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ACTIVITY IN PRELIMBIC CORTEX IS REQUIRED FOR ADJUSTING THE ANXIETY RESPONSE LEVEL DURING THE ELEVATED PLUS-MAZE RETEST

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Abstract—The prelimbic (PL) subregion of medial prefrontal cortex has been implicated in anxiety regulation. It is unknown, however, whether PL cortex also serves to fine-tuning the level of anxiety-related behavior exhibited on the next exposure to the same potentially threatening situation. To address this, we infused cobalt (1.0 mM) to temporarily inactivate the PL cortex during testing, post-testing or retesting in the elevated plus-maze (EPM). This protocol was chosen because it allowed us to concurrently investigate anxiety and the process of aversive learning and memory. PL cortex inactivation during the EPM testing increased the exploration of open-arms, substantiating its role in anxiety. PL cortex inactivation during the EPM retesting counteracted the further avoidance to open-arms exhibited by rats. Interestingly, as evidenced by min-by-min analysis, the cobalt-treated group behaved on EPM retesting as did the vehicle-treated group on EPM testing. This result may imply that activity in PL cortex is necessary for retrieving previously learned information that adjusts the anxiety response level on EPM retesting. Alternatively, a simple reduction in anxiety could explain the cobalt-induced increase in retest open-arms exploration. Neither test nor post-test PL cortex inactivation affected the further avoidance to open-arms observed on EPM retesting. To extend the investigation of PL cortex role in the regulation of open-arms avoidance, we infused other drugs prior to testing or retesting in the EPM. Antagonism of PL cortex adrenergic beta-1 receptors with atenolol (10 nmol), cholinergic muscarinic receptors with scopolamine (20 nmol) or glutamatergic N-methyl-D-aspartic acid (NMDA) receptors with AP5 (6.0 nmol) interfered with the level of open-arms exploration on testing, but not on retesting.

Key words: medial prefrontal cortex, emotional memory, fear conditioning, defensive behavior.

The medial prefrontal cortex has been associated with emotional processing. Excitotoxic or electrolytic lesion of its prelimbic (PL) subregion attenuates the expression of anxiety-related behaviors in rats exposed to potentially threatening situations such as the elevated plus-maze (EPM), the open-field, the social interaction, and the shock probe burying tests (Lacroix et al., 1998; Gonzalez et al., 2000; Shah and Treit, 2003). As lesion findings may be conflicting (Jinks and McGregor, 1997), owing to recruitment of other structures and/or compensatory mechanisms (Lomber, 1999), subsequent studies have substantiated the PL cortex role in anxiety by means of local infusion of drugs which temporarily inhibit the synaptic transmission such as the gamma aminobutyric acid type A ($GABA_A$) receptor agonist muscimol (Shah et al., 2004) and the cobalt (Resstel et al., 2008), which blocks voltage-dependent calcium channels responsible for neurotransmitter release and, consequently, affects the activity of postsynaptic elements as intrinsic neurons and cell processes (Kretz, 1984).

It is unknown, however, whether the PL cortex activity is also necessary for adjusting the anxiety response level on the subsequent exposure to the same potentially threatening siuation. Of particular relevance to this matter are those findings demonstrating that aversive learning and memory may be studied at the same time as anxiety in rodents exposed to the EPM test/retest (File, 1993; Rodgers et al., 1996; Lamprea et al., 2000; Wall and Messier, 2000; Bertoglio et al., 2006). Animals retested in the EPM exhibit a statistically significant decrease in open-arms exploration relative to their respective level on testing (Lee and Rodgers, 1990; Treit et al., 1993; Fernandes and File, 1996; Bertoglio and Carobrez, 2000). As evidenced by min-by-min analysis, this response of further avoidance to open-arms is gradually acquired throughout testing (Holmes and Rodgers, 1998; Bertoglio and Carobrez, 2004), and thought to reflect the retrieval of the aversive memory related to the initial EPM exploration (File, 1993; Lamprea et al., 2000; Carobrez and Bertoglio, 2005). In the test/retest protocol, animals may be infused with drugs at one of three time points: before testing, immediately after testing or before retesting. The rationale behind the choice of the moment of drug infusion depends on whether the experimenter wishes to (a) investigate drug effects on anxiety and/or aversive memory acquisition (Stern et al., 2008), (b) investigate drug effects on aversive memory consolidation (Vargas et al., 2006), or (c) investigate drug effects on aversive memory retrieval and/or anxiety (Bertoglio et al., 2006).

In the present study, we bilaterally infused cobalt to inactivate the rat PL cortex during testing (experiment 1), post-testing (experiment 2) or retesting (experiment 3) in the EPM. We found that processes in PL cortex are im-

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Abbreviations: ANOVA, analysis of variance; AP5, amino-5-phosphonopentanoic acid; EAE, enclosed-arms entries; EPM, elevated plus-maze; $GABA_A$ receptor, GABA type A receptor; NMDA receptor, N-methyl-D-aspartic acid receptor; OAE, open-arms entries; %OAT, percentage of open-arms time; PL cortex, prelimbic cortex; SAPs, stretched-attend postures.

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portant for the expression of anxiety on testing and for the aversive memory retrieval, which may adjust the anxiety response level, on retesting. It is of note that the cobalt-induced increase in retest open-arms exploration could alternatively be explained by a reduced anxiety expression, as shown in experiment 1. To extend the investigation of PL cortex role in the regulation of open-arms avoidance, other drugs were infused into the PL cortex before EPM testing or retesting. We found that the adrenergic beta-1 receptor antagonist atenolol (10 nmol), the cholinergic muscarinic receptor antagonist scopolamine (20 nmol) or the glutamatergic N-methyl-D-aspartic acid (NMDA) receptor antagonist AP5 (6.0 nmol) selectively interferes with the level of open-arms exploration on testing, but not on retesting.

