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ORIGINAL INVESTIGATION

Reciprocal activation/inactivation of ERK in the amygdala and frontal cortex is correlated with the degree of novelty of an open-field environment

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Abstract
Rationale Phosphorylated extracellular signal-regulated kinase (ERK) has been used to identify brain areas activated by exogenous stimuli including psychostimulant drugs.
Objective Assess the role of the amygdala in emotional responses.
Methods Experimental manipulations were performed in which environmental familiarity was the variable. To provide the maximal degree of familiarity, ERK was measured after removal from the home cage and re-placement back into the same cage. To maximize exposure to an unfamiliar environment, ERK was measured following placement into a novel open field. To assess whether familiarity was the critical variable in the ERK response to the novel open field, ERK was also measured after either four or eight placements into the same environment. ERK quantification was carried out in the amygdala, frontal cortex, and the nucleus accumbens.
Results After home cage re-placement, ERK activation was found in the frontal cortex and nucleus accumbens but was absent in the amygdala. Following placement in a novel environment, ERK activation was more prominent in the amygdala than the frontal cortex or nucleus accumbens. In contrast, with habituation to the novel environment, ERK phosphors declined markedly in the amygdala but increased in the frontal cortex and nucleus accumbens to the level observed following home cage re-placement.
Conclusions The differential responsiveness of the amygdala versus the frontal cortex and the nucleus accumbens to a novel versus a habituated environment is consistent with a reciprocal interaction between these neural systems and points to their important role in the mediation of behavioral activation to novelty and behavioral inactivation with habituation.

Keywords Novel environment · Amygdala · Frontal cortex · ERK · Habituation · Locomotion

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Introduction

With repeated exposure to a novel open-field environment, the locomotor activity of laboratory rats decreases. This well-documented phenomenon (Cerbone and Sadile 1994; Lubow and Kaplan 2005) has been extensively studied and is regarded as an adaptive behavioral response. Indeed, Carey and co-workers (1998) reported that even a single brief exposure to a novel environment can induce a reliable habituation effect selective to that specific environment. In as much as inhibitory habituation effects are sustained and are selective to a specific test environment, habituation is considered to be mediated by memory and has been utilized as an index of behavioral plasticity (Cerbone and Sadile 1994). In fact, this memory dimension of habituation has been used in numerous studies to assess the amnestic and mnemonic effects of drugs (de Angelis 1990; Izquierdo et al. 1993a, b; Lukaszewska 1993; Pellicano et al. 1993; Rosat et al. 1992; Sadile et al. 1979; Stoll et al. 1993; Tomaz et al. 1990; Ukai et al. 1994a,

b). While behavioral habituation to a novel environment is seemingly the most ubiquitous and simplest of associative behaviors to investigate, the neural systems that mediate this basic and critical adaptive behavior remain obscure.

Numerous studies have implicated the amygdala in the mediation of emotional memory processes particularly in fear and anxiogenic response mechanisms (Dunsmoor and Paz 2015; Gargiulo et al. 1996; Tomaz et al. 1993). Seemingly, the arousal and anxiogenic impact of placement in a novel open-field environment would be expected to activate the amygdala. In large part, however, the contribution of the amygdala to emotional behavior has been provided by studies using Pavlovian aversive conditioning protocols (Debiec et al. 2013; Fanselow and LeDoux 1999; Phillips and LeDoux 1992; Rogan et al. 1997; Schafe and LeDoux 2000). In order to expand the behavioral analysis of the environmental stimuli that activate the amygdala, we undertook to assess the response of the amygdala to the degree of novelty of an open-field environment. We used extracellular signal-regulated kinase protein (ERK) activation to assess the response of the amygdala to an open-field environment. ERK has been increasingly used to identify brain areas activated by various drug and environmental manipulations. The ERK underlies cellular response to stimuli, transmission of mitogenic signals, adaptive responses to environmental changes, gene expression, protein synthesis, and memory (Adams and Sweatt 2002; Girault et al. 2007; Radwanska et al. 2005; Shiflett and Balleine 2011; Valjent et al. 2004; Valjent et al. 2005). The phosphorylated ERK (ERK-P) antibody has been recently developed as a marker of cellular activity. The phosphorylated ERK has been used to assess activation of brain areas by psychostimulant drugs (DiRocco et al. 2009; Fricks-Gleason and Marshall 2011; Janes et al. 2009; Li et al. 2008; Marin et al. 2009; Radwanska et al. 2005; Salzmann et al. 2003; Valjent et al. 2004; Valjent et al. 2005; Valjent et al. 2006), and consistent with the involvement of the amygdala in aversive stimulus responses, ERK activation has been reported to occur in the amygdala in association with the elicitation of aversive conditioned behavior (Bertotto et al. 2011; Radwanska et al. 2002; Schafe et al. 2000).

