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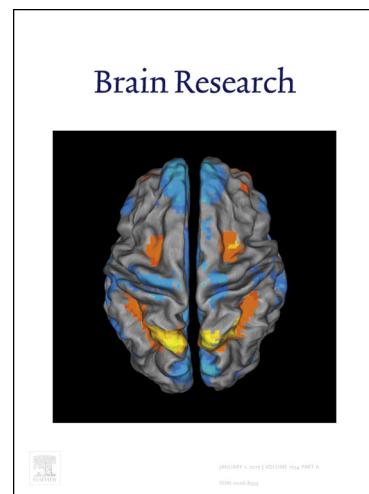Please cite this article as: L. Navigatore-Fonzo, A. Castro, V. Pignataro, M. Casais, M. Garraza, A.C. Anzulovich, Daily rhythms of cognition-related factors are modified in an experimental model of alzheimer disease, *Brain Research* (2017), doi: http://dx.doi.org/10.1016/j.brainres.2017.01.033

DAILY RHYTHMS OF COGNITION-RELATED FACTORS
ARE MODIFIED IN AN EXPERIMENTAL MODEL OF ALZHEIMER DISEASE

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ABSTRACT

The accumulation of amyloid-β (Aβ) peptides in the brain of Alzheimer disease patients is associated to cognitive deficit, increased oxidative stress, and alterations in the circadian rhythms. Brain-derived neurotrophic factor (BDNF) and Neurogranin (RC3), play an important role in the synaptic plasticity underlying memory and learning. Previously, we observed BDNF and RC3 expression follow a daily rhythmic pattern in the hippocampus of young rats. The objective of this study was to investigate the effects of an intracerebroventricular (i.c.v.) injection of aggregated β-amyloid peptide (1-42) on temporal patterns of ApoE protein, Bdnf and Rc3 mRNA, lipid peroxidation (LPO) and reduced glutathione (GSH) levels, in the rat hippocampus. We observed an i.c.v. injection of Aβ aggregates phase shifts daily BDNF and RC3 expression as well as LPO and decreased the mesor of GSH rhythms. ApoE protein levels vary rhythmically throughout the day. ApoE levels increase at ZT 03:39±00:22 in the hippocampus of control rats and at ZT 06:30±00:28 in the treated animals. Thus, elevated levels of Aβ aggregates, characteristic of AD, altered temporal patterns of cognition related-factors, probably, as a consequence of changes in the daily variation of ApoE-mediated Aβ aggregates clearance as well as in the 24h rhythms of the cellular redox state.

Key Words: Alzheimer disease, ApoE, GSH, BDNF, lipid peroxidation, daily rhythm.

1. Introduction

The increase in the life expectancy average leads to an increment in the incidence of diseases linked to old age, such as Alzheimer disease (AD). Nowadays, about 33.9 million people worldwide suffer of AD and this number will double every twenty years reaching almost 115,4 million cases by 2050 (WHO, 2012).

Alzheimer disease is characterized by a number of neuropathological changes including extracellular senile plaques, intracellular neurofibrillary tangles, synaptic dysfunction and neuronal death, particularly, in the cortex and hippocampus (Querfurth et al., 2010). Senile plaques are composed of extracellular insoluble aggregates of β-amyloid peptide (Aß) generated by enzymatic cleavages of the amyloid precursor protein (APP) by β- and γ-secretases (Selkoe 2001; Mattson 2004).

ApoE plays a key role in normal and physiological clearance of Aß, since it facilitates the peptide intra- and extracellular proteolytic degradation (Jiang et al., 2008). On the other hand, absence of ApoE has been correlated with diminished antioxidant capacity in animals (Law et al., 2003). Particularly, Honma et al (2013) observed that old ApoE-deficient mice show increased oxidative stress and reduced antioxidant capacity, in plasma and liver.

Oxidative damage plays an important role in the pathogenesis of AD (Barnham et al., 2004; Sayre et al., 2005). In vitro studies showed that incubation of 9–11-day-old primary hippocampal neuronal cultures with Aβ (1–42) leads to increased oxidative stress markers and neurotoxicity (Yatin et al., 1999; Boyd-Kimball et al., 2004). The Aβ(1–42) oligomers can insert into the lipid bilayer and initiate lipid peroxidation (LPO), followed by oxidative damage to proteins and other biomolecules (Butterfield et al., 2001).

Indeed, recent investigations revealed increased levels of lipid peroxidation and protein carbonylation, as well as decreased reduced glutathione (GSH) levels in the brain of an AD transgenic model (Subash et al., 2014). Numerous studies have shown that alterations in the antioxidant defense system produce learning and memory deficits as well as loss of hippocampal long-term potentiation (LTP), similar to that reported in patients with Alzheimer disease (Wu et al., 2004; Watson et al., 2006).

The brain-derived neurotrophic factor (BDNF), via its TrkB receptor, acts as a regulator of synaptic plasticity underlying memory and learning, in the hippocampus and cerebral cortex (Bekinschtein et al., 2007).

Abbreviations ANOVA= analysis of variance Bdnf=brain-derived neurothrophic factor ZT= zeitgeber times LTP=long-term potentiation APP=amyloid precursor protein LPO= lipid peroxidation GSH= reduced glutathione TBARS=thiobarbituric acid reactive substances MDA= malondialdehyde

Other molecular marker of neural plasticity associated to learning and memory is the neuron specific PKC substrate: neurogranin or RC3 (Pak et al., 2000; Husson et al., 2004; Zhabotinsky y col., 2006). Some investigators have reported reduced levels of RC3, BDNF and TrkB in AD patients, probably, underlying the characteristic cognitive deficits of AD (Ferrer et al., 1999; Peng et al., 2005; Kemppainen et al., 2012). Previously, we reported BDNF and RC3 expression oscillates on a daily basis in the hippocampus of young adult rats, providing a molecular basis for temporal occurrence of synaptic plasticity (Golini et al., 2012)

In addition to cognitive impairment, AD is characterized by circadian rhythm alterations. Examples of them include disturbances in thermoregulation, sleep-wake cycles and hormonal rhythms such as those of glucocorticoids levels (Van Someren et al., 1996; Giubilei et al., 2001; Harper et al., 2005; Bhatt et al, 2005). The desynchronization of circadian rhythms occurs in the most severe stages of the AD (Hatfield et al., 2004).

