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A Role for Anterior Thalamic Nuclei in Affective Cognition: Interaction With Environmental Conditions

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ABSTRACT: Damage to anterior thalamic nuclei (ATN) is a well-known cause of diencephalic pathology that produces a range of cognitive deficits reminiscent of a hippocampal syndrome. Anatomical connections of the ATN also extend to cerebral areas that support affective cognition. Enriched environments promote recovery of declarative/relational memory after ATN lesions and are known to downregulate emotional behaviors. Hence, the performance of standard-housed and enriched ATN rats in a range of behavioral tasks engaging affective cognition was compared. ATN rats exhibited reduced anxiety responses in the elevated plus maze, increased activity and reduced corticosterone responses when exploring an open field, and delayed acquisition of a conditioned contextual fear response. ATN rats also exhibited reduced c-Fos and phosphorylated cAMP response element-binding protein (pCREB) immunoreactivity in the hippocampal formation and the amygdala after completion of the contextual fear test. Marked c-Fos hypoactivity and reduced pCREB levels were also evident in the granular retrosplenial cortex and, to a lesser extent, in the anterior cingulate cortex. Unlike standard-housed ATN rats, enriched ATN rats expressed virtually no fear of the conditioned context. These results show that the ATN regulate affective cognition and that damage to this region may produce markedly different behavioral effects as a function of environmental housing conditions. © **2013 Wiley Periodicals, Inc.**

KEY WORDS: contextual fear; enrichment; anxiety; corticosterone; rat

INTRODUCTION

Damage to the anterior thalamic nuclei (ATN) and their connections is a major cause of diencephalic amnesia (Harding et al., 2000; Van der Werf et al., 2003). These clinical cases present memory deficits that resemble those of patients with hippocampal system injury, showing severe anterograde amnesia, temporally graded retrograde amnesia, and impaired subjective memory (Kopelman et al., 1998; Hampstead and Koffler, 2009). Diencephalic pathologies, such as the Korsakoff syndrome or vascular thalamic infarcts, frequently extend beyond the ATN to adjacent limbic thalamic loci, and experimental and clinical evidence show that the mediodorsal, intralaminar, and midline thalamic nuclei are also associated with memory processes (Bailey and Mair, 2005; Mitchell and Dalrymple-Alford, 2005; Vertes, 2006; Hampstead and Koffler, 2009; Lopez et al., 2009; Loureiro et al., 2012). However, animal studies have confirmed that the pattern of memory deficits after specific ATN lesions most closely corresponds with that produced by hippocampal system lesions for many spatial and nonspatial tasks (Warburton et al., 2001; Mitchell and Dalrymple-Alford, 2005; Gibb et al., 2006; Wolff et al., 2006, 2008a; Savage et al., 2011). The ATN thus appear to constitute the main thalamic contributor to hippocampal-dependent memory functions and are considered a critical node in an extended hippocampal system (Aggleton and Brown, 1999, 2006).

The ATN also provide one of the main sources of thalamic afferents to several regions considered to support affective cognition, including the anterior cingulate cortex (ACC; Gabriel et al., 1989; Shibata, 1993b) and the amygdala (van Groen et al., 1999; Reznikov et al., 2008). This pattern of efferent projections suggests that the role of the ATN extends beyond memory. Further support for this hypothesis arises from the observation that the ventral subdivision of the hippocampal formation is associated with emotion, stress, and affect (Esclassan et al., 2009b; Fanselow and Dong, 2010). For instance, lesions of the ventral hippocampus reduce anxiety, whereas lesions of the ventral subiculum have been suggested to have the opposite effect (Herman et al., 1998; Bannerman et al., 2004; Barkus et al., 2010). Early experimental studies suggested that the interconnected circuitry formed by cingulate cortex, limbic thalamus, and hippocampal formation has fundamentally similar functions in both approach and avoidance learning (Gabriel et al., 1987; Freeman et al., 1996). The

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unique position of ATN in this neural network suggests a role beyond memory for the ATN, which may therefore also directly influence affective cognition.

To examine the hypothesis that the functions of the ATN extend to the regulation of emotional processes, rats with ATN lesions received open field testing, exposure to elevated plus maze (EPM), and contextual fear conditioning. Plasma corticosterone was examined after open field exposure to provide an endocrine index of emotional reactivity to this mild stressor. Neuronal activation was assessed after a contextual fear memory test by immunohistochemical analyses of the phosphorylated cAMP response element-binding protein (pCREB) and c-Fos activity. We used these markers to examine neuronal activation in the hippocampus and the amygdala as both structures support contextual fear memory, as shown by lesion (Phillips and LeDoux, 1992, 1994; Anagnostaras et al., 1999; Gale et al., 2004; Esclassan et al., 2009b) and imaging studies (Hall et al., 2001; Hubbard et al., 2007; Trifilieff et al., 2007). Concerning the hippocampal formation, the dorsal CA1 is currently thought to convey contextual information, whereas the ventral CA1 may be associated with emotional processing. We also examined the ventral subiculum because, as opposed to CA1, it receives direct ATN afferents and may also support fear conditioning (Maren, 1999). For the amygdala, the basolateral amygdala (BLA) appears to be more specifically associated with contextual processing than the lateral amygdala (LA; Calandreau et al., 2005; Trifilieff et al., 2007; Onishi and Xavier, 2010; Sauerhofer et al., 2012) so that the latter may constitute an ideal control. Marked c-Fos hypoactivity within the retrosplenial cortex has been demonstrated as a prominent hallmark of ATN lesions (Jenkins et al., 2004; Poirier et al., 2008; Poirier and Aggleton, 2009; Amin et al., 2010; Dumont et al., 2012), and thus, this region was also carefully analyzed. Moreover, we provide a comprehensive description of c-Fos activity in different subregions of the medial prefrontal cortex and especially the ACC.

The expression of emotional behavior is strongly dependent on environmental conditions. Indeed, enriched environments (EEs) are known to downregulate emotional behaviors and to exert therapeutic and protective (anxiolytic) effects against impending threat or a wide range of stressors (Fox et al., 2006). EEs provide partial recovery of impaired spatial memory after ATN lesions (Loukavenko et al., 2007; Wolff et al., 2008b). We therefore examined the effects of ATN lesions under both standard and enriched conditions and tested whether emotional behaviors in ATN rats would also be influenced by an enriched postoperative environment.

METHODS

Animals, Housing Conditions, and Surgery

A total of 102 male Long Evans rats obtained from the Centre d'Elevage Janvier (France) were used (weight 275–325 g at surgery). Rats were initially housed in pairs [standard environment (Std)] and accustomed to the laboratory facility for 2 weeks before the beginning of the experiments. The facility was maintained at 21°C ± 1°C with lights on from 7 a.m. to 7 p.m., and rats were tested only during the light portion of the cycle. After surgery and the first session of open field testing, rats that were housed in EE (25 days) were grouped with 12 cage mates in large wire-mesh cages (85 cm × 60 cm × 30 cm high) with all the enrichment objects changed daily, and the position of food and water and cage placement in the colony room varied every 2–4 days (Loukavenko et al., 2007; Wolff et al., 2008b).

