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Nicotine modulates contextual fear extinction through changes in ventral hippocampal GABAergic function

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Abstract

Numerous studies have attributed the psychopathology of anxiety and stress disorders to maladaptive behavioral responses such as an inability to extinguish fear. Therefore, understanding neural substrates of fear extinction is imperative for developing more effective therapies for anxiety and stress disorders. Although several studies indicated a role for cholinergic transmission and nicotinic acetylcholine receptors (nAChRs) in anxiety and stress disorder symptomatology, very little is known about the specific contribution of nAChRs in the fear extinction process. In the present study, we first examined the involvement of several brain regions essential for fear extinction (i.e., dorsal and ventral hippocampus, dHPC and vHPC; infralimbic, IL, and prelimbic, PL of the medial prefrontal cortex, mPFC; basolateral nucleus of the amygdala, BLA) in the impairing effects of a nAChR agonist, nicotine, on contextual fear extinction in mice. Our results showed that systemic administration of nicotine during contextual fear extinction increased *c-fos* expression in the vHPC and BLA while not affecting dHPC, IL or PL. In line with these results, local nicotine infusions into the vHPC, but not dHPC, resulted in impaired contextual fear extinction. Interestingly, we found that local nicotine infusions into the PL also resulted in impairment of contextual fear extinction. Second, we measured the protein levels of the GABA synthesizing enzymes GAD65 and GAD67 in the dHPC and vHPC during contextual fear extinction. Our results showed that in the group that received acute nicotine, both GAD65 and GAD67 protein levels were downregulated in the vHPC, but not in dHPC. This effect was negatively correlated with the level of freezing response during fear extinction suggesting that the downregulated GAD65/67 levels were associated with disrupted fear extinction. Finally, using *c-fos*/GAD65/67 double immunofluorescence, we showed that nicotine mainly increased *c-fos*

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expression in non-GABAergic ventral hippocampal cells, indicating that acute nicotine increases vHPC excitability. Overall, our results suggest that acute nicotine's impairing effects on fear extinction is associated with ventral hippocampal disinhibition. Therefore, these results further our understanding of the interaction between nicotine addiction and anxiety and stress disorders by describing novel neural mechanisms mediating fear extinction.

Keywords

Nicotinic receptors; GABA; fear extinction; hippocampus; PTSD

Nicotine dependence is highly comorbid with anxiety and stress disorders such as Post-traumatic Stress Disorder (PTSD, Breslau *et al*, 2004)), which is characterized by inability to inhibit and extinguish learned fear (Rothbaum and Davis, 2003; VanElzakker *et al*, 2014). There is a bidirectional relationship between nicotine dependence and PTSD where nicotine increases one's risk for developing PTSD and one may be inclined to increase his/her nicotine intake after experiencing an event that triggers PTSD (Breslau *et al*, 2003, 2004; Koenen *et al*, 2005). Consequently, nicotine dependence is nearly 23% higher among PTSD patients (Ziedonis *et al*, 2008). Importantly, in parallel with these statistics, a growing body of literature suggests that nicotine may directly impact PTSD symptoms. Recent studies from our lab and others show that smokers have difficulty in learning cues associated with safety (Kutlu *et al*, 2018a) and nicotine intake increases the amount of intrusive memories associated with a traumatic event (Hawkins and Cougle, 2013). Therefore, nicotine exposure should be considered a contributing factor to PTSD symptoms in humans.

Animal studies have demonstrated that nicotine can alter fear memories via modulation of nicotinic acetylcholine receptors (nAChRs, Kutlu and Gould, 2015). Both hippocampus-dependent contextual and trace fear conditioning are enhanced by an acute dose of nicotine and these effects are dependent on high-affinity $\alpha 4\beta 2$ nAChRs (Davis *et al*, 2005, 2006; Davis and Gould, 2006; Gould, 2003; Gould *et al*, 2004; Gould and Higgins, 2003; Gould and Lommock, 2003; Gould and Wehner, 1999). However, the effects of nicotine are not limited to enhancing fear memories. Previous studies from our laboratory have demonstrated that nicotine administration impaired fear extinction and safety learning in mice (Connor *et al*, 2017; Kutlu *et al*, 2014, 2016a, 2017a, 2017b, 2018a; Kutlu and Gould, 2014). Specifically, acute nicotine administration was shown to cause deficits in encoding, consolidation, and retrieval of contextual fear extinction memories (Kutlu *et al*, 2016a, 2016b; Kutlu and Gould, 2014; Oliver *et al*, 2018), as well as deficits in contextual safety discrimination (Kutlu and Gould, 2014). These effects were specific to extinction and safety learning, as nicotine did not affect general freezing behavior (Kutlu and Gould, 2014). These results clearly suggest that nicotine is a strong modulator of fear extinction and inhibitory learning and may have negative consequences for psychopathologies associated with deficits in these processes.

There are several hippocampal processes that may be involved in the acute nicotine-induced deficits in fear extinction. Similar to the effects of acute nicotine on fear acquisition, we previously showed that high affinity $\alpha 4\beta 2$ nAChRs, which are expressed in the

hippocampus (Séguéla *et al*, 1993; Wada *et al*, 1989), were required for the impairing effects of acute nicotine on contextual fear extinction (Kutlu *et al*, 2016a). Further, we recently showed that hippocampal cell signaling cascades, such as the mitogen-activated protein kinase (MAPK) pathway, were altered by acute nicotine administration during fear extinction (Kutlu *et al*, 2017a). Nevertheless, the neural mechanism underlying the acute nicotine-induced deficits in fear extinction is still unknown. Therefore, in the present study, we defined how acute nicotine changes the activity of brain regions associated with fear extinction, including the dorsal (dHPC) and ventral (vHPC) hippocampus, infralimbic (IL) and prelimbic (PL) cortices, and basolateral amygdala (BLA), using *c-fos* immunohistochemistry. Then, we tested how nicotine affects fear extinction when locally infused into the dHPC, vHPC, IL and PL to isolate the main target of nicotine. Finally, we examined how acute nicotine alters GABA-synthesizing enzyme protein levels and spatial distribution of GABAergic activity in the hippocampus. Our results demonstrate that acute nicotine augments ventral hippocampal activity during fear extinction by reducing GABAergic activity in this brain region. Together these results define for the first time how acute nicotine controls hippocampal inhibitory/excitatory balance during fear extinction and provides a mechanism for its impairing effects.

Methods

Subjects

Eight-week-old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were group-housed in a colony room and maintained on a 12 hour light/dark cycle. Subjects had *ad libitum* access to food and water. Training and testing took place between 9:00 am and 6:00 pm. All behavioral procedures were approved by the Temple University and Penn State Institutional Animal Care and Use Committees.

