## Original Article
# Chronic pethidine treatment induces histone H3 acetylation in rat brain

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**Abstract:** Many gene transcriptional activities are modulated by histone acetylation at promoters. The present studies investigated histone H3 acetylation level following single and repeated pethidine treatment in different brain regions. Our data showed that repeated, but not single pethidine administration, induced a significant increase of histone H3 acetylation level in the subiculum of hippocampus. However, changes of histone H3 acetylation level was not detected in the CA1 of hippocampus or the medial prefrontal cortex (mPFC). Furthermore, a relationship between histone acetylaton and pethidine-induced place preference were discovered. Moreover, addiction-related gene transcriptional activations were regulated by H3 acetylation at specific gene promoters in the subiculum of hippocampus. Taken together, our data demonstrate that H3 acetylation in the subiculum of hippocampus may be involved in neural plasticity changes in opioid abuse.

**Keywords:** Histone acetylation, hippocampus, addiction, epigenetic, pethidine

## Introduction

Pethidine is a valuable drug in general practice. It is useful in the acute pain of trauma and renal or biliary colic. For much of the 20th century, pethidine was the opioid of choice for many physicians [1]. In fact, pethidine is no more effective than morphine at treating biliary or renal pain, and its low potency, short duration of action, and unique toxicity (i.e., seizures, delirium, other neuropsychological effects) relative to other available opioid analgesics have seen it fall out of favor in recent years for all but a very few, very specific indications. Several countries, including Australia, have put strict limits on its use [2]. Nevertheless, some physicians continue to use it as a first line strong opioid, for instance, pethidine is routinely used in our center in post cesarean pain treatment.

Unlike morphine, a potent mu opioid receptor agonist, pethidine exerts its analgesic effects by acting as an agonist at the kappa opioid receptor, primarily [3]. However, like other opioid drugs, pethidine has the potential to cause physical dependence or addiction [3, 4]. Pethi-

dine may be more likely to be abused than other prescription opioids, perhaps because of its rapid onset of action. The regulation of gene expression in the brain reward regions is known to contribute to the pathogenesis and persistence of drug addiction [5]. For example, chronic cocaine experience activate a set of genes in the nuclear accumbence (NAc), the Medial Prefrontal Cortex (mPFC) or the hippocampus, and the expression of these genes remains elevated for days to weeks [6-9]. Hence, the investigation on the adaptive change of the level of gene transcription caused by chronic opiate treatment is crucial for further understanding of the mechanisms of opiate addiction.

Recent evidence has prompted the notion that epigenetic mechanisms exert lasting effects on gene expression through the regulation of chromatin structure [10]. In general, increased histone acetylation level is correlative with DNA relaxation and elevated transcriptional activity, whereas decreased acetylation, results in tighter DNA coiling and gene silencing. The regulation of histone acetylation is recognized as a vital mechanism in several important phenom-

**Table 1.** Primers for PCR

| Method | Gene | Primer sequences |
|--------|------|------------------|
| RT-PCR | βactin | Fwd: TTACTGCCCTGGCTCCTA |
| | | Rev: ACTCATCGTACTCCTGCTTG |
| | c-fos | Fwd: GGAATTAACCTGGTGCTGGA |
| | | Rev: TGAACATGGACGCTGAAGAG |
| | | Rev: AGCCAGCTGTATTGCTCCTCCCT |
| | Cdk5 | Fwd: TGGCCGCTGCGATGCAGAAA |
| | | Rev: CCCGGAGGGCTGAACTTGGC |
| | BDNF | Fwd: TTGCCACAGCCCCAGGTGTGA |
| | | Rev: ACGCCTGTCACTGCGCCCTA |
| | FosB | Fwd: GGAGAAGCGTCGAATCCGGAGG |
| | | Rev: GGCCGTGAGCCACCAGCATGA |
| | CaMKIIa | Fwd: CCCCAATATCGTCCGACTC |
| | | Rev: CAGCCTCACTGTAATACTCCC |
| CHIP | BDNF | Fwd: TGAGGATAGGGGTGGAGTTG |
| | | Rev: GCAGCAGGAGGAAAAGGTTA |
| | FosB | Fwd: CCCTCTGACGTAATTGCTAGG |
| | | Rev: CACCTGCCCATAGTGTGACC |
| | CaMKIIa | Fwd: ACAGGGCGTTTCGG |
| | | Rev: TCGGGACTAGGACTGG |

ena in the brain, including neurodegeneration [11, 12], neuronal differentiation [13, 14], seizure [15, 16], memory formation [17-19], and drug addiction [20, 21]. However, the potential effects of pethidine treatments on addiction-related genes transcriptional activation were unclear.

