

ORIGINAL ARTICLE

Potential role for histone deacetylation in chronic diazepam-induced downregulation of $\alpha 1$ -GABA_A receptor subunit expression

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Funding information

National Institute on Alcohol Abuse and Alcoholism, Grant/Award Number: P50AA022538; NIH Blueprint for Neuroscience Research, Grant/Award Number: 1R01MH093348, 1R01MH101043

Abstract

Corroborating evidence indicate that the downregulation of GABA_A receptor subunit expression may underlie tolerance to the anticonvulsant and anxiolytic actions of benzodiazepine (BZ) ligands that act as full allosteric modulators (FAMs) of GABA actions at a variety of GABA_A receptor subtypes. We and others have shown that 10-14 days treatment with increasing doses of diazepam (a FAM) resulted in anticonvulsant tolerance and decreased the expression of the $\alpha 1$ GABA_A receptor subunit mRNA and protein in frontal cortex. In addition, we have also shown that long-term treatment with imidazenil, a partial allosteric modulator of GABA action at selective GABA_A receptor subtypes, fail to change the expression of the $\alpha 1$ subunit mRNA or induce tolerance to its anticonvulsant or anxiolytic action. However, little is known regarding the potential role of epigenetic mechanisms on long-term BZ-induced downregulation of GABA_A receptor subunit. Therefore, we examined the role of histone acetylation and DNA methylation mechanisms on long-term diazepam-induced downregulation of the $\alpha 1$ subunit mRNA expression in rat frontal cortex. We found that 10 days treatment with increasing doses of diazepam but not imidazenil decreased the expression of the $\alpha 1$ GABA_A receptor subunit mRNA and promoter acetylation in frontal cortex. In addition, we also found that 10 days treatment with diazepam but not imidazenil increased the expression of histone deacetylase (HDAC) 1 and 2 in frontal cortex. Thus, the increased expression of HDAC1 and HDAC2 (class 1 HDACs) and consequently increased histone deacetylation mechanism of this class 1 HDACs, may underlie long-term diazepam-induced decreased expression of the $\alpha 1$ GABA_A receptor subunit mRNA in frontal cortex.

KEYWORDS

diazepam, histone deacetylation, imidazenil, $\alpha 1$ GABA_A receptor subunit

Abbreviations: GABA, g-aminobutyric acid; BZ, benzodiazepines; FAM, full allosteric modulator; PAM, partial allosteric modulator; HDAC, histone deacetylase; MeCP2, methyl CpG binding protein 2; SAHA, Suberoylanilide hydroxamic acid; TSA, Trichostatin A; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; H3K9me2, histone H-3 dimethylated; DNMT, DNA methyl transferase; TET, ten-eleven translocas0065.

1 | INTRODUCTION

Long-term and repeated daily administration of benzodiazepines with full allosteric modulatory (FAM) (eg, diazepam, triazolam, and

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alprazolam) but not those with partial allosteric modulatory (PAM) actions (imidazenil and bretazenil) at a variety of GABA_A receptor subtypes has been well established to lead to tolerance and dependence liabilities.¹⁻³ Although considerable biochemical and electrophysiological evidence suggests that tolerance to the anxiolytic and anticonvulsant effects of FAM are associated with downregulation of receptor subunits expression and GABA_A receptor function,⁴⁻⁹ the precise molecular mechanism underlying the downregulation of GABA_A receptor subunits function is still not understood.

It is now widely recognized that epigenetic mechanisms such as DNA promoter methylation, histone acetylation, and methylation play significant roles in the pathophysiology of brain disorders including addictive behaviors, tolerance, and dependence liabilities.¹⁰⁻¹⁴ Histone tail lysine acetylation which involves the addition of acetyl groups to lysine residues on histone tails leads to a relaxed (open) chromatin structure and consequently the activation of target gene expression. In addition, the acetylation of histone tails is also regulated by histone acetyltransferases and histone deacetylases.¹⁵⁻¹⁷ Conversely, DNA promoter methylation which involves the covalent coupling of a methyl group to the C5 position of cytosine residues of CpG dinucleotide upstream of the transcription start site, is catalyzed by DNA methyltransferases [DNA methyltransferase 1 (Dnmt1), DNA methyltransferase 3a (Dnmt13a) DNA methyltransferase 3b (Dnmt3b) DNA methyltransferase 3 l (Dnmt3 l)] and is generally associated with gene silencing.^{11,12} Recent studies have also shown that DNA methylation is kept in a steady state by the action of a demethylase system which includes a first step 5-hydroxy methylation driven by ten-eleven translocase (TET) enzymes.¹⁸ The role of these enzymes is difficult to establish because of the lack of specific pharmacological agents targeting these enzymes.

