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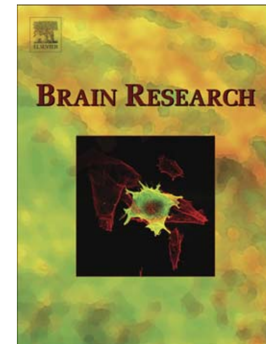

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PII: S0006-8993(11)00155-7
DOI: doi: 10.1016/j.brainres.2011.01.071
Reference: BRES 41223

To appear in: *Brain Research*

Accepted date: 20 January 2011

Please cite this article as: Filipa I. Baptista, Joana M. Gaspar, Armando Cristóvão, Paulo F. Santos, Attila Köfalvi, António F. Ambrósio, Diabetes induces early transient changes in the content of vesicular transporters and no major effects in neurotransmitter release in hippocampus and retina, *Brain Research* (2011), doi: 10.1016/j.brainres.2011.01.071

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Research Paper

Diabetes induces early transient changes in the content of vesicular transporters and no major effects in neurotransmitter release in hippocampus and retina

Filipa I. Baptista[1]*; Joana M. Gaspar[1,2]*; Armando Cristóvão[2,3]; Paulo F. Santos[2,3]; Attila Köfalvi[2]; António F. Ambrósio[1,2]

[1]Centre of Ophthalmology and Vision Sciences, IBILI, Faculty of Medicine, University of Coimbra, 3004-548 Coimbra, Portugal; [2]Center for Neuroscience and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal; [3]Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra, 3004-517 Coimbra, Portugal

* These authors contributed equally to this work

Corresponding author:

António F. Ambrósio

Centre of Ophthalmology and Vision Sciences

IBILI, Faculty of Medicine

Azinhaga de Santa Comba

3004-548 Coimbra, Portugal

Phone: + 351 239 480 222

Fax: + 351 239 480 280

Email: fambrosio@ibili.uc.pt

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1. Abstract

Diabetes induces changes in neurotransmitter release in central nervous system, which depend on the type of neurotransmitter and region studied. In this study, we evaluated the effect of diabetes (two and eight weeks duration) on basal and evoked release of [^{14}C]glutamate and [^{3}H]GABA in hippocampal and retinal synaptosomes. We also analyzed the effect of diabetes on the protein content of vesicular glutamate and GABA transporters, VGluT-1, VGluT-2 and VGAT, and on the α_{1A} subunit of P/Q type calcium channels, which are abundant in nerve terminals.

The protein content of vesicular glutamate and GABA transporters, and of the α_{1A} subunit, was differently affected by diabetes in hippocampal and retinal synaptosomes. The changes were more pronounced in the retina than in hippocampus. VGluT-1 and VGluT-2 content was not affected in hippocampus. Moreover, changes occurred early, at two weeks of diabetes, but after eight weeks almost no changes were detected, with the exception of VGAT in the retina. Regarding neurotransmitter release, no major changes were detected. After two weeks of diabetes, neurotransmitter release was similar to controls. After eight weeks of diabetes, the basal release of glutamate slightly increased in hippocampus and the evoked GABA release decreased in retina.

In conclusion, diabetes induces early transient changes in the content of glutamate and/or GABA vesicular transporters, and on calcium channels subunit, in retinal or hippocampal synaptosomes, but only minor changes in the release of glutamate or GABA. These results point to the importance of diabetes-induced changes in neural tissues at the presynaptic level, which may underlie alterations in synaptic transmission, particularly if they become permanent during the later stages of the disease.

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Research Highlights

- **Diabetes induces changes in vesicular transporters.**
- **In retina, diabetes induces a decrease in GABA release.**
- **In hippocampus, diabetes induces an increase in glutamate release.**
- **Imbalance between GABA/glutamate may lead to physiological changes or neurotoxicity.**

Key words: Diabetes, Hippocampus, Retina, Neurotransmitter release, Glutamate, GABA.

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2. Introduction

Diabetes mellitus is a metabolic disease resulting from impairment in insulin secretion and/or insulin resistance leading to hyperglycemia. Diabetes is associated with several diseases, such as diabetic encephalopathy and retinopathy (ADA, 2010), which are characterized by functional and structural alterations in brain and retina, respectively.

Several studies have demonstrated that diabetes impairs synaptic structure and function in hippocampus at presynaptic (Grillo et al., 2005; Gaspar et al., 2010) and postsynaptic levels (Biessels et al., 1996; Kamal et al., 1999), which can somehow underlie the development of cognitive impairments. In diabetic animals, changes in the content of exocytotic proteins and receptors involved in neuromodulation in hippocampal nerve terminals (Duarte et al., 2006; Duarte et al., 2007; Duarte et al., 2009; Gaspar et al., 2010), and a depletion of synaptic vesicles in hippocampal mossy fiber terminals (Magarinos and McEwen, 2000), have been detected. At the postsynaptic component, impairments in synaptic plasticity (Biessels et al., 1996; Artola et al., 2005) and in NMDA receptor subunit composition (Trudeau et al., 2004) were also observed. Furthermore, Chabot et al. (1997) reported a deficit in calcium-dependent processes modulating postsynaptic AMPA receptors during synaptic potentiation. Diabetes also triggers changes in neurotransmitter release in various brain regions, which depend on neurotransmitter and brain region studied (Guyot et al., 2001; Morris and Pavia, 2004; Miyata et al., 2007; Satoh and Takahashi, 2008). For instance, diabetes decreases the basal release levels of serotonin and dopamine in hippocampus (Yamato et al., 2004) and of glutamate in the dentate gyrus, but basal γ-aminobutyric acid (GABA) release is not affected (Reisi et al., 2009).

In the retina, the presynaptic component is also affected under diabetic conditions, since a decrease in the levels of several exocytotic proteins in retinal nerve terminals

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was recently reported (Vanguilder et al., 2008; Gaspar et al., 2010). In the retina of diabetic animals and in retinal neural cell cultures exposed to elevated glucose, we found an increase in the evoked release of [^{3}H]D-aspartate (marker of the glutamate transmitter pool) (Santiago et al., 2006a) and ATP (Costa et al., 2009). Moreover, elevated levels of GABA and glutamate were detected in the vitreous of patients with proliferative diabetic retinopathy (Ambati et al., 1997). We also reported that diabetes changes the content of ionotropic glutamate receptor subunits in the retina, which may account to retinal dysfunction (Santiago et al., 2009). In addition, it has been suggested that changes in GABA signalling may underlie the alterations in electroretinogram (ERG) responses in streptozotocin (STZ)-induced diabetic rats (Ramsey et al., 2006) and diabetic patients (Ambati et al., 1997). Diabetes modulates the properties of $GABA_C$ receptors in retinal bipolar cells, probably through alterations in the gene expression of GABA receptor subunits, which might therefore underlie changes in ERG of diabetic patients (Ramsey et al., 2007). In the retina of diabetic animals, changes in the content and localization of GABA are evident, with both amacrine and Müller cells accumulating high concentration of GABA (Ishikawa et al., 1996; Takeo-Goto et al., 2002). Moreover, diabetes leads to a generalized attenuation in the content of free amino acids in the retina and to a decrease in the uptake of aspartate, while GABA uptake is enhanced (Vilchis and Salceda, 1996). However, by nuclear magnetic resonance spectroscopy, we recently showed that the levels of the majority of the intermediate metabolites and amino acids present in retina is not affected by diabetes (Santiago et al., 2010).

