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# Glucocorticoid Receptor Blockade Normalizes Hippocampal Alterations and Cognitive Impairment in Streptozotocin-Induced Type 1 Diabetes Mice

**Yanina Revsin*[,1,2], Niels V Rekers[1], Mieke C Louwe[1], Flavia E Saravia[2], Alejandro F De Nicola[2], E Ron de Kloet[1] and Melly S Oitzl[1]**

*[1]Division of Medical Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden University Medical Center, Leiden, The Netherlands; [2]Laboratory of Neuroendocrine Biochemistry, IBYME, Buenos Aires, Argentina*

Type 1 diabetes is a common metabolic disorder accompanied by an increased secretion of glucocorticoids and cognitive deficits. Chronic excess of glucocorticoids *per se* can evoke similar neuropathological signals linked to its major target in the brain, the hippocampus. This deleterious action exerted by excess adrenal stress hormone is mediated by glucocorticoid receptors (GRs). The aim of the present study was to assess whether excessive stimulation of GR is causal to compromised neuronal viability and cognitive performance associated with the hippocampal function of the diabetic mice. For this purpose, mice had type 1 diabetes induced by streptozotocin (STZ) administration (170 mg/kg, i.p.). After 11 days, these STZ-diabetic mice showed increased glucocorticoid secretion and hippocampal alterations characterized by: (1) increased glial fibrillary acidic protein-positive astrocytes as a marker reacting to neurodegeneration, (2) increased c-Jun expression marking neuronal activation, (3) reduced Ki-67 immunostaining indicating decreased cell proliferation. At the same time, mild cognitive deficits became obvious in the novel object-placement recognition task. After 6 days of diabetes the GR antagonist mifepristone (RU486) was administered twice daily for 4 days (200 mg/kg, p.o.). Blockade of GR during early type 1 diabetes attenuated the morphological signs of hippocampal aberrations and rescued the diabetic mice from the cognitive deficits. We conclude that hippocampal disruption and cognitive impairment at the early stage of diabetes are caused by excessive GR activation due to hypercorticism. These signs of neurodegeneration can be prevented and/or reversed by GR blockade with mifepristone.
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## INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease that results in the permanent destruction of insulin-producing β-cells of the pancreas. To study the disease, a commonly used rodent model is generated by injecting streptozotocin (STZ). This toxin destroys the insulin-producing cells producing diabetes (Rees and Alcolado, 2005). The impact of T1D on the central nervous system is well documented. T1D patients show impairments in explicit memory, problem solving, and intellectual development (Ryan and Williams, 1993; Ryan *et al*, 1993; Kramer *et al*, 1998; Parisi and Uccioli, 2001; McCarthy *et al*, 2002; Schoenle *et al*, 2002). In the STZ-diabetic rodent model, the severity of the cognitive deficits is related to the duration of diabetes (Kaleeswari *et al*, 1986; Bellush and Rowland, 1989; Flood *et al*, 1990; Mayer *et al*, 1990; Biessels *et al*, 1996, 1998; Popovic *et al*, 2001). Some studies have revealed structural and functional abnormalities, particularly in the hippocampus, such as impaired long-term potentiation, synaptic alterations (dendritic spine densities and LTP), degeneration and neuronal loss (hamsters: Luse, 1970; rats: Bestetti and Rossi, 1980; Biessels *et al*, 1996; Gispen and Biessels, 2000; Magarinos and McEwen, 2000; McEwen *et al*, 2002, mice: Saravia *et al*, 2002). In the hippocampus of STZ mice, we have previously demonstrated: (1) astrogliosis, as evidenced by increased number of $GFAP^+$ cells, suggesting neuronal suffering, (2) increased immediate early gene expression such as elevated c-Jun immunoreactive neurons, a sign of neuronal activation, and (3) decreased neurogenesis in the dentate gyrus (DG), indicating that newborn cells are vulnerable to damage in diabetes (Saravia *et al*, 2002; Revsin *et al*, 2005). Overall, these alterations suggest mild hippocampal neurodegeneration in the STZ-treated mice.

In rodents, the effects of the glucocorticoid corticosterone on learning and memory processes are mediated by

*Correspondence: Dr Y Revsin, Division of Medical Pharmacology, Leiden/Amsterdam Center for Drug Research, Leiden University Medical Center. Einsteinweg 55, 2333CC Leiden, The Netherlands.
Tel: +31 71 527 6224, Fax: +31 71 527 4715,
E-mail: y.revsin@lacdr.leidenuniv.nl
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mineralocorticoid and glucocorticoid receptors (MR and GR), which are highly expressed in limbic brain areas. Glucocorticoids secreted after stress promote consolidation of a stressful event via transient activation of GR (Oitzl and de Kloet, 1992; Joëls, 2006). In contrast, sustained activation of GR by chronically elevated glucocorticoids impairs hippocampal function and memory processes (McEwen and Sapolsky, 1995). However, cognitive performance is improved during chronic blockade of GR with the glucocorticoid antagonist mifepristone (RU486) (Oitzl *et al*, 1998a, b; Conrad, 2006). MR is neuroprotective and stimulates hippocampal function (Joëls *et al*, 2008). It mediates the role of corticosterone in the appraisal of novel situations, behavioral reactivity, and affective responses (Oitzl and de Kloet, 1992; Oitzl *et al*, 1994; Rozeboom *et al*, 2007), and enhances the performance in spatial hippocampal-dependent cognitive tasks (Ferguson and Sapolsky, 2007; Lai *et al*, 2007). Thus, a balanced activation of MR and GR is crucial for optimal cognitive performance.

In diabetic animals, acute cerebral dysfunction resulting from hyper- and hypoglycemic episodes was described (Biessels *et al*, 1994; Cryer 1994). In addition, chronic hypercorticism triggered and maintained by enhanced adrenocortical sensitivity in STZ mice, and morphological alterations of predominantly the hippocampus were reported (Fitzpatrick *et al*, 1992; Durant *et al*, 1993; Scribner *et al*, 1993; Saravia *et al*, 2002, 2004; Revsin *et al*, 2005, 2008). A fundamental question in the neuropathology of T1D is, therefore, whether aberrant glucose metabolism and insulin deficiency and/or glucocorticoid excess and excessive GR activation may cause the functional and morphological signs of neurodegeneration and cognitive impairment. Previous reports described the central roles of glucose and insulin in the STZ-induced animal models of T1D (Sima and Li, 2005; Inouye *et al*, 2005; Chan *et al*, 2005; McNay *et al*, 2006).

