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Short-lived diabetes in the young-adult ZDF rat does not exacerbate neuronal Ca^{2+} biomarkers of aging

Shaniya Maimaiti, Chris DeMoll, Katie L. Anderson, Ryan B. Griggs*, Bradley K. Taylor*, Nada M. Porter, and Olivier Thibault

University of Kentucky Medical Center, Department of Pharmacology and Nutritional Sciences

Abstract

Results from clinical studies provide evidence that cognitive changes relatively late in life may be traced to antecedent conditions including diabetes, obesity, a sedentary lifestyle and an atherogenic diet. As such, several traits of Type 2 diabetes (T2DM) could be considered pathogenic factors of aging, contributing to age-dependent cognitive decline and our susceptibility to Alzheimer's disease. It appears that both the duration of metabolic condition and the age of the individual, together can contribute to the potential impact on peripheral as well as brain health. Because of robust evidence that in animal models of aging, Ca^{2+} dysregulation alters neuronal health, synaptic plasticity, and learning and memory processes, we tested the hypothesis that peripheral metabolic dysregulation could exacerbate Ca^{2+} dysfunction in hippocampal CA1 neurons. Using intracellular/ extracellular electrophysiological and Ca^{2+} imaging techniques, we show that Ca^{2+} levels at rest or during synaptic stimulation, the Ca^{2+} -dependent after hyperpolarization, baseline field potentials and short-term synaptic plasticity were not significantly altered in young-adult male Zucker diabetic fatty rats compare to their lean counterparts. Our observations suggest that early phases of T2DM characterized by high levels of glucose and insulin may be too transient to alter hippocampal CA1 physiology in this animal model of diabetes. These results are supported by clinical data showing that longer T2DM duration can have greater negative impact on cognitive functions.

Keywords

calcium; aging; diabetes; imaging; obesity; learning

1. Introduction

It has now become apparent that peripheral changes, particularly those associated with T2DM, energy metabolism, insulin and glucose levels, can contribute to cognitive decline in aging. In fact, metabolic syndrome, which is characterized by the clustering of insulin resistance, hyperglycemia and dyslipidemia, has been recognized to be an important risk

*Department of Physiology, 800 Rose Street, UKMC-MS313, Lexington, KY 40536.

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factor for the development of cognitive decline in aging and AD (Mielke et al., 2006; Whitmer et al., 2007; de la Monte and Wands, 2008; Whitmer et al., 2008; Huffman and Barzilai, 2009; Cheng et al., 2012; Yaffe et al., 2012; Craft et al., 2013; Biessels and Reijmer, 2014; Yarchoan and Arnold, 2014). In addition, glucose intolerance and insulin resistance are associated with varying degrees of cognitive and memory deficits, and aging is known to worsen the impact of T2DM on cognitive function. A recent meta-analysis shows that there is a strong positive correlation between diabetes progression and the onset of AD (Cheng et al., 2012). Clearly, insulin resistance associated with T2DM is an important pathogenic factor in dementia, including Alzheimer's (Watson and Craft, 2003; Biessels et al., 2006) and the term "new-age dementia" has been used to quantify the decrease in insulin sensitivity of the brain and its' impact on the development of AD (Williamson et al., 2012).

Among the numerous animal models of diabetes, those harboring mutations in the leptin receptor gene are often used to study the impact of obesity/hyperphagia on cognition (Funahashi et al., 2003; Harvey, 2003). In prior studies the fa/fa rat and the db/db mouse, animals harboring leptin receptor mutations have shown evidence of decreased learning through a mechanism involving decreased leptin receptor function in the hippocampus, and an underlying decrease in LTP induction (Li et al., 2002; Gerges et al., 2003; Oomura et al., 2010; Kamal et al., 2013). A more recent study, however, reports the same genetic manipulations combined with a more aggressive diabetic phenotype in the male Zucker diabetic fatty rat (ZDF), has no significant influence on spatial memory and/ or LTP induction or expression (Belanger et al., 2004). It is not clear how these relatively similar peripheral phenotypes can yield such different electrophysiological profiles associated with learning and memory processes. Some have argued for a role of different peripheral glucose levels (Winocur et al., 2005) while others claim that duration of the condition might be an important factor (Biessels and Gispen 2005).