Rats were anesthetized with 4% isoflurane and placed in a stereotaxic frame with atraumatic ear bars (Kopf, Tujunga, CA) in a flat skull position. Anesthesia was maintained with 1.5–2% isoflurane complemented by subcutaneous administration of diazepam (0.2 mL of Valium© per rat) and Carprophen (5 mg/kg; Norocarp©). Neurotoxic ATN lesions were made using multiple N-methyl-D-aspartate (NMDA) microinjections. NMDA (20 μg/μL; Sigma-Aldrich) in artificial cerebrospinal fluid (CMA Microdialysis AB, Solna, Sweden) was pressure injected into the brain through a glass micropipette (outside diameter: around 100 μm) and polyethylene tubing (Picospritzer, General Valve Corporation, Fairfield, NJ). Two lesion sites per side were used: AP −1.4 mm from bregma, laterality ±1.2 mm, and ventrality −5.4 mm from dura; and AP −1.5 mm, laterality ±1.5 mm, and ventrality −5.3 mm. Each site was injected with 0.12 μL of NMDA, and the pipette was left in place 3 min after injection before slow retraction. The Sham groups received similar surgery except that the micropipette was lowered only in the cortex and no injection was made (ventrality −2.5 mm). Rats were given at least 10 days of recovery before behavioral testing. The following four groups received behavioral testing: Sham-Std ($n = 19$), Sham-EE ($n = 18$), ATN-Std ($n = 22$), and ATN-EE ($n = 24$). An additional nine Sham-Std and 10 ATN-Std rats were used to determine baseline levels of plasma corticosterone (home-cage animals).

Apparatus and Behavioral Testing

Open field

Each rat was individually placed for a 10-min session in a circular open field filled with bedding (1 m diameter, 50 lux at the center). The 30-cm high wall was made of clear Perspex, and numerous distal and proximal cues were provided to encourage rats to explore this environment. A videotracking system (Viewpoint, Lyon, France) recorded the path length (in centimeters) travelled by rats, with activity analyzed using 1-min blocks. Based on the path length of the pre-enrichment test, matched pairs of rats within each group were allocated to the subsequent EE and Std groups. Rats were thus examined in the open field test before enrichment and then, after differential housing (either standard or enriched housing), exploration was compared either in the same ("familiar") or a novel room, using different groups of rats. Exploring an already familiar environment was expected to be less anxiety inducing and to reduce exploratory behaviors. Hence, there were two independent subsets of rats for the

postenrichment test: "Familiar": Sham-Std ($n = 12$), Sham-EE ($n = 8$), ATN-Std ($n = 13$), and ATN-EE ($n = 10$); "Novel": Sham-Std ($n = 7$), Sham-EE ($n = 8$), ATN-Std ($n = 9$), and ATN-EE ($n = 4$).

Elevated plus maze

A plus-shaped maze was elevated 70 cm above floor level with two open arms (10 cm in width × 48 cm in length) and two closed arms with 17-cm high walls. The light level was 65 lux at the 10 cm × 10 cm junction of the open and closed arms. Individual rats were placed on the center of the maze facing an open arm, and the time spent in open and closed arms as well as the path length were recorded during a 5-min period using the videotracking system.

Contextual fear conditioning

Conditioning was conducted in eight identical chambers (40 cm × 35 cm × 30 cm; Imetronic, Pessac, France) made of gray polyvinyl chloride on three sides and transparent perspex on the door. Each chamber was located inside a sound-attenuating cubicle with a roof-mounted loudspeaker and a ventilation fan providing background noise of ∼55 db. Behavior was recorded from above through a wide-angle (2.5) mini camera (SK-2005, Opto Vision, Toulouse, France). The grid floor (27 parallel 0.5-cm-diameter stainless-steel bars, 1.5 cm apart placed above a sawdust tray) delivered mild foot shocks (US, 1 s, 0.4-mA scrambled pulses). The computerized (Imetronic) conditioning procedure consisted of unpaired delivery of five tones (5,000 Hz, 70 dB, 10 s in duration) and five electric shocks (the contingency was irregular) over an 8-min period (no stimuli for the first 2 min). This procedure promotes conditioning to the context (Phillips and LeDoux, 1994; Esclassan et al., 2009a,b). The context test took place 24 h later by placing rats in the same conditioning chamber for 10 min without any stimuli. Automated video analysis of freezing behavior during both conditioning and test was performed using homemade software (Marchand et al., 2003; Esclassan et al., 2009a,b).

Blood sampling and corticosterone assay

Blood (∼400 μL) was collected either under basal conditions (rats from home cages with no behavioral testing) or 20 min after completion of the open field task (both before and after enrichment), only in rats that were tested in the familiar context test after enrichment. Blood was collected by tail incision on awake rats previously habituated to gentle restraint in a towel (Fluttert et al., 2000). Blood samples were centrifuged (10,000 rpm at 4°C), and the plasma was stored at −80°C for later enzyme immunoassay (Correlate-EIA, Assay Designs, Ann Arbor, MI) to measure plasma corticosterone.

Histology and immunohistochemistry

Animals received a lethal dose of sodium pentobarbital and were perfused transcardially with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The sections throughout the ATN region were collected onto gelatin-coated slides and dried before being stained with thionine. Histological analysis was performed under the microscope by an experimenter (M.W.) blind to both lesion and housing conditions. A subset of rats was killed 60 min after completion of the context test for immunohistochemistry, whereas the remainder of the rats was subsequently used in a separate behavioral experiment not reported here. After confirmation of lesion status, concurrent c-Fos and pCREB immunohistochemistry was conducted from Sham-Std ($n = 8$), Sham-EE ($n = 7$), ATN-Std ($n = 8$), and ATN-EE ($n = 5$), except that two rats in the ATN-Std group could not be processed for pCREB immunostaining due to a technical problem. Therefore, this group size was six instead of eight for pCREB immunohistochemistry.

Fifty-micrometer-thick sections were collected using a vibratome (Leica) and stored at −20°C in a cryoprotective solution (30% ethylene glycol, 30% glycerol, and 0.1 M PB) until processed. pCREB and c-Fos immunostaining followed the same protocol. After rinsing, free-floating sections were incubated with 0.5% H_2O_2 in 0.1 M Tris buffer (TB) with 0.9% of saline (TBS) to inhibit endogenous peroxidase. After blocking in TBS containing 3% bovine serum albumin, 2% normal goat serum, and 0.2% Triton X-100, tissue sections were incubated at 4°C for 48 h with rabbit primary polyclonal antibody for the active/phosphorylated form of CREB (anti-pCREB, 1:2,000; Upstate Biotechnology, Lake Placid, NY) or c-Fos protein (anti-c-Fos, 1:10,000; Oncogen). After TBS washes, sections were incubated in biotinylated goat anti-rabbit IgG (1:2,000; Jackson Immunoresearch) for 2 h at room temperature. After further rinsing in TBS, sections were incubated in avidin-biotinylated horseradish peroxidase complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) for 2 h at room temperature. After washing with TB, reaction products were revealed with diaminobenzidine (0.025% in TB) in the presence of 0.03% H_2O_2 for 10 min. The reaction was stopped by TB and 0.1 M PB washes. The sections were then mounted on gelatin-coated slides, dehydrated, and coverslipped with Eukitt.