In the present study, we undertook to use ERK activation to assess possible amygdala involvement in response to a novel environment. It has been long known that the placement of a rat in a novel open-field arena elicits both exploratory and anxiogenic responses (File et al. 1998; Genaro and Schmidek 2000; Treit and Fundytus 1988). In light of the importance of the amygdala in the memory of emotionally significant events, we ascertained if exposure to a novel open field would activate the amygdala. In our initial experiment, we assess whether exposure to a novel open-field environment would activate the amygdala as manifested by an increase in ERK-P in the amygdala. In order to evaluate the possible effects of factors not directly related to placement in a novel open-field environment, we employed a strategy of first adapting the animals to handling prior to the initiation of the testing protocols. Our control procedure was simply to handle the animals and subsequently place them back into their home cages so that the animals were exposed to handling, but the environment exposure was unchanged. After finding that placement into the novel arena but not placement back into the home cage increased ERK in the amygdala, we subsequently undertook a more extensive experiment to determine if this ERK activation initiated by placement in a novel environment would subside as the environment became familiar. In this experiment, there were four groups. The home cage control group and three additional groups that received one, four, or eight placements in the open field. In order to evaluate the habituation to the test environment, locomotor activity was measured during each open-field test. This present report details the effects of these test environment manipulations upon ERK activation in the amygdala when compared to the frontal cortex and another sub-cortical structure, the nucleus accumbens.

Methods

Subjects

Male Wistar albino rats provided by the State University of North Fluminense, initially weighing 200–300 g, were housed in individual plastic cages (25 × 18 × 17 cm) until the end of the experiment. Food and water were freely available at all times. The vivarium was maintained at a constant temperature (22 + 2 °C), humidity controlled, and a 12/12 h light/dark cycle (lights on at 0700 h and off at 1900 h). All experiments occurred between 9:00 and 14:00 h. For 7 days prior to all experimental procedures, each animal was weighed and handled daily for 5 min. All experiments were conducted in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Apparatus and environmental context of drug treatment

The behavioural measurements were conducted in a black open-field chamber (60 × 60 × 45 cm). A closed-circuit video camera (SONY, model IR575M), mounted 60 cm above the arena was used to record behavioral data. Locomotion, measured as distance traveled (m), was automatically analyzed by EthoVision software (Noldus, The Netherlands). The complete test procedure was conducted automatically without the presence of the experimenter in the test room. All behavioral testing was conducted under dim red light to avoid the possible aversive quality of white light and to enhance the contrast between the white subject and dark background of the test chamber. Testing under red light conditions is less stressful and also favors locomotor activation as the rats are transferred

from the ambient light of the vivarium to the red light of the testing room (Nasello et al. 1998). Masking noise was provided by a fan located in the experimental room that was turned on immediately prior to placing the animal in the experimental arena and turned off upon removal of the animal from the experimental arena (i.e., test chamber).

Experimental behavioral testing protocols

Experiment 1—home cage re-placement versus novel environment placement

One day after the 7-day handling protocol, one group of animals ($n=6$) was briefly removed from their home cage and then returned to the same home cage. Thirty minutes after being returned to their home cage, the animals were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.), perfused transcardially, and their brains removed and stored for immunohistochemical ERK-P analysis. The second group ($n=6$) was removed from their home cage and placed in a novel open-field environment and after 30 min were euthanized and their brains prepared for ERK-P analysis.

Experiment 2—repeated exposures to a novel test environment

Four groups of rats ($n=6$) were used. One group served as the home cage control group similar to experiment 1. Three separate groups of animals ($n=6$) were placed in the experimental arena, and locomotor activity was measured for 30 min. One group received one 30-min test in the novel open-field environment, the second group received four daily 30-min placements in the same open-field environment, and the third open-field group received eight 30-min tests in the same open-field environment. Immediately after completion of the 30-min home cage re-placement or the final arena test for the three open-field environment groups, the animals were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.), perfused transcardially, and the brains removed and stored for immunohistochemical ERK-P analysis.