Apparatus

Contextual fear conditioning training, testing, and extinction took place in 4 identical chambers (18.8 × 20 × 18.3 cm) within sound-attenuating boxes (MED Associates, St. Albans, VT). A ventilation fan located at the back of each box produced background noise (65 dB). A white noise conditioned stimulus (CS, 85 dB) was produced by a speaker within the right wall of the chambers. Each chamber was composed of Plexiglas walls and ceiling with metal grid floors (0.20 cm and 1.0 cm apart) connected to a shock generator, which produced a 2 s long, 0.57 mA foot-shock unconditioned stimulus (US). Both the CS and US were controlled by an IBM-PC compatible computer running MED-PC software. The open field testing took place within a Plexiglas arena (49.5 cm × 59.7 cm). Between subjects, the fear conditioning chambers and open field arena were cleaned with 70% ethanol.

Drugs and Administration

For all experiments, nicotine hydrogen tartrate salt (0.18mg/kg freebase, Fisher Scientific) dissolved in 0.9% physiological saline (saline) or saline alone was injected intraperitoneally (i.p.) 2–4 mins prior to each fear extinction session. Injection volumes were 10mL/kg as in previous studies (Kutlu and Gould, 2014). The 0.18mg/kg dose of acute nicotine was chosen

based on our previous reports showing impaired contextual fear extinction at this dose (Kutlu *et al.*, 2016a; Kutlu and Gould, 2014).

Behavioral Procedures

Following Kutlu and Gould (2014), mice were placed in the fear conditioning chambers and after a 120s baseline period, 2 conditioned stimulus-unconditioned stimulus pairings were delivered. A 30s white noise (85dB) served as the CS, and a 2s 0.57-mA foot shock served as the US. Twenty-four hours after training, the subjects were again placed into the fear conditioning chambers to assess their freezing behavior to the context (retention test). Both the fear conditioning training and retention test were 5min and 30s. Each day for the next five days, mice were placed into the chambers for 5min to assess their freezing behavior during contextual fear extinction. Prior to each extinction session, they received an i.p. injection of nicotine (0.18 mg/kg). Freezing behavior was defined as the absence of voluntary movement except respiration (Blanchard and Blanchard, 1969). Scores were then converted to percent freezing. Experimenters were blinded to drug conditions when scoring.

Immunohistochemistry and Double Immunofluorescence

To examine the effects of nicotine on the expression of *c-fos* in the dHPC, vHPC, IL, PL, and BLA, a cohort of male C57BL/6J mice underwent the same drug and behavioral procedures outlined previously and were placed in one of six groups. A group of nicotine and saline mice underwent training and extinction (Nic-Extinction and Sal-Extinction). To control for any effects of nicotine or injections, a group of mice received nicotine and saline injections in their homecages and did not receive any behavioral training (Nic-Homecage and Sal-Homecage groups). Mice were perfused using sterile phosphate buffered saline (PBS) and 4% paraformaldehyde (PFA) 1 hour following the last extinction session. After perfusion, brains were collected, stored in 4% PFA for 24 hours, and then incubated in a 30% sucrose solution for an additional 24 hours. Fixed brains were frozen and sectioned using a cryostat and coronal sections were collected.

The *c-fos* immunohistochemistry was performed using floating sections as described previously (Kutlu *et al.*, 2016b). Briefly, sections were incubated in a rabbit-host polyclonal antibody (*c-fos*, 1:1000 in PBS-TX-BSA with Sodium Azide; Santa Cruz Biotechnology) for two nights at room temperature. Following PBS-TX-BSA washes (3×10 min), the sections were incubated in the secondary antibody (biotinylated goat-anti rabbit, 1:200 in PBS-TX-BSA; Jackson Immuno-Research) for 90 min and incubated in avidin-biotin complex (1:600 in PBS; Vector ABC kit) for 30 min at room temperature. An oxidase-diaminobenzidine-nickel (DAB) method was used for the immunostaining reaction (5 min, diaminobenzidine, DAB Nickel Substrate Kit, Vector Laboratories). The sections were then mounted on slides and cover slipped using Permount (Fisher Chemicals).

The dorsal hippocampus (dHPC; CA1, CA3, and dentate gyrus, DG), ventral hippocampus (vHPC; CA1, CA3, and dentate gyrus), medial prefrontal cortex (prelimbic [PL] and infralimbic [IL] cortices), and basolateral nucleus of the amygdala (BLA) were selected as the regions of interest (ROI). The ROI coordinates were based on Paxinos and Franklin (2001), dHPC, 21.58 to 22.54 mm posterior to bregma; vHPC, 22.70 to 23.64mm posterior

to bregma; PL, 1.54 to 1.98mm anterior to bregma; IL, 1.54 to 1.98mm anterior to bregma; and BLA, 20.82 to 21.70mm posterior to bregma). Sections were visualized using a brightfield microscope (Leica), 20× images were taken for each brain region and processed using the ImageJ (1.48v) software. A custom ImageJ macro was used to count the *c-fos* immunoreactive (IR) neurons based on pixel intensity. Only *c-fos* signals that were above threshold were counted as *c-fos*-positive cells following previous studies (e.g., Zhao and Li, 2010). Three measurements from the given anterior–posterior coordinates for each brain region (every fourth section for IL and PL and every eighth section for dHPC, vHPC, and BLA) were averaged (Martinez et al. 2013) and expressed as the number of *c-fos*-positive cells per millimeter square (mm²).

Another group of mice were trained in fear extinction following saline and nicotine administrations and the brain tissue was collected following perfusion as described above. The sections from this cohort were stained for *c-fos*/GAD65/67 double immunofluorescence. Briefly, sections were incubated in 4 mL of 10% goat serum for one hour. Sections were incubated at room temperature with the primary antibodies, (anti-*c-fos* mouse mAb, Santa-Cruz, 1:100; anti-GAD65/67 rabbit pAb, Abcam, 1:100) for 48 hours in 2 mL of 1% goat serum. After incubation, the sections were washed three times in 4 mL of PBS-Triton X for 5 minutes per wash. In a dark room, sections were incubated in 2 mL of 1% goat serum and secondary antibodies (Alexa Fluor 488, anti-mouse, Invitrogen, 1:250; Alexa Fluor 647, anti-rabbit, Invitrogen, 1:250), for 3 hours. Sections were washed four times in PBS-Triton-X for 10 minutes per wash. Sections were then mounted onto slides using PBS and allowed to dry. A drop of Prolong Gold anti-fade was placed on each section prior to placing the coverslip on the slide. An epifluorescence microscope (Olympus) was used to take fluorescent images. *C-fos* and GAD65/67 IR cells in dHPC and vHPC ROIs as listed above were manually counted by 3 experimenters blinded to the experimental groups (see Supp Fig1 for counts from all 3 experimenters).