Recently, chronic hypercorticism was found to be responsible for the functional and morphological degeneration in hippocampus of rats and mice suffering from full-blown T1D (Stranahan *et al*, 2008). The study showed that impaired LTP, deficits in cognitive performance and reduced neurogenesis were normalized in diabetic rodents after adrenalectomy and corticosterone replacement at physiological concentrations (Stranahan *et al*, 2008). The objective of the present study is to evaluate whether these neurodegenerative effects of glucocorticoids in T1D mice were due to excessive activation of GR. We hypothesize that the STZ-induced hypercorticism of diabetic mice and the concomitant continuous activation of GR may change molecular markers of hippocampal morphology and cognitive performance. We have used the GR antagonist mifepristone (RU486) to clarify the role of continuous GR activation in the hippocampus of STZ-diabetic mice. Hippocampal integrity was based on markers for cellular proliferation and activation. For hippocampal function spatial memory tasks were used, specifically the novel object-placement recognition (NOPR) task. The data demonstrate protection of hippocampal integrity and improved cognitive performance by a brief GR antagonist treatment during the onset of diabetes in the STZ-diabetic mice.

## MATERIALS AND METHODS

### Animals

Twelve weeks old C57Bl/6 male mice (Janvier, NL) were group housed 3–4 per cage in humidity ($55 \pm 5\%$) and temperature ($23 \pm 2^{\circ}C$) controlled conditions, with 12–12 light-dark hours cycle (lights on at 0800 hours) at the animal facility of the LACDR, Leiden. Food and water was provided *ad libitum*. Animal experiments were performed in accordance with the European Communities Council Directive 86/609/EEC and with approval from the animal care committee of the Faculty of Medicine, Leiden University.

### Experimental Schedule

Mice received a single i.p. dose of 170 mg/kg of STZ (Sigma, St Louis, MO, USA) in 0.5 M sodium citrate buffer or vehicle; 48 h after injection glucose levels in urine (Diabur-Test 5000, Roche, Germany) indicated diabetes. Mice with blood glucose levels (Accu-Chek Compact, Roche) higher than 11 mM were classified as overtly diabetic. Six experimental groups ($n = 6$–8 mice per group) were designed as follows: (1) control and diabetic groups with 6 days of diabetes; (2) control and diabetic groups with 11 days of diabetes, vehicle treated; (3) control and diabetic groups with 11 days of diabetes, mifepristone treated. At 6 days after diabetes onset at 0900 hours, mice received every 12 h 100 µl of mifepristone (11$\beta$-(4-dimethyl-amino)-phenyl-17$\beta$-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-one, Corcept Therapeutics, CA, USA) for 4 consecutive days (200 mg/kg body weight, per o.s.) dissolved in 0.25% carboxymethyl-cellulose and 0.2% Tween-20 in 1 ml NaCl 0.9%, or vehicle. This dose of mifepristone ensures a complete blockade of the GR for at least 12 h (Dalm *et al*, 2008). Mifepristone displays antiglucocorticoid and antiprogestin activity. However, in conditions of chronic elevated glucocorticoid levels, such as occurring in T1D mice, the antiglucocorticoid activity is most prominent. Moreover, mifepristone has been used as antiglucocorticoid in animals models for stress (Krugers *et al*, 2007; Mayer *et al*, 2006; Oomen *et al*, 2007), drugs of abuse (Dong *et al*, 2006), and electroconvulsive therapy (Nagaraja *et al*, 2007). At day 11 of diabetes at 0800 hours, the behavioral experiment was started at a time GR blockade during the task is still ensured. Mice were killed between 1000 and 1200 hours for all the experiments (see Figure 1a).

### Novel Object-Placement Recognition Task

On the basis of locomotor activity and the time exploring the objects during the last habituation trial (each object was explored approximately equally long), mice were divided into two groups that received either STZ or vehicle injection.

*Apparatus and objects.* Object exploration was assessed in an open field ($50 \times 50 \times 40$ cm; gray polyvinyl chloride). Mirrors were placed on the walls of the open field to improve the visual analysis of the exploratory behavior of the mice. The illumination density was approximately 50 lux at the center of the open field.

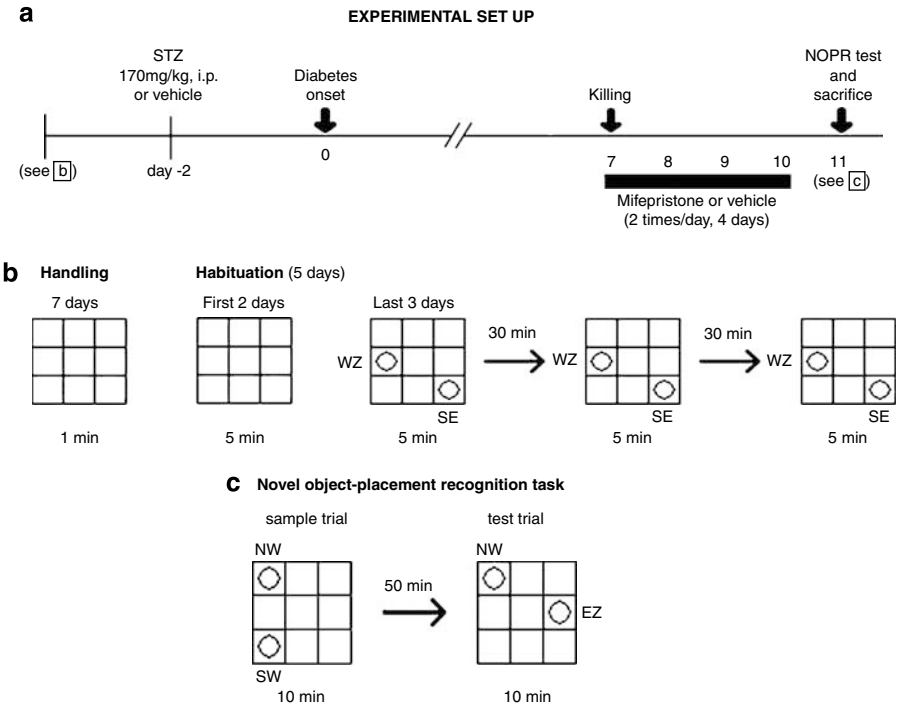


**Figure 1** Schematic representation of the methodology used. (a) Experimental design. (b) Handling and habituation for the novel object-placement recognition (NOPR) task before streptozotocin (STZ) or vehicle injections. (c) NOPR test trials at 11 days of diabetes.

We used two identical plastic bottles filled with water as objects (0.5 l without labels). After each trial, the apparatus and objects were thoroughly cleaned with a 75% ethanol solution to remove or spread odor cues.

*Procedure.* A scheme of the experimental design is presented in Figure 1. Figure 1b shows the handling and habituation procedure adapted from Dere *et al* (2005).

At the start of each trial, mice were picked up from the base of the tail and placed into an opaque cylinder (10 cm diameter; 20 cm high) in the center of the open field. After 10 s, the cylinder was lifted and the mice were allowed to explore the environment. Depending on the experimental condition, the two bottles were present or absent. At the end of each trial, the mice were removed from the open field by a grid and returned to the home cage. This procedure prevents the mice becoming stressed by the experimenter.

