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Epigenetic Mechanisms Within the Cingulate Cortex Regulate Innate Anxiety-Like Behavior

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Abstract

Background: Pathological anxiety originates from a complex interplay of genetic predisposition and environmental factors, acting via epigenetic mechanisms. Epigenetic processes that can counteract detrimental genetic risk towards innate high anxiety are not well characterized.
Methods: We used female mouse lines of selectively bred high (HAB)- vs low (LAB)-innate anxiety-related behavior and performed select environmental and pharmacological manipulations to alter anxiety levels as well as brain-specific manipulations and immunohistochemistry to investigate neuronal mechanisms associated with alterations in anxiety-related behavior.
Results: Inborn hyperanxiety of high anxiety-like phenotypes was effectively reduced by environmental enrichment exposure. c-Fos mapping revealed that hyperanxiety in high anxiety-like phenotypes was associated with blunted challenge-induced neuronal activation in the cingulate-cortex, which was normalized by environmental enrichment. Relating this finding with epigenetic modifications, we found that high anxiety-like phenotypes (compared with low-innate anxiety phenotypes) showed reduced acetylation in the hypoactivated cingulate-cortex neurons following a mild emotional challenge, which again was normalized by environmental enrichment. Paralleling the findings using environmental enrichment, systemic administration of histone-deacetylase-inhibitor MS-275 elicited an anxiolytic-like effect, which was correlated with increased acetylated-histone-3 levels within cingulate-cortex. Finally, as a proof-of-principle, local MS-275 injection into cingulate-cortex rescued enhanced innate anxiety and increased acetylated-histone-3 within the cingulate-cortex, suggesting this epigenetic mark as a biomarker for treatment success.
Conclusions: Taken together, the present findings provide the first causal evidence that the attenuation of high innate anxiety-like behavior via environmental/pharmacological manipulations is epigenetically mediated via acetylation changes within the cingulate-cortex. Finally, histone-3 specific histone-deacetylase-inhibitor could be of therapeutic importance in anxiety disorders.

Keywords: anxiolytic drug development, histone-3, immediate early gene, innate anxiety, prefrontal cortex

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Significance Statement

Anxiety disorders are the most prevalent mental disorders and disproportionately affect more women than men. There is little knowledge whether and how epigenetic modifications (which modulate activity of genes but do not change DNA sequence) contribute to pathological anxiety and whether targeting such mechanisms can effectively attenuate hyper-anxiety. Our results using high-anxiety female mice provide evidence that epigenetic mechanisms are indeed associated with high innate-anxiety and that positive behavioral interventions such as exposure to enriched-environment and/or pharmacological manipulations such as histone deacetylase inhibitors (HDACi) can normalize innate anxiety via epigenetic mechanisms. We identified a key brain region of this action, the cingulate cortex. In this brain area, we found that HDACi-induced increase in acetylation levels within histone-3 (acH3) correlated with the anxiolytic response, suggesting that acH3 may serve as a biomarker for treatment success.

Introduction

Anxiety is considered a polygenic, multifactorial trait wherein the continuum of physiological anxiety up to psychopathology is likely to be shaped by environmentally driven plasticity at the genomic level (Rutter et al., 2006; Bartlett et al., 2017; Schiele and Domschke, 2018). Recent literature supports the important role of animal models to unravel gene × environment interactions. However, many studies focus only on detrimental environmental effects such as chronic mild stress (Willner, 2017), whereas the absence of adversity is considered as the "good" end of the environmental continuum (Belsky et al., 2009). Thus, such studies have not assessed the effects of positive environmental factors and, therefore, fail to measure the full multi-faceted range of psychological and behavioral reactions (Sotnikov et al., 2014a). In the past, we have developed an animal model by using a selective breeding approach wherein the selective enrichment of genetic risk factors related to anxiety across generations produces a stable innate high anxiety-related behavior (HAB) compared with a low anxiety-related behavior (LAB) (Krömer et al., 2005). Interestingly, the high anxiety-related behavior in male HABs is attenuated by housing them in an enriched environment (EE) (Sotnikov et al., 2014a). Investigating beneficial environmental effects on anxiety disorders has considerable translational value, as positive stimuli like exercise, meditation, or social contact may indeed reduce the risk of developing an anxiety-disorder in humans (Tang et al., 2007; Goyal et al., 2014).

Although the underlying neuronal mechanisms for stress-induced anxiety disorders is well studied (for review, see McEwen et al., 2015; Tovote et al., 2015; Girotti et al., 2018), little is known about the neurobiological mechanisms of innate-anxiety disorders. HABs represent an ideal model to study these disorders. So far, we were able to show that HABs have evidence of dysfunction in neuronal activity processing in important anxiety-related circuits (Muigg et al., 2009; Schmuckermair et al., 2013; Sotnikov et al., 2014b) and that these neuronal aberrancies can be modulated by anxiolytic/antidepressant drugs in parallel with a positive behavioral readout (Muigg et al., 2007). More specifically using functional brain mapping studies, we have previously demonstrated that HABs display challenge-induced (e.g., light-dark [LD] test) aberrant activity within the limbic system such as blunted activity within the cingulate cortex (Cg1) (Kalisch et al., 2004; Salome et al., 2004; Singewald, 2007; Muigg et al., 2009). Interestingly, altered genomic copy number variations (Brenndorfer et al., 2015), altered proteome and metabolic profiles (such as amino acid metabolism, apoptosis) (Filiou et al., 2014), and changes in mitochondrial pathways, including oxidative phosphorylation and oxidative stress (Filiou et al., 2011), were observed in the Cg1 of HABs when compared with LABs. In line with our preclinical data, functional imaging studies have shown the involvement of the rostral anterior cingulate cortex in processing of negative stimuli including stress (Blair et al., 1999). Furthermore, it is quite clear that patients with generalized anxiety disorder show blunted rostral anterior cingulate cortex/dorsal ACC reactivity (Blair et al., 2012; Zhai et al., 2019) in fMRI studies. In line with these studies, trait anxiety has also been associated with reduced ACC activity (Klumpp et al., 2011). These preclinical as well as clinical data indicate Cg1 as a key node in the anxiety circuitry (Zeng et al., 2018). Thus, we wanted to investigate whether EE-induced attenuation of enhanced anxiety in HABs would be associated with normalization of aberrant activity within the Cg1.