Importantly, the synaptic level of GABA and glutamate is determined by two important factors: the integrity of the release and re-uptake systems. In nerve terminals, specific vesicular transporters (VGluT1-3 for glutamate, and VGAT for GABA) allow the

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incorporation of neurotransmitters into synaptic vesicles. These transporters have an essential role in transmitter recycling and homeostasis in the CNS, and several studies evidence their role in neurological disorders (Vemuganti, 2005; Benarroch, 2010).

Other key elements involved in neurotransmitter release are voltage-dependent calcium channels which mediate the entry of Ca^{2+} ions into nerve terminals. Calcium channels are multisubunit complexes composed of α_1, β, α_2/δ, and γ subunits. The channel activity is directed by the pore-forming α_1 subunit, whereas the others act as auxiliary subunits regulating this activity. The α_{1A} subunit is predominantly expressed in neuronal tissues (Evans and Zamponi, 2006). N- and P/Q-type Ca2+ channels are important in the presynaptic control of amino acid release in the hippocampus (Meir et al., 1999). These channels have an important contribution for endogenous glutamate release from hippocampal synaptosomes, being the contribution of P/Q-type channels for the release of glutamate more relevant than the contribution of N-type (Ambrósio et al., 1997).

Thus, our previous observations showing that diabetes changes the protein content of several exocytotic proteins in rat hippocampal and retinal nerve terminals (Gaspar et al., 2010) has prompted us to detect further alterations that occur at the presynaptic level and mapping the effect of diabetes on basal and evoked glutamate and GABA release in hippocampus and retina. In this study, we focused on retina and hippocampus not only because they are prone to diabetic neuropathy, but also based on our previous results, where we observed presynaptic changes and where one would expect impairment at the synaptic level. Moreover, since the release of glutamate and GABA depend on the transport of the amino acids into synaptic vesicles, we also evaluated the content of VGluT-1, VGluT-2 and VGAT, searching for possible

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impairments in these proteins under diabetic conditions, and aiming to correlate possible changes with alterations in neurotransmitter release.

2. Results

2.1 Animal weight and glycemia

Before diabetes induction, the body weight of animals assigned for control and diabetic groups was similar (289.9 ± 5.1 g for control and 284.6 ± 7.8 g for diabetic animals). The glucose levels were also similar in both groups (91.5 ± 2.4 mg/dL for controls and 86.0 ± 3.5 mg/dL for diabetic animals). Average weight and blood glucose levels for both diabetic and aged-matched control rats at the time of death are given in Table 1. A marked impairment in weight gain occurred in diabetic rats comparing with age-matched controls in both time points analyzed. Diabetic animals also presented significantly higher blood glucose levels comparing to age-matched controls (Table 1).

2.2 Diabetes changes the protein content of vesicular glutamate transporters in retinal synaptosomes

The protein levels of vesicular glutamate transporters were evaluated by immunoblotting in synaptosomes and total extracts of hippocampus and retina from both diabetic and age-matched control animals. In hippocampal synaptosomes, VGluT-1 and VGluT-2 content was not affected after two and eight weeks of diabetes (Figures 1A and 1B, respectively). In retinal synaptosomes, the protein content of VGluT-1 and VGluT-2 significantly decreased after two weeks of diabetes (reduction to 69.5 ± 8.8% and 77.5 ± 8.2%, respectively, compared to age-matched controls; Figures 2A and 2B). However, after eight weeks of diabetes no significant changes were observed in retinal synaptosomes. In total extracts from hippocampus (Figures 1C and 1D) and retina

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(Figures 2C and 2D) no significant differences were detected in the protein content of both vesicular transporters between diabetic and age-matched control animals.

2.3 Diabetes changes the protein content of vesicular GABA transporter in hippocampal and retinal synaptosomes

The protein content of VGAT significantly decreased after two weeks of diabetes, both in hippocampal and retinal synaptosomes (reduction to 75.8 ± 6.4% and 44.7 ± 3.3%, respectively; Figures 3A and 3B,). However, eight weeks after the onset of diabetes, the protein levels of VGAT in hippocampal synaptosomes were similar to those found in age-matched controls. Surprisingly, the protein levels of VGAT significantly increased in retinal synaptosomes (143.2 ± 3.9%; Figure 3B). In total extracts from hippocampus and retina, no significant differences were observed in the protein levels of VGAT between diabetic animals and age-matched controls (Figures 3C and 3D, respectively).

2.4 The protein content of the subunit α_{1A} of P/Q calcium channels decreased after two weeks of diabetes in hippocampal and retinal synaptosomes

The protein content of α_{1A} subunit of P/Q type calcium channels significantly decreased in hippocampal and retinal synaptosomes (reduction to 77.6 ± 6.4% and 55.2 ± 3.0% of age-matched controls, respectively) after two weeks of diabetes (Figures 4A and 4B). Conversely, eight weeks after the onset of diabetes, the protein levels of α_{1A} subunit in synaptosomes from both tissues were similar to those found in controls. In total extracts from hippocampus and retina, no significant changes were observed in the protein levels of this subunit (Figures 4C and 4D).

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2.5 The basal release of [^{14}C]glutamate increased in hippocampal synaptosomes after eight weeks of diabetes, but [^{3}H]GABA release was not affected by diabetes

The effect of diabetes on [^{14}C]glutamate and [^{3}H]GABA release from hippocampal synaptosomes was also evaluated. After two weeks of diabetes, no differences were found in the basal release of [^{14}C]glutamate between diabetic and age-matched control animals (Figure 5A). However, after eight weeks, a small but significant increase in the basal release of [^{14}C]glutamate was found in diabetic animals (2.42 ± 0.02 FR% for control and 2.74 ± 0.02 FR% for diabetic animals; Figure 5B). The amplitude of the repeated KCl-evoked release of [^{14}C]glutamate (S1 and S2) in synaptosomes isolated from two and eight weeks diabetic and control animals was not significantly different (Figures 5C and 5D). The S2/S1 ratio was also determined, but no differences were found between diabetic and control animals for both time points (Figure 5E).

Regarding [^{3}H]GABA release, no changes were observed in diabetic animals, either in basal or evoked release, as well as for S2/S1 ratio, at two and eight weeks of diabetes (Figure 6).

2.6 [^{3}H]GABA release decreased after eight weeks of diabetes in retinal synaptosomes

In retinal synaptosomes, we only measured the release of [^{3}H]GABA because the radioactive levels obtained for [^{14}C]glutamate release experiments were too low. The basal release of [^{3}H]GABA before the first stimulus was similar in both control and diabetic animals after two and eight weeks of diabetes (Figures 7A and 7B). After two weeks of diabetes, the KCl-evoked [^{3}H]GABA release (S1 and S2) was similar to the one observed in age-matched control animals (Figure 7A and 7C). However, after eight weeks of diabetes there was a significant decrease in [^{3}H]GABA release after the

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second stimulus (4.68 ± 0.26 FR% for control and 3.96 ± 0.31 FR% for diabetic animals, respectively; Figures 7B and 7D). Consequently, the S2/S1 ratio at eight weeks of diabetes, but not at two weeks of diabetes, was significantly decreased compared to control (Figure 7E).