Quantitative analysis (Biocom Visiolab 2000 image analysis, V4.50) was made by an experimenter blind to the groups. Each region was counted bilaterally with a 10× objective in at least three consecutive sections per animal (the rostrocaudal distance between consecutive sections was 0.2 mm) to generate an average for positively stained nuclei per rat. pCREB and c-Fos immunoreactivities were expressed as the mean number of immunopositive nuclei per millimeters squared. Immunoreactivity was examined in several regions of interest (Fig. 1), including the hippocampal formation [dorsal and ventral (CA1–dCA1) and ventral subiculum (vCA1)], the amygdala (LA and BLA), and the different subdivisions of the medial prefrontal cortex [including infralimbic (IL) and prelimbic (PL) cortices] with a particular emphasis on the ACC that was examined at the level of areas Cg1 and Cg2. We finally examined the superficial (Layer II and upper III) and deep laminae (Layers III and IV) of the granular retrosplenial cortex (RSGb) as the border between these laminae is easily recognized by an

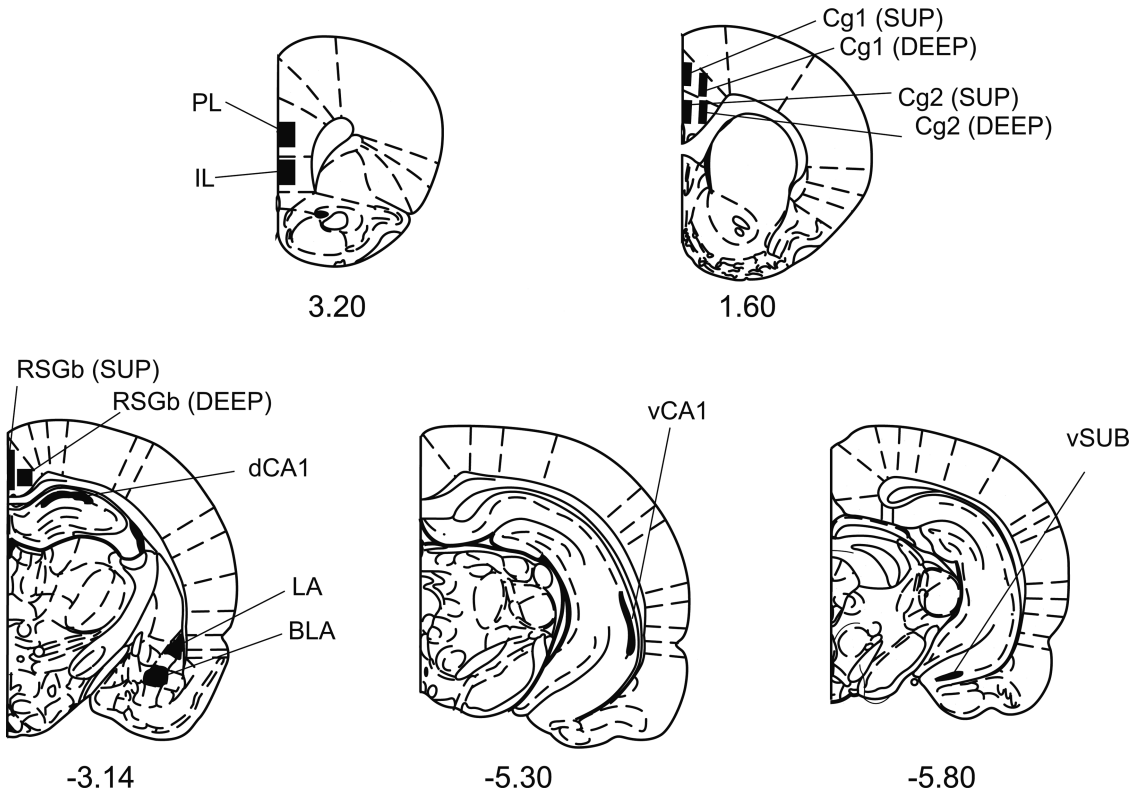

FIGURE 1. Regions of interest for c-Fos and pCREB immunohistochemistry. Anteroposterior coordinates are given relative to bregma (millimeters). Adapted from G. Paxinos and W. Watson, *The Rat Brain in Stereotaxic Coordinates*, 1998, Academic Press, San Diego. Abbreviations: PL/IL, prelimbic/infralimbic cortex; Cg1/Cg2, area Cg1 and Cg2 of the anterior cingulate cortex; RSGb, granular retrosplenial cortex; dCA1/vCA1, dorsal/ventral CA1; vSUB, ventral subiculum; BLA/LA, basolateral/lateral amygdala. (Adapted from *The Rat Brain in Stereotaxic Coordinates*, 4th edition, Paxinos and Watson. Copyright 1998, with permission from Elsevier.)

abrupt change in cell size and packing density (Jenkins et al., 2004; Poirier and Aggleton, 2009).

RESULTS

Histology

The ATN lesions are shown in Figure 2. Four ATN-Std (two from those used for baseline corticosterone assay) and eight ATN-EE rats had only minimal damage to the ATN and were discarded from further analysis. Two Sham-EE rats became sick and were euthanized during the experiment and were also discarded. The ATN lesions were highly specific and comparable with previous work (Loukavenko et al., 2007; Wolff et al., 2008b). Damage to other nontarget thalamic structures including midline nuclei was generally minimal, with the exception of the interanteromedial nucleus, the centromedial, and the parataenial nucleus; there was minor damage to the laterodorsal nucleus and negligible damage for paraventricular and posterior paraventricular nuclei, anterior paraventricular nucleus, reuniens and rhomboid nuclei. The thin glass micropipette caused no detectable mechanical injury to the fornix, and hence, the latter does not influence the behavioral profile of ATN rats reported here (see Fig. 2A). The final groups were as follows: Sham-Std (n = 19), Sham-EE (n = 16), ATN-Std (n = 22), and ATN-EE (n = 14). Another group of nine Sham-Std and eight ATN-Std rats were used for the specific baseline corticosterone assay.

Open Field

An ANOVA was performed on path length data using Lesion (Sham, ATN), Housing (Std, EE), Session (Open field session, before and after enrichment), Context (Familiar, New), and Time (10 min) as main factors. There was a significant interaction between Lesion, Housing, and Context [$F(1,63) = 5.13$, $P < 0.05$]. We therefore first analyzed open field activity prior to differential housing and then compared the two test conditions after enrichment. There was a Lesion × Context interaction across the latter two conditions [$F(1,63) = 6.63$, $P < 0.05$], and therefore, additional analyses examined activity in the familiar and the novel contexts separately across groups.

Before Enrichment

Prior to differential housing (Fig. 3A), ATN lesions substantially increased the overall level of activity in the open field [$F(1,63) = 21.00$, $P < 0.0001$]. However, all rats exhibited habituation over time [Lesion × Time, $F < 1.0$; Time, $F(9,567) = 42.15$, $P < 0.0001$]. As expected, there were no