Immunohistochemistry

The immunohistochemistry protocol was conducted by following an experimental protocol from Sanguedo and coworkers (2014). The animals were perfused with 0.9 % saline solution (100 ml) and then with 4 % paraformaldehyde (500 ml) in 0.1 M sodium phosphate buffer (pH 7.4) for 30 min. The brains were removed and post-fixed for 2 h in 4 % paraformaldehyde solution before transfer to 20 % sucrose in 0.1 M sodium phosphate buffer (pH 7.4) for 48 h at 4 °C. Brains were placed on an aluminum paper base and cryoprotected by a solution of water-soluble glycols and resins (Tissue Tek® O.C.T. Sakura Finetek®, USA). The brains were then frozen in liquid nitrogen, until being processed for immunohistochemistry. For immunohistochemistry, sections were rinsed three times for 10 min in phosphate-buffered saline (PBS) and placed in blocking buffer (3 % normal goat serum and 0.25 % Triton X-100 in PBS) for 1 h at 22 °C. Sections were then incubated for 24 h at 4 °C in 1:500 dilution of anti-phospho-ERK antibody diluted in blocking buffer as previously described (cat # 9101, Cell Signaling Technology®, Boston, MA, USA). After the end of the incubation time, sections were washed 3 × 10 min each in PBS and incubated at 22 °C with 1:100 dilution of biotinylated goat anti-rabbit IgG secondary antibody (BA-1000, Vector Laboratories®, CA, USA) in 1 % normal goat serum and 0.25 % Triton X-100 in PBS. Sections were then washed three times for 10 min in PBS, and they were processed using an ABC Elite kit (Vector Laboratories®, Burlingame, CA, USA). In the next step, sections were washed again in PBS and processed with DAB substrate kit for peroxidase (SK-4100, Vector Laboratories®, Burlingame, CA, USA) and incubated in DAB substrate at 22 °C for 3 min for color development of signal intensity. After drying, the slides were mounted with coverslips using DPX (Sigma®, USA).

Photomicrographs of brain sections were obtained using a CCD camera (Nikon Photometrics Cool Snap, Roper Scientific Inc., Trenton, NJ) attached to a Nikon microscope using ×40, ×100, and ×200 magnification. Four brain areas were used for the immunohistochemical analysis: prefrontal cortex (medial and lateral approximately +5.64 mm from bregma), the nucleus accumbens (core and shell approximately +1.70 mm from bregma), amygdala, and the lateral hypothalamus (approximately −2.40 mm from bregma). The coordinates adopted as the reference were obtained from Paxinos and Watson (2004). The labeled nuclei in two hemispheres per rat were observed in the areas of interest and quantified using the Image J® software "multi-point" tool. Negative control slices were incubated with normal serum instead of primary antibody (data not shown).

Statistics

For locomotion, the total time of locomotor activity in the arena was divided into 12 intervals of 2.5 min each. A two-way analysis of variance (ANOVA) was used to evaluate locomotion over the 12 intervals to assess the effect of repeated exposure to the test environment. For ERK activation, a one-way ANOVA was used to evaluate the activation of ERK in different brain areas as a function of environmental exposure. To assess the effect of the number of test environment exposures on ERK in different brain areas, a two-way ANOVA was used. When a significant effect was observed, the data were further evaluated by an independent t test or by one-way ANOVA followed by the Duncan post hoc test ($p<0.05$ was the criterion for statistical significance).

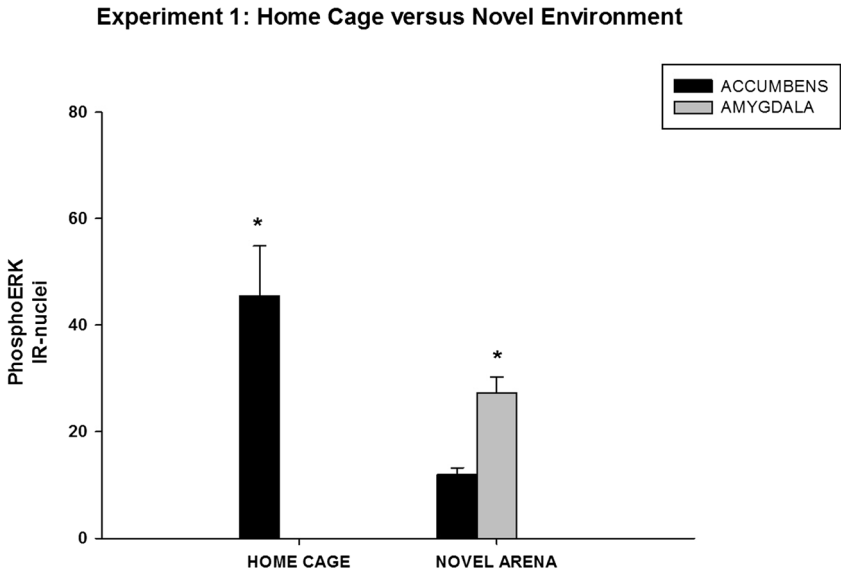

Fig. 1 Means and SEM of number of phosphor ERK immunoreactive nuclei in the nucleus accumbens and amygdala of rats 30 min after exposure to the home cage or a novel open-field test environment. *Asterisk* denotes elevated number of phosphor ERK immunoreactive nuclei ($p < 0.05$)

Results

Experiment 1

Figure 1 shows the number of phosphor ERK immunoreactive nuclei identified in the nucleus accumbens and amygdala of rats 30 min after either home cage re-placement or novel open-field environment placement. As can be seen in Fig. 1, a substantial number of ERK phosphors were identified in the nucleus accumbens but none were found in the amygdala. In contrast, Fig. 1 shows that after placement in the novel environment, a greater number of ERK phosphors were identified in the amygdala than in the nucleus accumbens ($p < 0.05$) and that the number of ERK phosphors in the nucleus accumbens decreased after novel environment exposure as compared to home cage re-placement ($p < 0.05$).

