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Hippocampal neurovascular and hypothalamic–pituitary–adrenal axis alterations in spontaneously type 2 diabetic GK rats

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ABSTRACT

Metabolic and vascular consequences of diabetes mellitus induce several CNS complications. The dentate gyrus of the hippocampus, a well-recognized target for diabetic alterations, is a neurogenic area associated with memory and learning processes. Here, we explored the hippocampal neurogenesis and its microenvironment (astrocytes, vascularisation and glucocorticoid influence) in a spontaneous model of type 2 diabetes, the Goto–Kakizaki rat. The number of proliferative Ki67$^+$ cells and young doublecortin$^+$ neurons was 2-fold higher in the hippocampus from diabetic rats than in normoglycemic control Wistar at 4 months of age. However, there was no difference in cell survival, studied 3 weeks after bromodeoxyuridine administration. Labeling of endothelial cells against von Willebrand factor, demonstrated a 50% decrease in the granular cell layer fractional area covered by blood vessels and a diminished capillary branching in diabetic rats. Finally, Goto–Kakizaki rats exhibited decreased glucocorticoid receptor immunolabeling in CA1, associated with higher corticosteronemia. In conclusion, diabetic rats showed increased cell proliferation and neuronal differentiation without concomitant survival modification. A high proliferation rate, potentially reflecting a compensatory mechanism for neuronal suffering, also exists in various pathological situations. However, endothelial alteration induced by chronic hyperglycemia, hyperleptinemia and insulin resistance and associated with deleterious glucocorticoid effects might impair effective neurogenesis in diabetic Goto–Kakizaki rats.

Introduction

Diabetes mellitus, regardless of the type, is associated with poor performance in cognitive functions, particularly learning and memory, and in complex information processing. These deficits are generally modest. Nevertheless, diabetic patients are known to present increased risk of depression, stroke, dementia and Alzheimer disease (AD) (Biessels et al., 2008; Biessels and Gispen 2005; Biessels et al., 1994). In diabetes, whether of type 1 (T1D) or type 2 (T2D), hyperglycemia can induce various alterations, such as glucose autooxidation, advanced glycation end-products, reactive oxygen species, vascular disturbances and neuroinflammation (Copeland et al., 2008; Ramasamy et al., 2005). As part of the limbic system, the hippocampus, is a crucial CNS target of diabetic alterations in both humans and experimental rodent models (Beauquis et al., 2006; Beauquis et al., 2008a; Jackson-Guilford et al., 2000; Magariños and McEwen 2000; Ott et al., 1999; Revsin et al., 2005).

In spontaneous and pharmacological mouse models of T1D, we previously described several hippocampal disturbances, including astrogliosis, abnormal neuronal activation, increased oxidative stress together with a marked deficit of dentate gyrus (DG) neurogenesis (Beauquis et al., 2006; Beauquis et al., 2008b). Indeed, the DG is one of the few brain areas, where new neurons are produced during adulthood. The generation of new neurons consists of proliferation, including neuronal fate and *specification* of progenitors, migration through the granular cell layer (GCL) and maturation and functional integration into neuronal circuits (Abrous et al., 2005; Gage 2002; Gould and Cameron 1996; Kempermann et al., 2004; Laplagne et al., 2006; Lie et al., 2004; Ming and Song 2005; Shors et al., 2002; van Praag et al., 2002). A large proportion of the newly generated cells die after the proliferation step and survival and functional integration are critical endpoints of this process. Newly generated hippocampal cells have been implicated in learning and memory processes (Shors et al., 2002).

Unexpectedly, despite the 90% prevalence of T2D as opposed to the 10% with T1D, there have been much more studies on brain deficits in

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models of T1D than T2D. The Goto–Kakizaki (GK) rat is a type 2 spontaneous diabetic model, produced by selective inbreeding of Wistar rats using glucose tolerance as a selection index. It provides an interesting polygenic model of diabetes without obesity (Goto et al., 1975; Portha 2005; Portha et al., 2009). These rats show the main features of the metabolic, hormonal, and vascular disorders usually described in T2D patients. They show mild basal hyperglycemia from weaning onwards (1 month of age), then develop hyperinsulinemia followed by deficient insulin secretion and peripheral insulin resistance later in life. Recently, neuronal abnormalities have been described in 2 spontaneous T2D models. First, Alzheimer-like changes, associated with cortical neurite degeneration and neuronal loss were observed in BBZDR/Wor-rats, which exhibit insulin resistance and hypercholesterolemia (Li et al., 2007). Second, diabetic GK rats showed a reduction of cognitive and exploratory activity, likely caused by learning impairments (Moreira et al., 2007). GK rats had also impaired adult neurogenesis and abnormal proliferation of cultured neural progenitors in response to growth factors (Lang et al., 2008). In addition to hyperglycemia, common features of these 2 spontaneous T2D models are insulin resistance and hypercholesterolemia (Li et al., 2007), which are involved in endothelial dysfunction and chronic vascular disease (Ramasamy et al., 2005). Notably, these metabolic alterations participate in the pathogenesis of atherosclerosis and Alzheimer's disease (Kivipelto et al., 2001; Li et al., 2007) and both represent pathological conditions strongly linked to T2D (Duron and Hanon 2008). Therefore, a vascular origin of neuronal alterations in T2D might be possible.

Moreover, hypothalamic–pituitary–adrenocortical (HPA) axis dysfunction, elevated basal levels of glucocorticoids and impaired stress responses are characteristics of diabetes (Harizi et al., 2007; Stranahan et al., 2008). A link between HPA axis alterations and diabetic "encephalopathy" can be suggested, more precisely involving limbic structures, known to be especially sensitive to stress and related hormones (Reagan et al., 2008; Revsin et al., 2008). Hippocampal neurogenesis is a complex event negatively affected by stress, aging, glucocorticoid administration, inflammation and, as previously mentioned, T1D (Beauquis et al., 2008a; Ekdahl et al., 2003; Eriksson and Wallin 2004; Gould et al., 2000; Karten et al., 2005; Kempermann 2002; Kuhn et al., 1996; Mirescu and Gould 2006). Factors, which positively regulate neurogenesis are: physical exercise, environmental enrichment, steroid hormones, antidepressants, insulin, leptin, growth factors (Jin et al., 2002; Saravia et al., 2007; van Praag et al., 1999) and some pathological situations such as trauma, epilepsy, cerebral ischemia and AD (Mohapel et al., 2004; Taupin 2006).

New neurons in the subgranular zone of the DG are produced in a microenvironment formed by blood vessels, glial cells, and granular cells with different stages of maturity (Palmer et al., 2000). Therefore, in the present study, we assessed the potential damage inflicted to newborn neurons, glial cells and vessels in 4-month-old type 2 diabetic GK rat hippocampus, compared to age-matched Wistar controls. We measured: a) basal fed glycemia, circulating levels of insulin, leptin and corticosterone; b) the ability for DG cell proliferation measured by Ki67 labeling; c) the differentiation of new neurons by doublecortin (DCX) marker; d) the capacity of newborn cell survival, via detection of 5-bromo-2′-deoxyuridine (BrdU), administered 21 days before killing; e) the astroglial density in the hippocampus using glial fibrillary acidic protein (GFAP) labeling; f) the vascular net of the DG, using the endothelial-specific marker, von Willebrand factor; and g) the hippocampal glucocorticoid receptor level, via specific immunohistochemistry.