Cannula surgeries

Mice were placed in a stereotaxic surgical apparatus after being anesthetized with isoflurane (5% induction, 2.5% maintenance). Bilateral guide cannulae were implanted into the dHPC (A/P −1.7, M/L ±3.0, D/V −2.3 mm), vHPC (A/P −2.8, M/L ±3.0, D/V −4.0 mm), IL (A/P +1.7, M/L ±0.5, D/V −3.8 mm) or PL (A/P +1.7, M/L ± 0.5, D/V −2.5 mm). All region coordinates were based on Paxinos and Franklin (2001). Mice were allowed to recover for at least five days prior to initiation of behavioral experimental procedures. Infusion sites were confirmed through cresyl violet staining and subjects with placements determined to fall outside of the target regions (8 mice total) were excluded from all analyses (see Supp Fig2&3 for placements).

Local Drug Infusions

We locally infused 0.18 ug/side of nicotine or saline into the dHPC, vHPC, IL and PL. All mice in this experiment were trained in fear conditioning and fear extinction. Nicotine or saline was administered prior to each extinction session. Due to potential risks of tissue damage after repeated drug administration, we employed 4 instead of 5 fear extinction sessions. Mice were momentarily restrained and the dummy cannula was removed. Using a

22 gauge internal cannula (dHPC and vHPC) and a 33 gauge internal cannula (IL and PL) attached to PE50 polyethylene tubing, the drug was infused directly at a 0.5 $\mu\text{L}/\text{min}$ rate with a dosing volume of 0.5 μL per side. A microinfusion pump (KD Scientific, New Hope, PA, USA) was utilized to control the infusion with a 10 μL Hamilton syringe (Reno, NV, USA). To allow diffusion of the drug, the internal cannulae were in place for one minute after infusion. Previous studies from our lab have shown that this local infusion procedure yielded a spread of infusion about 1 mm^3 (Davis *et al*, 2007; Lewis and Gould, 2007). Mice were placed in chambers immediately after infusion.

Western Blotting

Prior to each extinction session, subjects were given either nicotine (0.18 mg/kg, i.p.) or saline as described above. One hour following the 1st or 3rd extinction session, the hippocampal tissue was collected and dissected into dorsal and ventral sections (in a 1:1 ratio). Sections were frozen on dry ice immediately after dissection. Using a sonic dismembrator (Fisher Scientific), dorsal and ventral hippocampi were homogenized in RIPA buffer (Sigma) containing 1X HALT Protease and Phosphate Inhibitor Cocktail (Thermo Scientific). DCTM Protein Assay (Bio-Rad) was used to determine each sample's protein concentration. Samples were diluted with RIPA buffer to obtain 15 μg total protein. Each sample was diluted 1:1 using Laemmli sample buffer (Bio-Rad) containing 5% β -mercaptoethanol. Samples were loaded into TGX 4–20% gradient gels (Bio-Rad). Proteins were separated using electrophoresis conducted at 100v constant voltage for 80 min in 1X Tris-Glycine buffer (Bio-Rad). Proteins were transferred onto nitrocellulose membranes (Bio-Rad) for 2hr at 400mA constant current in ice-cold 1X Tris-Glycine buffer (Bio-Rad) containing 20% methanol. Membranes were then washed with Tris buffered solution (TBS) and blocked with 5% bovine serum albumin (BSA, fraction V, Omnipur) in 1X TBS with 0.1% Tween-20 (TBS-T) for 1hr at room temperature. Membranes were incubated in primary antibodies (anti-GAD67 rabbit pAb, Cell Signaling, 1:1000; anti-GAD65 rabbit pAb, Cell Signaling, 1:1000; anti-ChAT goat pAb, Sigma-Aldrich, 1:5000; anti- β -Actin mouse mAb, Sigma–Aldrich, 1:5000) in 5% BSA overnight at 4°C. The following day, blots were incubated in HRP-conjugated secondary antibodies (Vector Laboratories, anti-rabbit 1:1000 for GAD65 and GAD67; anti-goat 1:5000 for ChAT; anti-mouse 1:10,000 for β – actin in 5% BSA) for 1h in room temperature and enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific) was applied for 5min. To image the blots, a 60s exposure on a Kodak camera (Gel Logic 1500 Imaging System) was utilized. Using ImageJ software, bands were quantified as optical density values. Levels of GAD65, GAD67, and ChAT were normalized to β -actin loading control protein levels for final quantification (see Supp Fig4 for whole blot images).

Statistical Analysis

Behavioral fear extinction data following systemic and local nicotine administration was analyzed using a two-way mixed-design ANOVA that examined 2 levels of Drug across extinction sessions for each compound. To eliminate potential between-group baseline differences in contextual freezing, which may affect subsequent fear extinction curves, the dependent variable was percent freezing to the context normalized to the individual freezing levels at the initial testing session (freezing \times 100/ initial freezing; Kutlu *et al*, 2016a; Tian *et*

al, 2008). For *c-fos* immunohistochemistry, the number of *c-fos* IR cells was converted to number of cells per mm² (number of *c-fos* IR cells per area of the count; 0.31952 mm²). A multivariate ANOVA was run for all ROIs together to detect Drug (nicotine versus saline) × Training (extinction versus homecage) interaction, which was followed by Tukey post hoc tests to examine group differences. For the western blotting experiments, the optical density values from western blot experiments were represented as fold-changes relative to the respective saline control group and analyzed using a 2 × 2 × 2 ANOVA to test the interaction between Extinction Day, Brain Region and Drug as well as one-way ANOVAs for each brain region and protein. Finally, for the *c-fos*/GAD65/67 immunofluorescence experiments, we analyzed the total *c-fos* IR numbers as well as the *c-fos*+GAD65/7 to *c-fos* IR cell number ratios (*c-fos*+GAD/*c-fos*) for each brain region using one-way ANOVAs. We also analyzed correlations of *c-fos* IR cell numbers for all brain regions as well as normalized optical densities (OD) for GAD65, GAD67, and ChAT with normalized freezing responses using Pearson's r. Group sizes were indicated in figure captions. All statistical analyses were run using SPSS 21.

Results

Acute nicotine increases *c-fos* expression in the ventral hippocampus during contextual fear extinction

First, using *c-fos* immunohistochemistry, we examined general neural activity levels in the dHPC, vHPC, PL, IL, and BLA during fear extinction following systemic nicotine and saline administration. For the behavioral experiment, 3 contextual fear extinction sessions resulted in significant Drug × Trial interaction (Figure 1A, F(3,30)=4.973, p<0.01). Our *c-fos* immunohistochemistry results showed that the Drug x Training interaction was significant for each subregion of the vHPC (Figure 1B; total vHPC: F(3,20)=34.451, p<0.01; vCA1: F(3,20)=17.369, p<0.01; vCA3: F(3,20)=6.795, p<0.05; vDG: F(3,20)=7.755, p<0.05) as well as for BLA (Figure 1C; F(3,20)=7.489, p<0.05). Further, Tukey post hoc analysis showed that the number of Nic-Extinction *c-fos* IR cells was significantly higher than Sal-Extinction, Nic-Homecage, and Saline-Homecage controls for all vHPC subregions (ps<0.05). None of the mPFC (Figure 1D) or dHPC (Figure 1B) subregions showed significant Drug x Training interactions (ps>0.05). We also showed that individual number of *c-fos* IR cells and normalized %freezing response were correlated for the vHPC (r= 0.69, p<0.5), but not for dHPC, PL, IL, or BLA (Figure 1E; ps>0.05) suggesting that vHPC activation is the strongest predictor of the acute nicotine-induced impairment of contextual fear extinction. Overall, these results demonstrate that acute nicotine increases *c-fos* expression in the vHPC and BLA while not affecting mPFC subregions PL and IL or dHPC. This suggests systemic nicotine administration augments specific targets of the limbic system associated with fear expression and anxiety such as the vHPC and BLA, which may contribute to disrupted fear extinction observed following acute nicotine administration.