Living beings count on an internal timing mechanism (circadian system) which is entrained by external environmental factors (the main is the light-dark cycle) and generates endogenous rhythms with a period close to 24 hours (Franken et al., 2009; Coogan et al., 2011). The cellular and molecular basis of circadian rhythmicity consist of a complex interaction between several transcription factors including the heterodimers BMAL1:CLOCK and PER:CRY (Panda and Hogenesch, 2004). Some studies have reported a relationship between the circadian clock activity and the cellular redox state (Rutter et al., 2001; Hirayama et al., 2007). We and others have demonstrated 24-h patterns of lipid peroxidation and GSH levels in different brain areas. These could be, at least in part, responsible for the circadian oscillation of the cellular redox state (Baydas et al., 2002; Fanjul-Moles et al., 2009; Fonzo et al., 2009; Navigatore Fonzo et al., 2014). Additionally, it has been shown that the circadian system modulates the pathways involved in the production and utilization of GSH (Beaver et al., 2012).

Even though all above observations, to date, there is no report on temporal organization of cognition-related factors and the cellular redox state in the brain of AD patients or animal models. Thus, we aimed to study the consequences of an i.c.v. injection of aggregated β-amyloid peptide (1-42) on temporal patterns of BDNF, RC3, ApoE, lipid peroxidation and GSH levels in the rat hippocampus.

2.Results

2.1 Aβ protein levels in the hippocampus of i.c.v. injected rats

First of all, to test whether an i.c.v. injection of Aβ (1-42) aggregates increases the levels of amyloid beta peptide in the rat hippocampus, we analyzed Aβ protein levels, before and after the surgical intervention. As expected, we observed Aβ protein contents increased in the hippocampus of i.c.v. injected rats (p<0.05; see Figure 1A). After that, we studied the temporal patterns of Aβ peptide expression and observed Aβ levels vary significantly throughout a 24-h period in that brain area, with maximal Aβ levels occurring at ZT 08:24 ± 00:39 in the control group (p<0.05). Interestingly, the injection of Aβ (1-42) aggregates into the lateral ventricle did not eliminate the daily rhythmicity (acrophases: 07:09 ± 00:17, p<0.001, see Table 1 and Figure 1 A-C).

2.2 Apo E protein levels in the hippocampus of control and Aβ-injected rats in a period of 24 hours

As shown in the Figure 2 and Table 2, our results revealed that Apo E protein levels exhibit a daily variation in the hippocampus of control and Aβ-injected rats (p<0.001 and p<0.001, respectively). Apo E maximal levels occur at ZT 03:39 ± 00:22 in the control rats. The Aβ-injected group showed a phase delay in Apo E rhythm's, from ZT 03:39 ± 00:22 to ZT 06:30 ± 00:28 (p<0.01) and an increase in the mesor of rhythms (0.76 ± 0.05 vs 0.94 ± 0.02, p< 0.01) compared with controls.

2.3 Daily Bdnf and Rc3 expression in the rat hippocampus of control and Aβ-injected rats

When we analyzed the temporal expression of Bdnf, we found it exhibits a robust daily rhythmicity in the hippocampus of control rats (p <0.01) with the highest level occurring at ZT 03:27± 00:51 (p <0.001) (see Figure 3 and Table 3). Injection of Aβ aggregates (1-42) phase shifted daily patterns of Bdnf mRNA levels (from ZT 03:27± 00:51 to ZT 15:58± 00:23, p <0.01). On the other hand, we observed also Rc3 mRNA expression displays a 24-h rhythm in the rat hippocampus (p<0.05). Daily Rc3 mRNA levels peak at ZT 17:24±00:26 in the control rats. In this case, Aß peptide aggregates caused a phase shift specifically, an advance of the rhythm's acrophase (from ZT 17:24± 00:26 to ZT 11:15± 00:36, p <0.001) and reduced the Rc3 expression rhythm's mesor (0.69 ± 0.01 vs 0.51 ± 0.01, p <0.01).

2.4 Daily patterns of lipoperoxidation in the hippocampus of Aβ-injected rats

4

As shown in the Figure 4 and Table 2, our results revealed that MDA levels follow a robust diurnal rhythm in the hippocampus of control rats (p<0.01) peaking towards the end of the night (ZT 19:48 ± 00:54). The i.c.v. injection of Aβ aggregates phase-shifted daily rhythm of MDA (ZT 19:48 ± 00:54 to ZT 10:16 ZT ± 01:42 (p<0.001) and increased the rhythm's mesor (1.65 ± 0.05 vs 2.17 ± 0.09, p<0.01) in the rat hippocampus.

2.5 Reduced glutathione expression levels in the hippocampus of control and Aβ-injected rats in a period of 24 hours

We next analyzed whether GSH levels vary daily in the hippocampus of control and Aβ-injected rats (see Figure 5 and Table 4). Our results revealed that GSH levels oscillate rhythmically in this brain area (p <0.05), with a peak occurring at ZT 14:33 ± 00:33 (p<0.001). The i.c.v. injection of Aß aggregates not modified the rhythm's acrophase although decreased the mesor (3.32 ± 0.13 vs 2.79 ± 0.04) and increased the amplitude (0.41 ± 0.10 vs 1.10 ± 0.00) in the rat hippocampus

