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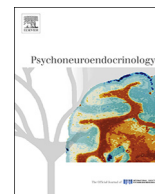
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Insulin treatment partially prevents cognitive and hippocampal alterations as well as glucocorticoid dysregulation in early-onset insulin-deficient diabetic rats



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ABSTRACT

The diagnosis of Type 1 Diabetes (T1D) in ever younger children led us to question the impact of insulin deficiency or chronic hyperglycemia on cerebral development and memory performances. Here, we sought abnormalities in these traits in a model of streptozotocin-induced diabetes in juvenile rats treated or not by insulin. We made the assumption that such alterations would be related, at least in part, to excessive glucocorticoid exposition in hippocampal neurons.

We have compared 3 groups of juvenile rats: controls, untreated diabetics and insulin-treated diabetics. Diabetes was induced by streptozotocin (65 mg/kg IP/day, 2 consecutive days), at postnatal days 21 and 22 and a subcutaneous pellet delivering 2 U of insulin/day was implanted in treated diabetic rats 3 days later. Three weeks after diabetes induction, cognitive performances (Y maze, object location and recognition tests), in vivo brain structure (brain volume and water diffusion by structural magnetic resonance imaging), and hippocampal neurogenesis (immunohistochemical labeling) measurements were undertaken. Corticosterone levels were evaluated in plasma under basal and stress conditions, and within hippocampus together with 11 β -dehydrocorticosterone to assess 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) activity.

The comparison of the three experimental groups revealed that, compared to controls, untreated diabetic rats showed decreased cognitive performances in Y-maze and object location test ($p < 0.05$), decreased brain and hippocampal microstructure ($p < 0.05$), and decreased maturation and survival of hippocampal newborn neurons ($p < 0.05$). These alterations were associated with increased plasma corticosterone at the baseline nadir of its secretion ($p < 0.001$) and during the recovery phase following a restraint stress ($p < 0.001$), as well as increased hippocampal corticosterone levels ($p < 0.01$) and 11 β -HSD1 activity ($p < 0.05$). As untreated diabetic rats, insulin-treated diabetic rats displayed decreased brain volume and water diffusion ($p < 0.05$ compared to controls) and intermediate memory performances and hippocampal neurogenesis (p value not significant compared to either controls or untreated diabetics). Moreover, they were similar to controls for basal plasma and hippocampal corticosterone and 11 β -HSD1 activity but show increased plasma corticosterone during the recovery phase following a restraint stress similar to untreated diabetics ($p < 0.001$ compared to controls).

Thus, insulin did not completely prevent several hippocampal-dependent behavioral and structural alterations induced by diabetes in juvenile rats which may relate to the higher cognitive difficulties encountered in T1D children compared to non-diabetic controls. Although insulin restored basal corticosterone and 11 β -HSD1 activity (in hippocampus and plasma), the negative feedback regulation of corticosterone secretion after stress

Abbreviations: T1D, type 1 diabetes; 11 β -HSD1, 11 β -hydroxysteroid dehydrogenase type 1; STZ, streptozotocin; DHC, dehydrocorticosterone; OLM, Object location memory; ORM, object recognition memory; FA, Fractional anisotropy; ADC, Apparent diffusion coefficient; DCX, doublecortin

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was still impaired in insulin-treated diabetic rats. Further characterization of insulin control on glucocorticoid regulation and availability within hippocampus is awaited.

1. Introduction

Incidence of type 1 Diabetes (T1D) in children younger than 15 years is increasing worldwide with an estimated overall annual increase of 3.9% (Maahs et al., 2010; Patterson et al., 2009). This increase is predominant in children younger than 5 years old, leading to predicted new cases of T1D in this age group that double in 2020 that of 2005 (Patterson et al., 2009). This is particularly alarming during this pivotal neurodevelopmental period (Biessels et al., 2008). Early-onset diabetes is associated with poorer learning and memory performances (Gaudieri et al., 2008) and considered as an important risk factor for cognitive decline in T1D (Biessels et al., 2008; Lin et al., 2010; Moheet et al., 2015).

The impact of T1D on brain functions has also been investigated in several rodent models, focusing on the hippocampus, a key structure for learning and memory processes (Kamal et al., 1999; Stranahan et al., 2008). However, the majority of these studies examined adults and not juvenile rodents although T1D-associated cognitive alterations are essentially observed in children. To our knowledge, there are only few papers on the brain and cognitive effects of insulin-deficient diabetes in juvenile rats (Rajashree et al., 2011; Sacai et al., 2014; Sasaki-Hamada et al., 2012). Furthermore, rodent models of T1D are generally compared to healthy controls, and a diabetic group receiving insulin treatment is lacking. In these studies on adults and untreated diabetic animals, enhanced levels of glucocorticoids appeared to be critical in T1D-associated impairment of hippocampal functions (Revsin et al., 2009; Stranahan et al., 2008; Zuo et al., 2011). Whether insulin treatment is sufficient to prevent excess glucocorticoid activity and the associated hippocampal alterations in juvenile animals remains unexplored.

In this study, we aimed to address these gaps by investigating the effects of type 1 diabetes on hippocampal-associated functions and the glucocorticoid system in juvenile rats treated or not with insulin. In order to obtain a thorough investigation of the hippocampal

consequences of diabetes, we performed hippocampal-dependent memory tasks and anatomical studies to measure hippocampal microstructure and neurogenesis. In parallel we evaluated glucocorticoid activity and reactivity in hippocampus and plasma.

2. Material and methods

2.1. Animals

All animal experiments were conducted according to our national research institute Quality Reference System, and to relevant French (Directive 87/148, Ministère de l'Agriculture et de la Pêche) and international legislation (Directive 2010/63/UE, Décret n° 2013-118) and approved by Institutional Regional Ethic Committee for animal experimentation (agreement# 5012063-A). The Guide for the care and use of laboratory animals, Eighth edition (2011) was followed. Since prepubertal girls and boys are equally affected with T1D in pediatric cases (Soltesz et al., 2007), males or female rats could have been used. We chose to study rat males only in order to reduce interindividual variability and to better compare with published studies that preferentially used males. Weaned male Sprague Dawley rats (3 week-old, 50–55 g) were purchased from Janvier (Le Genest Saint-Isle, France). Rats were housed two per cage (transparent plastic cages) in a room with a constant airflow system, controlled temperature (21–23 °C), and a 12 h light/dark cycle, lights on at 0700 h. We used gnawing wooden sticks as enrichment. Rats were given ad libitum access to food (standard A04 from Safe, Augy, France) and water. Body weight, and food and water intake were measured once a week. The experimental design of the study is presented in Fig. 1.

