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CHRONIC MATERNAL HYPERGLYCEMIA INDUCED DURING MID-PREGNANCY IN RATS INCREASES RAGE EXPRESSION, AUGMENTS HIPPOCAMPAL EXCITABILITY, AND ALTERS BEHAVIOR OF THE OFFSPRING

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Abstract—Maternal diabetes during pregnancy may increase the risk of neurodevelopmental disorders in the offspring by increasing inflammation. A major source of inflammatory signaling observed in diabetes is activation of the receptor for advanced glycation end-products (RAGE), and increased RAGE expression has been reported in psychiatric disorders. Thus, we sought to examine whether maternal diabetes creates a proinflammatory state, triggered largely by RAGE signaling, that alters normal brain development and behavior of the offspring. We tested this hypothesis in rats using the streptozotocin (STZ; 50 mg/kg; i.p.) model of diabetes induced during mid-pregnancy. Following STZ treatment, we observed a significant increase in RAGE protein expression in the forebrain of the offspring (postnatal day 1). Data obtained from whole-cell patch clamping of hippocampal neurons in cultures from the offspring of STZ-treated dams revealed a striking increase in excitability. When tested in a battery of behavioral tasks in early adulthood, the offspring of STZ-treated dams had significantly lower prepulse inhibition, reduced anxiety-like behavior, and altered object-place preference when compared to control offspring. In an operant-based strategy set-shifting task, STZ offspring did not differ from controls on an initial visual discrimination or reversal learning but took significantly longer to shift to a new strategy (i.e., set-shift). Insulin replacement with an implantable pellet in the dams reversed the effects of maternal diabetes on RAGE expression, hippocampal excitability, prepulse inhibition and object-place memory, but not anxiety-like behavior or set-shifting. Taken together, these results suggest that chronic maternal hyperglycemia alters normal hippocampal development and behavior of the offspring, effects that may be mediated by increased RAGE signaling in the fetal brain.
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Key words: gestational diabetes, hippocampus, neurodevelopmental disorder, behavioral flexibility, sensorimotor gating, recognition memory.

INTRODUCTION

Gestational diabetes (GDM) mellitus refers to the diagnosis of impaired glucose tolerance during pregnancy and accounts for 90% of all cases of maternal diabetes mellitus (Van Lieshout and Voruganti, 2008). GDM has varying effects on fetal development depending on its severity and time of onset (Van Lieshout and Voruganti, 2008). Of particular interest is the fact that GDM profoundly alters the normal trajectory of fetal brain development, thereby predisposing the offspring to neurodevelopmental disorders such as schizophrenia (Boksa, 2004; Van Lieshout and Voruganti, 2008) and autism (Gardener et al., 2009; Guinchat et al., 2012; Xiang et al., 2015). Children born to diabetic mothers may exhibit a range of neurodevelopmental abnormalities that parallel those in schizophrenia, including impairments in motor functioning, attention span and learning ability (Jones et al., 1994; Cannon et al., 2002).

While the mechanism(s) by which GDM predisposes the offspring to neurodevelopmental disorders remains to be determined, maternal hyperglycemia is one candidate as it induces immune activation, oxidative stress, hyperinsulinemia, chronic tissue hypoxia, and decreased iron levels in the fetus (Van Lieshout and Voruganti, 2008). A major source of inflammatory signaling in diabetes is activation of the receptor for advanced glycation end-products (RAGE) (Bierhaus and Nawroth, 2009; Xie et al., 2013). Interestingly, several studies have shown increased advanced glycation end-products (AGEs) and RAGE expression in people with psychiatric disorders (Steiner et al., 2009; Emanuele et al., 2011; Kouidrat et al., 2013). In addition to its contribution to neuropathology, RAGE is expressed in the developing brain under physiological conditions not associated with AGE formation (Hori et al., 1995), suggesting it may play a role in neurodevelopment. Thus, we sought to investigate the

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Abbreviations: AGEs, advanced glycation end-products; ANOVA, analysis of variance; EGTA, ethylene glycol tetraacetic acid; FR1, fixed ratio 1; GD, gestational day; GDM, gestational diabetes mellitus; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HMGB1, high-mobility group box-1; KCNK, K^+ currents generated by K^+ leak channels; LTP, long-term potentiation; PND, postnatal day; PPI, prepulse inhibition; RAGE, receptor for advanced glycation end-products; STZ, Streptozotocin; TTC, trials to criterion.

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role of hyperglycemia during pregnancy in altering RAGE expression and behavior in the offspring.

Animal models of diabetes during pregnancy include both surgical and chemical manipulations, each with different strengths and weaknesses (Jawerbaum and White, 2010). One common model involves the chemical ablation of pancreatic β-cells by streptozotocin (STZ) which causes insulin deficiency and hyperglycemia (Lenzen and Panten, 1988). Previous studies testing the effects of STZ treatment during pregnancy on the offspring have demonstrated both short- and long-term outcomes that depend on the timing of administration. Short-term outcomes of administration of STZ in pregnancy include neonatal morbidity and mortality, in particular when treatment occurs early in pregnancy (Jawerbaum and White, 2010). When the long-term effects of maternal diabetes during pregnancy on the offspring are considered, a range of behavioral, neurochemical, and molecular abnormalities are observed (Boksa, 2004; Van Lieshout and Voruganti, 2008; Jawerbaum and White, 2010). Increased anxiety-like behavior, reduced social interaction, and impaired learning and memory (female offspring only) have been reported in the offspring of dams treated with STZ (Ramanathan et al., 2000; Kinney et al., 2003). Elevated levels of dopamine and norepinephrine in the hypothalamus (Plagemann et al., 1998) and decreased brain weight (Yamano et al., 1986) have also been reported in the offspring of STZ-treated dams. These studies suggest that maternal diabetes subtly alters brain development and behavior; however, the consequences of maternal hyperglycemia on RAGE signaling and neural excitability in the offspring remain to be determined.

The present experiments used the STZ model in rats to examine the effect of severe maternal hyperglycemia initiated during pregnancy on the offspring. Pregnant rats were administered STZ on gestational day (GD) 13 to induce hyperglycemia and the male offspring were examined for changes in RAGE expression in the brain, hippocampal neuron excitability, and a battery of behavioral measures relevant to neurodevelopmental disorders (prepulse inhibition, elevated plus maze, recognition memory, and behavioral flexibility). We hypothesized that maternal hyperglycemia would induce RAGE-dependent developmental changes that would alter behavior in the offspring.

EXPERIMENTAL PROCEDURES

Subjects

Timed pregnant Long–Evans rats (GD 7; Charles River Laboratories, Quebec, Canada) were singly housed in transparent plastic cages in a colony room on a 12/12-h light/dark cycle (lights on at 07:00 h). Food and water was available *ad libitum* and the room was temperature controlled at 21 °C. All handling and testing was conducted between 0800 h and 1800 h. The experiments were conducted using two groups of dams (group 1, $n = 10$; group 2, $n = 21$). All experimental procedures were performed in accordance with the standards of the Canadian Council on Animal Care and were approved by the University of Saskatchewan Animal Research Ethics Board.

Gestational STZ treatment

Dams were weighed and handled for 2–3 days prior to treatment. On GD 13, the dams were individually transported to a procedure room, weighed, and the appropriate dose of either saline ($n = 10$) or STZ ($n = 21$; 50 mg/kg; reviewed by Jawerbaum and White, 2010) was administered (i.p.). The dams were then returned to the colony room in clean cages. Forty-eight hours following the injection, a diabetic state (blood glucose > 15 mM) was confirmed in STZ-treated dams via blood samples taken from the saphenous vein. STZ-treated dams that did not show elevated glucose levels (> 10 mM) received a second STZ injection at this time ($n = 7$). Four dams failed to develop a diabetic state and were excluded from the experiment. Some STZ-treated dams ($n = 7$) were pseudo-randomly assigned to receive subcutaneous sustained release insulin implants (2.5 mm length; Linplants, LinShin Canada, Inc., Toronto, Ontario) under isoflurane anesthesia on GD15, which provide a sustained release of insulin. Some of the saline- and STZ-treated dams received a palmitic acid control pellet ($n = 6$ dams in each group). Weights, blood glucose levels, and temperatures were monitored for the remainder of gestation. Data from the offspring of $n = 10$ saline-treated, $n = 10$ STZ-treated, and $n = 7$ STZ + Ins-treated dams were used in the final analysis.

Primary hippocampal cultures

Hippocampal neurons were cultured from newborn postnatal day (PND) 1 rats using a modified version of previously described methods (Falzone et al., 2009). Briefly, hippocampi (dorsal, rostral portion) were removed from the brain and enzymatically dissociated for 30 min at 37 °C in PBS solution containing papain (45 U; Worthington, Freehold, NJ, USA), 0.05% DNase I (Roche Diagnostics, Mannheim, Germany), TTX (1 μM; Alomone Labs Ltd., Jerusalem, Israel), DL-cysteine HCl (1.5 mM; Sigma, St. Louis, MO, USA), 0.025% bovine serum albumin (Sigma), and D-glucose (5 mM; Sigma). Hippocampi were then washed twice and triturated in DMEM media supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, USA) and L-glutamine (500 μM; Sigma). The resulting cell suspension was plated on laminin-coated coverslips and housed at 37 °C in a 95% air-5% CO_2 incubator. Cells were maintained in Neurobasal media (Invitrogen) containing 5 mM glucose and enriched with L-glutamine (500 μM; Sigma) and B27 (Invitrogen). Electrophysiological recordings from hippocampal pyramidal neurons were performed 7–12 days after plating. Cultures used for sodium and potassium currents and action potential experiments were generated from seven pups for the saline group, five pups for the STZ group, and seven pups for the STZ + Ins group. For the KCNK currents, cultures were generated from tissue obtained from five (saline group), three (STZ group), and seven pups (STZ + Ins). Hippocampi from several of

the pups were collected and plated at the same time; therefore, each neuron was used as the unit of analysis.

Whole-cell patch-clamp recording

Whole-cell current or membrane potentials were recorded with an Axopatch 200B amplifier (Molecular Devices, Union City, CA, USA) equipped with a 1-GΩ cooled head-stage feedback resistor and a Digidata 1400A analog-to-digital converter (Molecular Devices). Recordings were conducted at room temperature, sampled at 5 kHz, and stored on a personal computer. Current- and voltage-clamp protocols, data acquisition, and analysis were performed using pClamp 10 (Molecular Devices) and Origin 7.0 (OriginLab Corporation, Northampton, MA, USA) software. Action potentials were generated in current clamp mode by injection of a series of depolarizing current steps at 100-pA increments for 500 ms. All other experiments were carried out under voltage clamp mode. *I–V* plots were generated by comparing peak currents from the average of three recordings at each step potential, over the range of −100 to +90 mV (10 mV increments), from a holding potential of −60 mV. In all experiments, cells were continuously perfused using a gravity perfusion system. Recording electrodes were made from borosilicate glass (World Precision Instruments, Sarasota, FL, USA) using a vertical puller (PC 10; Narishige, East Meadow, NY, USA) and were fire-polished with a microforge (MF 900; Narishige). Patch pipettes had resistances of 5–11 MΩ when filled with intracellular recording solution and formed gigaseals between 1 and 10 GΩ. Recording electrodes were filled with the following solution unless otherwise stated (in mM): 65 KF, 55 KAc, 5 NaCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 10 EGTA, 2 MgATP and 10 HEPES, and pH was adjusted to 7.2 with KOH. In experiments recording action potentials and voltage-gated sodium and potassium currents, neurons were perfused with extracellular solution consisting of (in mM): 140 NaCl, 5.4 KCl, 1.3 $CaCl_2$, 25 HEPES, 5 glucose, and 5 μg/ml phenol red; pH was adjusted to 7.4 with NaOH. When recording KCNK currents, the extracellular solution contained (in mM): 105 NaCl, 5.4 KCl, 25 HEPES, 5 glucose, 1.3 Ni^{2+}, 30 TEA, 5 4AP, 0.001 TTX and 5 μg/ml phenol red; pH was adjusted to 7.4 with NaOH.