Local infusions of nicotine into the ventral hippocampus and mPFC impair contextual fear extinction

Following our *c-fos* expression experiment, we aimed to understand whether nicotine administration into the vHPC is sufficient to produce the fear extinction deficits we observed

following systemic injections. Therefore, we infused nicotine directly into the vHPC. We also locally infused nicotine into the dHPC and mPFC (i.e., IL and PL) as our control targets. Separate 2-way ANOVAs yielded that the Drug \times Trial interaction was not significant in the groups that received local nicotine infusions into the dHPC (Figure 2A; $F(4,48)=0.224$, $p>0.05$), IL (Figure 2C; $F(4,32)=1.709$, $p>0.05$), or PL (Figure 2D; $F(4,32)=1.948$, $p>0.05$). However, Drug \times Trial interaction was significant following vHPC local nicotine infusions (Figure 2B; $F(4,48)=5.953$, $p<0.05$). Further, Drug main effect was significant for vHPC ($F(1,12)=21.180$, $p<0.001$) and PL ($F(1,8)=21.070$, $p<0.001$) but not for dHPC or IL ($p>0.05$). Bonferroni corrected t-tests showed that the difference between Saline and Nicotine normalized % freezing was significant in Ext1, Ext2, Ext3 and Ext4 for vHPC and in Ext3 for PL ($p<0.05$). Therefore, nicotine infusion into the vHPC had the strongest impairment of fear extinction while dHPC or IL infusions did not produce an effect on extinction of contextual fear. These results parallel our *c-fos* immunohistochemistry experiment showing that the vHPC is critically involved in the impairing effects of nicotine on fear extinction. Interestingly, although *c-fos* expression was not increased in the mPFC as a response to nicotine injections prior to contextual fear extinction, local infusion of nicotine into the PL in isolation disrupted fear extinction. This suggests that activation of PL nAChRs may also be sufficient to produce impaired fear extinction, but they may not be necessary for the systemic effect.

Systemic administration of acute nicotine during contextual fear extinction decreases protein levels of GABA-synthesizing enzymes in the ventral hippocampus

Our initial immunohistochemistry results suggested that systemic nicotine injections increased overall activity in the vHPC as indicated by enhanced *c-fos* expression during contextual fear extinction. One possibility is that the nicotine-induced hyperactivation of the vHPC may be a result of decreased inhibitory signaling within the vHPC. Therefore, to test whether inhibitory GABAergic function is affected by nicotine, we measured the protein levels of GABA-synthesizing enzymes GAD65 and GAD67 in the dHPC and vHPC during fear extinction. We also tested ChAT protein levels to control for potential effects of nicotine on cholinergic function. For the immunoblotting data, we analyzed lysates collected for a previously published behavioral data showing acute nicotine-induced impaired fear extinction following 3 extinction, but not 1 extinction, session (Kutlu *et al*, 2017a).

Three $2 \times 2 \times 2$ repeated measures ANOVAs showed that the interaction between Extinction Day (1 vs. 3 days), Drug (Nicotine vs. Saline), and Brain Region (dHPC vs. vHPC) was not significant for GAD65 ($F(1,25)=2.562$, $p>0.05$), GAD67 ($F(1,25)=3.224$, $p>0.05$), or ChAT ($F(1,25)=0.173$, $p>0.05$). However, separate one-way ANOVAs yielded that after the 1st Extinction Day there was no significant Drug main effects for GAD65 (Figure 3A), GAD67 (Figure 3B), or ChAT (Figure 3C) in the dHPC or vHPC ($p>0.05$). However, after the 3rd Extinction Day, Drug main effect was significant for vHPC GAD65 (Figure 3D; $F(1,12)=6.382$, $p<0.05$) and GAD67 (Figure 3E; $F(1,12)=4.828$, $p<0.05$) but not ChAT (Figure 3F; $F(1,12)=1.512$, $p>0.05$) fold-changes. There were no significant Drug main effects for dHPC for any of the proteins ($p>0.05$). Together, these results show that nicotine administration during contextual fear extinction reduces GAD65 and GAD67 levels in the vHPC without affecting dHPC GAD65 and GAD67. ChAT protein levels were not affected

by acute nicotine in either the dHPC or vHPC. Further, the effects of acute nicotine on contextual fear extinction and vHPC GAD65 and GAD67 levels only appeared on the 3rd day of extinction, but not on the 1st day of extinction. Finally, we found that vHPC GAD67 ($r = -0.65$, $p < 0.5$) and GAD65 ($r = -0.65$, $p < 0.5$), but not ChAT ($r = -0.35$, $p > 0.5$), optical density values after the 3rd extinctions session were negatively correlated with the individual normalized %freezing responses (Figure 3G). This suggests that decreased levels of vHPC GABA synthesis may be a contributing factor for the increased activity patterns we observed in the vHPC. Further, the negative correlation between vHPC GAD65/67 levels and freezing response during fear extinction suggests that the decreased GAD65 and GAD67 protein levels may be associated with disrupted fear extinction.

Acute nicotine reduces active GAD to non-GAD ratio in vHPC during contextual fear extinction