On day 11 of diabetes, the NOPR test was performed (Figure 1c). In the sample trial, the two objects were present in the open field: one in the northwest zone and one in the southwest zone (SW). After 10 min exploration, the mouse was returned to its home cage for 50 min. For the consecutive 10 min test trial (T2), the SW object was relocated to the east zone.

*Computerized image analysis.* Exploration of an object is defined when the mouse is in the vicinity of an object at less than 2 cm and in physical contact with it. Contact was only counted as exploration when the mouse touched the object with the snout, forepaws, or vibrissae (Observer 4.1, Noldus Information Technology, Wageningen, The Netherlands). The parameters of exploration are based on Dere *et al* (2005).

Analysis of general locomotor activity and behavior was performed by EthoVision3.0 (Noldus Information Technology) using steps of five samples per second. The open field was divided into nine zones (Figure 1), and total distance moved (cm), mean velocity (cm/s), latency of first entry in each zone (seconds), and time per zone (percentage) were measured.

*Calculations.* The exploration of the two objects was calculated for the sample and test trials. The percentage of preference for exploring the relocated object was calculated as follows:

$$\frac{\text{Exploration time of relocated object (T2)}}{\text{Sum of exploration of both objects}} \times 100$$

## Immunocytochemistry for GFAP and Ki-67

Under pentobarbital sodium salt anesthesia (Nembutal 150 µl i.p., CEVA Sante Animale BV, The Netherlands), mice were intracardially perfused (20 ml of 0.9% NaCl followed by 40 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4), the brains were removed, kept for 2 h in the same fixative, and incubated overnight in 30% sucrose in PBS at 4°C. Thereafter, the brains were frozen on dry ice and stored at −80°C until processing.

Astrocytes and proliferative cell markers (GFAP and Ki-67) were measured in coronal brain cryosections (20 µm) from the dorsal hippocampus (bregma −1.7 to −2.06 mm; Paxinos and Frankling, 2001). Four to six sections from each brain were mounted per glass (SuperFrost Plus glasses,

Menzel-Glaser, Germany) and stored at −80°C. After washes with 0.1 M Tris-buffered saline pH 7.6 (TBS), the sections were placed in plastic jars filled with citrate buffer (0.1 M for GFAP and 0.01 M for Ki-67, pH 6), microwaved for 5 min at 800, 400, and 250 W, and cooled down to room temperature (RT) for 30 min. Ki-67 immunostaining follows the previously published by Heine *et al* (2004). For GFAP nonspecific binding was prevented by 10% normal goat serum (NGS)/0.3% Triton X-100 in 1 M TBS for 30 min at RT. Thereafter, sections were incubated with the primary antibody rabbit polyclonal anti-GFAP (1 : 600, DakoCytomation, The Netherlands) diluted in 2% NGS/0.2% Triton X-100 in 1 M TBS (TBS + ) for 1 h at RT and overnight at 4°C. For negative control, the first antibody was omitted. The next day after rinses, sections were incubated with biotinylated goat anti-rabbit immunoglobulin G (1 : 200, Jackson ImmunoResearch, The Netherlands) in TBS + for 1 h at 37°C. After 45 min incubation with ABC kit (1 : 800, Vector Laboratory, CA, USA), 3,3′-diaminobenzidine 0.5 mg/ml, 0.05% $H_2O_2$ at RT was used for development. After dehydration with graded ethanols and xylene, the sections were mounted with Entellant.

Sections stained for Ki-67 were additionally counterstained with 0.5% cresyl violet, with unbiased stereological sampling and quantification in every 10th hippocampal section according to Saravia *et al* (2004) and Oomen *et al* (2007). The number of cells expressing GFAP per area ($31 \times 10^4\,mm^2$) was determined in the *stratum radiatum* below the CA1 using computerized image analysis (Olympus Soft Imaging). Labeled cells were bilaterally counted in four to six sections per animal, using 4–8 animals per group as indicated.

### Western Blot for c-Jun

Western blot was performed as described by Vreugdenhil *et al* (2007) in decapitated separate groups. The same amount of protein (25 μg cell lysate) from mouse hippocampal tissue (six mice per group) was used. Polyclonal anti-c-Jun primary antibody (1 : 500, Santa Cruz Biotechnology, CA, USA) and monoclonal anti-α-tubulin DM1A (1 : 1000; Sigma-Aldrich, The Netherlands), as a control for the amount of protein loaded, were applied in different membranes (Millipore). Thereafter, the secondary antibody peroxidase conjugated (1 : 5000) was added. Films were developed and the intensity of the immunoreactive labeling was analyzed (Image analysis system, Image J, NIH Bethesda, USA). The values for each sample are expressed as a percentage of optical density (o.d.) obtained using α-tubulin.

### Corticosterone and ACTH Radio Immuno Assay

Trunk blood was collected individually in labeled potassium-EDTA-coated tubes (1.6 mg EDTA per ml blood, Sarstedt, Germany), kept on ice and centrifuged for 15 min at 3000 r.p.m. at 4°C. Plasma was transferred to clean tubes and stored frozen at −20°C until the determination of corticosterone or ACTH by the MP Biomedical RIA kits (ICN, Biomedicals Inc., CA).

### Data Analysis and Statistics

Data are expressed as mean ± SEM. Physiological and neuroendocrine parameters were analyzed by multifactorial analysis of variance (ANOVA) using SPSS software (version 7.5). Expression of molecular markers and behavioral measurements were analyzed by two-way ANOVA followed by Bonferroni-adjusted *post hoc* test using GraphPad Software (version 4). Differences were considered statistically significant at $p < 0.05$.

Behavior of separate groups was tested in open field, elevated plus maze, forced swim, and Morris water maze tasks (see Supplementary data). For each behavioral task, different groups of control and diabetic animals were used.

## RESULTS

### Physiological and Neuroendocrine Parameters

Diabetic mice showed the characteristic clinical features of the disease after 6 days of diabetes. Multifactorial ANOVA revealed significant main effects of group (diabetic *vs* control: $F(8,30)$ 61.176, $p < 0.001$) and treatment with mifepristone ($F(8,30)$ 18.485, $p < 0.001$ and a group × treatment interaction ($F(3,30)$ 5.191; detailed measurements and significances are given in Table 1). As we observed before (Revsin *et al*, 2008), diabetic mice showed hyperglycemia (> 11 mM), increased absolute adrenal weight, and adrenal/body weight ratio, and decreased body weight (BW), absolute thymus weight, and thymus/body weight ratio. Furthermore, basal plasma corticosterone levels were significantly increased, whereas ACTH concentration was significantly decreased compared to control mice. Untreated control and diabetic mice of 6 days of diabetes showed the same effects as the control and diabetic vehicle-treated groups, respectively (data not shown).