Western blot analysis

Whole-brain samples from PND 1 rat pups were lysed using a 1% NP-40 lysis buffer containing various protease inhibitors. Protein concentration was determined by Bradford assay and samples were resolved by 10% SDS–PAGE and then electro-transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with 5% blotting grade milk (Bio-Rad Laboratories), the resulting membrane was incubated with the following primary antibodies overnight at 4 °C: rabbit anti-RAGE (1:1000; Chemicon, Temecula, CA, USA), rabbit anti-NFκB (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-GAPDH (1:10,000; AbCam, Cambridge, MA, USA). The membrane was then washed three times with TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20) before being probed with either a horseradish peroxidase-conjugated goat anti-rabbit or mouse secondary antibody (1:20,000; Bio-Rad Laboratories). Protein signals were visualized using enhanced chemiluminescence reagents (Santa Cruz Biotechnology) and quantified by performing densitometry using Image J software (NIH, Bethesda, MD, USA).

Neonatal treatment

Parturition day was designated as postnatal day (PND) 0. On PND 1, litters were counted, weighed, and culled to a maximum of 10 pups per litter (six males and four females where possible). Two male pups per litter were sacrificed for primary hippocampal cultures and protein analysis immediately following culling on PND 1. To ensure that none of the male offspring were diabetic, one rat from each litter was tested for glucose levels on PND 7 and a subset of rats were tested again on PND 40. Besides normal animal husbandry (which included weighing at the same time as cage changing), the dams and pups were left undisturbed until PND 21 when pups were weaned. Only the male offspring were used in the subsequent behavioral tests.

Behavioral testing

General behavioral methods. Rats were handled for a minimum of 3 days during the week before behavioral testing was initiated. The elevated plus maze and object recognition memory tests were conducted in a rectangular room containing an empty plastic water maze. Four identical floor lamps provided background illumination during all tests. Litter mates were tested in two groups: one group was tested in prepulse inhibition (PPI) and object recognition memory and the second group was tested in elevated plus maze and the operant test battery. A one week interval separated repeated tests of the same subject unless otherwise noted. All testing equipment was thoroughly washed with 40% ethanol between trials to eliminate odor cues.

Prepulse inhibition (PPI). The PPI testing procedure was similar to previous protocols (Howland et al., 2004a, 2012; Ballendine et al., 2015). PPI testing was performed before rats reached puberty (PND 35 and 36) and again in early adulthood (PND 58 and 59). Testing was conducted in two standard SR-LAB startle boxes (San Diego Instruments, San Diego, CA, USA) containing a cylindrical Plexiglas chamber in which the rat is placed. Prior to testing, each box was calibrated in order to ensure consistent noise levels. Each PPI session began with a 5-min acclimatization period during which the rat was exposed to a 70-dB background noise that remained constant throughout the entire test session. This period was followed by six pulse alone trials (120 dB, 40 ms), in order to establish a relatively stable level of startle amplitude. Immediately after the pulse alone trials, 84

trials were presented in a pseudo-random order: no stimulus (six trials), pulse alone (six trials; 120 dB, 40 ms) or prepulse + pulse (6 trials × 4 prepulse–pulse time intervals × 3 prepulse intensities). Prepulse + pulse trials involved the presentation of a 20-ms prepulse of 3, 6, or 12 dB above background, followed by a pulse. Prepulse–pulse intervals were 30, 50, 80, or 140 ms from the onset of the prepulse to the onset of the pulse. Each PPI session ended with six pulse alone trials. The intertrial interval varied randomly, between 3 and 14 s (average 7.5 s), with each session lasting approximately 28 min. Two measures were calculated for each rat: startle amplitude and percent PPI. PPI was calculated by averaging the startle amplitudes for each trial type, and the percent PPI for each Prepulse Intensity was calculated using the formula: [100 − (100 × startle amplitude on prepulse + pulse trials)/(startle amplitude on pulse-alone trials)] (Howland et al., 2004a, 2012; Ballendine et al., 2015).

Elevated plus maze. The elevated plus maze procedure was designed in accordance with previous protocols (Hannesson et al., 2008). The elevated plus maze was constructed from plywood and corrugated plastic which lined all areas of the maze that would be exposed to a rat during a trial. The maze consisted of two sets of perpendicular interlocking arms 110 cm in length and 10 cm in width. The interlocking central region bisected the maze into two pairs of arms, one with 45-cm-high walls, the closed arms, and one without walls, the open arms. The entire maze was elevated on legs that were 45 cm high. Rats were brought to the testing room and tested individually. The test began with the rat being placed in the central region of the maze facing an open arm and continued for 5 min, at which point the rat was promptly removed and returned to the housing colony. The rat's behavior was recorded by an overhead camera and the time the rat spent in the two open arms and the two closed arms was scored by an experimenter blind to the treatment condition. Time spent in an arm was recorded when all four of the rat's paws crossed from the central region into an arm.

Recognition memory. The testing procedures for recognition memory were adapted from previous protocols (Howland and Cazakoff, 2010; Cazakoff and Howland, 2011; Howland et al., 2012). Recognition memory testing was conducted in an open-field area made from white corrugated plastic (60 × 60 × 60 cm). Objects were wiped clean with the 40% ethanol to ensure that animals could not use odor cues to help select objects. Subjects received three habituation sessions before the first test. During the first two, two rats were placed individually in separate arenas for 10 min. During the third habituation, rats were brought individually into the room and spent 10 min in an arena. The last habituation occurred 24–48 h before the first testing session. For subsequent recognition memory tests, subjects received only one habituation session, in pairs, 24–48 h before the test. Objects in all tests were constructed of glass, hard plastic, or porcelain and were all similar in size (∼10 cm in height and length). Unique objects with duplicate copies were used for all tests. Location of the novel object(s) was counterbalanced to eliminate the effect of any side preference in all tests. For both the sample and test phases, objects were located in the corners of the arena 10 cm from each of the nearest walls, while subjects were placed in the arena facing the wall opposite the objects. Two tests, the object recognition and object place tests, were conducted and consisted of a sample phase and test phase. During the sample phase, subjects explored two identical objects (A1 and A2) for 4 min. Following the 4 min of exploration, rats were then returned to the colony room. In the object recognition test, memory for the previously encountered objects was tested (105 min delay) during the 4-min test phase in which subjects explored a copy of the sample object (A3) and a novel object (B1). One week following the object recognition test, subjects were tested in the object-place test. The sample phase was identical to the object recognition sample phase in which rats explored two identical objects (C1 and C2). Twenty-four hours later, subjects received a test phase in which they explored two identical copies of the sample objects (C3 and C4), but with one object moved to a corner location at the front of the box and the other object in the original sample phase location. Object exploration was recorded using an overhead video camera connected to a computer. The time spent exploring each object was manually scored using stop watches using criteria described previously (Howland and Cazakoff, 2010; Cazakoff and Howland, 2011; Howland et al., 2012). Data are reported as discrimination ratios ((time spent exploring the novel object – time spent exploring the familiar object)/(total exploration time)).

Behavioral flexibility. The protocol for testing behavioral flexibility has been described previously (Floresco et al., 2008, 2009; Zhang et al., 2012; Thai et al., 2013; Ballendine et al., 2015).

Apparatus. All training and testing procedures were conducted in eight operant conditioning chambers located within a wooden sound-attenuating box (MedAssociates Systems, St. Albans, VT, USA). Each chamber included a food receptacle where food rewards (Dustless Precision Pellets, 45 mg, Rodent Purified Diet; BioServ, Frenchtown, NJ, USA) were delivered via a pellet dispenser. A retractable lever and a stimulus light were positioned on either side of the receptacle. A single house light (100 mA) located near the top and center of the wall opposite the levers illuminated the chamber. The chamber floor consisted of a removable metal grid which contained a detachable tray. A personal computer and interface box controlled the presentation of the trials and recorded all experimental data. Five to 7 days before operant training began, rats were weighed, individually housed, and put on a food-restricted diet to reduce their weight to 85% of their free feeding weight. A day before lever training commenced, rats were given 20 reward pellets in their home cage.

Lever training. Rats were initially trained using a fixed ratio 1 (FR1) schedule on one lever and then the other (starting lever counterbalanced between rats) until they made a minimum of 45 responses in 30 min. FR1 training typically took 4–5 days to complete. Following the successful completion of FR1 training on both levers, rats were familiarized with lever insertion on subsequent days. Rats were required to respond by pressing a lever within 10 s of it being inserted into the chamber. Training consisted of 90 trials each day. A new trial began every 20 s with illumination of the house light and insertion of one of the levers into the chamber. If a rat pressed the lever within 10 s following insertion, the trial was scored as a response, a food reward pellet was delivered to the food receptacle, and the house light remained on for an additional 4 s. Failure to respond within 10 s resulted in the trial being scored as an omission, retraction of the lever and the house light immediately being extinguished. The order of presentation of the left and right levers was randomized. However, each lever was presented once in every pair of trials such that the left and right levers were inserted for an equal number of trials (i.e., 45 trials each) during each training session. The criterion to pass retractable lever training was $\leqslant$5 omissions in a 90 trial session. Rats were trained in this phase of the experiment for a minimum of five days or until the criterion was reached. Upon reaching criterion, the lever side bias of each rat was determined.

Determination of side bias. Both levers were inserted into the chamber and pressing either lever resulted in delivery of a food reward pellet. Following the initial trial, rats were required to alternate pressing the left and right lever to obtain further food reward. The side preference session concluded when seven reward pellets had been delivered. Side preference was determined by comparing the total left and right lever presses. If the total presses were comparable ($<$2:1 ratio for total presses), then the lever pressed on the first trial was recorded as the rat's biased lever. If the total presses were not comparable ($\geqslant$2:1 ratio), then the lever that was pressed more was recorded as the biased lever. The stimulus lights above each lever were never used during training or side preference sessions. Three days of testing commenced the day following the final training session.

Visual-cue discrimination. The first phase of testing required rats to press the lever underneath the illuminated stimulus light to receive a food reward pellet. Visual-cue discrimination sessions began with neither lever inserted, and all lights extinguished (the inter-trial state). Every 20 s a trial began with one of the stimulus lights being illuminated. Three seconds later, the house light was illuminated and both levers were inserted. A correct response was scored if the rat pressed the lever underneath the illuminated stimulus light. This led to the retraction of both levers, extinguishing of the stimulus light, delivery of a food reward pellet, and the house light remaining on for an additional 4 s before the chamber returned to the inter-trial state. An incorrect response (error) was scored when the rat pressed the lever underneath the stimulus light that was not illuminated. This response caused the retraction of both levers, extinguishing of the stimulus and house lights, and an immediate return to the inter-trial state. Failure to press a lever within 10 s of insertion was scored as an omission and also resulted in the immediate return to the inter-trial state. The left and right stimulus lights were each illuminated once in every pair of trials with the order randomized within each pair of trials. The criterion to pass this phase required rats to make at least 10 consecutive correct choices in either the first 30 trials or within 150 trials. Failure to reach criterion within 150 trials resulted in rats being tested in visual-cue discrimination again during subsequent days until criterion was reached. During visual-cue discrimination testing, the lever that the rat chose, the illuminated stimulus light, and the latency to press a lever were recorded. The raw data were used to determine the trials to criterion (TTC), and the total errors committed by each rat.

