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Article

Memory deficits in a juvenile rat model of type 1 diabetes are due to excess 11β-HSD1 activity, which is upregulated by high glucose concentrations rather than insulin deficiency

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Abstract

Aims/hypothesis Children with diabetes may display cognitive alterations although vascular disorders have not yet appeared. Variations in glucose levels together with relative insulin deficiency in treated type 1 diabetes have been reported to impact brain function indirectly through dysregulation of the hypothalamus–pituitary–adrenal axis. We have recently shown that enhancement of glucocorticoid levels in children with type 1 diabetes is dependent not only on glucocorticoid secretion but also on glucocorticoid tissue concentrations, which is linked to 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) activity. Hypothalamus–pituitary–adrenal axis dysfunction and memory alteration were further dissected in a juvenile rat model of diabetes showing that excess 11β-HSD1 activity within the hippocampus is associated with hippocampal-dependent memory deficits. Here, to investigate the causal relationships between diabetes, 11β-HSD1 activity and hippocampus-dependent memory deficits, we evaluated the beneficial effect of 11β-HSD1 inhibition on hippocampal-related memory in juvenile diabetic rats. We also examined whether diabetes-associated enhancement of hippocampal 11β-HSD1 activity is due to an increase in brain glucose concentrations and/or a decrease in insulin signalling.

Methods Diabetes was induced in juvenile rats by daily i.p. injection of streptozotocin for 2 consecutive days. Inhibition of 11β-HSD1 was obtained by administrating the compound UE2316 twice daily by gavage for 3 weeks, after which hippocampal-dependent object location memory was assessed. Hippocampal 11β-HSD1 activity was estimated by the ratio of corticosterone/dehydrocorticosterone measured by LC/MS. Regulation of 11β-HSD1 activity in response to changes in glucose or insulin levels was determined ex vivo on acute brain hippocampal slices. The insulin

regulation of 11β-HSD1 was further examined in vivo using virally mediated knockdown of insulin receptor expression specifically in the hippocampus.

Results Our data show that inhibiting 11β-HSD1 activity prevents hippocampal-related memory deficits in diabetic juvenile rats. A significant increase (53.0±9.9%) in hippocampal 11β-HSD1 activity was found in hippocampal slices incubated in high glucose conditions (13.9 mmol/l) vs normal glucose conditions (2.8 mmol/l) without insulin. However, 11β-HSD1 activity was not affected by variations in insulin concentration either in the hippocampal slices or after a decrease in hippocampal insulin receptor expression.

Conclusions/interpretation Together, these data demonstrate that an increase in 11β-HSD1 activity contributes to memory deficits observed in juvenile diabetic rats and that an excess of hippocampal 11β-HSD1 activity stems from high glucose levels rather than insulin deficiency. 11β-HSD1 might be a therapeutic target for treating cognitive impairments associated with diabetes.

Keywords: Glucocorticoids, 11β-Hydroxysteroid dehydrogenase, Type 1 diabetes

Abbreviations

AAV	Adeno-associated virus
Cs	Corticosterone
CTL	Control group treated by vehicle
CTL+UE	Control group treated by UE
D	Diabetic group treated by vehicle

DHC	Dehydrocorticosterone
D+UE	Diabetic group treated by UE
fEPSP	Field excitatory post-synaptic potential
GC	Glucocorticoids
Hippo-CTL	Wild-type mice
Hippo-InsRKD	Mice with specific InsR knockdown in the hippocampus
11β-HSD1	11β-Hydroxysteroid dehydrogenase type 1
InsR	Insulin receptor
$InsR^{lox/lox}$	Insulin $receptor^{lox/lox}$
NMDG	*N*-Methyl-D-glucamine
OLM	Object location memory
PFA	Paraformaldehyde
PPP	Pentose phosphate pathway
STZ	Streptozotocin
UE	UE2316

Research in context

What is already known about this subject?

- Children with type 1 diabetes display cognitive alterations
- 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) activity in the brain modifies cognitive function
- 11β-HSD1 activity is enhanced in children with type 1 diabetes

What is the key question?

- Is excessive 11β-HSD1 activity responsible for the memory deficits associated with juvenile diabetes?

What are the new findings?

- Specific inhibition of 11β-HSD1 activity restores hippocampal-dependent memory
- Insulin has no effect on hippocampal 11β-HSD1 activity
- High glucose levels increase hippocampal 11β-HSD1 activity

How might this impact on clinical practice in the foreseeable future?

- These findings suggest that 11β-HSD1 might be a therapeutic target for treating cognitive impairments associated with diabetes

Introduction

People with type 1 diabetes show modest but well-recognised deficits on neuropsychological tests including cognitive tasks [1–3]. The mechanisms underlying cognitive dysfunction are related not only to diabetes manifestations such as acute hypoglycaemia and chronic hyperglycaemia, but also to diabetes-related CVD and microvascular dysfunction [4]. Children with diabetes are also affected by cognitive alterations even in advance of demonstrable vascular complications, suggesting an important role of blood sugar variations in brain function [5–7]. Besides these direct effects, variations in glucose levels together with relative insulin deficiency in treated diabetes have been reported to impact brain function indirectly through disturbances in the activity of the hypothalamus–pituitary–adrenal axis [8, 9]. Indeed, dysregulated glucocorticoids (GC) exposure is well known to lead to memory alterations [10]. In animal models, enhanced levels of GC are critical in type 1 diabetes-associated impairment of hippocampal-dependent memory processes [11–14].

Interestingly, we have recently shown that GC alterations in children with type 1 diabetes are dependent not only on GC secretion but also on GC metabolism, which is linked to 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) activity [15, 16]. This intracellular enzyme, which is widely distributed and highly expressed in the liver and the hippocampus, regulates the tissue response to cortisol at a pre-receptor step by converting inactive cortisone to biologically active cortisol (dehydrocorticosterone [DHC] to corticosterone [Cs] in rodents). In animal studies, we showed that GC levels and 11β-HSD1 activity were elevated in a juvenile rat model of diabetes in the absence of treatment with insulin. Subcutaneous administration of insulin, which normalised blood glucose levels, partially rescued several hippocampus-dependent

behavioural and structural changes in early-onset insulin-deficient diabetic rats and prevented GC dysregulation by decreasing 11β-HSD1 activity in the hippocampus [17], indicating that enhanced GC exposure within the hippocampus may be involved in type 1 diabetes-induced cognitive impairments.