## Materials and methods

### Antibodies and reagent

The primary antibodies used, and their dilutions were as follows: anti-acetylated H3 (rabbit polyclonal, Lys-9/Lys-14, 1 mg/ml, Upstate Biotechnology), anti-β-actin (Rabbit Polyclonal, 1 mg/ml, Cell Signaling). The secondary antibodies were horseradish peroxidase-conjugated goat anti-Rabbit IgG (1:200, Biotinylated Antibody, Vectastain), IRDye 800CW-conjugated anti-Rabbit antibodies (1:1000, Rockland Biosciences, Gilbertsville, PA), pethidine hydrochloride (Shenyang Pharmaceutical Company, 100 mg/ml).

### Animals and treatments paradigms

Male Sprague-Dawlay rats (Shanghai Center of Experimental Animal, Chinese Academy of Sciences) weighing 180-200 g were housed and maintained on a 12:12-hr reverse day/light cycle with free access to food and water. All procedures for the animals were performed in agreement with the National Institutes of Health guides. Rats were injected s.c. with 50 mg/kg pethidine hydrochloride (Shenyang Pharmaceutical Company, 100 mg/ml) once daily or an equal volume of normal saline (NS). The influence of acute or chronic pethidine hydrochloride treatment was evaluated by the following treatment paradigm. The NS-NS group received consecutive injections of NS for 10 days (control group); the NS-Pet group received single injection of pethidine on day 10 following consecutive injections of NS for 9 days (acute); the Pet-Pet group received consecutive injections of pethidine for 10 days (chronic). All animals were killed 1 h after the last injection.

### Immunohistochemistry

Brain sections were incubated overnight at 4°C with histone H3 acetylation (rabbit polyclonal, Lys-9/Lys-14, 1 mg/ml, Upstate Biotechnology) antibodies. Slices were then incubated for 1 hr at 20°C with biotinylated goat anti-rabbit antibody (1:200, Vector Laboratories) and processed with avidin-biotinylated horseradish peroxidase complex (Elite ABC kit, Vector Laboratories) for 45 min at room temperature. The reaction was visualized using diaminobenzidine (DAB, Sigma). Quantification of immunoreactive cells was performed with Image-Pro Plus 6.0. Counts above threshold were taken in a standard frame sample area from the 2 consecutive sections across both hemispheres per animal.

### Western blotting

Histone for western blotting was prepared as previously described [18]. In this analysis, the blots were incubated with IRDye 800CW-conjugated secondary antibody, the infrared fluorescence image was obtained using Odyssey infrared imaging system (Li-Cor Bioscience), and the optical density of the bands were quantified using the Image-Pro Plus 6.0 (Media Cybernetics), and the optical density of each histone H3 acetylation bands were normalized to the optical densities of the corresponding β-actin bands respectively. Data were obtained from at least two independent experiments.

Figure 1. Histone acetylation level induced by single or repeated pethidine administrations in the CA1, the subiculum of hippocampus, and the mPFC. A. Representative pictures of immunochemistry in brain slices. B. Immunohistochemistry data presented as mean ± s.e.m. *$P < 0.05$, compared with NS-NS control (n = 6-8 for each group).



Figure 2. Time-dependent changes of Histone acetylation level induced by repeated pethidine administrations in the subiculum of hippocampus, in day 1, day 5 and day 10 treatment groups. A. Representative immunochemistry pictures of the subiculum of hippocampus in brain slices. B. Immunohistochemistry data presented as mean ± s.e.m.

*Pethidine-induced place preference*

Place preference conditioning apparatus consisted of a shuttle chamber made of Plexiglas (30 cm×60 cm×30 cm) and was divided into two equal-sized compartments by insertion of a removable Plexiglas wall. The device was black, one compartment had white strips on the wall and a texture floor, and the other had walls with white dots and a smooth floor. The device was placed under conditions of dim illumination (40 lux) and masking noise. In preconditioning session, rats were in the middle of the apparatus, and then were allowed to freely explore the two compartments for 20 min. The time spent in each compartment was recorded by observation. Any rat spent less than 5 min (25% of total time) in either side was eliminated.
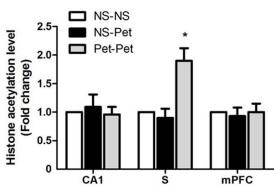
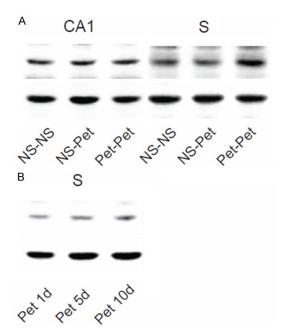**Figure 3.** Histone acetylation level detected by western blot. A. Histone acetylation level induced by repeated pethidine administrations in the CA1 and the subiculum of hippocampus. B. Time-dependent changes of Histone acetylation level induced by repeated pethidine administrations.