Strategy set-shift (shift to response discrimination). This next phase of testing required rats to ignore the stimulus light (visual cue) and only respond to the lever opposite of each rat's lever bias (spatial cue) to continue receiving a food reward. With the exception of how correct and incorrect responses were scored, trial format and criterion to pass were identical to that described for visual-cue discrimination day. Regardless of which stimulus light was illuminated, correct responses were scored when the rat pressed its non-biased lever. Incorrect responses were scored whenever the rat pressed its biased lever. In addition, errors during this phase of testing were divided into three categories. Perseverative errors were scored when a rat continued to follow the previously rewarding but currently unrewarding strategy (i.e., following the stimulus light). In this phase of testing, eight out of every 16 trials allowed a rat to make a response error (i.e., pressing the biased lever when the illuminated stimulus light was above it). With the exception of the first block of eight trials when all errors were scored as perseverative, perseverative errors were counted when a rat made six or more errors in every block of eight trials. If a rat made five or less errors in subsequent blocks of eight trials, these errors were then scored as regressive errors. Never-reinforced errors were scored when a rat made a response that was not previously rewarded (i.e., pressing the biased lever when the illuminated stimulus light was over the non-biased lever). These error subtypes, along with the total errors and TTC, were recorded.

Response reversal. During this final phase of testing, rats were required to shift responding from the non-biased lever to the biased lever to receive food rewards. With the exception of how correct and incorrect responses were scored, trial format and criterion to pass were identical to that of visual-cue and set-shift

discrimination days. A correct trial was now scored whenever a rat pressed its biased lever while an incorrect trial was scored when a rat pressed its non-biased lever. Scoring of perseverative and regressive errors was similar to that described for set-shift days. However, with the exception of the first block of 16 trials when all errors were scored as perseverative, perseverative errors were counted when a rat made 10 or more errors within a block of 16 trials. If a rat made fewer than 10 errors per block of 16 trials then subsequent errors were counted as regressive. Error subtypes were totaled for each rat along with the TTC and total errors.

Data analysis

Values are reported as mean ± standard error of the mean and p values less than or equal to 0.05 were considered statistically significant. To determine the statistical significance for differences between groups for immunoblots, *I–V* plots, action potential firing, and passive membrane properties, the nonparametric Kruskal Wallis Test was used followed by Dunn's post hoc analysis. Dam glucose levels were analyzed with a between groups two-way analysis of variance (ANOVA) (Time and Treatment as factors). Pups numbers and body weights were analyzed with ANOVAs as appropriate. The behavioral data was analyzed with between subjects ANOVA. Factors for each specific comparison are noted in the Results. Post-hocs (Tukey's test) were conducted where appropriate.

RESULTS

Effects of STZ treatment on dams and pups

Pregnant rats were treated with either saline or STZ on GD13. Rats treated with saline had blood glucose levels of 4.81 ± 0.24 mM 24–48 h after injection. Rats treated with STZ showed dramatically increased blood glucose 24–48 after STZ treatment (20.26 ± 1.37 mM). Blood glucose levels of the dams that went on to receive an insulin pellet (17.86 ± 2.13 mM) were similar to the dams that did not receive an insulin pellet (21.94 ± 1.68 mM). The insulin pellets dramatically reduced blood glucose levels in the dams measured 24 h after implantation (4.66 ± 0.91 mM) when compared to the dams that did not receive an insulin pellet (24.24 ± 1.75 mM). During the last week of pregnancy, STZ-treated rats continued to show high levels of blood glucose compared to the control dams and those that received an insulin pellet (Fig. 1A). A repeated measures ANOVA performed on the maternal glucose data confirmed these impressions with significant main effects of Time ($F(2,48) = 14.08$, $p < 0.001$) and Treatment ($F(2,24) = 90.00$, $p < 0.001$), and a significant Time by Treatment interaction ($F(4,48) = 32.93$, $p < 0.001$). Post-hoc analyses indicated that the saline group had significantly lower blood glucose levels 24–48 h after than the STZ and STZ + Ins groups ($p < 0.05$). At subsequent time points, the STZ-treated dams had significantly higher blood glucose levels than either of the other two groups ($p < 0.05$). Blood glucose was also measured from the dams on 7 and 21 days after parturition (Fig. 1B). The effects of STZ and the insulin treatment on blood glucose observed during pregnancy continued postnatally with the STZ-treated dams showing significantly higher blood glucose levels than the other two groups (main effect of Treatment: $F(2,24) = 34.68$, $p < 0.001$; main effect of Time and Time by Treatment interaction, both n.s.).

The average number of pups born per litter for each treatment are shown in Table 1. A one-way ANOVA demonstrated that there was no significant difference in the average number of pups per litter among treatments ($F(2,24) = 2.61$, $p = 0.10$). Averaged weight per pup for each treatment are summarized in Table 1. A repeated measures ANOVA (Day × Treatment) revealed a significant main effect of Day ($F(3,72) = 781.04$, $p < 0.001$), indicating that pups in all groups gained weight from PND 1 until the last measurement on PND 21. Interestingly, there was also a main effect of Treatment ($F(2,24) = 20.39$, $p < 0.001$). Post-hoc analyses indicated that pups from STZ-treated dams weighed significantly less than those from saline-treated and STZ + Insulin-treated dams. Lastly, there was significant Day by Treatment interaction effect ($F(6,72) = 19.00$, $p < 0.001$). Post-hoc analyses indicated that on PND 1 there were no significant differences in pup weight between groups; however, from PND 7 to 21, the pups from the STZ-treated dams weighed significantly less than pups from the other two groups ($p < 0.05$). Subsequently, weights of the male offspring tested in the operant test battery was taken on PND 84 and a one-way ANOVA revealed that these differences in weights were not significant ($F(2,16) = 2.32$, $p = 0.13$). Blood glucose measurements were also taken from a random sample of the first batch of pups (one per litter) from the STZ-treated and control dams on PND 7 and PND 40. No significant difference in glucose levels was noted between the pups (PND 7: $t(6) = 0.42$, $p = 0.30$; PND 40: $t(8) = 0.73$, $p = 0.49$) and none were in the diabetic range (PND 7: mean glucose = 7.31 ± 0.36 mM; PND 40: mean glucose = 7.50 ± 0.30 mM).

Increased RAGE expression in the brain of the offspring of diabetic dams

To test if RAGE expression was increased in the brain of offspring from diabetic mothers, westerns blots were performed. Proteins were extracted from neonatal (PND1) brain tissue excluding the cerebellum. Two RAGE bands were observed at approximately 37 kDa and were analyzed by normalizing the optical density of the RAGE band by the corresponding GAPDH band (Fig. 2A). For the bottom RAGE band (Band 2), offspring from STZ-treated mothers (2.92 ± 0.68) showed a significant twofold increase in RAGE expression relative to GAPDH compared to controls (1.38 ± 0.24; Kruskal–Wallis, $p = 0.018$). This increase in RAGE expression was not observed in offspring born from diabetic mothers treated with insulin (0.91 ± 0.33).

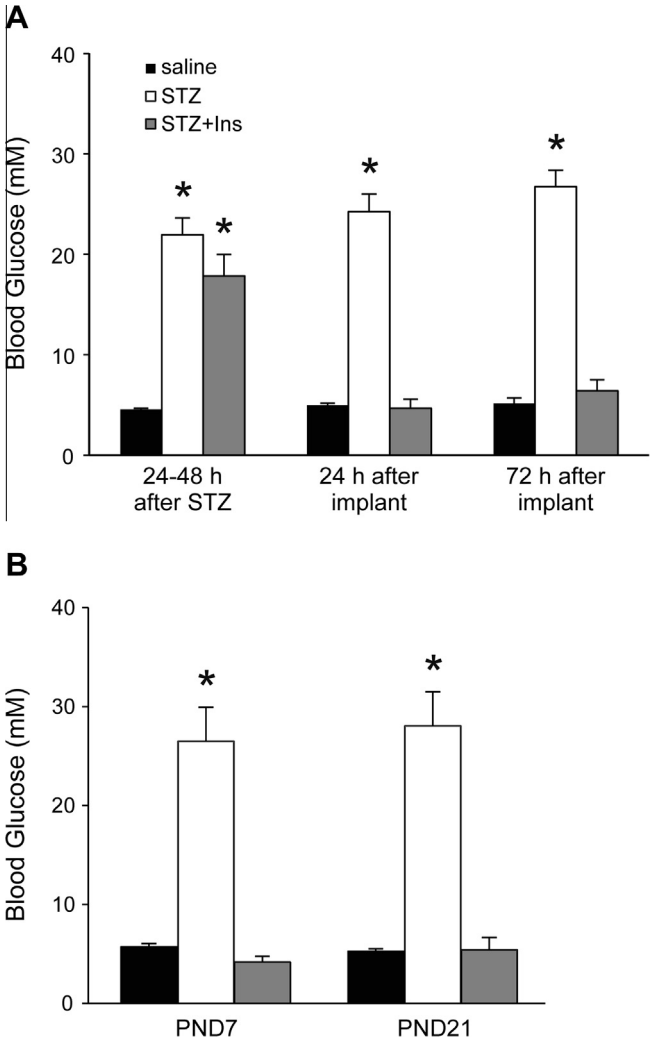

Fig. 1. Effects of STZ and insulin implants on glucose levels in the pregnant dams. (A) Blood glucose taken from the dams 24–48 h after STZ (gestational day 13–14), 24 h after the insulin or control implants were given (gestational day 16), and 72 h after the implants (gestational day 18). (B) Blood glucose taken from the dams on postnatal day (PND) 7 or 21. saline, $n = 10$ dams; STZ, $n = 10$ dams; STZ + Ins, $n = 7$ dams.

Table 1. Effects of STZ and insulin treatment on the weight (g) of the offspring over the course of development to the time of weaning. The number of litters are indicated in the table. The average weight of all pups for the litters is shown for PND 1–21. After PND 21, the average weights of only the male pups are indicated. Ins, insulin; PND, postnatal day

Treatment	Pups/litter	Weight/pup				
		PND 1	PND 7	PND 14	PND 21	PND 84
Saline ($n = 10$)	13.20 ± 1.2	7.00 ± 0.2	17.83 ± 0.6	35.66 ± 0.8	57.78 ± 1.0	477.58 ± 16
STZ ($n = 10$)	12.10 ± 0.7	6.54 ± 0.2	13.84 ± 0.5*	22.74 ± 1.3*	37.36 ± 2.6*	417.58 ± 14
STZ + Ins ($n = 7$)	9.57 ± 1.4	6.41 ± 0.2	15.82 ± 1.1	31.80 ± 2.7	53.05 ± 3.8	452.57 ± 20

* indicates significantly reduced weight relative to pups of the other two treatment groups.

Post hoc analyses confirmed that brain RAGE expression in pups from diabetic dams was larger than that of either of the other two groups (Fig. 2B; $p < 0.05$). For the RAGE band appearing slightly above (Band 1), the Kruskal–Wallis test revealed a significant effect between the treatment groups ($p = 0.003$). Pups from STZ-treated dams (1.81 ± 0.38) showed more RAGE protein relative to GAPDH compared to controls (0.92 ± 0.14); however, this effect was found to not be significant (post hoc, $p > 0.05$). Interestingly, post hoc analysis revealed a significant effect between STZ and STZ + insulin groups ($p < 0.01$).