Histone acetylation and DNA methylation are targeted by histone deacetylase (HDAC) inhibitors and DNA methyltransferase inhibitors, respectively.^{10,11,18} Thus, the epigenetic regulation of targeted gene expression with selective pharmacotherapeutic strategies is increasingly becoming a viable alternative for the design and development of new therapeutic strategies to treat specific types of malignancies and neuropsychiatric disorders such as Huntington disease, schizophrenia and depression.¹⁹⁻²⁴ Interestingly, it has also been reported that chronic antidepressant-induced increase acetylation status of histone proteins in frontal cortex and hippocampus contributes to the therapeutic effects of antidepressant interventions.²⁵ These findings raise the possibility that long-term diazepam exposure may lead to alterations of epigenetic mechanisms regulating the expression of α 1-GABA_A receptor subunit resulting to the downregulation of this receptor subunit, and a process that may underlie tolerance to the pharmacological actions of diazepam.

We and others have previously shown that long-term treatment of rats with increasing doses of diazepam or triazolam (FAMs) for 10-14 days induced anticonvulsant tolerance and decreased expression of mRNA and protein encoding for the α 1 GABA_A receptor subunit in prefrontal cortex.^{2,7,8,26,27} In contrast, long-term treatment with equipotent anti-bicuculline doses of partial allosteric modulators (PAM) of GABA_A receptors (imidazenil or bretazenil) fail to elicit

anticonvulsant tolerance or change the expression of mRNA and protein encoding for α 1 GABA_A receptor subunit.^{7,8,26,27} Furthermore, Rudolph et al.²⁸ demonstrated that the protective action of diazepam against pentylenetetrazole-induced tonic-clonic seizure is significantly reduced in α 1 GABA_A receptor subunit mutant mice when compared to their wild-type counterparts. It has also been suggested that α 1-containing GABA_A receptors probably mediate the sedative, amnesic and anticonvulsant actions of classical benzodiazepines.^{29,30} Taken together, these findings suggest that the downregulation of α 1-containing GABA_A receptors by FAM of GABA action might underlie anticonvulsant tolerance and dependence liabilities.

Recent studies in cerebral cortical cultured neurons have shown that the decreased α 1 GABA_A receptor subunit expression observed following chronic alcohol exposure is mediated by histone modifications.³¹ To the best of our knowledge there are no published studies which have examined the role of epigenetic mechanisms on long-term benzodiazepines-induced downregulation GABA_A receptor subunit expression. In light of these considerations, we examined the role of histone acetylation and DNA methylation on long-term imidazenil or diazepam-induced downregulation of α 1-GABA_A subunit mRNA expression in rat frontal cortex. Our results show that 10 days of repeated oral treatment with increasing doses of diazepam but not imidazenil resulted in decreased expression of α 1-GABA_A subunit mRNA and promoter acetylation in frontal cortex.

2 | MATERIALS AND METHODS

2.1 | Animals, drugs, and reagents

Adult male Sprague Dawley rats (Harlan, Indianapolis) were received and after 1 week of acclimatization they were housed individually, maintained on a 12/12-h light-dark cycle with standard food and water made available ad libitum. All experiments were carried out in accordance with the National Institute of Health Guidelines for the Care and Use Laboratory Animals and approved by the animal committee at the University of Illinois at Chicago.

Diazepam and imidazenil were obtained from Hoffman-La Roche (Nutley, NJ); TRIZOL reagent was obtained from Life Technologies (Life Technologies Corporation, USA); Qiagen RNeasy Kit from Qiagen (Valencia, CA, USA); Fermentas Maxima SYBR Green/ROX qPCR Master Mix obtained from Fermentas (Fermentas International Inc., Canada); Novex 4%-12% Tris-Glycine gels obtained from Invitrogen (Carlsbad, CA); HRP-conjugated secondary anti-mouse or anti-rabbit antibodies from GE Healthcare (Arlington Heights, IL); anti-GAPDH, Immobilon Western Chemiluminescent HRP Substrate, anti-H3K9me2, anti-MeCP2, anti-H3, and anti-acetyl-H3 from Millipore (Billerica, MA).

