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Alterations in fear conditioning and amygdalar activation following chronic wheel running in rats

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Abstract

Several convergent lines of evidence point to the amygdala as a key site of plasticity underlying most forms of fear conditioning. Studies have shown that chronic physical activity, such as wheel running, can alter learning in a variety of contexts, including aversive conditioning. The ability of chronic wheel running (WR) to alter both behavioral correlates of fear conditioning and indices of amygdalar activation, however, has not been simultaneously assessed. Here, rats were given constant access to either free-turning or–as a control–locked (LC) running wheels in their home cages. After 8 weeks of housing under these conditions, animals were exposed to a series of shocks in a separate testing chamber. Twenty-four hours later, the animals were returned to the shock chamber and freezing behavior was measured as an indicator of contextual fear conditioning. The animals were then sacrificed and their brains processed for immunohistochemical detection of Fos to assess patterns of putative neuronal activation. WR rats spent significantly more time freezing than their LC counterparts upon return to the shock-paired context. The enhanced conditioned freezing response was most pronounced in animals showing high levels of nightly wheel running activity. WR animals also had significantly higher levels of neuronal activation, as indicated by Fos expression in the central nucleus of the amygdala, but less activation in the basolateral nucleus, compared to sedentary controls. These data demonstrate the ability of chronic physical activity to alter contextual fear conditioning and implicate the amygdala as a potential site of plasticity underlying this phenomenon.

Keywords: Amygdala; Anxiety; Calcium/calmodulin kinase; Exercise; Fear conditioning; Fos

1. Introduction

The amygdala is essential for several forms of fear conditioning (Davis et al., 1994; Maren, 1999; McGaugh, 2002; Rosen, 2004). Exposure to both conditioned (CS) and unconditioned stimuli (US) increases expression of the immediate early gene (c-*fos*) product Fos in a variety of brain structures including the amygdala (Beck and Fibiger, 1995; Campeau et al., 1991; Radulovic et al., 1998; Rosen et al., 1998). Re-exposure to a contextual CS previously paired with a shock increases Fos expression in the central (CeA) and basolateral (BLA) nuclei, but not the medial nucleus (MeA) (Holahan and White, 2004) of the amygdala. The significance of these findings is underscored by a wealth of data implicating the BLA as the anatomical substrate for the formation of associations between the sensory stimuli from the CS and US, although other regions are known to be involved depending on the modality of the conditioned stimulus (McGaugh, 2002; Pare, 2003; Pare et al., 2004). The CeA, in turn, appears to be a crucial effector of amygdala-dependent behavioral and physiological responses to conditioned stimuli (Pare et al., 2004; Pitkanen et al., 1997). Collectively, these findings strongly suggest that subregion-specific changes in amygdalar activity underlie altered behavioral responses to a fear-conditioned CS.

Robust physical activity produces numerous neurochemical and physiological adaptations and alters behavioral responding in several tests that evaluate learning. Wheel running, for example, enhances hippocampal long-term potentiation and improves learning in the water maze (Fordyce and Wehner, 1993; van Praag et al., 1999). Furthermore, long periods of

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wheel running decrease footshock-elicited Fos expression in several regions involved in learned helplessness and decrease freezing in a shuttle box context after footshock (Greenwood et al., 2003). Chronic wheel running also modifies anxiety-related and defensive behaviors (Burghardt et al., 2004), which may involve amygdalar pathways. Finally, a recent study indicates that relatively short periods (3 weeks) of access to running wheels alters contextual fear-conditioned freezing in rats (Van Hoomissen et al., 2004). Since contextual fear conditioning is known to involve the amygdala and chronic wheel running alters Fos expression in response to a noxious US, these experiments examined the effects of long-term wheel running on the expression of contextually-conditioned freezing behavior and the induction of Fos-like immunoreactivity (Fos-li) in the amygdala. Double-label immunohistochemistry for Fos-li and calcium/calmodulin-dependent protein kinase II α-subunit [CaMKII], parvalbumin, and enkephalin was used to begin identifying the neuronal population(s) activated in the amygdala following fear-conditioning. We hypothesized that wheel runners would show enhanced levels of Pavlovian fear conditioning, along with altered patterns of Fos activation in the amygdala.

