

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. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

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 situation. 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-phosphopentanoic 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.

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 benzylpeni-

illin 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

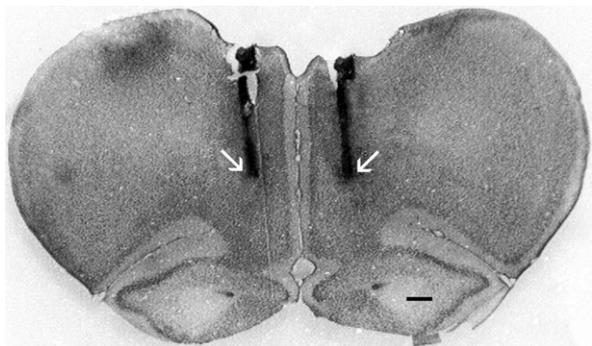


Fig. 1. Photomicrograph (scale bar=500 μ m) of representative infusion sites placement (indicated by arrows) in the rat prelimbic cortex (approximately 3.7 mm anterior to Bregma).

four groups ($n=8-11$ /group) according to the intra-PL cortex treatment (vehicle or 1.0 mM of cobalt) and the interval (1 or 10 min) between the drug infusion ending and the EPM testing beginning (Fig. 2). Rats tested 1 min after cobalt infusion showed an increase in % OAT ($F_{1,34}=6.55$; $P<0.01$; Fig. 2A) relative to controls. As a result, PL cortex inactivation decreases the avoidance to open-arms. This is corroborated by a trend ($P<0.10$) to increasing OAE (Fig. 2B) and reducing SAPs (Fig. 2C). The lack of an attenuation of anxiety-related behavior when a 10 min interval between cobalt infusion and EPM testing was adopted confirms the transitory effect of this drug (Kretz, 1984; Lomber, 1999). Importantly, these results were observed in the absence of changes in EAE (Fig. 2D), an EPM index of general exploratory activity (Carobrez and Bertoglio, 2005).

To investigate if the PL cortex activity is required for aversive memory acquisition, vehicle- and cobalt-treated groups were retested in the EPM 24 h later undrugged. As can be seen in Table 1, all groups reduced % OAT ($F_{3,68}=7.54$; $P<0.001$) on retesting when compared to their respective levels on testing. Because cobalt- and vehicle-treated rats behaved equally, demonstrating further avoidance to open-arms, it is suggested that aversive

memory acquired on testing took place entirely. Neither prior EPM experience nor test PL cortex inactivation interfered with OAE, SAPs and EAE on retesting (Table 1).

Experiment 2: PL cortex inactivation immediately after testing in the EPM does not interfere with the further avoidance to open-arms exhibited during the EPM retesting

To investigate if the PL cortex contributes to the consolidation of aversive memory acquired during the initial EPM experience, 17 rats were randomly allocated to two groups ($n=8-9$ /group) based on the treatment (vehicle or 1.0 mM of cobalt) given into the PL cortex immediately after EPM testing. As can be seen in Table 2, both groups of EPM-experienced rats reduced % OAT ($F_{1,15}=15.3$; $P<0.001$) during the EPM retesting. As their further avoidance to open-arms exhibited was equivalent, it is suggested that the post-test cobalt-induced PL cortex inactivation had no effect on aversive memory consolidation. The reduction of SAPs ($F_{1,15}=26.3$; $P<0.0001$) induced by prior EPM experience was unchanged by cobalt infusion. Neither prior EPM experience nor post-test PL cortex inactivation interfered with OAE and EAE on retesting (Table 2).