Our previous studies have indicated that genetic predisposition to trait anxiety can be manipulated by environmental manipulations via epigenetic processes such as altered methylation levels in candidate genes (Sotnikov et al., 2014a; Naik et al., 2018). Furthermore, challenge-induced alterations in neural activity coincide with epigenetic changes, particularly modulation of acetylation levels. For instance, we and others have shown enhanced lysine or histone acetylation in neurons activated during fear extinction (c-Fos positive or Zif268 positive) in brain regions such as the infralimbic cortex, basal amygdala, and the dorsal and ventral CA1 hippocampus (Whittle et al., 2016; Ranjan et al., 2017). Therefore, we wanted to investigate whether aberrant neuronal activity within the Cg1 is associated with altered epigenetic mechanisms, in particular lysine/histone acetylation. Identifying epigenetic mechanisms is of immediate therapeutic importance because histones can be targeted via different drugs such as with histone deacetylase inhibitors (HDACi) and, thus, could be suggested to be used for pharmacological manipulations of enhanced anxiety-like behavior in HABs. Furthermore, HDACi have previously been shown to rescue stress-induced enhanced anxiety (Han et al., 2014; Moloney et al., 2015). However, the role of HDACi in innate high-anxiety has been relatively less well explored.

A final aspect of the present study is the fact that stress-related psychiatric disorders disproportionally affect more women than men (Bangasser and Valentino, 2014). In contrast, rodent preclinical research on these disorders including our own has historically favored the use of males as experimental subjects (Holmes, 2017). Recently, the National Institutes of Health has recognized the gender gap in scientific knowledge and now mandates that studies be conducted in both sexes and to include gender as variables influencing physiological processes (http://orwh.od.nih.gov/sex-inscience/overview/pdf/NOT-OD-15-102_Guidance.pdf). Because we have already carried out enrichment studies in male HAB mice (Sotnikov et al., 2014a), we addressed the above-mentioned questions in female HAB mice.

Materials and Methods

Animals

In these experiments, virgin female HABs and LABs were used. They were selectively (in)bred in the animal facility of the Max Planck Institute of Psychiatry, Munich, from a CD-1 outbred population for high- and low anxiety-related behavior, respectively, for >45 generations. The selection criterion was the percentage of time spent on the open arms of an elevated plus maze with HAB <15% and LAB >60% open arm time. Food and water were provided ad libitum. All experiments on mice were approved by the Government of Upper Bavaria and conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Government of Upper Bavaria and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Standard Environment (Std) and Enriched Environment (EE)

Type II cages (207 x 140 x 265 mm) and type IV cages (380 x 200 x 590 mm) were used for std and EE, respectively (see supplementary Materials and Methods for full details).

Unpredictable Chronic Mild Stress

Unpredictable chronic mild stress, hereby denoted as stress, was applied to LABs at exactly the same time period as EE for HABs and comprised a series of alternating mild stressors to elicit an anxiogenic phenotype (see supplementary Materials and Methods for full details) (Sotnikov et al., 2014a).

LD Test

Anxiety-like behavior was assessed in the LD box. The LD box comprised a dark and light compartment illuminated with <20 Lux and 400 Lux, respectively (see supplementary Materials and Methods for full details) (Sartori et al., 2012).

Drug Administration

MS-275 (Entinostat, Selleck Chemicals; 10 mg/kg dissolved in saline + 25% DMSO vehicle) was administered 2 hours before the LD test (Simonini et al., 2006). Control animals received the respective vehicle. MS-275 or vehicle was administered i.p. in a volume of 10 mL/kg body weight (Whittle et al., 2016).

Immunohistochemistry

Mice were killed 2 hours after the LD test. Coronal sections were incubated with primary antibodies raised against c-Fos, *ac*LYS, acetylation of histone 3 (acH_3; acetylated at N-terminus), $ac\text{-}H_4K_{5,8,12,16}$ (*ac*H4), and/or neuronal nuclei (NeuN), followed by incubation with fluorescent-labeled secondary antibodies (see supplementary Materials and Methods for full details) (Whittle et al., 2016).

Bilateral Application of MS-275 into the Cg1

Mice were anesthetized with Isoflurane (Forene, ABBOTT, Wiesbaden, Germany) and quickly fixed into a stereotaxic apparatus (TSE, Bad Homburg, Germany). Guide cannulae (Microlance canula 21G, BD Bioscience, Heidelberg, Germany) were bilaterally implanted into the Cg1 from Bregma level: 1.94 mm rostral, ±0.20 mm lateral, and -1.25 mm ventral. Starting on postnatal day 70, MS-275 was injected bilaterally into the Cg1 (0.5 µL, 0.5 µL/min) (see supplementary Materials and Methods for full details) (Bahari-Javan et al., 2012).

Statistical Analysis

All data are presented as mean ± SEM and were first tested for homoscedasticity using Levene's test (using Statistica software, Statsoft Inc., OK). All behavior and immunohistochemistry experiments were analyzed using parametric tests (*t* test or 1-way ANOVA). Main effects and interactions for significant ANOVAs are described. Fischer least significant different post hoc test is listed for each condition examined. All *t* tests were 2-tailed. Throughout, $P < .05$ was considered significant.

Results

Level of Cg1 Lysine Acetylation in Cell Populations Activated Following Anxiety Test Correlates with Innate Anxiety-Like Behavior

We first wanted to assess whether hyperanxiety in std-housed female HABs is associated with aberrant activity processing within the Cg1 (experimental paradigm, Figure 1a). Indeed, female HABs displayed increased anxiety in LD, which was associated with blunted Cg1 activation. Specifically, std-housed HABs displayed reduced time spent in the lit compartment (Figure 1b), reduced number of entries into the lit compartment (Figure 1c), and reduced locomotor activity (Figure 1d) compared with LABs in the LD test. Importantly, mice of both lines displayed different estrous cycle stages; however, the anxiety parameters in mice of each line were similar irrespective of the mix of estrous cycle stages (Figure S1a), thus excluding a potential effect of estrous cycle on the quantified anxiety-like measures. Enhanced innate anxiety in the LD test in female HABs was associated with lower c-Fos positive cells, a surrogate marker for neuronal activation (Singewald, 2007), in the Cg1 compared with LABs (Figure 1f). Furthermore, HABs and LABs did not differ in the number of challenge-induced c-Fos positive cells in the prelimbic, infralimbic, and motor cortices (data not shown).