Mifepristone treatment increased adrenal weight, adrenal/BW ratio, and corticosterone levels in control as well as in diabetic mice. In addition, this mifepristone-treated diabetic group showed increased ACTH and gain in BW, but thymus weight and thymus/BW ratio remained as low as in diabetic vehicle-treated mice (Table 1).

### Mifepristone Effects on the Hippocampus

*Astrogliosis.* The number of astrocytes ($GFAP^+$ cells, Figure 2a) in the *stratum radiatum* of the hippocampus was measured in six experimental groups: control and diabetic mice killed 6 days after diabetes onset; control and diabetic mice treated with vehicle or mifepristone and killed at day 11 of diabetes. Only at 11 days of diabetes was the number of astrocytes significantly increased (Figure 2). Treating diabetic mice with mifepristone for 4 days prevented the increase in the number of $GFAP^+$ cells, which remained comparable to nondiabetic controls. Mifepristone treatment of nondiabetic mice did not modify this parameter.

*c-Jun protein expression.* Cellular activation was studied by Western blot using a specific antibody against c-Jun (Figure 3). To normalize c-Jun protein expression between groups, the endogenous nonregulated α-tubulin was

**Table 1** Effects of Diabetes and Mifepristone Treatment on Glycemia, Body, Adrenal and Thymus Weight, and Plasma Corticosterone and ACTH Levels

| | Vehicle | | Mifepristone | | | |
|---|---|---|---|---|---|---|
| | **Control** | **Diabetic** | **Control** | **Diabetic** | **Group** | **Group × treatment** |
| Glycemia (mM) | 6.8 ± 0.23 | 27.8 ± 1.3* | 6.5 ± 0.4 | 23.7 ± 3.6$^{\$}$ | 138.1 | NS |
| ΔBW | 3.1 ± 0.3 | −3.1 ± 0.5* | 2.3 ± 0.7 | −0.01 ± 0.5$^{\$/\#}$ | 51.4 | 11.7 |
| Adrenal weight (mg) | 1.5 ± 0.1 | 2.6 ± 0.1* | 2.1 ± 0.2* | 3.1 ± 0.2$^{\$}$ | 32.3 | NS |
| Adrenal/BW ratio | 57 ± 2.5 | 134.7 ± 6.5* | 88.7 ± 12.8* | 135.6 ± 15.2$^{\$}$ | 66 | 3.9 |
| Thymus (mg) | 22.9 ± 0.7 | 7.7 ± 0.7* | 19.5 ± 0.6 | 8.7 ± 0.9$^{\$}$ | 322.2 | 12.6 |
| Thymus/BW ratio | 0.8 ± 0.2 | 0.4 ± 0.04* | 0.8 ± 0.04 | 0.4 ± 0.04$^{\$}$ | 124.5 | NS |
| CORT (ng/ml) | 13.7 ± 3.1 | 321.0 ± 49.5* | 297.0 ± 79.3* | 836.9 ± 83.6$^{\$/\#}$ | 49.7 | 3.5 |
| ACTH (pg/ml) | 142.5 ± 15 | 70.9 ± 8.03* | 133.0 ± 22.5 | 148.8 ± 17.4$^{\#}$ | NS | 6.9 |

Abbreviations: BW, body weight; CORT, corticosterone, NS, nonsignificant.
*$p<0.05$ *vs* control+vehicle, $^{\$}p<0.05$ *vs* control+mifepristone, $^{\#}p<0.05$ *vs* diabetic+vehicle. Values are expressed as mean ± SEM. ΔBW: difference (in grams) in body weight at the time of vehicle or STZ injections and at killing. Adrenal/body weight ratio is expressed as absolute weight × 1000 (grams)/body weight (grams). F-values with d.f. = 1.37: between groups; d.f. = 1 for all the group × treatment interactions measurements; only significant values are given; right column, significant group × treatment interactions.

measured. The o.d. of c-Jun/α-tubulin ratio is shown in Figure 3a. The quantitative analysis reveals that hippocampal cellular activation takes place at 11, but not at 6 days after diabetes onset. Mifepristone treatment of nondiabetic mice did not alter c-Jun protein expression, but attenuated the c-Jun increase in diabetic mice. Comparable amounts of c-Jun protein expression were found in nondiabetic controls and mifepristone-treated diabetic mice.

*Cell proliferation.* Proliferative cells in the DG were detected by the endogenous proliferation marker Ki-67. The Ki-67 antigen is a protein complex present only in the $G_1$, S, $G_2$, and M, but not the $G_0$ phase of the cell cycle (Gerdes *et al*, 1984; Endl and Gerdes, 2000). Clusters of Ki-67-positive cells were shown to be similar to BrdU after short survival time and were found almost exclusively in the subgranular zone (SGZ; Kee *et al*, 2002; Oomen *et al*, 2007). Moreover, Ki-67 is a well-accepted proliferation marker (Gerdes *et al*, 1991; Heine *et al*, 2004; Krugers *et al*, 2007; Veenema *et al*, 2007).

After 6 days of diabetes, a significantly decreased cell proliferation in the SGZ of the DG was observed (diabetic day 6 *vs* control day 6, $p<0.05$, Figure 4). This reduction in the number of proliferative cells was maintained at 11 days of the disease: Ki-67-positive cell number of diabetic + vehicle group is significantly lower as compared to the control + vehicle ($p<0.001$). After mifepristone administration, the number of Ki-67-positive cells is significantly increased in diabetic mice (diabetic + mifepristone *vs* diabetic + vehicle, $p<0.05$). Mifepristone treatment of control mice did not modify the Ki-67-positive cell number in comparison to the control vehicle-treated group.

## Diabetes Effect on Cognition

Only the more subtle hippocampal-dependent task, the NOPR, revealed significant impairments in cognitive performance of diabetic mice at 11 days of the disease. In the sample trial, the percentage of time that both objects are explored is similar in both groups (control: 50.51 ± 5.34, diabetic: 57.84 ± 3.84, expressed as percentage of preference for the object that will be relocated in the test trial, $n=7$–8). In contrast to mice of the control group, diabetic mice had no preference for the relocated object in the test trial (control: 61.37 ± 5.54, diabetic: 44.41 ± 4.98%, of time exploring the relocated object, $p<0.05$).

## Mifepristone Effects on Cognition: the NOPR Task

*Locomotor activity.* Locomotor activity expressed as distance moved, duration of the movement, and mean velocity of the movement is presented in Table 2. During the sample trial of the NOPR task, both vehicle- and mifepristone-treated diabetic mice walked slower and shorter distances, with shorter duration of movement when compared to control groups. At 50 min later, during the test trial, the diabetic vehicle-treated group was still less active. However, diabetic mifepristone-treated mice significantly increased their locomotor activity. Locomotor activity of control groups remained comparable in sample and test trials.