As RAGE activation is known to increase the activity of the pro-inflammatory transcription factor NF-κB, western blots with a primary antibody detecting NF-κBp65 were performed (Fig. 2A, B). The relative expression of NF-κB was increased in the pups from STZ-treated dams (3.02 ± 1.03) compared to control

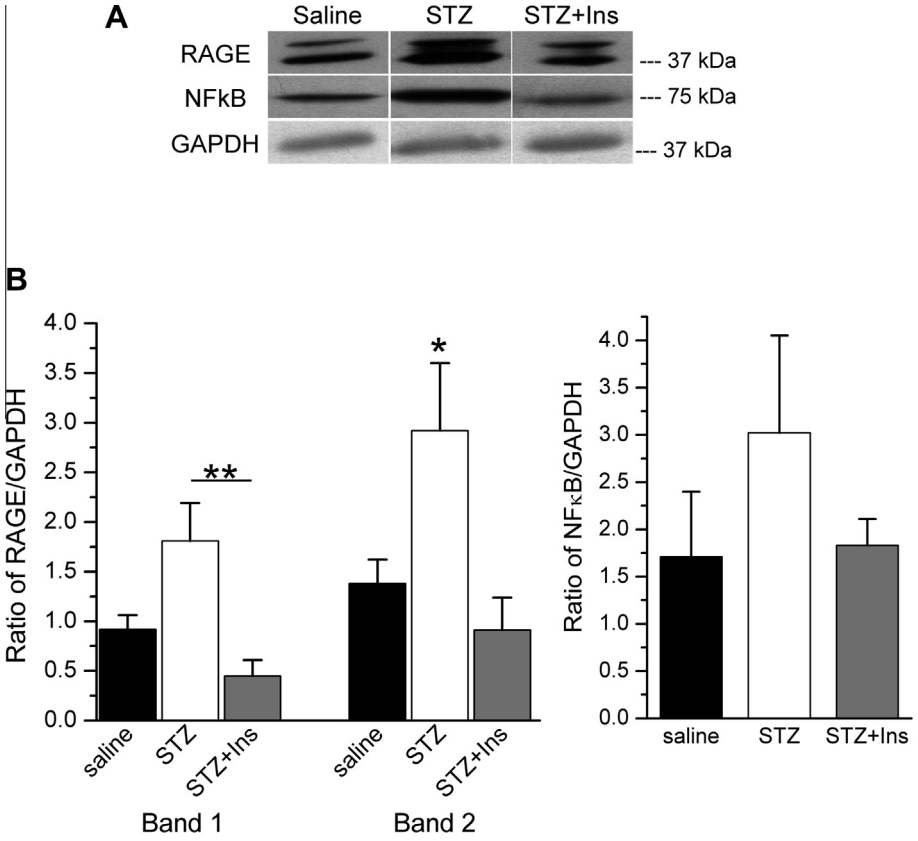

Fig. 2. Increased expression of RAGE in the brain of the offspring of diabetic dams. (A) Representative immunoblots showing RAGE, NFκB, and GAPDH protein expression from whole-brain samples of the postnatal day 1 offspring of saline-, STZ-, or STZ + Insulin-treated dams. (B) The bar graphs show the average integrated densities of RAGE (left) or NFκB (right) bands normalized to GAPDH for the brains of saline, STZ, or STZ and insulin pups ($n = 5$ brains for each group). Each bar represents the mean ± SEM; $^{*}p < 0.05$.

(1.71 ± 0.69) and insulin (1.83 ± 0.28) groups however, this difference was not significant (Kruskal–Wallis Test; $p = 0.37$). Despite this finding, the overall results show a significant up-regulation of RAGE in the brains of pups born from diabetic mothers.

Cultured hippocampal neurons from the offspring of STZ injected dams display altered neuronal excitability and action potential kinetics along with a more hyperpolarized resting membrane potential

In order to test how maternal hyperglycemia affects neuronal function in the offspring, we used whole-cell patch clamp techniques to characterize hippocampal neurons cultured from neonatal pups. Action potentials were recorded in current clamp mode from hippocampal neurons (neonatal) grown in culture for 1–2 weeks. To mitigate the effects of differences in resting membrane potentials between treatment groups, we held the cell at approximately −60 mV before application of depolarizing current steps. We observed a significant difference in the excitability of hippocampal neurons from the offspring of diabetic rats when action potentials were triggered by a series of depolarizing current steps at 100 pA increments for 500 ms (Fig. 3). Neurons from the offspring of STZ-treated dams generated more action potentials in response to stronger depolarization steps (> 150 pA) compared to saline-treated offspring. This increase in firing was prevented in neurons from the pups of diabetic dams receiving insulin treatment after STZ injection to maintain an euglycemic state. A Kruskal–Wallis test ($p < 0.001$) with post hoc analyses showed a significant increase in the number of action potentials fired in neurons from the offspring of STZ-treated dams compared to neurons from the offspring of saline-treated ($p < 0.01$) and insulin-implanted ($p < 0.001$) dams. Interestingly, this increase in excitability was not observed when the neurons were depolarized mildly with only 50 pA of current injection. Rather, the opposite was found where hippocampal neurons from the offspring of STZ-treated dams displayed hypoexcitabilty. This decrease in firing was confirmed statistically with the Kruskal–Wallis test ($p = 0.013$) and post hoc analyses showing a significant decrease in the number of action potentials fired in the STZ group compared to the saline ($p < 0.05$) and insulin ($p < 0.05$) groups. These findings implicate a maternal hyperglycemic environment as a major contributor to the altered hippocampal neuron excitability.

Along with the alterations in neuronal excitability observed, hippocampal neurons from STZ-treated dams also demonstrated changes in the action potential waveform. When analyzing the waveform kinetics of the first action potential evoked from the above

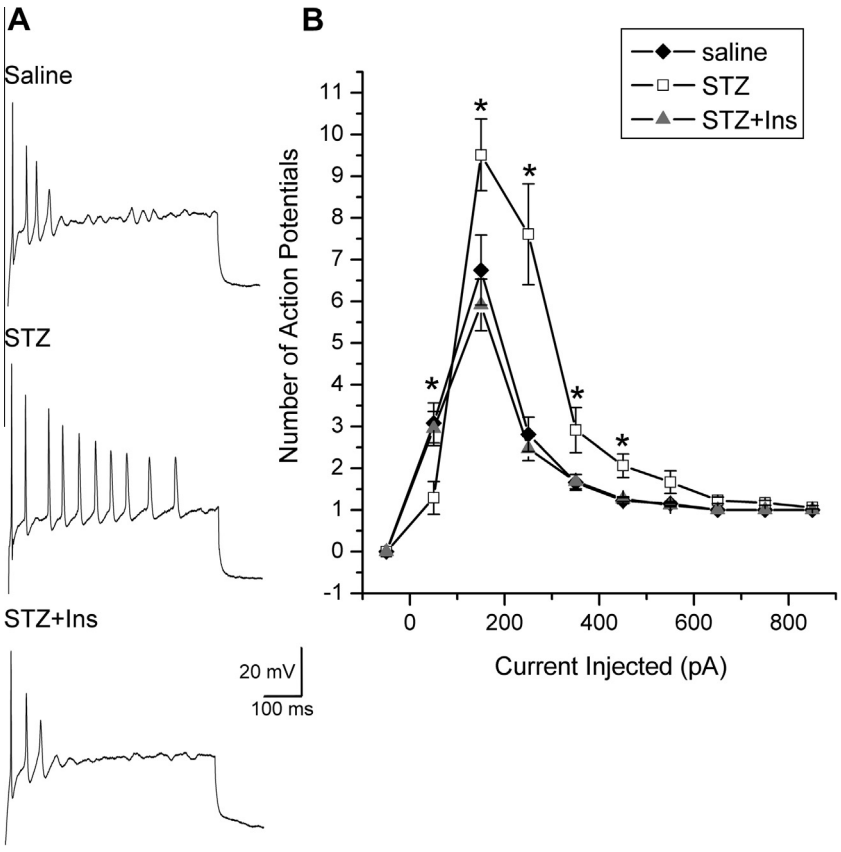

Fig. 3. Action potentials evoked from cultured hippocampal neurons. Cultured hippocampal neurons from the postnatal day 1 offspring of diabetic rats show hyper- or hypoexcitability depending on the magnitude of the depolarizing current. Increased excitability in cultured hippocampal neurons from the offspring of diabetic rats during stronger depolarizations. Using whole-cell current clamp techniques, action potentials were generated by a series of depolarizing current steps at 100-pA increments for 500 ms. (A) Example traces are from a 250-pA depolarizing current step. (B) The line graph summarizes the number of action potentials fired at each depolarizing current injection step for pyramidal hippocampal neurons cultured from pups born from saline- (n = 59 neurons), STZ- (n = 36 neurons), or STZ + Insulin (n = 76 neurons)-treated moms. Each point represents the mean ± SEM; $^{*}p < 0.05$.

experiments, offspring of STZ-treated mothers had action potentials with quicker decay times compared to control or insulin-treated groups (Table 2). The Kruskal–Wallis Test (p = 0.001) with post hoc analyses confirmed this observation as neurons from STZ-treated dams produced action potentials with an average decay time of 1.0 ± 0.04 ms, which was significantly shorter than neurons from saline-treated (1.39 ± 0.08; $p < 0.05$) and insulin-treated (1.56 ± 0.1; $p < 0.001$) dams. No significant differences were observed with the amplitude (Kruskal–Wallis Test; p = 0.53) or rise (Kruskal–Wallis Test; p = 0.078) of the action potential (Table 2). Maternal STZ treatment also seemed to cause a more hyperpolarized resting membrane potential (Vm) of cultured hippocampal neurons from the offspring (Table 2). The differences of Vm among treatment groups was confirmed statistically using the nonparametric Kruskal–Wallis Test (p = 0.0454) with Dunn's post hoc analyses ($p < 0.05$ for control vs STZ and $p < 0.05$ for STZ vs STZ + Ins).

Cultured hippocampal neurons from the offspring of diabetic rats show an augmented KCNK current at depolarized potentials beginning at −20 mV

To investigate the mechanism underlying the electrophysiological changes observed in hippocampal neurons from the pups of STZ-treated mothers, we studied Na^+ and K^+ currents. First, we tested for voltage-activated Na^+ and K^+ currents using voltage clamp techniques to generate current I–V plots of the corresponding currents elicited at various membrane

Table 2. Characteristics of the cultured neurons used for electrophysiology experiments. Vm, resting membrane potential; Rm, resistance; Cm, capacitance. See Results, Section 3.4 for details

	Vm (mV)	Rm (mΩ)	Cm (pF)	Amplitude (mV)	Rise (ms)	Decay (ms)
Saline	−48.31 ± 1.18	223.5 ± 15.42	35.92 ± 1.12	89.64 ± 1.21	3.74 ± 0.17	1.39 ± 0.08
STZ	−53.2 ± 1.22 *	186.57 ± 12.62	35.70 ± 1.29	92.03 ± 1.5	4.05 ± 0.19	1.0 ± 0.04 *
STZ + Ins	−48.52 ± 1.01	252.33 ± 16.16	33.47 ± 0.92	90.54 ± 1.42	4.3 ± 0.16	1.56 ± 0.1

potentials. Na^+ currents for all three treatment groups activated around -40 mV and showed no significant difference in peak current densities elicited at various voltage steps (Fig. 4A; Kruskal–Wallis test; $p > 0.05$). When analyzing the *I–V* plots for voltage-activated K^+ currents, no significant difference was also observed between the three groups (Fig. 4B; Kruskal–Wallis test; $p > 0.05$).