3. Discussion

In the present study, we demonstrated that diabetes induces transient changes in the protein content of vesicular glutamate and GABA transporters and also in the α_{1A} subunit of P/Q type calcium channels. Hippocampal and retinal nerve terminals, were differently affect by diabetes, however no changes were observed in total extracts. Moreover, our results show that after eight weeks of diabetes there is a slight increase in the basal release of glutamate in hippocampal synaptosomes and a slight decrease in the evoked release of GABA in retinal synaptosomes. A fine balance between GABA and glutamate is essential for a proper brain and retinal function and any imbalance may lead to physiological alterations. Although these changes, particularly those related with neurotransmitter release, are only slight changes, this study shows that diabetes can affect the pre-synaptic compartment in neuronal issues, and also that the balance between glutamate and GABA might be affected early under diabetic conditions in both tissues.

Alterations in memory and cognitive deficits (Biessels et al., 1996), and contrast sensitivity and color perception (Roy et al., 1986; Daley et al., 1987), induced by diabetes, might be due, at least in part, to changes in neurotransmission, at pre- and/or post-synaptic level. Recently, we demonstrated that diabetes induces changes in the content of several synaptic proteins involved in exocytosis in both hippocampal and retinal synaptosomes (Gaspar et al., 2010), suggesting that diabetes might impair

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neurotransmitter release early in the course of the disease. However, there are only three studies exploring the impact of diabetes on transmitter release in hippocampus. One shows that the basal release of monoamines is impaired in both STZ-injected and spontaneously diabetic rats (WBN/Kob rats) (Yamato et al., 2004). (Ramakrishnan et al., 2005)) reported an increase in the levels of dopamine. Other work shows that the basal release of glutamate in dentate gyrus is decreased after twelve weeks of diabetes (Reisi et al., 2009). Similarly, in the retina, there are a few studies where the effect of diabetes or hyperglycemic conditions on neurotransmitter release was analyzed. In the retina of diabetic animals and in high glucose-treated retinal neural cell cultures, we showed that the release of [^{3}H]D-aspartate is increased (Santiago et al., 2006a). Similarly, we found that the release of ATP in retinal cell cultures exposed to high glucose is also increased (Costa et al., 2009).

VGluT-1 and -2 are specific markers for glutamatergic neurons, and changes in their content may underlie changes in glutamatergic transmission (Benarroch, 2010; Phillips et al., 2010). The content of VGluT-1 and -2 was differently affected by diabetes in both tissues. No changes in VGluT-1 and -2 were detected in hippocampal synaptosomes, but a significant decrease in the content of both transporters was observed in retinal synaptosomes after two weeks of diabetes. The content of VGAT decreased in both hippocampal and retinal synaptosomes after two weeks of diabetes. These observations suggest that the retina appears to be more affected than hippocampus by diabetes, concerning the transport of neurotransmitters, namely glutamate and GABA, into the vesicles. The decrease in VGAT and VGluT-1 and -2 content in synaptosomes could lead to a slower packaging of GABA and glutamate which can contribute to changes in synaptic transmission. However, our results suggest that although the protein content of VGAT and P/Q proteins is affected at two weeks, the loading

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capacity of vesicular transporters appears not to be changed by diabetes, since the uptake of [^{3}H]GABA into synaptosomes was not affected. However, the decrease in the levels of the vesicular transporters was transient, at least in the early stages of diabetes, suggesting that after the initial stress conditions induced by diabetes both tissues are somehow able to react against diabetes-induced stress. In a previous work, we found that the content of synaptic proteins in retinal synaptosomes is affected mainly after two weeks of diabetes, recovering to control levels for longer periods (Gaspar et al., 2010), suggesting that changes can be plastic and reversible, and neural tissues are able to react against stress conditions, at least temporarily.

The P/Q type voltage-gated calcium channels are abundant in nerve terminals and play a predominant role in neurotransmitter release at central synapses (Ambrósio et al., 1997). P/Q type calcium channels co-localize densely with syntaxin-1 at the presynaptic nerve terminals (Westenbroek et al., 1995) and can be isolated as a complex with SNARE proteins (Bennett et al., 1992; Leveque et al., 1994). As for vesicular transporters, hippocampus and retina recovered the levels of α_{1A} subunit after eight weeks of diabetes, supporting the fact that both tissues are able to recover from changes occurring in synaptic proteins induced by diabetes, at least temporarily. Impaired Ca^{2+} regulation may result in synaptic dysfunction, impaired plasticity and neuronal degeneration (Mattson, 2007). Elevated glucose impairs calcium homeostasis in retinal neural cells, which may have implications for the mechanisms of vision loss in diabetic retinopathy (Santiago et al., 2006b). The observed decrease in the levels of α_{1A} subunit in diabetic animals might be considered a protective strategy against Ca^{2+} overload.

A few studies have demonstrated that diabetes induces changes in neurotransmitter release. In this study, after two weeks of diabetes, no differences were found in

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neurotransmitter release in hippocampus and retina. However, after eight weeks of diabetes, the evoked release of GABA is slightly, but significantly, decreased in retinal synaptosomes, and the basal release of glutamate is slightly, but also significantly, increased in hippocampal synaptosomes. We are aware of only three studies reporting changes in transmitter release in the diabetic hippocampus: while (Yamato et al., 2004) found a decrease in the basal levels of serotonin and dopamine, (Ramakrishnan et al., 2005) reported an increase in the levels of dopamine. The apparent contradiction may be explained by the different time-points of the disease investigated. Altogether, a growing body of evidence suggests that diabetes impairs hippocampal neurotransmission.

The accumulation of glutamate in the synaptic cleft can lead to excitotoxic neuronal damage due to excessive activation of glutamate receptors (Dong et al., 2009). The small increase in the basal release of glutamate can be responsible for glutamate accumulation in the synaptic cleft. If this small increase in basal glutamate release occurs during long periods, it may eventually contribute to neurotoxicity. In experimental models, it has been shown that diabetes does not induce neurodegeneration in the brain at early time points (eight weeks) of diabetes (Grillo et al., 2005). However, after longer periods of diabetes (eight months), neuronal apoptosis can occur (Li et al., 2002). In fact, small changes for longer periods of time might progressively conduct to neuronal cell dysfunction and death, as observed in the hippocampus of diabetic animals (Li et al., 2002; Jafari Anarkooli et al., 2008), which in turn might be associated with cognitive impairments. Reisi and colleagues (2009) recently reported that the basal glutamate release decreases in the dentate gyrus of STZ-induced diabetic animals, while we found an increase in whole hippocampus. This discrepancy may be due to the experimental approaches used. While we measured the

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release of glutamate and GABA in hippocampal synaptosomes in a superfusion system, Reisi and colleagues (2009) measured the release using a microdialysis system and only in dentate gyrus. However, concerning GABA release, no differences were found, similarly to what was observed by Reisi and colleagues. Notwithstanding, Li et al. (2000) have shown that hyperglycemia exacerbates the ischemia-triggered increase of extracellular glutamate concentration in the hippocampus and cortex. This is important because the removal of the neurotransmitters by the transporter systems is highly energy dependent. We recently found that the cerebral glucose uptake and metabolism in STZ-induced diabetic rats is also compromised (unpublished observation). Thus, it is likely that highly energy-dependent processes such as the reuptake of neurotransmitters may also be affected in type-1 diabetes. Some studies suggest that the impairments in glutamatergic neurotransmission, at postsynaptic level, involving the reorganization of post-synaptic receptors underlie the functional changes in synaptic plasticity that occur in the hippocampus of diabetic animals (Di Luca et al., 1999; Gardoni et al., 2002). Our findings suggest that diabetes can elicit alterations in excitatory neurotransmission, at presynaptic level, but additional experiments are needed to establish a clear correlation with changes in glutamatergic transmission and cell dysfunction or death in hippocampal neurons.