The latency to first exploration of the objects and to the zones, as well as the percentage of time in each zone did not differ between groups (data not shown). The number of mice at the beginning of the behavioral experiment was 12 per group. However, some diabetic and control + mifepristone mice died before the end of the experimental procedure. Moreover, two mice were discarded from the behavioral analysis of the diabetic + mifepristone group due to their inability to walk as a consequence of the metabolic deficits originated by the disease. The number of mice used was: control + vehicle, $n=12$; diabetic + vehicle, $n=10$; control + mifepristone, $n=8$; diabetic + mifepristone, $n=6$.

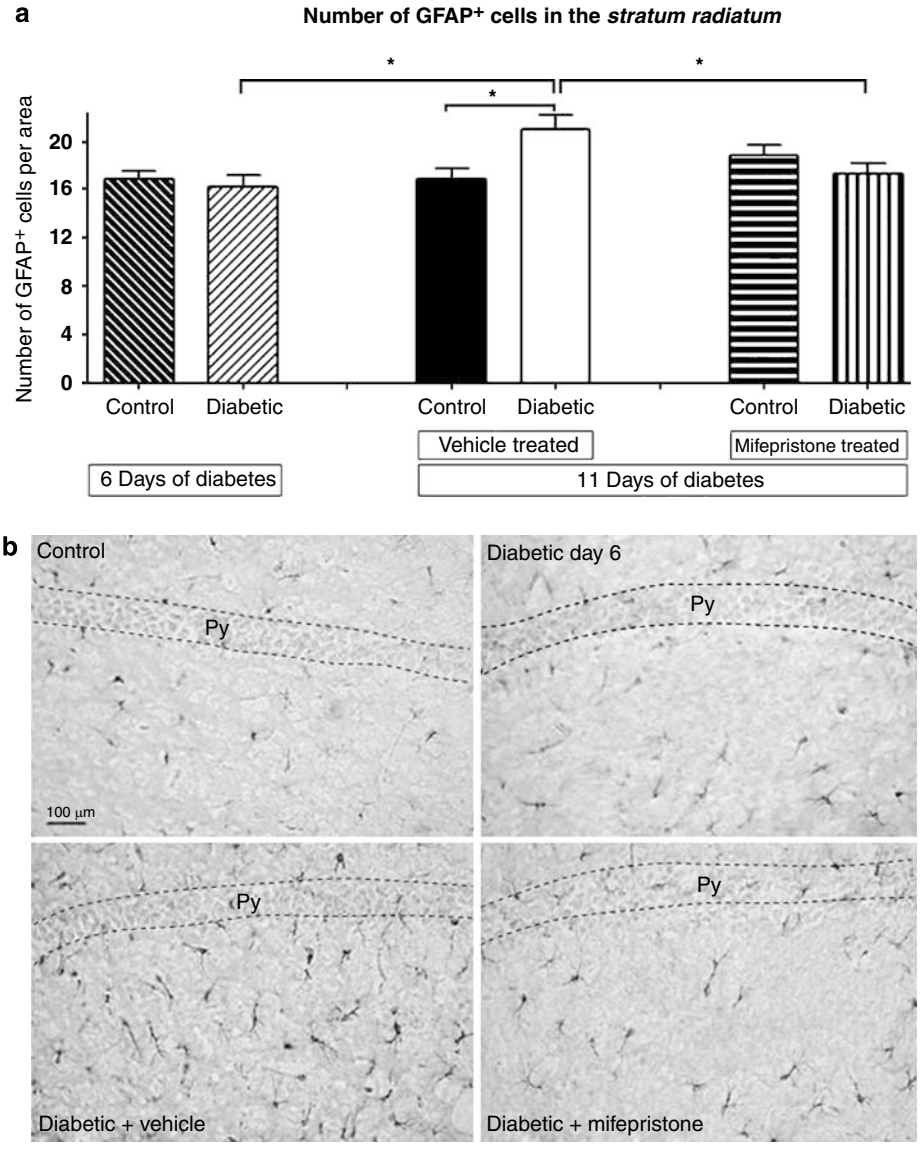


**Figure 2** Immunocytochemistry for glial fibrillary acidic protein (GFAP). (a) Quantification of GFAP$^+$ cell number in the *stratum radiatum* of the hippocampus. Values expressed mean ± SEM, $n = 6$–8, $^*p < 0.05$. (b) Microphotographs of the different experimental groups. Py = pyramidal cells of the CA1 area. Control microphotograph correspond to a control + vehicle mouse.

*Spatial memory: exploration of the relocated object.* During the sample trial, all groups spent a comparable percentage of time exploring both objects, expressed as percentage of preference for the object that will be relocated in the test trial (control + vehicle = 42.67 ± 2.07, diabetic + vehicle = 49.47 ± 5.17, control + mifepristone = 44.59 ± 4.32, diabetic + mifepristone = 51.03 ± 2.41). Figure 5 shows the percentage of preference for the object placed in the new location (ie the relocated object) during the test trial. As previously reported (Dere *et al*, 2005), the control vehicle-treated group preferred to explore the relocated object. The same effect was found in mifepristone-treated control mice. Conversely, diabetic mice explored both objects equally (50% of the exploration time for each object). The preference for the relocated object is restored when diabetic mice are treated with mifepristone. In addition, the difference in preference in this group is higher compared to its respective control ($p < 0.05$, Bonferroni-adjusted *post hoc* test).

## DISCUSSION

Our results show that: (1) hippocampal integrity is threatened in STZ-treated diabetic mice as revealed by astrogliosis, increased cellular activation, and decreased neuronal proliferation at 11 days of disease progression; (2) diabetic mice show spatial memory deficits as is indicated by the impaired performance in a specific hippocampal-dependent task (the NOPR task); and (3) 4 days of treatment with the GR antagonist mifepristone in the early phase of diabetes prevents the change in markers for hippocampal integrity and improves cognitive performance in the face of corticosterone hypersecretion.

