Brain, Behavior, & Immunity - Health 5 (2020) 100075

Contents lists available at ScienceDirect

Brain, Behavior, & Immunity - Health

journal homepage: www.editorialmanager.com/bbih/default.aspx

Full Length Article

Sex differences in offspring neurodevelopment, cognitive performance and microglia morphology associated with maternal diabetes: Putative targets for insulin therapy

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ARTICLE INFO

Keywords:
Maternal diabetes
Sex differences
Insulin therapy
Neurodevelopment
Recognition memory
Microglia

ABSTRACT

Diabetes during pregnancy has been shown to affect the central nervous system (CNS) of the offspring, resulting in short- and long-term adverse effects. Children of diabetic mothers are more likely to develop cognitive impairment, also having increased susceptibility to psychiatric disorders. Microglia, the immune cells of the CNS, work as sensors of environmental changes, namely metabolic challenges, as early as the intrauterine period. During this period, microglia is actively involved in processes of neurogenesis, synaptic pruning and detection of any environmental alteration that may impact brain development. The remarkable sex dimorphism in neurodevelopment, as well as sex differences in the morphology and immune function of microglia during development, led us to clarify if maternal diabetes affects specific behavioral traits and microglia morphology during infancy in a sex-specific manner. Another important goal of this study was to clarify if insulin, the gold standard treatment of diabetes during gestation, could prevent maternal diabetes-induced behavioral changes, as well as microglia morphology, also considering sex specificities. Other molecular and cellular players potentially involved in the link between changes in metabolism and behavior were also analyzed in the hippocampus, a brain region implicated in cognition and other behavioral outcomes. Diabetes during pregnancy globally delayed female and male offspring development and was associated with impairments in recognition memory, but only in female offspring. In line with these results, at early and late infancy, some molecular and cellular markers were altered in offspring hippocampus in a sex-specific manner. The strict control of glycemia by insulin during pregnancy prevented most of the negative effects induced by uncontrolled hyperglycemia. Notably, insulin administration to diabetic dams may also modulate offspring development in a way that differs from what is observed in physiological conditions, since it promoted the expedited acquisition of developmental milestones and of discrimination ability at memory test, also inducing a hyper-ramification of male and female hippocampal microglia. Importantly, this study highlights the importance of analyzing the impact of maternal diabetes and insulin therapy, taking into account sex differences, since male and female present different vulnerabilities to hyperglycemia in this critical period of life.

1. Introduction

Diabetes during pregnancy is one of the most common and most important pathological conditions affecting human pregnancies, being a major concern for public health. It has been associated with effects on children's health, originating short- and long-term adverse outcomes (Reece, 2010; Yamamoto et al., 2019), including increased risk of child mortality and morbidity, in particular central nervous system abnormalities (Hami et al., 2015). Children born from diabetic mothers are more likely to present neurodevelopmental problems, including impairments in

Abbreviations: CA, cornu ammonis; CTRL, offspring of control dams; EPM, elevated plus maze; GD, gestational day; NOR, novel object recognition; OPF, open field; P, postnatal day; SEM, standard error of the mean; STZ, offspring of streptozotocin-induced diabetic dams; STZ + INS, offspring of insulin treated-diabetic dams.

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https://doi.org/10.1016/j.bbih.2020.100075
Received 13 April 2020; Accepted 17 April 2020
Available online 19 April 2020

learning ability, activity level, attention span, motor functioning (Yamamoto et al., 2019; Nomura et al., 2012; Ornoy et al., 2001; Nielsen et al., 2010), poorer working ability/performance (Nielsen et al., 2010; DeBoer et al., 2005) and lower cognitive function (Temple et al., 2011; Rizzo et al., 1991). Several studies have shown that the offspring of diabetic pregnant women are more likely to develop attention deficit hyperactivity disorder (Ji et al., 2018), autism spectrum disorders (Xiang et al., 2015) and schizophrenia (Van Lieshout and Voruganti, 2008).

The hippocampus, a crucial brain region for short- and long-term memory formation and spatial navigation is particularly vulnerable to changes in glucose levels (Hami et al., 2015; Kamal et al., 1999). Experimental studies have shown that uncontrolled maternal diabetes increases hippocampal excitability and alters offspring behavior and memory in early adulthood (Chandna et al., 2015; Vuong et al., 2017), also inducing neuronal loss in specific subregions of rat hippocampus in early postnatal life (Golalipour et al., 2012).

Microglial cells, the immune cells of the brain, have an important role in newborn cell phagocytosis (VanRyzin et al., 2019) and synaptic pruning (Paolicelli et al., 2011; Schafer et al., 2012) during development, participating in both neurogenesis (Sato, 2015) and synaptogenesis (Konishi et al., 2019) by screening the brain parenchyma, using their cellular processes as sensors. The impact of metabolic challenges, namely maternal diabetes during the intrauterine period on offspring microglia morphology, remains to be elucidated.

Despite the insights on the impact of maternal diabetes in the offspring behavior, less is known about its impact in the infancy period. The present study aims to unveil neurodevelopment and behavioral changes during infancy induced by maternal diabetes, also uncovering possible underpinning molecular and cellular alterations in the hippocampus that may account for neurodevelopmental impairments and/or behavioral deficits in adulthood. Importantly, since diabetic intrauterine environment may also exert differential effects on offspring based on sex (Kinney et al., 2003), we assessed if maternal diabetes promotes a sexually dimorphic behavioral repertoire during infancy and if this is accompanied by sex-specific molecular and cellular hippocampal alterations.

Taking into account that insulin therapy is the gold standard therapeutic approach for diabetes, we also evaluated the therapeutic ability of insulin treatment to prevent the molecular, cellular and behavioral changes identified. Elucidating these important issues will open new avenues for preventing neurodevelopmental changes and/or therapeutically intervene early during development.

2. Materials and methods

2.1. Animals

All procedures involving animals were approved by the Animal Welfare Committee of the Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra. The animal experimentation was conducted in accordance with the European Community directive guidelines for the use of laboratory animals (2010/63/EU), transposed into the Portuguese law in 2013 (Decreto-Lei 113/2013). Assignment of experimental groups for each cage was performed in a random fashion by a technician blinded to the experimental details. Group sizes were determined based upon preliminary power analyses for gene expression, protein levels, and behavioral measurements.

2.2. Experimental design

2.2.1. Female dams

Female Wistar rats (Charles River Laboratories, Lyon, France), aged 8 weeks, were housed in certified facilities, in a temperature- and humidity-controlled environment under a 12 h:12 h light-dark cycle and with *ad libitum* access to water and food.

Female rats were randomly divided into control and diabetic groups. Pre-gestational type 1 diabetes was induced by a single intraperitoneal (IP) injection of streptozotocin (45 mg/kg; in 100 mM sodium citrate buffer, pH 4.5) in fasting (overnight) female rats (control females were injected with vehicle only). Glycemia was measured before and 2 days after STZ injection with a glucometer by pricking the tip of the tail. Animals were considered diabetic when blood glucose levels exceeded 250 mg/dL. Three days after streptozotocin injection, a subset of diabetic females received half of a 7 mm insulin implant (~1 U/day; Linplant®; Linshin, Canada), while control and diabetic animals received a blank one. Briefly, animals were anesthetized with 2.5% isoflurane (IsoFlo; Abbott Laboratories, Chicago, USA) in 1 L/min O2, and implants were inserted subcutaneously, under the dorsal skin accordingly to the manufacturer instructions.

Females of the 3 experimental groups were mated with non-diabetic males 1 week after diabetes onset. After mating, weight and glycemia of female dams were monitored regularly till they gave birth (gestation day 21; GD21). Both male and female offspring stayed housed with respective mothers during experimentation.

2.2.2. Offspring

In this work, in order to assess possible sex-dependent differences induced by maternal diabetes, both female and male offspring were analyzed.

After birth, offspring of control-CTRL, diabetic-STZ and diabetic treated with insulin-STZ + INS dams, were weighted at postnatal day (P) 0, P7, P14 and P21. Blood glycemia was measured with a glucometer and serum insulin levels were measured by ELISA assay according to manufacturer's instructions (Mercodia RAT Insulin ELISA kit, Uppsala, Sweden) at P0, P7 and P21.

2.3. Developmental behavioral testing

The offspring of the 3 experimental groups were submitted to a battery of tests from P5 to P17 for the assessment of behavior in early development, as previously described (VanRyzin et al., 2016; Baharnoori et al., 2012). Behavior was monitored each day on the light phase of the light cycle and under dim white light.

2.3.1. Surface righting reflex (P5–P10)

Pups were placed on their back on a flat soft surface and were held down for 5 s. After release, the time needed to return to its four limbs was taken as indicative of trunk control and vestibular system development. The cut-off time for this test was 5 s.

2.3.2. Negative geotaxis reaction (P5–P14)

Animals were placed face down on a platform with a 35-degree incline, covered with a sponge to enable traction. The time the animal took to turn from the upside down position to a point where both forepaws were top-oriented was evaluated, as an indication of coordination, balance and vestibular system development. Cut-off time for this test was 30 s.

2.3.3. Cliff aversion (P5–P10)

Pup ability to retract from the edge of a cliff was assessed. Animals were placed on an elevated flat surface only with their snout and forepaw digits hanging over the edge. The time for the pup to turn away from the cliff and move its paws and snout away from the edge was counted up to 30 s.

2.3.4. Wire suspension (P12–P14)

The pup's forelimb strength was assessed through the wire suspension test. The pups were placed against a horizontal wire rod and were allowed to grasp it with both forepaws. Time was scored after the release of the animal until it fell to a padded drop zone. The cut-off time for this test was 10 s.

2.3.5. Locomotion (P5–P14)

Pup locomotion was assessed by placing the animals on a flat surface in the center of a 13 cm diameter circle. The time for the animal to fully exit the arena with all four limbs was recorded up to 30 s.

2.3.6. Nest seeking (P5–P15)

The ability to identify and locate the maternal scent depends on the display of adequate olfactory, motor and discriminatory ability to discern nest bedding from home bedding (Baharnoori et al., 2012). Pups ability to discriminate their nest bedding by olfaction was determined by using a rectangular arena (26 × 14 cm) divided into 3 compartments: the center compartment where the pup was placed; the home bedding goal on one side, where there was home nest bedding; and a fresh bedding goal on the opposite side, where there was fresh clean bedding in a similar amount to home bedding. Each animal was placed in the central compartment at a 90° angle from the goal compartment for a first trial and after a 30 s intertrial interval the pup was again tested for a second trial. Each trial was performed with the animal facing opposite sides of the equipment to balance possible side turning preferences. The latency to goal was scored by the time the animal took to transpose the apparatus home bedding goal mark with both snout and forelimbs. The cut-off time of each trial was 120 s. Latency to goal was evaluated as an average between the two trials, resulting in one score per pup, per day.