Materials and methods

Experimental animals

Animal experiments were conducted on 4-month-old male GK and nondiabetic control Wistar rats from the Paris colonies (University Paris-Diderot, France) in accordance with accepted standards of animal care, established by the French National Centre for Scientific Research. Rats were housed under conditions of controlled humidity and temperature (22 ± 1 °C), with lights on from 07:00 am to 07:00 pm.

Metabolic parameters

For metabolic measurement, animals were bled as previously described (Amrani et al., 1998) and then weighed. Blood samples were kept on ice, centrifuged at 13,000 × *g* for 2 min at 4 °C and plasma (or serum) was removed and stored at −20 °C. Further determination of plasma D-glucose was done by the glucose oxidase method (Bergmeyer and Bernt, 1974) using a glucose analyzer (Beckman Instruments, Fullerton, CA) and plasma insulin by radioimmunoassay (Giroix et al., 1993). Leptin was assayed using Luminex technology (Millipore)(Ehses et al., 2009).Standard radioimmunoassay kit (ICN Biomedicals Inc., Sorin Biomedica, Antony, France) was used for corticosterone determinations.

Bromodeoxyuridine (BrdU) administration and procedures

In order to assess DG cell survival, GK and Wistar rats of 13 weeks of age were injected with a single i.p. dose of 250 mg/kg of BrdU (Sigma, 10 mg/mL stock, dissolved in NaCl 9 g/L) and killed 3 weeks later. At the time of experiment, rats were deeply anesthetized with an i.p. injection of tribromoethanol or "Avertin" (Aldrich-Chemie, Steilheim, Germany). Then, they were transcardially perfused with 30 mL of NaCl 9 g/L followed by 50 mL of 30 g/L paraformaldehyde in phosphate buffer, pH 7.4. Brains were removed and incubated overnight in 30 g/L paraformaldehyde at 4 °C. On the next day, they were transferred to Tris-buffered saline (TBS), pH 7.4, and sectioned frontally at 50 μm, using a vibrating microtome and finally stored in cryoprotectant solution at −20 °C until use.

Immunohistochemistry for Ki67

The number of proliferating cells in the DG was assessed by Ki67 labeling, a nuclear protein, which is expressed during the mitotic process and constitutes a well-recognized endogenous marker of cell proliferation (Kee et al., 2002). Antigen retrieval was done by incubating sections in 0.01 mol/L, pH 6 citrate buffer kept at 85 °C in a thermostatic bath for 40 min. Endogenous peroxidase activity was inhibited by incubation in H_2O_2 15 mL/L in TBS. Unspecific binding sites were blocked in TBS containing 20 g/L skim milk at room temperature for 30 min. Sections were incubated overnight at 4 °C with a rabbit polyclonal Ki67 antibody (1/2000, NCL-Ki67p, Novocastra, UK) in 5 mL/L Triton X-100 TBS and 10 mL/L goat serum followed by an incubation with anti-rabbit secondary antibody (Vector Labs, USA) and processing with the ABC Elite Kit (Vector Labs). Development was done by exposing sections to a solution containing 0.5 g/L 3,3′diaminobenzidine (DAB, Sigma, USA), 25 g/L Nickel ammonium, 0.5 mL/L H_2O_2 in 0.1 mol/L Tris buffer at room temperature (RT) for 5 min. Sections were finally mounted, dried, dehydrated and coverslipped with Permount (Fisher Chemical, USA). For all markers or antigens evaluated, nonspecific staining was assessed in the absence of primary antibody.

Immunohistochemistry for doublecortin (DCX)

DCX is a microtubule-associated protein used as a marker of neuroblast-like progenitor cells. DCX labeling allows quantification of absolute number of new neurons, which are generated in the DG over a 12-day period (Nacher et al., 2001). An antibody against this marker was employed to label differentiating cells by immunohistochemistry (He et al., 2005; Saravia et al., 2007). To this end, coronal brain

sections were first exposed to 500 mL/L methanol, H_2O_2 10 mL/L in phosphate buffered saline (PBS) during 10 min at room temperature, washed and blocked for 30 min in PBS containing 100 mL/L rabbit serum at 37 °C. Sections were incubated overnight at 4 °C with a goat polyclonal DCX antibody (1/250, sc-8066, Santa Cruz Biotechnology, USA) followed by a biotinylated anti-goat IgG made in rabbit (1/200, Sigma) and processed following the Vectastain Elite ABC kit instructions. For development, we used DAB at 0.25 g/L, 0.5 mL/L H_2O_2 in 0.1 mol/L Tris buffer at RT. Finally, sections were mounted, dried, dehydrated and coverslipped with Permount.

Immunohistochemistry for BrdU

For DNA denaturation and BrdU detection, sections from each rat were processed separately. They were incubated in pre-warmed 500 mL/L formamide/2×sodium citrate/sodium chloride buffer at 65 °C for 10 min, rinsed in 2×sodium citrate/sodium chloride buffer for 10 min, incubated in 2 mol/L HCl at 37 °C for 30 min, rinsed in 0.1 mol/L boric acid, pH 8.5, for 10 min and blocked for 30 min in TBS with 1 mL/L Triton X-100 and 10% goat serum. Sections were incubated for 48 h at 4 °C in a shaker with monoclonal anti-BrdU antibody made in mouse (1/200, G3G4, Hybridoma Bank USA) diluted in blocking solution. Sections were incubated with the secondary antibody, a biotinylated anti-mouse IgG (1/200, Sigma) in 1 mL/L Triton X-100-TBS for 2 h in a shaker at room temperature. Then, they were processed following the ABC kit instructions. For development, we used DAB at 0.25 g/L, 0.5 mL/L H_2O_2 in 0.1 mol/L Tris buffer at room temperature. Sections were mounted in gelatin-coated glass slides and air-dried. After counterstaining with cresyl violet for better identification of the brain region of interest, the slides were dehydrated and cleared with graded ethanol and xylene and mounted with Permount.

Immunohistochemistry for glial fibrillary acidic protein (GFAP), von Willebrand factor (vWF) and glucocorticoid receptor (GR)

Immunohistochemistry procedures were done following previously published protocols (Saravia et al., 2007). Briefly, after endogenous peroxidase inhibition, unspecific antigenic sites were blocked with normal goat serum in TBS-1 mL/L Triton X-100. Free-floating sections were exposed overnight to a rabbit anti-GFAP polyclonal antibody (1/300, G-9269, Sigma), rabbit anti-vWF polyclonal antibody (1/2000, A0082, Dako, USA) or rabbit anti-GR polyclonal antibody (1/200, SC-1004 Santa Cruz Biotechnology). Then, sections were incubated with a biotinylated goat anti-rabbit secondary antibody (1/200, Sigma) and development was completed with the ABC kit and DAB at 0.25 g/L in 0.5 mL/L H_2O_2 in 0.1 mol/L Tris buffer.

