LONG-TERM TREATMENT WITH STANDARDIZED EXTRACT OF *GINKGO BILOBA* L. ENHANCES THE CONDITIONED SUPPRESSION OF LICKING IN RATS BY THE MODULATION OF NEURONAL AND GLIAL CELL FUNCTION IN THE DORSAL HIPPOCAMPUS AND CENTRAL AMYGDALA

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Abstract—Our group previously demonstrated that short-term treatment with a standardized extract of *Ginkgo biloba* (EGb) changed fear-conditioned memory by modulating gene expression in the hippocampus, amygdaloid complex and prefrontal cortex. Although there are few controlled studies that support the long-term use of EGb for the prevention and/or treatment of memory impairment, the chronic use of *Ginkgo* is common. This study evaluated the effects of chronic treatment with EGb on the conditioned emotional response, assessed by the suppression of ongoing behavior and in the modulation of gene and protein expression. Male adult *Wistar* rats were treated over 28 days and assigned to five groups ($n = 10$) as follows: positive control (4 mg kg^{-1} Diazepam), negative control (12% Tween 80), EGb groups (0.5 and 1.0 g kg^{-1}) and the naïve group. The suppression of the licking response was calculated for each rat in six trials. Our results provide further evidence for the efficacy of EGb on memory. For the first time, we show that long-term treatment with the highest dose of EGb improves the fear memory and suggests that increased cAMP-responsive element-binding protein (CREB)-1 and glial fibrillary acidic protein (GFAP) mRNA and protein ($P < 0.001$) in the dorsal hippocampus and amygdaloid complex and reduced growth and plasticity-associated protein 43 (GAP-43) ($P < 0.01$) in the hippocampus are involved in this process. The fear memory/treatment-dependent changes observed in our study suggest that EGb might be effective for memory enhancement through its effect on the dorsal hippocampus and amygdaloid complex. © 2013 Published by Elsevier Ltd. on behalf of IBRO.

Key words: *Ginkgo biloba*, conditioned suppression, dorsal hippocampus, amygdala, CREB-1, GFAP.

INTRODUCTION

Herbs have been considered to be a promising source of novel therapeutic agents due to their reduced side effects, better efficacy, and greater structural diversity relative to synthetic chemistry. Although the biological functions of some herbal drugs have been investigated with different approaches, it remains unclear how these molecules contribute to these activities, and the cellular and molecular targets of herbs remain to be elucidated.

Recent studies from our laboratory (Oliveira et al., 2009) showed that short-term treatment with a standardized extract of *Ginkgo biloba* (EGb) modulates fear memory. However, unlike benzodiazepines, which are traditionally used to reduce anxiety, acute treatment with EGb did not cause deficits in the acquisition of a conditioned emotional response (CER). These behavioral changes were associated with neurochemical modifications in the hippocampus (Hp), prefrontal cortex (PFC) and amygdaloid complex (AC). Additionally, these findings raised new questions about the chronic effects of treatment with EGb in the CER and the neurochemical changes associated with this process.

Evidence from pre-clinical and clinical studies suggests that the chronic administration of EGb can improve the cognitive decline that occurs during aging (Allain et al., 1993; Elsabagh et al., 2005). EGb can also improve the speed of information processing, working memory and executive processing in healthy individuals (Le Bars and Kastelan, 2000; Stough et al., 2001) as well as in healthy rats (Satvat and Mallet, 2009). In addition, an anxiolytic effect was described after long-term treatment with EGb 761 (Woelk et al., 2007). Despite the lack of controlled studies to support the long-term use of EGb for the prevention and/or treatment of memory impairment, it is widely used.

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Abbreviations: AC, amygdala complex; BIS, behavioral inhibition system; CA1, regio 1 cornus ammonis; CER, conditioned emotional response; CG1 and CG2, cingulate cortex; CREB, cAMP-responsive element-binding protein; CS, conditioned stimulus; DH, dorsal hippocampus; EGb, extract of *Ginkgo biloba*; ERK, extracellular-signal-regulated kinase; GAP-43, growth and plasticity-associated protein 43; GFAP, glial fibrillary acidic protein; Hp, hippocampus; IL, infralimbic cortex; IR, immunoreactivity; LTM, long-term memory; mPFC, medial prefrontal cortex; PBS, phosphate-buffered saline; PFC, prefrontal cortex; PKC, protein kinase C; PrL, prelimbic cortex; SD, standard deviation; SEM, standard error of the mean; SHS, septo-hippocampal system; SR, suppression ratio; UC, unconditioned stimulus.

To elucidate aspects related to the effect of chronic treatment with EGb on fear memory, we used CER, which was previously employed to assess fear memory in rodents that are sensitive to anxiolytic drugs (Millenson and Leslie, 1974; George et al., 2009). In the CER procedure, stimuli consistently paired with shock become capable of suppressing ongoing behavior or elevating the amplitude of the startle reflex (fear-potentiated startle). The animal stops a behavior in progress to assess the level of threat of potentially dangerous stimuli, in which the motivated behavior of the animal is suppressed by the presentation of an aversive stimulus. Thus, while it plays a role in motivated behavior, the exposure of animals to an aversive stimulus suggests a conflict procedure. The conflict is established when two incompatible goals are present concurrently (Bond et al., 1973; Maren and Fanselow, 1998).