2.3.7. Eye opening (P12–P17)

Eye opening was monitored by observation of pups and the percentage of pups with eyes opened, per litter, per day, was calculated.

2.3.8. Auditory startle (P11–P14)

Auditory startle test was used as a measure of auditory system development, which indicates maturation of somatosensory, vestibular and/or proprioceptive function. Animals' capacity to produce full body startle response to a loud finger snap at a 10 cm distance was evaluated and the percentage of animals that responded per litter, per day was calculated.

2.4. Late infancy behavioral assessment

All animals were gradually adapted to manipulation over several days prior to experimentation. One hour before behavioral experiments all animals were habituated to the experimentation room under dimmed red light, controlled temperature and ventilation. Experiments were performed during the light phase of the light cycle.

2.4.1. Open field test-OPF (P20)

The OPF was performed to assess the locomotor behavior. Animals were placed facing the wall in the center of the arena and were left to explore the arena (45 × 45 × 40 cm) for 5 min. Post-hoc analysis was performed through the Any Maze software to evaluate the animals' locomotor pattern of exploration, average speed and distance traveled.

2.4.2. Elevated-plus maze-EPM (P21)

Animal anxious-like behavior was assessed with the EPM test, as previously described (Caetano et al., 2017). This test evaluates rodents' conflict between preferring protected areas (closed arms), and their innate motivation to explore new environments (open arms). For this test, animals were left at the maze for exploration during 5 min. Post-hoc analysis of animal performance in the test was achieved by "Observador" software (University of Athens, Medical School, Department of Pharmacology) analysis of the time and number of entries in the open arms.

2.4.3. Novel object recognition test-NOR (P21)

The NOR was performed in order to evaluate animal ability to distinguish between a familiar and a novel object, being an indicator of short-term recognition memory. The test is composed by a familiarization and a test trial. Each trial had the duration of 5 min with a 2 h intertrial interval. The animal performance was measured through the Recognition Index (RI): [RI = TN/(TN + TF)], and also by the Discrimination Index (DI): DI = (TN − TF)/(TN + TF) where TN is the time exploring the novel object, and TF the time exploring the familiar one (Antunes and Biala, 2012).

2.5. RNA extraction and reverse transcription

Total RNA was isolated from rat hippocampus at P7 and P21 using NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany), accordingly to manufacturer's instructions. RNA samples were dissolved in 60 μL of RNAse-free water, and the concentration and purity of total RNA were determined using NanoDrop ND1000 (Thermo Scientific, Waltham, MA, USA). Total RNA (0.5 μg) was reversed transcribed according to the instructions provided by the manufacturer (NZYTech, Lisbon, Portugal). The resultant cDNA was treated with RNAse-H for 20 min at 37 °C and samples were stored at −20 °C until qPCR analysis.

2.6. Real-time quantitative PCR

Quantitative PCR (qPCR) was performed using StepOnePlus (Applied Biosystems, Inc, Foster City, CA, USA) based on the real-time monitoring of fluorescent SYBR Green. The PCR conditions were as follows: iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 200 nM primers (Table 1), and 4 μL of 1:50 dilution cDNA, in a total volume of 10 μL. Cycling conditions were: 95 °C for 30 s, followed by 40 cycles at 95 °C for 15 s and 58-60 °C for 60 s. Melting curve generation analyses were also performed according to instrument settings (65 °C to 95 °C with 0.5 °C increment step, and 95 °C for 10 s). Ct values were converted to "Relative quantification" using the 2(-ΔΔ Ct) method previously described (Livak and Schmittgen, 2001). Actb, Hprt, Ywhaz, and B2m were evaluated as housekeeping genes using NormFinder Add-In for Microsoft Excel. Since Ywhaz was the most stable gene in all conditions, it was used as the reference gene. The results are expressed as the relative amount compared with control.

2.7. Western blot

2.7.1. Sample collection

At P21, female and male pups were anesthetized with 2.5% isoflurane (IsoFLO, Abbott Laboratories, Chicago, IL, USA) on O_2. Under anesthesia, animals were sacrificed by cervical dislocation. Decapitation was performed and brains were removed for hippocampal dissection.

2.7.2. Hippocampal total extracts preparation

Hippocampal total extracts were prepared as previously described (Gaspar et al., 2010). Briefly, after dissection, the offspring hippocampus was homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, supplemented with complete miniprotease inhibitor cocktail Tablets and 1 mM DTT). The homogenate was sonicated and centrifuged at 16,100×*g* for 10 min. The resultant supernatant was stored at - 80ºC until use.

2.7.3. SDS-PAGE western blot

Protein concentration was measured by colorimetric bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA). After protein concentration determination and denaturation, SDS-PAGE Western blot was performed as previously described (Gaspar et al., 2010). Primary antibodies against the proteins of interest are listed in Table 2. Membranes were processed for protein detection using Enhanced chemifluorescence system (ECF) on the Typhoon FLA 9000 (GE Healthcare, Chicago, IL, USA). Membranes were then reincubated for detection of the loading control protein, calnexin, and incubated with the secondary antibody coupled to horseradish peroxidase (HRP) and processed for protein detection using a commercial enhanced chemiluminescence (ECL) detection method kit on the ImageQuantTM LAS 500 (GE Healthcare). Subsequently, membranes' intensity was quantified

Table 1
List of primers of reference and target genes.

	Gene	Primer	Sequence (5'->3')	Product length (bp)
Reference	ywhaz	F	TCTGCAACGACGTACTGTCTC	117
		R	CCTCAGCCAAGTAGCGGTAG	
	b2m	F	TGCTTGCCATTCAGAAAACTCC	111
		R	TTTGAGGTGGGTGGAACTGAG	
	hprt	F	ATGGGAGGCCATCACATTGT	77
		R	ATGTAATCCAGCAGGTCAGCAA	
	actb	F	CCCGCGAGTACAACCTTCTT	90
		R	CGACGAGCGCAGCGATA	
Target	sox2	F	CTCTGTGGTCAAGTCCGAGG	146
		R	ATGCTGATCATGTCCCGGAG	
	ascl1	F	TCCTCCGACGAGGGATCCTA	124
		R	CCTGCCATCCTGCTTCCAAAG	
	rbfox3	F	CCACCACTCTCTTGTCCGTT	144
		R	ACCATAACTGTCACTGTAGGCTG	
	gfap	F	GTGGTATCGGTCCAAGTTTGC	129
		R	GACTCAAGGTCGCAGGTCAA	
	dcx	F	ACGACCAAGACGCAAATGGA	107
		R	CTTGTGCTTCCGCAGACTTC	
	msi1	F	GACGCCTTCATGCTGGGTAT	113
		R	TCGGGGAACTGGTAGGTGTA	
	iba1	F	GCAAGGATTTGCAGGGAGGA	96
		R	TGGGATCATCGAGGAAGTGC	

through the ImageQuant 5.0 software (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

2.8. Brain slice immunohistochemistry and tridimensional morphometric microglia analysis

Animals were deeply anesthetized with an IP injection of ketamine (80 mg/kg; Nimatek) and xylazine (5 mg/kg; Ronpum 2%) and transcardially perfused with phosphate buffer saline (PBS: 137 mM NaCl, 2.1 mM KCl, 1.8 mM KH2PO4 and 10 mM Na2HPO4, at pH 7.4) followed by 4% paraformaldehyde (PFA) in 1% PBS. Brains were postfixed in 4% PFA, transferred to a solution of 30% sucrose in PBS-1% and stored at −80 °C until processing.

Offspring brain slices (30 μm) containing the dorsal hippocampus (stereotactic coordinates of lambda 3.60 mm and bregma −1.80 mm for P7 and lambda 5.40 mm and bregma −2.20 mm for P21 (Khazipov et al., 2015);) were prepared as previously described (Duarte et al., 2019). Free-floating immunohistochemistry was performed using anti-Iba1 primary antibody (1:1000; Wako, Neuss, Germany), sections were examined with an LSM 710 Meta Confocal laser scanning microscope (Zeiss, Jena, Germany). Z-stack images with the spatial resolution of 0,264 μm x 0, 264 μm x 1000 μm and dimensions of x: 134.95 μm, y: 134.95 μm, z: 30.00 μm were obtained to perform the tridimensional microglia reconstruction using the Neurolucida Software (MBF Bioscience, Williston, VT, USA) (Duarte et al., 2019). The data related to the number and length of microglia processes as well as the number of processes intersections per radius (sholl analysis) was extracted using Neurolucida Explorer Software. Per animal, an average of 10 microglial cells was reconstructed.

A semi-quantitative analysis of the number of microglial cells was performed by counting the number of microglia in each image/Z-stack obtained (Dimensions: x: 134.95 μm, y: 134.95 μm, z: 30.00 μm). A total of 10 Z-stacks per animal were analyzed to assess microglia number at P7 on DG and at P21 on CA1.

Table 2
List of primary antibodies.

Primary Antibody	Sample	Dilution	Protein (μg)	Company
Rabbit Anti-NeuN	Total Extracts	1:1000	40	Abcam
Rabbit Anti-CX3CR1	Total Extracts	1:1000	40	Abcam
Rabbit Anti-PSD-95	Total Extracts	1:5000	10	Cell Signaling
Mouse Anti-Synapsin-1	Total Extracts	1:10,000	10	Synaptic Systems
Rabbit Anti-VGlut-1	Total Extracts	1:1000	40	Abcam
Goat Anti-Calnexin		1:5000		Sicgen

2.9. Statistical analysis

The results are presented as mean + standard error of the mean (SEM). Statistical analysis was performed with IBM SPSS Statistics 24 Software (IBM Software, Armonk, New York, USA). Graph illustrations presented were performed in GraphPad Prism 6 software (GraphPad Software, Inc, San Diego, USA). The normality of the data was assessed with Shapiro-Wilk normality test. Accordingly, data were analyzed with non-parametric Kruskal-Wallis test or with parametric one-way ANOVA followed by Tukey's post-hoc test. The data regarding eye opening and auditory startle was analyzed with Log-Rank Kaplan-Meier test. Differences were considered to be significant for $p < 0.05$.

3. Results

3.1. Metabolic characterization of pregnant dams and litter size evaluation

After mating, dam glycemia and weight were regularly monitored. At GD1 and GD21, diabetic dams presented, as expected, significant higher glycemic values comparing both with controls and diabetic dams treated with insulin (Table 3). Insulin treatment was effective in maintaining normoglycemia during the gestation period. Maternal diabetes did not induce significant changes in dam body weight and litter size (Table 3).

3.2. Effect of maternal diabetes on offspring body weight, glycemia and plasma insulin levels

3.2.1. Body weight

After birth, both male (Fig. 1A) and female (Fig. 1B) offspring were weighted at P0, P7, P14 and P21. At P0, the body weight of STZ male and female offspring was similar to CTRL animals (Fig. 1A), whereas both male and female STZ + INS offspring presented higher body weight comparing to both STZ and CTRL offspring (Supplementary Table 1).