Quantification of Ki67$^+$, DCX$^+$ and BrdU$^+$-cells

Ki67$^+$, DCX$^+$ or BrdU$^+$-cells were counted on every 8th section throughout the rostrocaudal extension of both halves of the DG, corresponding to Figs. 25 to 35, bregma −1.8 to −4.2 mm from the Stereotaxic atlas of the rat brain (Paxinos and Watson 1982). The same area and number of sections (6 per animal) were studied from each experimental group. All positive cells were counted at 400× magnification in an Olympus BH-2 microscope. Cell counts were restricted to the GCL and the subgranular zone (SGZ) of the DG. The SGZ was defined as a two-nucleus-wide band below the apparent border between the GCL and the hilus. To analyze migration of BrdU$^+$ and DCX$^+$ cells through the GCL, this region was divided into 3 equal parts: inner third (1/3, in contact with the SGZ), middle third (2/3) and outer third (3/3, adjacent to the molecular layer) and the number of cells was counted in each part. The total number of labeled cells was estimated as follows according to stereological methods (Coggeshall and Lekan 1996; West 1993): positive cells located in the uppermost focal plane were ignored, whereas immunopositive cells completely filled with DAB product were counted. Therefore, positive immunoreactive cells that came into focus and focusing down through the thickness of the section were considered for the study as already reported by others authors, (Cameron et al., 1998; Kempermann et al., 1997). The number of immunoreactive nuclei or cytoplasm counted in the SGZ or in each corresponding third of the GCL was multiplied by a factor of 8 to estimate the total number of Ki67$^+$, DCX$^+$ or BrdU$^+$-cells in this zone.

Quantification of DG DCX$^+$ processes

The length of DCX$^+$-processes was measured using the Optimas 6.5 image analysis software (Media Cybernetics, USA). The immunopositive processes were traced and measured in cells relatively isolated from neighbouring cells with a complete staining of the dendritic tree. A minimum of 15 cells were evaluated per animal. Results are expressed as the mean length of DCX$^+$-dendritic processes for each experimental group.

Quantification of GFAP immunohistochemistry

The number of GFAP$^+$-cells was assessed in the hilus of the DG and in the areas beneath the CA1 and CA3 regions using an Olympus BH-2 microscope at 400× magnification attached to a VT C330N CCD video camera set. A counting frame corresponding to 50 μm×50 μm was superimposed to the image on the screen and every cell inside the frame was counted except those on the uppermost focal plane and those in contact with the frame exclusion limits. Cells were counted in anatomically matched areas in 6 sections per animal to reach a criterion of a minimum of 150 cells counted per region per animal. Results are expressed as the mean number of labeled cells per mm^2. Additionally, the area of GFAP+-cells was determined in each of the hippocampal regions studied: hilus of the DG and stratum radiatum of CA1 and CA3 regions. A minimum of 30 GFAP+-cells per region were randomly chosen and analyzed in each animal under 600× magnification. Cell area was traced using thresholding and area finder tools (Optimas 6.5 software). Results are expressed in μm^2.

Quantification of vWF$^+$-blood vessel area in the GCL

The brain endothelium was labeled by immunocytochemistry against vWF, a large glycoprotein present in both endothelial and subendothelial matrix of the vessel wall (Ruggeri and Ware 1993). Images of the DG processed for vWF immunohistochemistry were obtained with a Panasonic GP-KR222 CCD camera on an Olympus BH-2 microscope at 40× magnification and analyzed using Optimas 6.5 software. Using this software, the contour of the GCL was manually traced in 6 sections per animal in both hippocampal halves and the reference area was determined. Using thresholding and automatic area finder tools, the vWF$^+$-blood vessels were selected and their area measured. The threshold level was determined by an operator blind to the experimental groups to allow positive elements to be included and to exclude background signal. The result is expressed as the percentage of the GCL area covered by blood vessels.

Determination of vWF$^+$ branching points in the hilus

The number of branching points of vWF+ vessels per area (mm^2) was assessed in the hilus from GK and W rats at 400× magnification using 40,000 μm^2 counting frames. Branching points were defined as the junction of three vessel segments according to (Tata and Anderson 2002). At least 100 branching points per animal were counted and results are expressed as the number of branching points/mm^2 per animal.

Table 1
Body weight and circulating morning basal nonfasting parameters in 4-month-old control Wistar and diabetic GK male rats. The insulinogenic index represents the paired ratio of plasma insulin/glucose. The number of animals is shown in parentheses. [a]$p<0.001$, [b]$p<0.005$, [c]$p<0.05$, using Student's t test.

	Wistar	GK
Body weight (g)	445 ± 11 (23)	311 ± 4 (38)[a]
Glycaemia (mmol/L)	7.9 ± 0.2 (23)	12.9 ± 0.6 (38) [a]
Insulin (ng/L)	2.9 ± 0.2 (23)	3.52 ± 0.11 (38)[b]
Insulinogenic index (mg/mol)	0.4 ± 0.0 (23)	0.30 ± 0.02 (38)[c]
Leptin (pmol/L)	239.2 ± 24.9 (6)	328.1 ± 47.3 (6)
Corticosterone (ng/mL)	71.7 ± 28.3 (7)	145.4 ± 18 (7)[c]

Quantification of glucocorticoid receptor (GR) immunohistochemistry

Pyramidal cells showing GR immunostaining were analyzed in the CA1 hippocampal area. GR^+-cells were counted using a modified version of the optical dissector method (Howart and Reed 1998) with 400× magnification. Images along the pyramidal cell layer in the CA1 region were obtained using the equipment previously described. A calibrated counting frame corresponding to 50 μm × 50 μm was superimposed on the video screen and cells inside the frame that came into focus through the section were considered for counting. Cells located on the uppermost focal plane or in contact with the frame exclusion limits were not considered. A minimum of 100 cells were counted per animal in 6 brain coronal sections with a 480 μm inter-sectional distance. Data are expressed as the number of GR^+-cells per counting frame.

Statistical analysis

Four or five animals were used in each experimental group, and 3 independent experiments were performed for this study. Results are expressed as mean ± SEM. For all studies, statistical analysis is performed using a Student's t-test with $p<0.05$ as the criterion for statistical significance.

Results

Circulating parameter disturbances in diabetic GK rats

As shown in Table 1, although being of the same age and sex, diabetic GK rats had a lower body weight than Wistar controls.