2.2 | Schedule for long-term treatment

For long-term diazepam, imidazenil or vehicle treatment, we used the same treatment schedule used in our previous studies in which

we demonstrated anti-bicuculline tolerance and downregulation of $\alpha 1$ GABA_A receptor subunit expression following long-term treatment with FAMs of GABA action at GABA_A receptors.^{7,8,26,27} In brief, diazepam or imidazenil was suspended in water containing 0.05% Tween-20 and administered in 1 mL volumes by oral gavage 3 times daily (at approximately 9:00 AM, 2:00 PM and 7:00 PM) for 10 days at increasing doses (diazepam: days 1-3, 17.6 $\mu\text{mol/kg}$; days 4-6, 35.2 $\mu\text{mol/kg}$ and days 7-10, 52.8 $\mu\text{mol/kg}$; imidazenil: days 1-3, 2.85 $\mu\text{mol/kg}$; days 4-6, 5.70 $\mu\text{mol/kg}$; and days 7-10, 8.55 $\mu\text{mol/kg}$). Control rats received vehicle (0.05% Tween 20 in water) treatment. Rats were sacrificed at least 18 hours after the last diazepam or imidazenil dose and frontal cortex dissected and stored at -80°C until used for RT-qPCR, ChIP assay or western blot.

2.3 | RNA extraction and real-time polymerase chain reaction (PCR) quantification

Total RNA was isolated from frontal cortices using TRIzol reagent and further purified using Qiagen RNeasy Kit. Total RNA was converted to cDNA using the Applied Biosystems (USA) High Capacity Archive Kit (4368813). Relative quantitative real-time polymerase chain reaction (qPCR) was performed with the Applied Biosynthesis Real-Time PCR system using Fermentas Maxima SYBR Green/ROX qPCR Master Mix (K0222; Fermentas International Inc., Canada). PCR mixtures were run on a Stratagene (USA) Mx3005P QPCR System. Primers were designed to cross over one intron to amplify cDNA and yield an amplicon of between 90 and 130 base pairs (primer sequences are provided in Table 1). Dissociation curves were conducted to establish the presence of a single amplicon at the predicted melting temperature and a lack of primer-dimer formation. A comparative threshold cycle (C_T) validation experiment was done to determine target and reference primer efficiency. For normalization of mRNAs expression, GAPDH was used as reference gene. C_T value was used for relative quantification of target gene expression and normalized to GAPDH and the relative expression levels were calculated as C_T .^{32,33}

2.4 | Chromatin immunoprecipitation assay

In order to establish a possible role for epigenetic mechanisms in long-term diazepam-induced downregulation of $\alpha 1$ GABA_A receptor subunit mRNA expression, we examined DNA methylation, histone acetylation, and methylation levels at $\alpha 1$ GABA_A receptor subunit promoter in frontal cortex. We used specific antibodies against H3K9me2, MeCP2, and acetylated histone H3 for chromatin immunoprecipitation (ChIP) assay as previously described.^{34,35} In brief, frontal cortices were fixed in methanol-free 1% formaldehyde by incubating on a rocking platform at room temperature for 15-20 minutes followed by homogenization in lysis buffer and DNA shearing by sonication. The resulting cross-linked DNA-chromatin complex was immunoprecipitated (IP) with antibodies directed at H3K9me2, MeCP2, or acetylated histone H3, and the precipitated DNA quantified by Real-Time PCR system using Fermentas Maxima

SYBR Green/ROX qPCR Master Mix. Input DNA was used as internal standard for normalization. After subtraction of input DNA Ct value from the Ct values of the respective samples, the $\Delta\Delta C_t$ method³² was used to determine the fold change in levels of H3K9me2, MeCP2, H3-total, or H3-acetylated at the $\alpha 1$ GABA_A receptor promoter. The percentages of IP DNA were calculated using the following formula: $\% (\text{IP}/\text{total input}) = 2^{(C_t (\text{10\% input}) - C_t (\text{IP}))} \times 100\%$. The primer sequences for $\alpha 1$ GABA_A subunit promoter were: Forward-5'-GTGCAAGTTAAATTGCGCTGCA-3', Reverse-5'-GCTTCCCAA TATCCAATCTGCAGC-3' (see Figure 1).