From P7 until P21, both male (Fig. 1A) and female (Fig. 1B) STZ offspring presented an inferior weight comparing with CTRL. Insulin administration to the dams was able to prevent the impairment in weight gain presented by STZ offspring (Supplementary Table 1). Interestingly, at P7, P14 and P21 STZ + INS female offspring also presented higher body weight levels comparing to CTRL, an effect that was not observed in STZ + INS males (Fig. 1A).

3.2.2. Glycemia

Offspring glycemia and insulin levels were assessed at P0, P7 and P21. At P0 STZ male (Fig. 1C) and female (Fig. 1D) offspring presented a tendency to present higher glycemia (mg/dl) values than CTRL (CTRL male vs STZ male: $p < 0.098$; CTRL female vs STZ female: $p < 0.191$), whereas insulin administration to diabetic dams was effective in preventing increased blood glucose levels in STZ offspring, being statistically different in females (STZ♀: 94.5 + 9.9, n = 6 vs STZ + INS♀: 69.9 + 3.5, n = 7) (Fig. 1C and D).

At P7 and P21 no changes regarding glycemia were detected

Table 3
Maternal metabolic characterization and litter size evaluation.

	Dams G1		Dams G21			Offspring P0		
	Glycemia	Weight	Glycemia	Weight		Pups	♂ Pups	♀ Pups
	(mg/dl)	(g)	(mg/dl)	(g)		nº	nº	nº
Control	109.0 ± 6.2	210.2 ± 5.4	87.7 ± 2.1	334.7 ± 7.3	*CTRL*	7.8 ± 0.7	3.9 ± 0.4	3.9 ± 0.5
Diabetic	391.9 ± 14.6***	210.6 ± 2.6	472.3 ± 19.7***	314.76 ± 5.1	*STZ*	7.5 ± 0.5	3.3 ± 0.4	4.1 ± 0.4
Diabetic + INS	103.0 ± 9.9###	200.4 ± 3.4	106.8 ± 10.8##	329.3 ± 7.9	*STZ + INS*	8.1 ± 0.9	3.8 ± 0.6	5.0 ± 0.5

Maternal glycemia and weight were measured at the beginning (GD1) and at the end (GD21) of pregnancy, and the number of pups per litter was noted at the day of birth. Data represent mean ± SEM values; n = 9–14. Statistical analysis was assessed by Kruskal-Wallis test. ***p < 0.001 Control vs Diabetic; ##p < 0.01 Diabetic vs Diabetic + INS; ###p < 0.001 STZ vs STZ-INS.

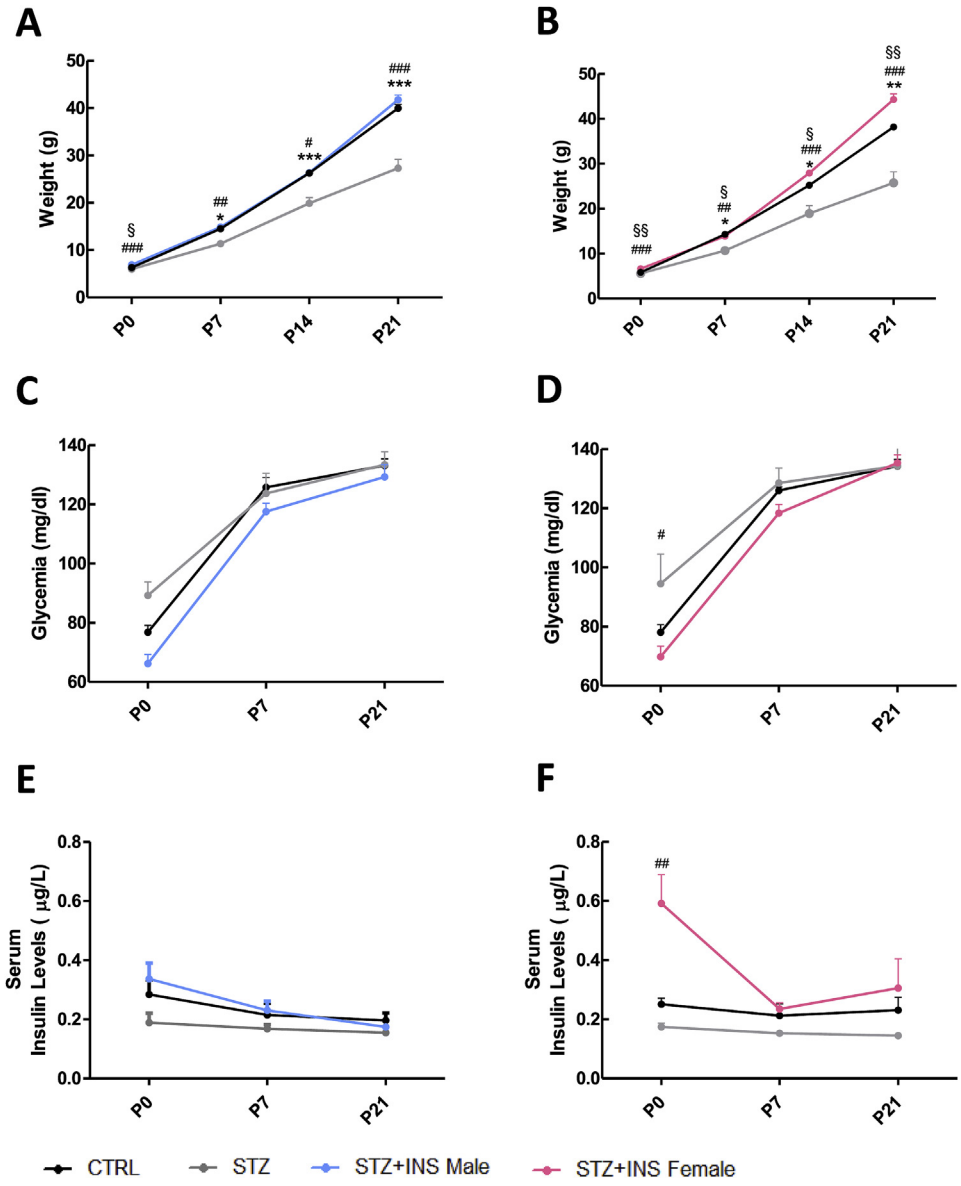

Fig. 1. Effect of maternal diabetes on offspring's body weight, glycemia and plasma insulin levels.
Male (A) and female (B) pups were weighted at P0, 7, 14 and 21. (C, D) Pup glycemia and (E, F) serum insulin levels were measured at P0, 7 and 21. Values are presented as the mean +SEM of 12–48 animals for weight data; 6–25 animals for glycemia data and 5–9 animals for insulin levels data. Statistical analysis was assessed with Kruskal-Wallis test; *p < 0.05, **p < 0.01, ***p < 0.001: CTRL (black) compared with STZ (grey) offspring; #p < 0.05, ##p < 0.01, ###p < 0.001: STZ compared with STZ + INS (blue and pink) offspring; §p < 0.05, §§p < 0.01: CTRL compared with STZ + INS offspring. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

between the 3 experimental groups in both male and female offspring (Fig. 1C and D).

3.2.3. Plasma insulin levels

Regarding plasma insulin levels, no statistically significant changes were detected in STZ male (Fig. 1E) and female (Fig. 1F) offspring comparing to CTRL at P0, P7 and P21, though STZ females presented a tendency to present lower levels comparing to CTRL (P0: p < 0.052; P7: p < 0.107; P21: p < 0.056). At P0, female STZ + INS offspring presented increased plasma levels comparing with STZ female offspring (STZ♀: 0.59 + 0.09, n = 5 vs STZ + INS♀: 0.25 + 0.02, n = 9), an effect only observed at birth day (Fig. 1F) and not observed in males (Fig. 1E).

3.3. Maternal diabetes induces a delay in male and female offspring development, an effect prevented by insulin administration to diabetic dams

Male and female offspring from the 3 experimental groups were subjected to several behavioral tests to assess any developmental delay (Fig. 2A). In the tests evaluating the development of the vestibular system, balance and coordination, namely righting reflex (Fig. 2B and C and Supplementary Table 2), negative geotaxis reaction (Fig. 2D and E and Supplementary Table 3) and cliff aversion (Fig. 2F and G and Supplementary Table 4), both male and female STZ offspring presented an impaired performance, taking more time to perform them comparing with CTRL. Insulin administration to diabetic dams was able to prevent STZ-related changes in development in righting reflex and negative geotaxis reaction tests.

Maternal diabetes also induced a marked impairment in both male (Fig. 2H) and female (Fig. 2I and Supplementary Table 5) STZ offspring performance in the locomotion test, being insulin administration to the diabetic dams able to prevent this delay.

Pups were also tested for their latencies to goal (home-bedding) on the nest seeking test. An impairment in this behavior was detected in both male (Fig. 2J) and female STZ (Fig. 2K and Supplementary Table 6) offspring, but only on the first days of testing.

In the wire suspension test, STZ male (Fig. 2L) and female (Fig. 2M) offspring also presented a worse performance comparing with CTRL, indicating less strength in their forelimbs. This effect was prevented by insulin administration in both male and female offspring at P14 (Fig. 2 L, M and Supplementary Table 7).

3.4. Maternal diabetes induces a delay in eye opening and in auditory startle response, whereas insulin administration anticipates the acquisition of these developmental milestones in both males and females

Similarly to what was observed in other developmental tests, STZ male and female offspring presented a delayed development concerning eye opening (Fig. 3A and B and Supplementary Table 8) and beginning of the auditory startle response (Fig. 3C and D and Supplementary Table 9), comparing to CTRL. Interestingly, insulin administration to diabetic dams, not only prevented these delays in the STZ offspring, but in fact expedited the acquisition of these developmental milestones comparing to physiological conditions in both male (Fig. 3A, C) and female (Fig. 3B, D) offspring.

3.5. Diabetes during pregnancy impairs short-term memory in female offspring

Since animal performance in EPM and NOR tests depend on the absence of locomotor problems, locomotion was assessed at P20 in the OPF by placing each animal in an open arena and leaving it to freely explore for 5 min (Fig. 4A). Regarding the distance traveled (Fig. 4B) and the mean speed (Fig. 4C), no statistically significant differences were observed between experimental groups in both sexes, indicating no changes in locomotor activity.

Anxious-like behavior was assessed at P21 by EPM test. Animals were left for 5 min in an elevated plus shape apparatus to explore the open and/or closed arms (Fig. 4A). Separated analysis of male and female offspring did not reveal any significant differences between STZ and CTRL offspring for the time spent in the open arms (Fig. 4D) and for the number of entries in the open arms (Fig. 4E).