Experiment 3: PL cortex inactivation during EPM retesting impairs the further avoidance to open-arms

To investigate if the PL cortex activity regulates the further avoidance to open-arms observed on EPM retesting, 35 EPM-experienced rats were randomly allocated to four groups ($n=7-10$ /group) according to the treatment (vehicle or 1.0 mM of cobalt) and the interval (1 or 10 min) between the ending of drug infusion into the PL cortex and the beginning of EPM retesting (Fig. 3). Vehicle-treated groups demonstrated further avoidance to open-arms on retesting, characterized by reduced % OAT ($F_{4,65}=7.94$; $P<0.0001$; Figs. 3A and 5) and OAE ($F_{4,65}=9.08$; $P<0.0001$; Fig. 3B) when compared to their respective levels on testing. However, the group in which the PL cortex was inactivated by cobalt 1 min, but not 10 min, before retesting performed differently: there was an in-

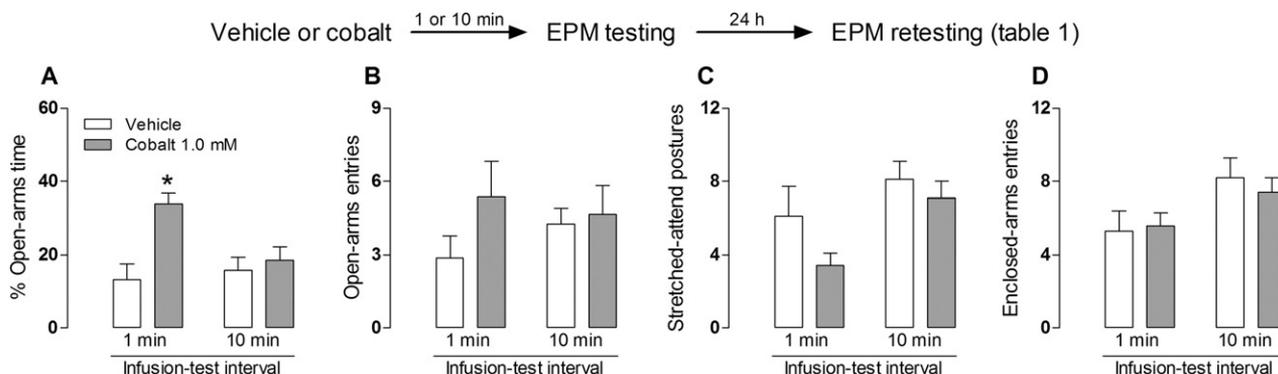


Fig. 2. Prelimbic cortex inactivation during the elevated plus-maze (EPM) testing attenuates the anxiety-related behavior. Rats tested 1 min after the cobalt infusion spent more time exploring the open-arms than respective controls (A, B, C). The inactivation of the prelimbic cortex by cobalt was temporary since rats tested 10 min after its infusion performed just as vehicle-treated subjects. Importantly, these results were observed in the absence of changes in enclosed-arm entries (D), an EPM index of general exploratory activity. Vertical bars represent the mean \pm SEM. The asterisk indicates a significant difference ($P<0.05$) from respective controls (two-way ANOVA followed by Newman-Keuls test).

Table 1. Retesting data from groups infused into the prelimbic cortex with vehicle or cobalt (1.0 mM) 1 or 10 min before testing in the elevated plus-maze. Data are presented as mean±SEM

	Vehicle 1 min (n=8)		Cobalt 1 min (n=11)		Vehicle 10 min (n=10)		Cobalt 10 min (n=9)	
	Testing	Retesting	Testing	Retesting	Testing	Retesting	Testing	Retesting
%OAT	13.2±4.2	1.3±1.0*	34.0±2.9	1.2±0.6*	15.8±3.4	1.6±1.2*	18.5±3.7	6.0±3.0*
OAE	2.9±0.9	0.5±0.4	5.4±1.5	0.5±0.3	4.3±0.7	0.6±0.4	4.7±1.2	2.1±1.0
SAPs	6.1±1.6	7.0±1.4	3.4±0.7	5.7±1.1	8.1±1.0	8.7±1.4	7.1±0.9	8.3±1.4
EAE	5.3±1.1	4.8±1.0	5.6±0.7	4.3±0.7	8.2±1.1	7.8±1.4	7.4±0.8	7.6±0.8