In the retina, we found no changes in the release of GABA at two weeks of diabetes. However, at eight weeks of diabetes we found a small, but significant, impairment in the evoked release of GABA, during the second stimulus. Previously, we demonstrated that the evoked release of [^{3}H]D-aspartate from the retina increases at four weeks of diabetes, increasing also in retinal neural cultures exposed to high glucose (Santiago et al., 2006a). These observations, namely the decrease in the evoked [^{3}H]GABA release and the increase in the evoked release of [^{3}H]D-aspartate (marker of glutamate

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transmitter pool) in the diabetic rat retinas suggests that diabetes can lead to the impairment of neurotransmission. Moreover, if these alterations in transmitter release persist for longer periods, they can somehow contribute to neuronal apoptosis detected in the diabetic retina (Barber et al., 1998; Park et al., 2003), which may be correlated to the hypothesis of glutamate-induced retinal neurodegeneration in diabetic retinopathy.

Taken together, and according to our previous findings, these results further demonstrate that diabetes induces molecular alterations in both retinal and hippocampal nerve terminals. We report changes in the density of vesicular glutamate and GABA transporters and of α_{1A} subunit of P/Q type calcium channels. Moreover, the alterations were more pronounced in the retina, and the majority of these changes were transient, which also suggests that retina and hippocampus are able to react against stress conditions, at least temporarily. The changes detected in nerve terminals at two weeks of diabetes did not correlate with changes in glutamate and GABA release. Changes in transmitter release, which were not very pronounced, were only detected at eight weeks of diabetes. If these changes persist or become more prominent, an imbalance between excitation and inhibition might take place in both tissues, which may lead to neuronal dysfunction, and ultimately to visual and memory impairments detected in diabetic animals and humans.

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4. Experimental Procedures

4.1 Materials

Reagents were acquired from Sigma, St. Louis, MO, USA, with the exception of those described along the text.

4.2 Animals

Male Wistar rats (Charles River Laboratories, Barcelona, Spain), eight weeks-old, were randomly assigned to control or diabetic groups. All animals were handled according with the EU guidelines for the use of experimental animals (86/609/EEC). Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ; 65 mg/kg, freshly dissolved in 10 mM sodium citrate buffer, pH 4.5). Hyperglycemic status (blood glucose levels exceeding 250 mg/dL) was confirmed two days later with a glucometer (Elite, Bayer, Portugal). Before sacrifice under halothane anesthesia, rats were weighted, and blood samples were collected to measure glucose levels.

4.3 Preparation of hippocampal synaptosomal extracts

Percoll purified synaptosomes were isolated as previously described (Köfalvi et al., 2007), with minor changes. The two hippocampi of each rat were dissected and homogenized in a sucrose-HEPES solution (0.32 M sucrose, 1 mM EDTA, 10 mM HEPES, 1 mg/mL BSA, pH 7.4). The homogenate was centrifuged at 3,000 x *g* for 10 min at 4ºC. The supernatant was collected and centrifuged at 14,000 x *g* for 12 min at 4ºC, and the resulting pellet was resuspended in 45% (v/v) Percoll solution prepared in Krebs–Henseleit Ringer (KHR) solution (in mM: 140 NaCl, 1 EDTA, 10 HEPES, 3 KCl, 5 glucose, pH 7.4). After centrifugation at 16,100 x *g* for 2 min at 4ºC, the top layer was

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removed (synaptosomal fraction). For release experiments, the synaptosomal fraction was collected and stored in a sealed container on ice until use. For synaptosomal extracts, pellet was resuspended in lysis buffer [RIPA: 150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate (DOC), 0.1% sodium dodecyl sulfate (SDS), supplemented with complete miniprotease inhibitor cocktail tablets (Roche, Basel, Switzerland) and 1 mM dithiothreitol (DTT)]. The samples were stored at -80ºC until use.

4.4 Preparation of retinal synaptosomal extracts

The two retinas of each diabetic and age-matched control rats were used for preparation of synaptosomes, as previously described (Vanguilder et al., 2008), with minor alterations. Immediately after animal sacrifice, both eyes were enucleated, and the two retinas were dissected and merged in 10 mL of ice-cold sucrose-HEPES solution. Retinas were washed three times combining gentle vortexing and buffer replacement to remove photoreceptor outer segments. Then, the retinas were homogenized in 4 mL of fresh sucrose buffer and then the homogenate was centrifuged at 200 x *g* for 10 min at 4ºC to pellet nuclear fraction. The supernatant was centrifuged at 800 x *g* for 12 min at 4ºC. The resulting supernatant was centrifuged at 16,100 x *g* for 20 min at 4ºC to obtain the synaptosomal fraction used in release experiments. For synaptosomal extracts the pellet was rinsed in a detergent-based extraction buffer (20 mM Tris, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 0.5% SDS, supplemented with complete miniprotease inhibitor cocktail tablets, pH 7.2), at 4ºC and stored at -80ºC until use.

4.5 Preparation of hippocampal total extracts

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After dissection, the two hippocampi were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, supplemented with complete mini protease inhibitor cocktail tablets and 1 mM DTT). The resulting homogenate was sonicated (4 pulses, 2 seconds each) and then centrifuged at 16,100 x *g* for 10 min. All procedure was done at 4ºC. The supernatant was stored at -80ºC until use.

4.6 Preparation of retinal total extracts

The eyes of diabetic and age-matched control animals were enucleated and placed in cold phosphate-buffered saline [(PBS), in mM: 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.4 at 4ºC]. The retinas were lysed in RIPA buffer supplemented with complete mini protease inhibitor cocktail tablets and 1 mM DTT, pH 7.2, at 4ºC. Then, the lysates were sonicated and centrifuged at 16,100 x *g* for 10 min at 4ºC. The supernatant was collected and stored at -80ºC until use.

4.7 [^{3}H]GABA and [^{14}C]glutamate release assays for hippocampal synaptosomes

Dual-label [^{3}H]GABA / [^{14}C]glutamate release experiments were performed as described by (Köfalvi et al., 2007), with some modifications. The synaptosomal pellet was ressuspended in 2 mL of Krebs solution [(in mM): 113 NaCl, 3 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 25 $NaHCO_3$, 10 glucose, oxygenated with 95% O_2 and 5% CO_2, pH 7.4]. The radioalabeled compounds [1 μCi/mL [^{14}C]glutamate and 2.3 μCi/mL [^{3}H]GABA (Amersham Pharmacia Biotech, Piscataway, NJ, USA)] were added to the synaptosomes for 10 min at 37ºC. All solutions contained the GABA transaminase/ glutamate decarboxylase inhibitor, aminooxyacetic acid (100 μM). Aliquots (400 μL) of the preloaded synaptosomes were transferred to 1 mL of oxygenated Krebs solution and then to perfusion chambers, being trapped in Whatman GF/C filters and

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superfused continuously at a rate of 0.75 mL/min until the end of the experiment. After a washout period (15 min), samples were collected (2 min perfusion) for liquid scintillation assay. The volume of sample (perfusate) that was collected in the vial for scintillation counting was 1.5 ml. All experimental procedures were performed at 37ºC. At the 4th and the 12th min of the sample collection period, release of transmitters was evoked with 20 mM KCl for 30 seconds each time (Köfalvi et al., 2007).