2.2. Diabetes induction

In each cohort, upon arrival, rats of the Diabetic groups (n = 28) were injected intraperitoneally (IP) with 65 mg/kg of streptozotocin

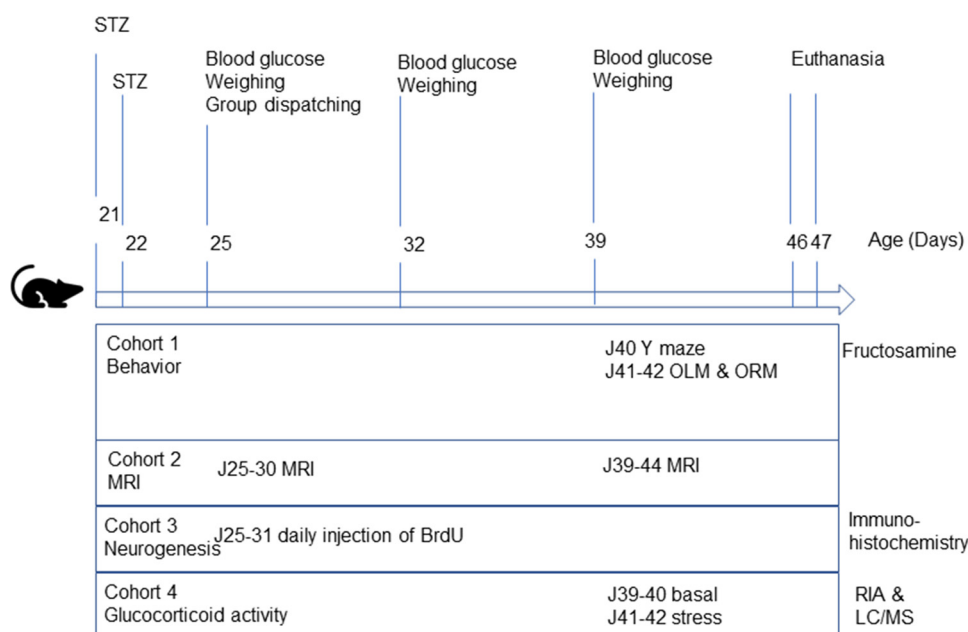


Fig. 1. Experimental design. STZ: Streptozotocin, OLM: Object location memory, ORM: Object recognition memory, MRI: Magnetic Resonance Imaging, LC/MS: liquid chromatography/Mass spectrometry.

(STZ)/day for 2 days. Control rats ($n = 8$) were injected IP with 100 μ l of the STZ buffer (citrate buffer 0.1 M pH 4.5). Blood glucose was measured from a tail blood sampling 3 days after STZ injection (Glucometer Freestyle, Abbott Diabetes Care Ltd, Alameda, USA), and rats that showed hyperglycemia > 200 mg/dl were considered diabetic. Rats that did not respond to STZ were used in the lab for other experiments or practices.

Half of the diabetic rats received a subcutaneous implant of insulin (2 U/day lasting 45 days, Linplant[®], Linshin, Ontario, M1T 2H5 Canada). The 3 experimental groups were submitted to the same isoflurane anesthesia (1.5%) for insertion of the subcutaneous identification chip and the insulin implant placement.

Blood samples on live animals were obtained either by a little incision of the tail for glycemia (5–10 μ l/puncture), or by puncturing the submandibular vein (30–50 μ l/puncture) for other biochemical measures. They were collected in EDTA-coated tubes and centrifuged at 4000g for 10 min at 4 °C in order to obtain plasma, then stored at –80 °C. All rats were killed by decapitation at 45 days of age (in the morning 0800h–1200 h), and the whole brain or the dissected hippocampi were rapidly collected and stored at –80 °C for the assays and measures described below. Truncal blood was also collected and treated as described above to measure plasma corticosterone and 11 dehydrocorticosterone by mass spectrometry (see section 6.2 below).

We measured blood glucose once a week as described above, and, in order to assess global glycemic control over the 3 weeks of experiments, we determined fructosamine plasma concentration, with a glycated serum protein test as described previously (Reusch et al., 1993; Zhu et al., 1993)

2.3. Behavioral testing

All the tests were conducted in the morning (0900h–1200 h) during the 3rd week after diabetes induction, i.e. at 6 weeks of age, in a quiet and dedicated room, under 40 ± 5 lx, and recorded with a camera. Sessions were video-tracked and analyzed with Viewpoint (Lyon, France) by an experimenter blind to the animal experimental group.

2.3.1. Y-maze

See Supplementary Material & Methods for details. Briefly, rats were exposed to two arms in a Y-maze for 10 min and 30 min later, they were exposed again to the Y-maze but with access to a new arm for 5 min. Spontaneous higher exploration of this new arm indicates spatial recognition and was used as a hippocampal-dependent test as previously described (Dellu et al., 2000; Labrousse et al., 2009).

2.3.2. Object location and recognition task

See Supplementary Material & methods for details. Briefly, two similar objects were first presented for 5 min in an open-field during a “training session”. One hour later, rats were allowed to explore during a 5-min test in the same open-field two objects (identical to the one used for training) but one object was moved to a new location. Longer investigation of the displaced object indicates intact object location memory (OLM) (Alteba et al., 2016) a function highly dependent on hippocampus (Ennaceur and Aggleton, 1997; Mumby et al., 2002). One hour after OLM test, rats were submitted to a second 5-min test in the same open-field where they were allowed to explore the familiar object (identical to the one used for training and OLM test) and a novel object of similar size and texture. Longer investigation of the new object indicates intact object recognition memory (ORM). With a short delay, this task is classically not dependent on the integrity of the hippocampus and will control for general or specific memory impairment.