% OAT, percentage of open-arms time; OAE, open-arms entries; SAPs, stretched-attend postures; EAE, enclosed-arms entries; * $P < 0.05$ versus the respective group on testing (two-way repeated-measures ANOVA followed by Newman Keuls test).

crease in % OAT ($F_{1,31}=6.19$; $P < 0.01$; Figs. 3A and 5) and OAE ($F_{1,31}=8.34$; $P < 0.01$; Fig. 3B) relative to controls. As the cobalt-treated group behaved on retesting as did the vehicle-treated group on testing (Fig. 5), this result may imply that activity in PL cortex is critical for aversive memory retrieval. A reduced anxiety expression, as demonstrated on testing (Fig. 2A), could alternatively explain the cobalt-induced increase in retest open-arms exploration. The reduction of SAPs ($F_{4,65}=14.2$; $P < 0.00001$; Fig. 3C) on retesting was unaffected by cobalt infusion. Neither prior EPM experience nor retest PL cortex inhibition affected EAE (Fig. 3D).

Experiment 4: Bilateral infusion of atenolol, scopolamine or AP5 into the PL cortex attenuates the avoidance to open-arms during the test, but not retest, in the EPM

Preceding results suggest that processes in PL cortex regulate the level of open-arms avoidance which rats exhibit during testing and retesting in the EPM. To extend these findings, 67 rats were allocated to eight groups ($n=7-10$ /group) based on the treatment given before testing or retesting in the EPM (Fig. 4). Antagonism of PL cortex adrenergic beta-1 receptors with atenolol (10 nmol), cholinergic muscarinic receptors with scopolamine (20 nmol), or glutamatergic NMDA receptors with AP5 (6.0 nmol) increased % OAT ($F_{3,59}=3.19$; $P < 0.05$) on testing of EPM-naive rats (Figs. 4A and 5), suggesting a role for these receptors in modulating anxiety-like behavior. This is

Table 2. Prelimbic cortex inactivation by 1.0 mM of cobalt immediately after testing in the elevated plus-maze did not interfere with the expression of further avoidance to open-arms on retesting performed 24 h later undrugged. Data are presented as mean±SEM

	Vehicle (n=8)		Cobalt (n=9)	
	Testing	Retesting	Testing	Retesting
%OAT	12.9±3.7	4.3±2.5*	15.6±3.4	5.0±1.8*
OAE	3.6±0.9	1.7±1.0	4.1±0.9	1.9±0.6
SAPs	10.5±1.0	5.9±0.8*	12.3±1.1	6.2±1.1*
EAE	7.4±0.9	8.1±1.3	9.9±0.9	9.4±1.3

% OAT, percentage of open-arms time; OAE, open-arms entries; SAPs, stretched-attend postures; EAE, enclosed-arms entries; * $P < 0.05$ versus the respective group on testing (one-way repeated-measures ANOVA followed by Newman Keuls test).

corroborated by a trend ($P < 0.10$) to increasing OAE (Fig. 4B) and reducing SAPs (Fig. 3C), in the absence of changes in EAE (Fig. 4D).

Moreover, regardless of the drug infused into the PL cortex before testing, all these groups demonstrated further avoidance to open-arms on retesting (Table 3). Thus, at least in the drugs' dose tested, the blockade of adrenergic beta-1, cholinergic muscarinic, or glutamatergic NMDA receptors located in the PL cortex did not affect the aversive memory acquisition.

In spite of infusing 10 nmol of atenolol, 20 nmol of scopolamine or 6.0 nmol of AP5 into the PL cortex, the open-arms exploration exhibited by EPM-experienced rats remained similar to controls during the EPM retesting. This suggests that the anxiolytic-like effect of these drugs is no longer present, a phenomenon known as one-trial tolerance and previously described to benzodiazepines and other drugs (File et al., 1990; Bertoglio and Carobrez, 2003; Carobrez and Bertoglio, 2005; Vargas et al., 2006; Nascimento Häckl and Carobrez, 2007; Albrechet-Souza et al., 2008). It is of note that this event has been associated with the retrieval of aversive memory acquired on EPM testing (Rodgers et al., 1996; Bertoglio and Carobrez, 2004; Stern et al., 2008). In view of the fact that all groups demonstrated further avoidance to open-arms on retesting, characterized by a reduction in % OAT ($F_{4,63}=10.6$; $P < 0.00001$; Figs. 4A and 5) and OAE ($F_{4,63}=12.7$; $P < 0.00001$; Fig. 4B) when compared to their respective levels on testing, the present results support this assumption. Neither prior EPM experience nor retest PL cortex drug infusion interfered with SAPs and EAE.