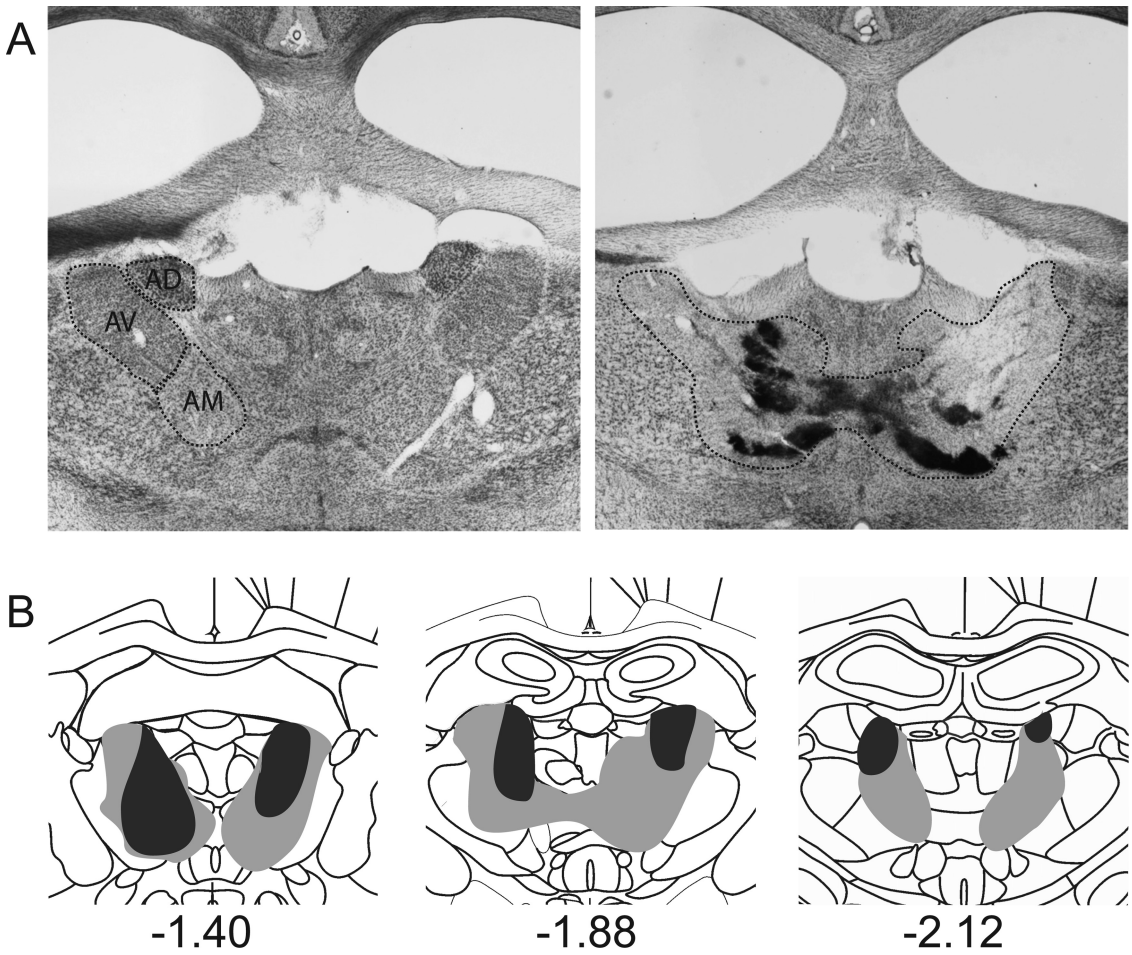

FIGURE 2. A: Photomicrographs showing the ATN region in a Sham (left) and a lesioned rats (right). Anterodorsal (AD), anteroventral (AV), and anteromedial (AM) damage is evident on that example (boundary of the lesion depicted by the dotted line), but the fornix is intact. B: Representation of the biggest and smallest ATN lesions indicated at three different levels of the anteroposterior axis (indicated in millimeters relative to bregma). These lesions both occurred in the standard-housed group.

differences between rats that were later placed in different housing conditions or between those to be tested later in either the familiar or novel room [Housing, $F < 1$; Context, $F(1,63) = 1,47$, $P > 0.2$; Context × Housing, $F(1,63) = 1.61$, $P > 0.2$; Lesion × Context, $F(1,63) = 1.41$, $P > 0.2$; Lesion × Context × Housing, $F < 1$].

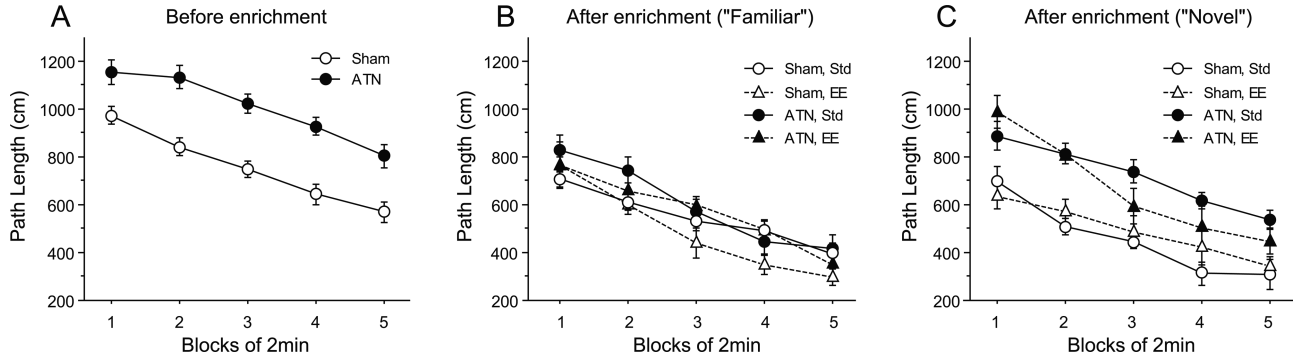

FIGURE 3. Exploration of an open field (10-min session). Habituation curves are expressed as distance travelled (path length, cm) over time (2-min blocks). Pre-enrichment exposure to the open field shows slower habituation for ATN rats (A). Postenrichment assessment of open field exploration was realized either in the same room (Familiar; B) or in a different room (Novel; C) on two independent subset of rats that were derived from the original batch (A).

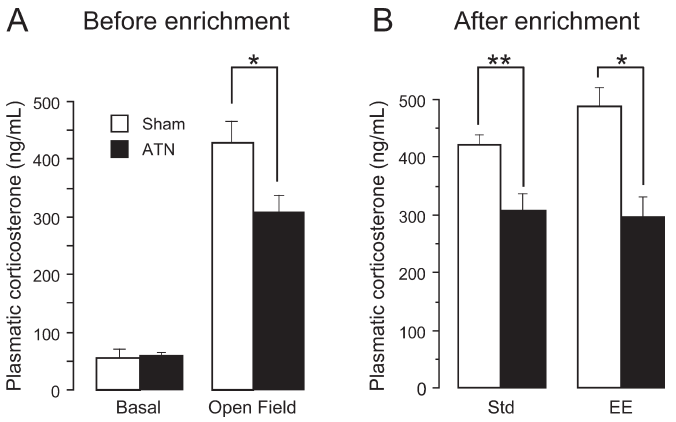

FIGURE 4. Plasma corticosterone (nanograms per milliliter) pre-enrichment (A) or postenrichment (B) 20 min after completion of open field exploration. Lesion effect: *$P < 0.05$; **$P < 0.01$.

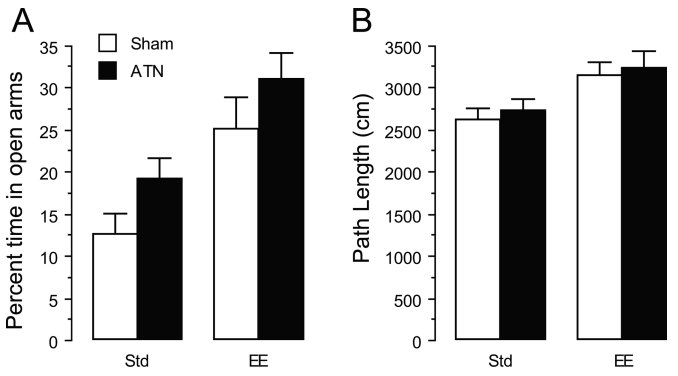

FIGURE 5. Performance during the 5-min session in the elevated plus maze. The anxiety index (A) corresponds to the proportion of time spent in the open arms as follows: (Time spent in open arms)/(Time spent in open arms + Time spent in closed arms) × 100. A low index indicates anxiety. Path length (centimeters) corresponds to the total distance travelled by the rat during the entire session (B).

After Enrichment

When tested in the same room after the period of differential housing (Fig. 3B), no significant differences emerged across the groups [Lesion, $F(1,39) = 3.02$, $P = 0.09$; Housing $F < 1$, Housing × Lesion, $F < 1$], and activity declined again across time in all groups [$F(9,351) = 34.27$, $P < 0.0001$].

For those rats tested in a novel context (Fig. 3C), however, increased exploratory activity was again evident in the ATN lesion rats [$F(1,24) = 34.86$, $P < 0.0001$], irrespective of housing [Housing, $F < 1$; Housing × Lesion interaction, $F(1,24) = 1.38$, $P > 0.2$]. Rats again showed marked habituation across time [$F(9,216) = 26.41$, $P < 0.0001$].

