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CLASSICAL AVERSIVE CONDITIONING INDUCES INCREASED EXPRESSION OF MATURE-BDNF IN THE HIPPOCAMPUS AND AMYGDALA OF PIGEONS

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Abstract—The expression of brain-derived neurotrophic factor (BDNF), which is found in the pro-BDNF, truncated-BDNF and mature-BDNF isoforms, changes with learning. Mature-BDNF shows a peak of late expression in the hippocampus that is involved in the persistence of aversive memory in rodents. However, the role of BDNF in the hippocampal synaptic mechanisms involved in the classical conditioning aversive memory in birds still needs clarification. This study investigated the late expression of BDNF in the hippocampus and amygdala of pigeons trained with tone-shock conditioning and the effects of intra-hippocampal infusion of anisomycin (Ani) in these changes. Seven days after implantation of intra-hippocampal microcannulae, adult pigeons trained with three tone-shock pairings were assigned to one of three groups: Conditioning and Ani (CondANI), Conditioning and saline vehicle (CondSAL) and Conditioning only (Cond). NAIVE group had no treatment or conditioning. Homogenates of tissues from the hippocampus and amygdala, obtained 12 h after training, were used to determine the content of mature-BDNF, truncated-BDNF and pro-BDNF using Western blotting. Higher values for mature-BDNF than for truncated- and pro-BDNF content were seen in the hippocampus of Cond and CondSAL birds, but not in the hippocampus of CondANI or NAIVE birds ($p < 0.05$). The values of mature-BDNF in the amygdala of all the three conditioned groups were higher than those observed for truncated- and pro-BDNF ($p < 0.05$), which indicates that the activation of this protein in the amygdala was not affected by the infusion of Ani in the hippocampus. The data indicate that the tone-shock conditioning induced the activation of molecular pathways of BDNF in the hippocampus and amygdala of the pigeons. The decreases in the content of truncated- and pro-BDNF isoforms found in conditioned pigeons may suggest cleavage mechanisms induced by the training. Our data confirm previous observations of rodent studies and extend these observations to pigeons, revealing that, in spite of the anatomical differences between the hippocampus of rodents and pigeons, there are functional and molecular mechanisms that are conservative between the species.

Key words: classical aversive conditioning, hippocampus, amygdala, pro-BDNF, truncated-BDNF, mature-BDNF.

INTRODUCTION

Exposure to aversive and threatening environmental events triggers defense responses and results in the formation of a memory of the aversive experiences that persists over time. Among such experiences, are those that result in classical aversive conditioning (Rescorla, 1968), a form of learning that occurs when an unconditioned aversive stimulus (US, such as a shock) is paired with a discrete neutral stimulus (for example, a tone). Following this pairing, the once neutral stimulus acquires conditioned aversive value and functions as a conditioned stimulus (CS) that can control defensive or fearful responses. Hence, when the animal is exposed to the CS alone, it will exhibit a conditioned fear response (CR; e.g., freezing), which is interpreted as the expression of aversive memory. Many studies have shown that classical aversive conditioning involves the occurrence of complex behavioral processes, which are organized in neural circuits that integrate different brain areas (Anagnostaras et al., 2001; Maren, 2001; Fanselow, 2010). Moreover, the process of classical aversive conditioning involves a sequence of molecular events, including the activation of gene transcription and the synthesis of various proteins which are implicated in the neuronal plasticity underlying the acquisition, consolidation, reconsolidation, persistence and extinction of the aversive memory (Brito et al., 2006, 2011; Bekinschtein et al., 2007; Mizuno et al., 2012).

The hippocampus participates in the aversive conditioning of both context and discrete stimuli, whereas the amygdala is indicated to be essentially involved in the conditioning to discrete stimuli (Maren and Baudry,

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Abbreviations: Ani, anisomycin; ANOVA, analysis of variance; AP, anteroposterior; BDNF, brain-derived neurotrophic factor; CAU, Careful Exploration; Cond, Conditioning only; CondANI, Conditioning and Ani; CondSAL, Conditioning and saline vehicle; CS, conditioned stimulus; EXP, Exploration; FRE, Freezing; L, lateral; LOC, Locomotion; MOV, Discrete Movements; PRN, Preening; RST, Resting; SEM, standard error of mean; tPA, tissue plasminogen activator; VIG, Vigilance.

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1995; LeDoux, 2000; Zhang et al., 2001; Bast et al., 2003; Brito et al., 2006; Mattson, 2008; An et al., 2012; Wang et al., 2013a,b). Despite the fact that many studies involve the hippocampus and the amygdala in learning and that extensive knowledge is available on the molecular mechanisms involved in the acquisition and consolidation of aversive memory (Hall et al., 2000; von Hertzen and Giese, 2005; Tyler et al., 2002; Liu et al., 2004; Lee et al., 2004; Brito et al., 2006, 2011; Lee and Hynds, 2013), few studies have examined issues related to the role of these structures in the persistence of long-term memory (Bekinschtein et al., 2007, 2010, 2013).