DISCUSSION

The main findings of the present study were: (i) PL cortex inactivation during the EPM testing increased open-arms exploration; (ii) Neither test nor post-test PL cortex inactivation interfered with the further avoidance to open-arms exhibited by rats during the EPM retesting; (iii) PL cortex inactivation during the EPM retesting counteracted the further avoidance to open-arms; and (iv) PL cortex infusion of atenolol, scopolamine or AP5 reduced avoidance to open-arms during the test, but not the retest, in the EPM.

Inactivating the PL cortex increases the open-arms exploration on EPM testing. This anxiolytic-like effect substantiates that of previous studies in which lesion or tem-

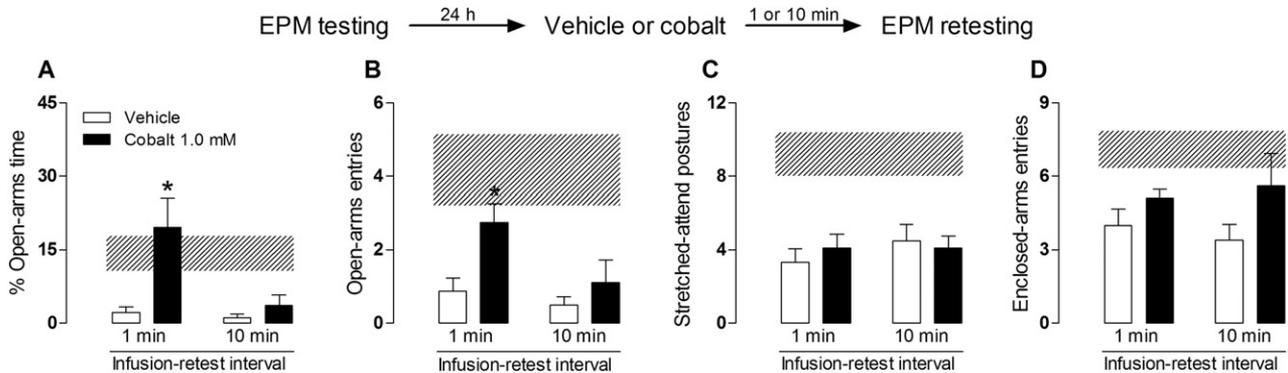


Fig. 3. Prelimbic cortex inactivation during the elevated plus-maze (EPM) retesting impairs the further avoidance to open-arms acquired on testing. Both groups infused with vehicle exhibited on retesting greater avoidance to open-arms than that found on testing (A, B, C). Infusion of cobalt 1 min, but not 10 min, before retesting in the EPM fully counteracted this pattern of result, without changes in general exploratory activity (D). Vertical bars represent the mean \pm SEM. Horizontal hatched bars represent the 95% confidence interval for the mean from these subjects on EPM testing. This way of data presenting was chosen because all groups performed equally ($F_{3,31} \leq 1.17$; $P \geq 0.33$). The asterisk indicates a significant difference ($P < 0.05$) from respective controls (two-way ANOVA followed by Newman–Keuls test).

porary inactivation of this region attenuated the expression of anxiety (Lacroix et al., 1998; Gonzalez et al., 2000; Shah and Treit, 2003; Shah et al., 2004; Resstel et al., 2008). Consistent with this are studies demonstrating Fos expression in the PL cortex of rats exposed to the EPM (Duncan et al., 1996) and the cat-odor (Staples et al., 2008), another test that makes use of a potentially threatening stimulus. Risk assessment is also considered an important behavioral response related to anxiety (Rodgers et al., 1997). PL cortex inactivation, however, had no effect on SAPs. Negative data such as these support the idea that critical neural substrates controlling these subtle acts and postures lie outside PL cortex, in the medial hypothalamus and the anterior cingulate cortex for instance (McNaughton and Corr, 2004; Albrechet-Souza et al., 2009).