Interestingly, data from several research groups show that in both animal models of diabetes, and in diabetic patients, intracellular Ca^{2+} homeostasis is disturbed across both peripheral and brain tissues (reviewed in Biessels et al., 2002; Thibault et al., 2007; Thibault et al., 2013). Given that LTP induction as well as learning and memory processes, are dependent on Ca^{2+} signaling (Lynch et al., 1983; Malenka et al., 1988; Disterhoft et al., 1993; Disterhoft et al., 1996; Thibault and Landfield, 1996; Thibault et al., 1998), we sought to test for the presence of Ca^{2+} dysregulation in the brain of male ZDF rats. Here we tested the hypothesis that peripheral hyperglycemia and hyperinsulinemia exacerbate hippocampal CA1 electrophysiological Ca^{2+} markers. Specifically, we focused on the Ca^{2+} -dependent AHP, a robust biomarker of brain aging, the paired-pulse ratio, the growth of the excitatory post-synaptic potential (EPSP) during repetitive synaptic activation at 7 Hz, and measures of neuronal Ca^{2+} levels at rest and during stimulation.

2. Methods

2.1 ZDFs

Ten week old male Zucker Diabetic Fatty rats (ZDF) were purchased from the Charles River colony and electrophysiology experiments were conducted at 19–20 weeks of age.

Originally described in the 1960's for its propensity to gain weight more readily than other rodents (Zucker and Zucker, 1963), the Zucker fatty rat was found to harbor a truncated leptin receptor with low affinity for leptin (Cusin et al., 1996), and was rederived at Lilly research Laboratories to include an exaggerated diabetic phenotype. "Diabetic" ZDF rats are homozygous for the loss-of-function "*fatty*" mutation in the leptin receptor ($ZDF^{fa/fa}$) that results in development of T2DM (Clark et al., 1983). "Control" ZDF rats are heterozygous ($ZDF^{fa/+}$) and do not develop a diabetic phenotype. Data presented here were obtained from 6 control and 6 diabetic ZDFs. All rats were housed in a temperature and humidity controlled room on a 12 hour light – 12 hour dark cycle. All rats were provided water and a diabetogenic diet of Purina 5008 chow given *ad libitum*. All procedures were carried out under an approved IACUC protocol granted by the University of Kentucky.

2.2 Measurement of blood glucose, insulin & HbA1c

Non-fasted animals were lightly restrained and the tail cleaned with an alcohol swab. Using a #11 scalpel blade a small nick was made at the distal tip of the tail, the initial blood drop wiped clean with gauze, and a subsequent drop of blood was placed on a glucose test strip and inserted into the glucose monitor (TrueTrack, Walgreens, Deerfield, IL). The average of three replicate measures is reported from data collected at 12 and 19 weeks. For glycated hemoglobin (HbA1c) measurements, the above procedure was repeated only the drop of blood was loaded into an HbA1c cartridge and measured using the DCA Vantage Analyzer (Siemens, Munich, Germany). Insulin levels were quantified by ELISA (at 19 weeks) according to manufacturer's recommendations (EMD Millipore; EZRMI-13K; Darmstadt, Germany) from serum obtained at time of sacrifice (non-fasted).

2.3 electrophysiology

2.3.1 Intracellular—Animals were briefly anesthetized (4% Isoflurane) and decapitated. Hippocampi were removed and transverse slices were prepared (350 μ m) in ice-cold low-calcium artificial cerebrospinal fluid (ACSF) composed of the following (in mM): 128 NaCl, 1.25 KH_2PO_4 , 10 Glucose, 26 $NaHCO_3$, 3 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$ using a Vibratome® (TPI, Saint Louis, MO). Slices were transferred to a heated (32°C) interface-type chamber and maintained in oxygenated (95% O_2 , 5% CO_2) normal-calcium ACSF containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ for at least 2 h prior to recording. Each hippocampal slice was placed in a recording chamber (RC22C, Warner Instruments, Co., Hamden, CT) and maintained in a continuous flow of oxygenated normal-calcium ACSF pre-heated at 32°C using a TC2Bip/HPRE2 in-line heating system (Cell Micro Controls, Norfolk, VA). The recording chamber was mounted on the stage of a Nikon E600FN inverted microscope. Cells were impaled with sharp microelectrodes filled with 10 mM Bis-Fura-2, 2M $KMeSO_4$ and 10mM HEPES, pH 7.4, pulled from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL).