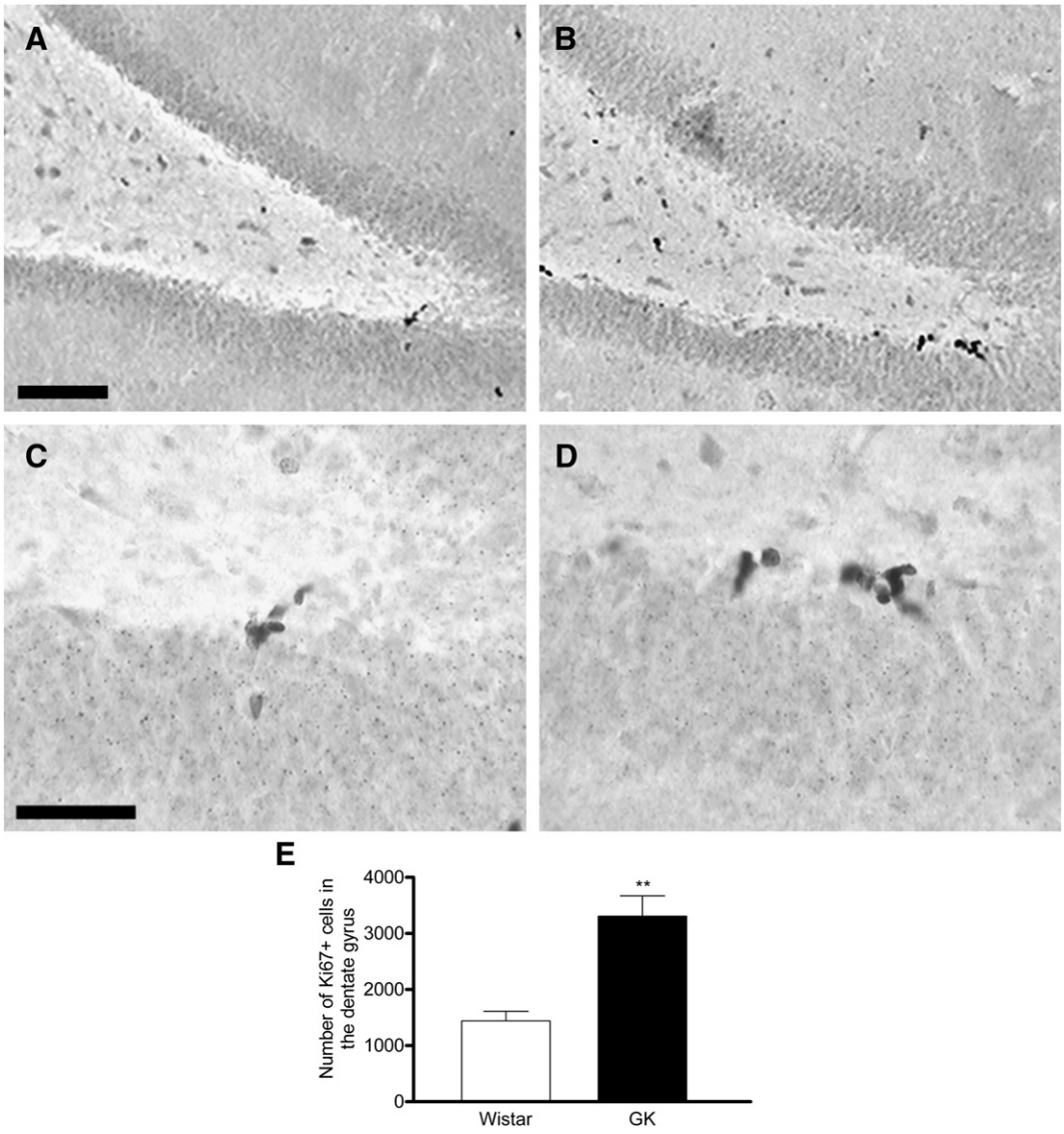

Fig. 1. Ki67 immunolabeling in the DG from Wistar controls (A and C) and diabetic GK rats (B and D). Scale bars in A and C correspond to 100 μm and 50 μm respectively. The number of $Ki67^+$ cells counted in the DG is shown in E, $^{**}p<0.01$.

When examined in the fed state, they exhibited a higher circulating plasma D-glucose concentration than control rats. Plasma insulin concentration was also higher in GK rats than in Wistar controls. However, the insulinogenic index (i.e., the paired ratio between plasma insulin and D-glucose concentration) was significantly lower in GK rats. Serum leptin level was elevated in 4-month-old GK rats, but the result did not reach statistical significance at this age. However, leptinemia was significantly higher in younger GK than age-matched Wistar rats: Wistar, 249.6 ± 32.8 *vs* GK, 359.8 ± 17.3 ($n = 7$, $p < 0.05$), and Wistar, 221.8 ± 44.8 *vs* GK, 412.5 ± 34.5 ($n = 6$, $p < 0.01$), at 2 and 3 months of age, respectively. Finally, serum corticosterone level was significantly higher in the 4-month-old diabetic GK than in the age-matched control rats.

More Ki67$^+$ proliferating cells in the diabetic GK DG

We found a significant increase in Ki67$^+$ cells in diabetic GK DG compared with that of controls (Wistar: 1442 ± 169.3 *vs* GK: 3309 ± 361.9 Ki67$^+$ cells/DG $p < 0.005$). A large part of the proliferating cells formed clusters, as shown in the representative microphotographs (Figs. 1C and D). Enhanced GK DG proliferation ability was also observed, in a separate experiment, after BrdU injection 2 h prior the sacrifice, and later immunodetection (data not shown).

More DCX$^+$ differentiated neuronal cells in the diabetic GK DG

Marked DCX immunoreactivity was found in the DG, where numerous neurons located in the hilar border of the GCL were labeled (Figs. 2A and B). The somata and dendritic trees entering the molecular layer also exhibited specific DCX immunoreactivity.

The number of DCX$^+$-cells was increased in diabetic GK DG compared with that of controls (Wistar: 9923 ± 1356; GK 15,776 ± 754.5 DCX$^+$ cells, $p < 0.01$, absolute numbers in SGZ-GCL) but, as expected, the labeled population was heterogeneous in maturation and migration terms (Figs. 2A–C). Indeed, the analysis of the migration through the GCL showed immature neurons mostly located in the SGZ in both groups. In the first third of the GCL (1/3, nearest to the SGZ), more DCX$^+$-cells were counted than in the second and third parts (2/3 and 3/3, respectively), where only a few immunopositive cells were found (Fig. 2C). However, the distribution, measured as the percentage of DCX$^+$-cells through the GCL, did not differ between both groups (SGZ: Wistar 94.10 ± 0.97 *vs* GK 94.26 ± 0.39; 1/3: Wistar 4.91 ± 0.39 *vs* GK 5.52 ± 0.35; 2/3: Wistar 0.68 ± 0.39 *vs* GK 0.19 ± 0.07; 3/3: Wistar 0.31 ± 0.24 *vs* GK 0.03 ± 0.029% of DCX$^+$-cells per region). Concerning the dendritic length, as shown in Fig. 2D, GK rats showed a tendency for shorter DCX$^+$-dendrites than Wistar controls.