2.4. Magnetic resonance imaging

Longitudinal in vivo nuclear magnetic resonance brain imaging (MRI) was performed in the 3 experimental groups at both 1 and 3

weeks after diabetes induction on a horizontal 4.7T Biospec 47/20 system (Bruker, Ettlingen, Germany) equipped with a 12-cm gradient system (660mT/m). Rats were anesthetized by isoflurane (4% induction, 1.5% maintenance) and respiration was monitored by a ventral pressure sensor. Warmed water circulating in the gradient coil system was used to maintain body temperature. Measurements were performed with Paravision 6.0 (Bruker BioSpin, Karlsruhe, Germany). Anatomical T2-weighted images of the brain were obtained using a RARE sequence (24 axial slices, 1-mm thick, TE 69 ms, TR 5785 ms). Water diffusion was assessed by magnetic resonance Diffusion Tensor Imaging (DTI: 24 axial slices of 0.7 mm, b-value 1000 s/mm², TE 24 ms, TR 2s, 30 directions, total duration 17 min 04 s). Apparent Diffusion Coefficient (ADC) and Fractional Anisotropy (FA) maps were computed using the Paravision 6.0 (Bruker BioSpin, Karlsruhe, Germany) diffusion tensor calculation module. For each rat, two regions-of-interest (ROI), hippocampus and total brain, were manually drawn, on each slice, to determine areas (and then volumes, each slide = 1 mm). ADC and FA were measured in the hippocampus.

2.5. Hippocampal neurogenesis

Rats were killed by decapitation 3 weeks after diabetes induction. The dissected brains were washed with 0.9% sodium chloride and fixed in 4% paraformaldehyde. After a 3-week postfixation period, 50 μ m coronal sections were cut on a vibratome (Leica, Nanterre, France) and stored at –20 °C in an anti-freeze cryoprotectant solution. Free-floating sections were processed with standard immunohistochemical procedures (Lemaire et al., 2006). Cell proliferation was evaluated with Ki-67 labeling using a mouse monoclonal anti-Ki-67 antibody (1:200, Dako by Agilent, Les Ulis, France) and the number of maturing neurons was quantified using doublecortin (DCX) labeling using a goat polyclonal antibody (1:1000, Santa Cruz Biotechnology by Sigma, Lyon, France). BrdU (Bromodesoxyuridine) was used in order to examine hippocampal proliferative cell survival over a 3-week period. All rats of the cohort 3 received a daily IP injection of BrdU (50 mg/kg, Sigma, Lyon, France), dissolved in phosphate buffer (0.1 M, pH 8.4), during 4 successive days upon arrival at 3 weeks of age. Cell survival was measured in the rats killed 3 weeks later, with a mouse monoclonal anti-BrdU antibody (1:200, Dako by Agilent, Les Ulis, France). See Supplementary Material methods for details.

2.6. Glucocorticoid activity

2.6.1. Plasma corticosterone concentration by RIA

Blood samples, obtained by puncturing the submandibular vein during the 3rd week after diabetes induction (i.e. at 6 weeks of age), were collected once in the morning at 0800 h (beginning of the resting period), and the day after in the evening at 1900 h (beginning of the active period). Two days later, rats were submitted to a restraint stress in plastic bags with a breathing hole (Harvard Apparatus, Ealing, Courtaboeuf, France) during 20 min between 9:00 h and 11:00 h. Blood was collected in basal (T0) and post-stress 30 min (T30), 60 min (T60) (peak) and 120 min (T120) (recovery). Times were chosen following our previous results (Marissal-Arvy et al., 2007). Total plasma corticosterone concentration was measured by RIA as previously described (Richard et al., 2010).

2.6.2. Estimation of 11 β -HSD1 activity in plasma and hippocampus by mass spectrometry

Each frozen hippocampus, dissected in basal conditions in the morning, was weighted before starting the extraction procedure to express the amounts of steroids measured as ng/g of tissue. Hippocampi were homogenized in acetonitrile-acetic acid (110:1 v/v, 1 ml) using ultrasonication and the supernatant recovered after centrifugation (10 min, 20 °C, 4500 rpm). Fifty μ l of an internal standard solution (deuterated cortisol, 500 ng/ml) was then added. A second extraction

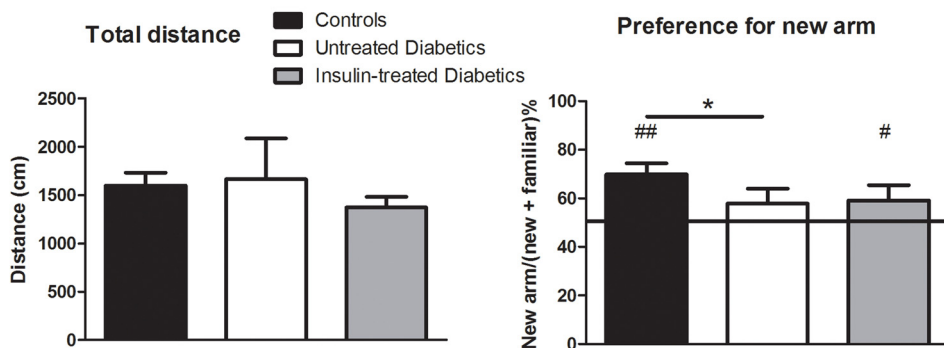
was done with 3 ml of hexane to dissolve the lipids. The upper phase was recovered after 10 min of shaking and centrifugation (5 min, 20 °C, 3500 rpm). The samples were then passed through a Captiva plate (Agilent, Les Ulis, France) and evaporated in a heated water bath (40 °C) under nitrogen flow, and re-dissolved in 100 µl of a 50:50 methanol-water solution. They were then subjected to LC/MS analysis (5500 QTRAP, Sciex) to determine corticosterone and dehydrocorticosterone (DHC) concentrations: peaks area ratios were calculated and compared with corresponding calibration samples. 11β-HSD1

activity was estimated using the corticosterone/DHC ratio. Plasmas were treated with the same protocol but omitting the extraction phase with hexane. See Supplementary Material & Methods for LC/MS details