2. Methods

2.1. Subjects and behavior

Male Sprague–Dawley rats (Harlan), weighing approximately 175 g upon arrival, were singly housed in an environmentally controlled animal facility on a 12:12 light/dark cycle with standard rat chow and water available ad libitum. All animal care and experimentation protocols were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of South Carolina Animal Care and Use Committee.

Rats were randomly assigned to wheel runner (WR, $n=20$) or locked wheel control (LC, $n=20$) groups upon arrival. Wheel runners were individually housed in standard polypropylene cages (45×28×20 cm) with 24 h access to a running wheel system (Nalgene); housing conditions were identical for LC animals, except the running wheel was locked (Burghardt et al., 2004). Running data was assessed using the Mini Mitter data collection system (Mini Mitter, Inc., Bend, OR) connected to a Windows-based PC. For each WR animal, distance run over a 24 h period was calculated from the summed number of turns per bin (20 min bins; lights on to lights on) multiplied by the running wheel circumference (108 cm). Animals were handled twice per week by the investigators during cage changing and were weighed weekly for the duration of the 8 weeks of wheel access.

The conditioned freezing protocol was adapted from that described by Fanselow (1980) on animals that had been tested 1 week earlier in the elevated plus maze and open field tests. This order of testing has been shown to impose the least amount of carryover between paradigms (McIlwain et al., 2001). The rats were placed in a box (46×24×22 cm; equipped with stainless steel floor rods spaced 1.9 cm apart) located inside a sound-attenuated chamber equipped with ventilating fans. After a 3-min exploration period a scrambled shock (1 mA for 1 s) was delivered to the floor grid by a solid-state shock source (Coulborn Instruments; Allentown, PA) followed by 2 additional shocks of equal duration and amplitude, each separated by a 60 s inter-shock interval (ISI). Rats were removed from the boxes after 6 min and returned to their home cages. Rats were returned to the context 24 h later for 8 min. Freezing behavior–defined as the absence of non-respiration related movements–was scored from videotape by a rater blind to treatment. As a control, a subset of animals from both WR and LC groups ($n=4$/group) received equivalent handling and exposure to the contextual conditioning environment, but without shock. All experiments were conducted during the light phase.

2.2. Immunohistochemistry

Two hours after re-exposure to the shock chamber animals were anesthetized with isoflurane and transcardially perfused with phosphate-buffered saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After post-fixation and cryoprotection, serial 45 μm sections through the rostro-caudal extent of the amygdalar complex were cut on a cryostat and processed for single (Fos) or double (Fos+calcium/calmodulin-dependent protein kinase II α-subunit [CaMKII], Fos+parvalbumin or Fos+enkephalin)-labeled immunohistochemistry using a two-color immunoperoxidase method (Fadel et al., 2002). All sections were initially incubated in goat anti-Fos antiserum (1:3000; 48 h at 4 °C; Santa Cruz Biotechnology, Santa Cruz, CA), followed by a biotinylated donkey anti-goat secondary antibody (1:1000; Jackson Immuno Research Laboratories, Inc., West Grove, PA) and horseradish peroxidase-conjugated streptavidin (1:1600; Jackson). Fos-li was visualized by developing the sections in a nickel–cobalt intensified diaminobenzadine (DAB) solution with hydrogen peroxide, yielding a blue-black reaction product in Fos-positive nuclei. One set of tissue from each brain was slide-mounted and analyzed for single-labeled Fos counts. Remaining sets of tissue from each brain were incubated at 4 °C for 48 h with one of the following primary antibodies: (1) mouse anti-CaMKIIinase α-subunit (1:500; Upstate, Waltham, MA), (2) mouse anti-parvalbumin (1:4000; Sigma, Saint Louis, MO) or (3) rabbit anti-enkephalin (1:2000; Immunostar, Inc. Hudson, WI). Sections were then incubated in species-appropriate unlabeled secondary antibodies (1:100; Jackson) followed by mouse or rabbit peroxidase anti-peroxidase (1:250; Sternberger Monoclonals, Inc., Lutherville, MD). CaMKII-, parvalbumin- or enkephalin-li was visualized by developing the sections in a plain DAB solution, resulting in light brown immunoreactive cell bodies. Single (Fos-li) and double-labeled (Fos+CaMKII, Fos+parvalbumin, Fos+enkephalin) cells were counted in two representative sections from the CeA (lateral and medial divisions) and BLA from each animal using a Nikon E600 microscope. The anterior-posterior levels of the portions of these nuclei (relative to Bregma) used for counting corresponded approximately to −2.3 to −2.8 mm for CeA and −2.8