Based on these findings, we wished to gain first insight into epigenetic processes to this altered neuronal activation in cell populations differently activated after the LD test between HABs and LABs. This was observed by quantifying the total expression of acetylated lysine-positive cells (a marker of transcriptional activation; Kouzarides et al., 2007; Whittle and Singewald 2014) and the percentage of acetylated lysine-positive cells co-localized in c-Fos-positive cells. The enhanced anxiety of HABs in the LD test was associated with a reduced number of acetylated lysine-positive cells (Figure 1g) in the Cg1. Additionally, HABs displayed a reduced percentage of co-localized acLys- and c-Fos-positive cells compared with LABs (Figure 1h). In contrast, no changes in the numbers of lysine acetylation-positive cells co-localized with $c\text{-}Fos^{+}$ co-localized cells were observed in the prelimbic cortex, infralimbic cortex, or motor cortices (supplementary Figure 2), which underscores the specificity of changes observed in the Cg1. These findings raise the possibility that reduced transcriptional activation in cell populations within the Cg1 may contribute to innate hyperanxiety-like behavior in HABs.

To further investigate this hypothesis, we reasoned that reducing hyperanxious behavior of HABs mice should increase lysine acetylation in activated cell populations in the Cg1, and, conversely, that increasing anxiety-like behavior in the low-anxiety LAB mice would reduce lysine acetylation in activated cell

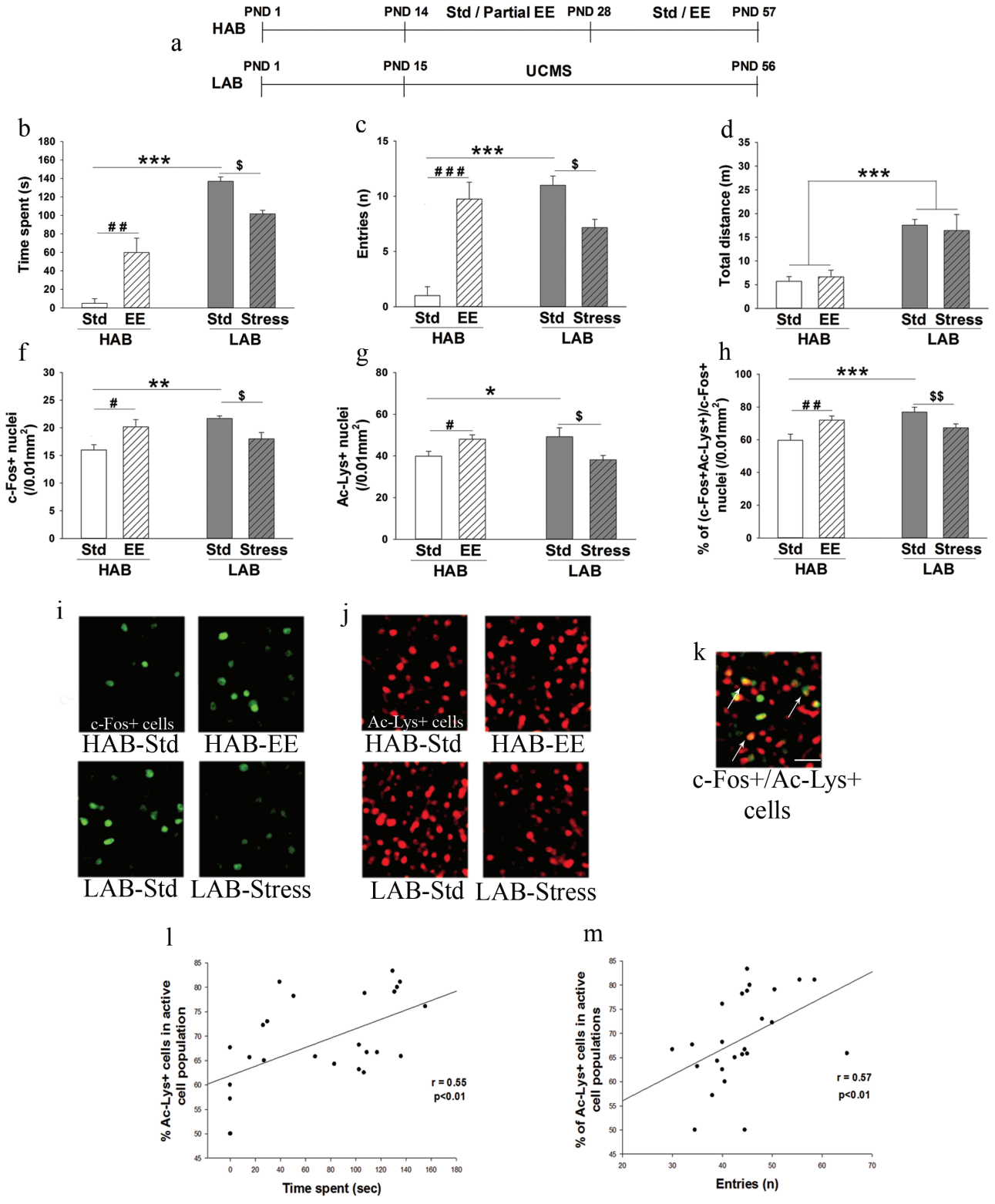

Figure 1. Behavioral effects of environmental modifications and alterations within the cingulate cortex (Cg1) following environmental modifications. (a) Experimental design. (b) Behavior: 1-way ANOVA revealed significant interaction on time spent following enriched environment (EE) in high anxiety-related behavior (HAB) and stress in low anxiety-related behavior (LAB) in the light-dark (LD) test ($F(3,21) = 13.61$, $P < .001$). EE significantly increased the time spent in the light compartment in the HABs ($P < .01$) whereas chronic mild stress (CMS) had an opposite effect ($P < .05$). Similar to time spent in the light compartment, environmental modification also elicited bidirectional interaction for (c) entries ($F(3,21) = 24.86$, $P < .001$; EE-HAB vs standard environment [std]-HAB, $P < .001$; stress LAB vs std LAB, $P < .05$). (d) Neither enrichment nor stress affected the overall locomotion in both the lines. (f) c-Fos mapping: 1-way ANOVA revealed a significant interaction for light dark-induced c-Fos expression following EE in HABs and stress in LABs ($F(3,21) = 4.41$, $P < .05$). HABs, in comparison with LABs, showed lower c-Fos expression within the Cg1 ($P < .01$). EE increased the c-Fos expression within the HABs ($P < .05$ vs std HAB) while stress reduced c-Fos expression within the LABs ($P < .05$ vs std LAB). (g) Mapping of acetylated-

populations. We chose environmental enrichment and chronic mild stress (Figure 1a) because these environmental manipulations have been shown to reduce or increase anxiety-like behavior in male HABs and LABs, respectively (Sotnikov et al., 2013).