Various studies have shown that the expression of brain-derived neurotrophic factor (BDNF), a protein that can be found in the pro-BDNF, truncated-BDNF and mature-BDNF isoforms, is involved in the persistence of memory (Pang and Lu, 2004; Bekinschtein et al., 2007, 2013; Nagappan et al., 2009; Sartori et al., 2009; Martínez-Moreno et al., 2011; Mercerón-Martínez et al., 2012; Mizuno et al., 2012; Leal et al., 2013). BDNF is synthesized in the endoplasmic reticulum as a precursor molecule (pro-BDNF; 32 kDa) and is then transported to the Golgi apparatus, where it is stored in vesicles (Yang et al., 2009; Cunha et al., 2010). These vesicles follow two distinct pathways. The first remains intracellularly, where the pro-BDNF is cleaved into two other isoforms: truncated-BDNF (28 kDa) and mature-BDNF (14 kDa). The second results in exocytosis, with pro-BDNF cleaved extracellularly by plasmin protease. This enzyme is expressed as an inactive zymogen known as plasminogen. Plasminogen activation involves another protease, the tissue plasminogen activator (tPA) (Lochner et al., 2008; Greenberg et al., 2009; Cunha et al., 2010). The pro-BDNF has a high affinity for the p75 neurotrophin receptor, triggering pro-apoptotic and anti-plasticity effects (Cunha et al., 2010; Xu et al., 2010; Sanchez et al., 2011; Dieni et al., 2012). In turn, mature-BDNF interacts predominantly with its membrane specific receptor, tyrosine kinase B (TrkB), and exerts its physiological effects on cell survival and neuronal plasticity, thus establishing the cellular and molecular alterations necessary for the reconsolidation and persistence of memory (Lu et al., 2005, 2008; Nagappan et al., 2009; Cardenas-Aguayo Mdel et al., 2013). However, the role of the truncated isoform has not yet been established (Seidah et al., 1999; Mowla et al., 2001; Carlino et al., 2011; Mizoguchi et al., 2011; Sartori et al., 2011; Garcia et al., 2012).

The mature-BDNF isoform shows increased expression after training in learning tasks (Hall et al., 2000; Sartori et al., 2009; Ou et al., 2010), and recent studies have reported a peak in mature-BDNF taking place approximately 12 h after training (Bekinschtein et al., 2007). This late post-training peak of mature-BDNF in the hippocampus of rodents has been implicated in the persistence of long-term memory of both inhibitory avoidance and contextual fear conditioning in rodents (Bekinschtein et al., 2007, 2010).

The relationship between the three BDNF isoforms (pro-BDNF, truncated-BDNF, mature-BDNF) in the process of learning and memory is not well understood, especially with regard to classical aversive conditioning. The present study has investigated whether tone-shock classical conditioning induces a late cycle of BDNF expression in the hippocampus and amygdala of pigeons. Hippocampal BDNF expression in a time-window of around 12 h was investigated by inhibiting protein synthesis with intra-hippocampal infusion of anisomycin (Ani). The changes in the expression of the BDNF isoforms (mature-BDNF, pro-BDNF and truncated-BDNF) were then analyzed in both the hippocampus and amygdala.

EXPERIMENTAL PROCEDURES

We used male pigeons from uncontrolled derivation of the species *Columba livia*, (350–500 g, 1–2 years of age). They were obtained from the São Francisco pigeon breeding center in Limeira, SP, Brazil. They were housed in a pigeon vivarium, in individual cages with food and water (mixture of corn grits, sunflower seed and poultry feed), and were maintained at 25 °C, in 12:12-h light–dark cycle (lights on at 06:00 a.m.). The pigeons had a 30-day period of adaptation to the conditions of the vivarium. The experimental procedures were approved by the Institutional Committee for Ethics in Animal Experimentation, UNICAMP (CEUA/IB-UNICAMP, protocol no. 2928-1).

Experimental groups

Pigeons were randomly allocated to one of four groups: (1) NAIVE (n = 6), of birds that were only habituated to handling and weighing, sacrificed 12 h after the last weighing on experimental Day 7; (2) Conditioning only (Cond; n = 6), of birds submitted only to training with tone-shock pairings; (3) Conditioning and treatment with saline solution (CondSAL; n = 6), of birds trained with tone-shock pairings, and after 11 h underwent intra-hippocampal infusion of saline solution (vehicle); (4) Conditioning and treatment with Ani (CondANI; n = 6), of birds trained with tone-shock pairings, and after 11 h, underwent intra-hippocampal infusion of Ani. The sequence of experimental events was initiated immediately after the 30-day period of adaptation to the conditions of the vivarium, and lasted for 8 days, as shown in the schematic representation of the experimental procedures in Fig. 1.

Surgical implantation of intra-hippocampal cannulae

On experimental Day 1, the pigeons were deeply anesthetized with a solution of xylazine and ketamine (0.1 mg/kg, 1:1, i.p.) and placed on a stereotaxic device (David Kopf, mod. 1204, with a Revzin adapter for pigeons). With a high-speed drill, two holes were made in the dorsal–lateral–medial regions, according to the anteroposterior (AP) and lateral (L) axis points of the Karten and Hodos (1967). The zero vertical point (V), determined by placing the tip of the cannula on the dura mater, was used to establish the experimental points: AP 6.75; V 1.5; L 0.8 (Karten and Hodos, 1967).