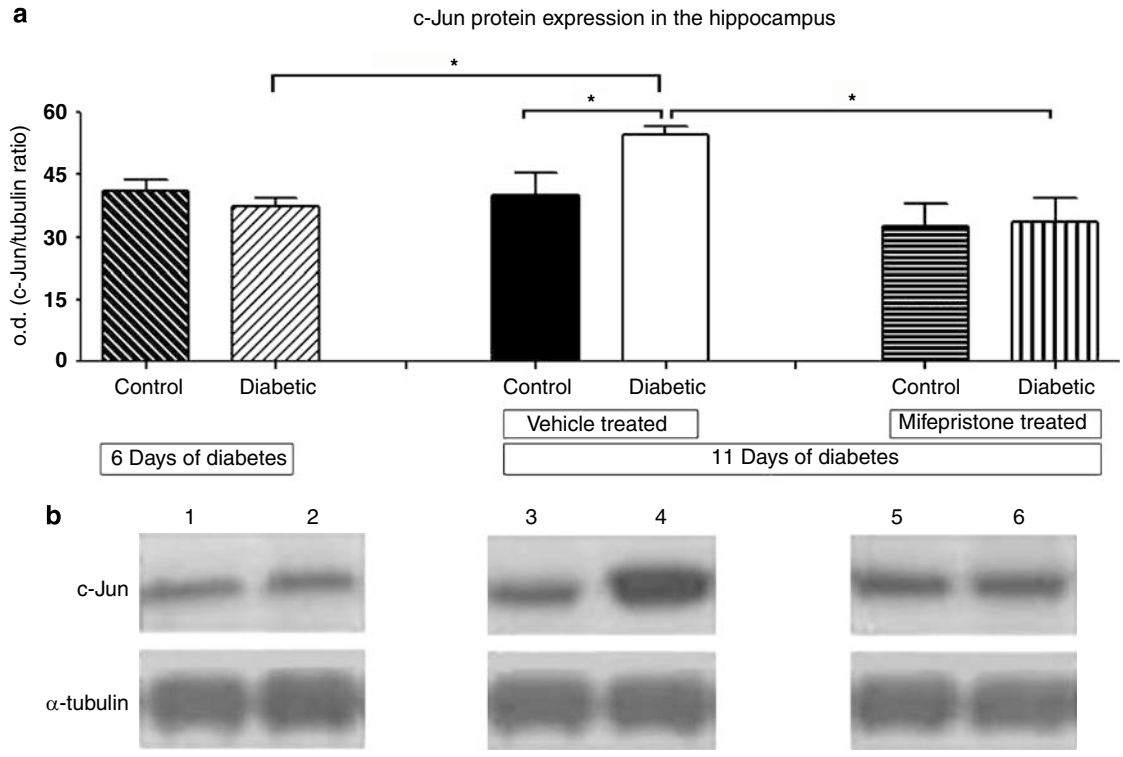


**Figure 3** Western blot for c-Jun. (a) Quantification of c-Jun/α-tubulin optical density (o.d.) from hippocampal homogenates. Values expressed mean ± SEM, $n = 6$, $^*p < 0.05$. (b) Autoradiograph for c-Jun and α-tubulin. 1: control day 6; 2: diabetic day 6; 3: control + vehicle; 4: diabetic + vehicle; 5: control + mifepristone; 6: diabetic + mifepristone.

### Mifepristone Effects on Hippocampal Alterations of STZ-Diabetic Mice

*Prevention of astrogliosis.* Increased GFAP immunoreactivity representing astrogliosis in the hippocampus was previously reported at day 28 of STZ diabetes in mice (Saravia *et al*, 2002). We found similar results already in 11-day-diabetic mice, whereas GFAP immunoreactivity was not affected at 6 days of diabetes. Treatment with the GR antagonist mifepristone from days 6 to 10, prevented the GFAP increase at day 11, indicating that blockade of excess glucocorticoid activation of the GR can prevent hippocampal astrogliosis.

Reactive, GFAP-expressing astrocytes provide neuroprotection during metabolic insults, stress, or injury to nearby neurons by secreting growth factors, substrate-bound neurite-promoting factors, and the removal of neurotoxins and excess glutamate (Kiessling *et al*, 1986; Liedtke *et al*, 1996; Lambert *et al*, 2000). Astrocytes may also protect neurons by increasing glucose uptake, metabolism, and transport (Magistretti and Pellerin, 1999; Vesce *et al*, 1999), in addition to being necessary for the preservation of myelin and normal white matter architecture (Louw *et al*, 1998). Moreover, the close connection of astrocytes to the blood–brain barrier makes them early sensors of variations of glucose homeostasis, which can be communicated to neurons (Magistretti and Pellerin, 1999). The astrogliosis therefore can be considered to be a neuroprotective response to brain damage inflicted by excessive GR activation, which is prevented by the GR antagonist.

*Prevention of cellular activation.* We previously reported that T1D also resulted in cellular activation, as shown by an increase of c-Jun- and c-fos-positive cells in neuronal populations of the CA1, CA3, and DG hippocampal areas (Revsin *et al*, 2005), and this is further supported by the current findings. Although some authors imply a functional role of early gene products in neuronal cell death (Eilers *et al*, 2001; Barone *et al*, 2008), others suggested that they might rather contribute to cellular repair and/or regenerating processes (Herzog and Morgan, 1996; Herdegen *et al*, 1997; Waetzig *et al*, 2006). As c-Jun was increased at day 11 but not at day 6 of STZ diabetes, we conclude that either corticosterone caused the hippocampal cellular activation or that increased c-Jun-positive cells are a response to an emerging corticosterone-induced neuronal derangement in the hippocampus of diabetic animals, which is prevented by the GR antagonist.

*Restoration of cell proliferation.* Previously, we demonstrated a strong reduction in cell proliferation rate in STZ-diabetic mice (Saravia *et al*, 2004). The results shown in the present study confirm these previous observations and demonstrate that significant suppression of cell proliferation already occurs at early stages of diabetes, at days 6 and 11, suggesting that newborn cells are particularly vulnerable to the detrimental effects of glucocorticoid excess. These data are in support of Stranahan *et al* (2008) showing that adrenalectomy and low-dose corticosterone replacement prevent the decreased proliferation and survival of newborn DG neurons in long-term STZ-induced diabetes.