To generate an after hyperpolarization (AHP), cells were held at -65 mV (baseline) and depolarized with a 100 ms intracellular current injection sufficient to generate 4 Na^+ action potentials. AHPs were elicited every 30 s and between 2 to 10 sweeps were averaged for each cell. The medium AHP (mAHP) was measured at the point of greatest hyperpolarization immediately after the offset of the depolarizing current injection, the slow

AHP (sAHP) was measured 800 ms after the end of the current injection (see Figure 2). AHP duration was measured from the end of the depolarizing step until return to baseline. Neurons with input resistance $>35\text{ M}\Omega$, holding current $<250\text{ pA}$, and overshooting action potentials were included for analysis. Action potential height, input resistance, current injection to elicit the AHPs, and recording electrode resistance were not different across groups (data not shown). Prior to repetitive synaptic activation, an *I/O* curve was established for each cell in order to determine the stimulation intensity necessary to trigger an action potential (SD9K stimulator; Astro Med Inc., Grass Instr., Warwick, RI). Repetitive synaptic stimulation was run at threshold for an action potential and for 10 s at 7 Hz (see Figure 2). Data were acquired using pClamp 8.0 (Molecular Devices) software through a Digidata 1320A A/D converter and an Axoclamp2B amplifier (Molecular Devices).

2.3.2 Extracellular—Similar procedures were used except hippocampal slices were cut at 400 microns and the standard recording ACSF was modified to include 2.5 mM CaCl_2 and 1.3 mM MgCl_2 . Slices were recorded from within a heated and oxygenated interface-type chamber (32°C) after at least 2 h of recovery. Recording electrodes were 5–10 $\text{M}\Omega$ (filled with ACSF), and the stimulating electrode was made from twisted insulated stainless steel wire (A-M Systems, Inc. Everett, WA). An SD9K stimulator attached to the stimulating electrode positioned in the Schaffer collaterals was used to elicit EPSPs recorded in stratum radiatum of area CA1. EPSP amplitudes were quantified at baseline (stimulated every 20 s), during paired-pulse facilitation protocols (50, 100, 150 and 200 ms), and during 10 s of 7 Hz stimulation at 33% of the max EPSP (measured on *I/O* curve). The slope of the *I/O* curve was also quantified (based on EPSP amplitude measures, see Figure 3). For quantification of EPSP growth during repetitive synaptic activation, the second through 5th EPSPs and the last four EPSPs were normalized to the first EPSP of the 10 s 7Hz stimulation.

2.4 Ca^{2+} imaging

For Bis-Fura-2 ratiometric Ca^{2+} imaging, excitation of the fluorophore was provided by a high-speed filter changer (Lambda DG4, Sutter instruments) equipped with 357 ± 20 and $380 \pm 20\text{ nm}$ exciter filters. Before being captured on a CCD camera (7 MHz MicroMax – Princeton Instruments, Trenton, NJ), emitted light was passed through a dichroic filter ($\sim 400\text{ nm}$ high pass) and an emitter filter ($520 \pm 30\text{ nm}$, Chroma technology). We used isosbestic imaging to increase imaging speed between ratio pairs. Binning of 3 to 4 was employed and typically, exposures of 500 ms were used. Axon Imaging Workbench (V2.1) was used to control light emission, acquisition and timing with synaptic activation during repetitive synaptic activation. Data are presented as ratio units (uncalibrated) derived from the 357 and 380 nm wavelengths each background subtracted from an area adjacent to the cell.

2.5 Statistics

Unpaired t-tests (GraphPad Prism, San Diego, CA) were used for some of the variables measured. Twoway RM ANOVAs were used for analyses on glucose levels, HbA1C levels, as well as paired pulse ratios and on quantification of EPSP growth (percent potentiation) during repetitive synaptic activation (StatView, SAS Inc., Cary, NC). Post-hoc analyses used the Bonferroni test. Serum measures were taken from 6 animals per group and electrophysiology data were derived from 4 animals per group. For extracellular physiology

analyses, 3–6 slices were recorded per day from each animal and data from all slices were averaged and presented per animal. Intracellular data is presented as the number of cells recorded. Outliers (> 2 SD from group mean) were removed from the analysis on all variables measured.