No survival modification in the diabetic GK DG, despite augmented proliferation

Survival of newly DG generated cells was studied by immunodetection of BrdU, administered i.p. 21 days before killing. The BrdU$^+$-cells, which display typical brown patches of DAB product in the nucleus (Figs. 3A and B), were counted in the SGZ-GCL. No significant differences were found between Wistar and GK rats (Wistar, 736 ± 109 *vs* GK, 852 ± 74, $p = 0.41$; Fig. 3C) and newborn cells from diabetic rats only showed a tendency for enhanced survival with the time in the DG. BrdU$^+$-cells were typically located in the SGZ in both groups. Regarding BrdU$^+$-cell percentages per zone, there was no difference in the SGZ between Wistar and diabetic GK rats (73.8 ± 2.1 and 75.8 ± 4.2%, respectively), but GK rats showed a significant decrease in the percentage of BrdU$^+$-cells in the 1/3 (Wistar: 18.7 ± 2.9 *vs* GK: 10 ± 1.7%), an increase in the 2/3 (Wistar: 2.5 ± 0.6 *vs* GK: 7.6 ± 1.7%) and no difference in the 3/3 (Wistar: 5 ± 0.6 *vs* GK: 6.6 ± 1.2%).

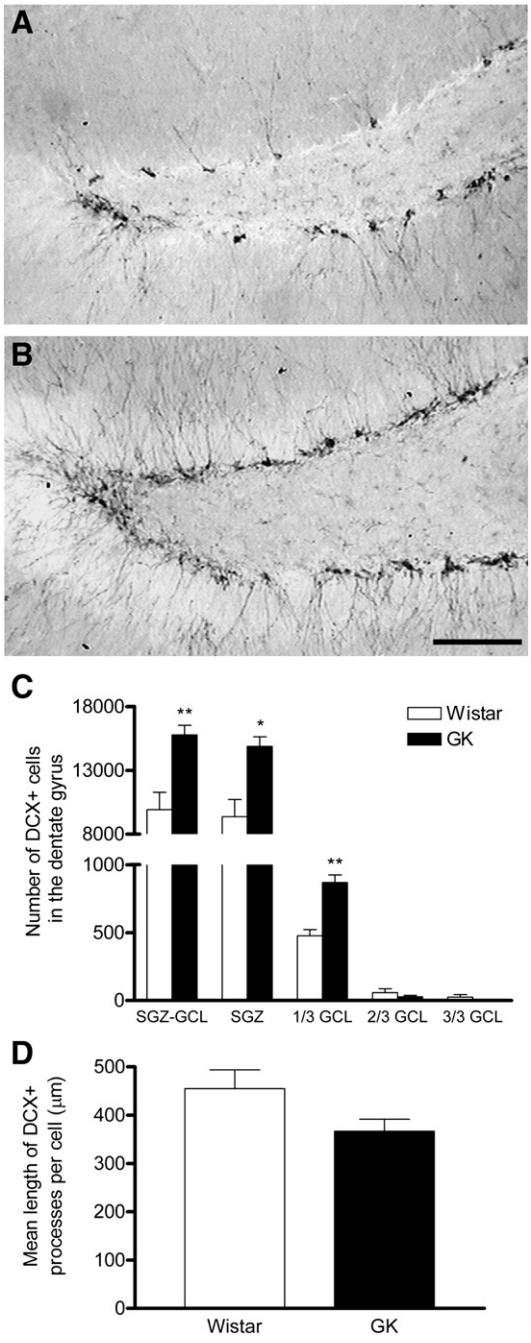

Fig. 2. Doublecortin (DCX) immunohistochemistry in the DG of Wistar (A) and GK (B) rats. Scale bar in B corresponds to 100 µm. (C) Number of DCX$^+$ cells in DG counted in subgranular zone (SGZ) and granular cell layer (GCL) and thirds (1/3, 2/3 and 3/3) corresponding to the GCL, *$p < 0.05$, **$p < 0.01$. (D) Length of DCX$^+$ processes per cell expressed in µm measured in both experimental groups.

Similar GFAP$^+$-astrocyte numbers in the hippocampal CA1, CA3 and DG areas of diabetic GK and control Wistar rats. No variations in GFAP area per cell

The density of glial cells was explored by immunohistochemistry against GFAP. The numbers of GFAP$^+$-astrocytes, quantified in CA1, CA3 and DG areas, were found to be similar in Wistar and GK rats. The cell density was homogeneous in the various hippocampal areas and no manifest differences were observed regarding astrocytic morphological structure, as shown in Fig. 4. In this line, the GFAP$^+$ cell area – as a measure of cell reactivity and size – was quantified, but only a

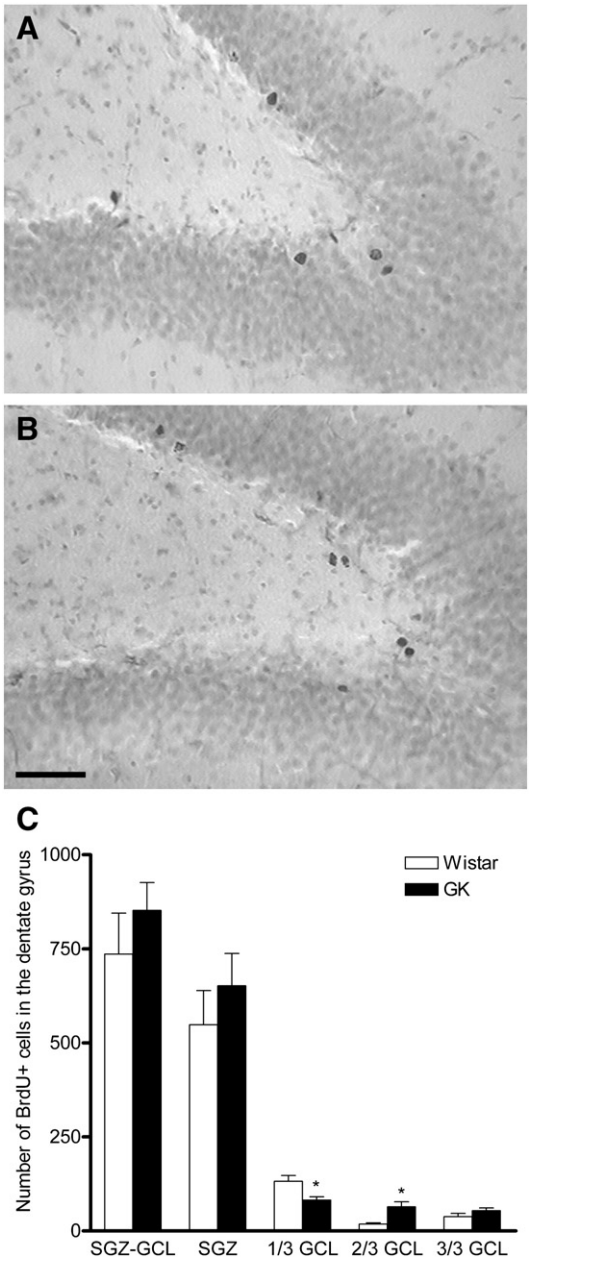

Fig. 3. 5-bromo-2′-deoxyuridine (BrdU) immunodetection in DG from Wistar (A) and GK (B) rats. BrdU (250 mg/kg) was administered i.p., 21 days before killing the animals in order to study cell survival in the hippocampus. Scale bar in B corresponds to 100 μm. (C) Number of $BrdU^+$ cells in the DG counted in subgranular zone (SGZ) and granular cell layer (GCL) and thirds (1/3, 2/3 and 3/3) corresponding to the GCL, *$p<0.05$.

modest trend to increase was found in astrocytes from diabetic rats in CA1 and DG regions as it is shown in Fig. 4D.