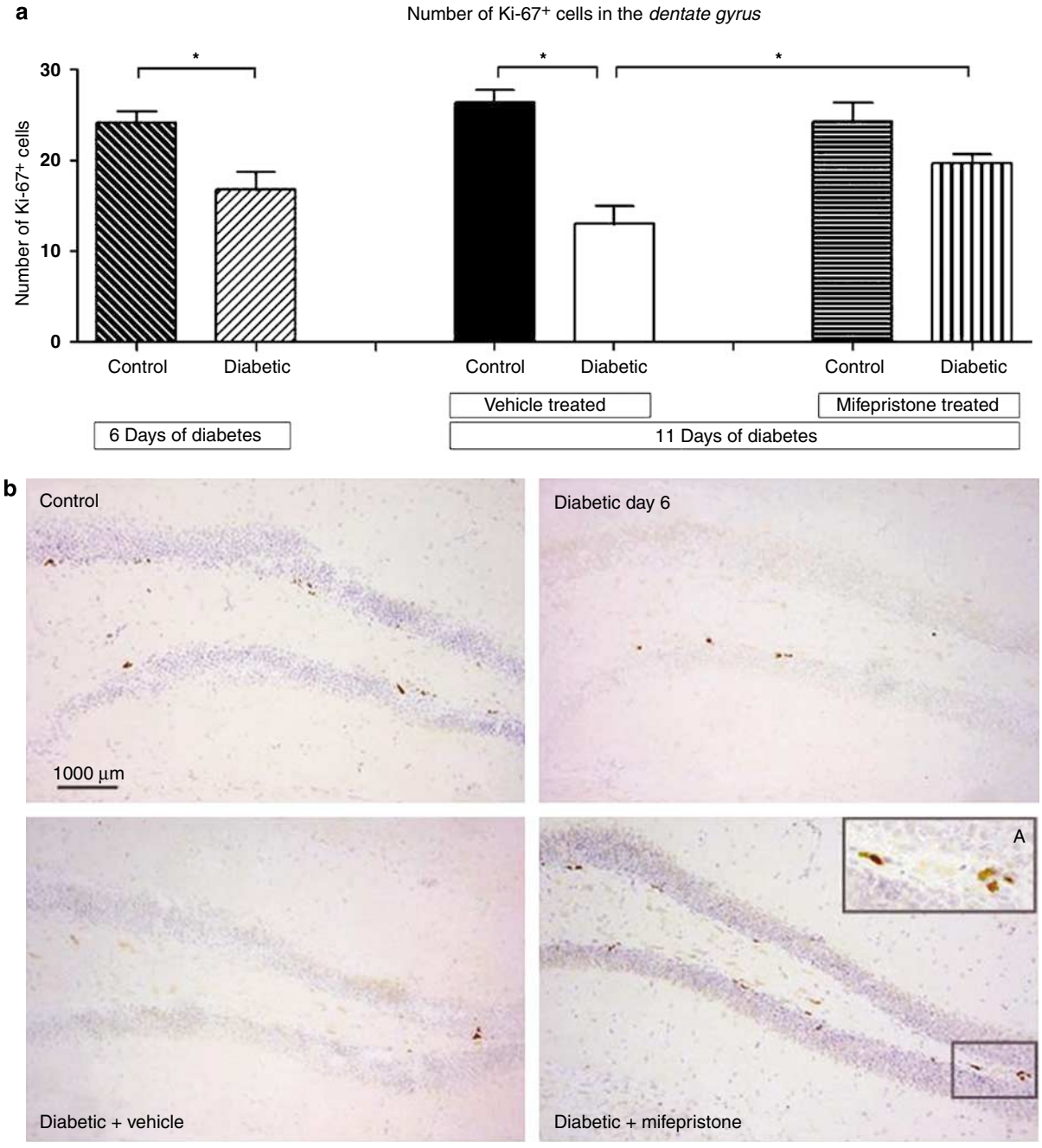


**Figure 4** Cell proliferation in the subgranular zone (SGZ) of the dentate gyrus. (a) Quantitative analysis of the immunocytochemistry for Ki-67 in the SGZ. Values expressed mean ± SEM, $n = 6$–8, $^*p < 0.05$. (b) Microphotographs of the different experimental groups. Insert A: four times magnification of Ki-67$^+$ cells. Control microphotograph correspond to a control + vehicle mouse.

The data also show that GR blockade in diabetic mice restores the number of Ki-67-positive cells in the DG toward control levels. Therefore, the suppression of proliferation in diabetes is mediated directly and/or indirectly by glucocorticoids via the GR. In other studies, neurogenesis was suppressed in high stress and corticosterone environments (Wong and Herbert, 2005; Mayer *et al*, 2006; Oomen *et al*, 2007), but not the Ki-67 marker observed in this study and by Stranahan *et al* (2008). Therefore, the proliferative response marker might be sensitive to a high corticosterone environment during hyperglycemia and this effect is reversible by blockade of the GR. Nevertheless, the action of glucocorticoids on proliferation is likely to be indirect as newly formed cells in the DG have been found not to express corticosteroid receptors (MR and GR; Cameron *et al*, 1993; Garcia *et al*, 2004; Wong and Herbert, 2005). Therefore, mature receptor-expressing dentate granule cells or glia surrounding the progenitors might determine proliferation and survival of these cells (Song *et al*, 2002; Hastings and Gould, 2003; Seki, 2003). However, a study from Garcia *et al* (2004) showed that a small proportion of newly formed cells (15%) does in fact express GR 24 h after division.

**Table 2** Effects of Diabetes and Mifepristone Treatment on Locomotor Activity

| | Vehicle | | Mifepristone | |
|---|---|---|---|---|
| | **Control** | **Diabetic** | **Control** | **Diabetic** |
| *Sample trial* | | | | |
| Distance moved (m) | 47.0 ± 2.5 | 22.0 ± 2.5* | 49.5 ± 2 | 25.5 ± 4.7$^{\$}$ |
| Mean velocity (cm/s) | 8.0 ± 0.4 | 3.7 ± 0.4* | 8.5 ± 0.4 | 4.3 ± 0.8$^{\$}$ |
| Duration movement (s) | 506 ± 9 | 335 ± 27* | 533 ± 6 | 356 ± 35$^{\$}$ |
| *Test trial* | | | | |
| Distance moved (m) | 44.2 ± 2.5 | 22.0 ± 2.5* | 46.3 ± 1.8 | 30.4 ± 2.4$^{\#}$ |
| Mean velocity (cm/s) | 7.4 ± 0.4 | 5.6 ± 0.5* | 7.8 ± 0.3 | 6.7 ± 0.4 |
| Duration movement (s) | 490 ± 12 | 415 ± 17* | 516 ± 10 | 474 ± 14$^{\#}$ |

*$p<0.05$ *vs* control+vehicle, $^{\$}p<0.05$ *vs* control+mifepristone, $^{\#}p<0.05$ *vs* diabetic+vehicle. Values are expressed as mean ± SEM.

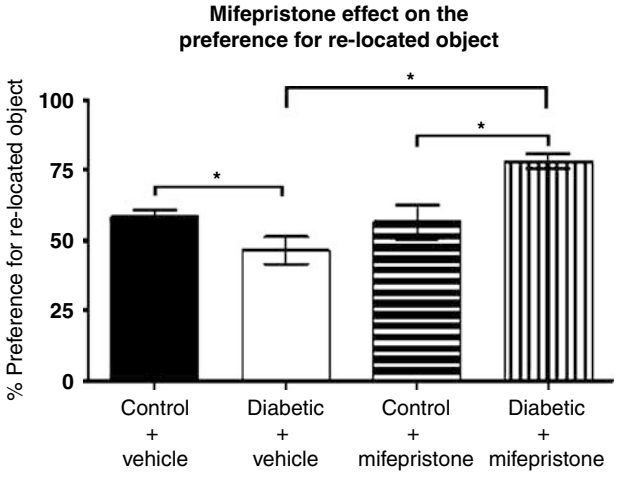


**Figure 5** Mifepristone effect on the novel object-recognition task. Percentage of preference for the relocated object during the test trial is shown for control and 11 days diabetic mice vehicle- and mifepristone treated. Values expressed mean ± SEM, *$p<0.05$ Bonferroni-adjusted *post hoc* test.