3. Discussion

In this study, we observed that an i.c.v. injection of Aβ aggregates phase shifts daily Bdnf and Rc3 expression as well as ApoE, LPO and decreased the mesor of GSH rhythms in the rat hippocampus. First, we proceeded to measure the relative levels of beta amyloid peptide in the rat hippocampus; as expected, Aß levels were significantly higher in the hippocampus of i.c.v. Aβ-injected rats in comparison to controls (p <0.05; Figure 1A-C). We investigated whether Aβ protein levels, showed a day-night oscillation in the rat hippocampus. Interestingly, we found Aβ protein levels displays a daily rhythmicity in that brain area, with Aβ levels peaking at the middle of the light period in control rats (Figure 1A-C and Table 1). Rhythms of Aβ protein have also been observed by Roh et al., (2012) and Lucey and Bateman (2014) in animal models. Interestingly, we show Aβ proteins oscillate in a daily pattern in the hippocampus of Aβ-injected rats, peaking at the half of the day, during the rest period in rats (Figure 1-C and Table 1). This last observation differs from that reported by Kang and collaborators (2009) who found that Aβ levels are higher in the interstitial fluid of APP transgenic mice during wakefulness, suggesting brain region-specific differences and in the animal model. Our findings led us to question whether the expression of Aβ as its clearance moves in time to the cellular clock in the hippocampus.

It is known ApoE modulates Aβ deposition and clearance (Jiang et al., 2008; Kanekiyo et al., 2014). Here, we observed Apo E protein levels vary throughout a 24h period, peaking at the

beginning of the day (p<0.01, Figure 2) in the control group. This is consistent with Shen and collaborators (2009) who demonstrated that Apo E protein oscillates on a daily basis in the rat hypothalamus. Interestingly, in the present study, we found, for the first time in our knowledge, that Aß peptide aggregates modify daily rhythmicity of Apo E protein in the rat hippocampus. The Apo E rhythm's acrophase was shifted at middle of the day and mesor was increased in the Aβ-injected animals (Figure 2, Table 2). Even though, to date, we did not find studies on the consequences of an i.c.v injection of aggregated Aβ (1-42) on daily rhythms of Apo E protein, some studies have shown that Apo E levels are increased by amyloid beta-protein in astrocytes (LaDu et al., 2000; Igbavboa et al.,2003). These observations suggested that such increase in Apo E levels may afford neuroprotection by reducing the inflammatory response against Aβ1-42 (Hu et al., 1998; LaDu et al., 2001). In our study, the increase of Apo E rhythm`s mesor in the hippocampus of Aβ-injected rats, might have a neuroprotective effect in the context of predictive homeostasis.

In addition to their effects on the Aβ clearance, Apo E isoforms affect synaptic plasticity in an isoform-dependent manner. Recent studies have shown that there is a strong association between Apo E and BDNF expression (Maioli et al., 2012; Alvarez et al., 2014; Neumann et al., 2015). The neurotrophin BDNF is vital for learning and memory and play an important role in the etiology of AD (Siegel and Chauhan, 2000; Murer et al., 2001; Fahnestock, 2001). Here, we found daily rhythms of Bdnf in the hippocampus of control rats (ZT 03:27± 00:51), coinciding with those previously reported by us and others (Berchtold et al., 1999; Pollock et al., 2001; Golini et al., 2012; Ikeno et al, 2013). Thus, we expect hippocampal BDNF-related synaptic plasticity to be maximal during the day (rest period for nocturnal rats) participating in memory-related processes during sleep (Figure 3A and Table 3). In addition, we also found that Rc3 gene displays a rhythmic expression in the hippocampus of the control group, with maximal mRNA levels occurring at the middle of the night (ZT 17:24 ± 00:26), similar to the observed by Golini et al. (2012) (Figure 3B and Table 3). Synaptic dysfunction is a pathologic feature of AD that precedes neuronal loss in several brain regions. Numerous studies indicate that neurogranin immunoreactivity is reduced in patients with early symptomatic AD, compared with controls (Davidsson et al., 1998; Masliah et al., 2001; Reddy et al., 2005). Moreover, in Alzheimer's disease, the Bdnf expression level is reduced in different brain areas (Phillips et al., 1991; Ferrer et al., 1999). Interestingly, we observed that injection of Aβ aggregates (1-42) exerts differential effects on the daily expression of Bdnf and Rc3. On one hand, caused a phase shift in daily oscillations of Bdnf transcript levels compared to the control group, peaking around the middle of the night in the hippocampus (Figure 3A and Table 3). We also found that Aβ protein peak

6

concurs with nadir in the oscillating Bdnf expression during the rest period in rats. Thus, knowing that the sleep plays a fundamental role in brain plasticity, memory, and learning (Diekelmann and Born, 2010), where the availability of BDNF, is crucial for synaptic plasticity (Kang and Schuman, 1995), a low level of this neurotrophin during sleep could affect learning and memory-related processes. On the other hand, i.c.v. Aβ aggregates phase-advanced day-night fluctuations of Rc3 mRNA, and reduced their mesor (Figure 3 A-B and Table 3). Although others have demonstrated the effects of Aβ peptide on protein and mRNA levels of Bdnf and Rc3 in vivo and in vitro models of Alzheimer's disease (Doi et al., 2013; George et al., 2010; Fukumoto et al., 2014), this would be, at least at our knowledge, the first published report on the effects of an i.c.v injection of aggregated Aβ (1-42) on the daily rhythmicity of Bdnf and Rc3 expression and its putative impact on temporal patterns of synaptic plasticity in the hippocampus.