Morning conditioning occurred between 8 and 11 am, animals received consecutive injections of pethidine (50 mg/kg) for 10 days and then confined for 50 min to the non-preferred side as determined in the preconditioning test. Afternoon conditioning occurred 4 h later. The animals received consecutive injections of saline, and were then kept in the preferred side as determined in the preconditioning test for 50 min. The above procedures were repeated on the second day of conditioning. Twenty-four hours after conditioning, the test was carried out exactly as described in the preconditioning phase (free access to each compartment for 20 min) and the time of each rat spent in each compartment was recorded. Preference for the drug-paired compartment was expressed as the difference between the mean of the testing and preconditioning scores.

*Reverse transcription and quantitative PCR (RT-PCR)*

Total RNA was prepared with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription of purified RNA (100 ng of total RNA) was performed using

superscript III reverse transcriptase (Invitrogen). The quantification of DNA was done by real-time PCR, using SYBRR Premix Ex TaqTM (Takara) and the ABI Prism 7700. Ct values from each sample were obtained using the operating software of the ABI Prism 7700 [22]. Each PCR reaction, run in triplicate for each brain sample, was repeated at least twice independently. ΔCt was obtained by normalizing to corresponding β-actin internal control. Primer pairs for real-time PCR are shown in the **Table 1**.

*Chromatin immunoprecipitation*

Chromatin immunoprecipitation (ChIP) was performed using the Upstate Biotechnology ChIP kit and following a modified protocol from the manufacturer. Briefly, brain tissue was fixed in 1% formaldehyde and the lysates were sheared by sonication in 1% SDS lysis buffer to generate chromatin fragments with an average length of 200-1000 bps. The chromatin was then immunoprecipitated overnight at 4°C with antibodies specific to histone H3 acetylation, or an equivalent amount of control IgG. Protein-DNA-antibody complexes were precipitated with protein A-agarose beads for 2 h at 4°C. Input or DNA in the complex was subjected to quantitative PCR. Primer pairs for specific promoter regions are shown in **Table 1**.

*Statistical analysis*

Data were analyzed by one-factor ANOVA, followed by Student-Newman-Keuls *post hoc* comparisons.

**Results**

*Histone acetylation level in the subiculum of hippocampus was induced by chronic pethidine administrations*

The level of histone H3 acetylation was evaluated whether change following the single and repeated administration of pethidine. As shown by immunochemistry analysis in **Figure 1A** and **1B**, seven of 11 animals H3 acetylation levels was increased specifically in the subiculum of hippocampus ($F_{2, 16}$ = 4.763, $P$ = 0.024; **Figure 1**), but not in the CA1 of hippocampus or the mPFC following repeated pethidine administrations. Furthermore, as is shown in **Figure 2**, time-dependent increases of histone acetyla-
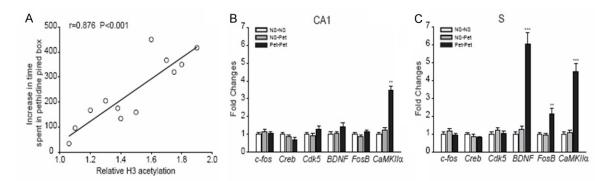
Figure 4. The correlation between histone acetylaton and pethidine-induced place preference (A), and addiction-related genes expression up-regulated by repeated pethidine administrations in the CA1 (B) and the subiculum of hippocampus (C). Data are expressed as mean ± s.e.m. **$P$ < 0.01, ***$P$ < 0.001, compared with NS-NS control (n = 6-8 for each group).
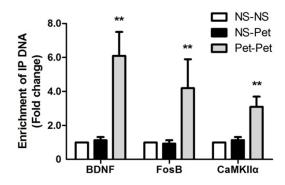


Figure 5. Changes of histone acetylation in specific gene promoter regions in the subiculum of hippocampus. Data are expressed as mean ± s.e.m. *$P$ <0.05, ***$P$ < 0.001, compared with NS-NS control (n = 6-8 for each group).

tion in the subiculum of hippocampus in response to repeated pethidine administrations were uncovered ($F_{2, 16}$ = 3.672, $P$ = 0.050; **Figure 2**). These results were further verified by western blotting (**Figure 3**). Therefore, the result showed that the chronic, but not acute pethidine use, induced the elevation of histone H3 acetylation in the subiculum of hippocampus, in consistence with that the other drug use, such as cocaine, morphine etc. [20, 21].