4.8 [^{3}H]GABA release assay for retinal synaptosomes

Release experiments were performed as previously described by (Köfalvi et al., 2007), with some modifications. The synaptosomal pellet was resuspended in 1 mL of Krebs solution. The radioalabeled compound [4.2 µCi/mL [^{3}H]GABA (Amersham Pharmacia Biotech, Piscataway, NJ, USA)] was added to the synaptosomes for 10 min at 37ºC. All solutions contained the GABA transaminase/ glutamate decarboxylase inhibitor, aminooxyacetic acid (100 mM). Aliquots (200 µL) of the preloaded synaptosomes were transferred to 1 mL of oxygenated Krebs solution and then to perfusion chambers, being trapped in Whatman GF/C filters and superfused continuously at a rate of 0.75 mL/min until the end of the experiment. After a washout period (15 min), samples (2 min perfusion) were collected for liquid scintillation assay. The volume of sample (perfusate) that was collected in the vial for scintillation counting was 1.5 ml. All experimental procedures were performed at 37ºC. At the 4th and the 12th min of the sample collection period, release of transmitters was evoked with with 30 mM KCl for 1 min each time. In these release experiments, a 30 mM KCl stimulus was chosen because the evoked response triggered with 20 mM KCl in retinal synaptosomes was weak.

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4.9 Western blot analysis

The protein concentration of each sample was determined by the BCA protein assay (Pierce Biotechnology, Rockford, IL, USA). The samples were denaturated by adding 6x concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M DTT, 0.012% bromophenol blue) and heating for 5 min at 95ºC. Equal amounts of protein were loaded into the gel and proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 4%-8% gels. Then, proteins were transferred electrophoretically to PVDF membranes (Millipore, Billerica, Massachusetts, USA). The membranes were blocked for 1 h at room temperature, in Tris-buffered saline (137 mM NaCl, 20 mM Tris-HCl, pH 7.6) containing 0.1% Tween-20 (TBS-T) and 5% low-fat milk. The membranes were incubated with the primary antibody VGluT-1 1:10,000 and VGluT-2 1:5,000 (Sigma Aldrich, St.Louis, MO, USA); VGAT (1:2,000 from Synaptic Systems, Goettingen, Germany) and α_{1A} P/Q Type calcium channel (1:200 from Alomone Labs, Jerusalem, Israel) overnight at 4ºC. After washing for 1 h in TBS-T with 0.5% low-fat milk, the membranes were incubated for 1 h at room temperature with the respective alkaline phosphatase-linked secondary antibody (1:20,000; GE Healthcare, Buckinghamshire, UK), prepared in TBS-T with 1% low-fat milk. The membranes were processed for protein detection using the Enhanced Chemi-Fluorescence system (ECF; GE Healthcare, Buckinghamshire, UK) and a Storm device (Molecular Dynamics, GE Healthcare, Buckinghamshire, UK). Digital quantification of bands intensity was performed using ImageQuant 5.0 software (Molecular Dynamics, Inc., Sunnyvale. CA, USA). The membranes were then reprobed and tested for β-actin (1:20,000; Sigma, St.Louis, MO, USA) immunoreactivity to prove that similar amounts of protein were applied in the gels.

4.10 Radioactivity assay and calculations

The radioactivity released from the synaptosomal preparations was measured with a Packard 2900 Tricarb (Canberra, Australia) liquid scintillation spectrometer, equipped with Dynamic Color Corrected DPM Option providing absolute activity (disintegrations per minute, DPM) calculation and correction for different color quenching. The release of the transmitters was calculated as the percentage of the amount of radioactivity in the tissue at the sample collection time point. We expressed the radioactivity value from each sample (perfusate) as the % of the filter content at the period of time corresponding to the release of each sample, which is called as fractional release (FR%).

4.11 Statistical Analysis

Results are presented as mean ± SEM. Statistical comparisons between diabetic animals and respective age-matched controls were performed using the unpaired Student's *t*-test. Differences were considered significant for $p<0.05$.

Acknowledgments

This work was supported by Faculty of Medicine, University of Coimbra, Portugal (GAPI 07/09). Joana M. Gaspar and Filipa I. Baptista acknowledge a fellowship from Fundação para a Ciência e a Tecnologia, Portugal (SFRH/BD/32949/2006 and SFRH/BD/35961/2007, respectively).

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Figure 1: Diabetes does not affect the protein content of vesicular glutamate transporters in the hippocampus. The protein levels of VGluT-1 and VGluT-2 were analyzed by immunoblotting in extracts of hippocampal synaptosomes (A and B) and in hippocampal total extracts (C and D) isolated from control and STZ-induced diabetic animals. Representative Western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded into the gel. The results are expressed as percentage of age-matched controls, and data are presented as the mean ± SEM of at least 5 animals.

Figure 2: Diabetes induces changes in the protein content of vesicular glutamate transporters in the retina. The protein levels of VGluT-1 and VGluT-2 were analyzed by immunoblotting in extracts of retinal synaptosomes (A and B) and in retinal total extracts (C and D) isolated from control and STZ-induced diabetic animals. Representative Western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded into the gel. The results are expressed as percentage of age-matched controls, and data are presented as the mean ± SEM of at least 5 animals. * $p < 0.05$, compared to age-matched control animals.

Figure 3: Diabetes induces changes in the protein content of vesicular GABA transporter in hippocampal and retinal synaptosomes. The protein levels of VGAT were analyzed by immunoblotting in hippocampal (A) and retinal (B) synaptosomes,

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and also in hippocampal (C) and retinal (D) total extracts, from diabetic animals and aged-matched controls. Representative western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded in the gel. The results are expressed as percentage of age-matched controls, and data are presented as the mean ± SEM of at least 4 animals. * $p < 0.05$, *** $p < 0.001$, compared to age-matched control animals.

Figure 4: The content of α_{1A} subunit of P/Q type calcium channels decreases at two weeks of diabetes in hippocampal and retinal synaptosomes. The protein levels of α_{1A} P/Q type calcium channels were analyzed by immunoblotting in hippocampal (A) and retinal (B) synaptosomes, and also in hippocampal (C) and retinal (D) total extracts, from diabetic animals and aged-matched controls. Representative western blots are presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein from control and diabetic samples were loaded in the gel. The results are expressed as percentage of age-matched controls, and data are presented as the mean ± SEM of at least 4 animals. * $p < 0.05$, ** $p < 0.01$, compared to age-matched control animals.

Figure 5: Diabetes increases the basal release of glutamate after eight weeks of diabetes. Synaptosomes were simultaneously loaded with [^{14}C]glutamate (besides [^{3}H]GABA), and after 15 min of washout, 2 min perfusate samples were collected and radioactivity counted. Results are expressed as fractional release % (FR%). Synaptosomes were stimulated twice (S1 and S2) with 20 mM KCl for 30 sec each. (A) Glutamate release at two weeks of diabetes; (B) Glutamate release at eight weeks of

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diabetes; (C-D) S1 and S2 at two and eight weeks time points for diabetic and age-matched control animals; (E) S2/S1 ratio at two and eight weeks time points for diabetic and age-matched control animals. All data points represent the mean ± SEM of at least 8 animals; * $p < 0.05$, compared to age-matched control animals.