Numerous studies suggest that brain tissues of AD patients are exposed to oxidative stress during the development of the disease (Hensley y col., 1995; Wang y col., 2005; Lovell y col., 2011; Bradley y col., 2012). Additionally, it has been shown that oligomers of Aβ are the principal toxic substances which induce oxidative stress, cognitive dysfunction and impairing memory (Lesne et al., 2006; Bulbarelli et al., 2009). In the present study, we observed, for the first time in our knowledge, that an i.c.v injection of aggregated Aβ (1-42) modifies daily rhythmicity of oxidative stress-related parameters. Zafrilla et al (2006) have reported that analysis of AD brains demonstrates an increase in lipid peroxidation products compared with age-matched controls. On the other hand, investigations made by Yamada et al (1999) and Cioanca et al (2014) have shown that an injection of Aβ(1–42) causes oxidative stress in the rat brain. In our study, we investigated whether lipoperoxidation displays a daily rhythmicity in the hippocampal homogenates of control and Aβ-injected rats maintained under 12h-light:12h-dark conditions. Interestingly, our results reported that TBARS levels showed a daily oscillation in the hippocampus of control rats (p <0.001), with a peak of MDA levels towards the end of the night (Figure 4 and Table 2). Thus, this peak in the level of lipid peroxides, could be explained as a consequence of an increase in free radicals during the active phase of nocturnal rodents, as seen by Baydas et al. (2002) in rat cerebral tissue. These observations are also consistent with temporal patterns of lipoperoxidation reported by others and us in the brain (Subramanian et al. 2008; Pandi-Perumal et al. 2008 and Fonzo et al 2009). Interestingly, we found that an i.c.v injection of Aβ aggregates caused a phase shift, and increase the rhythm's mesor of TBARS levels in the rat hippocampus. (Figure 4 and Table 2). As expected, we observed MDA peak (ZT 10:16 ± 01:42) follows maximal levels of Aβ protein (ZT 07:09 ± 00:17) in the hippocampus of Aβ-injected rats. This is consistent with results obtained by Yatin et al. (1999) and Pratico et al.

(2001). The first group demonstrated that Aβ promoting ROS production and lipid peroxidation in neuronal culture, whereas Pratico's group in an animal model of Alzheimer amyloidosis. Although recent research by Sharma et al (2016) has shown that a single intracerebroventricular (icv) injection of protofibrillar Aβ 1-42 induced lipid peroxidation, in hippocampus, cortex, and striatum regions of rat brain, this would be, the first published report on the effects of amyloid aggregates via i.c.v on temporal patterns of lipoperoxidation.

In this study, we also determined the levels of reduced GSH, a key component in the cascade of antioxidant defense against oxidative stress. We found that GSH levels oscillate rhythmically in the hippocampus of control rat (p <0.001). GSH levels are higher at the beginning of the night in control animals, when it would generate a more reduced redox environment during the animal activity period (Figure 5 and Table 4). This coincides with that observed by us and other groups in different tissues of rodents maintained under light: dark cycles (Manikonda et al., 2012; Ponce et al., 2012; Xu et al., 2012). Investigations by Puertas et al (2012) observed reduced plasma levels of GSH in Alzheimer's patients. Interestingly, we observed that i.c.v. injection of Aß aggregates does not modify the phase of GSH rhythmicity but decreased the mesor and increased the rhythm's amplitude in the rat hippocampus (Figure 5 and Table 4). Interestingly, we also found that Aβ protein peak concurs with the lowest level of GSH during the rest period in rats, which could explain, at least in part, the alterations in memory-related processes during sleep. Our results are supported by Abramov et al.(2003), whose findings suggest that Aβ might initiate a cascade of events resulting in a severe depletion of GSH. They are also consistent with those observed by Also Bermejo et al. (2008) and Cristalli et al. (2012) who show in subjects with Alzheimer type dementia (ATD) a decrease in total GSH content in plasma. Thus the antioxidant defense system could be unable to respond to the oxidative stress generated by the amyloid aggregates, stimulating the appearance of cognitive disorders characteristic of the disease.

In conclusion, the results presented here show that the expression of memory-and learning related genes (Bdnf and Rc3) is rhythmic in the rat hippocampus. Particularly, Bdnf expression is in phase with daily patterns of Apo E, however, such temporal organization is altered by an i.c.v. injection of Aβ aggregates. In our model, an i.c.v. injection of Aβ aggregates also reduced the mesor, and changed the phase of lipoperoxidation and modified mesor and amplitude of GSH rhythm in the rat hippocampus. These alterations in the daily rhythmicity of markers of oxidative stress might be responsible for changes in the temporal organization of the redox state in this brain area.

Given the relevant role of oxidative stress and the consequent alterations in expression of factors related to cognition and clearance of Aβ on the pathophysiology of the disease Alzheimer. We would expect emerging data from these and future studies will also highlight Apo E, cognition-related factors, redox signaling pathways as potential novel therapeutic targets for circadian rhythms disorders.

4. Experimental Procedure

4.1 Preparation of aggregated Aβ (1-42)

Lyophilized synthetic Aβ(1–42) (Sigma-Aldrich, St Louis, MO, USA) was dissolved in sterile saline solution at a concentration of 2 g/L and stored at –20°C. To obtain the neurotoxic (aggregated) form of Aβ1-42, the peptide solution was placed in an incubator at 37°C for 7 consecutive days following Zhang et al. (2013).

4.2 Animal Model

Male Holtzman rats bred in our animal facility (National University of San Luis, Argentina) were housed in cages (four/cage) with water and food *ad libitum,* under a 21–23°C controlled environment and 12h-light:12h-dark conditions, from weaning and during the whole experimental period. Four-month old rats were randomly assigned to the groups: control (CO) and Aβ-injected (Aβ) (n=16/group). First group received an i.c.v injection of sterile saline solution and the second group received an i.c.v injection of aggregated Aβ (1-42) (10 μg / 5 μl). Sterile saline solution was used as control, instead of Aβ peptides, since even nontoxic Aβ derivatives, such as the scrambled Aβ, usually employed as control in *in vivo* models, may themselves produce free radicals (Hensley et al., 1994). On the day of surgery, animals were anesthetized with an intraperitoneal injection of 0.2 ml of a mixture of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg). They were then stereotaxically injected into the lateral ventricles (at coordinates AP:-1 mm, L: 1.5 mm, and DV: -3.5 mm according to Paxinos and Watson, 1997) and immediately taken to individual cages. Seven days after surgery, four rats were euthanized by decapitation every 6 hours during a 24-hour period, at the *zeitgeber* (from German *zeit*: time and *geber*: giver) times (ZT) ZT2, ZT8, ZT14 and ZT20, (ZT0 = lights on at 07:00, ZT12 = lights off).Rats were killed under dim red light at ZT14 and ZT20 to avoid acute effects of light. All experiments were carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and the National University of San Luis Committee' s Guidelines for the Care and Use of Experimental Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