to −3.1 mm for BLA (Paxinos and Watson, 1998). Single-labeled Fos nuclei were counted using a reticle encompassing an area of 0.1225 mm^2 at 20× magnification. For double-labeling, total numbers of phenotypically-described neurons were counted in two sections for each area; data were then expressed as the percentage of those neurons with a Fos-positive nucleus. Digital images were captured with a Photometrics CF monochrome CCD camera (Roper Scientific, Trenton, NJ, USA) and IP Lab Software and imported into Adobe Photoshop where minor adjustments for contrast and brightness were made.

2.3. High and low levels of spontaneous wheel running

Running data and conditioned freezing data from several groups of WR ($n=27$) and LC ($n=20$) animals were combined and statistically analyzed in a separate experiment. Wheel running data was subjected to a median split to reveal the possibility of clustered variations in amounts of daily spontaneous wheel running. The conditioned freezing responses were analyzed based on this median split of wheel running activity. These WR and LC animals were subjected to the same wheel running and conditioned fear paradigm as described above, but only a subset of these animals was used for Fos immunohistochemistry.

2.4. Statistics

Group variations in freezing behavior were determined by analysis of variance (ANOVA), with wheel running and fear conditioning (shock history) as between-subjects factors and time as a within-subjects repeated measure. ANOVA was also used to determine the effect of wheel running and fear conditioning on numbers of single- or double-labeled cells in CeA and BLA following re-exposure to the shock-paired context. The source of significant main effects or interactions was determined by post-hoc *t*-tests. All data are reported as group means±S.E.M. Significance for all statistical analyses was set at $p<0.05$.

3. Results

3.1. Wheel running behavioral data

After 8 weeks of unrestricted access to running wheels, WR rats were running an average of 7.7±0.7 km/24 h during the last 4 weeks of the experiment and weighed significantly less than LC rats (335±7 g vs. 389±11g; $t_{18}=4.2$; $p<0.001$) at the conclusion of the study. These observations and the pattern of running were consistent with those reported previously by Burghardt et al. (2004). Rats given free access to running wheels gradually increase their level of running, to plateau at 3–4 weeks. Other wheel-running studies have suggested that at least 4 weeks are necessary for animals to reach their maximum running activity (Burghardt et al., 2004; Greenwood et al., 2003; Lambert and Jonsdottir, 1998). ANOVA revealed significant effects of both wheel running ($F_{1,26}=10.5$; $p=0.003$) and fear conditioning ($F_{1,26}=165.3$; $p<0.001$) on total amount of time spent freezing when rats were returned to the shock box, with previously shocked WR rats spending a greater proportion of their time freezing compared to LC rats ($F_{3,208}=203.0$; $p<0.001$; Fig. 1). WR rats tended to have a shorter latency to begin freezing and continued to spend nearly the entirety of each 1-min post-shock interval in a freezing posture throughout the duration of the 8-min trial (Fig. 1). During the conditioning phase, the initial freezing response during the ISI following each shock did not differ as a function of wheel running history (data not shown). In control groups exposed to the context on both days (acquisition and retention) but never shocked, WR also showed more freezing than LC groups (Fig. 1; $F_{1,48}=12.8$, $p<0.001$).

3.2. Context-elicited Fos expression in the amygdala

There were significant, but opposite, effects of wheel running on conditioned fear-elicited Fos expression in CeA and BLA (Fig. 2), with WR animals showing higher numbers of Fos-li neurons in the CeA ($F_{1,8}=10.3$; $p=0.012$) and LC animals showing a greater Fos response in BLA ($F_{1,8}=39.0$; $p<0.001$) following re-exposure to the shock-paired context. Control (non-shocked) groups of WR and LC animals showed a similar divergence of Fos expression in these two nuclei as a function of running history. In both groups of animals, CaMKII was the only phenotypic marker that resulted in significant double-labeling, with the large majority of Fos expression occurring in CaMKII-positive cells in BLA and CeA (Fig. 2E and F). Neither enkephalin- (within CeA) nor parvalbumin-positive neurons (both regions) showed significant levels of Fos expression. Although a recent report has shown an exercise-induced increase in the number of detectable parvalbumin-immunoreactive neurons in the dentate gyrus (Arida et al., 2004), the absolute numbers of CaMKII-, enkephalin- or parvalbumin-li neurons in the present study did not differ as a function of wheel running history (all p's>0.35).