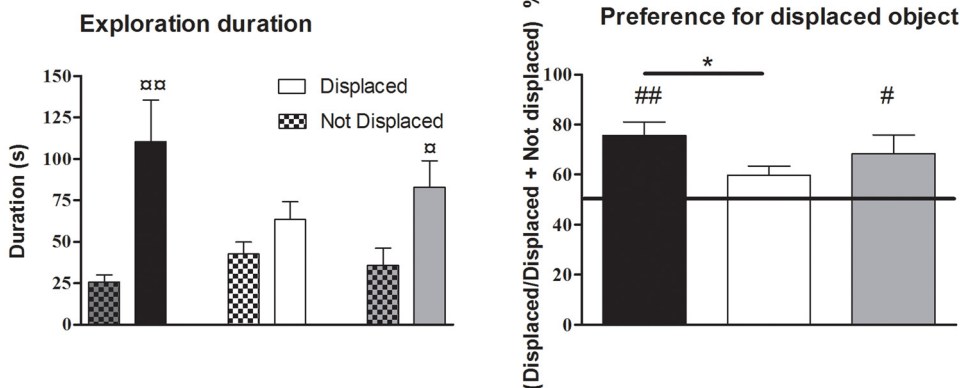
2.7. Statistical analysis

All results were expressed as mean ± SEM. For comparing the 3 groups (controls, untreated diabetics and insulin-treated diabetics) data were submitted to a one-way analysis of variance (ANOVA) with

a. Y-maze Spatial Memory



b. Object Location Memory



c. Object Recognition Memory

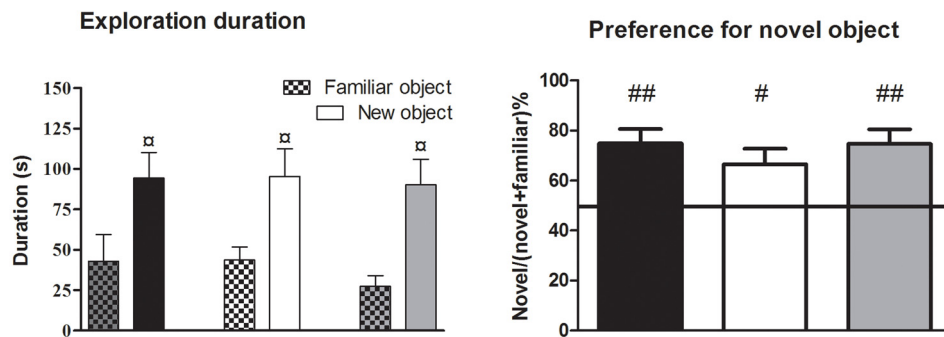


Fig. 2. Hippocampal-dependent memory performances deficits are not prevented by insulin in diabetic juvenile rats. Y-maze test (a), Object location memory test (b) and object recognition memory tests (c). * p < 0.05 different from the control group (One-way ANOVA followed by Fisher LSD post-hoc test), # p < 0.05, ## p < 0.01 compared to chance level (t-test vs. 50%), α p < 0.05, αα p < 0.01 (paired t-test). The horizontal bar represents the chance threshold (50% preference). N = 8 per group.

experimental group as factor. When appropriate (group effect $p < 0.05$), post-hoc comparisons were performed using Fisher LSD test. For the behavioral tasks (Y maze, OLM or ORM tests) in addition to one-way ANOVA that compare groups, each experimental group was assessed for a value of preference, analyzed by a one-sample t -test between the group mean and 50%, i.e. chance level). Two-way ANOVA with treatment as the between factor and time as the repeated factor was used for the kinetics of corticosterone levels. A non-parametric test (Kruskall-Wallis) was used for mass spectrometry measures since the data were not normally distributed; these analyses were followed by Dunns post hoc test.

3. Results

3.1. Does insulin treatment improve memory performances in juvenile diabetic rats?

To validate the animal model, weight, food intake, water intake and glucose levels were recorded during the three weeks of the experimental period and at sacrifice, fructosamine was measured from plasma samples. The untreated diabetic rats displayed a lower weight (One way ANOVA, $F_{2,21} = 60.13$, group effect $p < 0.001$, post-hoc Fischer LSD untreated diabetic vs. controls $p < 0.001$), despite much higher food (One way ANOVA, $F_{2,9} = 175.69$, group effect $p < 0.001$, post-hoc Fischer LSD, untreated diabetic vs. controls $p < 0.001$) and water intake (One way ANOVA, $F_{2,9} = 1224.57$, group effect $p < 0.001$, post-hoc Fischer LSD, untreated diabetic vs. controls $p < 0.001$) than controls, and as expected very high circulating glucose (One way ANOVA, $F_{2,21} = 138.70$, group effect $p < 0.001$, post-hoc Fischer LSD, untreated diabetic vs. controls $p < 0.001$) and fructosamine (One way ANOVA, $F_{2,21} = 187.64$, group effect $p < 0.001$, post-hoc Fischer LSD, untreated diabetic vs. controls $p < 0.001$, Supplementary Material Fig. S1). Diabetic rats treated with insulin displayed values not significantly different from controls except for plasma fructosamine, which was increased. Still, insulin-treated diabetic rats have significantly lower fructosamine than untreated diabetic rats (One-way ANOVA, $F_{2,21} = 187.64$, group effect $p < 0.001$, post-hoc Fischer LSD, insulin

treated vs. untreated diabetics, $p < 0.001$, Fig. S1).

3.1.1. Y-maze spatial memory

Locomotor activity estimated by the total distance was not different between groups (One-way ANOVA, $F_{2,21} = 3.43$, group effect NS, Fig. 2a, left). Preference value for the new arm was significantly lower in untreated diabetic rats than control rats (One-way ANOVA, $F_{2,21} = 6.20$, group effect $p < 0.05$, and Fisher LSD post-hoc test $p < 0.05$, Fig. 2a right) and insulin-treated diabetic group presented intermediary levels as compared to untreated diabetic and control rats (Fisher LSD post-hoc test following one-way ANOVA not significant between insulin-treated diabetic and controls or untreated diabetic rats).

The control and insulin-treated diabetic groups showed a clear preference for the new arm (preference different from chance, Fig. 2a right, one-sample t -test against 50% $p < 0.01$) contrary to untreated diabetic rats which showed no significant preference.

3.1.2. Object location and recognition memory

During training, all groups similarly explored both objects (Supplementary Material Fig. S2). During OLM test performed one hour later, preference value for the novel object was significantly lower in untreated diabetic rats than control rats (One way ANOVA $F_{2,21} = 4.97$, group effect $p < 0.05$, and Fisher LSD post-hoc test $p < 0.05$, Fig. 2b right) and insulin-treated diabetic group presented intermediary levels as compared to untreated diabetic and control rats (Fisher LSD post-hoc test following one-way ANOVA not significant between insulin-treated diabetic and controls or untreated diabetic rats).

The control and insulin-treated diabetic groups showed a clear preference for the moved object (Fig. 2b, one-sample t -test against 50% $p < 0.01$ and $p < 0.05$ respectively) while untreated diabetic rats explored the two objects equivalently (no difference with 50% chance level).