4.3 Hippocampus dissection

Hippocampus samples were isolated every 6 hours starting at ZT2 from CO and Aβ groups. Hippocampi isolation was carried out as described in Babu et al. (2011). Briefly, following animal decapitation, the head was recovered and the skull was opened with sterile scissors. Brain was carefully removed, quickly washed in ice-cold sterile saline solution and put on an ice-chilled plate. Immediately, it was cut along the longitudinal fissure to divide both hemispheres. The diencephalon was removed with sterile microsurgical forceps and scissors and the exposed hippocampus was resected from the neocortex and immediately placed in liquid nitrogen.

4.4 Immunobloting assays

Protein extracts were prepared from hippocampus samples isolated from CO and Aβ rats, at every time point, ZT2, ZT8, ZT14 and ZT20, by homogenizing 50 mg in 1/5 (w/v) dilution of 120 mM KCl, 30 mM phosphate buffer, 1 µg/ml leupeptin and 1 µg/ml of pepstatin pH 7.2 at 4°C. Suspensions were centrifuged at $800 \times g$ for 10 minutes at 4°C to remove nuclei and cell debris. The pellets were discarded and supernatants were used to determine Aβ and Apo E protein contents. Total protein concentration was measured by the Lowry method (Lowry et al., 1951), using bovine serum albumin as standard. Aliquots containing 25 µg of total protein were subjected to electrophoresis in 12% polyacrylamide gels, and then transferred to Immobilon-PTM transfer membranes (Millipore, Bedford, MA). Immunoblot analyses were performed as described in the manufacturers' protocols for the detecting antibodies. Membranes were blocked in Blotto (5% nonfat dry milk, 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) overnight at 4°C in constant shaking. To reveal the specific bands, membranes were incubated overnight at 4°C with either rabbit anti-Aβ peptide, anti-Apo E or anti-ACTIN antibodies (Abcam, USA), diluted 1:8000 in Blotto containing 0.05% thimerosal. After incubation with primary antibody, the membranes were washed in TBS (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) containing 0.05% Tween-20, before incubation with goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:5,000 in Blotto, for 2 hours at room temperature. After washing, antibody/protein complexes were detected using a chemiluminescence method (Embiotec, Argentina) following the manufacturer's indications. The mean of intensity of each band was measured using the NIH ImageJ software (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/) and the relative abundance of each band was normalized to ACTIN, calculated as the ratio of the mean of gray value of amyloid beta peptide and Apo-E band to that of actin.

4.5 Lipid peroxidation measurement

Hippocampal tissue homogenates obtained as in 2.4, were used to determine daily variation of lipoperoxidation in the hippocampus of CO and Aβ groups. Lipid peroxidation was quantified spectrophotometrically by measuring MDA (malondialdehyde) levels as thiobarbituric acid reactive substances (TBARS) according to Draper and Hadley (1990). All reagents were from Sigma-Aldrich (St Louis, MO, USA).

4.6 Reduced glutathione determination

GSH levels were determined in hippocampus samples isolated from CO and Aβ rats, following Akerboom and Sies (1981). Briefly, GSH was measured in neutralized acid extracts by a kinetic assay using 5,5 '-dithiobisnitrobenzoic acid. Reduced glutathione values were expressed as micromoles/gr of tissue. All reagents were from Sigma-Aldrich (St Louis, MO, USA).

4.7 RNA isolation and Reverse Transcriptase (RT) reaction

RNA isolation and RT reaction techniques were carried out as described in Navigatore-Fonzo et al. (2014). Briefly, total RNA was extracted from hippocampus samples by using the Trizol reagent (Invitrogen Co) as directed by the manufacturers. Gel electrophoresis and Gel-Red staining confirmed the integrity of the samples. Quantification of RNA was based on spectrophotometric analysis at 260 nm. Three micrograms of total RNA were reverse-transcribed with 200 units of MMLV Reverse Transcriptase (Promega) using random hexamers, in a 25-µl reaction mixture and following manufacturer's instructions.

4.8 PCR amplification

Transcript levels of Bdnf and Rc3 were determined by RT-PCR and normalized to 28S expression as endogenous control. Fragments coding for those genes were amplified by PCR in 50 µl of reaction solution containing 0.2 mM dNTPs, 1.5 mM MgCl2, 1.25 U of Taq polymerase, 25 pmol of each rat specific oligonucleotide primer and RT generated cDNA (1/5 of RT reaction). The sequences of the specific primers used were: for Bdnf, forward primer 5'-GGGTCACAGGGGGCAGATAAA-3' and reverse primer 5'-CGATTGGGTAGTTCGGCATT-3' (fragment size 200 bp) and for Rc3, forward primer 5'- GCCAGACGACGATATTCTAGACATC-3' and reverse primer 5'- CACACTCTCCACTCTTTATCTTCTTCCT -3' (fragment size 122 bp).

For Bdnf expression, samples were heated in a thermalcycler (My Cycler, BioRad) to 94°C for 2 min, followed by 30 cycles of: (1) denaturation, 94°C for 1 min; (2) annealing, 59°C during 1 min; (3) extension, 72°C for 1min. After 30 reaction cycles, the extension reaction was continued for

another 5 min a 72℃. In the case of Rc3, samples were heated under the following conditions: 94℃ during 4 min followed by 28 cycles of: (1) denaturation, 94 ℃ for 45 seg; (2) annealing, 59℃

during 45 seg; (3) extension, 72℃ for 45 seg; After 28 reaction cycles, the extension reaction was continued for another 10 min. PCR products were then electrophoresed on 2% (w/v) agarose gel with 0.01% (w/v) Gel-Red. The amplified fragments were visualized under ultraviolet (UV) transillumination and photographed using a Cannon PowerShot A75 3.2MP digital camera. The mean of gray value for each band was measured using the NIH ImageJ software (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/) and the relative abundance of each band was normalized according to the housekeeping 28S gene, calculated as the ratio of the mean of gray value of each product to that of 28S.