Next, we tested for voltage-insensitive K^+ currents generated by K^+ leak channels (KCNK). In recent

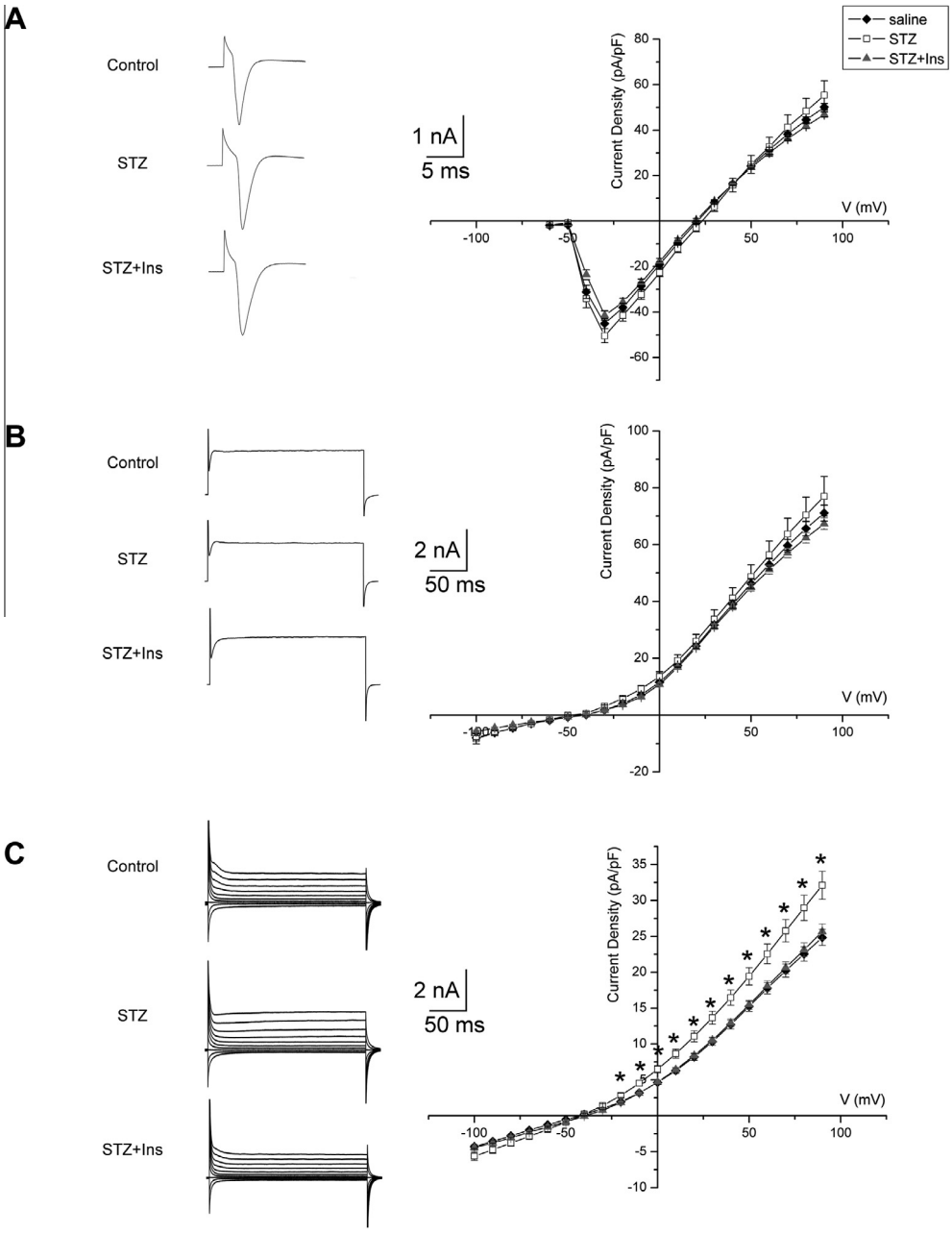

Fig. 4. Current–voltage plots of KCNK and voltage-activated Na^+ and K^+ currents. (A) Traces showing a representative example of voltage-activated Na^+ currents during a step to -30 mV from cultured hippocampal neurons from the postnatal day 1 offspring of saline, STZ, or STZ + Insulin-treated rats. Current–voltage (*I–V*) plots of the current density for all three groups (saline: $n = 66$ neurons, STZ: $n = 39$ neurons, STZ + Insulin: $n = 89$ neurons) are shown on the right. No significant differences were found between treatment groups. The *I–V* plot shows the mean ± SEM current density. (B) Traces showing a representative example of voltage-activated K^+ currents during a step to +60 mV. *I–V* plots for all three treatment groups (saline: $n = 67$ neurons, STZ: $n = 40$ neurons, STZ + Insulin: $n = 89$ neurons) are shown on the right. No significant differences were found between treatment groups. The *I–V* plot shows the mean ± SEM current density. (C) Traces showing a representative example of background or 'leak' K^+ channels (KCNK) recorded in asymmetrical K^+ extracellular solution (5.5 mM) containing TTX (1 μm), 4-AP (5 mM), TEA (30 mM) and Ni^{2+} (1.3 mM; substituted for Ca^{2+}), during various voltage steps from −100 to +100 mV. The corresponding *I–V* plots are shown on the right for all three treatment groups (saline: $n = 53$ neurons, STZ: $n = 35$ neurons, STZ + Insulin: $n = 53$ neurons). The *I–V* plot shows the mean ± SEM current density; $^*p < 0.05$.

years, considerable research has shed on the integral role KCNK channels play in controlling neuronal excitability by influencing duration, frequency, and amplitude of action potentials as well as its strong influence in determining the resting membrane potential of a neuron (Goldstein et al., 2001). Our above findings showing a more hyperpolarized membrane potential and a quicker decay time of action potentials in neurons from the offspring of STZ-treated dams lead us to hypothesize that KCNK channels may underlie these changes, ultimately altering neuronal excitability. To enhance the magnitude of KCNK currents and facilitate quantification (Campanucci et al., 2003), voltage-clamp recordings were carried out in extracellular solution containing 1 μm TTX, 30 mM TEA, 5 mM 4-AP and 2 mM Ni^{2+} to block voltage-dependent currents (Fig. 4C). Hippocampal neurons from the offspring of STZ-treated dams showed a significant increase in KCNK current density ($p < 0.05$; $n = 35$) compared to saline ($n = 53$) and insulin ($n = 53$) groups at potentials greater than or equal to −20 mV. For example, a step to +40 mV produced a mean outward KCNK current density of 12.70 ± 0.62 pA pF^{-1} for saline, 16.46 ± 1.06 pA pF^{-1} for STZ, and 12.93 ± 0.53 pA pF^{-1} for STZ + Insulin. A nonparametric ANOVA (Kruskal–Wallis Test) demonstrated a significant difference between treatment groups ($p = 0.007$). Post hoc analyses revealed that neurons from the offspring of STZ-treated dams had a significantly larger KCNK current density compared to saline ($p < 0.01$) and insulin-implanted groups ($p < 0.05$). These findings demonstrate an enhanced outward rectification of KCNK currents at positive test potentials in hippocampal pyramidal neurons cultured from the offspring of diabetic moms.

Effects of maternal STZ treatment on PPI and the acoustic startle response

Prepuberty. As depicted in Fig. 5A, rats from all treatment conditions showed an expected pattern of startle amplitudes, with higher responses during the initial pulse-alone trials than those during presentation of the PPI trials and after the session. Consistent with this observation, a repeated measures ANOVA revealed a significant main effect of Pulse Block ($F(2,116) = 80.70$, $p < 0.001$), with post hoc analyses indicating that the initial pulse-alone startles were significantly higher than the other two blocks ($p < 0.05$). A main effect of Treatment ($F(2,58) = 4.42$, $p = 0.016$) and a significant Pulse Block by Treatment effect were also observed ($F(4,116) = 5.17$, $p = 0.001$). Post-hoc analyses indicated that offspring from dams treated with STZ alone had significantly lower startle amplitudes than offspring from the other two treatments ($p < 0.05$).

Percent PPI was assessed for the longer time intervals (50, 80, and 140 ms) in a repeated measures ANOVA (Fig. 5B,C). As expected, significant main effects of Interval ($F(2,116) = 11.03$, $p < 0.001$) and Prepulse Intensity ($F(2,116) = 172.73$, $p < 0.001$), along with a significant Interval × Prepulse Intensity interaction ($F(4,232) = 6.10$, $p < 0.001$), were observed. The main effect of Treatment was not significant ($F(2,58) = 2.29$, $p = 0.11$), nor were any other interactions (statistics not shown). Post-hoc analyses indicated that the 80-ms interval resulted in significantly higher PPI than the 50-ms interval ($p < 0.05$), while significantly higher PPI was observed for each increasing Prepulse Intensity ($p < 0.05$). When a repeated measures ANOVA was conducted for the data for trials with a 30-ms interval (Fig. 5D), a significant main effect of Prepulse Intensity ($F(2,116) = 56.65$, $p < 0.001$) was observed while neither the main effect of Treatment ($F(2,58) = 1.08$, $p = 0.35$) nor the Prepulse Intensity by Treatment interaction effect ($F(2,116) = 0.63$, $p = 0.65$) reached statistical significance.

Early adulthood. Fig. 5E shows startle amplitudes for the adult rats. A repeated measures ANOVA revealed significant main effects of Pulse Block ($F(2,112) = 63.02$, $p < 0.001$) and Treatment ($F(2,56) = 4.40$, $p = 0.017$). The Pulse Block by Treatment interaction was also significant ($F(4,112) = 3.20$, $p = 0.016$). Post-hoc analyses indicated that the first block of pulse-alone trials generated significantly higher startle responses than the other two blocks and offspring from STZ-treated dams had significantly lower startle than offspring from either of the other two groups ($p < 0.05$). Post-hoc tests on the interaction term demonstrated that the between-group effects of treatment were only significant for the first two blocks of pulse-alone trials ($p < 0.05$), and the startle responses among the groups were not significantly different for the last block of pulse-alone trials.

Percent PPI levels are shown in Fig. 5F, G. For the long intervals (50, 80, 140 ms), the expected main effect of Prepulse Intensity was significant ($F(2,112) = 91.79$, $p < 0.001$), with stronger prepulse intensities eliciting stronger PPI. The main effect of Interval was not significant ($F(2,112) = 2.04$, $p = 0.16$), although there was a significant Prepulse Intensity × Interval interaction ($F(4,224) = 9.91$, $p < 0.001$). Importantly, the main effect of Treatment was significant ($F(2,56) = 3.82$, $p = 0.028$). No other interaction terms were significant (statistics not shown). Post-hoc analyses revealed that the offspring from STZ-treated litters demonstrated significantly lower PPI than the saline-treated offspring ($p < 0.05$). The difference between the STZ- and STZ + Insulin-treated offspring just failed to reach significance. Analysis of the 30-ms prepulse–pulse interval (Fig. 5H) revealed significant main effects of Prepulse Intensity ($F(2,112) = 54.14$, $p < 0.001$) and Treatment ($F(2,56) = 3.30$, $p = 0.044$), along with a significant Prepulse Intensity by Treatment interaction ($F(4,112) = 2.51$, $p = 0.045$). Post-hoc analyses revealed that the control offspring displayed significantly more PPI than the offspring of STZ-treated dams, which demonstrated significant paired pulse facilitation at the short interval ($p < 0.05$). Analyses of the interaction revealed that the offspring of the insulin-implanted dams demonstrated significantly greater PPI for trials with a 3-dB prepulse ($p < 0.05$).