Altogether these results thus clearly indicate a strong lesion effect with ATN rats being much more active than their Sham counterparts when the spatial context is new, both on a first occasion and when tested for a second time but in a novel environment after 25 days of differential housing. This effect was not affected by enrichment and appeared to be specifically associated with the exploration of a novel environment.

Plasma Corticosterone Assay

Basal values of corticosterone in home-cage rats not given any behavioral tests (Fig. 4A, Basal) were assessed in an independent experiment with a different batch of rats. Basal values were low and did not differ as a function of lesion status ($F < 1$). The analysis of rats tested in the open field (Fig. 4A, Open Field) revealed lower corticosterone levels in the ATN group when compared with Sham rats [$F(1,38) = 5.23$, $P < 0.05$].

After enrichment (Fig. 4B), the reduced corticosterone response in rats with ATN lesions was even more evident [Lesion, $F(1,36) = 22.83$, $P < 0.0001$], whereas housing had no apparent effect [$F < 1$; Lesion × Housing, $F(1,36) = 1.42$, $P > 0.2$]. The plasma corticosterone response to open field exploration was therefore consistently reduced in ATN rats but not further modified by enrichment.

Elevated Plus Maze

ATN lesions increased the relative time spent in the open arms, consistent with reduced anxiety [Lesion effect, $F(1,67) = 4.77$, $P < 0.05$; Fig. 5A], irrespective of Housing condition (Lesion × Housing interaction, $F < 1$). Enrichment also significantly elevated the time spent in the open arms [Housing effect, $F(1,67) = 19.75$, $P < 0.0001$]. Although ATN lesions did not increase the overall activity in the EPM ($F < 1$), enriched rats showed an increase in this measure [Housing effect, $F(1,67) = 12.45$, $P < 0.001$] irrespective of lesion status (Lesion × Housing, $F < 1$; Fig. 5B).

Contextual Fear Conditioning

Conditioning

As shown in Figure 6A, all groups showed acquisition of freezing behavior during the conditioning session [$F(7,469) = 311.56$, $P < 0.0001$]. ATN lesions slowed acquisition of this task [Lesion, $F(1,67) = 18.66$, $P < 0.0001$; Lesion × Time, $F(7,469) = 7.51$, $P < 0.0001$], as did enrichment [Housing, $F(1,67) = 10.54$, $P < 0.01$; Housing × Time, $F(7,469) = 8.01$, $P < 0.0001$]. There was no Lesion × Housing or Lesion × Housing × Time interactions (All Fs < 1), which suggests that both the lesion and enrichment elicited similar but independent effects on freezing behavior.

Context test

Although both Sham and ATN Std groups initially showed substantial levels of freezing and a gradual extinction of this response, EE rats exhibited marked behavioral differences on this occasion (Fig. 6B). The Sham-EE group began with the same level of freezing shown by both Std groups; however, this initial response was followed by a much faster rate of extinction. Surprisingly, the ATN-EE group exhibited only low level of freezing from the start of the session. These observations were confirmed

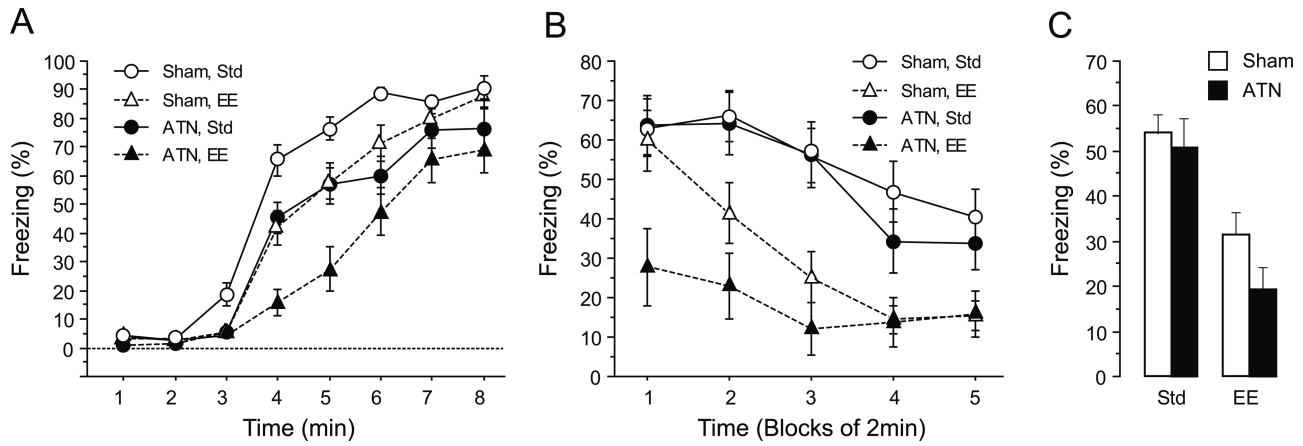

FIGURE 6. Contextual fear conditioning. During the conditioning procedure, freezing responses are represented over time (8 min; A). The 10-min context test occurred 24 h later. Freezing is expressed either as 2-min blocks (B) or overall means (C).

by the lack of an overall Lesion effect [$F(1,67) = 1.85$, $P > 0.1$] but a highly significant effect of Housing [$F(1,67) = 19.71$, $P < 0.0001$] as well as significant Time × Housing [$F(9,603) = 2.14$, $P < 0.05$] and Time × Lesion × Housing [$F(9,603) = 2.89$, $P < 0.01$] interactions. When the effect of the lesion was assessed separately in standard and enriched groups, the Time × Lesion interaction was highly significant for enriched rats [$F(9,252) = 4.18$, $P < 0.0001$]. However, this interaction was absent for rats housed in standard conditions ($F < 1$), confirming that the effect of ATN lesions was highly dependent on housing conditions. The observation that the initial freezing response from the ATN-EE group differed from that of the three other groups was confirmed by an analysis restricted to the first block of 2 min of the test, which showed a significant effect of Housing [$F(1,67) = 5.80$, $P < 0.05$] and Lesion × Housing interaction [$F(1,67) = 4.05$, $P < 0.05$]. Thus, housing did not affect the initial freezing response of Sham rats ($F < 1$); however, it had a pronounced influence on that of ATN rats [$F(1,34) = 8.71$, $P < 0.01$]. The ATN-EE group exhibited a unique behavioral profile at this occasion as these rats expressed virtually no fear of the conditioned context.

Immunohistochemistry

Immunohistochemistry for c-Fos and pCREB activities were examined 60 min after completion of the context test (Fig. 7). Representative pictures of c-Fos and pCREB immunostainings within the medial prefrontal cortex, dCA1, and the amygdala are provided in Figure 8.

Amygdala (BLA/LA)

c-Fos and pCREB levels in the amygdala were examined at the level of the basolateral (BLA) and the lateral nuclei (LA). Both markers produced a very similar pattern of staining even though c-Fos was only weakly activated. Although the lesion reduced both c-Fos [$F(1,24) = 15.42$, $P < 0.001$; Fig. 7A] and pCREB [$F(1,22) = 4.44$, $P < 0.05$; Fig. 7B] counts in the BLA, these effects were not significant in the LA [c-Fos: $F(1,24) = 2.97$, $P > 0.1$; pCREB: $F(1,22) = 2.76$, $P > 0.1$]. Enrichment did not affect c-Fos or pCREB immunoreactivity in either region [BLA, c-Fos: $F < 1$, pCREB: $F(1,22) = 1.2$, $P > 0.2$; LA, c-Fos: $F(1,24) = 1.64$, $P > 0.2$, pCREB: $F < 1$]. There were no Lesion × Housing interactions [c-Fos: All Fs < 1; pCREB: LA, $F < 1$, BLA, $F(1,24) = 2.71$, $P > 0.1$).