4.9 Statistical Analysis

Time point data were expressed as mean ± standard error of the mean (SEM) and pertinent curves were drawn. Time series were computed first by one-way analysis of variance followed by Tukey post hoc test for specific comparisons; a `p< 0.05 was considered to be significant. The daily rhythm was assessed by the Chronos-Fit program, using a combination of a partial Fourier analysis and a stepwise regression technique, with a single fundamental period set to 24 h (Zuther et al., 1996). The Chronos-Fit, is a specialized, comprehensive program to analyze 24 h rhythm data from humans and animals. The following parameters were calculated for each of the fitted curves: mesor (24-hour rhythm-adjusted mean), acrophase (peak concentration time, is estimated span of time to reach the crest of the detected rhythm for the period [24 hours] under consideration), and amplitude (the difference found between the maximum value and the average (or Mesor) value adjusted of rhythm. The percentage of rhythm (%rhythm, an index of the amount of variance accounted for) of the fitted curve, and the significance of rhythmicity, testing the null hypothesis of the amplitude being equal to zero, was performed using an F test (>3.5; $p < 0.05$). A cosine-fitted curve was generated with GraphPad Prism® 3.0 software (CA, USA). Student´s t-test was used for comparison of mesor, amplitude or acrophase between CO and Aβ groups, with $p<0.05$ for significant differences.

Figure legends

Figure 1. **Protein levels of Aβ in the hippocampus of control and Aβ-injected groups**. A) Basal protein levels were determined by western blot and normalized to actin. Each bar represents the mean ± SEM of n=4 samples in triplicates with *p<0.05, in comparison to

controls. B) Temporal profiles of Aβ protein levels were determined by western blot on hippocampus samples isolated from control and Aβ-injected rats at zeitgeber times ZT2, ZT8, ZT14, and ZT20. Horizontal bars represent the distribution of light (open) and dark (closed) phases of a 24 h (ZT0-ZT24) photoperiod. Each point represents the mean ± SEM of four hippocampus samples. C) Representative Immunoblot analysis

Figure 2. **Daily variation of ApoE protein levels in the hippocampus of control and Aβ-injected rats**. A) Each point on the graphs represents the mean ± SEM of n=4 hippocampus samples. Horizontal bars represent the distribution of light (open) and dark (closed) phases of the 24h photoperiod. ZT is *zeitgeber* time, with ZT=0 when lights on in the animal facility. B) Representative Immunoblot analysis

Figure 3. **Daily BDNF and RC3 expression in the hippocampus of control and Aβ-injected rats**. A) Each value on the curves represents the mean ± SEM of n=4 hippocampus samples. Horizontal bars represent the distribution of light (open) and dark (closed) phases of the 24 h photoperiod. ZT is *zeitgeber* time, with ZT=0 when lights on in the animal facility. B) Representative patterns of PCR products at different ZTs throughout a daynight cycle.

Figure 4. **Daily levels of MDA in the hippocampus of control and Aβ-injected rats**. Each value on the curves represents the mean ± SEM of n=4 hippocampus samples. Horizontal bars represent the distribution of light (open) and dark (closed) phases of the 24 h photoperiod. ZT is *zeitgeber* time, with ZT=0 when lights on in the animal facility.

Figure 5. **Reduced glutathione levels in the hippocampus of control and Aβ-injected rats during a 24 h period**. Each value on the curves represents the mean ± SEM of n=4 hippocampus samples. Horizontal bars represent the distribution of light (open) and dark (closed) phases of the 24 h photoperiod. ZT is *zeitgeber* time, with ZT=0 when lights on in the animal facility.

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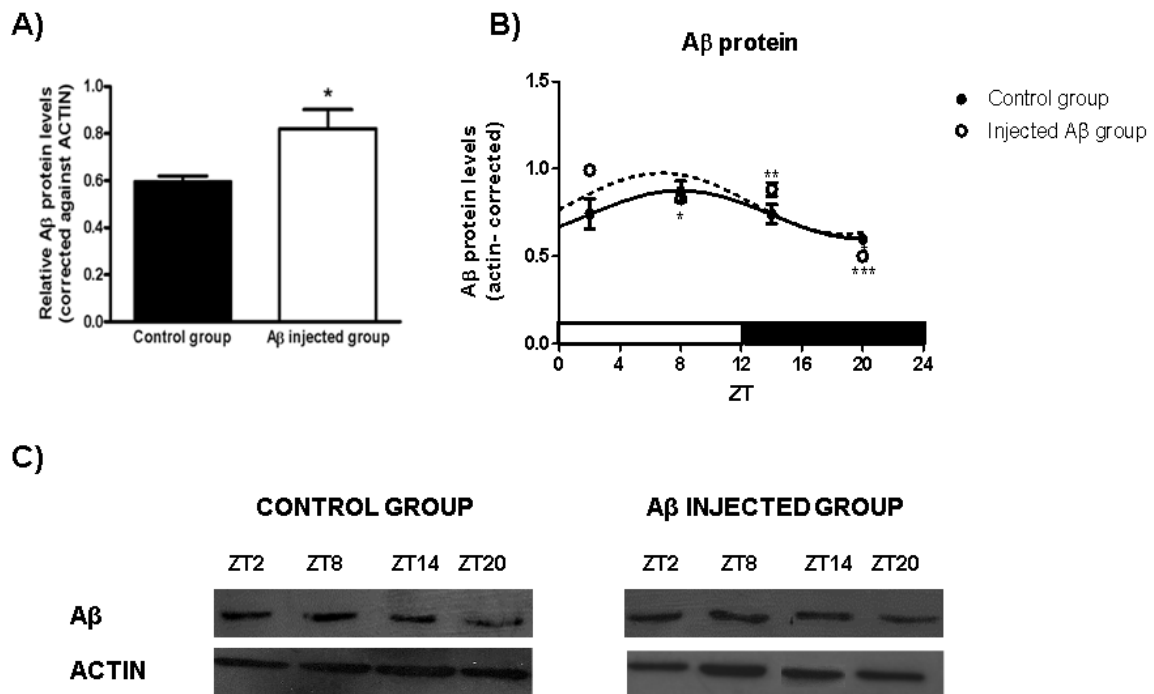
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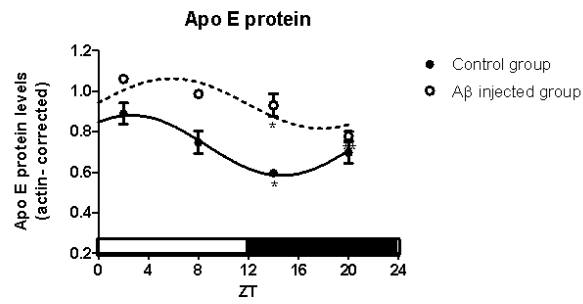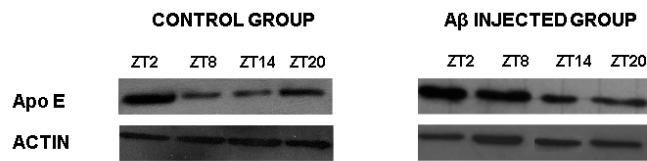
Figure 1