Behavior suppression has been observed in anxious children (Rosenbaum et al., 1988) and in rodent models of anxiety (George et al., 2009). Anxiolytic compounds were effective in reducing or abolishing the inhibition response of animals against the aversive stimulus to reduce the suppression of CER (McNaughton and Gray, 2000; Miyamoto et al., 2000; George et al., 2009).

In recent years, great advances have been made toward elucidating the neural systems that mediate the acquisition of CER. The Hp, along with other structures, seems to have an important role in the resolution of conflict when an aversive stimulus is presented during a performance in progress through its role in integrating information in the environment with those stored. Changes in the hippocampal activity have been described in the acquisition, recall and extinction of contextual fear conditioning (Hall et al., 2001; Quirk, 2002; Frankland et al., 2004; Matus-Amat et al., 2004; Maren, 2008; Brito et al., 2006). The regio 1 cornus ammonis (CA1) region of the dorsal hippocampus (DH) was shown to be associated with the consolidation of fear memory, as evaluated by a one-trial inhibitory avoidance test (Izquierdo and Medina, 1997; Roesler et al., 1998). Furthermore, Cammarota and colleagues suggested that the Hp function in the memory formation of inhibitory avoidance could be extended to other tasks. This statement correlates with previous data from our laboratory (Oliveira et al., 2009) that suggested that the Hp is involved in the acquisition of the suppression of lick responses.

Besides the Hp, both the PFC and AC have been implicated in the fear memory process (Cammarota et al., 2008). Substantial effort is being directed to develop a better understanding of the role of the PFC in the expression and extinction of conditioned fear (Sierra-Mercado et al., 2006; Cammarota et al., 2007; Oliveira et al., 2009; Sotres-Bayon et al., 2009). Likewise, understanding the role of the AC in conditioned fear and aversive memories is important because a number of anxiety disorders are thought to result from dysregulated fear acquisition, retrieval or extinction. AC is crucial for the acquisition, consolidation, retrieval and extinction of the CER, as it

maintains attention and alertness with respect to potentially dangerous stimuli (Fendt and Fanselow, 1999; Amorapanth et al., 2000; Maren, 2001; Stork et al., 2001; Cammarota et al., 2007; Knapska et al., 2007). Although the AC and Hp control two independent memory systems, emotional and cognitive memories, they interact when emotion and memory come together (Izquierdo and Medina, 1997; Phelps and LeDoux, 2005).

Some of the cellular changes described in the nervous system subjacent to memory formation are related to changes in synaptic efficacy and, thus, the transmission of impulses between neurons. The cellular changes observed in memory formation were also associated with the function of glial cells (Franke et al., 1997; Fawcett and Asher, 1999; Cerutti et al., 2003).

Thereby, the brain's ability to adapt and change its structure, connectivity and functional properties over time characterizes neural plasticity (Kolb and Whishaw, 1998; McClung and Nestler, 2008), which can be investigated using a combination of morphological, molecular, pharmacological, electrophysiological and behavioral approaches (McEwen et al., 2011). Memory formation has been recognized as a major stimulant of brain plasticity, which is often characterized by neuronal and glial adaptations that enable the central nervous system to generate variations in individual behaviors due to its own activity (Benowitz and Routtenberg, 1997). Thus, memory reflects the persistence of such changes over time (Johansen et al., 2011). These changes are mediated by different neurochemical substances, which are involved in the structural expression of neuroplasticity. Molecular changes have been shown to contribute to the understanding of the mechanisms underlying fear memory. Gene and protein expression can be used as an indicator of the patterns of activated cells that reflect active information processing in the system.

The most well characterized transcription factor in the context of neural plasticity is the cAMP-responsive element-binding protein (CREB). The activation of CREB has been recognized to be critical for many important functions in the nervous system, including memory formation (Silva et al., 1998; Cammarota et al., 2000; Kida et al., 2002; Barco et al., 2003). Increasing CREB expression is essential in the amygdala and in the Hp for the formation of fear memory (Bernabeu et al., 1997; Silva et al., 1998; Sweatt, 2001). To date, numerous studies on invertebrates and vertebrates have shown that gene and protein expression are necessary for long-term plasticity and, therefore, long-term memory (LTM) (Izquierdo et al., 1999; Kandel and Pittenger, 1999; Lamprecht, 1999). Furthermore, CREB expression may be affected by behavioral and pharmacological interventions (Kida et al., 2002; Barco et al., 2003; Oliveira et al., 2009). In this study, we sought to investigate whether the expression of genes that were previously associated with neuronal and glial remodeling and memory formation and that are modulated following short-term EGb treatment are also regulated following CER learning and long-term EGb treatment in the Hp, AC and PFC. We initially tested