Experiment 2

Figure 2 presents the total locomotor activity scores for the three groups tested in the open-field arena on the first test day. The statistical comparison using a one-way ANOVA showed that the groups were not statistically different ($p > 0.05$). This result indicated that the groups had comparable initial responses to the open-field environment.

Figure 3 shows the number of phosphor ERK immunoreactive nuclei in the frontal cortex, nucleus accumbens, and amygdala of rats exposed to the two environments (home cage and arena test environment) and the within session locomotor activity of the three groups tested in the open-field arena. As is shown in Fig. 3, the home cage group ERK results are similar to experiment 1 in that substantial ERK phosphors were observed in the nucleus accumbens and a comparable number

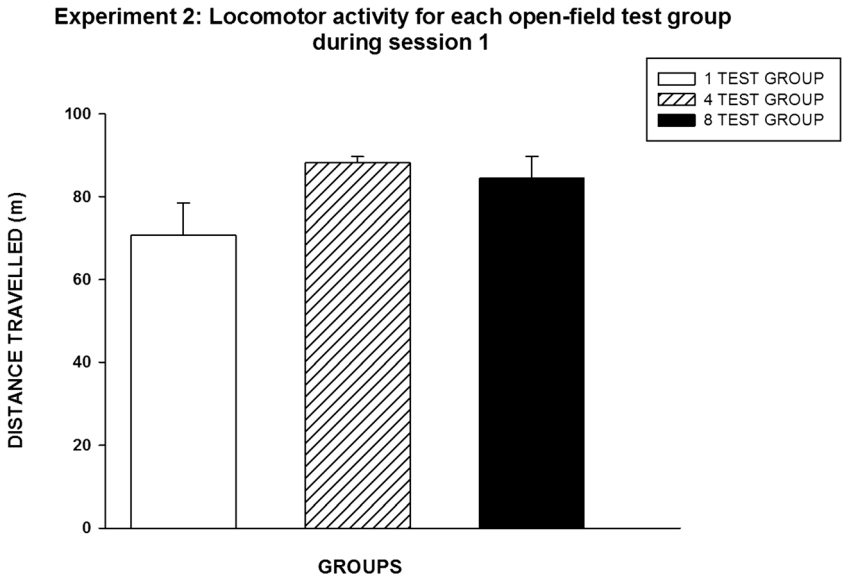

Fig. 2 Means and SEMs of locomotor activity (m) in the initial 30-min open-field test for the one, four, and eight test groups ($p > 0.05$). In this test, the open-field was a novel environment for each group