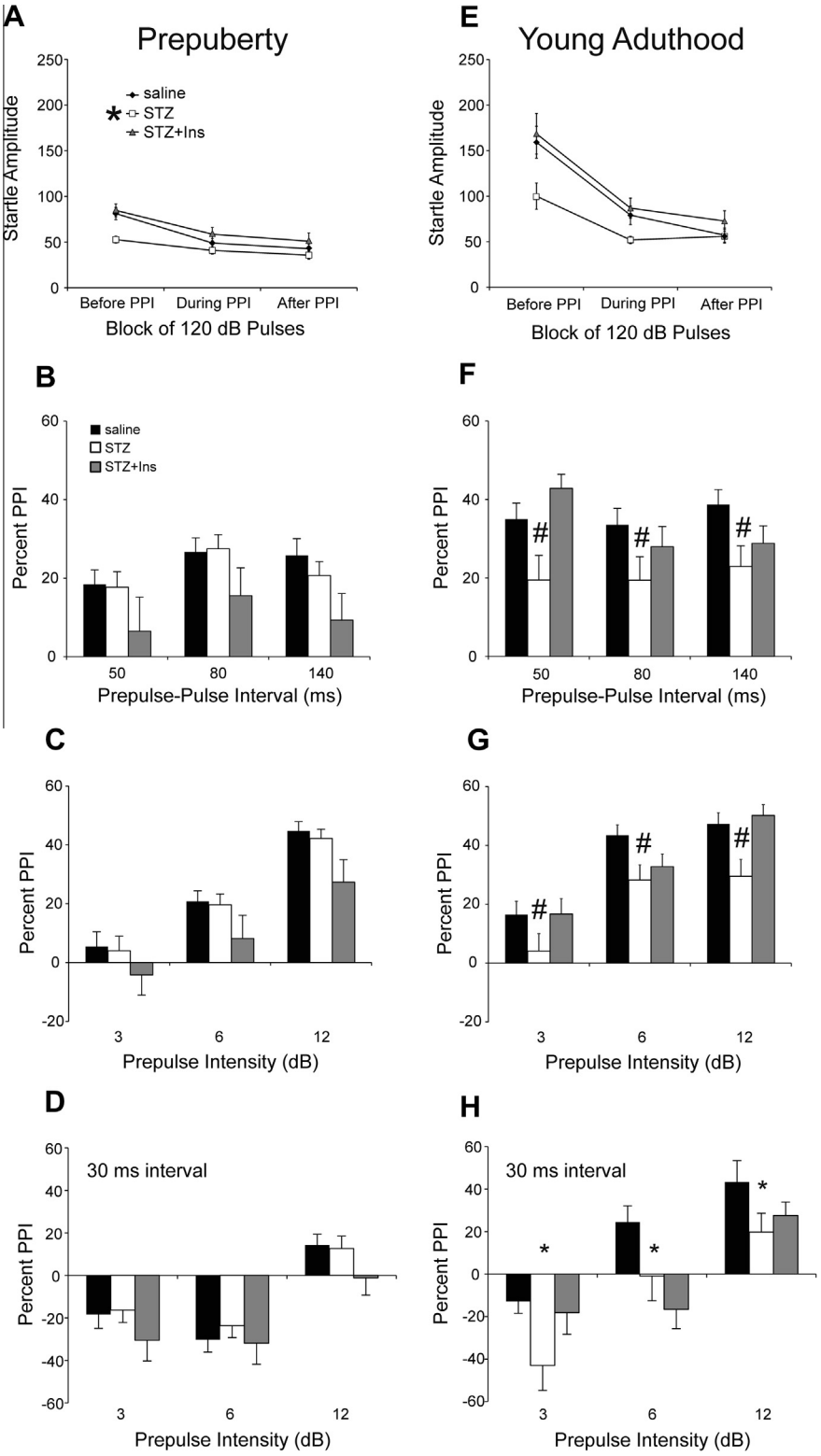

Fig. 5. Effects of maternal STZ and insulin treatment on the acoustic startle response and prepulse inhibition. Acoustic startle response before puberty (A) and in early adulthood (E) for pulse alone trials conducted before, during, and after the PPI trials (6 per time point). Percent PPI sorted by prepulse–pulse interval or Prepulse Intensity for the tests conducted before puberty (B–D) and in early adulthood (F–H). Note that a negative score reflects an *increase* in startle in the presence of the prepulse. Before puberty: saline, $n = 24$ from 10 litters; STZ, $n = 23$ from 10 litters; STZ + Ins, $n = 14$ from 7 litters. In early adulthood: saline, $n = 23$ from 10 litters; STZ, $n = 23$ from 10 litters; STZ + Ins, $n = 14$ from 7 litters. $^{*}p < 0.05$ vs all other groups. # $p < 0.05$ for saline group compared to the offspring of STZ-treated dams.

Effects of maternal STZ on behavior in the elevated plus maze

The effects of STZ on anxiety-related behavior were measured using the elevated plus maze. Ratios of dwell time in all arms to the total time of the test (300 s) and dwell time in the open arms to total time in are depicted in Fig. 6A and B, respectively. Analysis of the means using an ANOVA showed a significant difference among the groups for open arm ratio ($F(2,55) = 8.81$, $p < 0.001$) but not all arms ($F(2,55) = 0.13$, $p = 0.88$). Post-hoc analysis indicated that pups from saline-treated dams spent significantly less time in the open arms than pups from either of the other two groups ($p < 0.05$).

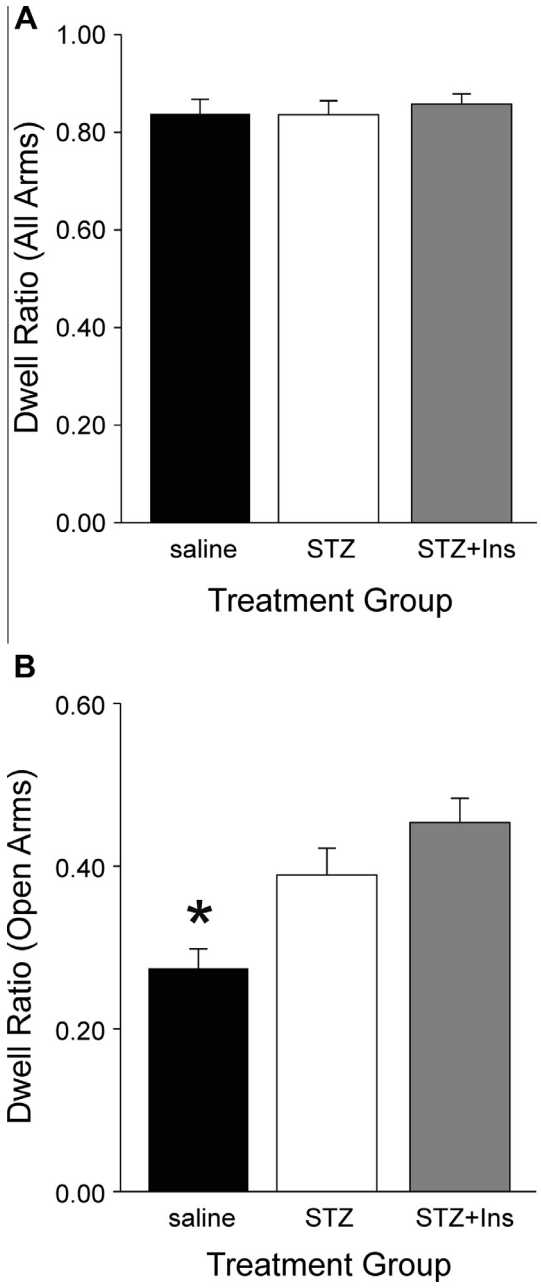

Fig. 6. Behavior of rats on the elevated plus maze following maternal treatment with saline, STZ, or STZ + Insulin. A. The ratio of time the rats spent in the closed and open arms relative to the total time of the trial (300 s). B. Ratio of time the young adult offspring spent in the open arms relative to the total time. Offspring of saline-treated dams spent significantly less time exploring the open arms than those from the other two groups, indicative of increased anxiety-like behavior. Saline, $n = 22$ from 10 litters; STZ, $n = 22$ from 10 litters; STZ + Ins, $n = 14$ from 7 litters. $^{*}p < 0.05$ vs the other groups.

Effects of maternal STZ treatment on recognition memory

Average total exploration times during the sample (4 min) and test phases (2 min) of both the object and object-place recognition tests are depicted in Table 3. During the object recognition test, rats from the three treatment groups explored the objects for similar amounts of time during the sample phase ($F(2,55) = 1.03$, $p = 0.36$); however, a significant difference among the groups was noted for the test phase ($F(2,55) = 10.25$, $p < 0.001$). Post-hoc analyses indicated that the control offspring explored significantly less than the other two groups ($p < 0.05$). For the object place test, significant differences in total exploration time for the sample ($F(2,55) = 4.34$, $p = 0.018$) and test phases ($F(2,55) = 3.53$, $p = 0.036$) were noted among the groups. The control offspring explored the objects significantly less than the other two groups during the sample trial while in the test trial, the control offspring explored significantly less than STZ + Ins offspring ($p < 0.05$).

Analyses of the discrimination ratios for the test phases of both tests revealed that rats in all treatment groups demonstrated significant memory (Fig. 7; one-sample *t*-tests, statistics not shown). ANOVA for analysis of between-group differences revealed no significant differences for the object recognition test ($F(2,55) = 1.51$, $p = 0.23$), while memory did differ significantly among the groups in the object-place test ($F(2,55) = 4.47$, $p < 0.016$). Offspring of the STZ-treated dams displayed significantly higher discrimination ratios than the other two groups (post hoc, $p < 0.05$).

Effects of maternal STZ treatment on behavioral flexibility

Visual cue discrimination. The mean numbers of TTC and total errors for each group are shown in Fig. 8A, B. A one-way ANOVA showed no significant difference in TTC between groups ($F(2,52) = 1.07$, $p = 0.35$), suggesting that rats in each condition learned the visual cue discrimination at about the same rate. There was also no significant difference in the total number of errors between groups ($F(2,52) = 0.73$, $p = 0.49$), indicating that all rats learned the discrimination task. No differences in average response latencies were observed among the groups ($F(2,52) = 0.81$, $p = 0.45$).

Set-shifting. The mean TTC and total errors for the set-shifting task are represented in Fig. 8C,D. Unlike the visual cue discrimination task, there was a significant

Table 3. Effects of STZ and insulin treatment on exploration times (s) in the object (OR) and object-place (OP) recognition tests. OR, object recognition; OP, object-place

Treatment	Sample – OR	Test - OR	Sample – OP	Test – OP
Saline (n = 22)	55.73 ± 3.9	30.55 ± 2.0*	59.90 ± 4.0*	33.11 ± 2.5#
STZ (n = 22)	63.34 ± 4.0	42.69 ± 2.1	73.05 ± 3.27	37.64 ± 2.0
STZ + Ins (n = 14)	62.59 ± 4.9	42.18 ± 2.5	72.10 ± 3.0	42.77 ± 1.7

* Indicates significantly reduced exploration relative to rats from the other two treatment groups.
# Indicates significantly reduced exploration relative to the STZ + Ins group.