2.5 | Western blot

Frontal cortex of vehicle, imidazenil and diazepam-treated rats were homogenized in HEPES-buffered sucrose (320 mmol L⁻¹ Sucrose, 4 mmol L⁻¹ HEPES pH 7.4, 1% SDS). We used Pierce® Bicinchoninic acid (BCA) assay to determine protein concentration (Thermo Fisher Scientific, Waltham, MA). Lysates were resuspended in Laemmli reducing buffer and 30 μg of each sample were separated by electrophoresis on Novex 4%-12% Tris-Glycine gels. Proteins were then transferred to PVDF membrane in a buffer containing 35 mmol L⁻¹ Tris, 192 mmol L⁻¹ glycine, and 20% methanol at 4°C. Membranes were blocked using 5% milk and later incubated with the primary antibody, anti-H3 (1:1000) or anti-acetyl-H3 (1:1500). Protein levels were normalized with GAPDH (1:5000). HRP-conjugated secondary anti-mouse or anti-rabbit antibodies (1:10 000) were used. Membranes were developed with Immobilon Western Chemiluminescent HRP Substrate and densitometric analysis performed with Image Studio™ (LI-COR, Bioscience). The expression levels for the respective proteins were normalized to GAPDH protein levels.

2.6 | Statistical analysis

Data were analyzed by one-way ANOVA followed by Tukey's post hoc comparison.

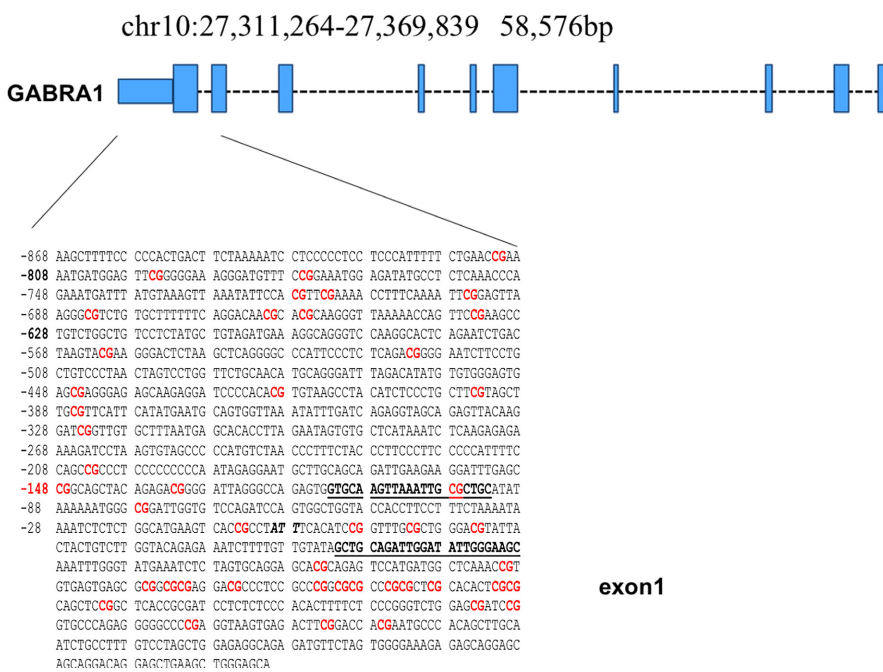
3 | RESULTS

3.1 | Long-term diazepam treatment alters the expression of $\alpha 1$ -GABA_A receptor subunit and Dnmt3b in frontal cortex

In previous studies, we demonstrated that 5-10 days treatment with diazepam but not imidazenil resulted to anticonvulsant tolerance against bicuculline-induced tonic convulsion²⁶ in Sprague Dawley rats. In subsequent studies we also demonstrated that this same diazepam treatment schedule consistently elicited downregulation of $\alpha 1$ GABA_A receptor subunit mRNA and cognate protein expression in rat frontal cortex.^{7,8,27,36} Here, we measured the expression of mRNAs encoding for $\alpha 1$ -GABA_A receptor subunit, MeCP2, Dnmt1 and Dnmt3b in frontal cortex 18 hours after the last dose of 10 days diazepam, imidazenil, or vehicle treatment. As previously