Figure 6: Diabetes does not affect GABA release from hippocampal synaptosomes. Results were obtained from the same experiments shown in Figure 5. Synaptosomes were simultaneously loaded with [^{3}H]GABA (besides [^{14}C]glutamate), and after 15 min of washout, 2 min perfusate samples were collected and radioactivity counted. Results are expressed as fractional release % (FR%). Synaptosomes were stimulated twice (S1 and S2) with 20 mM KCl for 30 sec each. (A) GABA release at two weeks of diabetes; (B) GABA release at eight weeks of diabetes; (C-D) S1 and S2 at two and eight weeks time points for diabetic and age-matched control animals; (E) S2/S1 ratio at two and eight weeks time points for diabetic and age-matched control animals. All data points represent the mean ± SEM of at least 8 animals.

Figure 7: Diabetes affects the release of GABA in retinal synaptosomes. Synaptosomes were loaded with [^{3}H]GABA, and after 15 min of washout, 2 min perfusate samples were collected and radioactivity counted. Results are expressed as fractional release % (FR%). Synaptosomes were stimulated twice (S1 and S2) with 30 mM KCl for 1 min each. (A) GABA release at two weeks of diabetes; (B) GABA release at eight weeks of diabetes; (C-D) S1 and S2 at two and eight weeks time points for diabetic and age-matched control animals; (E) S2/S1 ratio at two and eight weeks time points for diabetic and age-matched control animals. All data points represent the mean ± SEM of 11 animals. * $p < 0.05$ compared to age-matched control animals.

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Table 1: Average weight and blood glucose levels of diabetic and aged-matched control rats.

Diabetes duration		Weight (g)	Blood Glucose (mg/dL)
2 Weeks	Control (n=15)	312.3±13.6	97.0±6.6
	Diabetic (n=15)	240.1±8.2***	467.8±26.9***
8 Weeks	Control (n=15)	401.1±8.1	94.1±2.7
	Diabetic (n=15)	278.5±7.9***	431.0±19.9***

Measurements were made immediately before the sacrifice of the animals. ***p <0.001

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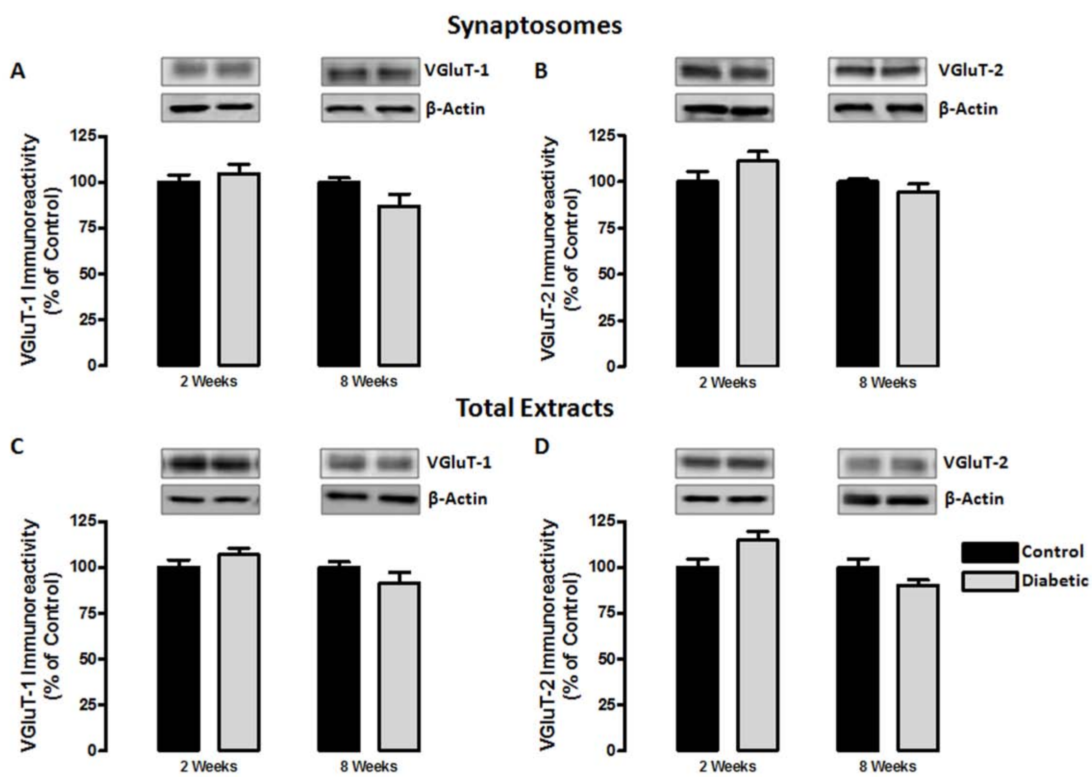

Figure 1

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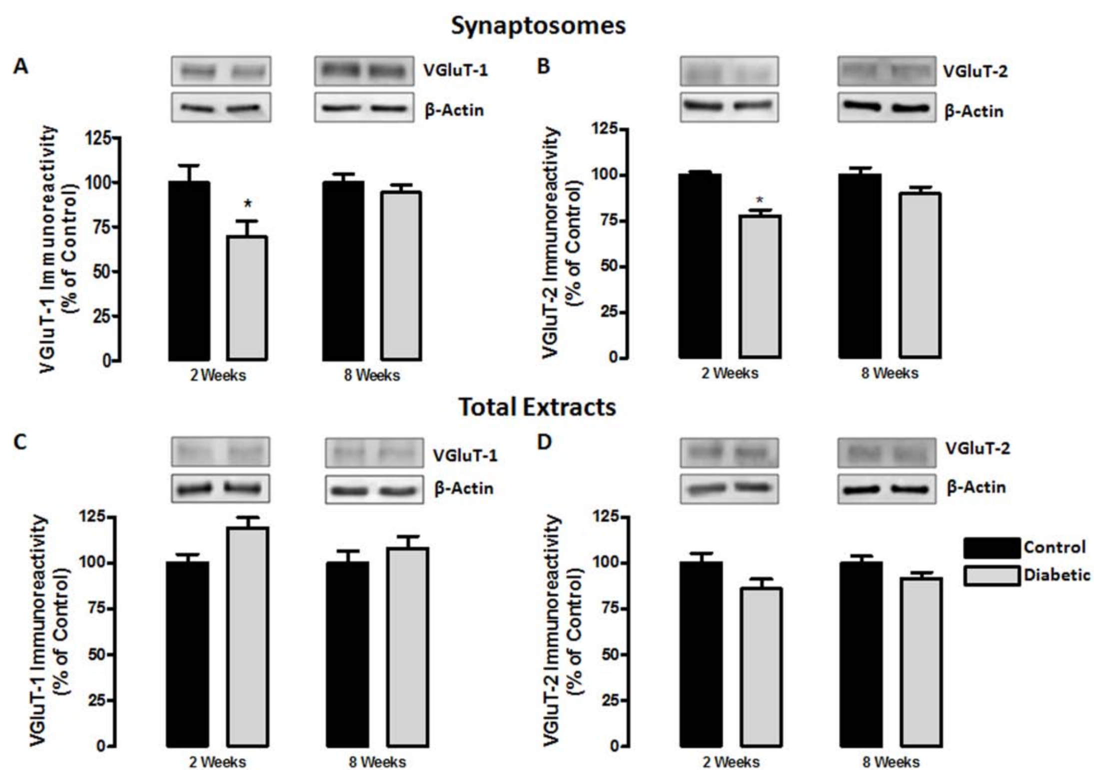