We show for the first time to our knowledge that EE attenuated the high anxiety-like behavior in female HABs compared with std-housed HABs as indicated by an increased time spent in the lit compartment (Figure 1b) and number of entries into the lit compartment (Figure 1c) during the LD test. Becuase no difference in locomotor activity between std and EE HABs (Figure 1d) and a similar mixture of estrous cycle stages between HABs groups (Figure S1a) were observed, it is suggested that the anxiolytic effects of EE were independent of locomotor activity or estrous cycle stage. At the cellular level, the reduced anxiety-like behavior following EE was accompanied by acetylated lysine changes in cell populations in the Cg1 activated by the anxiety test challenge in HABs. In detail, compared with std-housed HABs, EE HABs displayed increased numbers of c-Fos-positive cells (Figure 1f) and acetylated lysine-positive cells (Figure 1g) and an increased percentage of acetylated lysine cells localized with c-Fos-positive cells (Figure 1h) in the Cg1. No such changes were observed in the prelimbic, infralimbic, or motor cortices (supplementary Figure 2a–c). Conversely, unpredictable chronic mild stress increased the low innate anxiety-like behavior of LABs because the time spent in the lit compartment (Figure 1b) and number of entries into the lit compartment (Figure 1c) were reduced compared with the nonstressed conditions. No differences in locomotor activity between no-stress and chronic mild stress LABs (Figure 1d) and a similar mixture of estrous cycle stages between LABs groups (Figure S1a) were observed, which suggests that the increase in anxiety following stress was independent of locomotor activity or estrous cycle stage. At the cellular level, and compared with control nonstressed LABs, chronic mild stress in LABs reduced the number of c-Fos-positive cells (Figure 1f), acetylated lysine-positive cells (Figure 1g) and co-localized acLys- and c-Fos-positive cells in the Cg1 (Figure 1h), but, again, not in the prelimbic cortex, infralimbic cortex, or motor cortex (supplementary Figure 2a–c). Together, the bidirectional effects of environmental manipulations on innate levels of anxiety-like behaviors and acetylated lysine suggest a dynamic relationship between anxiety state and level of epigenetic processes in the Cg1.

To gain evidence whether levels of acetylated lysine in anxiety-induced active cells can predict individual innate anxiety levels, we correlated behavioral measures to the total number of acetylated lysine-positive cells and also to the percentage of acetylated lysine levels in active cell populations. Results revealed that, although the total number of ac-Lys cells did not correlate with any behavioral measure (time spent in lit arena, R = 0.26, $P > .05$; number of entries into the lit arena, R = 0.29, $P > .05$), the percentage of acetylated lysine-positive cells in cells activated during the LD challenge was positively correlated with both the time spent in the lit arena (R = 0.55, $P < .01$) (Figure 1l) and number of entries into the lit arena (R = 0.57, $P < .01$) (Figure 1m). Underscoring the specificity of these correlations was the finding that no significant correlations were observed for either the total number of acetylated lysine or the percentage of ac-Lys-positive cells in active cell populations in the prelimbic cortex (time spent in lit arena, R = 0.22, $P > .05$; number of entries into the lit arena, R = 0.11, $P > .05$), infralimbic cortex (time spent in lit arena, R = –0.002, $P > .05$; number of entries into the lit arena, R = –0.06, $P > .05$), or motor cortex (time spent in lit arena, R = –0.24, $P > .05$; number of entries into the lit arena, R = –0.24, $P > .05$). Collectively, these results reveal that lysine acetylation in cell populations activated during the LD test can predict innate anxiety and, moreover, that increased lysine acetylation in active cell populations is correlated with reduced anxiety-like behavior.

Intra-Cg1 HDAC Inhibition Elicited an Anxiolytic Effect in HABs Correlated With Increased Histone H3 Acetylation

To demonstrate a causal mechanistic role that enhancing histone acetylation within the Cg1 can reduce the innate hyperanxiety in HABs, we administered MS-275 (an HDAC-1,2,3 and 9 isoform inhibitor) (Khan et al., 2008; Bantscheff et al., 2011) that has been shown to enhance brain lysine acetylation (Nebbioso et al., 2005) directly into the Cg1 and assessed anxiety-like behavior in the LD test (Figure 2a,b). Indeed, MS-275-treated HABs displayed increased time spent in the lit arena compared with vehicle-treated HAB controls (Figure 2c) as well as increased number of entries into the lit arena (Figure 2d). No differences in locomotor activity between MS-275- and vehicle-treated controls was observed (Figure 2e), negating a potential effect of altered locomotor activity influencing changes in time spent and entries into the lit arena.

Following assessment of global alterations in lysine acetylation, we wanted to be more specific and investigated changes in histone acetylation. Gene transcription is regulated by histone acetylation involving particularly acetylation of lysine residues in histone H3 and H4 proteins (Grunstein, 1997; Woo et al., 2017). Additionally, MS-275 has been shown to alter the acetylation of H3 and H4 levels (Simonini et al., 2006; Drogaris et al., 2012). Our findings reveal that, compared with vehicle-treated controls, intra-Cg1 MS-275-treated HABs displayed enhanced levels of acetylated histone H3 in neuronal (Figure 2f), but not nonneuronal (Figure 2g), cell populations. No alterations in acetylated histone H4 levels were observed (Figure 2h,i). Moreover, the level of acetylated histone H3 in neuronal cell populations was positively correlated with both the time spent in the lit arena (R = 0.49 $P < .05$) (Figure 2l) and number of entries into the lit arena (R = 0.42, $P = .07$) (Figure 2m). Therefore, the present results show that reducing hyperanxiety via administration of MS-275 is associated with enhanced histone acetylation, particularly in neuronal localized histone H3 within the Cg1. These data add further congruent evidence highlighting a mechanistic role of epigenetic processes in modulating anxiety-like behavior.