One hour later, in the ORM test, there was no group difference in preference value (One-way ANOVA $F_{2,20} = 0.64$, no group effect) and the three groups spent significantly more time exploring the novel object in comparison to the familiar object (one-sample t -test against 50%,

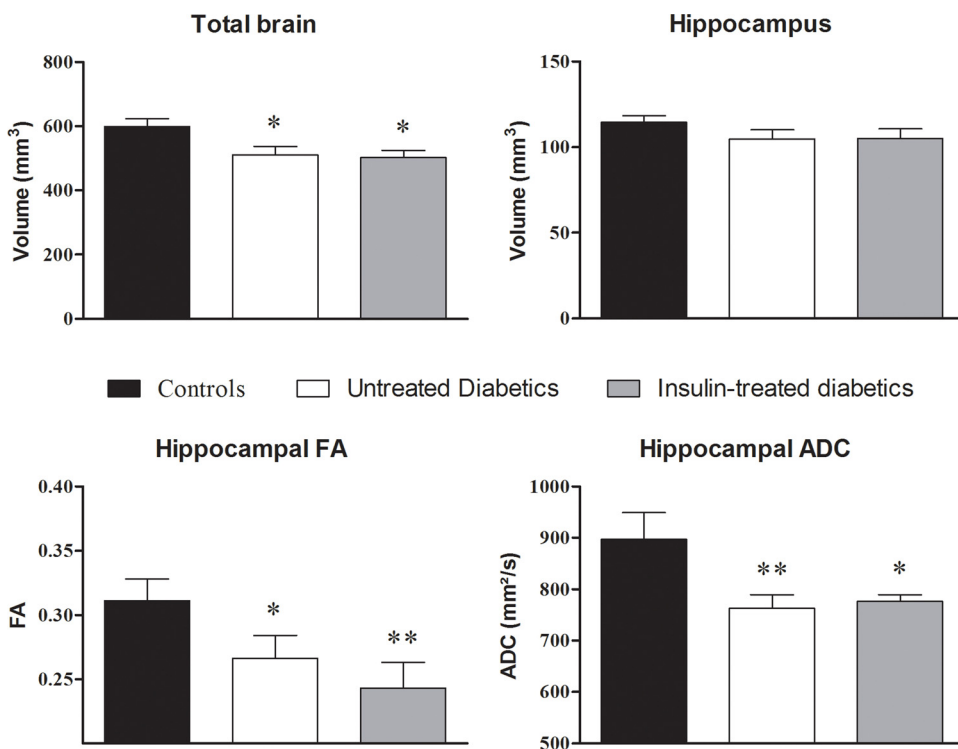


Fig. 3. Hippocampal structural abnormalities are not prevented by insulin in juvenile diabetic rats. In vivo brain MRI data 3 weeks after diabetes induction. * $p < 0.05$, ** $p < 0.01$ compared to the control group (one-way ANOVA followed by Fisher LSD post-hoc test). FA: fractional anisotropy, ADC: apparent diffusion coefficient. N = 5 Controls, N = 6 Untreated diabetics, N = 6 Insulin-treated diabetics.

$p < 0.01$ or $p < 0.05$ for control and insulin-treated, and untreated diabetics respectively, Fig. 2c) indicating intact ORM.

3.2. Does diabetes and insulin treatment affect hippocampal microstructure in juvenile rats?

Total brain and hippocampal volume, ADC and FA, measured by MRI one week after diabetes induction, were similar between groups (Supplementary Material Fig. S3). After three weeks of diabetes, untreated and insulin-treated diabetic rats showed lower total brain volume than control rats (One-way ANOVA $F_{2,14} = 4.70$, group effect $p < 0.05$, and Fisher LSD post-hoc test, $p < 0.05$ for both diabetic groups compared to controls) (Fig. 3). In hippocampus, FA was decreased in both untreated and insulin-treated diabetic groups compared to the control group (One-way ANOVA, $F_{2,14} = 5.14$, group effect $p < 0.05$, post-hoc Fischer LSD, $p < 0.05$ and $p < 0.01$ respectively).

Similarly, ADC was decreased in both untreated and insulin-treated diabetic groups compared to the control group (One-way ANOVA, and $F_{2,14} = 4.80$, group effect $p < 0.05$, Fisher LSD post-hoc test, $p < 0.01$ and $p < 0.05$ respectively) while hippocampal volume was unchanged (Fig. 3).

3.3. Does insulin treatment restore hippocampal neurogenesis in juvenile diabetic rats?

Cell proliferation estimated 3 weeks after diabetes induction, using Ki-67, was found equivalent between the three groups (One-way ANOVA $F_{2,22} = 0.39$, no group effect, Fig. 4a).

However, untreated diabetic rats presented a reduced DCX labeling in neurons with dendritic arborization, indicating a reduced number of neurons in maturation (One-way ANOVA $F_{2,22} = 4.37$, group effect $p < 0.05$, post-hoc Fischer LSD $p < 0.01$). Insulin-treated diabetic