Marked decreased blood vessel area in the diabetic GK GCL

Blood vessels of a vast range of gauges were specifically labeled in brain slices using vWF immunohistochemistry. The positive area for this marker was measured in the GCL of both groups. As shown in Figs. 5A–C, the percentage of the GCL area covered by vWF^+-vessels was nearly 67% lower in GK than in Wistar rats (Wistar, 7.8 ± 1.3 *vs* GK, 3.3 ± 0.4% of GCL area occupied by vWF^+-blood vessels, $p<0.05$). GCL reference areas were similar between the 2 groups (Wistar, $116{,}268 \pm 15{,}062$ μm^2 *vs* GK, $114{,}461 \pm 4087$ μm^2, $p=0.91$), indicating that the differences relative to vWF were attributable specifically to this parameter only.

Decreased branching points of vWF vessels in the diabetic GK hilus

The number of vWF vessels branching points per mm^2 was notably decreased in GK rats compared with controls (Wistar 133.4 ± 10.04 vs GK 73.4 ± 6.05, $p<0.005$) as it is shown in Fig. 5E.

Decreased glucocorticoid receptor (GR) number in diabetic GK CA1 hippocampal area

The density of pyramidal cells positively labeled by GR immunohistochemistry was calculated in CA1 hippocampal area from Wistar controls and GK rats. As shown in Fig. 6, the diabetic strain exhibited a 25% decrease in the number of GR-expressing

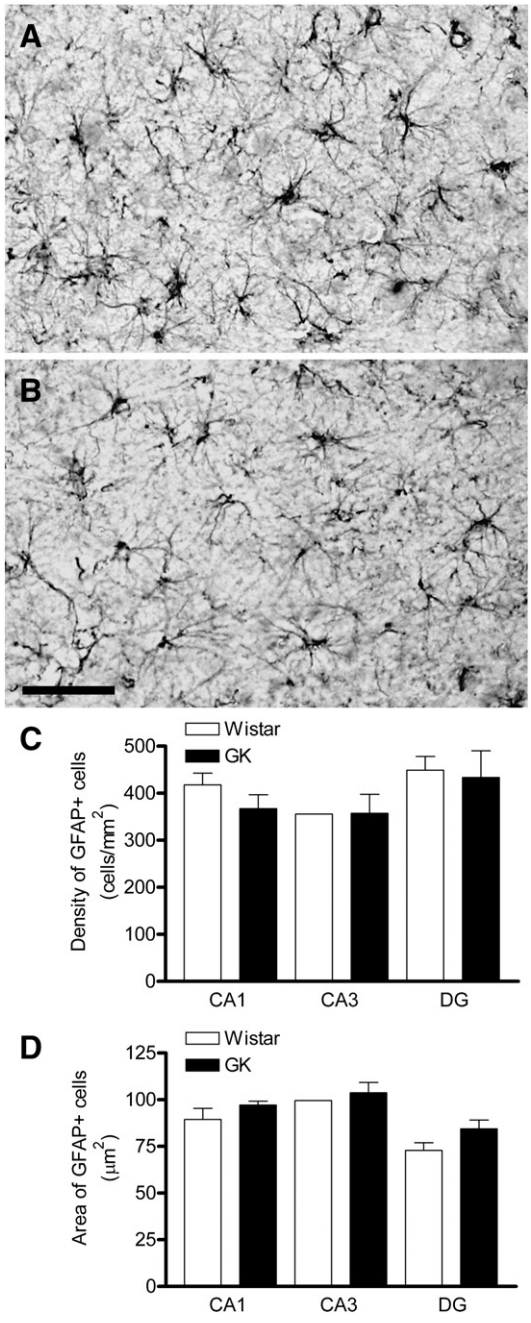

Fig. 4. Glial fibrillary acidic protein (GFAP) immunolabeling in the DG of Wistar (A) and GK (B) rats. Scale bar in B corresponds to 100 μm. (C) Density of $GFAP^+$ cells/mm^2 counted under CA1, CA3 region and DG. (D) GFAP+ cell area (in μm^2) corresponding to cells under CA1, CA3 and DG areas. No significant differences were found between experimental groups.

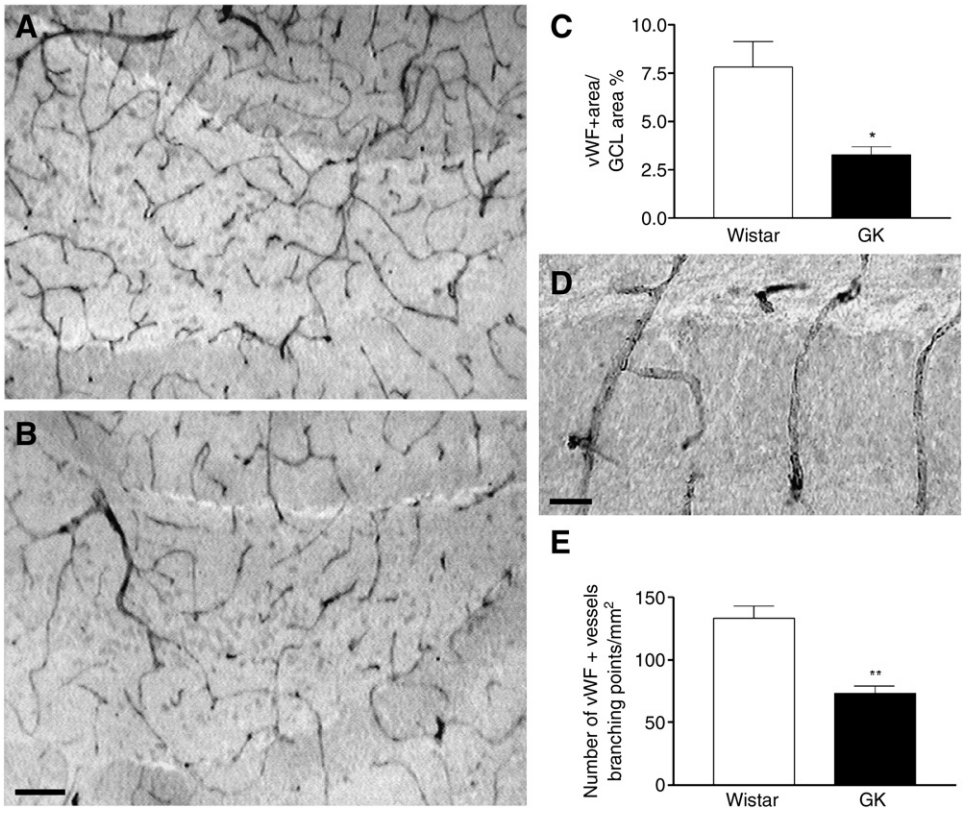

Fig. 5. von Willebrand Factor (vWF) immunohistochemistry in the DG from Wistar (A and D) and GK (B) rats. Scale bars in B and D correspond to 100 and 20 μm respectively. C vWF$^+$ area per GCL in percentage in the experimental groups, *$p<0.05$. (E) Number of vWF+ vessels branching points per mm^2 in the GCL expressed measured as it is explained in the Materials and methods section, **$p<0.01$.

cells in comparison to control rats (number of GR$^+$-cells per counting frame: Wistar, 7.6 ± 0.3 *vs* GK, 5.7 ± 0.2, $p<0.001$).