NOR test was performed to evaluate recognition memory in both male and female offspring (Fig. 4F and G). The Discrimination Index in the testing trial allows assessing the discrimination between the novel and the familiar objects, where a positive score indicates more time spent with the novel object, whereas a negative score indicates more time spent with the familiar object, and a zero score indicates a null preference. Our results show that in physiological conditions, although CTRL female offspring were able to discriminate between the novel and familiar object (CTRL♀: $0.30 + 0.08$, $n = 11$), CTRL male offspring discrimination ability was impaired or not fully developed at P21 (CTRL♂: $0.02 + 0.10$, $n = 10$). Regarding STZ offspring, both male and female STZ offspring presented a negative score, close to zero, demonstrating they did not have a preference for the novel object. Male STZ + INS offspring presented positive scores, indicative of more time spent with the novel object, which were statistically different comparing with zero (zero score indicates a null preference), with CTRL or with STZ offspring (CTRL♂: $0.02 + 0.10$, $n = 10$ vs STZ♂: $-0.14 + 0.15$, $n = 8$ vs STZ + INS♂: $0.53 + 0.07$, $n = 10$). Female STZ + INS offspring also presented positive scores statistically different of zero and of STZ female offspring (STZ♀: $-0.08 + 0.11$, $n = 8$ vs STZ + INS♀: $0.63 + 0.07$, $n = 8$), showing that they were able to discriminate between familiar and novel object and that they spent more time exploring the novel object (Fig. 4F).

The analysis of Recognition Index (RI), as an index of memory retention, showed that STZ female offspring spent less time exploring the novel object relative to the total time of objects investigation, having insulin administration a tendency (STZ female Novel vs STZ + INS female Novel: $p < 0.105$) to prevent this effect (CTRL♀: $0.64 + 0.04$, $n = 11$ vs STZ♀: $0.46 + 0.06$, $n = 8$ vs STZ + INS♀: $0.64 + 0.07$, $n = 8$) (Fig. 4G).

3.6. Diabetes during pregnancy induces changes in the expression of neural markers in the hippocampus at early infancy, but does not impact microglia morphology in hippocampal dentate gyrus

The expression of neural markers was evaluated on offspring hippocampus at P7. Although we analyzed the whole hippocampus, P7 is reported to be a very active period for neurogenesis in the hippocampal dentate gyrus (Rice and Barone, 2000). Maternal diabetes did not induce changes in the expression of neural markers gfap and sox2 (neural stem cell markers, though gfap can also be expressed by astrocytes), but led to an increase in msi1 expression (neural stem cell marker) in male (CTRL♂: $1.00 + 0.05$, $n = 8$ vs STZ♂: $1.26 + 0.08$, $n = 6$) (Fig. 5A), but not in female offspring hippocampus (Fig. 5B). Ascl1 is involved in the proliferation, neuronal commitment and differentiation of progenitor cells (Urban and Guillemot, 2014). Fluctuations in the expression of this gene promote the proliferation of neural progenitors, while its stable expression drives differentiation (Imayoshi et al., 2013). No changes in ascl1 expression were found both in STZ male (Fig. 5A) and female (Fig. 5B) offspring hippocampus at P7. The expression of doublecortin (dcx), which is expressed in migrating neuronal precursors, non-migrating neuroblasts and also in immature neurons, increased both in male (CTRL♂: $1.00 + 0.07$, $n = 8$ vs STZ♂: $1.47 + 0.11$, $n = 6$) (Fig. 5A) and female (CTRL♀: $1.00 + 0.07$, $n = 7$ vs STZ♀: $1.41 + 0.11$, $n = 7$) (Fig. 5B) offspring of diabetic dams at P7. Concerning rbfox3 expression (encodes for NeuN protein), a marker for post-mitotic neurons, no changes in its expression and protein levels were found both in male (Fig. 5A) and female (Fig. 5B) offspring at P7. Interestingly, insulin administration to diabetic dams induced an increase in the expression of ascl1 (CTRL♀: $1.00 + 0.05$, $n = 8$ vs STZ♀: $1.09 + 0.09$, $n = 7$ vs STZ + INS♀: $1.54 + 0.15$, $n = 5$) comparing both with CTRL and STZ offspring, but only in females. Likewise, in STZ + INS females, rbfox3 expression presented a tendency (STZ female vs STZ + INS female: $p < 0.07$) for increased expression comparing with CTRL and STZ offspring (CTRL♀: $1.00 + 0.05$, $n = 8$ vs STZ♀: $0.90 + 0.08$, $n = 7$ vs STZ + INS♀: $1.48 + 0.21$, $n = 6$) (Fig. 5B).

Microglia cells have regulatory effects on neurogenesis, by governing the proliferation and differentiation of progenitor cells, also controlling the resulting number of newborn neurons via phagocytosis (Sato, 2015). Since microglia play a critical role during development, and being microglia function dependent on their morphology, we assessed microglial morphologic changes in hippocampal dentate gyrus (DG) at P7, in the 3 experimental groups both in male (Fig. 5C) and female (Fig. 5K) offspring.

Maternal diabetes did not induce changes in microglia morphology in terms of number of process per branch order (Fig. 5D, L), total number of processes (Fig. 5E, M), length per branch order (Fig. 5F, N), total length

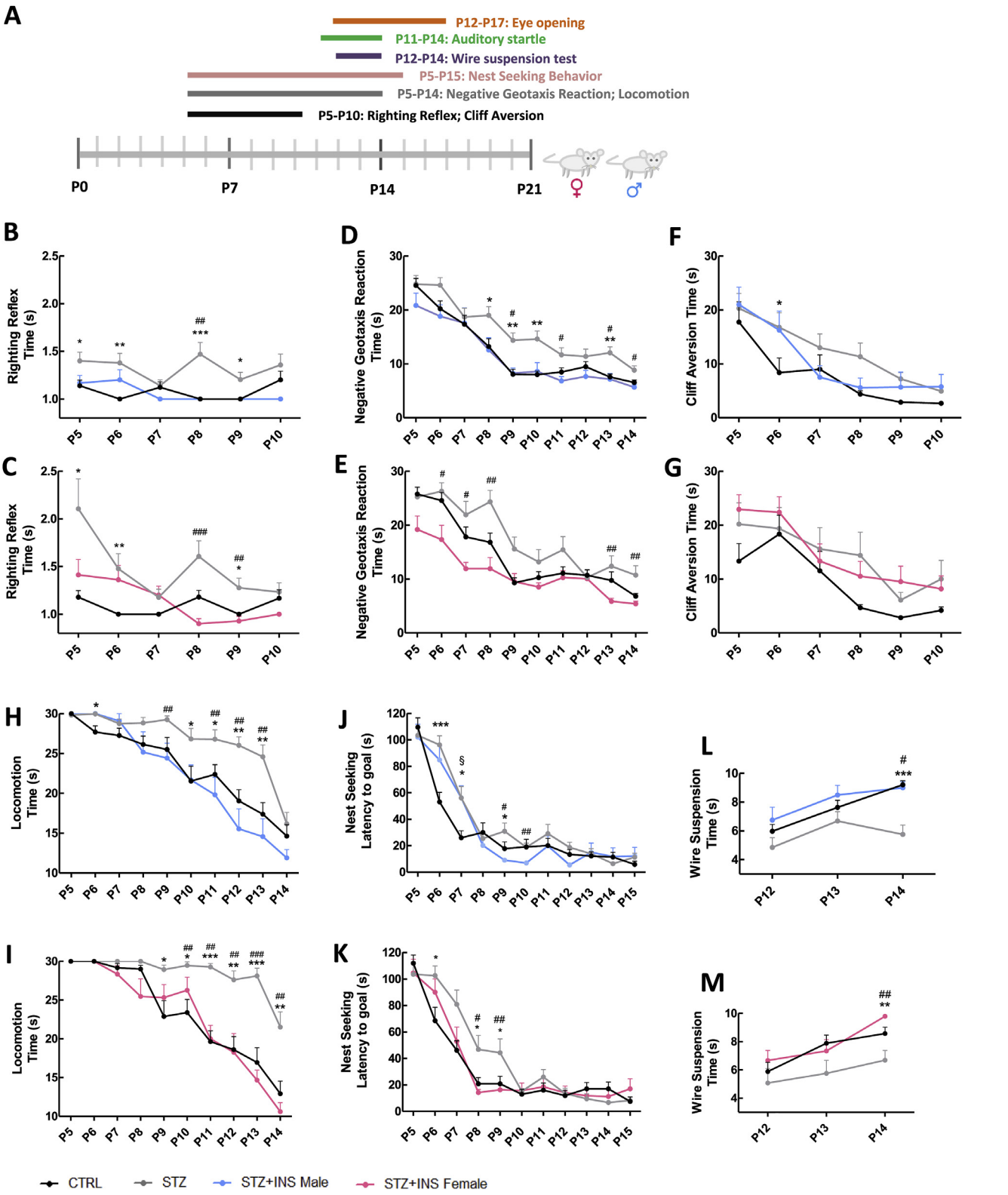

Fig. 2. Maternal diabetes induces a delay in male and female offspring's development: prevention by insulin administration to diabetic dams.
(A) Male and female offspring were subjected to several behavioral tests from P5 until P17. (B, C) Rat pups were tested for surface righting reflex (s) from P5–P10 and (D, E) tested for the latency (s) to reverse orientation and face upwards on an inclined plane from P5–P14. (F, G) The time (s) pups took to retract from an edge of a platform was evaluated from P5–P10. (H, I) Pup locomotor activity was assessed from P5–P14 and (J, K) pups were tested for their latency to achieve goal (home-bedding) on the nest seeking test (s) from P5–P15. (L, M) Forepaws strength was evaluated in the wire suspension test from P12–P14. Values are presented as the mean +SEM of 8–37 animals (detailed n values regarding each test and timepoint can be observed at Supplementary Tables 2-7). Statistical analysis was assessed with Kruskal-Wallis test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$: CTRL (black) compared with STZ (grey) offspring; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$: STZ compared with STZ + INS (blue and pink) offspring; $^{\S}p < 0.05$: CTRL compared with STZ + INS offspring. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