The lack of effect of either test or post-test PL cortex inactivation on the further avoidance to open-arms exhibited by rats during the EPM retesting agrees with results from studies in which the lesion of this region did not

disrupt acquisition or consolidation of conditioned fear in rats (Morgan et al., 1993; Kim and Jung, 2006; Sierra-Mercado et al., 2006). Altogether, these findings suggest that the PL cortex activity appears to be unnecessary for acquisition and consolidation of learned responses related to anxiety (inhibitory avoidance) and fear (freezing). In contrast to PL cortex, either permanent or reversible inactivation of the amygdala impairs the acquisition and the consolidation of conditioned fear in rats (Blanchard and Blanchard, 1972; Kim and Davis, 1993; Helmstetter and Bellgowan, 1994; Cousens and Otto, 1998; Goosens and Maren, 2001; Anglada-Figueroa and Quirk, 2005; Wilensky et al., 2006). It is unknown, however, whether activity in the amygdala is also fundamental to acquire and/or consolidate emotional information that leads to the further avoidance to open-arms during the EPM retesting. Moreover, because the memory consolidation of learned fear lasts longer than 10 min (Schafe et al., 2001), it would have been interesting to have evaluated whether the intra-PL cortex infusion of longer last-

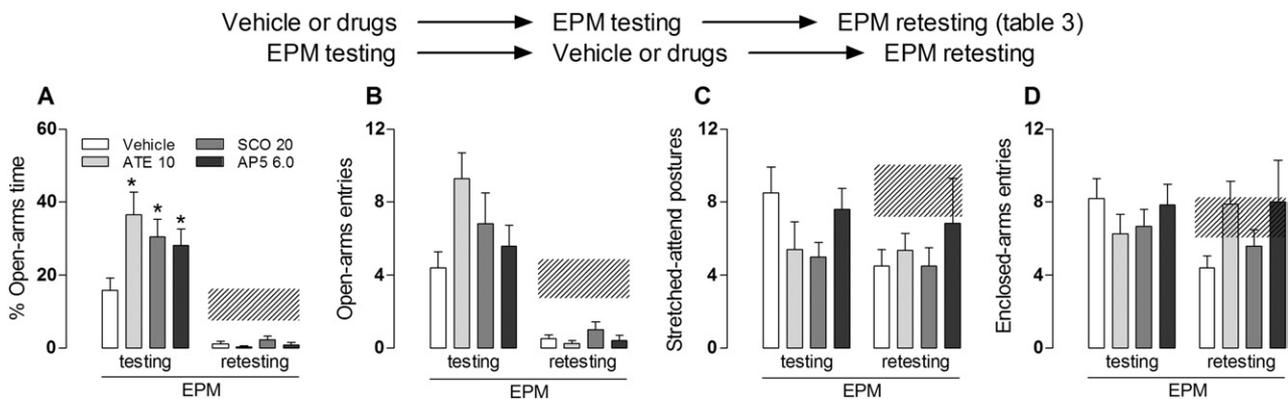


Fig. 4. Infusing 10 nmol of atenolol (ATE 10), 20 nmol of scopolamine (SCO 20) or 6.0 nmol of AP5 (AP5 6.0) attenuates the anxiety-related behavior on testing of elevated plus-maze (EPM) naive rats relative to respective controls (A). The same treatment, however, did not affect the further avoidance to open-arms exhibited by rats on retesting (A, B, C). These results were observed in the absence of changes in the EPM index of general exploratory activity (D). Vertical bars represent the mean \pm SEM. Horizontal hatched bars represent the 95% confidence interval for the mean from these subjects on EPM testing. This way of data presenting was chosen because all groups performed equally ($F_{3,29} \leq 1.21$; $P \geq 0.32$). The asterisk indicates a significant difference ($P < 0.05$) from respective controls (two-way ANOVA followed by Newman–Keuls test).