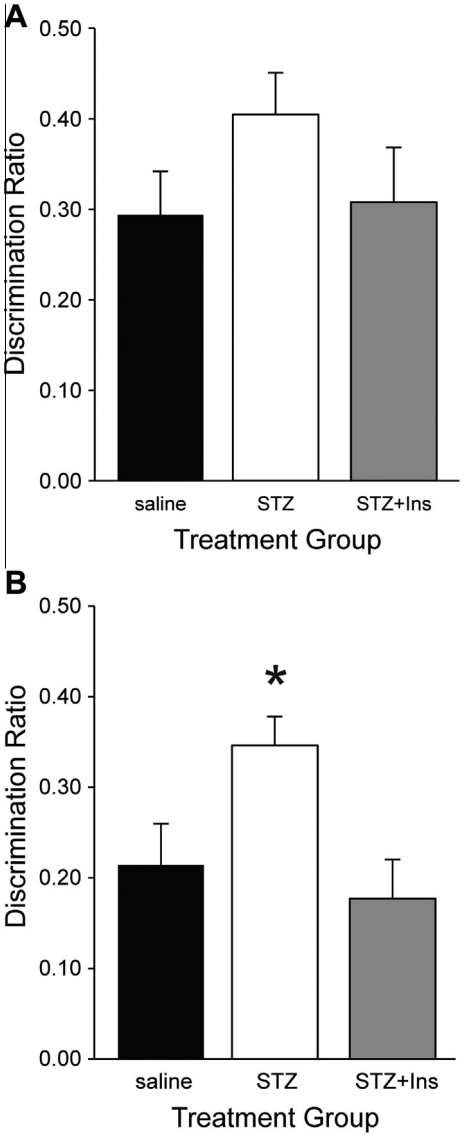

Fig. 7. Performance of the rats on the spontaneous object and object-place recognition memory tests. A. Young adult offspring in all three groups displayed significant memory in the object recognition memory test. B. Young adult offspring from the STZ-treated group displayed a significantly greater bias for exploring the object that was position in a novel location in the object-place test. Saline, n = 22 from 10 litters; STZ, n = 22 from 10 litters; STZ + Ins, n = 14 from 7 litters. *$p < 0.05$ vs the other groups.

difference between groups ($F(2,52) = 3.73$, $p = 0.031$). Specifically, animals in the STZ and in the STZ + Ins condition did worse than those from saline-treated dams. A one-way ANOVA revealed no significant difference for the total number of errors between groups ($F(2,52) = 2.04$, $p = 0.14$), and further analysis also indicated no difference in the type of errors (perseverative, regressive or never-reinforced) committed between groups (Fig. 9A). While a repeated measures ANOVA showed a significant main effect of Error Type ($F(2,104) = 23.70$, $p < 0.001$), the main effect of Treatment ($F(2,52) = 1.70$, $p = 0.19$) and the Error Type by Treatment interaction effect ($F(4,104) = 1.16$, $p = 0.34$) were not significant. A significant effect of treatment on response latency was noted for the set-shifting day ($F(2,52) = 3.32$, $p = 0.044$). Posthoc analyses revealed that the offspring of STZ-treated dams (0.58 ± 0.04) had significantly shorter latencies than the other two groups (control: 0.80 ± 0.09; STZ + Ins: 0.84 ± 0.11).

Reversal learning. Mean TTC and total errors are shown in Fig. 8E,F. Similar to the visual cue discrimination task, rats from all three conditions were able to learn the reversal task at similar rates: a one-way ANOVA indicated no significant difference in TTC between groups ($F(2,52) = 1.21$, $p = 0.31$). Furthermore, there was also no significant difference in the number of total errors between treatment groups ($F(2,52) = 1.00$, $p = 0.38$). Again, a repeated measures ANOVA examining error type (perseverative or regressive; Fig. 9B) showed no significant main effect of Treatment ($F(2,52) = 1.65$, $p = 0.20$) or Error Type by Treatment interaction effect ($F(2,52) = 0.13$, $p = 0.88$). However, the main effect of Error Type was significant ($F(1,52) = 136.59$, $p < 0.001$), with rats committing more perseverative errors (33.37 ± 15.81) than regressive errors (3.46 ± 4.29). No differences in average response latencies were observed among the groups ($F(2,52) = 0.78$, $p = 0.46$).

DISCUSSION

We reported the effects of maternal diabetes induced during mid-pregnancy on the offspring. Expression of RAGE in the brain and the excitability of hippocampal neurons cultured from PND 1 offspring were both increased. Behavioral studies revealed that offspring from STZ-treated dams display impaired PPI, increased exploration of the open arms of an elevated plus maze, increased preference for a novel object in an object-place recognition memory test, and impaired strategy set-shifting in an operant test battery of behavioral flexibility. Some, but not all, of these effects were

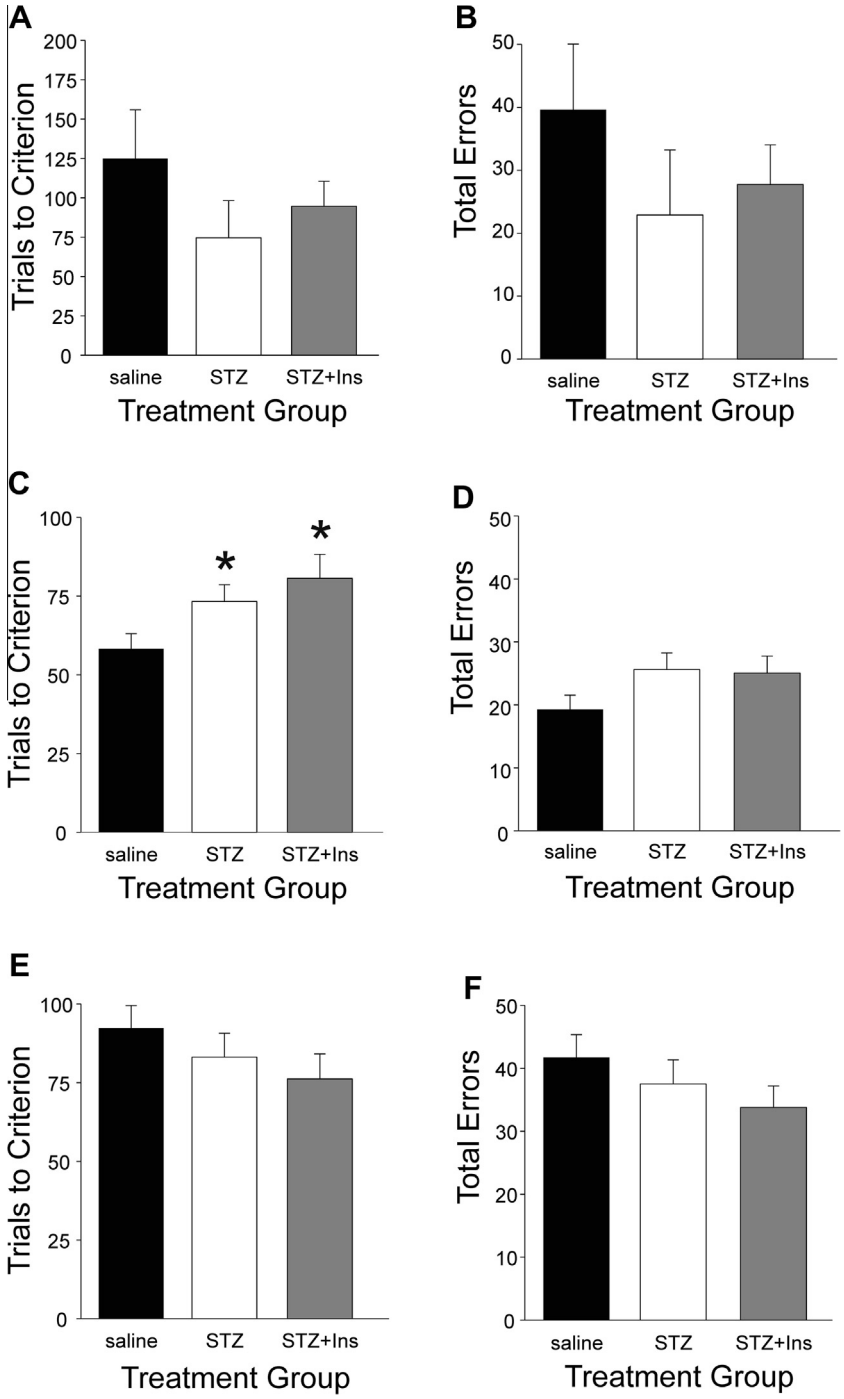

Fig. 8. The effects of STZ treatment on visual cue discrimination (A, B), strategy set-shifting (C, D), and reversal learning (E, F). Trials to criterion are shown in panels A, C, and E. Total errors committed are shown in panels B, D, and F. Saline, $n = 19$ from 10 litters; STZ, $n = 21$ from 10 litters; STZ + Ins, $n = 15$ from 7 litters. Offspring were tested in early adulthood. $^{*}p < 0.05$ vs the saline-treated group.

reversed when glucose was controlled with a slow release insulin pellet implanted in the pregnant dams treated with STZ.

Short-term effects of maternal diabetes during mid-gestation on the offspring

STZ treatment during mid-pregnancy produced a severe and enduring hyperglycemic state in the dams that was normalized by slow release insulin pellets. We chose to administer STZ at this time because it coincides with neurogenesis in the hippocampus and entorhinal cortex (Bayer et al., 1991, 1993) and we wanted to prevent the severe abnormalities in the offspring commonly observed as a result of a severe Type 1 diabetic state induced before or near the start of pregnancy (Jawerbaum and White, 2010). In addition, as gestational diabetes typically arises later in pregnancy, we sought to mimic this aspect

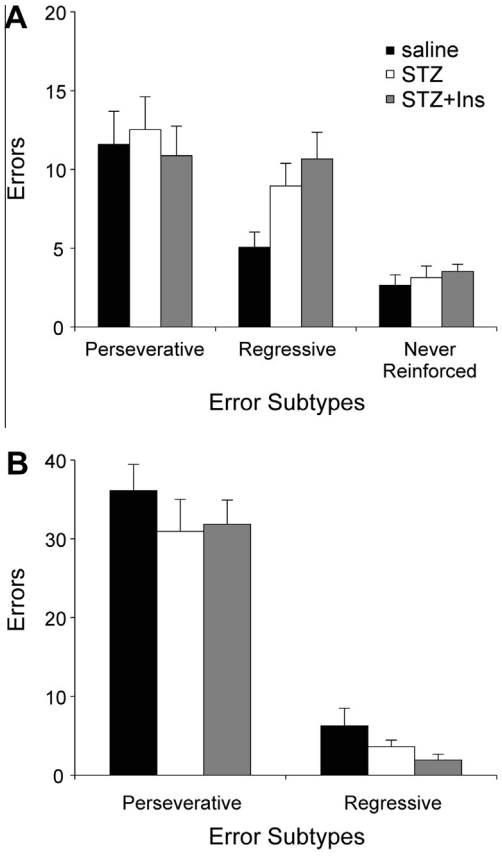

Fig. 9. Error subtypes committed by the rats for strategy set-shifting (A) and reversal learning (B). Errors were broken down into perseverative, regressive, and never reinforced subtypes for set-shifting. Significant differences in the number of errors classified as each subtype were found (see Results for details), although between-group differences in number of errors of each subtype were not statistically significant. For reversal learning, rats from all groups showed high rates of perseveration, with significantly fewer regressive errors. Offspring were tested in early adulthood. Group *n*'s are as noted in the caption for Fig. 8.

of the human condition. A concern is the potential for direct effects of STZ on the developing fetuses, as it can cross the placenta at least in primates (Reynolds et al., 1974) and induce changes in fetal glucose transporter expression in rats (Schroeder et al., 1997). Direct effects of STZ on behavior unrelated to maternal hyperglycemia may have been caused if it entered the fetal brain and affected neurodevelopment. While we cannot exclude this possibility, we do not believe that the fetuses were directly affected by STZ for a number of reasons: (1) litter size was not altered at birth; (2) pup weights were not altered at birth; (3) the offspring showed no evidence of hyperglycemia when tested on PND 7 and 40. However, significantly reduced postnatal weight gain was noted in the pups from STZ-treated dams. As offspring from STZ + Insulin-treated dams gained weight at rate similar to controls, it is likely that continued hyperglycemia in the STZ-treated dams after parturition (Fig. 1B) contributed to the reduced growth (Thamotharan et al., 2003). While the insulin implants were used during pregnancy to control blood glucose levels after STZ injection, it is imperative that future studies either cross-foster litters, use healthy surrogates to raise pups from STZ-treated dams (Thamotharan et al., 2003), or treat diabetic dams with insulin after birth to rule out the postnatal effect of hyperglycemia on pup behavior. It is worth noting that the biochemical and electrophysiological experiments were conducted on the brain tissue collected from the offspring on PND 1 – before these changes were observed.