Discussion

In contrast to T1D, where marked hippocampal astrogliosis, loss of hilar neurons and poor neurogenesis ability have been reproducibly described in different rodent models together with cognitive impairment (Alvarez et al., 2009; Biessels and Gispen 2005; Revsin et al., 2008; Revsin et al., 2009), little is known about potential brain alterations in T2D. Here, we demonstrated anomalies of hippocampal neurogenesis and its microenvironment in type 2 diabetic 16-week-old GK rats.

Our first finding was that DG neurogenesis in diabetic GK rats is characterized by increased proliferation of progenitor cells and differentiation into immature neurons, but deficient survival ability: indeed, while the proliferation rate in GK DG exhibited near 130% increase *vs* Wistar, the cell survival capacity was only 15% higher. However, altered neurogenesis in the GK DG is not associated with changes in the number or shape of astrocytes. Our data on DG neurogenesis confirm those obtained in 18-week-old diabetic GK rats (from Taconic) (Lang et al., 2008). These authors also showed that neurogenesis is not modified in younger normoglycemic GK rats, indicating the deleterious effect of chronic hyperglycemia itself. In addition, they demonstrated that diabetic GK progenitors fail to respond *in vitro* to growth factors. These alterations might be responsible for longer-lasting cognitive impairments, which have been observed following ischemia in diabetic GK rats than in Wistar controls (Moreira et al., 2007).

Evidence for stimulated progenitor proliferation in neurogenic areas has been observed after brain insults (Grote and Hannan 2007; Kaneko and Sawamoto 2009; Ming and Song 2005; Verret et al., 2007). Immediately after an ischemic lesion, the expression of angiogenesis-related genes, including vascular endothelial growth factor (VEGF), is markedly increased in the damaged region (Hayashi et al., 2003). These factors are known to promote the production of new neurons (for review, see Ruiz et al., 2009). Based on these data, adult neurogenesis might be seen as an intrinsic compensatory response for self-repair, which depends upon angiogenesis.

Alterations of replicating neuronal progenitors exist also in AD. In an AD mouse model with a genetic mutation causing β-amyloid peptide accumulation, stem cell proliferation and survival/differentiation is decreased in the DG and SVZ, while neurogenesis is increased in another model (Jin et al., 2004a; Verret et al., 2007). In humans with AD, increased numbers of proliferating cells and immature neurons have been observed in a post-mortem study of senile AD brains (Jin et al., 2004b), while proliferating cells were found to be non-neuronal in presenile AD brains (Boekhoorn et al., 2006). There is also evidence that the cerebral vasculature, which undergoes changes during normal aging (Spangler et al., 1994), might participate in AD development. In AD mice, vessel density is reduced in vulnerable brain regions before amyloid plaques appear, suggesting that vessel defects might contribute to the pathogenesis of the disease (Niwa et al., 2002).

As developed above in various pathophysiological situations, neurogenesis appears to be tightly linked to vasculature, and particularly to angiogenesis capability (see for review, (Zacchigna et al., 2008). We hypothesized that the defective DG survival/differentiation in the diabetic GK rat could be linked at least partly, to vessel alterations. Our second main finding was a net reduction in blood vessel area and branching in dentate gyrus from diabetic GK rats. Our hypothesis was based on the following: 1) cerebrovascular diseases are among the principal causes of death and disability in human diabetes (Brownlee 2001; Creager et al., 2003) 2) vessel abnormalities are found in numerous neurological disorders and often parallel neuron loss; these abnormalities