Fig. 5. Min-by-min analysis of cobalt (experiments 1 and 3), atenolol, scopolamine or AP5 (experiment 4) effects on the percentage of time spent in the open-arms of rats tested or retested in the elevated plus-maze (EPM). On EPM testing, all drugs infused into the prelimbic cortex increased this parameter relative to respective controls. Its lower level exhibited on EPM retesting, however, was only affected by cobalt. Interestingly, the cobalt-treated group behaved on retesting as did the vehicle-treated group on testing. Lines represent the mean. The asterisk indicates a significant decrease ($P < 0.05$) from the first min in the same vehicle-treated group (one-way repeated-measures ANOVA followed by Newman–Keuls test).

ing drugs than cobalt (e.g. muscimol) might have interfered with the EPM aversive memory consolidation.

Although not hindering acquisition or consolidation of aversive memory, PL cortex inactivation virtually abolished the further avoidance to open-arms exhibited by rats during the EPM retesting. Interestingly, as the cobalt-treated group behaved on retesting as did the vehicle-treated group on testing, this result may imply that activity in PL cortex is critical for retrieving previously learned information that serves to fine-tuning the anxiety response level on the subsequent exposure to the same potential threat situation. Alternatively, a simple reduction in anxiety expression, as demonstrated on testing, could explain this result. In any case, consistent with our result are those showing a significant increase in Fos expression in this brain region of

rats retested in the EPM when compared to the level found on testing (Albrechet-Souza et al., 2008, 2009). Besides PL cortex, another study has associated the dorsal hippocampus with this process (Bertoglio et al., 2006). Participation of these regions would be expected, as long as they deal with contextual, motivational, and mnemonic information required for determining circumstances in which it is proper to exhibit defensive learned responses. Activity in PL cortex and dorsal hippocampus also appears to be decisive for retrieval of learned fear since their selective lesion reduces freezing to a tone and/or a context that had been previously paired with footshocks (Sanders et al., 2003; Matus-Amat et al., 2004; Blum et al., 2006; Sierra-Mercado et al., 2006; Corcoran and Quirk, 2007). Another important neural substrate for this latter process is

Table 3. Retesting data from groups infused into the prelimbic cortex with vehicle, atenolol (10 nmol), scopolamine (20 nmol) or AP5 (6.0 nmol) prior to elevated plus-maze testing. Data are presented as mean \pm SEM

	Vehicle (n=10)		Atenolol (n=8)		Scopolamine (n=9)		AP5 (n=7)	
	Testing	Retesting	Testing	Retesting	Testing	Retesting	Testing	Retesting
%OAT	15.8 \pm 3.4	1.6 \pm 1.2*	36.6 \pm 6.0	1.3 \pm 0.6*	30.5 \pm 4.9	2.3 \pm 0.9*	28.2 \pm 4.6	1.9 \pm 1.0*
OAE	4.4 \pm 0.9	0.6 \pm 0.4*	9.3 \pm 1.4	0.6 \pm 0.3*	6.8 \pm 1.7	1.0 \pm 0.4*	5.6 \pm 1.2	0.6 \pm 0.4*
SAPs	8.5 \pm 1.4	8.7 \pm 1.4	5.4 \pm 1.5	9.7 \pm 0.9	5.0 \pm 0.8	4.5 \pm 1.0	7.6 \pm 1.2	5.1 \pm 1.3
EAE	8.2 \pm 1.1	7.8 \pm 1.4	6.3 \pm 0.7	9.0 \pm 1.1	6.7 \pm 0.9	5.6 \pm 0.9	7.9 \pm 1.1	5.0 \pm 0.8

% OAT, percentage of open-arms time; OAE, open-arms entries; SAPs, stretched-attend postures; EAE, enclosed-arms entries; * $P < 0.05$ versus the respective group on testing (two-way repeated-measures ANOVA followed by Newman Keuls test).

amygdala (Maren, 2001; Paré et al., 2004). However, its role in the further avoidance to open-arms observed on EPM retesting remains elusive.