To investigate the causal relationships between diabetes, 11β-HSD1 activity and hippocampus-dependent memory deficits, this study examined the beneficial effect of 11β-HSD1 inhibition on spatial hippocampal memory deficits in a juvenile diabetic rat model. Furthermore, to understand the origin of the excess 11β-HSD1 activity we evaluated whether the enhancement of hippocampal 11β-HSD1 activity is due to an elevation of brain glucose levels and/or a deficit in hippocampal insulin signalling.

Methods

Animals

All animal experiments were conducted according to our national research institute Quality Reference System and relevant French (Directive 87/148, Ministère de l'Agriculture et de la Pêche) and international (Directive 2010/63/UE, Décret no. 2013-118) legislation, and were approved by the Ministry of Higher Education, Research and Innovation (APAFIS no. 2016100616143057 and no. 12898-2017122216179189). The Guide for the care and use of laboratory animals, 8th edition (2011), was followed.

Rats used for diabetes and 11β-HSD1 inhibitor treatment As prepubertal girls and boys are equally affected with type 1 diabetes [18], male or female rats could have

been used in this study. However, because of the low quantity of 11β-HSD1 inhibitor available to us, we chose to use male rats only. Weaned male Sprague Dawley rats (3 weeks 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, a controlled temperature (21–23°C) and a 12 h light/dark cycle, with lights on at 07:00. 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.

Insulin receptor floxed mutant mice Insulin receptor$^{lox/lox}$ (InsR$^{lox/lox}$) mice [B6.129S4(FVB)-Insr^{tm1khn}/J, The Jackson Laboratory, Bar Harbor, USA] were bred in our animal facility in groups of six to eight in the same condition as the rats, with plastic houses as enrichment in addition to gnawing wooden sticks. After surgery mice were kept two per cage for 3 days.

Ex vivo experiments in mice Naive 6-week-old C57Bl/6J mice (Janvier, Le Genest Saint-Isle, France) were used to perform ex vivo experiments.

Diabetes induction, insulin treatment and 11β-HSD1 inhibitor treatment

Two cohorts of rats were produced. Diabetes was induced in 25 rats in each cohort by i.p. injection of streptozotocin (STZ; Sigma-Aldrich, Merck, Darmstadt, Germany) at 65 mg kg^{-1} day^{-1} for 2 consecutive days. Blood glucose was measured from a tail blood sampling 3 days after the last STZ injection (Glucometer Freestyle, Abbott Diabetes Care, Alameda, USA). Rats that showed hyperglycaemia (>11 mmol/l [*n*=16]) were considered diabetic. Of the 50 STZ-injected rats, 16 were successfully rendered diabetic in cohort 1 and 20 in cohort 2. Control rats were injected

intraperitoneally with 100 µl of STZ buffer (citrate buffer 0.1 mol/l, pH 4.5) [17]. The rats were randomly subdivided into four groups (electronic supplementary material [ESM] Table 1): two control groups treated by vehicle or by 11β-HSD1 inhibitor (UE2316 [UE]) (CTL and CTL+UE) and two diabetic groups treated by vehicle or by 11β-HSD1 inhibitor (D and D+UE). The 11β-HSD1 inhibitor UE was supplied by the University of Edinburgh as previously described [19] and administered 4 days after diabetes induction and until euthanasia at a dose of 10 mg/kg twice daily (08:30 and 18:30) by gavage, that is, 500 µl of a solution of UE (diluted in a vehicle solution of [vol/vol] 38% polyethylene glycol [PEG], 60% NaCl at 0.9% wt/vol and 2% DMSO; Sigma-Aldrich, Merck, Darmstadt, Germany). The dose of treatment administered was adjusted based on weight gain throughout the experimental period. Rats were submitted to an object location memory (OLM) test and the Y-maze (primary experimental outcome) before they were killed by decapitation 3 weeks after diabetes induction in basal conditions in the morning to evaluate hippocampal 11β-HSD1 activity (secondary experimental outcome) and plasma glucose and fructosamine concentrations (tertiary outcomes).

Behavioural testing

All the tests were conducted in the morning (09:00–12:00) during the third week after diabetes induction, that is, at 6 weeks of age, in a quiet and dedicated room, under 40±5 lux. Sessions were video-tracked and analysed with ViewPoint technology (Lyon, France) by an experimenter blind to the animal experimental group.

OLM task An OLM task was performed as previously described [17]. 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 with two objects (identical to the ones used for training), but with one object moved to a new location. Longer investigation of the displaced object indicates good OLM [20], a function highly dependent on the hippocampus [21, 22]. Animals that explored both objects for less than 20 s during the 10 min exposure were excluded. Rats showing lateralisation, that is, a significant preference for one of the two objects (whatever its location), during the training phase were also excluded.

Y-maze memory task A Y-maze task was performed as previously described [17]. Briefly, rats were exposed to two arms in a Y-maze for 10 min; 2 h 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. Animals that explored one of the two arms for less than 20 s during the 10 min exposure of the training phase were excluded.

Hippocampal insulin receptor knockdown mice

Stereotaxic microinjection of adeno-associated virus vectors Thirty seven (one for GFP fluorescence, three per group for western blotting, 15 per group for hippocampal 11β-HSD1) 8-week-old $InsR^{lox/lox}$ mice from the same litter were randomly injected with either an adeno-associated virus (AAV) AAV2/9-CBA.nls-myc-Cre/eGFP or the control AAV2/9-CMV-GFP (homemade by Laboratory Therapie Genique, Inserm U1089, University of Nantes, Nantes, France), allowing comparison between mice with specific InsR knockdown in the hippocampus (Hippo-InsRKD) and wild-type mice (Hippo-CTL), respectively. AAVs were injected bilaterally within the hippocampus (1.5 µl per side of 6×10^{11} viral genome particles per ml at 0.1 µl/min) using a 33 G needle and a stereotaxic frame with blunt ear bars. To accommodate the larger volume of the hippocampus, viruses were injected bilaterally into both

anterior and posterior sites. Coordinates (from bregma) according to the Mouse Brain Atlas [23] were as follows (A, rostrocaudal; L, mediolateral; V, dorsoventral): Hippocampus: A, −2; L, ±2; V, −1.5 and A, −2; L, ±2; V, −2. Anaesthesia was initiated by injection of a mix of ketamine (80 mg/kg) and xylazine (20 mg/kg) administered intraperitoneally. Buprenorphine (0.05 mg/kg, subcutaneously) was injected 30 min before the anaesthesia for pain management. The positions of injection and extent of coverage in hippocampus were confirmed by GFP fluorescence on hippocampus slices 5 weeks after the virus injection. The choice of a ubiquitous promoter guaranteed to target a maximum of cells including those expressing insulin receptor (InsR) and 11β-HSD1. Five weeks after infection, Hippo-InsRKD and Hippo-CTL mice were housed in individual cages 24 h before euthanasia. Animals were randomly killed in the evening, 30 min after a soft contention stress, using isoflurane anaesthesia, to obtain tissues on which to undertake western blot analysis, measurement of GFP fluorescence or quantification of 11β-HSD1 activity in hippocampus (primary experimental outcome).