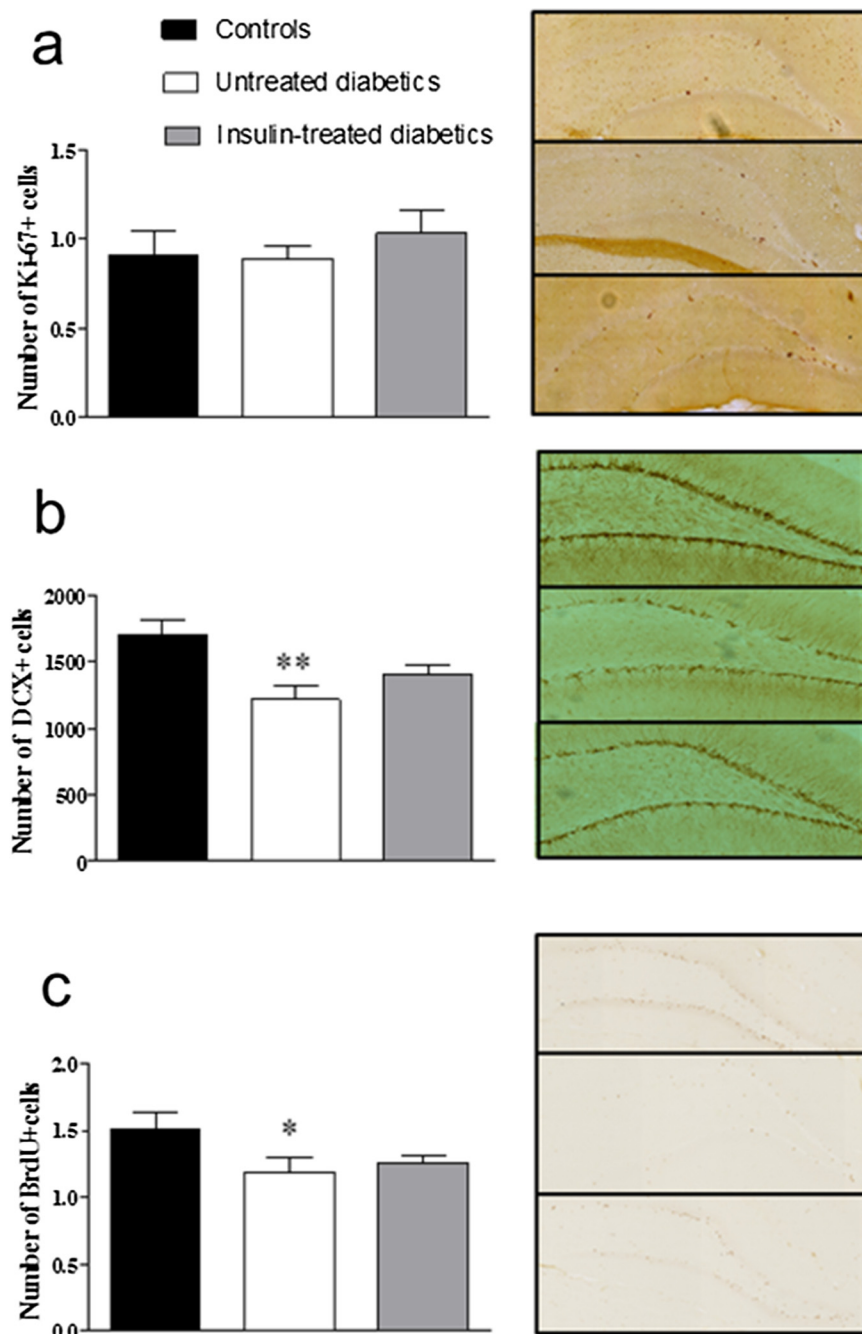


Fig. 4. Reduced neurogenesis is not prevented by insulin in juvenile diabetic rats. Number of Ki-67 positive cells as estimation of neuronal proliferation (a), number of DCX positive neurons with a dendritic arborization, index of neuronal maturation (b), and number of BrdU positive cells over 3 weeks, index of cell survival (c). * $p < 0.05$, ** $p < 0.01$ compared to the control group (one-way ANOVA followed by Fisher LSD post-hoc test). Representative images corresponding to each immunostaining; for each block top: controls, middle: untreated diabetics, bottom: insulin-treated diabetics. N = 8 Controls, N = 8 Untreated diabetics, N = 9 Insulin-treated diabetics.

rats presented only partial restoration of DCX labeling since there was no significant difference when compared to either control or untreated diabetic rats (Fischer LSD post hoc test not significant for insulin-treated group compared to the 2 other groups, Fig. 4b)

Most of the 3-week-old surviving BrdU-immunoreactive cells within the dentate gyrus were isolated, round and large. They were less numerous in untreated diabetic than in control rats (One-way ANOVA $F_{2,22} = 3.18$, group effect $p < 0.05$, and Fischer LSD post-hoc test $p < 0.05$, Fig. 4c). The number of BrdU-labeled cells in insulin-treated diabetic rats was intermediate compared to control and untreated diabetic groups (Fischer LSD post hoc test not significant for insulin-treated group compared to either controls or untreated diabetic rats) (Fig. 4c).

3.4. Does insulin treatment normalize glucocorticoid activity in juvenile diabetic rats?

We found a similar corticosterone concentration, measured by RIA, in our three experimental groups when blood was collected in the evening, i.e. during the circadian peak of corticosterone secretion. However, in the morning, i.e. at the circadian nadir, a higher

concentration of corticosterone was found in untreated diabetic in comparison to control or insulin-treated diabetic rats (one-way ANOVA $F_{2,21} = 14.18$, group effect $p < 0.001$, post-hoc Fischer LSD, $p < 0.001$, Fig. 5a left). After a restraint stress (Fig. 5a right), the peak of plasma corticosterone response was similar between the groups (T30 and T60 min), (two-way ANOVA, group effect $F_{2,15} = 1.46$ not significant, time effect $F_{3,45} = 110.05$, group effect $p < 0.001$, interaction $F_{6,45} = 1.01$ not significant). The lack of interaction is due to similar profiles of the immediate response to stress between groups, that dilute the effect of group differences at T120. When considered independently, the point T120 shows a delayed recovery in untreated and insulin-treated diabetic groups in comparison to control (one-way ANOVA $F_{2,17} = 5.02$, group effect $p < 0.05$, Fischer LSD post-hoc $p < 0.001$).

Using mass spectrometry, we confirmed that plasma corticosterone in the morning (at sacrifice) was increased in untreated diabetic rats, and restored to control levels in insulin-treated diabetic group (Kruskall-Wallis $H = 9.63$, group effect $p < 0.01$, followed by Dunns post-hoc test, $p < 0.05$ untreated vs. insulin treated diabetics, Fig. 5b). Similarly, the concentration of corticosterone in hippocampus was increased in untreated diabetic rats, and restored to control levels in