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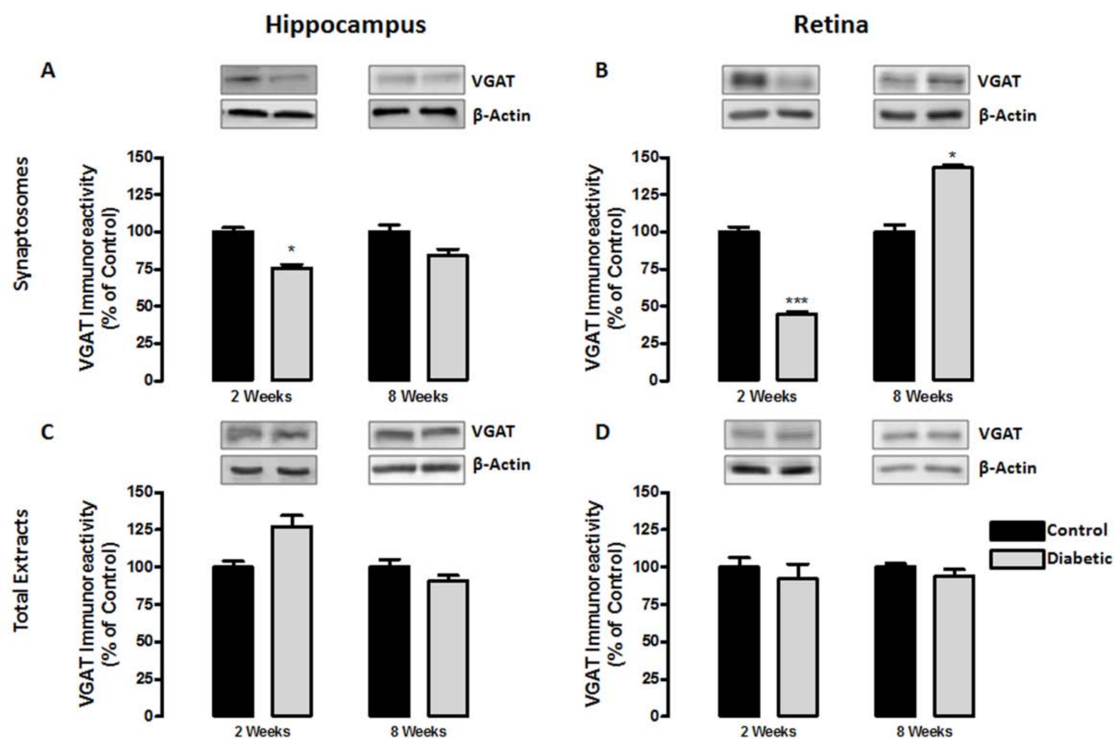

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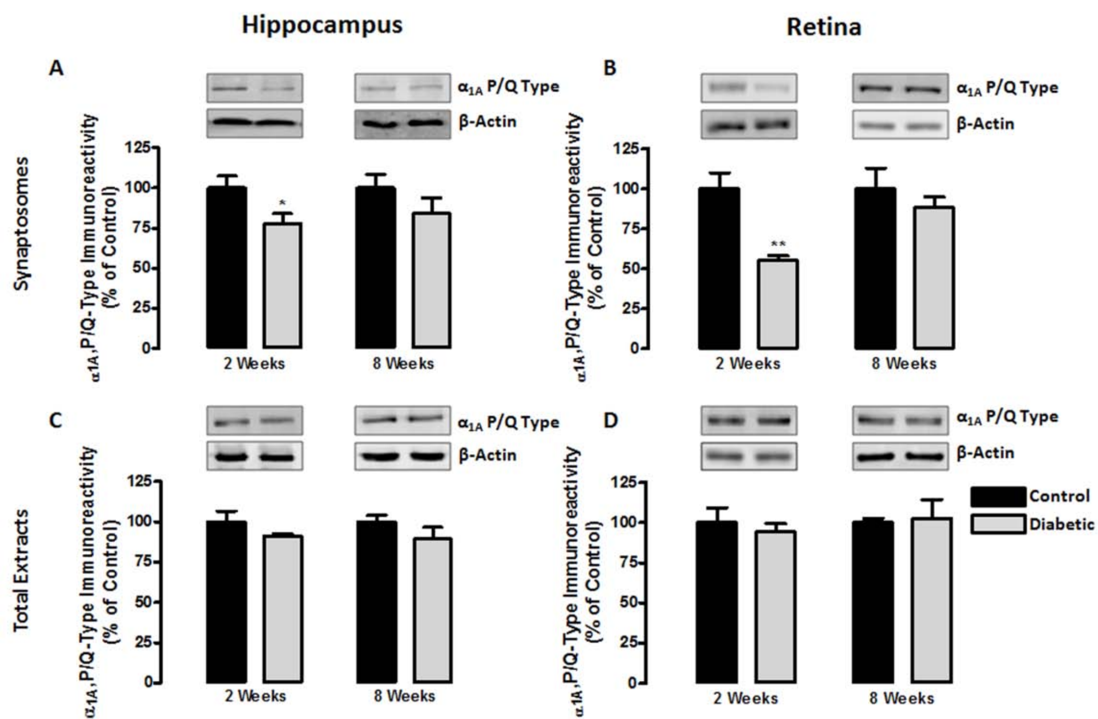

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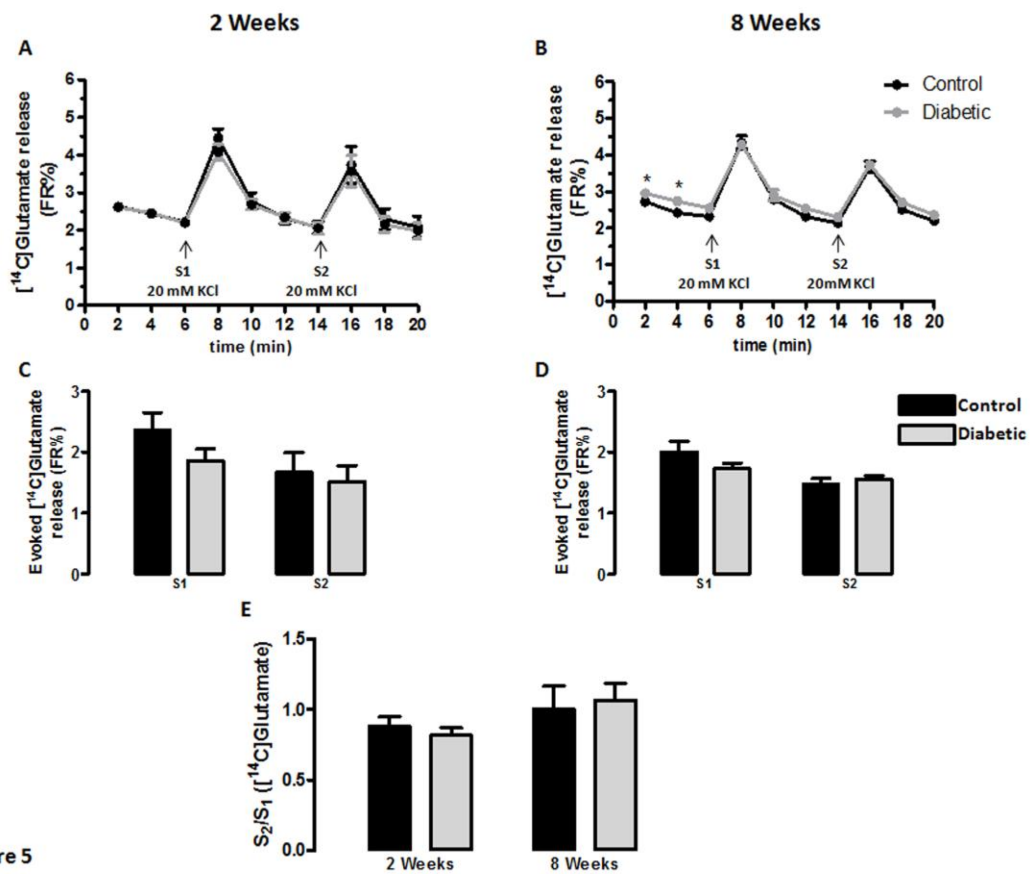