lysine (Ac-Lys). A significant interaction for Ac-Lys expression following EE in HABs and stress in LABs was observed ($F(3,21) = 4.37$, $P < .05$). HABs, in comparison with LABs, showed lower Ac-Lys expression within the Cg1 ($P < .05$). EE increased the Ac-Lys expression within the HABs ($P < .05$ vs std HAB) whereas stress reduced Ac-Lys expression within the LABs ($P < .05$ vs std LAB). (h) Mapping of Ac-Lys and c-Fos. Finally, a significant interaction for activated neurons expressing Ac-Lys expression (c-Fos+/Ac-Lys+ co-localized cells) following EE in HABs and stress in LABs was observed ($F(3,21) = 8.19$, $P < .001$). HABs, in comparison with LABs, showed lower c-Fos+/Ac-Lys+ nuclei within the Cg1 ($P < .001$). EE increased the c-Fos+/Ac-Lys+ nuclei within the HABs ($P < .01$ vs std HAB) whereas stress reduced Ac-Lys expression within the LABs ($P < .01$ vs std LAB). (i) Representative photographs showing c-Fos expression in each group. (j) Representative photographs showing Ac-Lys expression in each group. (k) Examples of c-Fos+ nuclei also showing Ac-Lys expression indicated by white arrows. (l) A significant positive correlation was observed between time spent and c-Fos+/Ac-Lys+ nuclei ($P < .01$). (m) A significant positive correlation was observed between entires and c-Fos+/Ac-Lys+ nuclei ($P < .01$).$^{***}P < .001$ std HAB vs EE HAB; $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ std HAB vs std LAB; $^{\$}P < 0.05$, std LAB vs stress LAB. Data are represented as mean ± SEM.