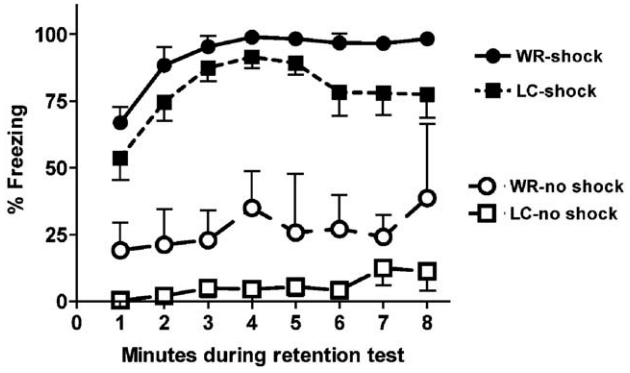

Fig. 1. Twenty-four hours following exposure to the conditioning chamber, with or without footshock, rats were tested for freezing behavior upon an 8-min re-exposure to the chamber. Rats given unrestricted access to running wheels (WR) for 8 weeks spent a greater proportion of time freezing compared to rats housed with a locked running wheel (LC), regardless of shock history.

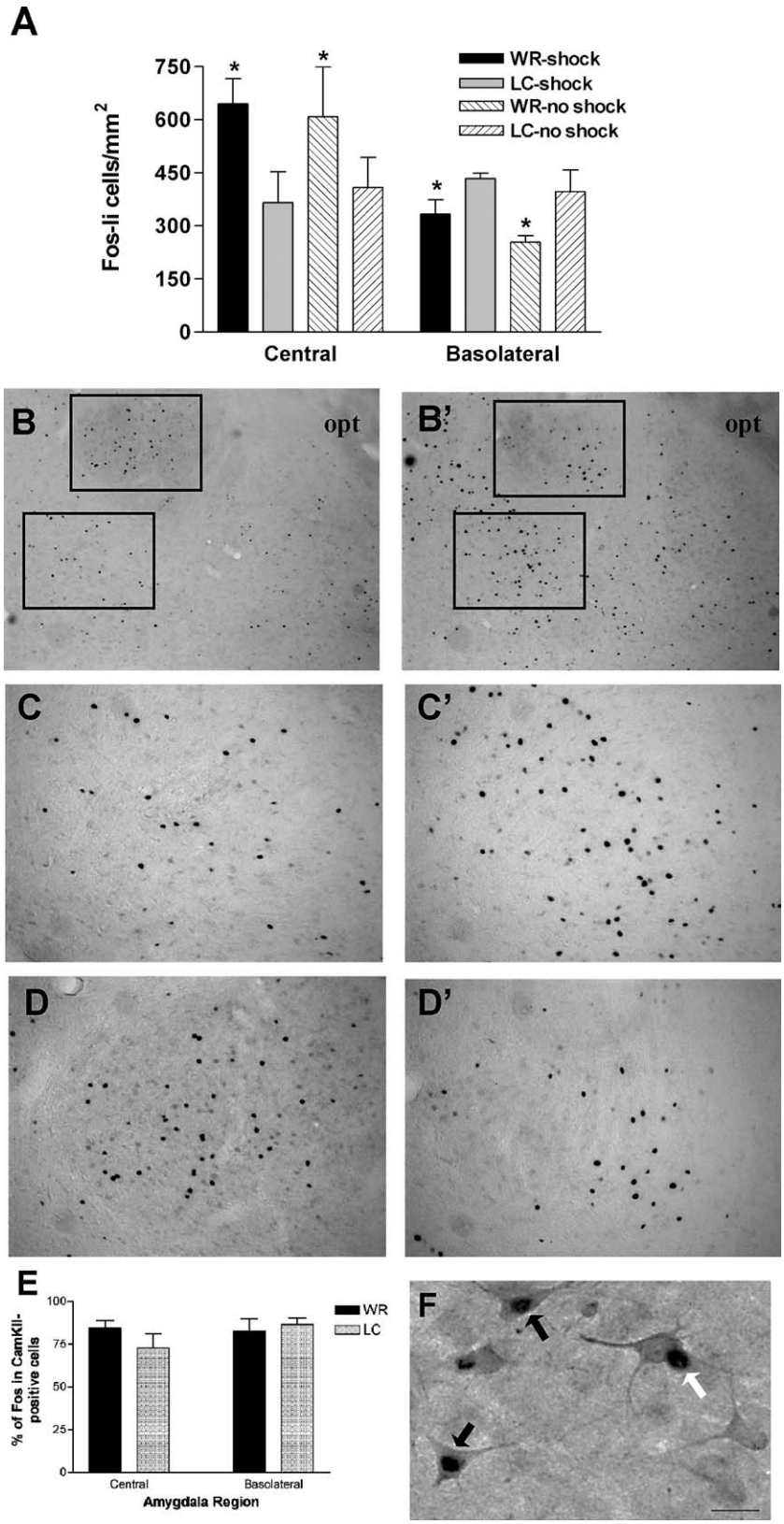

Fig. 2. Fos expression in the amygdala in wheel runner (WR) and locked control (LC) animals following re-exposure to the conditioning chamber. (A) WR rats showed higher levels of Fos expression in the central amygdala, and lower levels in basolateral amygdala, relative to their LC counterparts. These differences were observed regardless of shock history. *All p's ≤ 0.05. (B) Photomicrograph of Fos-li in CeA and BLA from a WR (B) and LC (B′) animal. opt, optic tract. Fos-li is visible as discrete nuclear labeling in individual neurons. Areas indicated by the outlined boxes are shown at higher magnification in (C) and (C′) (BLA) and (D) and (D′) (CeA). (E) In both WR and LC animals, and in both amygdalar regions, the large majority of Fos-li was colocalized with CaMKII. (F) Fos and CaMKII immunohistochemistry reveals numerous double-labeled neurons (black arrows). Infrequently, a Fos-positive nucleus was observed without a visible CaMKII-labeled soma (white arrow). Scale bar, 20 μm of the BLA. Photomicrographs taken at approximately 2.8 mm posterior to bregma (Paxinos and Watson, 1998); medial is right and lateral is left.

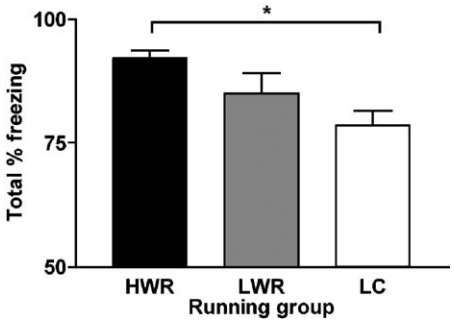

Fig. 3. Context-conditioned freezing behavior in high-running (HWR), low-running (LWR) and locked control (LC) animals. A median split of WR animals into high and low groups of spontaneous running reveals that only HWR animals show significantly enhanced freezing responses relative to sedentary controls. *$p<0.05$.