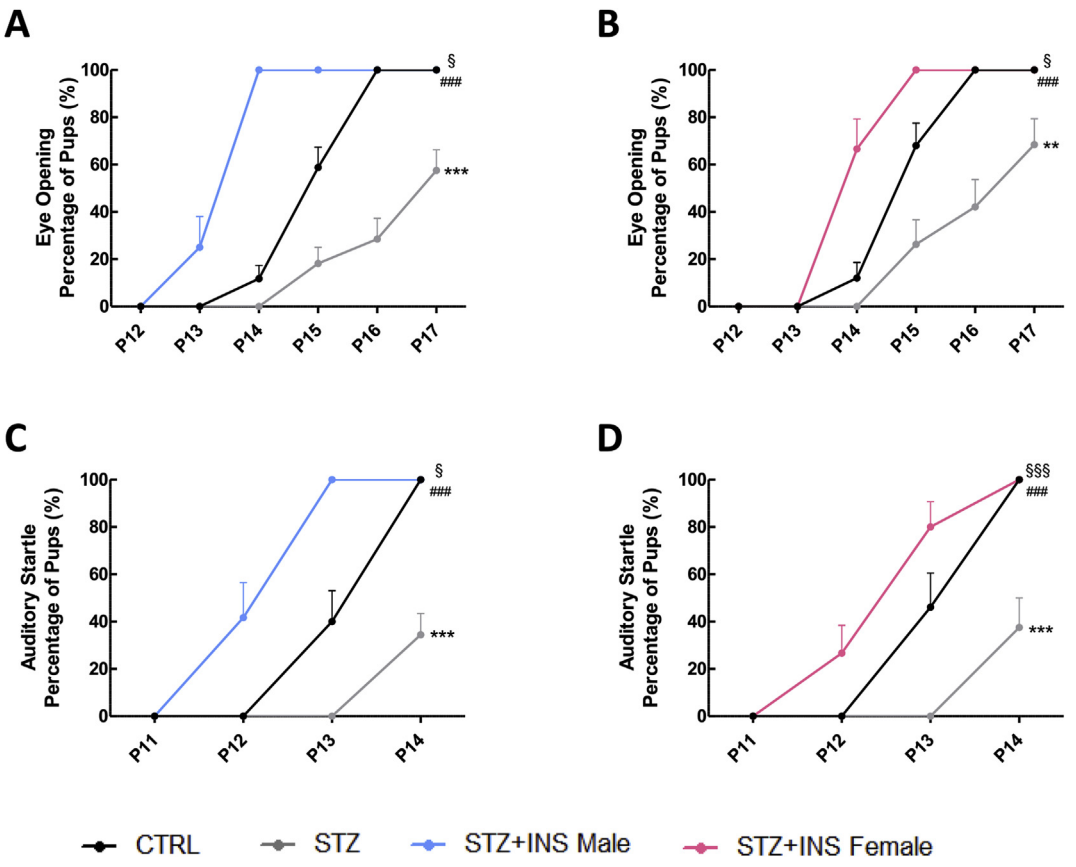

Fig. 3. Maternal diabetes induces a delay in eye opening and in the presentation of auditory startle response: insulin administration anticipates the acquisition of these developmental milestones.

(A, B) Pups were evaluated for the day of eye opening from P12–P17, and tested for (C, D) auditory startle response from P11–P14. Values are presented as the mean +SEM of 12–34 animals (detailed n values regarding each test and timepoint can be observed at Supplementary Tables 8-9). Statistical analysis was assessed with Log-Rank Kaplan-Meier test; **p < 0.01, ***p < 0.001: CTRL (black) compared with STZ (grey) offspring; $^{###}$p < 0.001: STZ compared with STZ + INS (blue and pink) offspring; §p < 0.05, §§§§p < 0.0001: CTRL compared with STZ + INS offspring. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 5G, O), and number of processes intersections per radius (Fig. 5H, P and Supplementary Table 11) both in male and female offspring at P7. Notably, STZ + INS male (Fig. 5D and E and Supplementary Table 10) and female (Fig. 5L and M and Supplementary Table 10) offspring microglia were hyper-ramified compared to STZ and CTRL ones (CTRL♂: 16.5 + 1.1, n = 5; STZ♂: 16.3 + 1.3, n = 3; STZ + INS♂: 30.5 + 1.5, n = 3/CTRL♀: 21.6 + 1.3, n = 6; STZ♀: 23.9 + 1.7, n = 4; STZ + INS♀: 29.6 + 1.3, n = 3). Additionally, male STZ + INS offspring total process length increased comparing to CTRLs (CTRL♂: 122.8 + 7.2, n = 5; STZ + INS♂: 151.7 + 7.6, n = 3) (Figure E). STZ + INS female offspring presented an increase in process length, but only in the most proximal branch order (Fig. 5N and Supplementary Table 10) comparing to CTRL and STZ offspring (CTRL♀: 17.8 + 1.6, n = 6; STZ♀: 20.7 + 1.8, n = 3; STZ + INS♀: 29.5 + 2.1, n = 3), without changes in the total length (Fig. 5O and Supplementary Table 10), also presenting increased number of intersections of processes at 10 μm of radial distance from soma (Fig. 5P and Supplementary Table 11B) comparing with CTRL (CTRL♀: 4.8 + 0.4, n = 6; STZ♀: 5.8 + 0.6, n = 4; STZ + INS♀: 7.8 + 1.0, n = 3). Regarding microglia cell number, maternal diabetes did not induce changes in male and female microglia density in hippocampal DG. Nevertheless, STZ + INS female offspring presented increased density of microglia comparing both with CTRL and STZ offspring (CTRL♀: 2.9 + 0.4, n = 5; STZ♀: 2.3 + 0.4, n = 5; STZ + INS♀: 6.8 + 0.2, n = 4). No changes were detected in Iba1 RNA expression levels in male (Fig. 5J) and female (Fig. 5R) offspring hippocampus at P7.

3.7. Diabetes during pregnancy decreases rbfox3 mRNA expression and fractalkine receptor protein levels in male offspring hippocampus at late infancy

Aiming to associate memory impairments with cellular and molecular changes, we started by evaluating rbfox3 gene expression in male and female hippocampus, as well as synaptic protein content. At P21, maternal diabetes was associated with a decrease in rbfox3 RNA expression levels (CTRL♂: 1.0 + 0.08, n = 6; STZ♂: 0.62.2 + 0.09, n = 6; STZ + INS♂: 1.2 + 0.08, n = 6) (Fig. 6A), only in STZ male offspring hippocampus, which was prevented by insulin administration to the diabetic dam. This effect was accompanied by a tendency (CTRL male vs STZ male: p < 0.08) for decreased NeuN protein levels (CTRL♂: 100.0 + 9.7, n = 12; STZ♂: 63.9 + 12.9, n = 7; STZ + INS♂: 97.6 + 9.3, n = 9) also only in male offspring (Fig. 6B). Both rbfox3 expression levels (Fig. 6C), and NeuN protein levels (Fig. 6D) remained unchanged in STZ female offspring. (Fig. 6A and B). Postsynaptic PSD-95 as well as presynaptic synapsin-1 and vesicular glutamate transporter-1 (VGlut-1) protein levels at male and female offspring hippocampal total extracts were not changed by maternal diabetes (Supplementary Fig. 1A-F), though STZ male offspring showed a tendency to present decreased VGlut-1 levels comparing to CTRL (p < 0.09).

Since in a previous study, using an animal model of gestational diabetes, cognitive changes observed were associated with reduced density and derangement of the CA1 pyramidal neuronal layer (Vuong et al., 2017) in male adult offspring, and considering that alterations in behavior

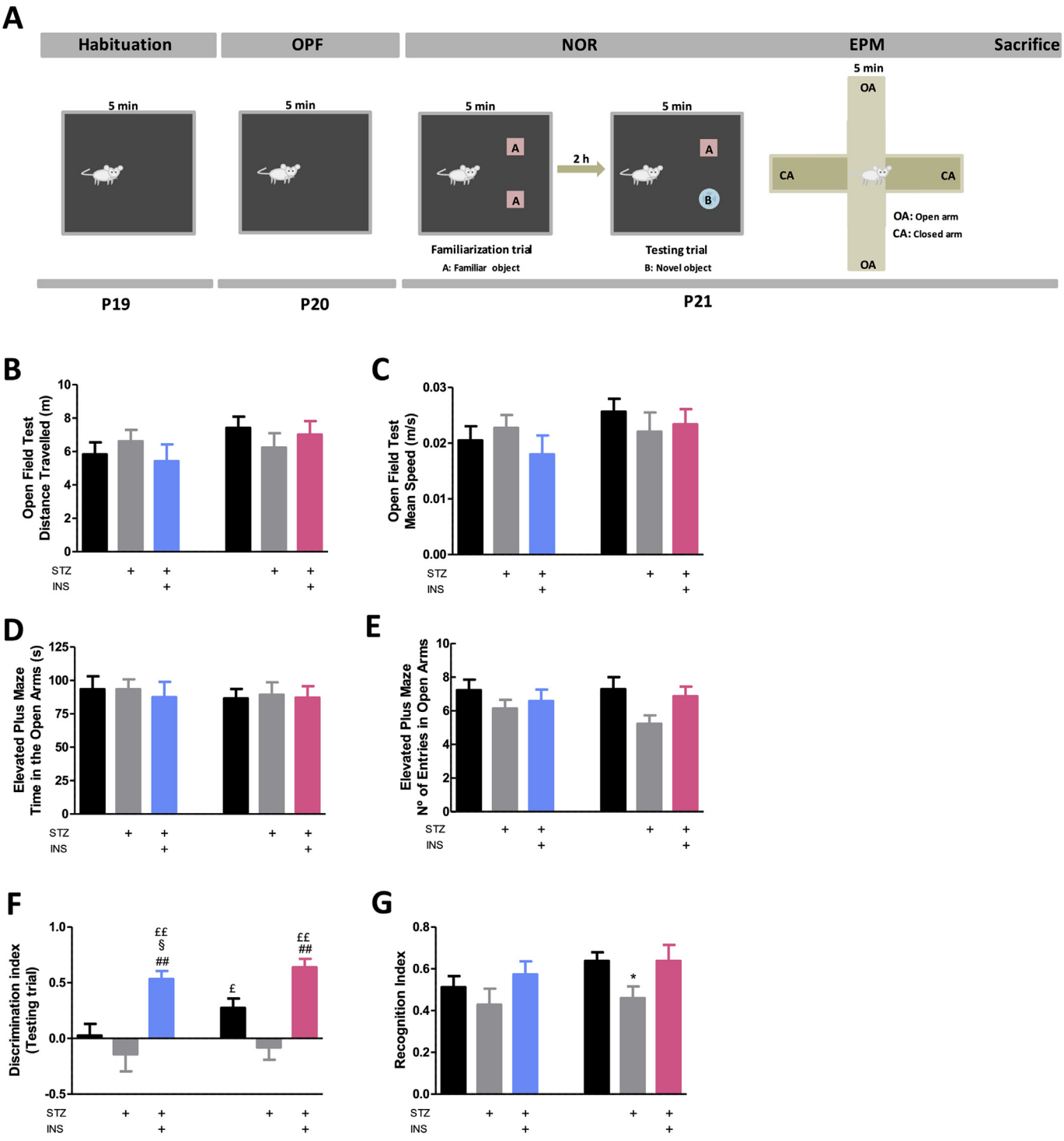

Fig. 4. Diabetes during pregnancy impairs female offspring short-term memory.
(A) Offspring were tested in the OPF test at P20 and in the NOR test and EPM at P21. (B) The distance traveled (m) by the offspring in the open field and the (C) mean speed (m/s) were recorded. (D) The time spent by the offspring in the open arms of the EPM test and the (C) number of entries in the open arms at P21 was noted. (F, G) Offspring were tested in the NOR test for the assessment of (F) Discrimination Index and of (G) Recognition Index. Values are presented as mean +SEM of 12–21 animals for OPF data; 12–21 animals for EPM data and 8–11 animals for NOR levels data. Statistical analysis was assessed with Kruskal-Wallis test; *$p < 0.05$: CTRL (black) compared with STZ (grey) offspring; $^{\#\#}p < 0.01$: STZ compared with STZ + INS (blue and pink) offspring; $^{\S}p < 0.05$: CTRL compared with STZ + INS offspring; $^{£}p < 0.05$: CTRL compared with zero (zero score indicates a null preference); $^{££}p < 0.01$: STZ + INS compared with zero. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

may also be associated with a reshaping of microglia cytoarchitecture (Caetano et al., 2017), we performed a morphometric analysis of microglia in both male and female offspring at late infancy, in the CA1 hippocampal subregion, a critical brain region for memory processes (Fig. 6E, N). Maternal diabetes did not induce changes in microglia morphology regarding the number of process per branch order (Fig. 6F, O and Supplementary Table 12), total number of processes (Fig. 6G, P), length per branch order (Fig. 6H, Q and Supplementary Table 12), total length