Analysis of whole-brain tissue revealed a significant increase in expression of RAGE in the offspring on PND 1. Our findings are consistent with recent reports showing fetal neurodevelopmental abnormalities and elevated RAGE expression in brain regions of the offspring of gestational diabetic dams (Luo and Yang, 2012; Tang et al., 2015), which were associated with increased maternal serum AGE levels (Luo and Yang, 2012). Although the RAGE-associated mechanisms underlying cognitive impairment in the offspring are still unknown, we have identified electrophysiological changes which potentially relate to the behavioral abnormalities. Hippocampal neurons from the offspring of gestational diabetic rats had a more hyperpolarized resting potential, an apparent shift in threshold voltage, and increased firing frequency. The latter was accompanied by a faster decay in the action potential repolarizing phase, suggesting these functional changes could reside in either modulation or change in expression of ion channels. Indeed, while no significant changes were observed in voltage-gated Na^+ and K^+ channels, we detected an increase in the voltage-insensitive outwardly rectifying KCNK current. KCNK channels play a key role in setting the resting membrane potential and input resistance of the cell (Lesage and Lazdunski, 2000; Goldstein et al., 2001; Patel and Honore, 2001). Therefore, they are important determinants of the magnitude and kinetics of synaptic inputs in neurons and help shape neuronal excitability. Although hippocampal neurons co-express multiple members (KCNK-1,-3, -4, -9 and -10; Talley et al., 2001) of the seven known KCNK families, the molecular identity of the KCNK channel linking RAGE signaling and behavioral abnormalities in the offspring of gestational diabetic rats remains to be studied.

Although we are unsure of the mechanisms of activation and up-regulation of RAGE in the offspring of STZ-treated dams, the generation of AGEs and other proinflammatory signals are likely involved. AGE levels in maternal circulation are reported to follow those of blood glucose (Luo and Yang, 2012; Tang et al., 2015). However, AGEs are not expected to cross the placenta barrier, which is particularly tight for most proteins other than transferrin (Srai et al., 2002) and immunoglobulin G antibodies (Palmeira et al., 2012). Even if some glycated proteins crossed the placenta, it is doubtful that they would be the only source of RAGE ligands in the offspring. RAGE-related activity in human placenta of women with GDM (Liu et al., 2012) and RAGE expression in the placenta of diabetic rats are reported to be increased (Tang et al., 2015); thus, it is possible that RAGE signaling from the placenta and/or AGEs formed in the embryos exposed to maternal hyperglycemia *in utero* contribute to the brain abnormalities of the offspring. In addition to

AGEs, other proinflammatory signals such as S100 and high-mobility group box-1 (HMGB1) proteins may contribute to electrophysiological abnormalities in the offspring of diabetic dams by binding to RAGE. For example, activation of RAGE by S100 protein has been linked to the modulation of hippocampal synaptic plasticity. Infusion of brain slices with S100 decreases long-term potentiation (LTP), while $S100^{-/-}$ mice showed stronger LTP and enhanced spatial and conditioned fear memory (Nishiyama et al., 2002). Further support for a role of RAGE in modulating synaptic activity was provided by Sakatani et al. (2007); these authors revealed that kainate-induced seizures in $S100^{-/-}$ mice were weaker than in wild type controls, and that this effect was mediated by RAGE-S100 interactions. More recently, a series of reports linking RAGE activation and NMDA receptor function aimed to explain the detrimental effects of RAGE on memory (Mazarati et al., 2011). For example, activation of RAGE by HMGB1 impairs memory encoding in mice independently of the known HMGB1 binding to toll-like receptor 4 (TLR4) (Mazarati et al., 2011). It has been proposed that the previously described excitatory effect of HMGB1 in the hippocampus of mice may underlie the memory impairments. HMGB1 induces phosphorylation of GluN2B-containing NMDA receptors (Maroso et al., 2010), resulting in increased calcium conductance in hippocampal neurons (Viviani et al., 2003). Since GluN2B-containing NMDA receptors inhibit cell surface expression of the GluA1 subunit of AMPA receptors (Kim et al., 2005), which are critical for synaptic plasticity and memory (Sanderson et al., 2008), excessive HMGB1-induced phosphorylation of GluN2B-containing receptors could impair learning and memory. As discussed by Mazarati et al. (2011), it is conceivable that the same mechanism mediating the seizure-facilitating effect of HMGB1 (i.e., activation of GluN2B-containing NMDA receptors) may be concurrently involved in learning deficits. However, the role of GluN2B-containing NMDA receptors in memory remains controversial since these subunits are also suggested to positively modulate learning and memory, including novel object recognition, and a reduction of GluN2B expression has been shown in the hippocampus of patients with Alzheimer's disease (Loftis and Janowsky, 2003). In addition, in the adult brain, activation of GluN2B-containing NMDA receptors shortens the duration of Ras/ERK activation (Kim et al., 2005), which may also contribute to memory impairments (Weeber and Sweatt, 2002).

In the current study, we also showed that the increased expression of RAGE in the offspring is accompanied by a possible increase in expression of NFkB. In fact, RAGE signaling often leads to sustained activation of the pro-inflammatory transcription factor NFkB (Yan et al., 1994; Bierhaus et al., 2004), which suggests that the deleterious effects of RAGE may be linked to inflammation. Interestingly, the RAGE promoter, isolated from the 50-flanking region of a human lung RAGE genomic clone, contains binding sites for NFkB, implicating NFkB in a positive feedback regulation of RAGE expression (Li and Schmidt, 1997). In addition, in the hippocampus of temporal lobe epilepsy patients (Teocchi et al., 2013), NFkB is up-regulated and suggested to be responsible for the dysregulation of synaptic transmission and neuronal excitability characteristic of epilepsy. Although the NFkB levels in the offspring of STZ-treated dams were not significantly different from controls, the apparent increase suggests RAGE signaling through NFkB in the hippocampus of the offspring of diabetic dams could be linked to the development of cognitive impairments later in life. We are unsure of the role played by NFkB in this pathology and which cellular mechanisms may be involved. Therefore, further research is required to study the potential link of NFkB to neuronal excitability and cognitive impairment in the context of maternal diabetes. Given that the behaviors affected in the offspring depend on cortico-striatal-limbic circuits, care should be taken to specify the brain regions in which altered RAGE and NFkB levels occur following maternal diabetes.

Behavioral effects of maternal diabetes on the offspring during puberty and early adulthood

The offspring were tested on a battery of behavioral tasks commonly used to measure sensorimotor gating, anxiety, learning and memory, and behavioral flexibility in rodents, as these behaviors relate to the symptoms of neurodevelopmental disorders. Performance of the control rats was similar to previous reports from our laboratory (Howland et al., 2004a, 2012; Howland and Cazakoff, 2010; Zhang et al., 2012; Thai et al., 2013; Ballendine et al., 2015) and others (Floresco et al., 2008, 2009; Hannesson et al., 2008). PPI was assessed before and after puberty. While the offspring of STZ-treated dams showed reduced startle at both ages, PPI was only impaired in young adulthood (PND 58 and 59). The impairment was quite profound, as it existed for all prepulse–pulse intervals and prepulse intensities. The STZ-induced disruption of PPI in young adulthood resembles the deficits observed in several studies examining other environmental factors that may contribute to neurodevelopmental psychiatric disorders, including maternal immune activation during pregnancy (Wolff and Bilkey, 2008, 2010; Meyer et al., 2008; Howland et al., 2012; Ballendine et al., 2015). It is also worth noting that a significant increase in prepulse facilitation to short interval (30 ms) prepulse–pulse interval trials was observed in the offspring from STZ-treated dams. Interestingly, Howland et al. (2012) reported similar results in a model of maternal immune activation during pregnancy.

Offspring of STZ-treated dams displayed more open arm exploration in the elevated plus maze test without changes in total time spent in all arms of the maze, suggesting reduced anxiety without a gross change in exploratory behavior. Previous studies have noted increased anxiety-like behavior (Ramanathan et al., 2000) and no change in behavior on the elevated plus maze (Kinney et al., 2003) in the offspring of diabetic dams. However, in these previous studies, the dams were rendered diabetic before breeding. We also noted an apparent increase in preference for novel location in the object-place paradigm without significant effects in object recognition memory. Previous studies have noted

impairments in learning and memory in some paradigms, including the Lashley III maze and inhibitory avoidance paradigm in the offspring of dams diabetic for the entire pregnancy (Kinney et al., 2003). Interestingly, these effects were more pronounced in females. As only males were tested in the present experiments, testing both sexes will be important to consider in future studies.

The ability of rats from all groups to learn a visual discrimination strategy to obtain food reward in the operant task shows that simple forms of learning were not impaired in the offspring. However, when the offspring were forced to adopt a new strategy to solve the task during the set-shifting day, offspring from STZ- and STZ + Insulin-treated dams took more trials to reach criterion. Inspection of the data suggests that the impairment was the result of an inability of the rats to maintain correct responding during a streak (10 correct responses) as the effect on total errors was not significantly different between groups.

The pattern of behavioral alterations noted in the STZ offspring may provide insight into the neural circuits altered as a result of maternal hyperglycemia. As the electrophysiological experiments conducted demonstrate a striking change in hippocampal excitability early in postnatal life, it is possible that long-lasting alterations of hippocampal function and related circuits caused the behavioral changes observed. Examining neuronal excitability in hippocampal slices from the young adult offspring would enable a direct assessment of this hypothesis. PPI is regulated by a complex network of brain areas termed the cortico-striato-pallido-pontine circuitry (Swerdlow et al., 2001), which includes the hippocampus (Bast and Feldon, 2003; Howland et al., 2004b). The strategy set-shifting task is typically associated with the prelimbic region of medial prefrontal cortex (Floresco et al., 2008), although the importance of the hippocampus has also been suggested in other studies with different task designs (Cholvin et al., 2013). Increased open arm time in the elevated plus maze has also been noted following inactivation of either the hippocampus (Degroot and Treit, 2004) or medial prefrontal cortex (Shah et al., 2004). The lack of effect of the insulin treatment on the behavioral effects of maternal STZ on the elevated plus maze and set-shifting task suggest that mechanisms distinct from those which caused the changes in prepulse inhibition and object-place memory may be involved. Given that mPFC and hippocampal lesions recapitulate these effects (impaired set-shifting and reduced anxiety-like behavior in the elevated plus maze), a more thorough examination of the effects of STZ treatment on this circuit may increase our understanding of the behavioral changes in the offspring.

Relevance of the results for diabetes during pregnancy

The practicalities of recapitulating GDM in animals, particularly rodents, warrants discussion. Because STZ induces diabetes by chemical ablation of β-cells in the pancreas, it generates a maternal diabetic state related to type 1 diabetes (Jawerbaum and White, 2010). Therefore, despite the fact that STZ is administered during pregnancy, it does not truly reflect the state in GDM, which develops due to a lack of adaptation of the β-cells to the metabolic changes that take place during pregnancy (Jawerbaum and White, 2010). Unfortunately, given current knowledge and resources, the short duration of rodent gestation makes it very difficult to induce a state closely resembling GDM in rats, particularly without the use of genetic manipulations. A further consideration is that, once diagnosed, GDM is subsequently treated in humans, meaning that our animal model may not reflect the mechanisms through which GDM affects neural development in humans. A more accurate model of severe GDM would involve treating diabetic rats with insulin during pregnancy; however, the fact that the time of diagnosis and treatment varies in human pregnancies makes it difficult to assess how long GDM should remain untreated in an animal model. The severity of hyperglycemia induced in the present study is also not typical of human GDM. This limitation affects the construct validity of the present study and calls for the development of new models of GDM that are able to recapitulate the pathological condition in humans more closely. One option for future research would be to assess the effects of a lower dose of STZ that produced a milder hyperglycemic state during pregnancy.

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