3. Results

As previously shown, the male Zucker diabetic fatty rat displays robust peripheral markers of glucose dysregulation when compared to its control littermate (Schmidt et al., 2003; Belanger et al., 2004; Campbell et al., 2010). In 6 control and 6 ZDF rats, blood glucose levels taken at 12 and 19 weeks of age were significantly elevated as a function of genotype ($F_{(1,20)}=67$; $p<0.001$) and time between measures ($F_{(1,20)}=12$; $p<0.005$), as seen in Figure 1. Similar results were seen on measures of glycated hemoglobin, showing a robust genotype ($F_{(1,20)}=111$; $p<0.0001$) and time effects ($F_{(1,20)}=30$; $p<0.0001$). A significant increase in insulin levels was also noted on measures taken at the same time point ($p<0.005$). While these numbers were derived from non-fasted animals, the size of the effect presented here is not likely to reflect changes in recent feeding activity (glucose levels were greater than 400 ng/mL across all animals in the ZDF groups). Also, glucose levels were, on average near the 200 ng/mL mark, and significantly elevated in the ZDF groups at 12 weeks of age (Fig. 1A) suggesting diabetes was present when animals were delivered to our facility.

Following approximately 7 weeks of hyperglycemia, hippocampal CA1 Ca^{2+} -dependent electrophysiological assessment was initiated using intracellular and extracellular recording techniques. Ratiometric measures of Ca^{2+} taken during intracellular recordings using the low affinity Ca^{2+} indicator Bis-Fura-2, show that neither the baseline Ca^{2+} ratio ($p=0.6$; $n=6-9$ cells/group), nor the peak Ca^{2+} ratio ($p=0.3$; $n=6-9$ cells/group) taken at the maximal height of the Ca^{2+} transient during a 10 s, 7 Hz synaptic burst of activity, were significantly different across groups (Figure 2). The growth of the EPSP during this synaptic stimulation run (7 Hz repetitive synaptic activation, Fig. 2B), a measure of short term plasticity, was not different across groups (data not shown). We also quantified the amplitude of the EPSP measured at threshold for an action potential and found no difference across groups ($p=0.5$; $n=5-9$ cells/group). As shown in figure 2F, the AHP, a robust biomarker of Ca^{2+} homeostasis and aging also was not altered. The amplitude of fast mAHP ($p=0.5$; $n=6-11$ cells/group), the slow sAHP ($p=0.5$; $n=6-11$ cells/group), or the duration of the AHP were not changed in the control and ZDF animals ($p=0.6$; $n=6-11$ cells/group). Measures of input resistance, holding current at -70 mV, and height of the action potential also were unchanged across groups ($p>0.5$; data not shown). These intracellular recording data do not support the presence of neuronal Ca^{2+} dysregulation at rest or during synaptic activation, in the ZDF at 19–20 weeks of age.

Extracellular field potential measures during baseline and short-term plasticity experiments showed no significant alteration in EPSPs measured (Figure 3). EPSP amplitudes measured at 33% of the *I/O* curve ($p=0.6$; $n=4$ animals/group; Fig. 3D), and the slope of the *I/O* curve ($p=0.5$; $n=3-4$ animals/group; Fig. 3C and E) for each animal, were not significantly altered by the genotype. While the paired-pulse facilitation, another measure of short term plasticity, was found to be dependent on interpulse interval ($F_{(3,20)}=63$; $p<0.001$; $n=4$

animals/group; Fig. 3B), the resulting paired pulse ratios were not significantly altered across groups ($F_{(1,20)}=1.7$; $p>0.05$; $n=4$ animals/group; Fig. 3B). As with intracellular recordings, extracellular field recordings fail to highlight a robust genotype effect on hippocampal CA1 physiology measured at this time point in the disease and under these conditions in the ZDF rat. It should be emphasized, however, that extracellular recordings were acquired in the absence of any selective receptor blockers and reflect net excitatory as well as inhibitory neurotransmission. While we cannot address the presence of a selective impact on NMDA receptors, as previously reported using a different animal model of diabetes (Chabot et al., 1997; Kamal et al., 1999), it should be emphasized that similar results reporting no effect of the genotype (ZDF) on I/O slope and EPSP amplitude as been previously reported (Belanger et al., 2004). Clearly, greater characterization of the impact of diabetes on hippocampal function is needed (Gispen and Biessels, 2000; Artola et al., 2005; Kamal et al., 2006).