Western blot analysis In total, 30 µg of 100°C-heated protein from dorsal and ventral hippocampus was subjected to 10% SDS-PAGE and electroblotted on a nitrocellulose membrane (Amersham, Björkgatan, Sweden). The membranes were then cut according to the molecular mass scale at 55 kDa to hybridise separately InsR and GAPDH. After 1 h of saturation (Intercept [TBS] Blocking Buffer, LI-COR, Lincoln, NE, USA) at room temperature, membranes were incubated overnight at 4°C with anti-mouse InsR antibody diluted 1/2000 (sc-57342; Santa Cruz Biotechnology, Santa Cruz, CA, USA [24, 25]) and anti-rabbit GAPDH diluted 1/50,000 (D16H11; Cell Signaling Technology, Beverly, MA, USA). Specific fluorescent secondary antibodies (IRDye 800CW anti-rabbit and IRDye 680RD anti-mouse, LI-COR,

Lincoln, NE, USA) were incubated for 1 h at room temperature. Fluorescence was captured with the Odyssey Imaging system (LI-COR, Lincoln, NE, USA) and quantified using Image Studio Lite Quantification software version 5.2 (LI-COR, Lincoln, NE, USA). The InsR densitometry signal was normalised by dividing it by the densitometry signal obtained with GAPDH antibody on the same sample.

Virus-induced GFP fluorescence within hippocampus Hippo-InsRKD and Hippo-CTL mice were anaesthetised and transcardially perfused with physiological saline (0.9% NaCl [wt/vol]) solution followed by 4% (wt/vol) paraformaldehyde (PFA). Brains were dissected and postfixed in 4% PFA at 4°C and then transferred to 30% (wt/vol) sucrose overnight and frozen in tissue freezing medium. Next, 50 µm coronal sections were cut on a vibratome (Leica, Nanterre, France) and stored at −20°C in an anti-freeze cryoprotectant solution (PBS 1X, 30% [wt/vol] ethylene glycol, 30% [wt/vol] glycerol). Free-floating sections were washed with PBS 1X, mounted on slides with mounting medium (DAPI fluoromont-G, Clinisciences, Nanterre, France) and cover slipped. Spontaneous fluorescence of GFP was imaged with a ×10 objective on a widefield microscope (DM5000, Leica, Nanterre, France) and with a greyscale sCMOS camera for fluorescence (Hamamatsu Flash4.0 V2, Hamamatsu Photonics France, Massy, France).

Ex vivo hippocampal 11β-HSD1 activity analysis

We developed an ex vivo method to study glucose and insulin effects on hippocampal 11β-HSD1 activity. We incubated 350 µm coronal hippocampal slices in six separate wells of a brain slice keeper (Brain Slice Keeper, BSK 6, Automate Scientific, Berkeley, USA) for 3 h at 37°C (Fig. 1a). Briefly, mice were intracardially perfused during euthanasia (exagon/lidocaine: 300/30 mg/kg, i.p.) with ice-cold *N*-

methyl-D-glucamine (NMDG) solution containing the following (in mmol/l, provided from Sigma-Aldrich, Merck, Darmstadt, Germany): 1.25 NaH_2PO_4, 2.5 KCl, 7 $MgCl_2$, 20 HEPES, 0.5 $CaCl_2$, 28 $NaHCO_3$, 8 D-glucose, 5 L(+)-ascorbate, 3 Na-pyruvate, 2 thiourea, 93 NMDG and 93 HCl; pH 7.3–7.4; osmolarity: 305–310 mOsM, bubbled with carbogen gas. After decapitation of naive mice, brains were quickly removed from the skull and 350 µm coronal slices containing the hippocampus were cut at 4°C with a vibroslice (Leica VT1000S, Leica, Nanterre, France). Hippocampus was dissected on each slice and was then transferred to NMDG solution at 34°C for 12 min before being placed in a well of a brain slide keeper at 37°C. Each well contained five hippocampal slices in 4 ml of an artificial cerebrospinal fluid solution containing the following, in mmol/l: 124 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2.5 $CaCl_2$ and 25 $NaHCO_3$, with 2.8 mmol/l (normal glucose) or 13.9 mmol/l (high glucose) of glucose, without or with 20 nmol/l of insulin and with DHC (1.72 µg/ml). 11β-HSD1 activity was estimated by the conversion rate of DHC to Cs after 3 h incubation of the hippocampal slices. To avoid inter-individual variability between each mouse used for the experiment, slices of the left hemisphere were all incubated in the same well and compared, in a different condition, with the well containing slices of the right hippocampus of the same mouse.

In order to control the viability of slices after 3 h of incubation, electrophysiological measurements were performed with recording of field excitatory post-synaptic potentials (fEPSPs) between CA3 and CA1 regions. Electrophysiological measurements were performed as already published [26]. We verified with extracellular field recording that hippocampal slices were functional after 3 h of incubation in incubation medium. Stimulation of the CA3 region induced a clear excitatory response (fEPSP) in the CA1 region in every recorded slice (mean peak

amplitude: −0.64±0.12 mV, *n*=9; Fig. 1b). Paired stimulation with 50 ms interval induced paired-pulse facilitation without difference between high glucose and normal glucose conditions, confirming the viability after 3 h of incubation.