TABLE 1 Primer Sequences used for real-time PCR

Gene	Forward	Reverse
α 1GABAR	5'-GTGCAAGTTAAATTGCGCTGCA	5'-GCTTCCCAATATCCAATCTGCAGC
DNMT1	5'-AAGCCAGCTATGCGACTTGAAAC	5'-ACA ACC GTTGCTTTCTGAGTGA
DNTM3b	5'-TGTGCAGAGTCCATTGCTGTAGGA	5'-GCT TCCGCCAATACCAAGTCAA
MeCP2	5'-ACCAGCTCCAACAGATTCCAT	5'- TCTGCAGAATGGTGGGCTGA
HDAC1	5'-GACATGCCAAGTGTGTGGAGT	5'- CACAGCTGTCTCGTAAGTCCAG
HDAC2	5'-GTGAAGCTGAACCGTCAACAGA	5'-CTCAGGACAGCAAGCACAA
HDAC3	5'-TGGTGGTCTACATCATGCCAA	5'-CGAGGGTGGTATTGAGCAG
GAPDH	5'-ACCAGGGCTGCCTTCTCT	5'-ATCTCGCTCCTGGAAGATGGT

**FIGURE 1** Sequence and genomic location for Gabra1 promoter region of interest including the first exon. ChIP assays were performed using the primer pair indicated in underlined bold letters and the transcription start site in italicized bold letters

reported, we have confirmed that long-term treatment with diazepam but not imidazenil in doses that induced anticonvulsant tolerance²⁷ resulted in a significantly decreased expression of mRNA encoding for the α 1-GABA_A receptor subunit. In addition, 10 days of diazepam treatment also elicited a significantly increased the expression of mRNA encoding for Dnmt3b but failed to alter the expression of mRNAs encoding for Dnmt1 and MeCP2 in frontal cortex (Figure 2).

3.2 | Decreased α 1GABA_A receptor subunit mRNA expression is associated with decreased acetylated histone H3 without altering the level of H3K9me2

To investigate a possible role for epigenetic mechanisms on long-term diazepam-induced downregulation α 1GABA_A receptor subunit expression, we examined whether the decreased expression of this subunit mRNA in frontal cortex is associated with increased DNA methylation (MeCP2 occupancy), increased occupancy of H3K9me2 or decreased histone H3 acetylation at the α 1 GABA_A receptor

subunit promoter. In Figure 3, we show that long-term treatment with diazepam but not imidazenil significantly decreased the occupancy of acetylated histone H3 at α 1GABA_A receptor subunit promoter. In addition, diazepam treatment also resulted in a significantly increased of the occupancy of MeCP2 in the absence of expression changes but failed to alter the level of H3K9me2 at this GABA_A receptor subunit promoter while imidazenil treatment had no effect. These results indicate that only long-term diazepam treatment resulted in a significant reduction of the level of acetylated histone H3 at α 1GABA_A receptor subunit promoter thereby leading to a decreased expression of this receptor subunit in frontal cortex. Thus long-term diazepam exposure will most likely result to the induction of chromatin and synaptic remodeling in frontal cortex.

3.3 | Long-term diazepam treatment alters the expression of HDAC1 and HDAC2 but not HDAC3

Since long-term diazepam treatment decreased the levels of acetylated histone H3 at α 1-GABA_A receptor subunit promoter, we also