Our results show that nicotine increases *c-fos* expression and decreases the GABA synthesizing enzyme in the vHPC during fear extinction. Therefore, we hypothesized that nicotine may decrease the GABAergic to non-GABAergic cell activation ratio within the vHPC. To test this hypothesis, we ran a *c-fos*/GAD65/67 double immunofluorescence and identified the *c-fos* expressing vHPC cells following contextual fear extinction (Figure 4A&B). For total *c-fos* IR counts, we found no changes in *c-fos* IR counts in the dHPC subregions (Figure 4C; $ps > 0.05$). However, the Drug main effect was significant for all vHPC subregions (Figure 4D; vHPC total: $F(1,11) = 15.387$, $p < 0.05$; vCA1: $F(1,11) = 20.404$, $p < 0.05$; vCA3: $F(1,11) = 20.351$, $p < 0.05$; vDG: $F(1,11) = 6.351$, $p < 0.05$). This result replicates our findings using *c-fos* immunohistochemistry described above. Similarly, the dHPC subregions showed no significant Drug main effects for *c-fos*+GAD/*c-fos* ratios (Figure 4E; $ps > 0.05$). The main effect of Drug for *c-fos*+GAD/*c-fos* ratios was significant for all vHPC subregions (Figure 4F; vHPC total: $F(1,11) = 15.387$, $p < 0.05$; vCA1: $F(1,11) = 20.404$, $p < 0.05$; vCA3: $F(1,11) = 20.351$, $p < 0.05$; vDG: $F(1,11) = 6.351$, $p < 0.05$). Interestingly, we found no significant main effects of Drug for the number of *c-fos* expressing GAD65/67 cells for the dHPC and vHPC or any of the subregions ($ps < 0.05$). This demonstrates that acute nicotine did not alter the number of active GAD65/67 cells but increased *c-fos* expression in the non-GAD65/67 cells in the vHPC. Overall, our results show that although the number of active vHPC GABAergic cells were not decreased, acute nicotine increased the number of active excitatory neurons. Together with our immunoblotting results showing decreased GAD65/67 synthesis, these results suggest that nicotine increases vHPC activation during fear extinction by decreasing GABAergic transmission. Thus, acute nicotine may disinhibit excitatory vHPC neuronal populations, which may result in disrupted fear extinction learning.

Discussion

The results of the present study demonstrate that systemic injections of acute nicotine prior to contextual fear extinction increases neural activity in the limbic brain regions critical for fear learning and anxiety. Specifically, acute nicotine increased *c-fos* expression in the vHPC and BLA while not affecting dHPC, IL or PL subregions of the mPFC. Further, local infusions of nicotine into the vHPC and PL, but not into the dHPC and IL, prior to

contextual fear extinction also impaired fear extinction. These results suggest that vHPC and PL nAChR activation is sufficient for the acute nicotine-induced impairment of contextual fear extinction. We also showed that the acute nicotine administration during fear extinction reduced vHPC GAD65 and GAD67 protein levels. This effect was accompanied by increased *c-fos* activation in non-GABAergic vHPC cells. Overall, our results suggest a novel mechanism that underlies the acute nicotine-induced impairment of contextual fear extinction. That is, acute nicotine augments activity in the brain regions that regulate fear expression and anxiety by decreasing the protein levels of GABA-synthesizing enzymes and disinhibiting excitatory neuronal populations.

Previous literature on fear extinction supports an important role of the ventral hippocampus in fear-related behaviors. Importantly, in contrast to the dorsal hippocampus, the ventral hippocampus directly innervates the amygdala, which has a role as a general mediator of fear conditioning (Pitkänen *et al*, 2000). This way the ventral hippocampus is mainly involved in emotional processing as opposed to the dorsal hippocampus' role in cognitive function (Fanselow and Dong, 2010). For example, the projections from the ventral hippocampus to the amygdala are necessary for the formation and retrieval of successful context-shock associations (Hunsaker and Kesner, 2008; Maren and Fanselow, 1995). In contrast to fear conditioning, only a few studies have directly investigated the role of the ventral hippocampus in fear extinction. Nevertheless, evidence from those studies suggests that the ventral hippocampus may be critically involved in several stages of extinction learning (Hobin *et al*, 2006; Kutlu *et al*, 2016b; Orsini *et al*, 2011). Specifically, failure to retrieve extinction memories was associated with increased activation in the ventral hippocampus (Kutlu *et al*, 2016b; Orsini *et al*, 2011). Moreover, ventral hippocampal inactivation enhanced encoding (Sierra-Mercado *et al*, 2011) and retrieval (Hobin *et al*, 2006) of fear extinction memories. We recently showed that retrieval of previously acquired contextual fear extinction memories induced *c-fos* expression in the vHPC and acute nicotine increased *c-fos* expression and enhanced spontaneous recovery of extinguished contextual fear (Kutlu *et al*, 2016b). Importantly, the enhancing effects of acute nicotine on spontaneous recovery was also accompanied by decreased *c-fos* expression in the IL, an effect we did not observe for the encoding of contextual fear extinction in the present study. The differential IL activation pattern in the acute nicotine-induced impairment of encoding and retrieval of fear extinction memories are in line with previous results suggesting that IL is mainly involved in fear extinction retrieval (Milad and Quirk, 2002). Overall, these studies support our conclusion that the ventral hippocampus is a key region for the nAChR-mediated effects on contextual fear extinction.

In addition to identifying the vHPC as the critical site for the impairing effects of nicotine on contextual fear extinction, our results suggest that the nicotine-induced enhancement of vHPC activation may result from decreased GABAergic transmission in this region. Specifically, acute nicotine administration during contextual fear extinction downregulated GABA-synthesizing enzymes GAD67 and GAD65 in the vHPC, but not in dHPC. In support, multiple studies showed that GABA is required for contextual fear extinction (Harris and Westbrook, 1998; Hobin *et al*, 2006; Shumyatsky *et al*, 2002). Importantly, nAChRs modulate several aspects of GABAergic transmission. For example, nicotine leads to GABA release in the hippocampus (Alkondon *et al*, 1999), reverses GABAergic inhibition

of hippocampal long-term potentiation (Fujii *et al*, 2000), a process that may underlie formation of long-term memory, and impairs LTP of GABAergic neurons (Niehaus *et al*, 2010). Thus, nAChRs may both downregulate and upregulate GABA signaling in the hippocampus. In support of this hypothesis, several studies have shown that nAChR-induced increase in the inhibitory GABAergic transmission may result in both inhibition and disinhibition of the excitatory pyramidal neurons in the hippocampus depending on the activated population of interneurons (Alkondon *et al*, 2000; Ji *et al*, 2001; Ji and Dani, 2000). That is, if nicotine activates inhibitory interneurons that form synapses with pyramidal neurons, it results in inhibition of those neurons, whereas if nicotine activates interneurons that form synapses with other inhibitory interneurons, it leads to disinhibition of the pyramidal neurons. Therefore, it is possible that nicotine may cause disinhibition of the hippocampal network resulting in over-activation of the ventral hippocampus and thus impair contextual fear extinction. Nevertheless, it is important to note that other mechanisms may be in play to compensate for the nicotine-induced changes we have observed in GABAergic function, which requires further investigation of this effect.