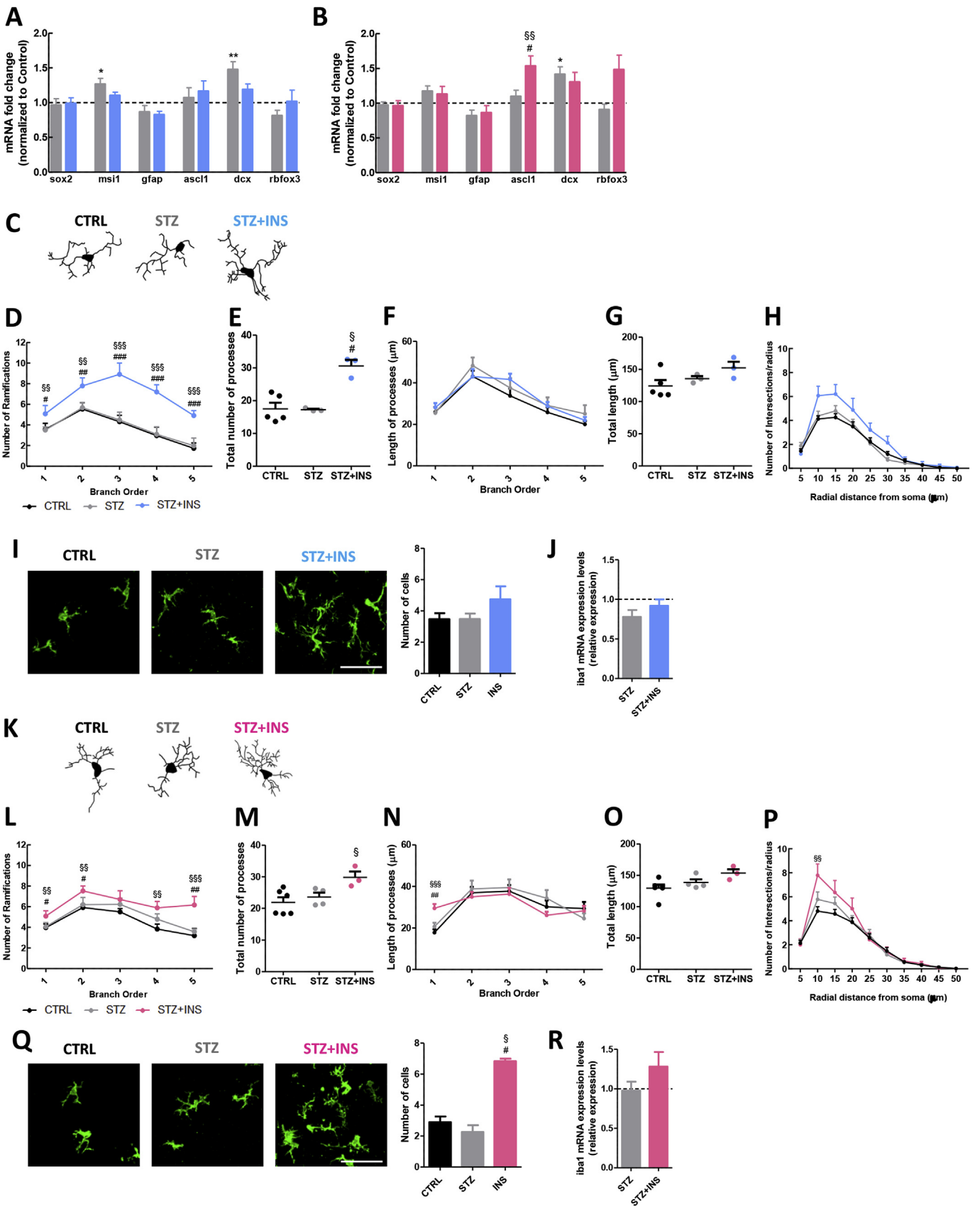

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Fig. 5. Diabetes during pregnancy induces changes in the expression of neural markers in hippocampus at early infancy, but does not impact microglia morphology in hippocampal dentate gyrus.

The expression of several neural markers was evaluated in the hippocampus of (A) male and (B) female offspring at P7. Microglial morphometry was assessed through manual reconstruction of Iba-1 stained microglia from (C) male and (K) female offspring DG at P7. (D, E, L, M) Microglia number of processes and (F, G, N, O) length were evaluated in male and female offspring DG subregion of the hippocampus at P7, as well as the (H, P) number of process intersections per radius. (I, Q) The number of microglial cells per z-stack image (Dimensions: x: 134.95 μm, y: 134.95 μm, z: 30.00 μm) was assessed (scale bar: 50 μm). (J, R) Iba-1 RNA expression levels were evaluated by qPCR. Values are presented as the mean +SEM of 6–8 animals for PCR data; n of 3–6 regarding microglia morphology (detailed n values concerning each morphometric parameter can be observed at Supplementary Tables 10 and 11). Statistical analysis was assessed with Kruskal-Wallis test; *$p < 0.05$, **$p < 0.01$: CTRL (black) compared with STZ (grey) offspring; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$: STZ compared with STZ + INS (blue and pink) offspring; $^{§}p < 0.05$, $^{§§}p < 0.01$, $^{§§§}p < 0.01$: CTRL compared with STZ + INS offspring. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 6I, R), and number of processes intersections per radius (Fig. 5J, S and Supplementary Table 13) both in male and female offspring at P21.

Regarding microglia cell number, maternal diabetes did not induce changes in male and female microglia density in hippocampal CA1 subregion (Fig. 6K, T).

Similarly to what was observed at P7, at late infancy, STZ + INS male (Fig. 6F, G) and female (Fig. 6O, P) offspring microglia, were hyperramified compared to STZ and CTRL ones (CTRL♂: 110.0 + 7.2, n = 4; STZ♂: 108.3 + 6.6, n = 4; STZ + INS♂: 180.6 + 6.0, n = 3/CTRL♀: 97.6 + 12.7, n = 4; STZ♀: 97.7 + 9.0, n = 4; STZ + INS♀: 149.8 + 8.5, n = 3). Additionally, male STZ + INS offspring microglia presented processes with increased length, mainly in the more distal branch orders comparing with STZ and CTRL ones (Fig. 6H and Supplementary Table 12). Additionally, both male and female STZ + INS offspring microglia presented increased number of radii intersections by their processes comparing with CTRL and STZ offspring (Fig. 5J, S and Supplementary Table 13). At P21, insulin administration to diabetic dams did not affect male and female offspring microglia density at CA1 hippocampal subregion (Fig. 6K, T).

Regarding Iba1 RNA expression levels, no changes were observed in male (Fig. 6L) and female (Fig. 6U) offspring hippocampus at P21.

One of the signaling pathways that is important for the communication that microglia establish with neurons is the fractalkine/CX3CR1. Fractalkine receptor (CX3CR1) is important in mediating microglia activity, namely by modulating synaptic pruning by these cells during postnatal development (Paolicelli et al., 2014). Fractalkine signaling has also been described to play a role in the electrophysiological properties of excitatory synapses in the hippocampus (Ragozzino et al., 2006; Bertollini et al., 2006). The content of CX3CR1 in the hippocampus of STZ male offspring was significantly decreased compared with CTRL (Fig. 6M), an effect that was not observed in female offspring (Fig. 6V) and that was prevented by insulin administration to the diabetic dams (CTRL♂: 100.0 + 7.4, n = 10; STZ♂: 67.4 + 6.6, n = 9; STZ + INS♂: 102.3 + 9.6, n = 7).

4. Discussion

The adaptation that fetus undergoes due to suboptimal intrauterine conditions creates long-term health consequences (Barker et al., 1993). In diabetes, the intrauterine environment has been evidenced to create an adverse environment for fetal growth and development, impacting several outcomes later in life (Van Assche et al., 2001).

The animal model used in this work (injection of 45 mg/kg STZ, IP) is described to induce moderate diabetes (Vafaei-Nezhad et al., 2016). Nevertheless, our results are in accordance with what is described for more severe cases of diabetes (Van Assche et al., 2001). Both female and male STZ offspring were microssomic during infancy. Similar findings were already reported at this age and also at adolescence (Piazza et al., 2019) being the animals small up to adulthood (Aerts et al., 1990). However, results also revealed that pregnant diabetic dams did not differ in terms of weight comparing to controls, while the literature describes specifically for a severely diabetic rat that during pregnancy fat deposits are mobilized and animals hardly gain weight during pregnancy (Aerts et al., 1990). We also did not find any statistically significant differences in the number of pups born from diabetic dams comparing with control ones, which could influence dams' weight at GD21. Interestingly, STZ + INS female offspring were heavier than CTRL and STZ offspring from P0 until P21, an effect not observed in STZ + INS males. In humans, babies (no sex differences were assessed) of insulin-treated gestational diabetic mothers, are reported to be heavier than diet control ones (Wong and Jalaludin, 2011). Maternal diabetes changes the placental structure, which may alter the transport of nutrients to the fetus (Araujo et al., 2015). Although a low insulin dose was administered to the dams to prevent it to cross the placenta, it can bind to insulin receptors present in the trophoblast membrane, activating signaling pathways, thus contributing to the placental metabolism of nutrients (Hiden et al., 2006) and, therefore to placental and fetal development (Ruiz-Palacios et al., 2017).

