups; ** $p < 0.01$; chance level: 50%. (c) Effect of anisomycin administered in dorsal hippocampus 45 min before Cort-3CMO-BSA on delayed alternation performance. Anisomycin (128 μ g per side) failed to block the deleterious effects of Cort-3CMO-BSA (1 mg/ml) bilaterally injected in the dorsal hippocampus 15 min before the test session. * $p < 0.05$.

(85.7 $\pm$ 9.7%, 78.6 $\pm$ 11.4%, 80.0 $\pm$ 13.3%, and 88.7 $\pm$ 6.3% for non-operated, vehicle, MR, and GR antagonists groups, respectively; $F(3, 44) < 1.0$).

Effects of intra-hippocampus MR or GR antagonists administered before Cort-3CMO-BSA on delayed alternation rates. Data are represented in Figure 4b. The data revealed a significant between-group difference ($F(3, 58) = 5.48$; $p = 0.002$). Significant between-group differences were observed between performance of vehicle + Cort-3CMO-BSA-injected mice ($N = 12$; 33.3 $\pm$ 14.2%) as compared to vehicle + vehicle group ($N = 12$; 70.8 $\pm$ 12.9%; $p < 0.02$) and to MR antagonist + Cort-3CMO-BSA group ($N = 16$; 84.4 $\pm$ 6.0%; $p < 0.002$). In contrast, no significant difference was observed between the GR antagonist + Cort-3CMO-BSA group ($N = 22$; 43.2 $\pm$ 8.9%) and vehicle + Cort-3CMO-BSA-injected mice (33.3 $\pm$ 14.2%; NS).

All groups exhibited similar short-term alternation rates evaluated 30 s after the 24-h delayed test session (83.3 $\pm$ 11.2%, 75.0 $\pm$ 13.0%, 81.8 $\pm$ 8.4%, and 87.5 $\pm$ 8.5% for vehicle + vehicle, vehicle + Cort-3CMO-BSA, GR antagonist + Cort-3CMO-BSA, and MR antagonist + Cort-3CMO-BSA groups, respectively; $F(3, 58) < 1.0$).

Fourth Experiment: Effects of Intra-Hippocampus MR Antagonist Administered before Acute Stress Delivery on Delayed Alternation Evaluated Either 15 or 60 min After Stress Delivery

Data are represented in Figure 5. The data revealed a significant between-group difference ($F(3, 90) = 5.78$; $p = 0.001$). Significant between-group differences were observed between the non-stressed vehicle group ($N = 21$; 80.9 $\pm$ 8.7%) and the vehicle + stress group ($N = 20$; 30.0 $\pm$ 10.5%; $p < 0.001$). Moreover, the performance in 'MR antagonist + stress 15 min' group ($N = 25$; 68.0 $\pm$ 9.5%) was significantly different as compared to the vehicle stressed group ($p < 0.01$), but did not differ from that of the vehicle-injected mice (NS). However, performance of the MR antagonist + stress 60 min group ($N = 28$; 39.2 $\pm$ 9.3%) did not differ from that of stressed mice (NS), but were significantly lower as compared to both the MR antagonist + stress 15 min group ($p = 0.02$) and the non-stressed vehicle one ($p = 0.002$).

All groups exhibited similar short-term alternation rates evaluated 30 s after the delayed test session ($F(3, 90) < 1.0$; vehicle: 66.6 $\pm$ 10.5%; vehicle + stress: 70.0 $\pm$ 10.5%; 'MR antagonist + stress 15 min': 68.0 $\pm$ 9.5%; 'MR antagonist + stress 60 min': 67.8 $\pm$ 8.9%).

Figure 4 (a) Effects of glucocorticoid receptor (GR) antagonist (RU-38486 or mifepristone) and mineralocorticoid receptor (MR) antagonist (RU-28318) on delayed alternation rates. Both antagonists were injected bilaterally in the dorsal hippocampus at the dose of 20 μ g/ml each 15 min before behavioral testing. Control groups were either non-operated animals or animals receiving vehicle only. The administration of both antagonists did not significantly modify alternation rates as compared to control groups. Chance level: 50%. (b) Effects of GR antagonist (RU-38486 or mifepristone) and MR antagonist (RU-28318) and Cort-3CMO-BSA (corticosterone-3-O-carboxymethyloxime-BSA conjugate) on delayed alternation rates. Both antagonists were injected bilaterally in the dorsal hippocampus at the dose of 50 ng/ml each 15 min before the Cort-3CMO-BSA injection (1 mg/ml). Behavioral testing occurred 15 min after Cort-3CMO-BSA injection. Control groups were either animals receiving two vehicle injections separated by 15 min or animals receiving vehicle, followed 15 min later by Cort-3CMO-BSA injection. Results showed that the MR antagonist blocked the memory impairment observed in animals receiving Cort-3CMO-BSA only; in contrast, the GR antagonist did not block the deleterious effects of Cort-3CMO-BSA injection on alternation rates. Comparisons to Cort-3CMO-BSA-treated animals: * $p < 0.05$; ** $p < 0.01$; chance level: 50%.