Quantification of steroids by LC/MS

The estimation of 11β-HSD1 activity was performed by calculating the ratio of Cs and DHC concentrations as previously described [17]. All solvents were provided by VWR (VWR International, West Chester, PA, USA). Each frozen hippocampus was weighed before starting the extraction procedure, to express the amounts of steroids measured as ng/g of tissue. Hippocampi were homogenised in acetonitrile/acetic acid (99:1 v/v, 1 ml) using ultrasonication and the supernatant recovered after centrifugation (10 min, 20°C, 3000 *g*). Then, 50 µl of an internal standard solution (^{2}H-labelled cortisol, 500 ng/ml; Steraloids, Newport, USA) was 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, 2000 g). 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. Plasma samples were treated with the same protocol but omitting the extraction phase with hexane. They were then injected to LC/MS analysis (Prominence liquid chromatography system, Shimadzu, Nakagyo, Japan; 5500 Qtrap detector, Sciex, Framingham, USA) to determine Cs and DHC concentrations: peak area ratios were calculated and compared with corresponding calibration samples (provided by Steraloids Newport, USA). For the estimation of 11β-HSD1 activity in the ex vivo study, 1 ml of the hippocampal slice incubation medium was extracted with 3 ml of dichloromethane. The upper phase was removed

after 10 min of shaking and centrifugation (5 min, 20°C, 2000 g) and the organic phase was evaporated in a heated water bath (40°C) under nitrogen flow, and re-dissolved in 100 µl of a 50:50 methanol/water solution before injection into the LC/MS system.

Metabolic studies

Blood glucose levels (measured in tail vein blood, Glucometer Freestyle, Abbott Diabetes Care) were measured weekly after diabetes induction or AAV injection. In order to assess global glycaemic control over the 3 weeks during the diabetes induction experiments, we determined the fructosamine plasma concentration with a glycated serum protein test as described previously [27]. An i.p. GTT (2 g/kg) was performed in Hippo-InsRKD and Hippo-CTL mice 8 weeks after AAV injection and after a 4 h fasting period to control for the absence of impact of the hippocampal InsR deletion on glucose homeostasis. Blood glucose was collected from the tail prick at t=0, 15, 30, 45, 60, 90 and 120 min (Accu-Chek Performa glucometer, Roche, Basel, Switzerland).

Statistical analysis

Statistical analysis was performed using Prism GraphPad 9 (San Diego, CA, USA). All results are expressed as mean ± SEM and individual values are plotted on graphs where possible. A two-way ANOVA, followed by Tukey's post hoc test, was used when comparing data for the four groups (CTL, CTL+UE, D and D+UE), and a two-way repeated measures ANOVA was used when analysis accounted for the kinetics of the i.p. GTT. For the behavioural task (OLM and Y-maze), each experimental group was assessed for a value of preference, analysed by a one-sample t test

between the group mean and 50%, that is, chance level. A Mann–Whitney *U* test was chosen to compare two population samples. Statistical analyses for each comparison are detailed in Table 1. For more details about the exclusion of some animals or data, see ESM Table 2.

Results

11β-HSD1 inhibition prevents hippocampus-related memory deficits in juvenile diabetic rats

Treatment with the 11β-HSD1 inhibitor UE had no substantial effect on blood glucose levels in the type 1 diabetes model. Control or diabetic rats treated with UE displayed similar glucose and fructosamine levels to vehicle-treated controls. However, as expected, diabetic rats with or without UE displayed very high circulating glucose and fructosamine levels compared with non-diabetic controls (Fig. 2a,b), with a significantly altered body weight gain (ESM Fig. 1) and a polyuro-polydipsic syndrome. For this reason, the animals' health was checked on a daily basis.

The hippocampal ratio of Cs to its inactive metabolite (Cs/DHC), for estimation of 11β-HSD1 activity by LC/MS, was dramatically increased in diabetic rats without UE treatment (by nearly a factor of 5) and restored to control levels in UE-treated diabetic rats (Fig. 2c, significant diabetes and UE effect). Plasma 11β-HSD1 activity was also increased in diabetic rats without UE treatment and decreased in UE-treated diabetic rats but did not reach control levels (Fig. 2d, significant diabetes effect but no UE effect).

We evaluated whether specific inhibition of 11β-HSD1 influenced the behaviour of diabetic rats using hippocampal-dependent OLM and a Y-maze. As expected, the untreated control group explored the displaced object more (different from 50%, $p<0.05$). Control mice treated with UE displayed impaired memory for both tasks. Diabetic rats untreated with the 11β-HSD1 inhibitor did not show any preference, confirming previous data showing OLM and Y-maze deficits in diabetic juvenile mice (Fig. 2e,f). However, UE treatment prevented diabetes-induced deficits in both OLM (high tendency with $p=0.052$; Fig. 2e) and Y-maze ($p<0.01$; Fig. 2f) analysis.

Hippocampal 11β-HSD1 activity is not influenced by insulin signalling

To assess the role of insulin in hippocampal 11β-HSD1 activity, we knocked down InsR expression in the hippocampus through the use of AAV-Cre-GFP injection in $InsR^{lox/lox}$ mice. The injection was verified by visualising the immunofluorescence of hippocampal slices from the injected mice (Fig. 3a). Knockdown of InsR decreased InsR protein levels by 70±9.6% in the hippocampus of mice injected with AAV-Cre-GFP (Hippo-InsRKD) in comparison with mice injected with control virus AAV-GFP (Hippo-CTL; Fig. 3b). Change in hippocampal InsR expression was not associated with changes either in glucose tolerance (Fig. 3c) or in 11β-HSD1 activity (Fig. 3d).

Glucose rather than insulin concentrations impact hippocampal 11β-HSD1 activity

To assess the respective role of glucose or insulin, we estimated hippocampal 11β-HSD1 activity ex vivo from acute hippocampal slices incubated for 3 h with or without insulin (20 nmol/l) in the presence of either normal (2.8 mmol/l) or high (13.9 mmol/l) glucose concentrations (Fig. 1a). A significant increase (53.0±9.9%) in hippocampal

11β-HSD1 activity was found in slices incubated without insulin in the high glucose condition compared with the normal glucose condition (Fig. 4a). However, the activity was not affected by variations in the insulin concentration in the normal condition (Fig. 4b) or the high glucose condition (Fig. 4c). Together, these data show that 11β-HSD1 activity is influenced by glucose levels rather than decreased insulin levels or signalling.