In studies where STZ injection was administered to dams in mid-gestation, offspring are reported to be hyperglycemic from birth to P21 (Chandna et al., 2015). In our study, both male and female STZ offspring showed a tendency to present higher glycemia values compared to CTRL at birth, similarly to what was reported in other studies (Vafaei-Nezhad et al., 2016; Sadeghi et al., 2018), being insulin treatment able to prevent this effect. In severe maternal diabetes, the increased glycemia may lead to an intense activity of the fetuses β-cells that deplete their stores, as they are not able to biosynthesize insulin at the rate that it is secreted (Van Assche et al., 2001), leading to a reduction in fetal glucose uptake. Consequently, fetal protein mass growth and protein synthesis are consistently lower than in controls (Canavan and Goldspink, 1988), which may account for the decreased weight presented by STZ offspring. It is described that after birth, β-cells are able to restore their insulin pool and respond with effectiveness to circulating levels of glucose. Although that seems to be the case, since at P7 and P21 STZ offspring glycemia is similar to CTRL, STZ pups remained microssomic.

Regarding plasma insulin levels, STZ female offspring presented a tendency to lower insulin levels comparing to CTRLs, suggesting that low *in utero* growth induced by maternal diabetes may be correlated with decreased number of pancreatic β-cells and decreased capacity to produce insulin. STZ + INS female offspring, but not males, presented a transient increase in serum insulin levels comparing with STZ and CTRL at birth, suggesting a sex-different response.

Since low birth weight has been associated with poor cognitive development at infancy (Linsell et al., 2015) and psychiatric disorders at adolescence (Costa Machado et al., 2018), and maternal diabetes has been implicated in developmental susceptibility to psychomotor delays in early ages in humans (Rizzo et al., 1995), we evaluated offspring neonatal development taking into account sex-differences, and also in response to insulin therapy, given that developmental alterations in the offspring may influence behavioral changes later in life (Caetano et al., 2017; Duarte et al., 2019). Both STZ male and female performed worse than CTRLs in all the neurodevelopmental tests performed. The results suggest that STZ offspring present vestibular system deficits, as well as impaired motor coordination and balance. This may indicate a delay in the maturation of somatosensory, vestibular and/or proprioception function, which may be associated with a delayed maturation (neurogenesis) of these systems or hindered functional organization of the synaptic circuitry underlying these reflexes in the brain. STZ offspring also presented less strength in forelimbs and marked locomotion deficits.

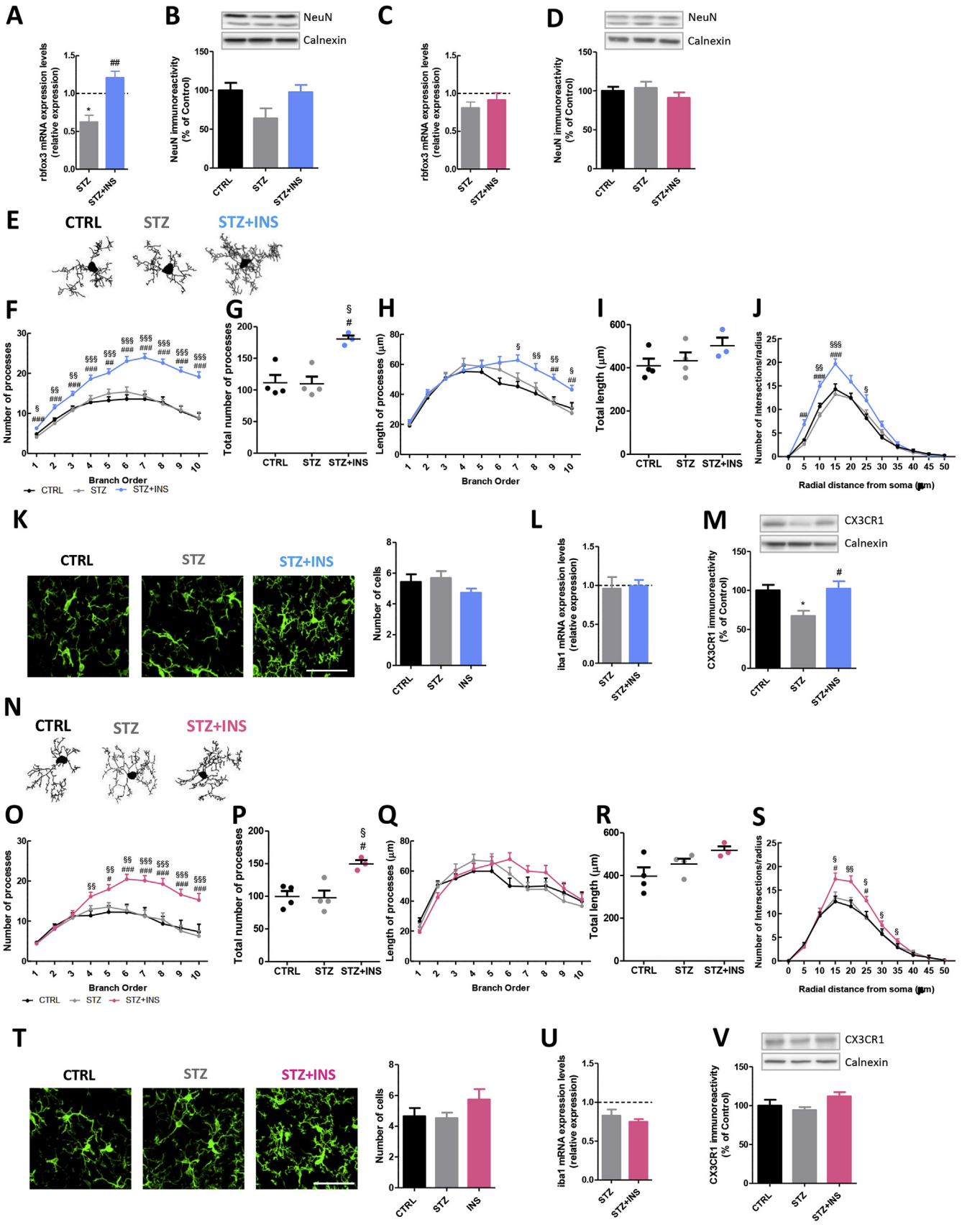

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Fig. 6. Diabetes during pregnancy decreases rbfox3 mRNA expression and fractalkine receptor protein levels in male offspring hippocampus at late infancy.

The expression of rbfox3 mRNA levels was evaluated in the hippocampus of (A) male and (C) female offspring at P21, as well as (B, D) NeuN protein levels in hippocampal total extracts.

The number (F, G, O, P), length (H, I, Q, R) of microglial processes, as well as the number of process intersections per radius (J, S) were evaluated in male (E) and female offspring (N) CA1 subregion of the hippocampus at P21. The number of microglial cells per z-stack image (Dimensions: x: 134.95 μm, y: 134.95 μm, z: 30.00 μm) was assessed in male (K) and female (T) hippocampus (scale bar: 50 μm). (L, U). Iba-1 RNA expression levels were assessed by qPCR and (M, V) CX3CR1 protein levels by Western blot. Values are presented as the mean +SEM of 5–6 animals for qPCR data; n of 7–10 animals for Western blot data; n of 3–4 regarding microglia morphology (detailed n values concerning each morphometric parameter can be observed at Supplementary Tables 12 and 13). Statistical analysis was assessed by Kruskal-Wallis test; *$p < 0.05$: CTRL (black) compared with STZ (grey) offspring; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$: STZ compared with STZ + INS (blue and pink) offspring; $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$: CTRL compared with STZ + INS offspring. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Nest seeking behavior was also impaired in STZ offspring. Since performance in nest seeking test is also dependent on locomotion to achieve the home bedding, components evaluated in this test as mother-pup relation and odor discrimination of home bedding may be biased by impaired locomotion. Nonetheless, it was described that the offspring of STZ dams at P4 and P6 emitted higher numbers of ultrasound calls compared to control offspring (Johansson et al., 1991), showing an aversive affective state of STZ pups.

In an animal model of gestational diabetes it was recently described that the offspring of diabetic dams also present a worse performance in neurodevelopmental tests. Yet, this was only observed in some of the tests, and the offspring performed positively the tasks much sooner than what we report in this study. Additionally, the authors did not evaluate the impact of insulin administration in preventing the effects, whereas we show that insulin treatment to STZ dams was able to prevent most of the delay impairments presented by STZ offspring. Likewise, it was demonstrated that infants from mothers with insulin-dependent diabetes present a normal neurodevelopment if it is maintained a good glycemic control throughout pregnancy (Sells et al., 1994). Contrarily to what was described in an animal model of gestational diabetes in which male offspring performed worse than females in some of the behavioral tests (Piazza et al., 2019), in our study (pregestational type 1 diabetes) we report a similar magnitude of developmental delay in both sexes.

Regarding the evaluation of the eye opening day and auditory startle response, STZ offspring had a clear delay in the achievement of these developmental milestones, which is in agreement with results obtained in a previous study using an animal model of gestational diabetes (Piazza et al., 2019). Interestingly, comparing to the aforementioned study, in our work an expedited opening of pup eyes and of auditory startle response was observed in both male and female STZ + INS offspring. These findings may be associated with insulin and the levels of other growth factors that are prominent determinants of fetal development. Although we did not find marked plasma insulin changes between experimental groups after birth, as previously referred, insulin can bind to insulin receptors present in the trophoblast membrane, activating signaling pathways that contribute to fetal development (Ruiz-Palacios et al., 2017). In fact, recently it was described the critical involvement of placental insulin receptors in sex-specific neurodevelopment and their implications in maternal diabetes (Bronson et al., 2017).

Maternal diabetes can induce cognitive impairment in both humans (Nelson et al., 2000) and animal models (Vuong et al., 2017; Kinney et al., 2003; Ramanathan et al., 2000), also increasing the susceptibility to neuropsychiatric disorders (Van Lieshout and Voruganti, 2008; Xiang, 2017). We evaluated the performance of the offspring in OPF at late infancy in order to exclude any locomotion impairment that could interfere with their performance at EPM and NOR tests. Contrarily to what was observed during early stages of development, at P20 no locomotor changes were observed between STZ and CTRL offspring regarding the distance traveled and mean speed, showing that there are no locomotor impairments at this age. Remarkably, in a pregestational STZ-induced diabetic rat model, authors report increased ambulation of the 8 weeks-old offspring of diabetic dams, although not making sex distinction (Ramanathan et al., 2000). In the same study, authors reported that the offspring of diabetic dams displayed an anxious-like behavior in EPM (Ramanathan et al., 2000). In the present study, both STZ male and female offspring did not exhibit an anxious-like behavior at EPM. Aligned with our results, a study performed at P60 on both male and female offspring of diabetic dams reported no alterations in anxious-like behavior assessed by EPM test relative to control ones (Kinney et al., 2003). Interestingly, in a gestational diabetes animal model, male offspring of STZ-injected dams and STZ-injected dams treated with insulin dwelled more in the open arms of the EPM at P40 showing a disinhibition compared to control ones (Chandna et al., 2015). The contrasting results reported may be explained by differences in the animal model of maternal diabetes implemented, strain and postnatal day assessed. As anxiety-related brain structures are undergoing maturation, variations could emerge when comparing results in infancy, adolescence and adulthood.