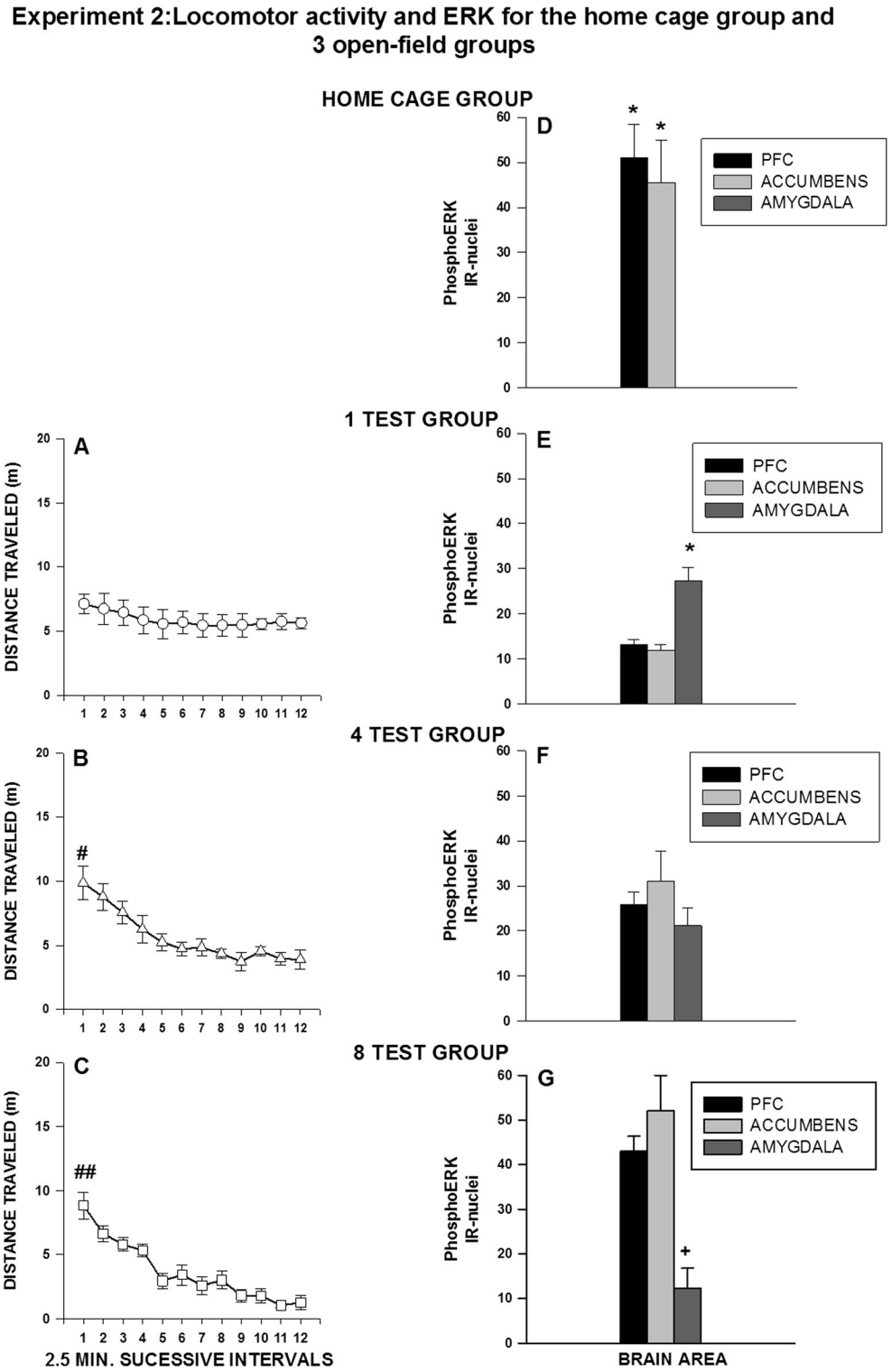

Fig. 3 The *column on the left side* of Fig. 3 (**a**, **b**, **c**) presents the means and SEMs of within session locomotion (m) for the one, four, and eight test session groups on their respective euthanasia days. The *column on the right side* of Fig. 3 presents the means and SEMs of the number of phosphor ERK immunoreactive nuclei in the prefrontal cortex, nucleus accumbens, and amygdala in the four experimental groups. The *top graph* (**d**) shows the ERK results for the home cage re-placement group and in descending order the one, four, and eight open-field placement groups (**e**, **f**, **g**). In terms of activity scores, the *number sign* denotes decreased activity relative to the 1-day group ($p < 0.01$) and *double number sign* denotes decreased activity relative to all groups ($p < 0.01$). For the ERK results, *asterisk* denotes elevated number of phosphor ERK immunoreactive counts ($p < 0.05$; ANOVA followed by Duncan's test)

were counted in the frontal cortex tissue sample but no phosphors were identified in the amygdala. A very different picture emerged for the groups placed in the open-field arena. In the group that received one placement, the highest ERK count was in the amygdala whereas the number of ERK phosphors in the frontal cortex and nucleus accumbens markedly declined from the level following home cage re-placement. As can be seen in Fig. 3, the ERK counts in the amygdala declined with repeated placements in the open-field but the counts in the frontal cortex and nucleus accumbens increased such that after eight placements, their ERK counts in the frontal cortex and nucleus accumbens returned to the levels