One possible way for nAChRs to modulate GABA-synthesizing enzymes during contextual fear extinction is altering cell signaling cascades responsible for inhibitory plasticity in the ventral hippocampal interneurons. We recently found that the impairing effects of nicotine on fear extinction are absent in $\alpha 4$ and $\beta 2$ knockout mice (Kutlu *et al*, 2016a) and inactivation and desensitization of high-affinity $\alpha 4\beta 2$ nAChRs via systemic injections of antagonists and partial agonists prior to each extinction session enhanced fear extinction learning in mice (Kutlu *et al*, 2018b). Importantly, activation of nAChRs triggers GABA release in pre-synaptic interneurons (Alkondon *et al*, 2000). The nAChR-induced GABA release can be mediated by phosphorylation of protein kinase A (PKA) and extracellular signal regulated kinases (ERK)1/2 through calcium (Ca^{2+}) influx into the cell. In support, an interaction between the mitogen-activated protein kinase (MAPK) cascade and GABA receptors has been documented (Cui *et al*, 2008; Matsumoto *et al*, 2006). Specifically, these results showed that inhibition of ERK1/2 resulted in a decrease in GABA release (Cui *et al*, 2008). However, it is possible that the nAChR-induced GABA release from the pre-synaptic interneurons leads to a decrease in intercellular Ca^{2+} levels through GABA receptor activation. Consequently, the decreased Ca^{2+} levels will result in downregulation of PKA and ERK1/2, which in turn, will reduce GABA release and disinhibit the excitatory pyramidal neurons. In line with this model, recent studies from our lab showed that acute nicotine administration prior to contextual fear extinction sessions downregulated ERK1/2 levels in the ventral but not in dorsal hippocampus compared to saline controls (Kutlu *et al*, 2017a). Together with our results showing that acute nicotine decreases GAD67 and GAD65 protein levels and enhances ventral hippocampal activity, the downregulation of ERK1/2 in the ventral hippocampus may explain the nicotine-induced hyperactivity observed in the ventral hippocampus during contextual fear extinction.

Our results have several implications for anxiety and stress disorders in humans. In conjunction with the results of our previous studies (e.g., (Connor *et al*, 2017; Kutlu *et al*, 2014, 2016a, 2016b; Kutlu and Gould, 2014), the results of the present study strongly suggest that nicotine exposure might be a contributing factor to fundamental maladaptive behaviors such as inability to inhibit and extinguish fear observed in these

psychopathologies. In line with this hypothesis, we recently showed that chronic nicotine exposure both in mice and humans is associated with a decreased ability to learn cues signaling safety (Kutlu *et al*, 2018a). Nevertheless, the nicotinic action on GABAergic signaling and fear extinction may also offer novel ways to treat fear-related symptoms in anxiety and stress disorders. For example, we found that acute nicotine requires high-affinity $\alpha 4\beta 2$ subtype of nAChRs to exert its effects on fear extinction (Kutlu *et al*, 2016a), and inactivation of these receptors via direct antagonists or partial-agonists enhances fear extinction in mice (Kutlu *et al*, 2018a). Therefore, in future clinical trials, hippocampal nAChR mechanisms may be utilized to alleviate deficits in fear extinction in anxiety and stress disorder patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights:

- Systemic administration of nicotine during contextual fear extinction increased *c-fos* expression in the vHPC and BLA
- Local nicotine infusions into the vHPC, but not dHPC, resulted in impaired contextual fear extinction
- Acute nicotine downregulated both GAD65 and GAD67 protein levels in the vHPC, but not in dHPC

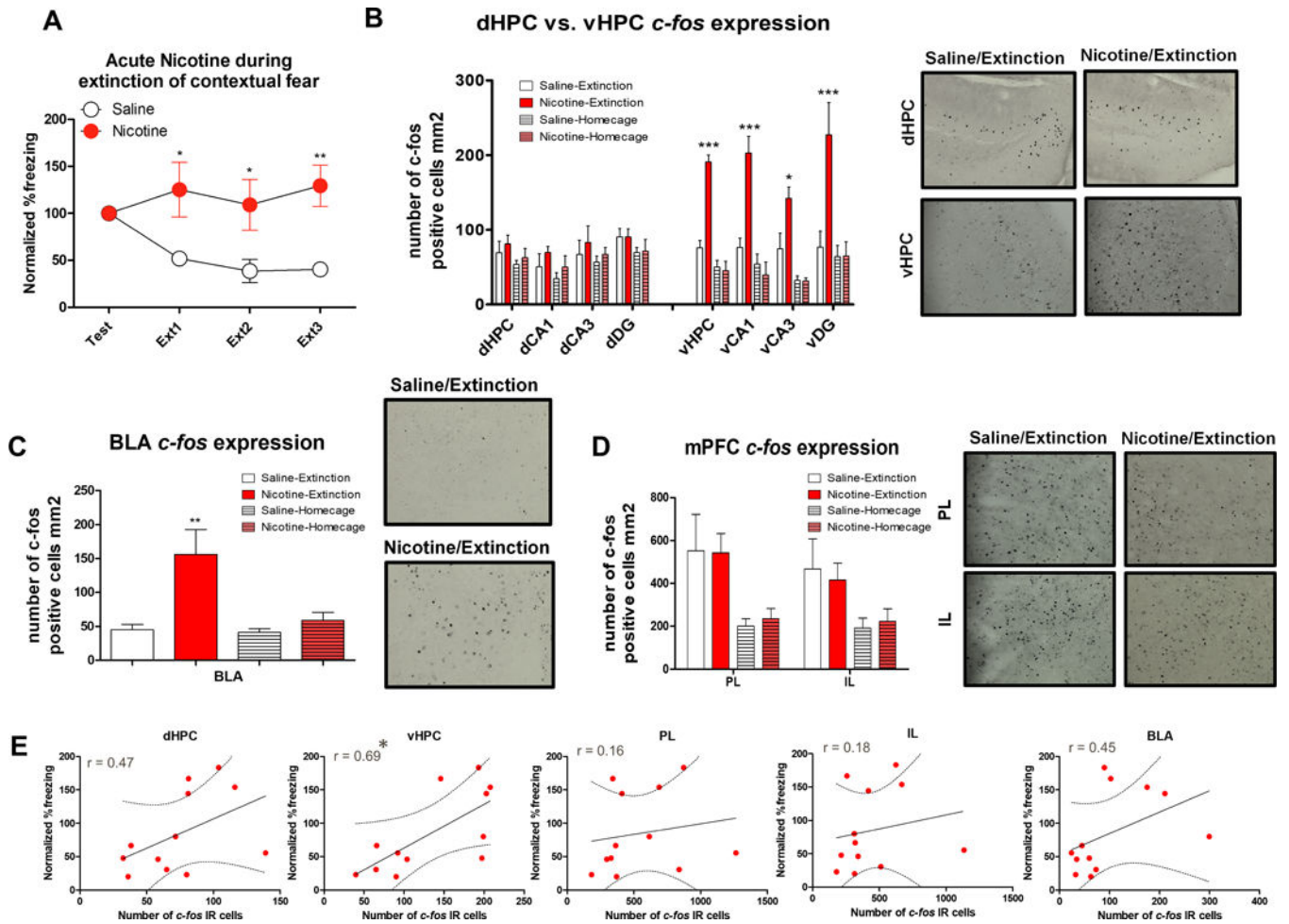


Figure 1. Acute nicotine administration during contextual fear extinction increases vHPC and BLA *c-fos* expression.