Discussion

This study follows up on our previous preclinical and clinical reports that revealed the association between hippocampal GC levels, elevated 11β-HSD1 activity and type 1 diabetes-associated cognitive impairments [15–17]. Here, we examined the causal role of excess 11β-HSD1 activity in hippocampal-dependent memory deficits in a rat model of juvenile diabetes, and investigated the origin of this excess hippocampal 11β-HSD1 activity. By treating diabetic juvenile rats with a specific inhibitor of 11β-HSD1 activity, we were able to prevent hippocampal-dependent memory deficits, revealing the important role of this enzyme. Although systemic variation might have contributed to some of the variation in the hippocampus, the Cs/DHC ratio is about twice as high in the hippocampus as it is in plasma in the diabetic rats. Then, using in vivo decreases in InsR in hippocampal cells of wild-type mice and ex vivo modulation of insulin levels in live hippocampal slices, we demonstrated that higher glucose levels, but not lower insulin levels, increase 11β-HSD1 activity, suggesting a potential mechanism underlying elevated 11β-HSD1 activity in type 1 diabetes.

Our data demonstrating a causal role of 11β-HSD1 activity in diabetes-related cognitive impairments in a juvenile diabetes rat model were expected from our previous studies [15–17] but also from works of others. Indeed, as previously shown in a mouse model of ageing, this enzyme is involved in central nervous system dysfunction, amplifying intracellular GC concentrations and hence their deleterious effects on memory [28, 29]. A role for 11β-HSD1 in cognitive deficits was also reported in human pathological contexts such as post-traumatic stress disorder, Alzheimer's disease, obesity and type 2 diabetes [30–33]. In these pathologies, excessive 11β-HSD1 activity is proposed to contribute to cognitive dysfunction through the exposure of brain tissue to excessive GC levels. Thus, 11β-HSD1 constitutes an interesting therapeutic target for treating cognitive impairments associated with type 1 diabetes. Indeed, several clinical studies have reported on the efficacy of 11β-HSD1 inhibitors at reducing peripheral and brain enzyme activity. Several studies have shown that the inhibitors improved cognitive performance in individuals with type 2 diabetes or with Alzheimer's disease, as reviewed in [34, 35].

From a pathophysiological point of view, it is interesting to better understand the mechanisms by which type 1 diabetes leads to excess 11β-HSD1 activity. Thus, we investigated the origin of excess 11β-HSD1 activity in diabetic juvenile rodents, characterised by variations in glucose and insulin levels. Insulin and glucose levels have already been shown to regulate 11β-HSD1 activity but only in peripheral tissues such as liver and adipocyte cells. Insulin downregulates 11β-HSD1 expression or activity in the liver [36–38] while this activity is increased by glucose in hepatic and adipocyte cells [37, 39]. To our knowledge, only one study has found an increase in hippocampal 11β-HSD1 protein expression in diabetic rats, but the authors neither explored enzyme activity nor separated the action of glucose from that of insulin [40].

In addition, it is important to note that glucose can enter brain cells even if insulin is absent, mainly through the insulin-independent transporters GLUT1 and GLUT3 [41]. Here, we dissected further the role of insulin deficiency and/or high glucose concentrations in the enhancement of hippocampal 11β-HSD1 activity.

Using knockdown of InsR specifically in the hippocampus (Hippo-InsRKD), we demonstrated that deficient insulin signalling in the hippocampus does not affect 11β-HSD1 activity. This lack of insulin modulation was observed while blood glucose levels were controlled, as basal glucose or glucose measured during an i.p. GTT was not different between Hippo-InsRKD and Hippo-CTL mice. In addition, using an ex vivo preparation, we confirmed that insulin has no effect on 11β-HSD1 activity in fresh hippocampus slices. By contrast, we detected a 50% increase in 11β-HSD1 activity when the slices were incubated in a high glucose concentration compared with a physiological brain glucose concentration. Together, these data provide evidence that elevated hippocampal 11β-HSD1 activity during type 1 diabetes is due to increased brain glucose levels rather than insulin deficiency.

A question that remains to be answered is how glucose can modulate 11β-HSD1 activity. It is well known that 11β-HSD1 activity depends on the cofactor NADPH [42, 39]. A large part of the NADPH cellular pool is provided by the reduction of $NADP^+$ during the first oxidative step of the pentose phosphate pathway (PPP). This metabolic pathway involves oxidation of glucose in parallel with glycolysis. Adipocyte and hepatic microsome studies showed that 11β-HSD1 activity depends on the flux of glucose through the PPP: hyperglycaemia increases NADPH levels via the PPP and as a consequence enhances 11β-HSD1 activity [3, 38]. Whether the same mechanism operates in brain cells remains to be demonstrated. Of note, there is a

positive correlation between the PPP flux and glucose levels in brain cells [43], favouring the hypothesis that 11β-HSD1 activity enhancement in the brain is linked to increased glucose levels via the NADPH production of the PPP flux. As the brain glucose concentration is the key element of this mechanism, it may be interesting to consider in future studies the role of brain 11β-HSD1 activity in people with type 2 diabetes. Characterised by systemic insulin resistance, there is a large amount of literature on the importance of the role of insulin in brain function in this context. However, the role of glucose uptake and metabolism in type 2 diabetes is still poorly described [41]. Therefore, based on the preclinical data collected here and in previous clinical studies, treatment with a specific 11β-HSD1 inhibitor may be considered in various hyperglycaemia-related disorders to attenuate cognitive alterations. However, our results were obtained in male models only. Future studies are needed to confirm that the same mechanism is involved in female models.

To conclude, our data contribute to a better understanding of how detrimental effects of type 1 diabetes on brain function can occur in children. High glucose and low insulin levels are known to alter memory performance [9]. This study, together with previous studies [11, 12], supports the hypothesis that elevated GC levels may be one of the major contributors to type 1 diabetes-related cognitive impairments. This may be particularly helpful in finding new therapeutic strategies by utilising 11β-HSD1 inhibitors. Here, we show that 11β-HSD1 is a potential therapeutic target that should now be tested in clinical trials.

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Authors' relationships and activities BRW and SPW are inventors on relevant patents owned by the University of Edinburgh and licensed to Actinogen Medical Ltd. The authors declare that there are no other relationships or activities that might bias, or be perceived to bias, their work.

Contribution statement JB, CB-B, XF, PB, J-BC and M-PM contributed to the conception and design of the study. JB, J-CH, NM-A, M-NC-L, CB-B and M-PM acquired the data. JB, CB-B, SPW, BRW, XF, GF and M-PM analysed and interpreted the data. JB, CB-B, J-CH, XF and M-PM wrote the drafted manuscript and JB, CB-B, NM-A, M-NC-L, SPW, BRW, GF, PB, J-BC and M-PM revised it. All authors approved the manuscript. JB and M-PM are the guarantors of this work.