3.3. Effects of high- and low-wheel running on fear conditioning

Examination of the pattern of wheel running activity and freezing behaviors suggested a bimodal distribution pattern. We therefore combined all of our subjects (WR and LC) that had been analyzed in contextually conditioned freezing using this paradigm, including subjects included in earlier studies (Burghardt et al., 2004). A median split (6.2 km/day) revealed significantly different subpopulations of WR activity, with a high running group (HWR) that averaged 9.6±0.4 km/day and a low running group (LWR) that averaged 3.2±0.3 km/day ($t=12.4$, $p<0.001$) during the last 4 weeks of running. These groups began to diverge 3–4 weeks into the experiment, when HWR animals began to run more each night while LWR animals reached plateau. ANOVA conducted on the freezing data following return to the shock context in these three groups of animals (HWR; LWR; LC) revealed an overall group difference ($F_{2,48}=5.0$; $p<0.01$) and post-hoc analysis demonstrated that only the HWR group spent significantly more time freezing than the LC animals following return to the shock-paired context ($t=3.4$, $p<0.01$; Fig. 3). Analysis of all rats tested after 4–8 weeks of running ($n=25$) indicated a trend toward a correlation between nightly running level for the weeks just prior to testing and percent freezing when returned to the context ($r=0.38$, $p=0.06$; one rat eliminated from statistical analysis). Due to the low number of subjects tested in each individual experiment, however, correlations between running distance and freezing behavior were not significant within each experimental group.