EXPERIMENTAL PROCEDURES

Subjects

All procedures were approved by the Institutional Ethical Committee for the care and use of laboratory animals of the Federal University of Santa Catarina (068/CEUA/PRPe/2008) in compliance with Brazilian Society of Neuroscience and Behavior guidelines. Male Wistar rats weighing 300–350 g, aged 14–16 weeks at the time of testing, were housed in groups of four to five per cage (50×30×15 cm) in a temperature-controlled room (22±1 °C), under standard laboratory conditions with free access to food and water, and with a 12 h light/12 h dark cycle (lights on at 7:00 AM).

Drugs

Cobalt chloride hexahydrated (cobalt; Sigma-Aldrich, USA), (RS)-atenolol (atenolol; Tocris Bioscience, UK), (−)-scopolamine hydrobromide (scopolamine; RBI, USA), and (±)-2-amino-5-phosphonopentanoic acid (AP5; Tocris Bioscience, UK) were dissolved in phosphate buffered saline, which alone served as a vehicle control. The dose selection of these drugs was based on both pilot and previously published studies (Kretz, 1984; Nascimento Häckl and Carobrez, 2007; Resstel et al., 2008; Kincheski and Carobrez, 2010).

Elevated plus-maze (EPM) apparatus

It was made of wood and consisted of two opposite open-arms (50×10 cm) surrounded by a 1 cm high Plexiglas ledge, and two enclosed-arms (50×10×40 cm), set up 50 cm above the floor. The junction area of the four arms (central platform) measured 10×10 cm (Carobrez and Bertoglio, 2005).

Stereotaxic surgery and drug infusion

Rats were intraperitoneally anesthetized using 1.0 ml/kg of a solution containing xylazine (10 mg/mL; Carlier, Brazil) and ketamine (100 mg/mL; Sespo, Brazil), associated with local anaesthesia (3.0% lidocaine with norepinephrine 1:50000; Dentsply, Brazil), and positioned in a stereotaxic frame. Two stainless steel guide cannulas (length=11.0 mm and outer diameter=0.6 mm) were implanted bilaterally aimed at the PL cortex following the coordinates from the rat brain atlas by Paxinos and Watson (2009), and fixed to the skull with acrylic resin and two stainless steel screws. The cannula tips were 2.2 mm above the site of drug injection. A stylet was introduced inside each guide cannula to prevent occlusion. For post-surgery analgesia, subjects were injected subcutaneously with flunixin meglumine (2.5 mg/kg; Schering-Plough, Brazil), a drug with analgesic, antipyretic and anti-inflammatory properties. An antibiotic association of benzylpenicillin and streptomycin (1.0 ml/kg; Fort Dodge, Brazil) was administered intramuscularly to prevent possible infections.

One-week after surgery, rats received a bilateral infusion into the PL cortex with dental needles (outer diameter=0.3 mm) introduced through the guide cannulas until their tips were 2.2 mm below the cannula end. A 0.2 μl/side of either vehicle or drug was injected during 1 min, using two microsyringes connected to an infusion pump. A polyethylene catheter was interposed between the upper end of the dental needles and the microsyringes. The displacement of an air bubble inside the polyethylene was used to monitor drug flow. Needles were removed 30 s after the end of drug infusion.

General conditions and data collection

All experiments were carried out in a low illumination (40-lux) condition room, during the diurnal phase, between 1:00 and 5:00 PM. EPM sessions last for 5 min, and were recorded by a video camera while a monitor and a DVD-recording system were installed in an adjacent room. After each EPM session, the apparatus was cleaned with 10% ethanol solution (v/v) and dry towels to avoid urine impregnation.

A trained observer blind to the experimental design scored the following behavioral measures from the DVD: the number of open- (OAE) and enclosed-arms entries (EAE) with the four paws, as well as the time spent in open- and enclosed-arms. Raw data were used to calculate the percentage of time spent in open-arms {%OAT; [(time in open-arms/300)×100]}. The number of stretched-attend postures (SAPs), defined as a posture in which the subject stretches forward and then retracts to its original position, performed from the central platform or enclosed-arms towards open-arms, was also recorded. This latter response is categorized as risk assessment, and has also been considered closed related to anxiety (Rodgers et al., 1997; Carobrez and Bertoglio, 2005).

Histology

After the conclusion of each experiment, rats were intraperitoneally anesthetized using 1.0 ml/kg of a solution containing xylazine (10 mg/mL; Carlier, Brazil) and chloral hydrate (2.3 mg/mL; Vetec, Brazil), injected through the cannulas with 0.2 μl/side of Evans Blue to mark the sites where drugs were previously infused, and then transcardially perfused with 0.9% of NaCl followed by 10% of formalin solution. Each rat brain was removed and immersed in a 10% formalin solution. Slices (50 μm thick) were obtained in a cryostat (Leica, Germany), mounted on glass microscope slides, and stained with Giemsa to anatomically localize the Evans Blue marks in diagrams from Paxinos and Watson's (2009) rat brain atlas. Their location was mostly in the PL cortex bottom, and ranged from 3.7 to 2.7 mm anterior to Bregma. Fig. 1 shows a photomicrograph of representative infusion sites placement into the PL cortex. Rats receiving drug infusion outside this region were excluded from the analysis.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA). Following significant ANOVA results, post-hoc comparisons using Newman–Keuls test were performed. The level of statistical significance adopted was $P<0.05$.

RESULTS

Experiment 1: PL cortex inactivation during EPM testing reduces the avoidance to open-arms

To substantiate that the PL cortex serves a critical role in anxiety, 38 EPM-naive rats were randomly allocated to