A)

B)

Aβ protein

- ● Control group
- ○ Injected Aβ group

C)

Figure 2
A)

Apo E protein

- Control group
- Aβ injected group

B)

CONTROL GROUP   Aβ INJECTED GROUP

ZT2   ZT8   ZT14   ZT20   ZT2   ZT8   ZT14   ZT20

Apo E

ACTIN

23

Figure 3

A)

mRNA Bdnf

B)

mRNA Rc3

CONTROL GROUP   Aβ INJECTED GROUP   CONTROL GROUP   Aβ INJECTED GROUP

Figure 3

A) mRNA Bdnf

B) mRNA Rc3

Figure 4

MDA in hipocampal tissue

Figure 5

GSH
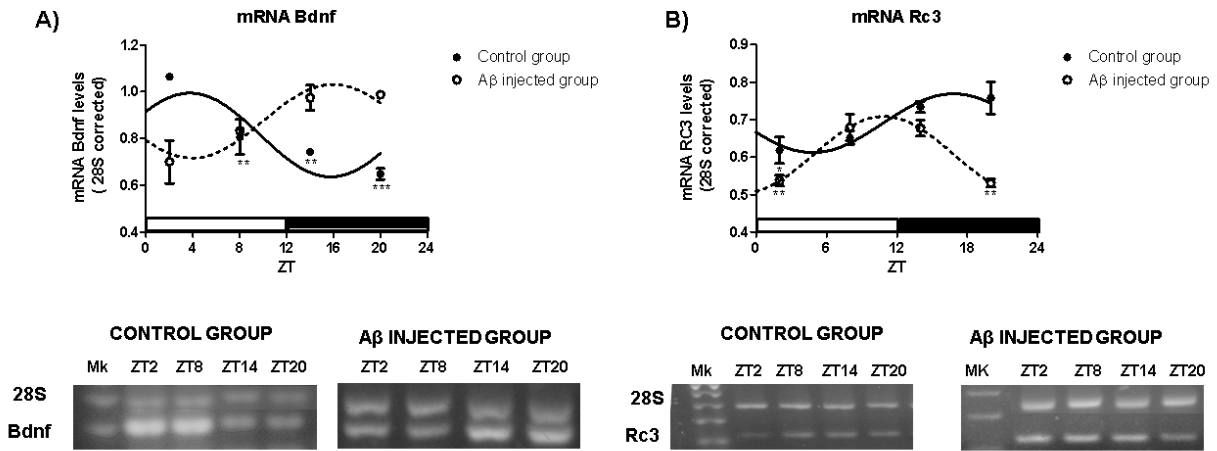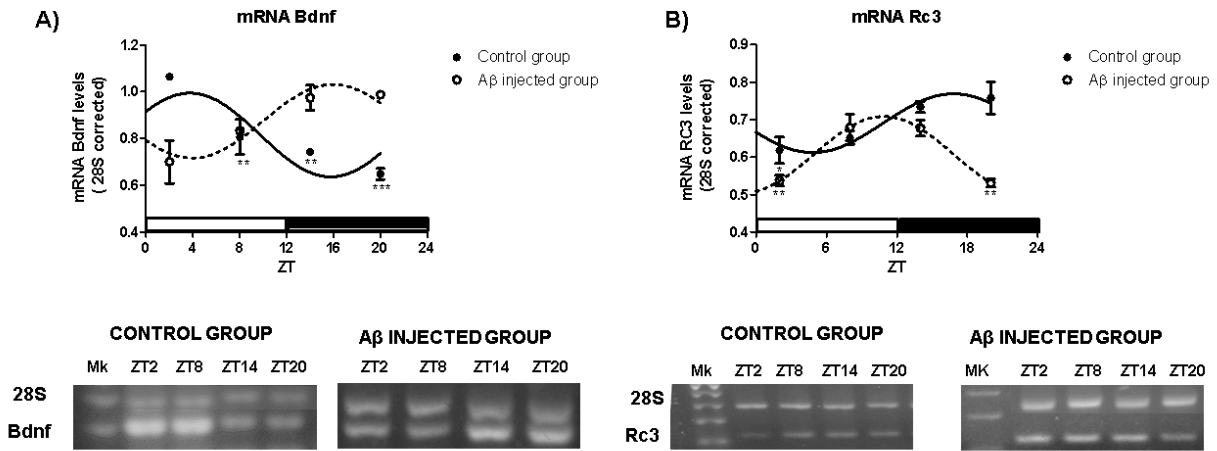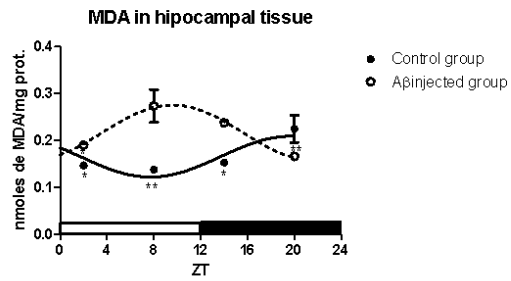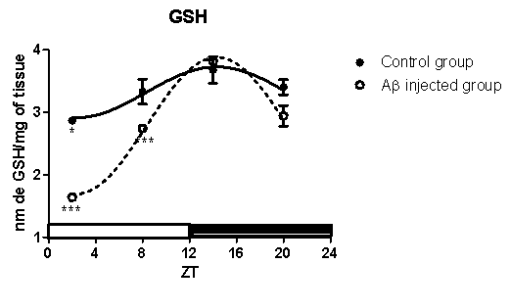
Table 1 Rhythms' parameters of oscillating Aβ protein levels in hippocampal samples of