4. Discussion

Our study was designed to test the hypothesis that peripheral hyperglycemia and hyperinsulinemia could impact neuronal Ca^{2+} homeostasis in area CA1 of the hippocampus of young-adult animals. We used intracellular and extracellular electrophysiology combined with Ca^{2+} imaging techniques to measure postsynaptic Ca^{2+} changes during repetitive synaptic activation (inferred from ratiometric measures of changes in bis-Fura-2 fluorescence), short-term synaptic plasticity, paired-pulse facilitation, the Ca^{2+} -dependent AHP, and field potentials. None of the central outcome variables analyzed was altered by the peripheral phenotype (hyperglycemia and hyperinsulinemia). From the lack of evidence for brain Ca^{2+} dysregulation in young-adult diabetic and hyperinsulinemic animals, we suggest that longer periods of peripheral metabolic dysregulation may be needed to have an impact on hippocampal CA1 Ca^{2+} physiology. Alternatively, interactions with aging factors may also be necessary, as evidenced by clinical and preclinical data showing that cognitive dysfunction in T2DM is more pronounced in the elderly (Gispen and Biessels, 2000; Ryan and Geckle, 2000; Ryan et al., 2006). These interpretations are framed in the context of our experiments, without measures of Ca^{2+} dysregulation in response to peripheral diabetes in aged ZDF animals (but see below).

Recently, genetically manipulated animal models of T2DM have been characterized and have shown some degree of cognitive dysfunction compared to wild-type littermates, however, the condition is difficult to study concomitantly with aging given the shortened lifespan of these animals. Perhaps because of the early onset in the inherited mutation, the lep^{ob} mouse on a C57Bl background dies early, and Lep^{db} homozygotes die between 10 and 18 months of age (depending on the background strain). In these monogenic models, a loss of function of the leptin pathway leads to hyperphagia and obesity (e.g., Lep^{ob} ; Lep^{db} ; Tub^{tub} mice; Zucker fatty (fa/fa), Goto-Kakizaki, and Agouti rats). Among these animals, the Zucker fatty rat and the db/db mouse show decreased learning by 7 weeks of age (Morris water maze) and a decrease in hippocampal LTP induction (Li et al., 2002). In contrast, 5–8 weeks of confirmed hyperinsulinemia and hyperglycemia (animals approximately 6.5 months of age) did not alter spatial memory and/or LTP in ZDF rats (Belanger et al., 2004). Clearly, these differences might be due to several factors, some of which include age of the

animal, duration of the diabetic condition, and perhaps differences in LTP induction paradigms. One important limitation of these models, however, is their short lifespan and hence, it is unclear how cognition is altered in these models of obesity with aging. Unfortunately, however, T2DM is a disease of aging as evidenced by its increased incidence with age (CDC Statistics; Ogden et al., 2007), and significant associations between T2DM, vascular dementia and AD which manifest readily in the elderly (Cheng et al., 2012; De Felice et al., 2014; Yarchoan and Arnold, 2014).

In several studies, higher plasma insulin but lower brain insulin levels are correlated with a decline in cognitive status (Craft et al., 1998; Ott et al., 1999; Janson et al., 2004; Watson and Craft, 2004; Steen et al., 2005; Cole and Frautschy, 2007). The mechanisms underlying the relationship between peripheral and central insulin levels, in both aging and diabetes, propose that following prolonged exposures to hyperinsulinemia and hyperglycemia, down-regulation of insulin and glucose transporters at the blood brain barrier is critical (Baura et al., 1996; Korol and Gold, 1998; Simpson et al., 1999; Duelli et al., 2000; Craft, 2005; McNay, 2005). It should be emphasized, however, that brain insulin resistance has been demonstrated in AD cases without evidence of overt diabetes (Talbot et al., 2012). Clearly, however, strategies that acutely increase insulin occupation of brain insulin receptors in aging and/or AD have been very successful in the clinic (Benedict et al., 2004; Craft et al., 2012; de la Monte, 2013; Freiherr et al., 2013; Heni et al., 2014).