In summary, the molecular changes described above indicate that mifepristone treatment interferes with the disruption of hippocampal integrity at early stages of STZ diabetes. Whether mifepristone's prevention and/or reversal of the disturbances caused by diabetes is directly on hippocampal astrocytes, neurons, and proliferative cells, or by changing its surroundings (ie their inputs) requires further investigation. It is worth noting that 4 days mifepristone treatment can affect not only the functioning of these cells (present study, Karst and Joëls, 2007; Wu *et al*, 2007; Mayer *et al*, 2006; Wong and Herbert, 2005), but also the innervating projections from other brain regions (Witter, 2007). Moreover, in the present study, we reported that reversion of hippocampal alterations takes place in the face of hypercorticism. Recently, Oomen *et al* (2007) and Mayer *et al* (2006) reported similar beneficial effects of mifepristone in rats subjected to chronic unpredictable stressors and to high doses of exogenous corticosterone, respectively.

## Cognitive Performance

The previously observed HPA axis dysregulation in the type 1 diabetic mice (Revsin *et al*, 2008) suggested that hypercorticism and excessive GR activation might impair cognitive performance. Therefore, we assessed whether the learning and memory capabilities of STZ-induced diabetes mice were altered at different early states of the disease in a series of behavioral tasks (open field, forced swim test, Morris water maze, and elevated plus maze). Surprisingly, diabetic and nondiabetic control mice performed equally well in these early stages of diabetes (Supplementary Figures S1–S3 and Supplementary Table S1), although during a more prolonged state of diabetes behavioral deficits were established (Stranahan *et al*, 2008). It is possible that in our study these tasks are insensitive to the moderate hippocampal disturbances observed at early diabetes. For this reason, we decided to adopt another behavioral task for hippocampus-specific memory, the NOPR task (Dere *et al*, 2005).

The NOPR task uses the spontaneous exploration of objects in novel locations (Li *et al*, 2004; Dere *et al*, 2005), and thus allows the study of mild hippocampal alterations. In accordance with the literature, nondiabetic control mice preferred exploring the object placed in the novel location. The diabetic mice, however, exhibit less exploration of the object in the novel location compared to controls (Figure 5). This reduced exploration displayed by the diabetic mouse indicates impaired spatial object-placement memory. It is noteworthy that although diabetic mice showed a significantly decreased locomotor activity and exploration time of the objects than nondiabetic controls, the influence of these parameters on the preference for the objects was prevented by the method used to calculate the percentage of preference for the relocated object.

*Mifepristone restores spatial memory deficits of STZ-diabetic mice.* Mifepristone treatment of diabetic mice restored the preference of these animals to explore an object in a novel location. This effect exerted by the GR antagonist occurred the day after termination of the treatment. In the studies of Stranahan *et al* (2008), the reinstatement in long-term adrenalectomized T1D animals with exogenous corticosterone in physiological concentrations levels was a prerequisite to prevent deterioration of cognitive functions and impairment of LTP. The current study shows that similar results are achieved during the early stages of diabetes when the mild cognitive impairments are ameliorated by pharmacological blockade of the GR in an environment of extremely high-circulating corticosterone. This observation raises a number of interesting issues:

First, the GR-mediated action of corticosterone on hippocampal function overrides the influence of insulin and glycemia (Stranahan *et al*, 2008). The current study also shows that chronic mifepristone causes extreme increases in corticosterone, and reverses some parameters (body weight, hippocampal function) but not others (thymus weight; Table 1; van Haarst *et al*, 1996). The treatment schedule was based on the efficacy of mifepristone in the treatment of psychosis (van der Lely *et al*, 1991) and psychotic depression (Flores *et al*, 2006), and previous animal studies using corticosterone injections and chronic stress (Mayer

*et al*, 2006; Oomen *et al*, 2007). How precisely mifepristone exerts its lasting antagonistic central action through GR requires further investigation.

Second, the blockade with the antagonist becomes only effective when the GRs are fully occupied with high levels of corticosterone. At low levels of corticosterone GR is hardly occupied, and GR blockade would be less effective (Ratka *et al*, 1989), and hence mifepristone is not effective in the nondiabetic controls.

Third, during GR blockade the MR is still freely accessible for corticosterone. In the NOPR test, we specifically address MR-mediated functions, ie behavioral reactivity in the acquisition and retrieval of memory (Oitzl and de Kloet, 1992; Oitzl *et al*, 1994). Overexpression of MR could block some of the impairing behavioral effects mediated by GR (Ferguson and Sapolsky, 2007) and perhaps this explains why the GR antagonism in our diabetic mice not only rescued cognitive performance, but also even enhanced it. GR antagonism also allows a more prominent function of the MR-mediated action in preserving integrity and stability of the hippocampus (Joëls *et al*, 2008). MR is neuroprotective (Lai *et al*, 2007), promotes neuronal survival, and facilitates hippocampus function (Joëls *et al*, 2008). Hence this would predict that during GR blockade in the face of high-circulating glucocorticoids, the maintenance of hippocampal integrity is a necessary condition for improved performance in the NOPR task. Further experiments with administration of GR and MR antagonists are needed to test this possibility.

Fourth, understanding the mechanism would require the identification of the functional recovery of plasticity related genes downregulated by the excess of glucocorticoids. Such genes are related to synaptic plasticity and glutamate transmission, alterations in dendrite and spine morphology, neurogenesis in the DG, and to the rapid functional responses underlying information processing in the NOPR test (Joëls *et al*, 2007; Morsink *et al*, 2007). Obviously, future research should be focused on the question how the molecular and cellular changes during diabetes and anti-glucocorticoid treatment are linked to behavior.

In conclusion, in the present study, we have provided evidence that glucocorticoid excess and continuous activation of the GR compromise hippocampal integrity and function in T1D regardless of the hyperglycemic state. This deleterious effect of excess glucocorticoid can be abolished by a brief treatment with the GR antagonist mifepristone. The beneficial effect of mifepristone on molecular markers of hippocampal plasticity and spatial recognition is exerted in a high-corticosterone environment and probably depends on blockade of excess GR activation, perhaps, facilitated by more prominent MR-mediated actions preserving hippocampal integrity.

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## DISCLOSURE/CONFLICT OF INTEREST

The authors Yanina Revsin, Niels V Rekers, Mieke C Louwe, Flavia E Saravia, Alejandro F De Nicola, and Melly S Oitzl have nothing to declare. E Ronald de Kloet has been a member of the Scientific Advisory Board of Corcept Therapeutics Inc., the manufacturer of mifepristone over the past 3 years.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)