Hippocampal formation (dCA1/vCA1/ventral subiculum)

Experimental treatments differentially affected c-Fos and pCREB immunostainings in the CA1 region of the dorsal and the ventral hippocampus. In the dorsal CA1, the lesion did not affect c-Fos levels ($F < 1$; Fig. 7C); however, enrichment significantly reduced c-Fos levels [$F(1,24) = 5.14$, $P < 0.05$; Fig. 7D] in both Sham and ATN rats. c-Fos in the ventral CA1 was not affected either by the lesion [$F(1,24) = 1.51$, $P > 0.2$] or by enrichment ($F < 1$). By contrast, pCREB levels were reduced by the lesion in both areas [dCA1: $F(1,22) = 9.57$, $P < 0.01$; vCA1: $F(1,22) = 5.49$, $P < 0.05$]. The Housing main effect was not significant for either hippocampal area; however, the Lesion × Housing interaction was close to significance for dCA1 [$F(1,22) = 3.97$, $P = 0.06$]. Although enrichment substantially increased pCREB counts in Sham rats [$F(1,13) = 5.33$, $P < 0.05$], we found no evidence of such an effect in ATN rats ($F < 1$). These observations are consistent with the view that pCREB expression in the dorsal hippocampus may be differentially affected by enrichment in Sham and ATN rats.

However, in the ventral subiculum, a lesion effect was evident with c-Fos [$F(1,24) = 7.94$, $P < 0.01$], and a similar nonsignificant trend was also observed with pCREB expression [$F(1,22) = 3.37$, $P = 0.08$]. Enrichment did not significantly affect those markers [c-Fos, Housing, Lesion × Housing, $F < 1$; pCREB, Housing, $F(1,22) = 3.03$, $P = 0.09$, Lesion × Housing, $F(1,22) = 1.06$, $P > 0.3$].

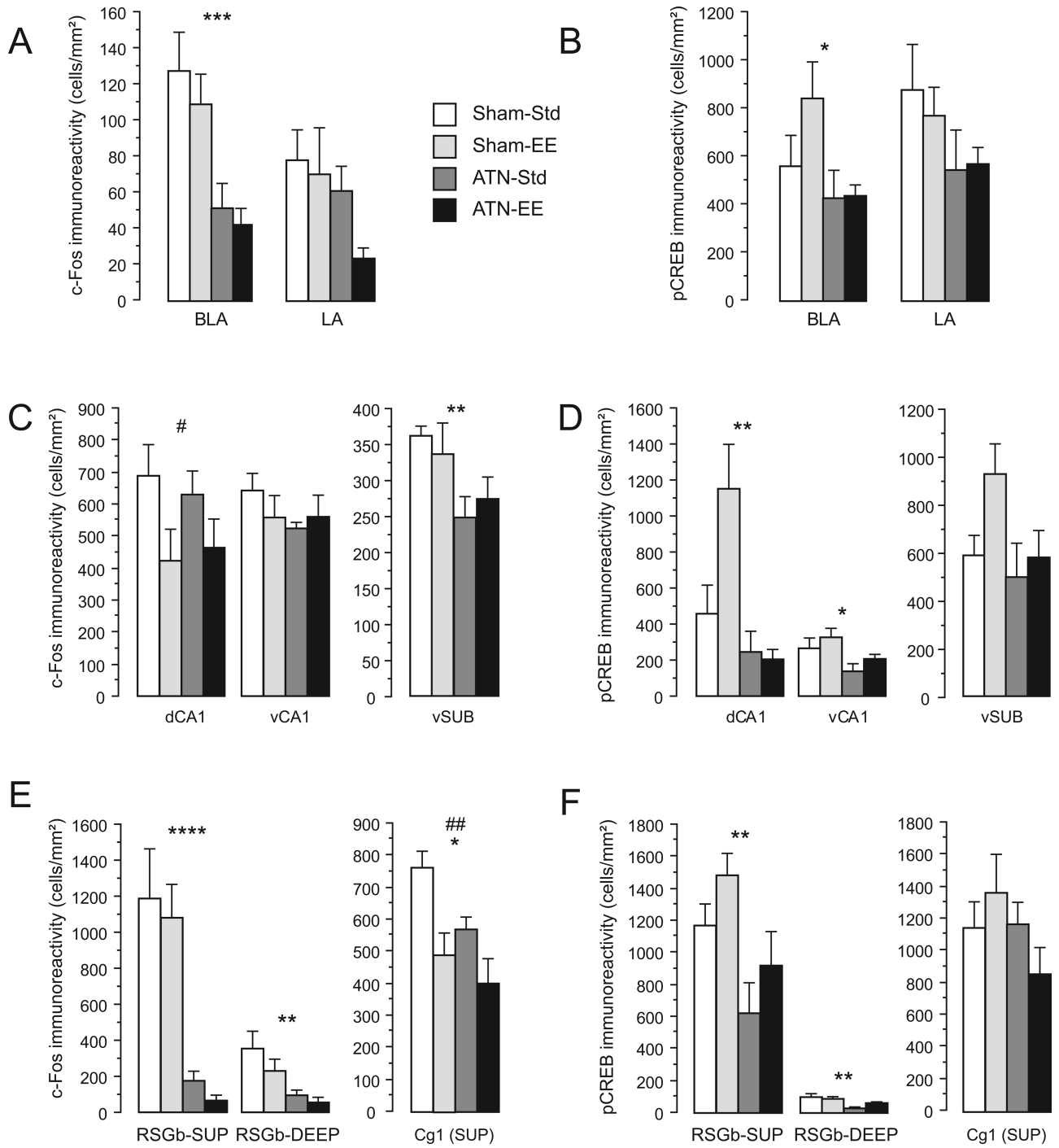

FIGURE 7. c-Fos (A, C, and E) and pCREB (B, D, and F) immunoreactivity expressed as numbers of positive cells per millimeters squared. Values correspond to means for at least three sections taken at the level of the amygdala (lateral and basolateral nuclei; A and B), the hippocampal formation (dorsal and ventral CA1 and ventral subiculum; C and D), granular retrosplenial (RSGb), and anterior cingulate cortices (E and F). Abbreviations: LA, lateral nucleus; BLA, basolateral nucleus; dCA1/vCA1, dorsal/ventral CA1; vSUB, ventral subiculum; RSGb, granular retrosplenial cortex (SUP/DEEP, superficial/deep laminae); Cg1, area Cg1 (superficial lamina) of the anterior cingulate cortex. Main effect of lesion: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$). Main effect of housing: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

Retrosplenial cortex (superficial and deep laminae)

Patterns of c-Fos (Fig. 7E) and pCREB (Fig. 7F) stainings were examined in the superficial (SUP) and the deep (DEEP) laminae of the retrosplenial granular cortex. c-Fos levels were dramatically reduced in both laminae by the lesion, especially in the superficial lamina [Lesion effects; SUP: $F(1,24) = 29.48$, $P < 0.0001$; DEEP: $F(1,24) = 9.50$, $P < 0.01$; Fig.