Histological Analysis

Figure 6 shows the anterograde extent and the localization of the cannulae tips in the dHPC of mice. Black zones: main implantation sites; and black hashed areas: antero-retrograde extent of the cannulae tips implantation.

Figure 5 Effects of mineralocorticoid receptor (MR) antagonist (RU-28318) administered into the dorsal hippocampus 15 min before acute stress on delayed alternation rates. Behavioral test session occurred either 15 or 60 min after stress. Control groups were animals receiving vehicle only, followed or not by acute stress. The administration of the MR antagonist at the dose of 20 μ g/ml blocked the stress-induced impairment on alternation rates 15 min but not 60 min after stress delivery. Comparisons to vehicle stressed animals: ** $p < 0.01$; *** $p < 0.001$; chance level: 50 %.

Figure 6 Representative localization of the sites of drug injections into the dorsal hippocampus. Reconstruction of the main sites of drug injections (black areas) and antero-posterior extent of the sites of injections (black hashed areas). Stereotaxic rostro-caudal coordinates are mentioned in mm from bregma.

DISCUSSION

The main findings of the study are as follows: *in a first experiment*, we evidenced that an acute stress (electric footshocks) induced both a dHPC corticosterone rise measured by microdialysis and a memory retrieval impairment in a non-rewarded spontaneous delayed alternation task. In addition, both the endocrinal and memory retrieval effects of stress were blocked by metyrapone (a corticosterone synthesis inhibitor; de Quervain *et al*, 1998; Roozendaal *et al*, 2001). *In a second experiment*, we showed that bilateral dHPC injections of either corticosterone or Cort-3CMO-BSA 15 min before memory retrieval produced impairments similar to those resulting from acute stress. Furthermore, we showed that anisomycin (a protein synthesis inhibitor) failed to block the deleterious effect of Cort-3CMO-BSA on memory. *In a third experiment*, we demonstrated that bilateral intra-hippocampal injection of RU-28318 (an MR antagonist) but not of RU-38486 (a GR antagonist) totally blocked the Cort-3CMO-BSA-induced memory retrieval deficit. *In a fourth experiment*, we found that the MR antagonist RU-28318 administered 15 min before acute stress blocked the stress-induced memory impairments when test occurred 15 min but not 60 min after stress delivery.

Overall, this study provides the first *in vivo* evidence that the dHPC membrane GRs involved in the rapid and non-genomic effects of acute stress on memory retrieval are of MR type.

The findings of the first two experiments are congruent with our previous study showing memory retrieval impairments associated to dHPC corticosterone rise 15 min but not 120 min after acute stress delivery (Chauveau *et al*, 2010; Tronche *et al*, 2010). Moreover, earlier studies stemming from our research team showed that stress effects on memory are only transient and were observed 15 min but not 120 min after stress delivery (Celerier *et al*, 2004). As Cort-3CMO-BSA—a high MW complex that cannot cross biological membranes and stable in physiological conditions—produced deleterious effects on memory retrieval similar to those resulting from acute stress or dHPC corticosterone injections, we can conclude that the rapid effects of stress are mediated by membrane GRs (see Chauveau *et al*, 2010). This finding is sustained by the fact that anisomycin (a protein synthesis inhibitor) was unable to block the deleterious effect of Cort-3CMO-BSA on memory retrieval (see Figure 3b and c). The lack of deleterious effect of anisomycin itself on memory retrieval is in agreement with studies showing that anisomycin impairs consolidation and reconsolidation processes that involve transcriptional factors (Stafford and Lattal, 2009). In contrast, in our experiment, anisomycin was administered just before the retrieval session, that is, 24 h after the acquisition of the to-be remembered information. Moreover, a genomic effect of stress or Cort-3CMO-BSA was unlikely because we evaluated memory retrieval 15 min after treatments, a time interval that excludes a genomic action that requires more than 30 min to develop (Sapolsky *et al*, 2000).

Surprisingly, corticosterone covalently bound to a protein has been seldom used to evaluate the membrane effects of GCs on cognitive processes (Chauveau *et al*,

2010; Barsegyan *et al*, 2010). In this study, we synthesized, purified, and characterized Cort-3CMO-BSA. Repetitive dialysis was applied to eliminate non-reacted corticosterone and MALDI-MS was used to determine the binding efficiency of corticosterone–protein conjugate (number of covalently bound corticosterone entities per protein molecule, that is, 30:1). This well-characterized home-made Cort-BSA complex, totally devoid of free corticosterone and found to be stable in physiological conditions, allowed us to investigate the effects of membrane GRs activation.