control and Aβ-injected groups

Apo E PROTEIN LEVELS			
Rhythm Parameters	**Control group (mean±SEM)**	**Aβ injected group (mean±SEM)**	**p**
MESOR	**0.73 ± 0.04**	**0.78 ± 0.00**	**N/S**
AMPLITUDE	**0.15 ± 0.03**	**0.18 ± 0.02**	**N/S**
ACROPHASE	**08:24 ± 00:39**	**07:09 ± 00:17**	**N/S**

Note: Data are presented as mean ± SEM (n = 4 per group). p-levels were obtained for the corresponding control vs. Aβ-injected groups comparison using Student's t-test.

N/S = not significant

Aβ levels (% rhythm Control group: 47.52 Aβ injected group: 37.29)

Table 2 Rhythms' parameters of oscillating Apo E protein and MDA levels in the

hippocampus of control and Aβ-injected groups

Apo E PROTEIN LEVELS			
Rhythm Parameters	Control group (mean±SEM)	Aβ injected group (mean±SEM)	p
MESOR	0.76 ± 0.05	0.94 ± 0.02	< 0.01
AMPLITUDE	0.15 ± 0.02	0.13 ± 0.03	N/S
ACROPHASE	03:39 ± 00:22	06:30 ± 00:28	< 0.01
HIPPOCAMPAL MDA LEVELS			
Rhythm's Parameters	Control group (mean±SEM)	Aβ-injected group (mean±SEM)	p
MESOR	1.65 ± 0.05	2.17 ± 0.09	<0.01
AMPLITUDE	0.45 ± 0.04	0.63 ± 0.13	N/S
ACROPHASE	19:48 ± 00:54	10:16 ± 01:42	<0.001

Note: Data are presented as mean ± SEM (n = 4 per group). p-levels were obtained for the corresponding control vs. Aβ-injected groups comparison using Student's t-test.

N/S = not significant

Apo E levels (% rhythm Control group: 58.10 Aβ injected group: 47.4)

MDA levels (% rhythm Control group: 45.8 Aβ injected group: 62.5)

Table 3 Rhythms' parameters of Bdnf and Rc3 oscillating mRNA levels in the hippocampus of control and Aβ-injected groups

Bdnf mRNA			
Rhythm Parameters	**Control group (mean±SEM)**	**Aβ-injected group (mean±SEM)**	**p**
MESOR	0.81 ± 0.09	0.87 ± 0.03	N/S
AMPLITUDE	0.19 ± 0.03	0.16 ± 0.01	N/S
ACROPHASE	03:27 ± 00:51	15:58 ± 00:23	< 0.01
Rc3 mRNA			
Rhythm Parameters	**Control group (mean±SEM)**	**Aβ-injected group (mean±SEM)**	**p**
MESOR	0.69 ± 0.01	0.51 ± 0.01	< 0.01
AMPLITUDE	0.08 ± 0.03	0.09 ± 0.00	N/S
ACROPHASE	17:24 ± 00:26	11:15 ± 00:36	< 0.001

Note: Data are presented as mean ± SEM (n = 4 per group). p-levels were obtained for the corresponding control vs. Aβ-injected groups comparisons using Student's t-test.

N/S = not significant

(% rhythm Control group: 60.10 Aβ injected group: 60.5)

Table 4. Rhythms' parameters of GSH oscillating levels in the hippocampus of control

and Aβ-injected groups

GSH			
Rhythm's Parameters	**Control group (mean±SEM)**	**Aβ-injected group (mean±SEM)**	**p**
MESOR	**3.32 ± 0.13**	**2.79 ± 0.04**	**<0.05**
AMPLITUDE	**0.41 ± 0.10**	**1.10 ± 0.00**	**<0.05**
ACROPHASE	**14:33 ± 00:33**	**14:18 ± 00:17**	**N/S**

Note: Data are presented as mean ± SEM (n = 4 per group). p-levels were obtained for the corresponding control vs. Aβ-injected groups comparisons using Student's t-test.

N/S = not significant

(% rhythm Control group: 60.6 Aβ injected group: 96.5)

Highlights

- Daily rhythms of Apo E and Aβ protein are observed in the rat hippocampus.
- Bdnf and Rc3 expression display a daily variation.
- MDA and GSH levels vary rhythmically in this brain area
- An injection of Aβ aggregates phase shifts daily BDNF, RC3 and Apo E rhythms
- The Aβ group showed a phase shift in MDA and decrease in mesor of GSH rhythms.