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Table 1 Statistical results obtained in this study
H

Figure number	Statistical test	*n* per group		Statistical result
Fig. 1				
Interval induced paired-pulse facilitation	Mann–Whitney *U* test	*n*=5 for normal glucose *n*=4 for high glucose		*p*>0.05
Fig. 2				
Blood glucose	Two-way ANOVA	*n*=10 for CTL group *n*=9 for CTL+UE group *n*=8 for D group *n*=7 for D+UE group		Diabetes effect, *F*(1,30)=1238 CTL vs D groups ***p<0.001
Plasma fructosamine	Two-way ANOVA	*n*=10 for CTL group *n*=9 for CTL+UE group *n*=8 for D group *n*=7 for D+UE group		Diabetes effect, *F*(1,30)=677 CTL vs D groups ***p<0.001
Hippocampus 11β-HSD1 activity	Two-way ANOVA followed by Tukey's post hoc test	*n*=10 for CTL group *n*=9 for CTL+UE group; *n*=8 for D group *n*=7 for D+UE group		Diabetes effect, *F*(1,30)=9.65 UE treatment effect, *F*(1,30)=8.91 Interaction, *F*(1,30)=4.83 CTL vs D §§p<0.01, D vs D+UE §§p<0.01
Plasma 11β-HSD1 activity	Two-way ANOVA followed by Tukey's post hoc test	*n*=10 for CTL group *n*=9 for CTL+UE group *n*=8 for D group *n*=7 for D+UE group		Diabetes effect, *F*(1,30)=12.77 UE treatment effect, *F*(1,30)=3.722 Interaction, *F*(1,30)=1.206 CTL vs D §p<0.05
OLM	One-sample *t* test vs 50%	Cohort 1	*n*=8 for CTL group *n*=7 for CTL+UE group *n*=6 for D group *n*=5 for D+UE group	‡p<0.05 compared with chance level
		Cohort 2	*n*=10 for CTL group *n*=10 for CTL+UE group *n*=9 for D group *n*=7 for D+UE group	
Y-maze spatial memory	One-sample *t* test vs 50%	Cohort 1	*n*=8 for CTL group *n*=9 for CTL+UE group *n*=8 for D group *n*=6 for D+UE group	‡‡p<0.01 compared with chance level
		Cohort 2	*n*=10 for CTL group *n*=10 for CTL+UE group *n*=10 for D group *n*=10 for D+UE group	
Fig. 3				
Western blot InsR	Mann–Whitney *U* test	*n*=3 for each group		**p<0.01
GTT	Repeated measure two-way ANOVA	*n*=14 for Hippo-CTL group *n*=13 for Hippo-InsRKD group		Group effect *p*=0.51
Hippocampus 11β-HSD1 activity	Mann–Whitney *U* test	*n*=14 for Hippo-CTL group *n*=12 for Hippo-InsRKD group		Group effect *p*=0.29
Fig. 4				
11β-HSD1 activity of hippocampus slices	Mann–Whitney *U* test	*n*=9 for each group		Normal vs high glucose ***p<0.001 Normal glucose and 0.0 vs 20.0 nmol/l of insulin *p*=0.71 High glucose and 0.0 vs 20.0 nmol/l of insulin *p*=0.75

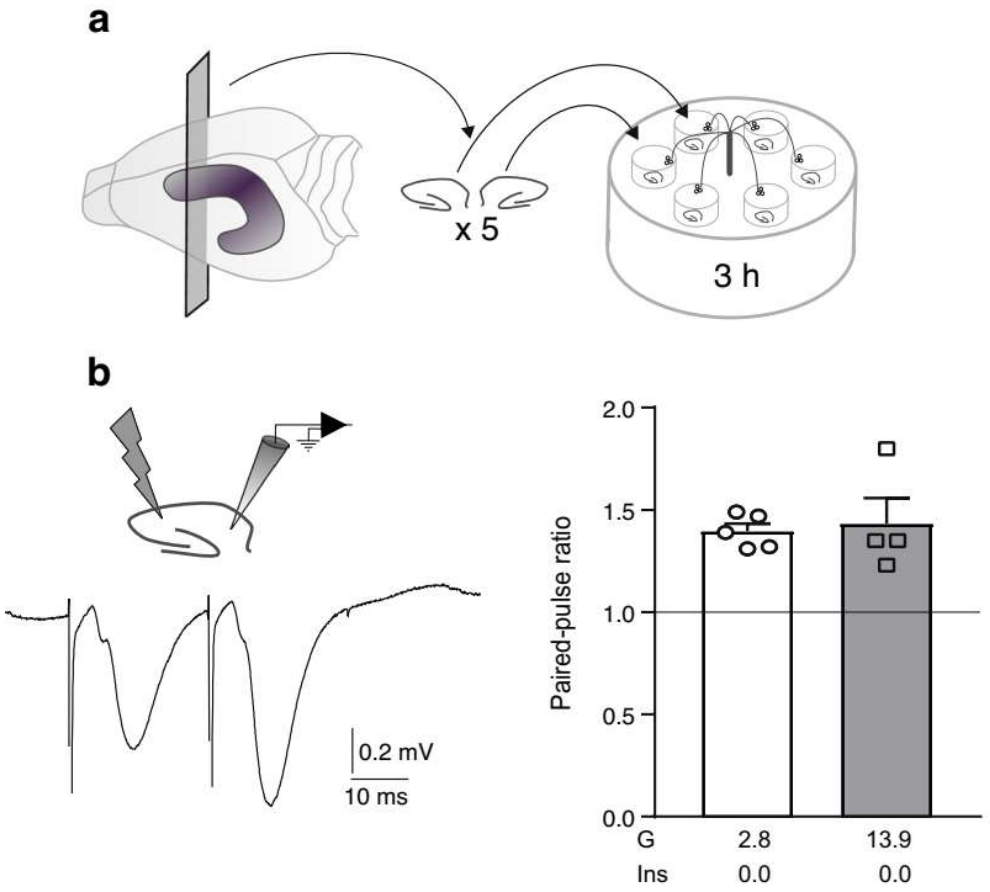

Fig. 1 Experimental methods. (**a**) Ex vivo hippocampal analysis method: coronal hippocampal slices in six separate wells of a brain slice keeper. (**b**) Electrophysiological measurements with the recording of fEPSPs between the CA3 and CA1 regions of the hippocampus in order to control the viability of slices after 3 h of incubation, and paired stimulation with 50 ms interval induced paired-pulse facilitation, confirming the slices' viability after 3 h of incubation. Data are presented as mean ± SEM. G, glucose; Ins, insulin