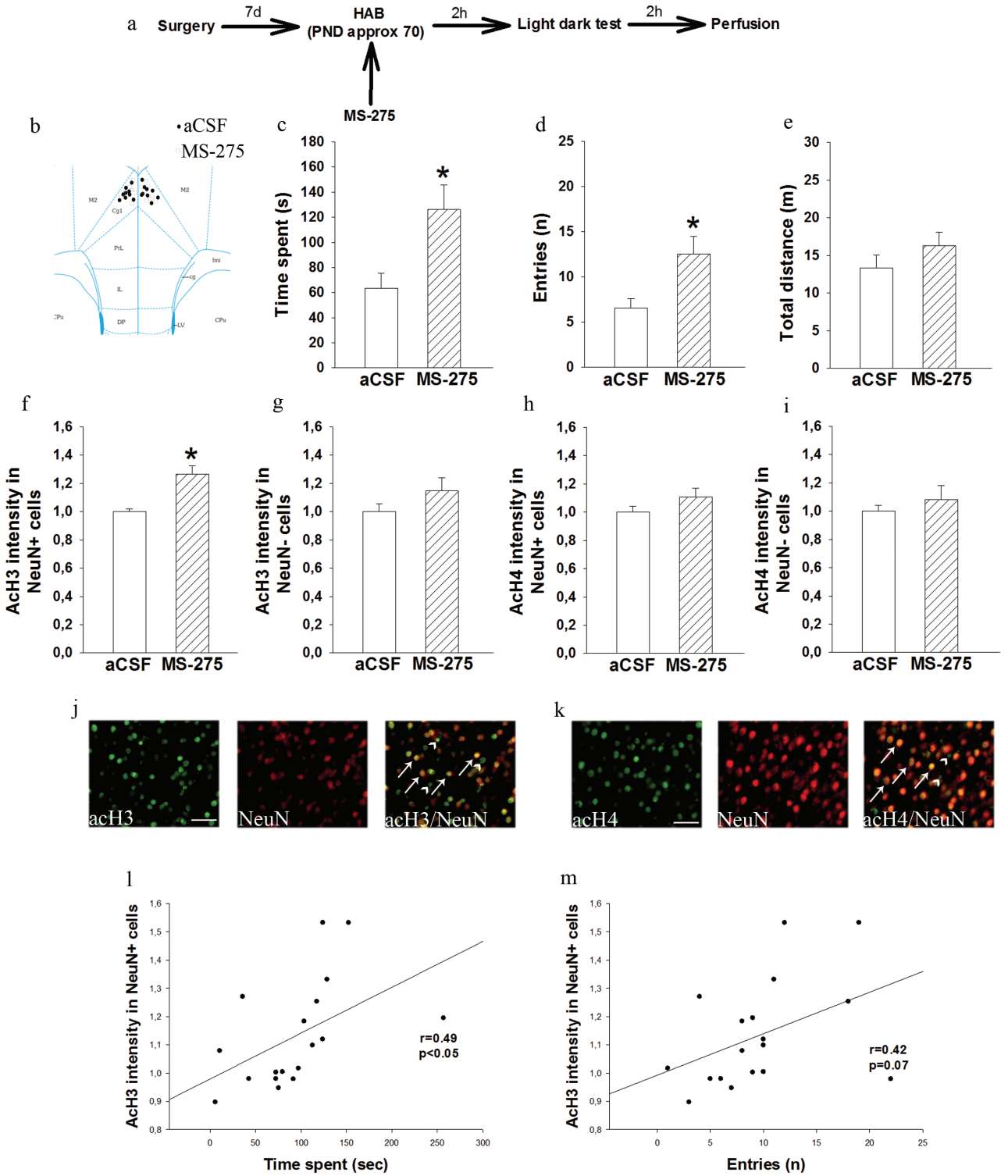

Figure 2. Intra-cingulate cortex (Cg1) MS-275 administration enhances histone acetylation and modulates anxiety-like behavior. (a) Experimental design. (b) Schematic illustration of the sites of Cg1 hits for animals of the artificial cerebrospinal fluid (aCSF) group (black dots) and MS-275 group (grey rectangle). (c) Behavior. Unpaired t test revealed that MS-275 increased the time spent in the light compartment ($P < .05$) as well as (d) increased entries into the light compartment ($P < .05$) in the light-dark (LD) test. (e) The locomotor distance was not affected ($P > .05$). (f) Acetylated histone-3 (AcH3) mapping: MS-275-treated high anxiety-related behavior (HAB) showed higher intensity of AcH3 within the neuronal, (g) but not within the nonneuronal, cell population. (h and i) AcH4 mapping. No differences in acH4 intensity were observed within the neuronal and nonneuronal population. (j) Representative photographs showing acH3 (green), neuronal nuclei (NeuN, red), and colocalized acH3/NeuN cells. Bold arrows indicated acH3+NeuN+ cells and dotted arrow indicates acH3+NeuN– cells. (k) Representative photographs showing acH4 (green), NeuN (red), and colocalized acH4/NeuN cells. Bold arrows indicate acH4+NeuN+ cells and arrowheads indicate acH4+NeuN– cells. (l) A significant positive correlation was observed between time spent and acH3+NeuN+ nuclei ($P < .05$). (m) A trend towards positive correlation was observed between entries and acH3+NeuN+ nuclei ($P = .07$).*$P < .05$ saline vs MS-275. Data are represented as mean ± SEM.

Peripheral Administration of MS-275 Reduces Innate Anxiety-Like Behavior in HABs Correlated With Increased Histone H3 Acetylation in Cg1

To ascertain a more general role of histone acetylation enhancement in reducing innate anxiety, we administered the histone deacetylase inhibitor MS-275 peripherally prior to an LD test (Figure 3a). Indeed, we observed an increased time spent in the lit arena (Figure 3b) and number of entries into the lit arena (Figure 3c) of HABs treated with MS-275 compared with vehicle-treated controls, indicating reduced anxiety-like behavior. This anxiolytic effect was independent on locomotor activity because the distance travelled was the same between MS-275- and vehicle-treated controls (Figure 3d). At the cellular level, although we observed no change in the total numbers of ac-Lys-positive cells in the Cg1 (vehicle-treated controls, 43.2 ± 1.6; MS-275, 41.6 ± 1.8), we observed an increased lysine acetylation in the c-Fos-positive cells activated via the LD test in the Cg1 (Figure 3e) in the MS-275-treated group. These changes were not observed in the prelimbic cortex, infralimbic cortex, or motor cortex (supplementary Figure 3a–c). In addition, the lysine acetylation within the activated cell populations was positively correlated with both the time spent in the lit arena ($R = 0.78$, $P < .001$) (Figure 3f) and the number of entries into the lit arena ($R = 0.74$, $P < .01$) (Figure 3g). This finding is again consistent with our above finding revealing that ac-Lys levels in anxiety test-activated cell populations is positively correlated with reductions in anxiety-like behavior.

Mirroring what we observed with the intra-Cg1 administration of MS-275, systemically treated HABs also displayed enhanced levels of acetylated histone H3 in neuronal (Figure 3h), but not nonneuronal (Figure 3i), cell populations. No alteration in acetylated histone H4 levels were observed in either neuronal or nonneuronal cell populations (Figure 3j,k). Moreover, the levels of acetylated histone H3 in neuronal cell populations was positively correlated with both the time spent in the lit arena ($R = 0.78$, $P < .001$) (Figure 3m) and number of entries into the lit arena ($R = 0.74$, $P < .01$) (Figure 3n). This result showing that reducing innate hyperanxiety via pharmacologically enhancing histone acetylation, particularly in neuronally localized histone H3 within the Cg1, adds further congruent evidence highlighting a mechanistic role of epigenetic processes in rescuing hyperanxiety behavior.

Discussion

The continuum of physiological anxiety up to psychopathology is orchestrated by the interplay of genetic predisposition and environment interactions involving epigenetic modifications, including histone acetylation. Both preclinical and clinical studies have shown that anxiety- and trauma-related disorders such as PTSD and panic disorder are accompanied by epigenetic modifications of the genome (Daskalakis et al., 2018) (Matosin et al., 2017), whereas there is little knowledge whether epigenetic modifications contribute to generalized anxiety disorder including high innate anxiety. We here took advantage of the extreme genetic predisposition of the selectively bred high- (HAB) and low-anxiety (LAB) mouse model exhibiting stable high vs low anxiety-related behavior. Together, we support and extend previous findings that environmental manipulations can shape anxiety-like behavior in both male and female mice. We now show in female mice that this effect is accompanied by modulation of epigenetic mechanisms in challenge-induced activation neurons within the Cg1. Next, our findings reveal that the class I HDAC inhibitor (MS-275) can attenuate innate hyper-anxiety. Finally, our study for the first time to our knowledge identified the Cg1 as the locus for modulating hyperanxiety states by HDAC inhibition.

It is noteworthy that the male representatives of the HAB and LAB mouse lines have been extensively studied in terms of behavior and neurobiological dysfunctions underlying their phenotypes (Krömer et al., 2005; Muigg et al., 2009; Sah et al., 2012; Schmuckermair et al., 2013), whereas the female sex has, to date, been relatively understudied (Sah et al., 2012) despite that stress-related psychiatric disorders including anxiety disorders affect women more than men (Pedersen et al., 2014). Our present finding of increased anxiety-like behavior of female HAB compared with LABs in the LD test is congruent with their male counterparts (Krömer et al., 2005; Sotnikov et al., 2014a) and, as we could demonstrate, is independent of the estrous cycle stage (Hoeijmakers et al., 2014; Fritz et al., 2017). Similar to males, both positive housing environment (EE) and negative housing environment (chronic mild stress) was able to shift the behavior of female HABs and LABs. These data reflect the dynamics of gene-environment interactions, suggesting that environmental influences via epigenetic mechanisms can indeed shape the anxiety phenotype in both males and females (Belsky and Pluess, 2009).