The analysis of discrimination index and recognition index in the NOR showed that STZ female offspring presented impaired recognition memory at infancy, having insulin therapy a preventive effect. Regarding STZ male offspring, animals presented a negative score, but close to zero, in the discrimination index, demonstrating that they did not have a preference for the novel object. Both STZ + INS male and female offspring presented positive scores indicating that they spent more time exploring the novel object. Their ability to discriminate the novel object was in fact greater that the one presented by respective CTRL. The results also demonstrated that CTRL female offspring presented the expected performance in NOR test, being able to discriminate between the familiar and novel object and spending significantly more time exploring the novel object. Notably, CTRL male offspring were not able to accurately discriminate between the familiar and novel object. Consequently, we were not able to properly conclude that STZ male offspring present impaired recognition memory, though there is a trend for STZ male offspring to present negative scores in the discrimination index assessment, and for CTRL male offspring to present a positive one. Importantly, we cannot exclude the possibility that at this early age, offspring may still not have fully developed their exploration and learning skills, since the emergence of spatial learning capability and active exploration develops around P21 and continues to mature between P30 and P90 (Wills et al., 2014; Tan et al., 2017). Therefore the results may suggest that in physiological conditions female offspring develop these skills before males, also suggesting that insulin therapy may in fact promote the emergency of spatial learning and development of active exploration. Impaired recognition memory was described in young adult male offspring subjected to gestational diabetes induced by high sucrose and fatty acids diet (Vuong et al., 2017). On the other hand, in an STZ-induced gestational diabetes animal model, it was reported that young adult male offspring did not display deficits in the NOR, but presented increased preference for the novel object in a NOR displacement test (Chandna et al., 2015). Additionally, it was reported that only female offspring of STZ-diabetic dams displayed learning deficits at P60 by using the Lasheley III maze and the inhibitory avoidance task (Kinney et al., 2003). These studies further reinforce the importance of sex-differential analysis of behavior in the offspring of diabetic dams.

Further aiming to associate postnatal behavior with changes in the formation and maturation of brain circuitry, we analyzed the expression of several neural markers in whole offspring hippocampus at early infancy. Increased expression of msi1 in STZ male and dcx expression both in male and female offspring may suggest an increase in migrating neuronal precursors. Nevertheless, caution in interpreting these results must be taken, since we analyzed gene expression at the whole hippocampus and not only at DG, and dcx may also be expressed by non-migrating neuroblasts and immature neurons. On the other hand, and in agreement with our qPCR findings, a previous study reported a significant higher hippocampal dcx expression levels in STZ male offspring (Sadeghi et al., 2018). Also, and though the authors only evaluated male offspring and the age analyzed was different (P14) from the one in our study (P7), they associated this dcx mRNA expression increase in the whole hippocampus with an increase in the mean number of dcx + neurons at hippocampal DG (assessed by stereological methods) in male STZ pups (Sadeghi et al., 2018). Concerning the increased expression of ascl1, in STZ + INS female offspring observed in this study, it could be related to an increased proliferation or survival of progenitor cells, but it can also indicate an increase in neuronal differentiation of STZ + INS female hippocampus.

Since microglia regulates brain development through newly-born/dying cells phagocytosis, impacting neuronal and synapse formation/elimination, we analyzed the number of microglial cells and morphology in the DG at P7. Maternal diabetes did not induce changes in the density of microglia, number and length of microglia processes at DG in both male and female STZ offspring at P7, suggesting there are no morphological alterations in the hippocampal DG induced by diabetes during early neonatal period. In an animal model of gestational diabetes a reduction in the proportion of ramified microglia and increased amoeboid morphology was observed, concomitant with increased levels of pro-inflammatory cytokines in the hippocampus of prenatal (E20) male offspring (Vuong et al., 2017). In the same study, no changes in Iba-1 levels were found at P0 (Vuong et al., 2017), similarly to what we found at P7. Insulin treatment to diabetic dams induced a significant increase in the number of microglia processes, both in females and males comparing with CTRL and STZ offspring. In females, STZ + INS offspring also presented increased number of microglial cells at DG and increased number of processes intersecting sholl radius at 10 μm. Together these results suggest that at DG, STZ + INS offspring microglia are more complex than CTRL and STZ microglia, occupying and surveilling more area of DG parenchyma.

Aiming to associate the memory impairments observed at late infancy (P21) with cellular and molecular changes, we evaluated rbfox3 gene expression/NeuN protein levels in male and female hippocampus, as well as synaptic protein content. Although a decrease in rbfox3 gene expression was observed in STZ male offspring, (no statistically significant differences were detected regarding NeuN protein levels, though a tendency for decrease was observed), this reduction in expression levels may not be indicative of a decrease in the number of mature neurons in STZ offspring hippocampus since the number of expressing NeuN + cells may remain unchanged and only the expression levels per cell may be reduced. Nonetheless, a previous study reported a decrease in NeuN expression levels at whole hippocampus of STZ males at P14 (Sadeghi et al., 2018), which was associated with a reduction on the number of NeuN + cells at STZ offspring at DG hippocampal subregion. Also, in a model of uncontrolled gestational diabetes a reduced number of pyramidal neurons was detected in the hippocampus of male offspring at P7 and P21 (Golalipour et al., 2012), suggesting that maternal diabetes may in fact induce a loss of mature neurons or an impairment of their maturation in male offspring.

Additionally, in a model of gestational diabetes, a reduction in the number of BrdU + cells at hippocampal DG was detected at P40, indicating an impairment on the survival of newborn cells (Piazza et al., 2019). Nevertheless, since the authors did not check the initial amount of BrdU + cells, this reduction may also be due to decreased proliferation instead of decreased survival.

Regarding synapse integrity, previous studies reported, in male offspring, a downregulation of the presynaptic marker synaptophysin at P14 in a model of pregestational diabetes and in a model of gestational diabetes at 15 weeks of age (Vuong et al., 2017; Vafaei-Nezhad et al., 2016). In this study we did not observe changes in the synaptic proteins PSD-95, synapsin-1 and VGlut-1 protein levels, though STZ male offspring presented a tendency to have reduced levels of VGlut-1, a marker of glutamatergic synapse integrity.

The fractalkine/CX3CR1 signaling is known to modulate the activity and plasticity of developing and mature synapses, brain functional connectivity, as well as learning and memory processes (Paolicelli et al., 2014). At P21, a reduction in CX3CR1 in STZ male offspring hippocampus was observed. The decrease in rbfox3 expression, together with the reduction in CX3CR1 content, and the tendency to decreased levels of NeuN and VGlut-1 specifically in male STZ offspring suggest a different vulnerability to maternal diabetes between males and females. Nevertheless, the mechanisms involved in the crosstalk between neuron and microglia that may contribute to synaptic alterations under maternal diabetes and to behavioral outcomes remain to be further elucidated.

Similarly to what was observed at offspring DG at P7, we did not observe changes in microglia cell number and morphology in the hippocampal CA1 of STZ males and females at P21. Nevertheless, both at P7 and P21, insulin treatment to diabetic dams induced a significant increase in the number of microglia processes, both in females and males comparing with CTRL and STZ offspring. Microglia hyper-ramification has been observed in several studies depending on the stimuli/stressor. Footshock exposure has been shown to promote microglia hyper-ramification concomitant with reductions in dendritic spines in the medial prefrontal cortex (mPFC) and dorsal hippocampus (dHIP) (Smith et al., 2019). It was also described a hyper-ramification of retinal microglia after ionotropic glutamatergic neurotransmission (Fontainhas et al., 2011). We have previously reported that prenatal exposure to dexamethasone induces microglia hyper-ramification at the dHIP of adult female offspring (Duarte et al., 2019), and an atrophy in females and hyper-ramification in males offspring at the mPFC (Caetano et al., 2017). This evidence highlights the sensor capability of microglia and its ability to respond to different stimuli.

Insulin regulates placental function during gestation, altering the levels and function of insulin receptors along time. (Hiden et al., 2006). Alterations in placental insulin signaling may modulate offspring intracellular metabolism and gene expression regulation (Hiden et al., 2006). We speculate that the hyper-ramification observed in STZ + INS may be due to a microglia specific insulin response, but future studies are needed to reconcile the hyper-ramified microglial cell morphology observed in the STZ + INS offspring with metabolic and behavioral changes.

Most of the negative impacts induced by maternal diabetes on the offspring, namely the developmental delay and impaired memory were prevented by insulin administration to diabetic dams, demonstrating the importance of strict glycemic control during pregnancy. Furthermore, this study also demonstrated that insulin administration may have an impact on the offspring, namely in expediting the developmental physical milestones, in inducing microglia hyper-ramification and possibly also promoting the emergency of offspring spatial learning and the development of active exploration.

Further studies will be important to dissect the underpinning mechanisms responsible for the negative effects of maternal diabetes on the offspring. Clarification of such mechanisms could allow delineating future therapeutic strategies to prevent and treat offspring neurobehavioral impairments induced by maternal diabetes. Also, since we found that maternal diabetes impacts differently male and female offspring, further understanding of gender differences in the etiology and clinical presentation of pathologies will be fundamental for the efficient prevention of offspring impairments.

5. Conclusions

Taken together, the findings of the current study highlight the negative effects of uncontrolled hyperglycemia during pregnancy on the offspring, also emphasizing the importance of insulin therapy in preventing those effects. Moreover, insulin therapy promoted the advancement of the achievement of developmental milestones and induced a hyper-ramification of male and female hippocampal microglia. Most notably, the impact of maternal diabetes on offspring behavior and hippocampus is sex-specific emphasizing the importance of taking into account gender-specificities in experimental studies and in clinics.

Funding

This work was supported by Foundation for Science and Technology (PEst UID/NEU/04539/2013, UID/NEU/04539/2019, UIDB/04539/2020 and UIDP/04539/2020), COMPETE-FEDER (POCI-01-0145-FEDER-007440), and Centro 2020 Regional Operational Programme (CENTRO-01-0145-FEDER-000008: BrainHealth, 2020). Filipa I. Baptista acknowledges a fellowship from Foundation for Science and Technology, Portugal (SFRH/BPD/86830/2012).

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors acknowledge Raquel Boia and Célia Aveleira for assistance in PCR data analysis, and also Bárbara Oliveiros from the Laboratory of Biostatistics and Medical Informatics of the Faculty of Medicine of the University of Coimbra for assistance in the statistical analysis of the data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2020.100075.

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