4. Discussion

These results indicate that chronic wheel running alters the behavioral response to an environment previously paired with an aversive stimulus. WR animals also exhibited a qualitatively different pattern of neuronal activation in the amygdala following this re-exposure, implicating the amygdala as a neuronal substrate underlying wheel running-induced changes in fear conditioned behavior.

4.1. Use of Fos as a neuronal activity marker

Expression of Fos protein or c-*fos* mRNA is a widely-used marker for neuronal activation; its utility stems from its ability to allow simultaneous assessment of the effects of a stimulus on multiple brain regions and the fact that Fos can be combined with other immunohistochemical markers to provide detailed phenotypic descriptions of neuronal populations activated by such a stimulus. It has been extensively used to map functional circuitry underlying both contextual and cued fear conditioning (Campeau et al., 1997; Milanovic et al., 1998; Radulovic et al., 1998; Rosen et al., 1998). Dissociations between CeA Fos expression and fear-elicited freezing behavior have been reported (Beck and Fibiger, 1995; Holahan and White, 2004), suggesting that activation of the CeA may generally reflect responses to aversive conditioned or unconditioned stimuli. Consistent with this interpretation, we did not observe a main effect of fear conditioning on Fos expression in either the CeA or BLA, with animals showing similar levels of Fos expression following return to the context regardless of shock history. An additional explanation is that context-elicited Fos expression reflects previous learning about the context regardless of the behavioral manifestation of this association.

4.2. Significance of enhanced CeA activation in fear conditioning

WR animals returned to the shock-paired context showed significantly higher levels of freezing behavior and Fos expression in the CeA than their LC counterparts (Fig. 2). The positive association between these two variables is consistent with a long-hypothesized role of the CeA as a mediator of autonomic and behavioral correlates of conditioned fear. Recent anatomical and pharmacological evidence, however, has implicated the CeA as not just as a passive downstream effector of conditioning-associated changes in lateral and basolateral amygdalar activation, but as a site of plasticity itself (Pare et al., 2004). The CeA appears to receive direct input from brainstem and thalamic areas related to nociceptive and other sensory processing (Bernard et al., 1996; Linke et al., 2000). Furthermore, infusion of NMDA antagonists into the CeA impairs the acquisition of both auditory and contextual fear conditioning (Goosens and Maren, 2003). Finally, another recent report (Samson and Pare, 2005) has demonstrated NMDA-dependent LTP in the CeA following stimulation of thalamic inputs to CeA, suggesting a potential anatomic and physiological basis for enhanced CeA activation following fear conditioning. Our data are consistent with a model whereby wheel running may directly influence conditioning-related plastic changes in CeA output. In contrast to the enhanced CeA Fos expression, WR animals showed *decreases* in BLA Fos expression relative to LC animals. A similar phenomenon was observed in the lateral nucleus (data not shown), which also plays a crucial role in fear conditioning. This suggests that chronic physical activity may involve direct activation of the CeA by extra-amygdalar (e.g., thalamic, brainstem or cortical) regions related to sensory processing of conditioned or unconditioned aspects of even mildly aversive stimuli, in addition to modified

intra-amygdalar synaptic regulation and BLA processing of contextual cues.

4.3. Amygdalar cell populations underlying changes in fear conditioning

The major amygdalar neuronal population affected by re-exposure to the shock-paired environment, irrespective of wheel running condition, was CaMKII-positive cells. Approximately 75–80% of amygdalar Fos expression was observed in these cells, and this figure may actually represent an underestimate as the double-labeling procedure may bias against the detection of weakly-immunoreactive CaMKII neurons. CaMKII labels glutamatergic cell bodies in the BLA (McDonald et al., 2002); widespread expression of CaMKII in the CeA has also been reported (McDonald et al., 2002), although the neurotransmitter phenotype of CaMKII-positive CeA neurons has not been established. We did not see colocalization of Fos with enkephalin (a major output cell type) or parvalbumin, suggesting that Fos expression may have occurred in another major output cell population, such as the corticotropin releasing factor (CRF) neurons, of the CeA (Day et al., 1999). CRF neurons of the CeA project widely to hypothalamic and brainstem regions implicated in behavioral responses to fear-conditioned stimuli (Gray, 1993). This speculation awaits confirmation, as currently available antibodies to CRF do not reliably label CeA cells without colchicine injections, and no studies on CRF and CaMKII colocalization within the amygdala have been performed. While an exhaustive description of Fos expression within all known amygdalar cell types was beyond the scope of these experiments, the role played by CaMKII-dependent processes in behavioral and physiological correlates of amygdala-dependent learning (Maren, 1999; Moriya et al., 2000; Rodrigues et al., 2004) strongly implicates its potential involvement in the altered expression of fear conditioning seen in WR animals in the present study.