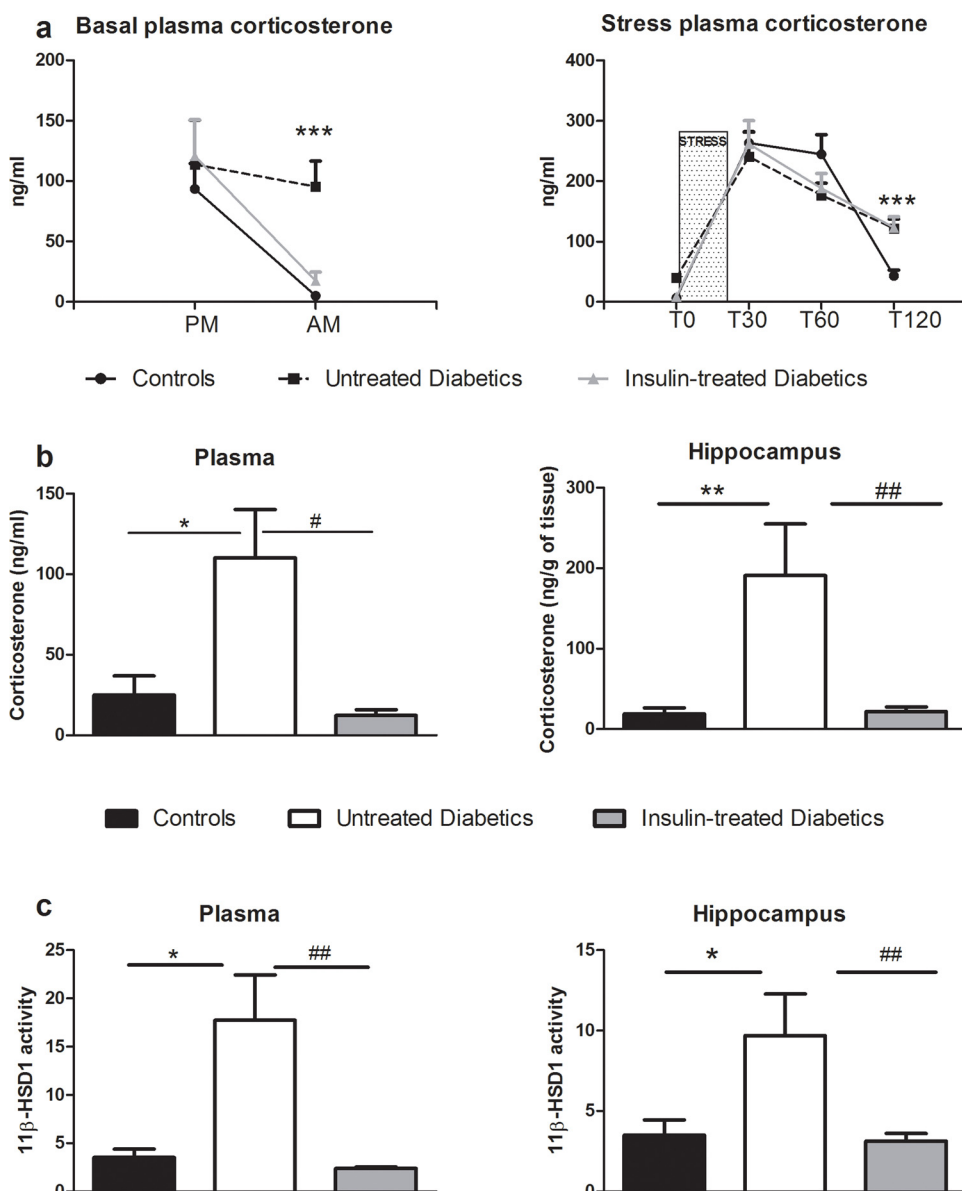


Fig. 5. Excess glucocorticoid and 11β-HSD1 levels are prevented by insulin, except after stress, in juvenile diabetic rats. a. left: in basal condition (PM: measure done in the evening (active phase), AM: measure done in the morning (inactive phase)), right: in response to a 20 min restraint stress. *** $p < 0.001$ compared to the Control group. Plasma corticosterone was analyzed by a two-way ANOVA with treatment as the between factor and time as the repeated factor. $N = 8$ per group. b: Concentration of corticosterone and c: activity of 11β-HSD1 (estimated by the ratio of corticosterone on its derivative 11β-dehydrocorticosterone) in plasma and in hippocampus, quantified by mass spectrometry. * $p < 0.05$, ** $p < 0.01$ Control vs. untreated diabetics, # $p < 0.05$, ## $p < 0.01$ Untreated diabetics vs insulin-treated diabetic rats (Kruskall-Wallis followed by Dunns post-hoc test). The measures were done on the samples collected at euthanasia (in the morning). $N = 6$ controls, $N = 8$ Untreated diabetics, $N = 8$ Insulin-treated diabetics.

insulin-treated diabetic rats (Kruskall-Wallis $H = 13.3$, group effect $p < 0.01$, followed by Dunns post-hoc test, $p < 0.01$ untreated vs. either controls or insulin treated diabetics, Fig. 5b). The corticosterone/dehydrocorticosterone ratio, estimation of 11β -HSD1 activity, was increased in untreated diabetic rats, and restored to control levels in insulin-treated diabetic rats both in plasma and hippocampus (Kruskall-Wallis $H = 13.18$ and $H = 12.17$ respectively, for both plasma and hippocampus group effect $p < 0.01$, followed by Dunns post-hoc test, $p < 0.05$ untreated diabetics vs. controls, $p < 0.01$ vs. insulin treated diabetics, Fig. 5c).

4. Discussion

We studied the detrimental effects of STZ-induced diabetes in juvenile rats on hippocampus and glucocorticoid system, as well as the potential therapeutic effect of insulin on these alterations. After validating our rodent model of diabetes, we found that insulin-deficient juvenile rats displayed alterations in hippocampal-dependent memory tasks. We dissected further these hippocampal effects of diabetes by detecting a similar hippocampal volume but a modified microstructure as indicated by lower fractional anisotropy and a decreased water diffusion using MRI analysis. At the cellular level we found lower hippocampal neurogenesis and survival of newborn cells. Interestingly, these alterations are not all prevented by insulin treatment. These hippocampal alterations are also associated with increased glucocorticoid levels and 11β -HSD1 activity in the blood and within hippocampus and impaired glucocorticoid feedback control after stress. Insulin treatment prevented all these alterations except glucocorticoid feedback control after stress.

The first interest of this study is the analysis of juvenile animals and the inclusion of an insulin-treated diabetic group. Most of researches on STZ-induced diabetes are conducted in adult rather than juvenile rodents, and, surprisingly, do not often include an insulin-treated group, although this aspect is of utmost importance to model clinical observations. Insulin was provided through a subcutaneous pellet which delivered constant levels of insulin contrary to clinical conditions of T1D where insulin is injected several times during the day and according to the children's needs. Nevertheless, we believe that our experimental groups of rats were relevant because the measure of fructosamine, an index of albumin glycation linked to glucose level, indicated that the untreated diabetic group displayed chronic hyperglycemia, as expected in uncontrolled diabetes, while the insulin-treated diabetic group presented decreased fructosamine levels vs untreated diabetics, but significantly over the control group, reflecting a sub-optimal glycemic control as often encountered in T1D children (Supplemental FigS1). Furthermore, the major symptoms of uncontrolled T1D, i.e. decreased body weight and growth, and increased water intake, were totally prevented by insulin treatment (Supplemental FigS1).