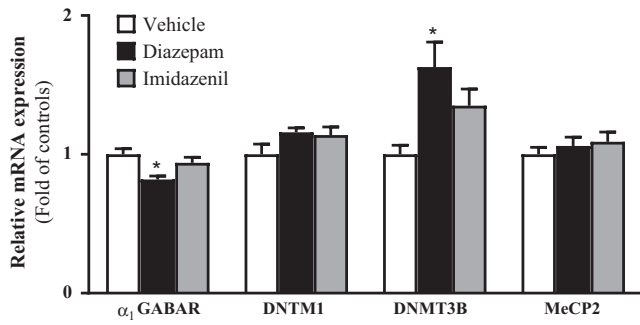


FIGURE 2 Effects of long-term diazepam and imidazenil treatment on mRNA profiling in frontal cortex. Frontal cortices of rats treated with increasing doses of diazepam, imidazenil or vehicle for 10 days were sacrificed 18 hours after the last dose, brains collected and frontal cortices dissected for mRNA analysis. Values are expressed as mean \pm SEM of the fold change in controls, $n = 5$ rats. * $P < .05$ significantly different from controls, one-way ANOVA followed by post hoc Turkey's test

examined whether the decreased levels of acetylated histone H3 was mediated by increased expression of histone deacetylase (HDAC) enzymes. It has been suggested that HDAC2 plays a significant role in the development of alcohol dependence in rodents,^{10,37-39} therefore we examined the expression of class 1 HDACs (HDAC 1, 2, and 3), which are localized in the nucleus.⁴⁰ In Figure 4, we show that long-term treatment with diazepam but not imidazenil significantly increased the expression of HDAC1 and HDAC2 without changing the expression of HDAC3. These results suggest that the decreased levels of acetylated histone H3 at the α_1 -GABA_A receptor subunit promoter and consequently the decreased α_1 -GABA_A receptor subunit expression may be due to increased expression and most likely, the enzymatic activity of HDAC1 and HDAC2.

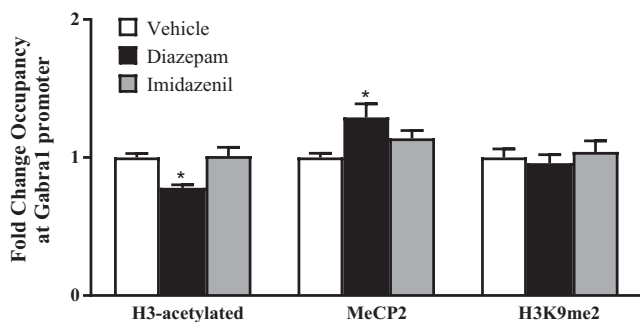


FIGURE 3 Effects of long-term diazepam and imidazenil treatment on histone H3-acetylated, histone H-3 dimethylated (H3K9me2) and MeCP2 occupancy at Gabra1 gene promoter as shown in Figure 1. Frontal cortices of rats treated with increasing doses of diazepam, imidazenil or vehicle for 10 days were used to measure occupancy at the Gabra1 promoter by chromatin immunoprecipitation (ChIP) assay. Values are represented as the mean \pm SEM of the fold change in controls, $n = 5$ rats. * $P < .05$ significantly different from controls, one-way ANOVA followed by post hoc Turkey's test

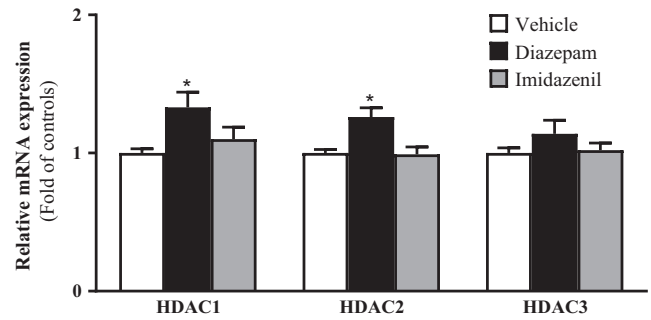


FIGURE 4 Effects of long-term diazepam and imidazenil treatment on class 1 HDACs mRNA profiling in frontal cortex. Long-term diazepam but not imidazenil increased HDAC1 and HDAC2 expression in frontal cortex. Frontal cortices of rats treated with increasing doses of diazepam, imidazenil or vehicle for 10 days were sacrificed 18 hr after the last dose, brains collected and frontal cortices dissected for mRNA analysis. Values are expressed as mean \pm SEM of the fold change in controls, $n = 5$ rats. * $P < .05$ significantly different from controls, one-way ANOVA followed by post hoc Turkey's test

3.4 | Long-term diazepam treatment does not alter the expression of total histone H3 or acetylated histone H3 in frontal cortex