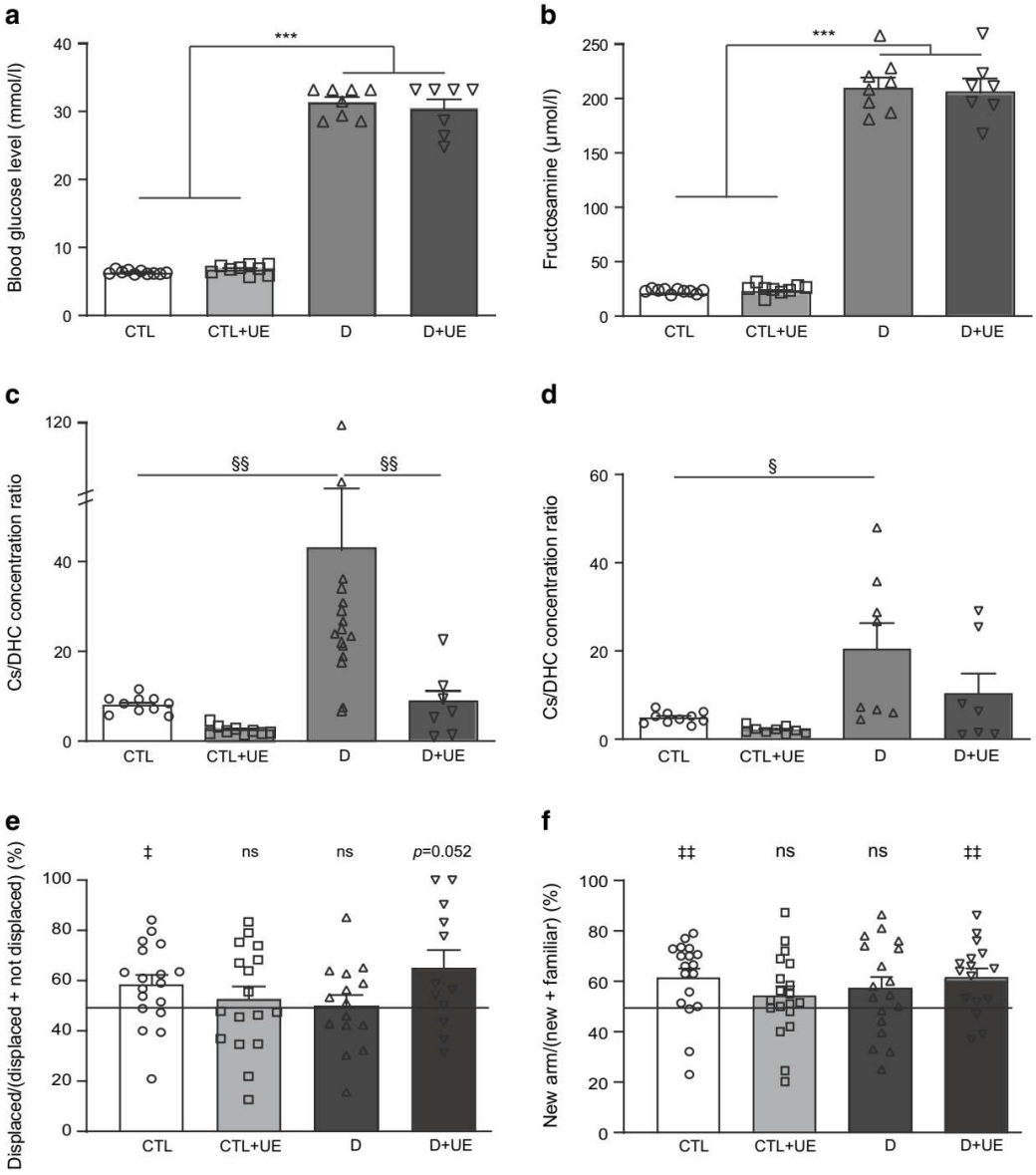

Fig. 2 Inhibition of 11β-HSD1 activity prevents hippocampal-dependent memory performance deficits in diabetic juvenile rats. (**a**) Blood glucose and (**b**) plasma fructosamine levels 3 weeks after diabetes induction by STZ. (**c**) Hippocampal 11β-HSD1 activity estimated by Cs/DHC concentration ratio and (**d**) plasma 11β-HSD1 activity estimated by Cs/DHC concentration ratio. (**e**) Preference for displaced object in an OLM test or (**f**) preference for new arm in a Y-maze test in the control group (CTL) compared with the diabetic (D) group of juvenile rats treated with vehicle or UE. ***p<0.001 and $^{§§}p$<0.01 after post hoc tests of two-way ANOVA. $^{‡}p$<0.05 and

‡‡p<0.01 compared with chance level (50%) by one-sample t test. The horizontal bar represents the chance threshold (50% preference). Data are presented as mean ± SEM. ns, not significant