Memory performances in the Y maze and OLM tests, were impaired in untreated diabetic juvenile rats while their behavior was not affected in the ORM test (Fig. 2). The first two tasks are strongly dependent on hippocampus, while ORM mainly depends on the integrity of the perirhinal cortex (Okuda et al., 2004). This suggests that the hippocampus is particularly sensitive to STZ-induced diabetes in juvenile rats. These results corroborate previous results obtained in adult rodents, in which insulin-deficient rats were also found to have decreased memory performances in hippocampal-dependent task such as the Morris water maze (Stranahan et al., 2008; Zuo et al., 2011) and novel object-placement recognition (similar to OLM) (Revsin et al., 2009). Thanks to the insulin-treated diabetic group, our study revealed that insulin treatment did not completely prevent the diabetes-induced impairments of hippocampal-dependent memory. Indeed, although the insulin-treated diabetic rats show a significant preference for the new arm of the Y maze and for the displaced object in the OLM test, their performances were intermediate between controls and untreated

diabetic juvenile rats (Fig. 2). Thus, insulin-treated diabetic juvenile rats constitute a good model to study the cognitive alterations observed in T1D children.

To further investigate diabetes-induced alterations of the hippocampus we analyzed hippocampal structure, water diffusion and neurogenesis. The hippocampal volume was not affected by diabetes, unless this measure was normalized to total brain volume or to body weight (Fig. 3). Rather than a relative hypertrophy of the hippocampus, a cellular oedema would be possible given the reduced ADC in the diabetic rats. Studies in children with T1D show significant regional differences in gray and white matter volume, related to cumulative hyperglycemia and glucose variability (Mauras et al., 2015), these effects being more important in children with early-onset of diabetes (Ferguson et al., 2005; Marzelli et al., 2014). Furthermore, parameters of water diffusion, ADC and FA, were decreased by diabetes in our rats, and this decrease was not prevented by insulin treatment suggesting dramatic changes of hippocampal microstructure (Fig. 3). Usually, FA increases with brain maturation, a phenomenon observed in controls rats, however this was not the case in untreated and insulin treated diabetic rats between one and three weeks after diabetes induction. This may be due to a delay in myelination as the latter was found responsible for progressive elevation of FA with age (Beauvieux et al., 2013). Interestingly, reduced electrophysiological properties were detected in the hippocampus of juvenile and adult insulin-deficient rats (Sasaki-Hamada et al., 2012; Stranahan et al., 2008). Regarding cellular changes, we found important changes within the dentate gyrus with a lower number of newborn neurons presenting a dendritic arborization, as shown by the DCX labeling, and of newborn cells survival (Fig. 4). In adult rodent studies, cell survival was also found decreased in insulin-deficient animals. However, cell proliferation estimated with Ki-67 was also found decreased in adult rodents (Revsin et al., 2009; Stranahan et al., 2008) contrary to our finding in juvenile animals. This could be related to the drastically higher levels of proliferation of progenitors in the dentate gyrus of juveniles compared to adults (Cushman et al., 2012; He and Crews, 2007).

Because glucocorticoid excess has been directly linked to decreased hippocampal neurogenesis and cognitive deficits in STZ-induced diabetes of adult rodents (Revsin et al., 2009; Stranahan et al., 2008; Zuo et al., 2011), it was important to evaluate glucocorticoid regulation in juvenile models of T1D. We detected elevated plasma concentration of corticosterone in untreated diabetic rats, as expected with such a chronic metabolic stress, but only at the nadir of secretion (Fig. 5a). In response to a restraint stress, the peak of secretion of corticosterone was similar between experimental groups, contrary to the recovery that was delayed in untreated diabetic rats, suggesting impaired feedback mechanism. Although insulin treatment prevented the increased levels of corticosterone in basal conditions, there was no beneficial effect under stress conditions (Fig. 5a). Within hippocampus, corticosterone concentration was also increased at the nadir in untreated diabetic rats, as well as 11β -HSD1 activity (Fig. 5b & c). Hyperactivation of plasma and hippocampal 11β -HSD1 confirms data we obtained previously in another cohort of insulin-deficient juvenile rats (Rougeon et al., 2017), suggesting that this enzyme elevation may be the cause of glucocorticoid excess and consequently of altered hippocampal function. The impaired negative feedback observed after stress may involve an altered neuronal environment that may be related to our microstructural and neurogenesis data. We found that insulin treatment prevented both corticosterone and 11β -HSD1 excess in our experimental conditions (Fig. 5c), which is congruent with our previous study in which insulin was injected IP or subcutaneously (Rougeon et al., 2017). However, in a clinical study, we found elevated 11β -HSD1 activity in the morning urine of T1D children even in the presence of insulin treatment (Barat et al., 2013). Thus, the regulation of 11β -HSD1 activity by insulin should be investigated further, for example by investigating the effect of varying doses of insulin on 11β -HSD1 activity in vitro. An interesting perspective would be to evaluate the potential beneficial effect of 11β -

HSD1 inhibition in untreated and insulin-treated diabetic juvenile rats in order to decipher the role of 11 β -HSD1 in the hippocampal and cognitive alterations of diabetes in juvenile rats. Of note, the important role of hippocampal 11 β -HSD1 was recently shown in rodent models of aging (Sooy et al., 2010; Wheelan et al., 2015), suggesting that it may be the case also in T1D.

To conclude, insulin treatment did not completely prevent several diabetes-induced alterations in juvenile rats (hippocampal-dependent memories, hippocampal neurogenesis and microstructure, as well as glucocorticoid response to stress). Ultimately, these data should facilitate the evaluation and development of new pharmacological interventions that could successfully prevent the detrimental effects of diabetes on brain function in children.

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Contribution statement

NMA, PB, JBC, JB, GF & MPM contributed to conception and design, analysis and interpretation of data, writing and revising the article

NMA, MNC, AS, CDC, MCB, JCH, KT, SV, AKBS contributed to acquisition of data, analysis and interpretation and revising the article.

All authors provided final approval of the version to be published.

Conflict of interest

P Barat has been paid lecture and conference fees by Eli Lilly and Novo Nordisk, but reports no commercial sponsorships or industry-based research supports. All other authors declare no conflicts of interest that might bias this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.psyneuen.2018.04.016>.

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