A. Normalized %freezing scores across initial testing and 3 extinction sessions (n=6 per group). **B.** Acute nicotine increased the number of *c-fos* IR cells within the vHPC and vCA1, VCA3, and vDG but did not affect *c-fos* expression in the dHPC and its subregions. Right panel shows representative *c-fos* immunohistochemistry images from dHPC and vHPC of the Saline-Extinction and Nicotine-Extinction groups. **C.** BLA *c-fos* expression was increased in the group that received acute nicotine during contextual fear extinction. Right panel shows representative *c-fos* immunohistochemistry images from BLA of the Saline-Extinction and Nicotine-Extinction groups. **D.** Acute nicotine did not affect IL or PL *c-fos* expression, but extinction training increased *c-fos* expression in these brain regions. Right panel shows representative *c-fos* immunohistochemistry images from IL and PL of the Saline-Extinction and Nicotine-Extinction groups. **E.** Correlation plots between dHPC, vHPC, PL, IL, and BLA *c-fos* IR cell numbers and normalized %freezing scores for each subject. Error bars show standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to Saline-Extinction controls.

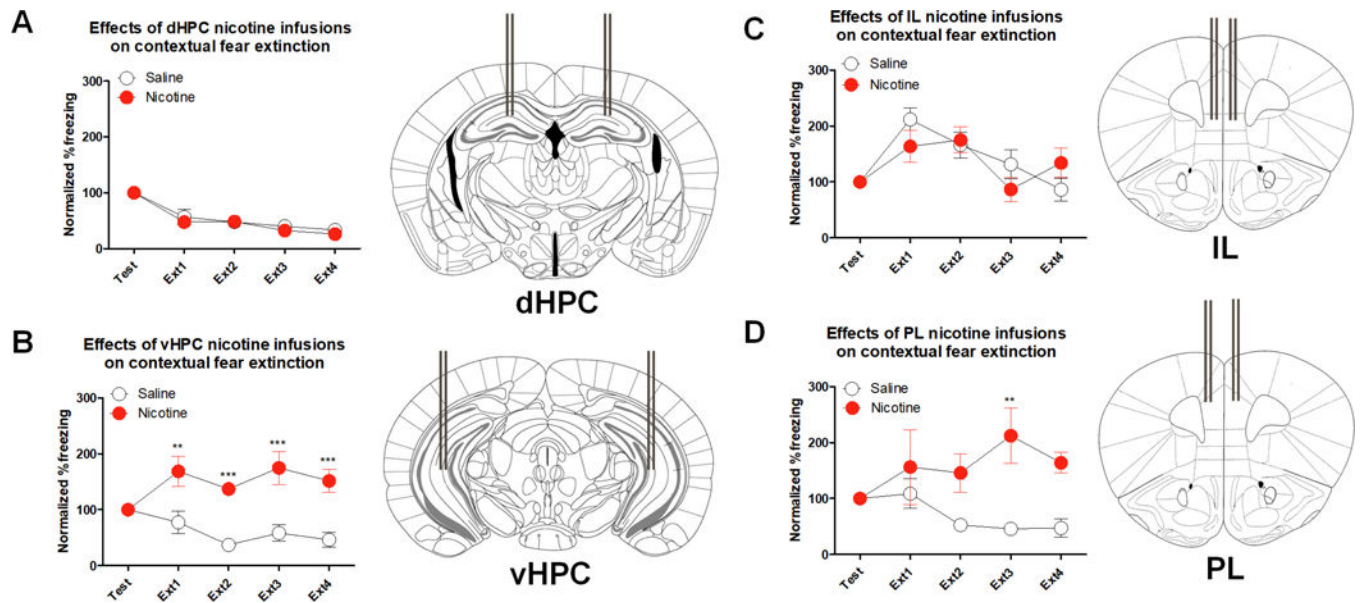


Figure 2. Local nicotine infusion into the vHPC and PL result in contextual fear extinction deficits.

A. Local nicotine infusion into the dHPC did not affect normalized %freezing scores across 4 extinction sessions (n=7 per group). **B.** vHPC local nicotine infusions disrupted contextual fear extinction (n=7 per group). **C.** IL nicotine infusions had no effect on contextual fear extinction (n=4–6 per group). **D.** Local nicotine infusions into the PL also resulted in impaired extinction of contextual fear. (n=5 per group). Right panels show placement of guide cannulas. Error bars show standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to Saline controls.