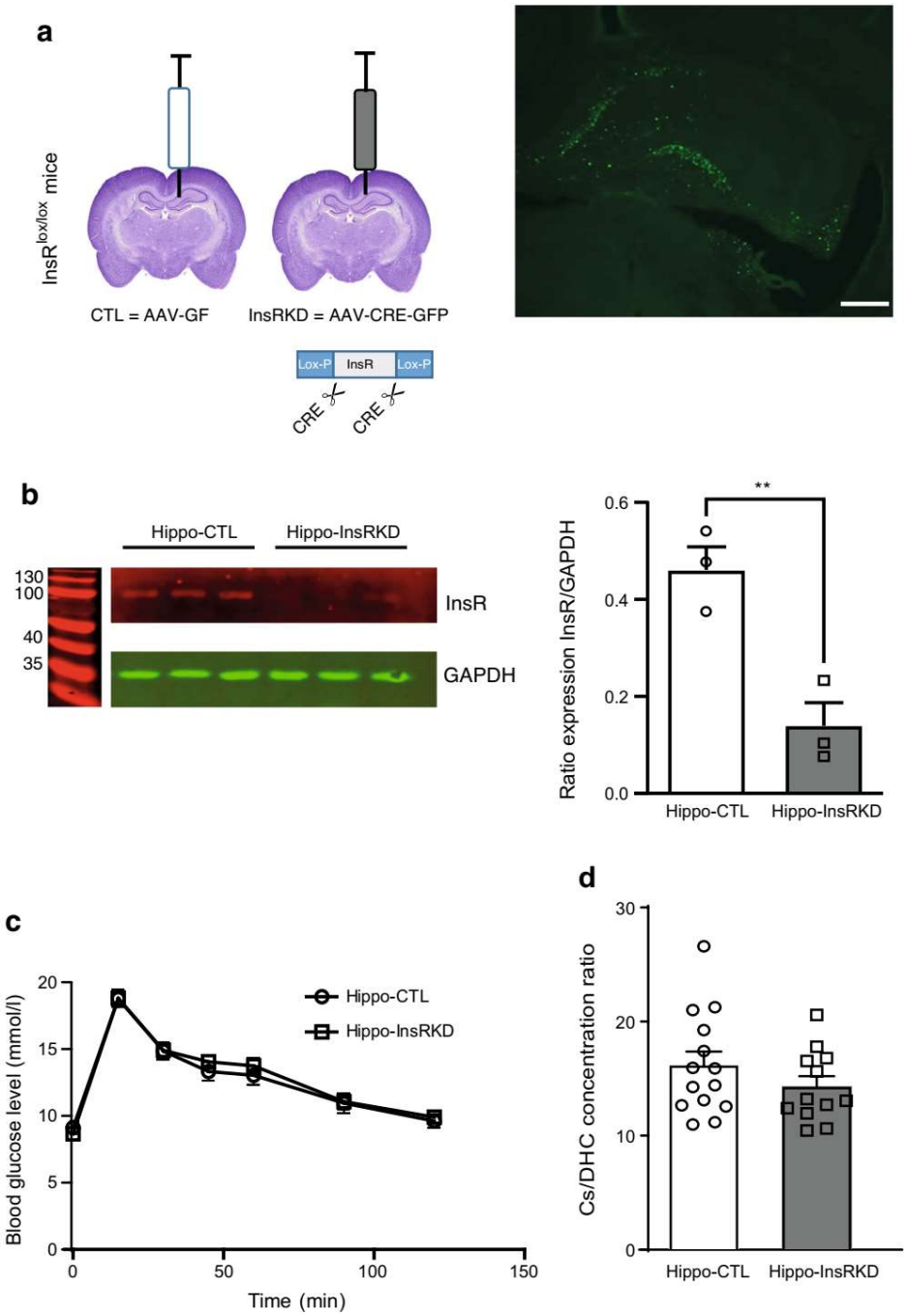

Fig. 3 Hippocampal 11β-HSD1 activity is not affected by specific InsR knockdown in the hippocampus (Hippo-InsRKD) compared with the wild-type group (Hippo-CTL).

(**a**) Bilateral AAV injection sites of the anterior and posterior hippocampus and representative image of GFP fluorescence in the hippocampus after AAV infection. (**b**) Western blot of InsR protein levels in the hippocampus of $InsR^{lox/lox}$ mice injected with AAV-GFP (Hippo-CTL) or AAV-Cre-GFP (Hippo-InsRKD) (**p<0.01, Mann–Whitney t test). (**c**) Comparison of the capillary glucose concentration during an i.p. GTT between Hippo-InsRKD and Hippo-CTL mice (repeated two-way ANOVA, NS). (**d**) Hippocampal 11β-HSD1 activity in Hippo-InsRKD mice compared with Hippo-CTL mice (Mann–Whitney t test, NS). Data are presented as mean ± SEM. Scale bar, 400 µm. CRE, tyrosine recombinase enzyme; Ins, insulin

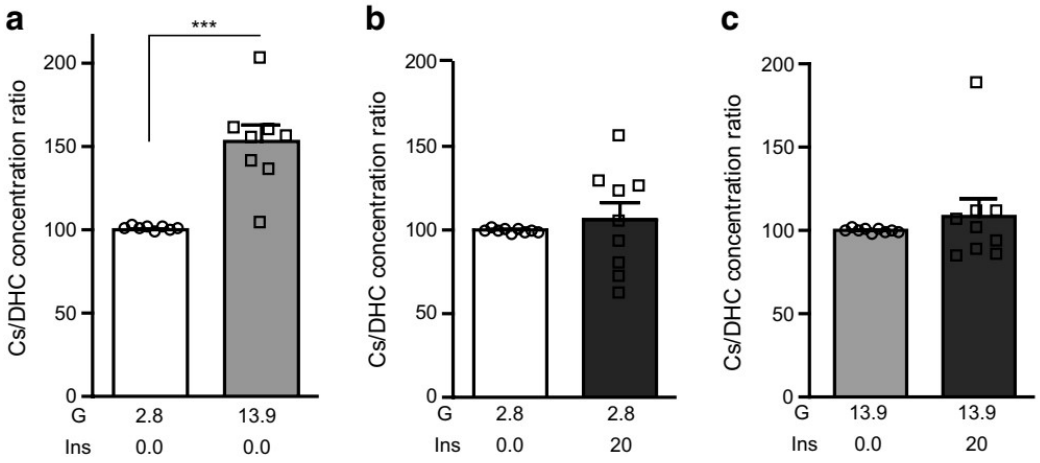

Fig. 4 High glucose concentration increases 11β-HSD1 activity in fresh hippocampus slices while insulin has no effect. (**a**) Glucose effect on 11 β-HSD1 activity. Comparison of 11β-HSD1 activity of hippocampus slices maintained for 3 h in an artificial cerebrospinal fluid containing 2.8 or 13.9 mmol/l of glucose and no insulin (***p<0.001, Mann–Whitney t test). (**b**) Insulin effect on 11 β-HSD1 activity (normal glucose condition). Comparison of 11β-HSD1 activity of hippocampus slices maintained for 3 h in an artificial cerebrospinal fluid containing 2.8 mmol/l of glucose and either 0.0 or 20.0 nmol/l of insulin (p=0.71, Mann–Whitney t test). (**c**) Insulin

effect on 11 β-HSD1 activity (high glucose condition). Comparison of 11β-HSD1 activity of hippocampus slices maintained for 3 h in an artificial cerebrospinal fluid containing 13.9 mmol/l of glucose and either 0.0 or 20.0 nmol/l of insulin (p=0.75, Mann–Whitney t test). Data are presented as mean ± SEM. G, glucose; Ins, insulin