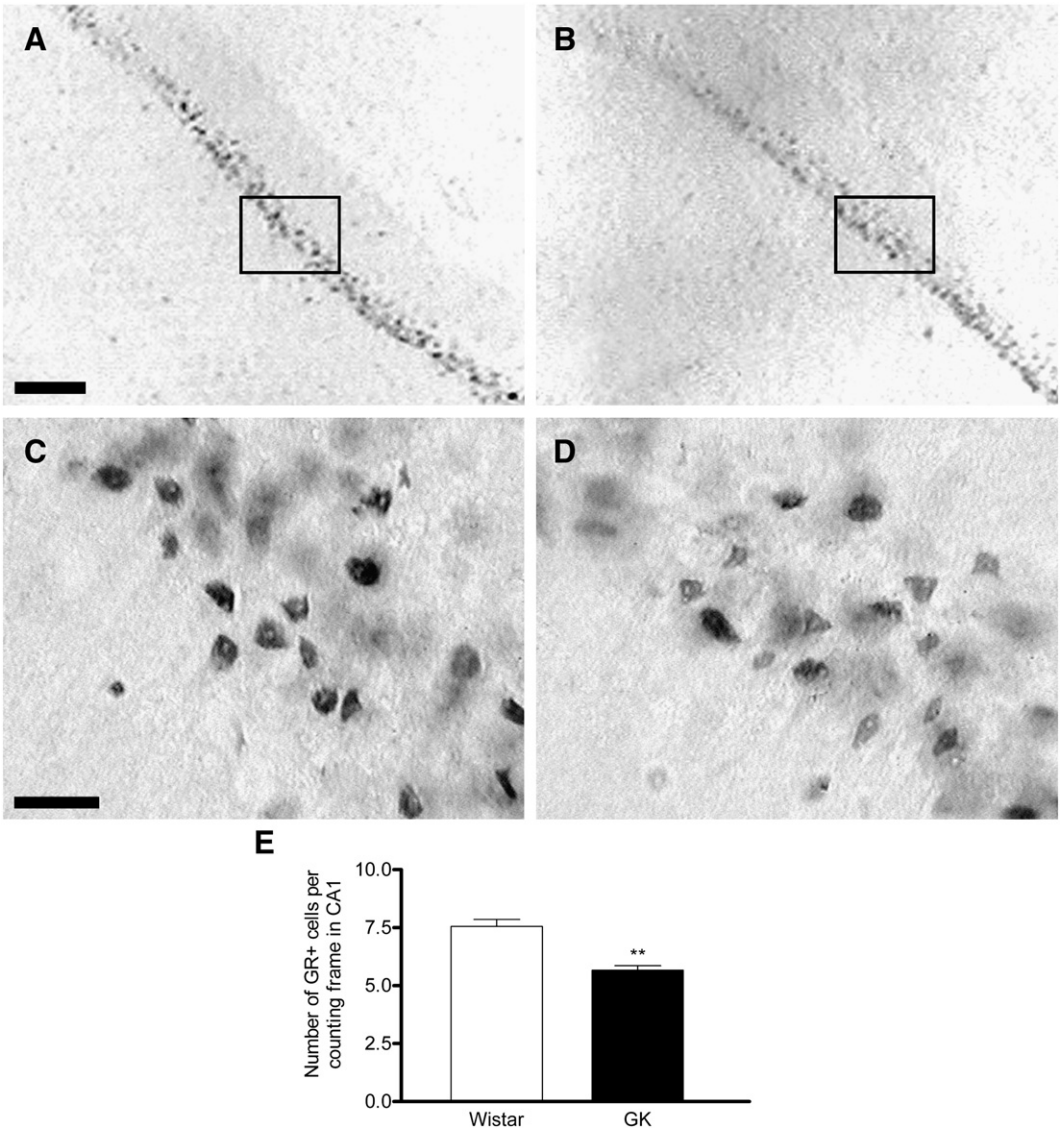

Fig. 6. Glucocorticoid receptor immunohistochemistry in the hippocampal CA1 area of Wistar (A and C) and GK (B and D) rats. Scale bars in A and C correspond to 100 and 20 μm respectively. (E) Number of GR^+ cells per counting frame in CA1, $^{**}p<0.01$.

include vessel regression, hypoperfusion, endothelial degeneration, abnormal vessel growth, size and shape (Zacchigna et al., 2008); 3) vascular disorders increase the risk of dementia, being diabetes associated with high risk of vascular dementia and, probably, AD (Nelson et al., 2009; van den Berg et al., 2006; Xu et al., 2009); 4) neuroimaging data suggest that the increased risk of cognitive decline and dementia in diabetic patients is due to dual pathological processes involving cerebrovascular damage and neurodegenerative changes (van Harten et al., 2006); and 5) in diabetic GK rats, endothelial dysfunction is a characteristic of macrovessels (Harris et al., 2005) and microangiopathy is also present, particularly at the islet level (Lacraz et al., 2009).

Pathophysiologically, the contributors to brain alterations in T2D are: hyperglycemia; hyperinsulinemia and its cause, insulin resistance: indeed, hyperinsulinemia and insulin resistance induce several deleterious effects in the brain interfering with the central role that insulin plays in the CNS; hyperleptinemia; obesity; advance glycation end-products, oxidative stress and inflammation; and HPA axis dysfunction. These alterations are commonly observed during aging, and T2D is more prevalent with increasing age (Wrighten et al., 2009). GK rats, a nonobese model of T2D, exhibit successively, from birth onwards, hyperlipidemia (particularly hypercholesterolemia, hypertriglyceridemia and elevated free fatty acids), mild hyperglycemia and hyperleptinemia, hyperinsulinemia and insulin resistance, before β-cell exhaustion (Portha et al., 2009). There is also growing literature to support the modulating effect of leptin on the CNS and, particularly, the facilitation of hippocampal synaptic plasticity (Morrison 2009). Interestingly, chronic leptin administration to adult mice increase hippocampal cell proliferation without effect on survival via Akt and STAT 3 signalling pathway (Garza et al., 2008). In GK rats, where hyperglycemia, hyperleptinemia and insulin resistance correlate with hippocampal damage and impaired learning (Moreira et al., 2007), hyperleptinemia might stimulate their DG proliferation, as long as they do not become leptin-resistant.

The last contributor to brain alterations in diabetic GK rats could be a dysregulation of the HPA axis, as our main findings are stress-compatible circulating levels of corticosterone, associated with a down-regulation of glucocorticoid receptor (GR) in the hippocampus. The low affinity GR type facilitates the termination of a stress response in the brain via a negative feedback loop (de Kloet et al., 1998). Glucocorticoids elicit insulin resistance in the hippocampus and many pathophysiological conditions are associated with HPA axis dysregulation, including aging, affective disorders, and metabolic diseases, particularly T2D. Concerning neurogenesis it is well

established that high corticosterone levels suppress proliferation and regulate survival in the DG while aged rodents, which have reduced GR expression in the CA1 region, exhibit attenuation of brain glucocorticoid negative feedback, high circulating corticosterone and decreased hippocampal neurogenesis (Ferrini et al., 1999; Mizoguchi et al., 2009; Saravia et al., 2007); moreover, the lowered neurogenic potential of old rats has been recently associated to a reduced vascular density (Hattiangady and Shetty 2008). GR are implicated in DG neurogenesis alterations and cognitive impairment in the insulin-dependent (T1D) streptozotocin-induced model and in the insulin-resistant (T2D) obese db/db mouse model, both of which have high glucocorticoid levels. These alterations are reversed when physiological levels of corticosterone are maintained in both diabetic models (Revsin et al., 2009; Stranahan et al., 2008). In nonobese GK rats, it should be noted that, at variance with *db/db* mice, we found an elevated rate of DG proliferation and differentiation into immature neurons despite high corticosterone levels, probably because db/db mice, due to their leptin receptor defect, lack potentially leptin-stimulating effect on neurogenesis, as GK rats might have. Additionally, serum leptin levels are decreased in T1D patients and animal models (Motyl and McCabe 2009). Therefore, in GK rats, a "compensatory" response, induced by leptin among other factors, might partly mask the glucocorticoid-mediated inhibition on DG proliferation, leading to proliferation rather than inhibition.

Finally, taking into account the tight relationship between neural stem cells and blood vessels (Kuhn et al., 2001), glucocorticoids can affect neurogenesis indirectly, via their effects on endothelial cells. In this regard: 1) in the hippocampus, chronic stress inhibits the proliferation of vascular-associated cells more strongly than that of cells not associated with vessels, together with VEGF and Flk-1 protein expression (Heine et al., 2005); and 2) there is a differential inhibition of neurogenesis and angiogenesis by corticosterone in rats stimulated with electroconvusive seizures, suggesting that corticosterone mediated-inhibition results in a vascular network incapable of sustaining enhanced neurogenesis for an extended period of time (Ekstrand et al., 2008). In addition, in the context of T2D and atherosclerosis, defects in endothelial precursor cells have been recognized (Adams et al., 2007). In this line, diabetic GK rats present a myocardial microvascular endothelial deficiency linked to decreased expression of VEGF and its receptors that might be responsible for impaired cardiac angiogenesis (Wang et al., 2009). Therefore, in GK rats, both deleterious glucocorticoid effects and defective angiogenesis may explain the vessel rarefaction seen in hippocampus and adapted neuronal survival capability.

In conclusion, our results show that diabetic GK rats have an aberrant DG neurogenesis, which could be secondary to mild neurodegeneration, acting as a brain compensatory strategy. Despite a small initial upregulation of cell proliferation, complex interactions of metabolic and endothelial dysfunctions render the neurogenic GK microenvironment unable to preserve the survival of newborn cells.

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