Because 10 days diazepam treatment significantly decreased the occupancy of acetylated histone H3 at the promoter of the α_1 GABA_A receptor subunit, we examined whether long-term treatment with diazepam or imidazenil led to changes in the expression of total or acetylated histone H3 protein in frontal cortex. The data expressed as the ratio of the optical density of the target protein (total histone H3 or acetylated histone H3) and GAPDH indicated that there were no statistical significant differences between the levels of total histone H3 (vehicle 0.818 ± 0.079 , diazepam 0.557 ± 0.070 , imidazenil 0.614 ± 0.082 ; $F_{(2,11)} = 3.491$, $P = .067$) and acetylated histone H3 (vehicle 0.742 ± 0.051 , diazepam 0.576 ± 0.049 , imidazenil 0.564 ± 0.044 ; $F_{(2,11)} = 4.245$, $P = .063$) in frontal cortex of long-term diazepam or imidazenil-treated groups when compared with the vehicle-treated group. These results indicate that the decreased acetylated histone H3 bound to the α_1 GABA_A receptor subunit is not due to changes in the levels of histone H3 proteins resulting from long-term diazepam treatment. Thus, the decreased levels of histone H3 acetylated at α_1 -GABA_A receptor subunit promoter resulting from long-term diazepam treatment may underlie the decrease expression of this GABA_A receptor subunit in frontal cortex.

4 | DISCUSSION

The outcomes of in vivo and in vitro studies on the regulation of GABA_A receptor subunit expression show that the magnitude and direction of long-term benzodiazepine-induced alterations of GABA_A receptor subunit expression is subunit-specific, brain region-specific,

and treatment duration dependent. In addition, overwhelming evidence show a consistent downregulation of $\alpha 1$ GABA_A receptor subunit expression following long-term treatment with FAMs of GABA action at a variety of GABA_A receptor subtypes.^{2,41} Moreover, the concurrent decreases in $\alpha 1$ subunit mRNA and protein expression which occurs following chronic diazepam treatment,⁸ suggests that transcriptional mechanisms might play a significant role in regulating the expression of GABA_A receptor subunits during long-term benzodiazepine exposure. This study focuses on the role of epigenetic mechanisms in long-term diazepam-induced downregulation of $\alpha 1$ GABA_A receptor subunit expression. To the best of our knowledge, this is the first study reporting association between alterations of acetylated histone H3 and long-term benzodiazepine-induced downregulation of GABA_A receptor subunit expression. Specifically, we report here that the resulting decrease in the expression of $\alpha 1$ GABA_A receptor subunit in frontal cortex associated with long-term treatment with diazepam (a FAM) but not imidazenil (a PAM) might be due to decreased levels of acetylated histone H3 at the promoter of $\alpha 1$ -containing GABA_A receptors. Furthermore, the increased occupancy of MeCP2 may recruit HDAC1 and HDAC3 to this promoter complex and thus augment histone deacetylation mechanisms and indirectly DNA methylation mechanisms at this promoter.⁴² Taken together, the increased expression of HDAC1 and HDAC2 and consequently histone deacetylation mechanism in frontal cortex may underlie long-term diazepam-mediated decrease in the levels of acetylated histone H3 at this GABA_A receptor subunit promoter.

Chronic exposure to ethanol, a positive allosteric modulator of GABA action at GABA_A receptors results in decreases in mRNA and protein expression for $\alpha 1$ GABA_A receptor subunit both in vitro and in vivo.⁴³⁻⁴⁵ Most importantly, Arora et al.³⁷ reported that treatment with HDAC inhibitors attenuated the decrease in $\alpha 1$ protein expression induced by chronic ethanol exposure. In a different but related study, Di et al.⁴⁶ reported that $\alpha 1$ (A322D) gene mutation leads to the decrease expression of $\alpha 1$ subunit found in epilepsy. Together, the results of these studies suggest a possible involvement of histone acetylation and/or DNA methylation mechanisms in the regulation of $\alpha 1$ GABA_A receptor subunit expression. The results of our studies indicate that long-term diazepam-induced downregulation of $\alpha 1$ subunit expression might in part be mediated by alterations of the histone acetylation mechanisms associated with the $\alpha 1$ GABA_A receptor subunit promoter. It has been established that the deacetylation of histone core wrapped around promoters leads to a closed or condensed chromatin structure, which has been associated with reductions in gene transcription and in rare cases gene silencing precludes changes in histone acetylation.⁴⁷