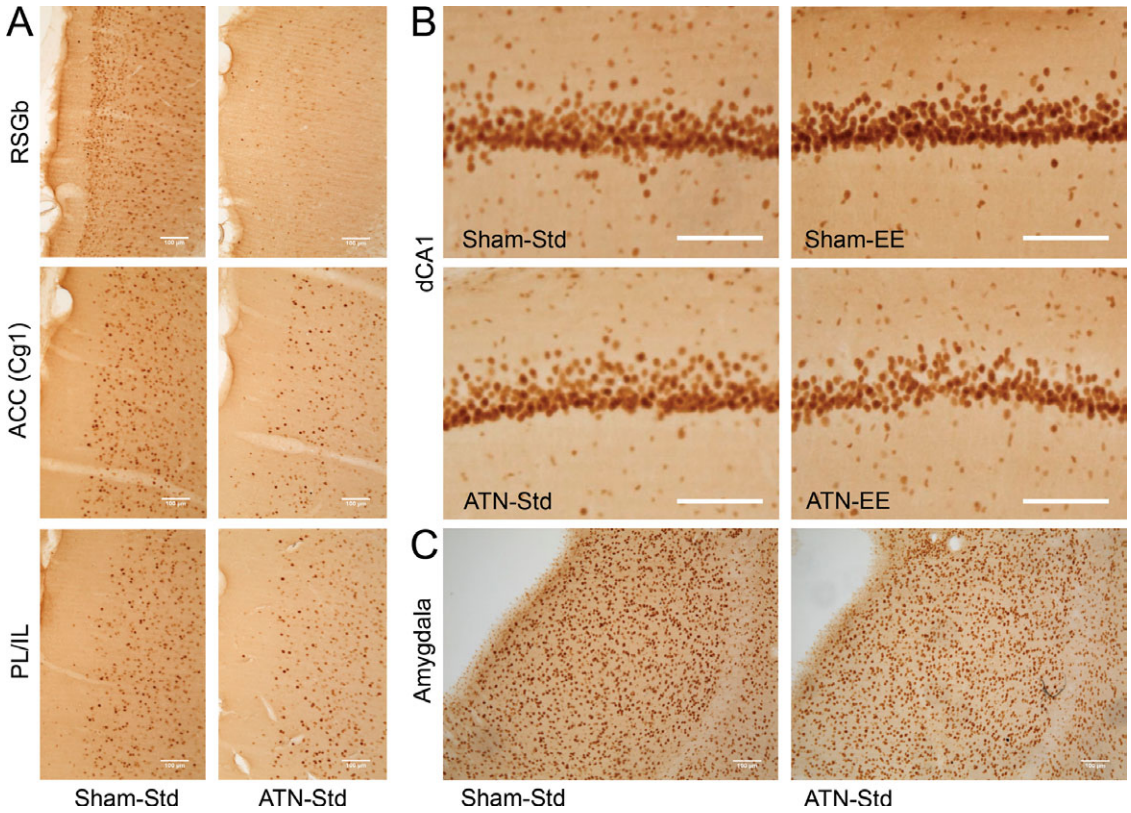

FIGURE 8. Representative pictures showing c-Fos (A) and pCREB (B and C) immunostainings. c-Fos activity is shown at the level of the retrosplenial, the anterior cingulate, and the prelimbic/infralimbic cortices from the same Sham-Std (left) and ATN-Std (right) rats. The lesion effect is particularly evident in the RSGb, moderate in the ACC, and not significant in the PL–IL area. pCREB expression in the dorsal CA1 (B) is greater in the Sham-EE rat. pCREB activation is also slightly decreased in the amygdala (C) of ATN-Std versus Sham-Std rats. All scale bars: 100 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

6A]. Enrichment did not affect c-Fos levels [SUP: $F < 1$; DEEP: $F(1,24) = 1.47$, $P > 0.2$] and failed to restore c-Fos activity in ATN rats as demonstrated by the lack of any significant interaction (All Fs < 1). These results were associated with a generally similar pattern of pCREB in the retrosplenial cortex. Although the effect of the lesion was less striking than that observed with c-Fos, pCREB levels were significantly diminished in ATN rats for both regions [SUP: $F(1,22) = 10.32$, $P < 0.01$; DEEP: $F(1,22) = 9.47$, $P < 0.01$; Fig. 6B]. Again, this effect was not significantly affected by housing conditions although there was a nonsignificant trend toward increased pCREB in both groups of enriched rats for the superficial lamina [Housing, SUP: $F(1,22) = 3.3$, $P = 0.08$, DEEP: $F < 1$] with no Housing × Lesion interaction [SUP: $F < 1$, DEEP: $F(1,22) = 1.98$, $P > 0.1$].

To summarize, both markers showed that neuronal activity was compromised within the retrosplenial cortex of ATN rats and that enrichment did not produce any further change or reverse such a dysfunction.

Medial prefrontal cortex

We compared c-Fos activity in the ACC that receives ATN (anteromedial) afferents and in the prelimbic–infralimbic (PL–IL) area that is devoid of such thalamic projections (Figs. 7E and 7F). Concerning the ACC, we examined the areas Cg1 and Cg2 at both the level of superficial versus deeper laminae (as per the RSGb). We found a significant lesion effect in the superficial lamina of Cg1 only [$F(1,24) = 5.61$, $P < 0.05$], with no significant alteration of c-Fos activity in all other medial prefrontal area examined [PL, IL, Cg1-DEEP, All Fs < 1; Cg2-SUP, $F(1,24) = 1.20$, $P > 0.2$; CG2-DEEP, $F(1,24) = 2.72$, $P > 0.1$]. In general, enrichment had a tendency to decrease c-Fos counts in these areas [Cg1-SUP, $F(1,24) = 13.63$, $P < 0.01$; Cg1-DEEP, $F(1,24) = 9.72$, $P < 0.01$; Cg2-SUP, $F(1,24) = 2.98$, $P = 0.09$; PL, $F(1,24) = 4.89$, $P < 0.05$; and IL, $F(1,24) = 3.44$, $P = 0.08$], with the exception of the deep lamina of Cg2 that was clearly unaffected ($F < 1$). There was no evidence of any Lesion × Housing interaction in these areas [Cg1-SUP/DEEP, Cg2-SUP/DEEP, All Fs < 1; IL, $F(1,24) = 1.54$, $P > 0.2$; PL, $F(1,24) = 1.61$, $P > 0.02$], suggesting that both Lesion and Housing exerted independent effects. pCREB activity in prefrontal regions appeared less sensitive to these treatments than c-Fos activity. In particular, we found no significant change in pCREB levels in the superficial lamina of Cg1 [Lesion, $F(1,22) = 1.45$, $P > 0.2$, Housing, $F < 1$; Lesion × Housing, $F(1,22) = 1.86$, $P > 0.1$].

DISCUSSION

This study provides clear evidence that emotional reactivity is modified in rats with localized ATN lesions. Affective cognition was reduced in ATN rats in terms of a reduced anxiety response in the EPM, increased activity and reduced corticosterone levels when exploring an open field, and delayed acquisition of conditioned fear. These changes were also associated with dysfunctions in the hippocampal–amygdalar circuit as revealed by reduced c-Fos and pCREB immunoreactivity in the CA1, the ventral subiculum, and the BLA. Similar reductions were also evident in the retrosplenial and ACCs. Enrichment produced a marked hypoanxiety in the EPM but no change in open field activity. ATN lesions in standard-housed rats did not affect retrieval of contextual fear memory; however, this response was almost absent in enriched ATN rats.

This study shows that ATN lesions produce symptoms that extend beyond traditional memory tasks. We report decreased emotional reactivity in ATN rats when exploring an EPM and an open field. Although increased open field activity could reflect an inability to habituate to the context (Maren et al., 1998), the EPM is currently the preferred choice for screening anxiolytic drugs (Ramos, 2008). Moreover, intertest overlap between these two different behavioral assays is thought to reflect "true" measures of anxiety (Ramos et al., 1997; Ramos, 2008). As locomotor activity was similar between ATN and Sham rats during the EPM test, differences in this task do not appear to be the result of differences in habituation. The fact that increases in open field were evident only when tested in a novel environment also suggests that these effects were not due to increased locomotor activity. ATN lesions therefore decreased anxiety-like behaviors, which is consistent with the fact that this region occupies a central position in a distributed network of cortical and subcortical areas relevant for emotional behaviors.