4.4. Mechanism of wheel running effects on fear conditioning

The enhanced behavioral (freezing) response to the shock-paired context seen in WR animals is suggestive of more robust associative learning in these animals. The neurobiological substrates of enhanced amygdala-dependent fear learning following chronic wheel running are unclear. One candidate is the locus coeruleus (LC), which plays an important role in fear conditioning and has reciprocal connections with the amygdalar complex, particularly the central nucleus. Noradrenergic modulation of amygdala-dependent learning has long been recognized (Davies et al., 2004; Gallagher et al., 1977; McGaugh, 2002) and chronic running alters indices of noradrenergic activity (Dishman, 1997). Furthermore, recent data have shown that wheel running-associated changes in contextual fear conditioning are blocked by chronic systemic administration of the β-adrenergic receptor antagonist propranolol (Van Hoomissen et al., 2004). In vivo electrochemistry or microdialysis experiments may yield additional insights as to whether chronic wheel running increases aversive conditioning-elicited norepinephrine release in the amygdala or other stress-related LC targets (e.g., the prefrontal cortex) during the acquisition and/or expression of contextual fear conditioning. These findings, however, must be interpreted with caution since enhanced freezing behavior was also seen in WR compared to LC groups that did not receive shock-context pairings but were re-exposed to the context. This may suggest that a generalized increase in freezing behavior is induced by wheel running.

A final possibility is that wheel running-induced alterations in pain sensitivity (Kanarek et al., 1998) contribute to enhanced behavioral expression of contextual fear conditioning; increased sensitivity to the shock stimulus could thus underlie the increased freezing response in WR animals. Arguing against this possibility, however, is the lack of difference between WR and LC animals in freezing during the acquisition phase of contextual fear conditioning (data not shown).

4.5. Implications of activity-associated changes in fear conditioning

The increased freezing response seen in WR animals suggests that chronic exercise promotes anxiety-like behaviors. Indeed, we have previously shown that rats given prolonged access to running wheels spend less time in the open arm of the elevated plus maze or in the center of an open field (Burghardt et al., 2004). An alternative explanation is that chronic wheel running facilitates the learning and/or expression of aversively-motivated adaptive behaviors or the attentional processing of fear-associated stimuli; this interpretation would be consistent with other reports showing positive effects of exercise in a variety of different cognitive tasks–such as those assessing spatial learning–with a less explicit fear component (Fordyce and Wehner, 1993; van Praag et al., 1999). The increased freezing in WR re-exposed to a context that had not been paired with shock might also support this conclusion. This interpretation is also in accord with the observed shifts in Fos expression, which suggest amygdalar processing associated with both contextual fear conditioning and re-exposure to a novel context is altered by chronic wheel access.

4.6. Individual variations in wheel running activity

Rodent studies suggest there is significant individual variation in the motivation to run and wheel running has been suggested as a model of natural reward (Sherwin, 1998). This variation appears to impact changes in conditioned fear responses following chronic wheel running, since only HWR rats showed significant differences in freezing compared to LC animals. Interestingly, this may explain our earlier results that showed a non-significant difference between WR and LC in conditioned freezing (Burghardt et al., 2004). It has been suggested that individual variations in running involve the opioid system, since transgenic mice overexpressing deltaFosB in either dynorphin- or enkephalin-containing neurons of the striatum show altered wheel running behavior (Werme et al., 2002). Studies examining Fos activation in mice bred for high voluntary running behavior, however, have suggested that different neuronal substrates might underlie

reinforcing and locomotor aspects of these individual variations (Rhodes et al., 2003). Our experimental design only tested Fos-li after the conditioned freezing test, so the influences of running alone on expression of Fos or related antigens in the amygdala cannot be assessed directly. This will be an important area of future investigation.

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