Antagonizing the PL cortex adrenergic beta-1 receptors with atenolol (10 nmol), cholinergic muscarinic receptors with scopolamine (20 nmol), or glutamatergic NMDA receptors with AP5 (6.0 nmol), increases the open-arms exploration on EPM testing. This anxiolytic-like effect corroborates that of previous studies in which the central infusion of these antagonists, in the dose range including those tested here, selectively attenuated the expression of anxiety (Nascimento Häckl and Carobrez, 2007; Do Monte et al., 2008). Scopolamine effects on anxiety-related behavior, however, are not well understood. For instance, systemic or intra-hippocampal administration of scopolamine increased anxiety-related behaviors in the light–dark test (Smythe et al., 1996; Hughes et al., 2004), while it reduced fear responses in the olfactory fear conditioning paradigm (Kroon and Carobrez, 2009). Once it is assumed that central infusion of any drug in animals offers information with respect to the function of a neurotransmitter in a specific brain region (Klinkenberg and Blokland, 2010), our results suggest that this dose of scopolamine produced a selective anti-aversive effect when infused into the PL cortex. This assumption is supported by the fact that vehicle- and scopolamine-treated rats behaved equally during the EPM retesting, exhibiting further avoidance to open-arms (Table 3).

There was no change in open-arms exploration of rats administered with atenolol (10 nmol), scopolamine (20 nmol), or AP5 (6.0 nmol) into the PL cortex prior to the EPM retesting. This suggests the occurrence of one-trial tolerance, a phenomenon which has been associated with the retrieval of aversive memory acquired on EPM testing (Rodgers et al., 1996; Bertoglio and Carobrez, 2004; Stern et al., 2008). Because these groups demonstrated further avoidance to open-arms on retesting, present results support this idea. It is also suggested that the recruitment of other receptors than those antagonized may be relevant to regulating the further avoidance to open-arms exhibited by rats. Although the latter assumption does not exclude the involvement of adrenergic beta-1, cholinergic muscarinic and glutamatergic NMDA receptors in other brain regions mediating the expression of learned anxiety (Bertoglio and Zangrossi, 2006; Do Monte et al., 2008; Kincheski and Carobrez, 2010), it contrasts with which one observed after PL cortex inactivation. This pattern of response would be supposed, in so far as the antagonism of specific receptors not always causes a similar effect as impairing the whole synaptic transmission by means of cobalt infusion (Kretz, 1984). In any case, it agrees with findings from studies in which the dorsal hippocampus was investigated: temporary inactivation of this region counteracts the further avoidance to open-arms demonstrated on EPM retesting (Bertoglio et al., 2006), but antagonism of NMDA receptors by AP5 (6.0 and 24 nmol) has no effect on it (Nascimento Häckl and Carobrez, 2007). Concerning the aversive memory acquisition, intra PL-cortex infusion of atenolol, scopolamine or AP5 before testing in the EPM did not

interfere with the further avoidance to open-arms exhibited by rats on retesting. Given that adrenergic and cholinergic mechanisms have been associated with this process (Bertoglio and Carobrez, 2004; Stern et al., 2008), our findings suggest that the critical brain region responsible for these effects may lie outside PL cortex.

CONCLUSION

The present results substantiate the evidence implicating the PL cortex in anxiety, and suggest that activity in this brain region also appears to be necessary for adjusting the level of open-arms avoidance demonstrated by rats retested in the EPM. We have further shown that PL cortex recruits adrenergic beta-1, cholinergic muscarinic and glutamatergic NMDA receptors to regulate open-arms exploration in the EPM.

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