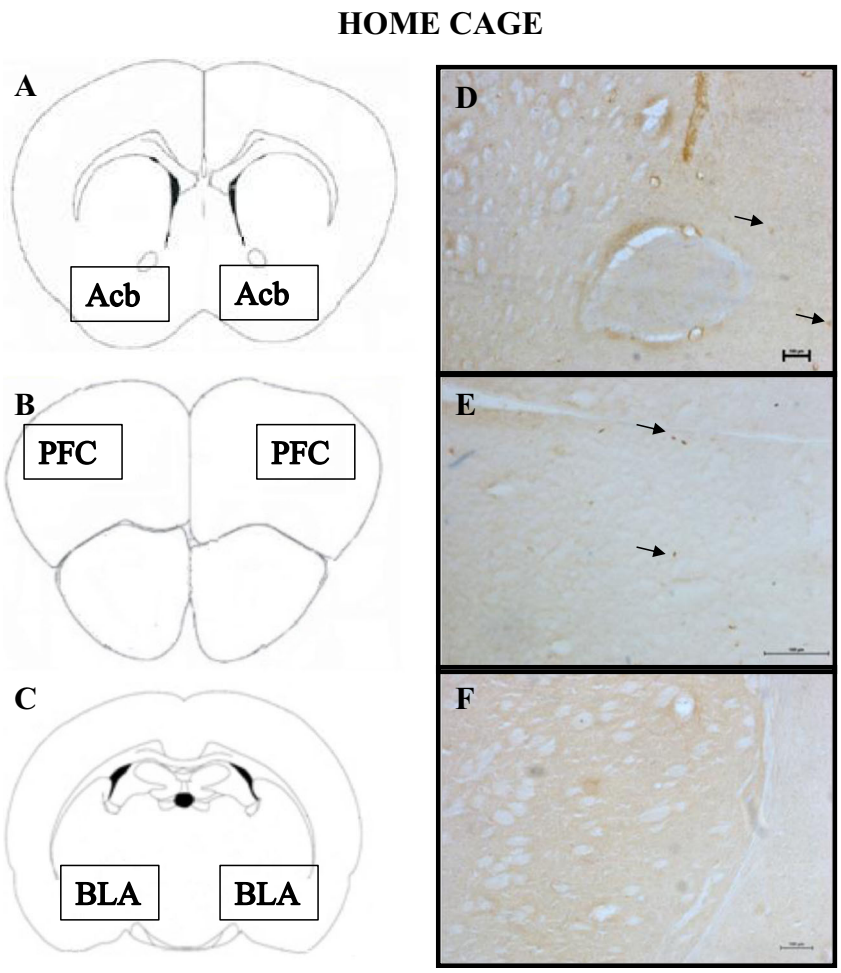

Fig. 4 Schematic representation of brain regions and immunohistochemistry images obtained after re-placement into the home cage. Schematic representation of coronal sections employed in immunohistochemistry detection for **a** nucleus accumbens, **b** prefrontal cortex, and **c** amygdala. Drawings of coronal sections and coordinates were obtained from the atlas of Paxinos and Watson (2004). The *rectangle* represents the area used for protein quantification. Representative examples of ERK-P-immunoreactive cells observed in **d** the nucleus accumbens, **e** prefrontal cortex, and **f** amygdala. *Arrows* indicate immunoreactive cells

observed after home cage re-placement. The changes in ERK counts in the different brain areas as a function of number of open-field placements were evaluated using a two-way ANOVA. The statistical analysis showed an interaction of day of test versus brain areas [F (6, 72) = 10.40; $p < 0.01$], an effect of brain areas [F (2, 72) = 18.20; $p < 0.01$], and an effect of day of test [F (3, 72) = 7.25; $p < 0.01$]. To further analyze the effect of habituation on the number of phosphor ERK immunoreactive nuclei in different brain areas, a one-way ANOVA was used for each brain area across the days of testing. For the prefrontal cortex, the results showed that the number of phosphor ERK immunoreactive nuclei increased with habituation to the environment, i.e., the number of phosphor ERK immunoreactive nuclei were statistically not different in the home cage and the 8-day arena groups but both groups had higher ERK counts than the 1- and 4-day arena groups ($p < 0.05$). For the nucleus accumbens, the results also showed a similar pattern of results. In contrast, for the amygdala, the results showed that the number of phosphor ERK immunoreactive nuclei was highest in the 1-day open-field group when the environment was maximally novel but declined with repeated exposure to the environment ($p < 0.05$).

Figure 3 also shows the scores for locomotor activity of the one, four, and eight arena placement groups. In order to present the habituation as expressed in locomotion that occurred with repeated placements in the open field, the within session activity for day 1 of the one exposure group and the fourth and eighth placements for the four and eight placement groups are shown. As is apparent in Fig. 3, the four and eight placement groups exhibited a progressive habituation to the open-field as manifested in the within session decreases in activity on sessions 4 and 8 relative to the within session activity scores for the one exposure test group. A repeated two-way ANOVA indicated a day X interval interaction [F (22, 165) = 2.70; $p < 0.01$], an effect of days [F (2, 15) = 6.70; $p < 0.01$], and an effect of intervals [F (11, 165) = 19.0; $p < 0.01$].

Figures 4 and 5 show the schematic number of ERK-P-immunoreactive cells in the prefrontal cortex, nucleus accumbens, and amygdala of rats exposed to the home cage and after one, four, and eight placements in the open-field arena.