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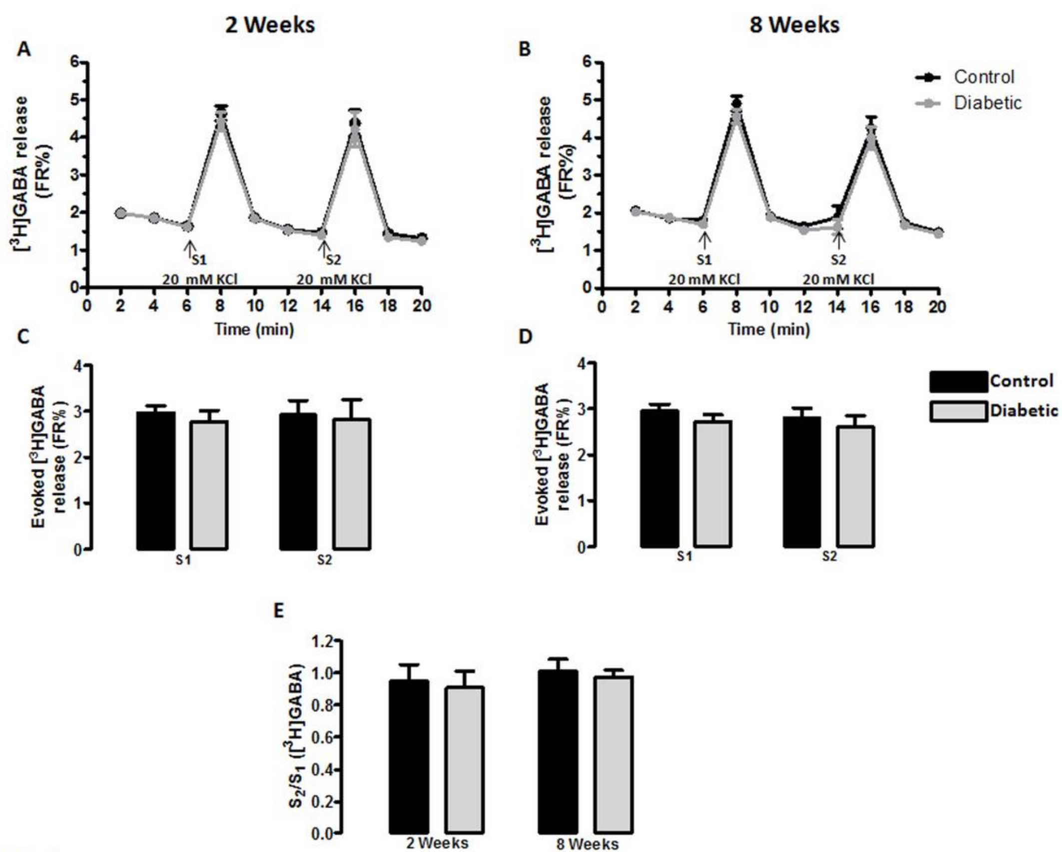

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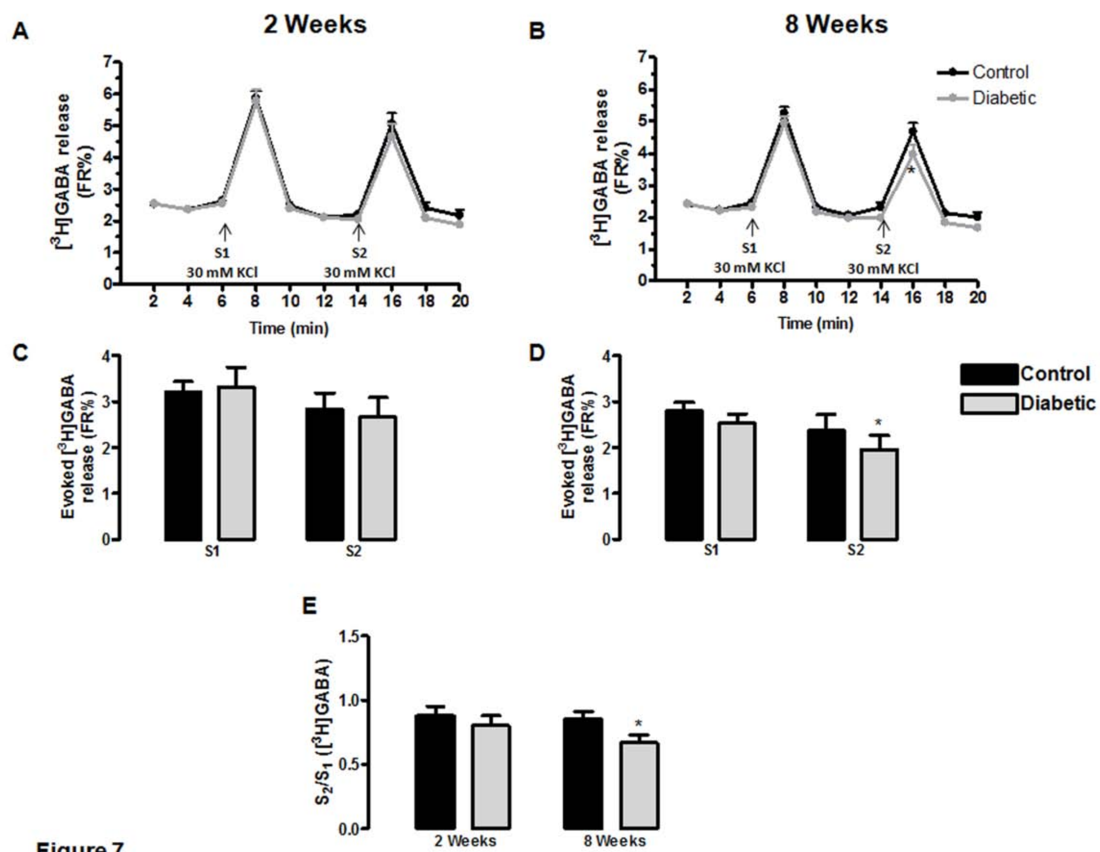

Figure 7