Because EE has been linked with epigenetic processes (Zhang et al., 2018) and particularly acetylation of lysine residues on histone proteins is a key mechanistic step in the regulation of transcription (Choudhary et al., 2009), we assessed whether such epigenetic processes are altered in relevant cell populations activated by different behavioral responses. Thereby, we focused our analysis on the Cg1 because recent findings have shown that deregulated amino acid expression, including amino acids that modulate epigenetic processes mediated by acetylated histones, is observed in the Cg1 between HAB and LABs (Filiou et al., 2011). In line with these data, in the present study we firstly observed that high anxiety in the LD test was associated with reduced numbers of activated cells in the Cg1. This result, combined with a prior finding showing reduced numbers of active cells following anxiety challenge in the Cg1 of male HABs (open-arm of an elevated plus maze) (Kalisch et al., 2004; Salome et al., 2004; Muigg et al., 2009), suggests that deregulated cellular activity in the Cg1 following anxiety challenge is similar across both male and female HABs. Moreover, we were able to show that modulating anxiety-like behavior from both poles of the anxiety continuum resulted in changes in the number of activated cells in the Cg1; shifting the high anxiety in HABs towards low anxiety (by EE) increased the number of active cells and shifting the low anxiety in LABs (by stress) reduced the number of active cells. These results highlight the plasticity of Cg1 responses across the anxiety spectrum. Mechanistically, ablation of somatostatin interneurons within the Cg1/prelimbic cortices has been found to reduce anxiety in the elevated plus maze and, congruent with our findings, is associated with increased numbers of activated cells (c-Fos-positive cells) in the Cg1/prelimbic cortices (Soumier and Sibille, 2014). Therefore, the study by Soumier and Sibille (2014) provides the impetus for future follow-up studies investigating how local inhibitory circuits can modulate innate anxiety levels in our pathological genetic models of extremes in anxiety. Interestingly, a lesion study within the rCg1/rCg2 in “normal” rats showed no changes in anxiety-like behavior as assessed by the elevated plus maze. However, the authors observed a relatively high baseline anxiety in the test, thereby suggesting that a floor effect may have masked potential anxiogenic effects in the elevated plus maze (Bissiere et al., 2006).

The rodent Cg1 is homologous to rostral anterior cingulate cortex (Brodmann area 24) in humans (Bicks et al., 2015), which

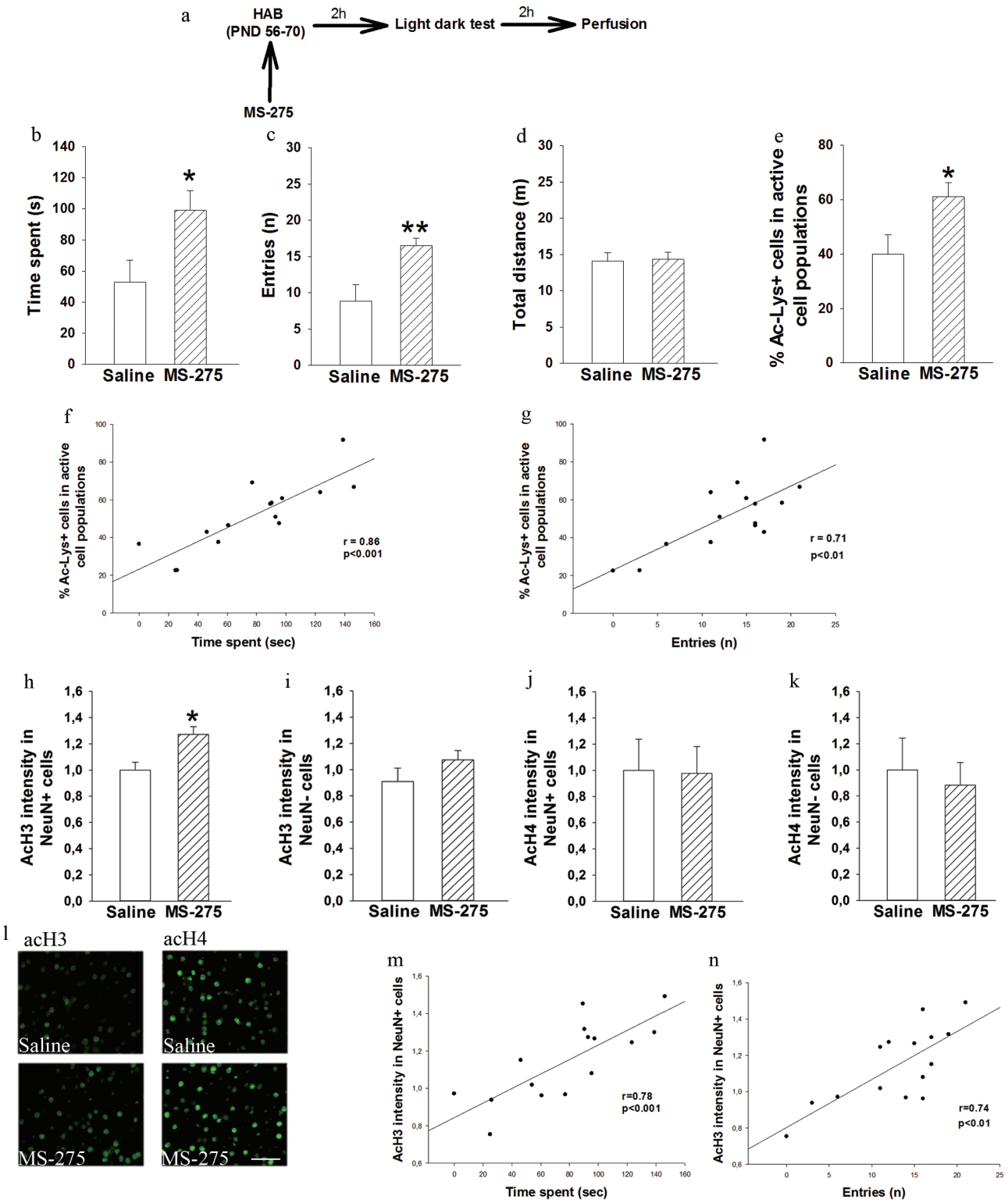

Figure 3. Systemic MS-275 enhances acetylation in the cingulate cortex (Cg1) and attenuates anxiety-like behavior in high anxiety-related behavior (HAB). (a) Experimental design. (b) Behavior. Unpaired *t* test revealed that MS-275 increased the time spent in the light compartment in the light-dark (LD) test ($P < .05$) as well as (c) increased entries ($P < .05$). (d) The locomotor distance was not affected ($P > .05$). (e) Mapping of acetylated-lysine (Ac-Lys) and c-Fos: MS-275-treated HABs showed a greater number of activated neurons expressing Ac-Lys expression (c-Fos+/Ac-Lys+ colocalized cells) ($P < .05$). (f) A significant positive correlation was observed between time spent and c-Fos+/Ac-Lys+ nuclei ($P < .01$). (g) A significant positive correlation was observed between entries and c-Fos+/Ac-Lys+ nuclei ($P < .01$). (h) Acetylated histone-3 (AcH3) mapping: MS-275-treated HABs showed higher intensity of AcH3 within the neuronal, (i) but not within the nonneuronal, population. (j and k) AcH4 mapping. No differences were observed within the acH4 intensity within the neuronal and nonneuronal population. (l) Representative images showing acH3 expression within the saline (top, left) and MS-275 (bottom, left) treated groups. Also representative images showing acH4 expression within the saline (top, right) and MS-275 (bottom, right) treated groups. (m) A significant positive correlation was observed between time spent and acH3+NeuN+ nuclei ($P < .001$). (n) A significant positive correlation was observed between entries and acH3+NeuN+ nuclei ($P < .01$). *$P < .05$ saline vs MS-275. Data are represented as mean ± SEM.