The ACC has abundant reciprocal connections with the anteromedial nucleus of the ATN (Shibata, 1993b; Shibata and Naito, 2005). Beyond its involvement in avoidance learning (Gabriel et al., 1983, 1987, 1989; Freeman et al., 1996), the ACC supports the affective component of pain (Yan et al., 2012), social interactions (Rudebeck et al., 2008), and fear conditioning (Bissiere et al., 2008). This last study provided evidence that elemental but not contextual fear memory acquisition is delayed by ACC lesions. When contextual processing is favored by the use of an unpaired procedure, as in this study, most evidence suggest that the role of ACC is the retrieval of remote fear memory (Frankland et al., 2004; Goshen et al., 2011). Interestingly, ACC lesions do not modify anxiety (Rudebeck et al., 2007; Bissiere et al., 2008). Taken together, the behavioral profile of ATN rats therefore appears to differ from that of ACC rats, presumably because the ATN may integrate emotional information not only from the ACC but also via its reciprocal connections with the ventral subicular complex, which has been specifically associated with stress and anxiety (Herman et al., 1998; Maren, 1999; O'Mara et al., 2009). Within the ATN, only the anterodorsal and the anteroventral nuclei are bilaterally connected with the ventral subicular complex (Shibata, 1993a; Van Groen and Wyss, 1995; Wright et al., 2010). As lesions restricted to the anterodorsal thalamic nuclei generally fail to modify anxiety-related behavior (Suarez et al., 2004; Molina et al., 2006; Esquivel et al., 2009), it is therefore possible that connections between the anteroventral nucleus and the ventral subiculum may be particularly relevant for emotional processing.

Contextual fear conditioning is the archetypal behavioral task thought to engage affective cognition. Retrieval of contextual fear memory 24 h later is known to be supported by the joint involvement of the amygdala and the hippocampus (Fanselow, 2010). Our study indicates that ATN lesions delayed the acquisition of freezing during conditioning, but, at least in standard-housed rats, without affecting retrieval of contextual fear memory. This lack of lesion effect in ATN rats was postulated by an earlier study (Ward-Robinson et al., 2002) and provides an unusual dissociation between the effects of ATN versus hippocampal lesions. To date, many behavioral tasks that are sensitive to hippocampal dysfunctions also show impairment after ATN lesions. This apparent discrepancy (see Wiltgen et al., 2006) may be attributable to the fact that we only investigated contextual fear in rats that received ATN lesions before conditioning. Dorsal hippocampal lesions have also been reported to spare contextual fear conditioning when given before training, which has led to the suggestion that contextual fear can be learned with a nonhippocampal strategy (Maren et al., 1997; Fanselow, 2010). It is therefore possible that such a strategy may also survive ATN damage.

However, the behavioral profile of enriched ATN rats was totally different. Enrichment delayed acquisition of the freezing response for all rats. Although Sham-EE animals showed a fast rate of extinction during the retrieval test, ATN-EE rats expressed virtually no fear to the conditioned context. This latter finding suggests that ATN lesions have specific effects on affective cognition and may be associated with markedly distinct behavioral effects, depending on environmental conditions. The dramatic contrast between the performance of Std-ATN and EE-ATN rats during this task may be due to the fact that both the lesion and enrichment produced substantial changes in terms of emotionality. EPM exploration and contextual fear conditioning were indeed particularly sensitive to enrichment as reported in the literature (Barbelivien et al., 2006; Fox et al., 2006). Such an effect is generally long lasting (Cirulli et al., 2010), can be elicited even after the occurrence of a traumatic event (Hendriksen et al., 2010), and can be demonstrated not only in intact subjects but also in pathological animal models (Gortz et al., 2008). Beyond the wide range of neurobiological changes elicited by enrichment such as increases in cortical thickness, synaptic size, or more recently hippocampal neurogenesis (Rosenzweig and Bennett, 1996; Sale et al., 2009), more specialized studies have suggested that enrichment may reduce the reactivity to stress of the prefrontal dopaminergic and cholinergic systems (Segovia et al., 2009). With respect to the activity of the hypothalamic–pituitary–adrenal axis, mixed results have been reported on the effect of enrichment. Plasma levels of corticosterone have been found to be either decreased or increased in enriched animals, and studies

reporting no effect as in this work also exist (for a review of these studies, see Fox et al., 2006). However, it is suspected that behavioral mechanisms may account for the reduced anxiety in enriched animals. Such animals have opportunities to structure and organize their environment, which may reduce overall stress level (Wiepkema and Koolhaas, 1993). Alternatively, enrichment may represent a mechanism of stress inoculation because the continuous renewal of the environment may be comparable with repeated mild stress exposures (Larsson et al., 2002), which may render enriched animals more resilient to challenge (Konkle et al., 2010). Therefore, it is possible that in ATN rats, enrichment may have further reduced emotionality at a level where expression of fear memory could hardly be detected.

These results are also likely to originate from the severe neuronal dysfunctions that were apparent in ATN rats after the contextual fear test as revealed by marked c-Fos and pCREB hypoactivity in several limbic areas. In general, c-Fos activity was reduced by the lesion in areas that receive direct ATN afferents (RSGb, ACC, BLA, and vSUB, with the only exception of the LA) with stronger reductions in areas receiving dense thalamic afferent as particularly evident at the cortical level (i.e., the effect is severe in RSGb, mild in ACC, and absent in PL–IL; Fig. 8). By comparison, selective pCREB dysfunctions were more evident in brain regions that critically support contextual fear memory, not necessarily in relation with ATN afferents. For instance, pCREB hypoactivation was most evident in CA1 that does not receive direct ATN afferents, whereas the ventral subiculum that receives such an innervation did not show significant changes. Interestingly, c-Fos and pCREB hypoactivities perfectly overlap in the BLA, indicative of a fundamental neuronal dysfunction that could correspond to reduced emotionality in ATN rats, irrespective of memory impairment (Reznikov et al., 2008; Butler et al., 2011). In the hippocampal formation, c-Fos activity reflected sensitivity to environmental conditions in CA1, whereas pCREB activity was reduced by the lesion. However, the ventral subiculum showed reduced c-Fos but not pCREB counts. These mixed results are consistent with the fact that ATN rats, despite a slower acquisition, appear to be able to process contextual information.

When compared with previous studies, we also reproduced a new behavioral situation (contextual fear test) the very striking c-Fos hypoexpression in the retrosplenial cortex (Jenkins et al., 2004; Poirier et al., 2008; Poirier and Aggleton, 2009; Amin et al., 2010), and we show that pCREB expression was, to a lesser extent, also diminished in that area, consistent with a recent report (Dumont et al., 2012). Interestingly, enrichment consistently failed to rescue neuronal activation in regions affected by the lesion and even produce further c-Fos reductions especially in the medial prefrontal cortex and dCA1. The reason for this downregulation of c-Fos activity is currently unknown; however, it is possible that everyday exposure to novelty in EE rats may have reduced neuronal excitability (Struthers et al., 2005). However, the pattern of pCREB expression was different. Enrichment increased pCREB expression in CA1 for Sham rats, consistent with a previous study (Huang et al., 2006); however, this effect was not observed in ATN rats. This last result further suggests that enrichment produced differential effects in Sham and ATN rats at the neuronal level.

In summary, the present set of data provides evidence that the ATN play an important role in the regulation of affective cognition. ATN lesions produced specific neuronal dysfunctions in several limbic areas relevant to cognition and emotion, consistent with the behavioral changes observed. Both behavioral changes and neuronal dysfunctions triggered by ATN lesions were differently affected by enrichment, which suggests that diencephalic pathology, as modeled by ATN lesions, may produce markedly different behavioral effects as a function of environmental conditions. These data may be relevant for human studies as they prompt the necessity to apprehend the emotional dimension in the clinical evaluation of diencephalic patients in relation with their life environment.

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