In a further step, we attempted to characterize the type (MR vs GR) of GC membrane receptors involved in the rapid modulation of memory retrieval induced by stress or Cort-3CMO-BSA. Thus, this study demonstrated that GRs of MR type in the dHPC are responsible for these effects. Indeed, RU-28318, an MR antagonist, blocked the deleterious effects of Cort-3CMO-BSA or stress-induced dHPC corticosterone rise on memory retrieval. In contrast, RU-38486 (mifepristone), a GR antagonist, was unable to counteract such effects. Thus, this study provides pharmacological direct evidence that the membrane dHPC GRs responsible for the rapid cognitive impairments of stress and corticosterone on memory retrieval are of MR but not GR type (for a review, see Joels *et al*, 2007).

It has already been suggested that central MRs are indispensable for corticosterone-induced impairment of memory retrieval in rats. Thus, Khaksari *et al* (2007) evidenced a non-genomic effect of corticosterone on memory retrieval using anisomycin. However, in contrast to this study, their data did not directly demonstrate the involvement of MR at membrane level. Moreover, in their study, the GC antagonists were injected intracerebroventricularly, precluding identification of the brain areas sustaining the pharmacological effects, as opposed to this study. Our current data however apparently contrast with recent findings from Barsegyan *et al* (2010), who showed that Cort-BSA injected into the medial prefrontal cortex facilitated memory consolidation of emotional experiences and concurrently impaired working memory in a rewarded sequential alternation task in rats. In their study, these effects were antagonized by GR but not MR antagonists. Their pharmacological design differs from ours however as regards the brain site of drug injections and the time of drug injections before memory testing, that is, 60 min. Indeed, this delay can neither exclude a non-genomic mechanism nor the possible involvement of GRs. The results provided by the fourth experiment of this study clearly indicate that the injection of MR antagonist is unable to block the stress-induced memory retrieval impairment when the test session occurred 60 min after stress delivery. Interestingly, we showed in the first experiment (see Figure 2c) that the maximum level of corticosterone rise in dHPC is observed about 60 min after stress delivery. Therefore, one can suggest that the lack of effect of the MR antagonist RU-28318 on memory retrieval performance in stressed animals at the 60 min interval could rely on either an action of corticosterone on cytosolic receptors or an involvement of GRs, which exhibit low affinity for corticosterone, thus requiring high corticosterone concentration to be activated (for a review see Joëls, 2008). The latter hypothesis fits well with the high corticosterone level observed in the dHPC 60 min after stress delivery. Indeed,

our data obtained with the MR antagonist at 60 min is in agreement with the findings of Barseganyan *et al* (2010), which evidenced that MR receptors are not involved on memory performance at this delay interval. In agreement with the study of Joels *et al* (2007), it can be suggested that low-affinity membrane version of the MR contributes to the initial phase of the stress reaction, followed by the GR involvement, which terminates the stress response.

Another finding within our study is that short-term memory (assessed by the second free choice trial of the test session procedure) was unaffected by stress, corticosterone, or GC antagonists administration. The effects of GCs on short-term memory are variable and depend on several factors such as the kind of task, the type of stressors, the brain structures sustaining task performances, and so on (Barseganyan *et al*, 2010; Yehuda *et al*, 2007; Brunner *et al*, 2006). In this study, the lack of deleterious effects of corticosterone on short-term memory may result from the different strength of memory for the to-be remembered information, which critically depends on the time interval elapsed between the acquisition and test trials. Moreover, the addition at the test session of the second short-term trial constitutes a shift in the behavioral procedure, which possibly engages the subject in a cognitive processing of information different from that sustaining memory retrieval performance upon the first trial. Thus, both short-term memory testing and the shift of behavioral procedure (forced choices *vs* discrete trial) may involve different brain circuitries, which could compensate the corticosterone-induced hippocampus dysfunction. Whatever the explanations, the high level of alternation rates observed in the short-term trial with the experimental mice show that both stress and the pharmacological treatments did not impair the ability to alternate *per se*. Thus, the deficit observed in the 24 h delayed test trial in experimental mice cannot be ascribed to an intrinsic impairment of alternation behavior.

In conclusion, our study provides strong *in vivo* evidence that the *membrane* GRs in the dHPC mediating the rapid and non-genomic effects of stress and GCs on memory retrieval are of MR but not GR type. Given the present results, we intend to investigate in a further study the functional role of the GRs of the ventral hippocampus in relation to the time-course evolution of the stress-induced corticosterone rise and associated memory retrieval impairments for short (15 min) and long (60 min) delays. Indeed, it has been shown that the dorsal and ventral hippocampus are functionally distinct structures (Fanselow and Dong, 2010), mainly as regards the density and balance of MRs and GRs and brain connectivity (Segal *et al*, 2010).

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DISCLOSURE

We wish to extend the following statements: except for income received from my primary employer, no financial support or any compensation has been received from neither any individual nor corporate entity over the past 3 years for either research or professional service. Further, no single personal financial holding may exist or be perceived as constituting a potential conflict of interest.

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