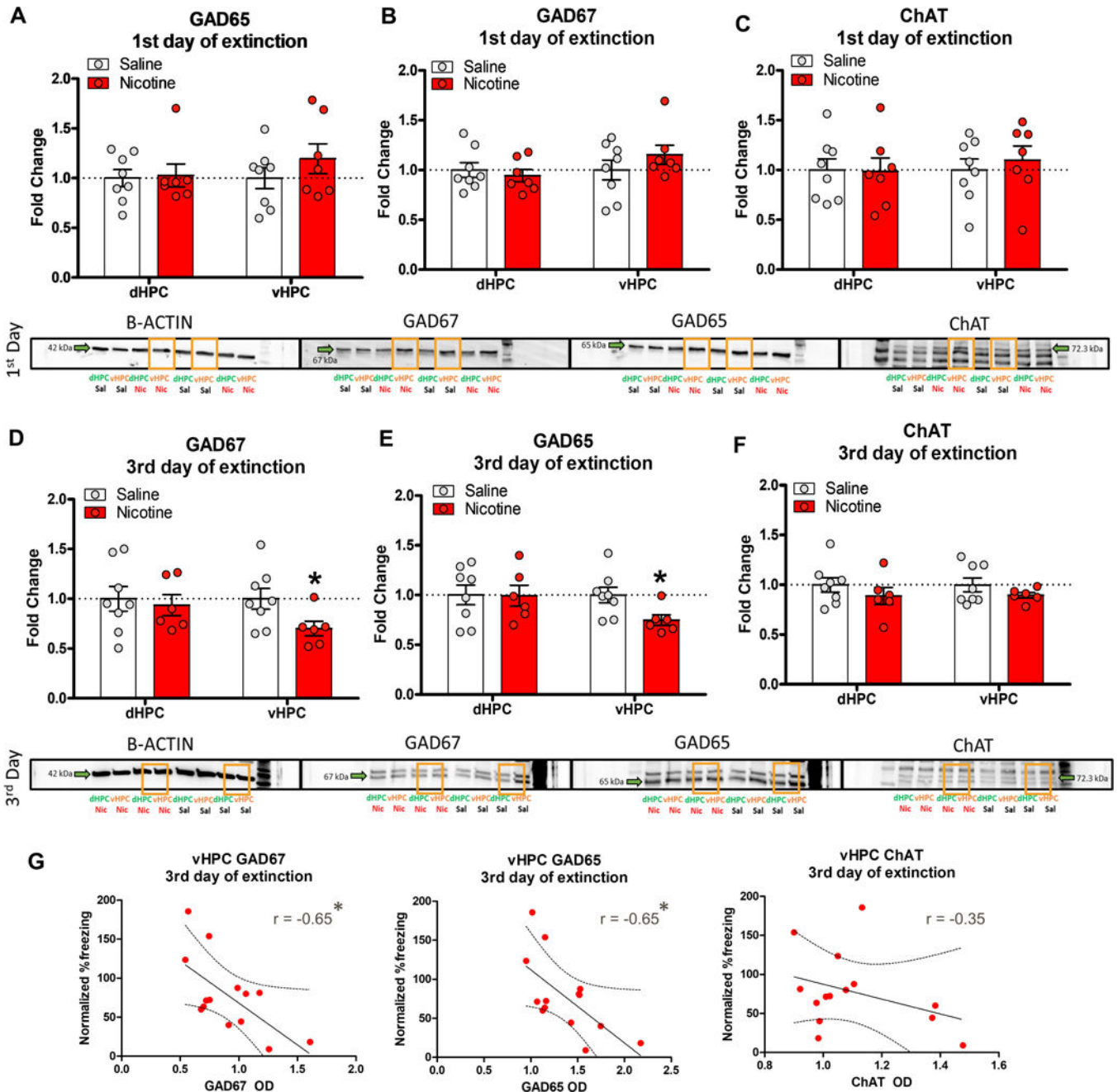


Figure 3. Acute nicotine decreased protein levels of vHPC GAD65 and GAD67 following the 3rd extinction session.

A. GAD65, **B.** GAD67, and **C.** ChAT levels in the dHPC and vHPC were not altered by acute nicotine administration after the 1st contextual fear extinction session (n=7–8 per group). Acute nicotine decreased vHPC levels of **D.** GAD65 and **E.** GAD67, but not **F.** ChAT (n=6–8 per group). dHPC levels of these proteins were not altered. Lower panels show representative immunoblots for each target protein and brain region in the Nicotine and Saline treated groups. **G.** Correlation plots between vHPC GAD67, GAD65, ChAT optical density values and normalized %freezing scores after the 3rd extinction session for

each subject. Error bars show standard error of the mean. * $p < 0.05$ compared to Saline controls.

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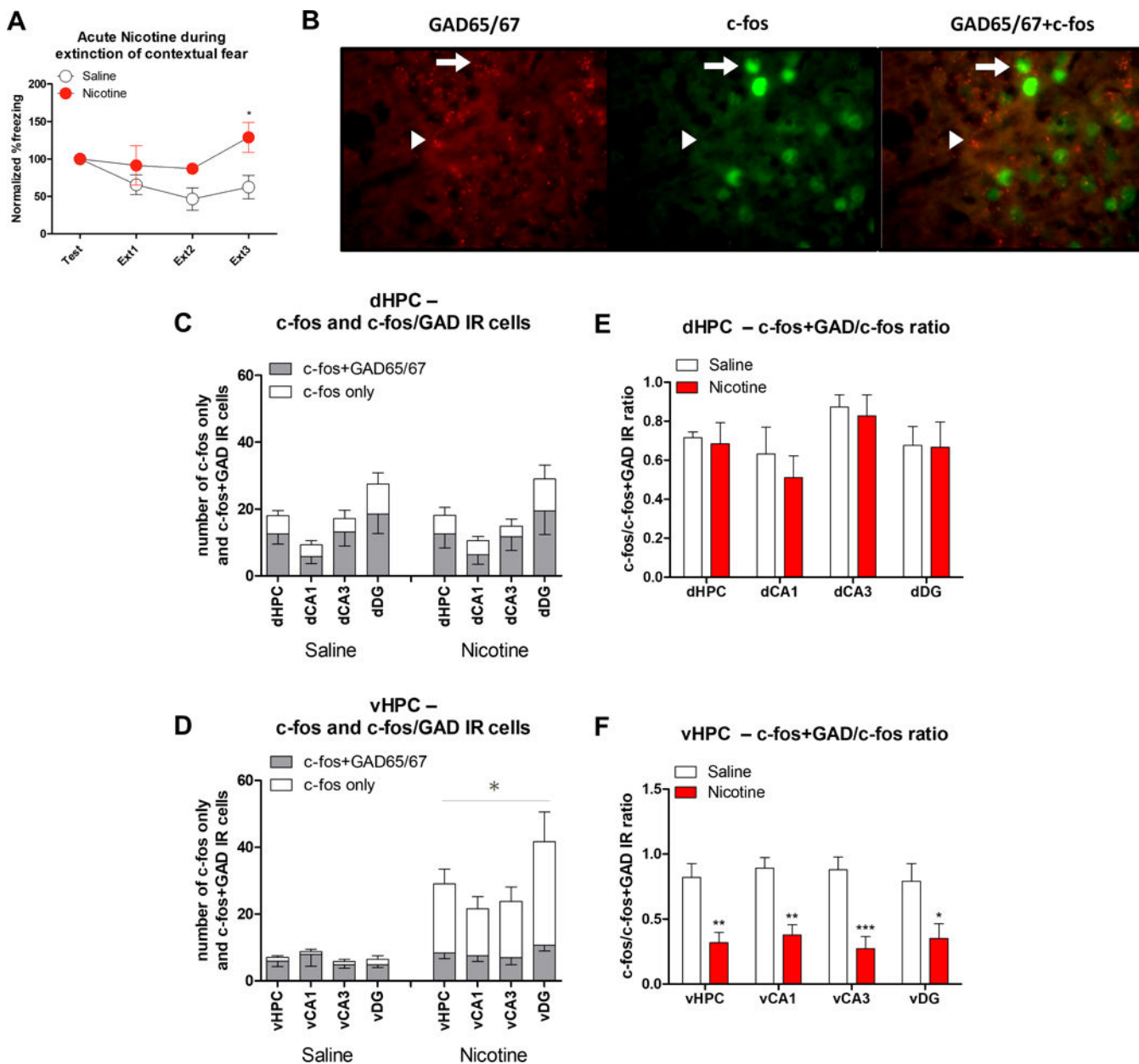


Figure 4. vHPC c-fos expression in non-GAD cells was enhanced by acute nicotine administration during contextual fear extinction.

A. Behavioral results showing that acute nicotine injections increased normalized %freezing response to the context and impaired contextual fear extinction. **B.** Representative image showing *c-fos*-GAD65/67 double immunofluorescent labeled cells in the hippocampus. Arrows show cells double labeled for GAD65/67 and *c-fos*, while arrowheads show cells labeled for only GAD65/67. **C.** Number of dHPC *c-fos* and *c-fos*+GAD65/67 labeled cells was not altered by acute nicotine during contextual fear extinction (n=6–7 per group). **D.** Acute nicotine increased non-GAD65/67 *c-fos* expression in the vHPC without altering *c-fos* expression in the GAD65/67 cells. **E.** dHPC *c-fos*+GAD/*c-fos* ratios were not affected by acute nicotine. **F.** Acute nicotine decreased *c-fos*+GAD/*c-fos* ratios in the vHPC during

contextual fear extinction. Error bars show standard error of the mean. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to Saline controls.