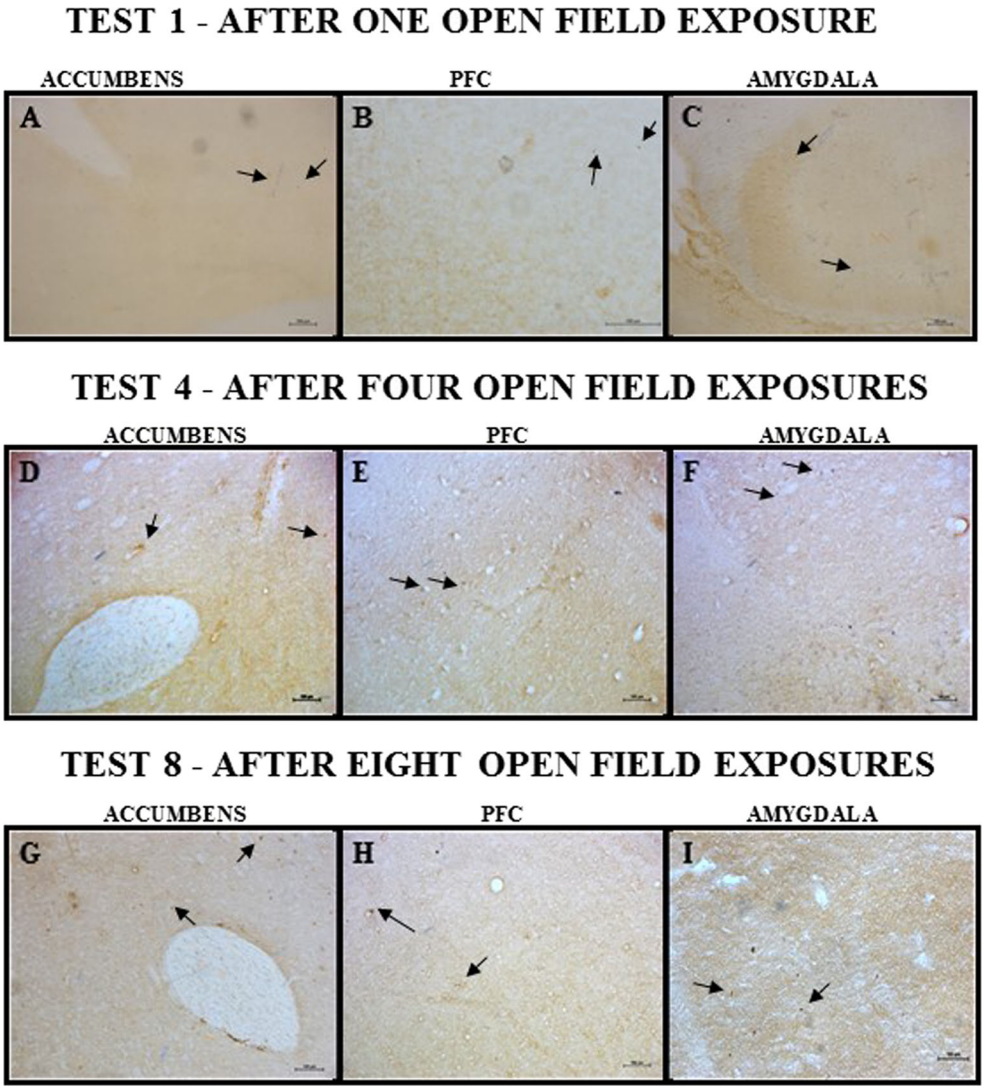

Fig. 5 Schematic representation of brain regions and immunohistochemistry images obtained for the one, four, and eight test groups. Representative examples of ERK-P-immunoreactive cells obtained for **a** the nucleus accumbens, **b** prefrontal cortex, and **c** amygdala. *Arrows* indicate immunoreactive cells

Discussion

The ERK results showed that the amygdala was highly sensitive to a novel environment. Whereas prominent ERK activation was observed 30 min following handling and return to the familiar home cage in the other two brain areas sampled namely the frontal cortex and nucleus accumbens, no detectable ERK activation was found in the amygdala. However, 30 min following exposure to a novel open-field environment, ERK activation was most prominent in the amygdala. With repeated exposures to the open field, locomotor activity decreased indicative of habituation. In parallel with the increased familiarity with the open-field environment, ERK activation in the amygdala declined but increased in the frontal cortex and the nucleus accumbens. In fact, the increase in ERK in the frontal cortex and nucleus accumbens by the eighth placement in the open field was comparable to the level observed following placement in the home cage. This latter finding is consistent with a high degree of familiarity to the open field by the eighth exposure.