It is important to note that long-term treatment with imidazenil, a PAM which does not elicit anticonvulsant tolerance or the downregulation of the $\alpha 1$ subunit following chronic treatment,^{2,7,8,26,27,48,49} fail to downregulate $\alpha 1$ subunit expression, change the levels of acetylated histone H3 at the $\alpha 1$ GABA_A receptor subunit promoter or change expression of HDAC1 and HDAC2 in frontal cortex (Figures 2 and 4). Therefore one can surmise that HDAC1 and HDAC2 mediated histone deacetylation of the $\alpha 1$ GABA_A receptor subunit promoter

might regulate the downregulation of the $\alpha 1$ subunit expression during long-term diazepam treatment. This inference is in agreement with the studies by Arora et al.,³⁷ in which they found that blockade of the upregulation of HDAC2 with HDAC inhibitors [Suberoylanilide hydroxamic acid (SAHA) or Trichostatin A (TSA)] normalized the hyposensitivity of dopaminergic ventral tegmental area GABAergic neurons to GABA. In addition, it has been suggested that HDAC2 is involved in the development of alcohol dependence.^{38,39} It is also interesting to note that HDAC1 and HDAC2 which are typically localized in the nucleus, are structurally identical and are often found in the same repressive complexes with MeCP2.⁴⁰ MeCP2, which is often found in a complex together with class I HDACs acts as a transcriptional repressor by recruiting HDACs to active gene promoters^{50,51} and may also repress the transcription of the $\alpha 1$ receptor subunit by recruiting HDAC1 and HDAC2 to this GABA_A receptor subunit promoter complex. Thus, HDAC1 and HDAC2-mediated histone deacetylation at the $\alpha 1$ GABA_A receptor subunit promoter may trigger the signaling mechanisms that underlie the downregulation of the $\alpha 1$ subunit that is associated with long-term benzodiazepine treatment. Accordingly, pretreatment or concurrent treatment with selective HDAC1 and HDAC2 inhibitors might prevent the decrease in $\alpha 1$ GABA_A receptor subunit expression associated with chronic benzodiazepine treatment. Therefore future experiments will examine whether concurrent treatment with selective HDAC inhibitors will attenuated the decrease $\alpha 1$ subunit expression associated with anxiolytic and anticonvulsant benzodiazepines tolerance and/or dependence.

In conclusion, the outcome of our study points to a new and important role for histone deacetylation in chronic benzodiazepine-induced downregulation of $\alpha 1$ GABA_A receptor subunit expression. However, further studies will be needed to establish whether the decreased $\alpha 1$ subunit expression elicited by long-term diazepam can be attenuated by pretreatment or concurrent treatment with class 1 HDAC inhibitors. Nonetheless, our study presents a central role for epigenetic mechanisms in the transcriptional regulation of GABA_A receptor subunit expression during chronic benzodiazepine exposure. Furthermore, understanding the role of HDAC inhibitors in benzodiazepine tolerance and the downregulation of GABA_A subunit expression may uncover new therapeutic targets to prevent benzodiazepine tolerance, dependence and/or withdrawal syndrome.

ACKNOWLEDGEMENT

This study was supported by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) grant P50AA022538 to Alessandro Guidotti and Subhash Pandey, Senior Research Career Scientist award from the Department of Veterans Affairs to Subhash Pandey, 1R01MH101043 and 1R01MH093348 to Alessandro Guidotti.

AUTHORS' CONTRIBUTIONS

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Contributed reagents and analytical tools: A. Guidotti, S.C. Pandey, J. M. Davis.

Performed data analysis: J. Auta, E. Gatta, A. Guidotti.

Wrote or contributed to the writing of the manuscript: J. Auta, S.C. Pandey, A. Guidotti.

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How to cite this article: Auta J, Gatta E, Davis JM, Pandey SC, Guidotti A. Potential role for histone deacetylation in chronic diazepam-induced downregulation of $\alpha 1$ -GABA_A receptor subunit expression. *Pharmacol Res Perspect*. 2018; e00416. <https://doi.org/10.1002/prp2.416>