While the ZDF rat may not harbor all characteristics of late T2DM due to the presence of sustained hyperinsulinemia together with hyperglycemia, the animal model may mimic earlier stages of the disease when both conditions coexist. Continuing to study this model at later ages, when pancreatic beta cell function begins to fail and a more clinically relevant T2DM-like phenotype prevails (hyperglycemia with hypoinsulinemia) is challenging, as maintaining diabetic states for longer periods (e.g., 12–16 months) is difficult. Clearly, therefore, the model, while valuable for studies on the impact of peripheral metabolic dysregulation, lacks the impact of aging which is known to alter brain Ca^{2+} homeostasis (Oh et al., 2010; Thibault et al., 2013). Here we present data on animals approximately 4.5 months of age and show normal brain Ca^{2+} signaling in CA1 pyramidal neurons. The Belanger study reported no effects on learning and memory of LTP maintenance while using slightly older animals (approximately 6.5 months of age) with greater plasma glucose and insulin levels. Thus, it seems possible that more prolonged periods of peripheral dysregulation, perhaps in combination with aging, or that conditional knockin/ knockout approaches should be considered when attempting to model clinical settings and test the impact of peripheral metabolic dysregulation on the aging brain.

Overall therefore, our results do not support the hypothesis that short-lived peripheral hyperinsulinemia and hyperglycemia have an impact on neuronal Ca^{2+} dysregulation in area CA1 of the hippocampus in young-adult ZDF rats. Our data, combined with the results of others showing little effect of the genotype on learning and memory (Belanger et al., 2004) or on peripheral neuronal structural integrity (Schmidt et al., 2003), suggest that hyperglycemia *per se* may not be a key pathogenic factor of diabetes-induced cognitive decline in younger animals. This is well supported by the literature showing that hypoglycemia is more likely to alter cognitive function (McNay and Sherwin, 2004; McNay,

2005). Alternatively, hypoinsulinemia could well be the trigger element underlying cognitive decline in aging and/ or AD. This is currently supported by evidence that brain insulin sparing strategies (*e.g.*, intranasal insulin) in AD are very successful at reversing cognitive decline in the clinic (Craft et al., 2013; de la Monte, 2013; Freiherr et al., 2013).

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Highlights

- Neuronal Calcium dysregulation is a hallmark of aging and AD.
- The AHP, a neuronal calcium biomarker of aging is increased with age.
- Male ZDF rats with sustained hyperglycemia show no signs CNS Ca²⁺ dysregulation.
- Our results suggest aging is an important factor in diabetes-mediated cognitive decline.

Figure 1. Peripheral hyperglycemia and hyperinsulinemia in the ZDF rat

Serum glucose levels measured at 12 weeks and 19 weeks of age in control and male ZDF rats show genotype and time effects (A). Asterisks and pound signs indicate significance of genotype and time between measures (respectively). Measures of glycated hemoglobin (B) and insulin levels (C) also show increases in the ZDF compared to control rats. Means $\pm$ SEM are presented.

Figure 2. Intracellular neuronal Ca^{2+} status in the ZDF rat

A pseudocolor photomicrograph of Bis-Fura-2 fluorescence in a cell at rest (left) and during the peak Ca^{2+} response (right), is shown in (A). A representative example of the growth of the EPSP and cellular hyperpolarization during repetitive synaptic activation at 7 Hz used to trigger postsynaptic rises in Ca^{2+} , is shown in (B). Data quantification across groups shows no change in resting Ca^{2+} measures (baseline ratio) taken at rest (C) and during the peak of activation by synaptic stimulation (D), in controls compared to diabetic Zucker fatty rats. The amplitude of the intracellularly recorded EPSP at threshold for an action potential (measured from the 1st EPSP in B) was unchanged across groups (E). The Ca^{2+} - dependent afterhyperpolarization (AHP) following 4 action potentials (truncated) triggered postsynaptically is shown in (F). Neither the amplitude of the mAHP (G), the sAHP (H), nor the duration (I) of the AHP was altered. Means $\pm$ SEM are presented.

Figure 3. Extracellular measures of hippocampal CA1 physiology in the ZDF rat

Representative field excitatory postsynaptic potential (EPSP) recordings (A) taken during paired-pulse facilitation (interpulse intervals of 50, 100, 150, and 200 ms) in a ZDF control animal. Quantification of paired-pulse facilitation, expressed as the ratio of the 2nd, 3rd, 4th, and 5th EPSP amplitude to the first EPSP (bold), is presented in (B). Paired pulse facilitation ratios, while dependent on interpulse interval (ms), were not different between control and ZDF animals. The amplitude of the EPSP at 33% of the input output (I/O) curve (C) was not significant across groups (D), neither was the slope of the I/O curve (E). Asterisk indicates interpulse interval effect. Means $\pm$ SEM are presented.