In that repeated exposure to an open field typically leads to decreased activity and behavior indicative of adaptation, acclimation, and diminished stress and anxiety (Carey et al. 2005a, b), the decline we observed in ERK activation in the amygdala suggests that the relative decrease in amygdala activation with repeated exposures to the open field may reflect a diminished anxiogenic response (Davis 1992; File 1985; Graeff et al. 1993; Killcross et al. 1997; LeDoux 2007; Perez de la Mora et al. 2012) to the increasingly familiar open-field environment. In the present study, the open-field testing was conducted under conditions of red light illumination in order to diminish the aversive impact of the open field (Nasello et al. 1998). Interestingly, the red light environment favors locomotion over the typical indicator of fear (freezing) (Nasello et al. 1998). In many reports of amygdala involvement in emotional behavior, the amygdala involvement is

associated with freezing behavior (LeDoux 2000; Pape and Pare 2010). It is also possible to consider the high level of locomotion as a manifestation of a flight response. Seen in this light, the freezing/flight responses can be viewed as fear responses, and when considered in this way, the present results could be integrated into the established role of the amygdala, a key brain structure mediating an animal's response to a fear stimulus.

In the present report, we observed strong locomotor response that persisted throughout the first exposure to the novel environment and this behavior was associated with the maximal ERK response in the amygdala. This result indicates that amygdala activation is not specific to response inhibition. An alternative possibility is that this locomotor activation represents a vigorous exploratory response. In that there is evidence indicating that the amygdala can be activated by novel stimuli (Schwartz et al. 2003), ERK activation of the amygdala observed in the present study could be as a response to exposure to the novelty feature of the open-field environment. Indeed, in CPP testing, a novel environment is preferred to a familiar environment even when possible anxiogenic factors are eliminated (Klebaur and Bardo 1999). Another way to assess the response of an animal to novel stimuli in an open-field test environment conducted using red light is to habituate the animals and then activate the animals using white light (Amato et al. 2015; Pum et al. 2011). In future studies, it will be important to induce habituation under red light conditions and then introduce white light to ascertain if the light-induced behavioral activation induces ERK activation in the amygdala. In that this light induced activation has been shown to be non-aversive, this would point to an important role for the amygdala in an animal's response to a novel sensory stimulus (Pum et al. 2009). If ERK activation is selectively increased in the amygdala by white light illumination, this line of evidence would be critical information pointing to the amygdala in the mediation of behavioral responses to novel stimuli and thereby implicate the amygdala in non-fear-related processes. Thus, the open-field methodology in combination with ERK offers new opportunities to assess the involvement of the amygdala more broadly in behavior.

The neuroanatomical connections between the frontal cortex and the amygdala have been well documented (McDonald 1998). and there have been a number of reports of the interaction between the amygdala and the frontal cortex (Fisher et al. 2009; Laviolette et al. 2005; Quirk et al. 2003; Stevenson 2011). The inverse relationship between amygdala ERK activation and frontal cortex ERK activation is certainly consistent with an interaction between the frontal cortex and the amygdala. Seemingly, the high level of arousal evoked by the novel environment can be seen as activating the amygdala and basic instinctual behavioral responses, but that with repeated exposure to the environment without any untoward consequences, this instinctual response activation extinguishes. In fact, the frontal cortex has been implicated in the mediation of extinction of fear responses by inhibition of the amygdala (Pape and Pare 2010; Phelps et al. 2004) so that the decrease in amygdala ERK in conjunction with an increase in frontal cortex ERK is consistent with the frontal cortex inhibition of amygdala activation as a contributing mechanism to permit adaptation and habituation to a new environment. We also observed that the nucleus accumbens ERK changes mirrored those for the frontal cortex and as has been shown by Bassareo and co-workers (2002), there is a close relationship between the frontal cortex and the nucleus accumbens shell.

In conclusion, the present report demonstrated the activation of the amygdala as manifested by ERK to a novel environment. Undoubtedly, a novel environment evokes a strong emotional response. This finding of amygdala activation with exposure to a novel open field is consistent with the importance of the amygdala in emotional behavior. Whether this emotional response reflects an intense exploratory response, a flight response, or a complex mix of exploratory behavior and anxiety remains to be determined. Regardless of the uncertainty of the emotional label assigned to behavior in a novel open field, the evident activation of the amygdala by a novel open field provides a new perspective regarding neural mechanisms implicated in an animal's response to a novel environment and suggests that the interplay between the amygdala and the frontal cortex plays a critical role in an animal's initial response and subsequent adaptation to new environments.

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Compliance with ethical standards The experiments were performed in compliance with the recommendations of Brazilian Society of Neuroscience and Behavior (SBNeC), which are based on the US National Institutes of Health Guide for Care and Use of Laboratory Animals.

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