seems to be an important hub in anxiety processing in humans (Rauch et al., 1997) including high trait anxiety (Imperatori et al., 2018). Similar to our findings in the HAB mice showing blunted Cg1 activation, patients with generalized anxiety disorder (Ball et al., 2013) including trait anxiety (Klumpp et al., 2011) also show blunted rostral anterior cingulate cortex activation under challenged conditions. Furthermore, reduction in rostral anterior cingulate cortex connectivity with limbic structures is observed during emotional processing in anxious individuals (Szekely et al., 2017). Interestingly, interventions to reduce anxiety, such as meditation, increased cingulate cortex activity in anxiety patients (Zeidan et al., 2014), similar to what we observed with EE.

Importantly, within the activated cell populations in the Cg1 the amount of lysine acetylation correlated with anxiety-like levels. Specifically, increase in lysine acetylation within the activated cell population was observed when the anxiety-like behavior was reduced. The observed changes in the Cg1 were remarkably specific given that we observed no such alteration in lysine acetylation in activated cell populations in surrounding areas (prelimbic cortex, infralimbic cortex, or motor cortex). Our finding that the level of lysine acetylation in activated cell populations following an anxiety challenge can predict the level of anxiety response in the LD test is a novel finding and led us to investigate this in greater detail. Indeed, our results revealed a functional role of lysine acetylation in the Cg1 in modulating innate anxiety levels. Enhancing lysine acetylation by administration of MS-275 (Entinostat, a HDAC-1, 2, 3 and 9 isoform inhibitor (Khan et al., 2008; Bantscheff et al., 2011) directly into the Cg1 was sufficient to reduce the innate hyperanxiety behavior of HAB mice. This result highlights an important mechanistic role of the Cg1 in modulating anxiety levels because applying MS-275 in the prelimbic/infralimbic cortices, nucleus accumbens, or hippocampus fails to modulate anxiety levels (Covington et al., 2009, 2015; Yuan et al., 2015). Our present data extend this finding to innate hyperanxious individuals.

We further narrowed our findings and demonstrated that reduction of high anxiety following MS-275 administration was associated with a dichotomy in the acetylation of 2 key histone protein isoforms that gate epigenetic regulation of gene transcription. We observed increased acetylation of histone 3, but not histone 4, within the neurons of the Cg1. Unlike the specific increase in acetylation of histone 3 (in the current study), the HDAC inhibitor Trichostatin A normalized innate anxiety in alcohol-preferring rats compared with nonpreferring rats via increasing histone H3 and H4 acetylation within the amygdala (Sakharkar et al., 2014). This difference could be attributed to the selectivity of MS-275, which is restricted to Class I HDACs unlike trichostatin A, which targets both class I and II HDACs, or the fact that different brain regions (Cg1 vs amygdala) were quantified. Collectively, these studies highlight the specificity of MS-275 in modulating innate anxiety via altering acetylation of H3.

Acetylation and/or methylation of H3 was also altered in response to challenges such as conditioned anxiety (Fischer et al., 2007; Bredy et al., 2010; Malvaez et al., 2010; Monsey et al., 2011; Stafford et al., 2012; Matsumoto et al., 2013; Chaudhury et al., 2014; Pizzimenti and Lattal, 2015) and chronic stress (Nasca et al., 2015; Lomazzo et al., 2017), thereby modulating state anxiety. These studies together with the present study suggest that epigenetic alterations within H3 could be used as a biomarker to predict anxiety levels as well as the anxiolytic response.

To highlight the potential therapeutic value of our result, we systemically administered MS-275 in high-anxiety mice and revealed that this treatment is sufficient to shift the high anxiety toward a lower anxiety level. Interestingly, a selective increase in colocalization of c-Fos and ac-Lys+ cells was observed following the anxiolytic effect by systemic administration of MS-275 within the Cg1, but not other mPFC regions, suggesting that Cg1 is particularly recruited in the MS-275-induced anxiolytic-like effect. Because no general increases in ac-Lys level or c-Fos+ cells were observed within the Cg1 (data not shown), this result indicates that the increase in acetylation was observed specifically in activated cells. These data support the notion that HDAC inhibitors prime the genome such that the anxiolytic modulation of neuronal activity within the Cg1 can enhance acetylation. Additionally, increased histone-3 acetylation was observed only in the neuronal but not nonneuronal populations within the Cg1, further supporting the epigenetic priming theory. Therefore, our data indicate that reduction in innate anxiety via HDAC inhibitor involves epigenetic priming within the Cg1 (Graff et al., 2014). This idea, however, needs further support in future investigations. Finally, our study suggests that histone 3-specific HDAC inhibitor(s) selectively increasing histone-3 acetylation could be of immediate therapeutic importance.

Conclusions

Our study using female mice reveals for the first time, to our knowledge, that epigenetic mechanisms via altered acetylation levels are associated with enhanced innate anxiety. Subsequently, positive behavioral interventions such as EE and/or pharmacological manipulations targeting histone acetylation (such as HDACi) can normalize innate hyperanxiety via acetylation changes within the Cg1. In this brain region, the HDACi-induced increase in acetylation levels within histone-3 correlated with the anxiolytic response, suggesting that epigenetic marks within histone-3 may serve as a biomarker for treatment success. Finally, histone-3-specific HDACi could be of therapeutic importance in anxiety disorders.

Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

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Statement of Interest

None.

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