*Addiction-related genes expression were up-regulated by chronic pethidine administrations*

The correlation between histone acetylaton and pethidine-induced place preference were analyzed. As was shown in **Figure 4A**, the increased time spent in pethidine paired box correlated positively with the global acetylation level of H3. It has been demonstrated that chronic drug use induces many gene changes.

Reverse transcription and quantitative PCR (RT-PCR) was performed to test the addiction-related genes expression up-regulated by repeated pethidine administrations. As is shown in **Figure 4**, the mRNA levels of *BDNF*, *FosB*, and *CaMKIIa* were 6.03, 2.13 and 4.47-fold increases in the subiculum of hippocampus respectively. However, only *CaMKIIa* mRNA level was increased in the CA1 of hippocampus (3.46-fold). The number of gene expression could be correlated positively with the global acetylation level of H3. These data indicated that more gene expression may be up-regulated by chronic pethidine administrations in the subiculum of hippocampus, compare with the CA1 of hippocampus.

*Chronic-pethidine-induced gene transcriptional activation is modulated H3 acetylation*

More and more evidence suggests that the regulation of gene transcription is mediated by alter histone acetylation at specific gene promoters. To further clarify the relationship between histone acetylation and gene expression, the levels of H3 acetylation at the promoters of these genes were analyzed in the subiculum of hippocampus by chromatin immunoprecipitation (ChIP) following repeated pethidine administrations. As shown in **Figure 5**, in the subiculum of hippocampus, chronic pethidine use caused a robust (more than 3-fold) increase in H3 acetylation at promoters *BDNF*, *FosB*, and *CaMKIIa*. These data are consistent with the correlation analysis results and indicate that Chronic-pethidine-induced gene transcriptional activation was occurred in the subiculum of hippocampus and modulated by H3 acetylation.

## Discussion

Pethidine is indicated for the treatment of moderate to severe pain, and is delivered as a hydrochloride salt in tablets, as a syrup, or by intramuscular or intravenous injection. For much of the 20th century, pethidine was the opioid of choice for many physicians; in 1983 60% of doctors prescribed it for acute pain and 22% for chronic severe pain [1]. Despite guidelines for acute or chronic pain management, availability of many other more suitable opioids, and surveillance by government authorities, the prescribing of meperidine remains a concern [23-27]. As a result, pethidine may be more likely to be abused than other prescription opioids [2]. In our study, we show that histone H3 acetylation level changes and addiction-related gene expression were examined with single and repeated pethidine treatment in different brain regions. Chromatin immunoprecipitation (ChIP) assays were performed to investigate the chromatin remodeling by which gene expression is regulated with dramatic cocaine regulation. Our results demonstrate that H3 acetylation in the subiculum of hippocampus may be involved in neural plasticity changes in opioid abuse.

The rat hippocampal formation is a C-shaped structure that is situated in the caudal part of the brain. Three distinct subregions can be distinguished: the hippocampus proper (consisting of CA1, CA2 and CA3), the dentate gyrus (DG) and the subiculum [28, 29]. The sequence of molecular mechanisms underlying long-term potentiation (LTP) in the CA1 region of the hippocampus has been proposed to underlie memory consolidation in that region [30, 31]. CA1 projects to the subiculum as well as sending information along the aforementioned output paths of the hippocampus. The subiculum is the final stage in the pathway, combining information from the CA1 projection and EC layer III to also send information along the output pathways of the hippocampus. Recently studies reveal that the subiculum may also have a role in some of the neurobiological processes underlying motivation [28, 29]. In our experiment, the CA1 and subiculum of hippocampus were investigated as two distinct regions. Our results show that the chronic, but not acute pethidine uses, induced the elevation of histone H3 acetylation in the subiculum of hippocampus. However, changes of histone H3 acetylation level was not detected in CA1 of hippocampus. This indicates that chronic pethidine uses may activate more genes and induce stronger transcriptional activation in the subiculum of hippocampus. This finding was consistent with the attitude of the neurobiological processes underlying motivation, and uncovered the epigenetic mechanism of the phenomenon in the subiculum of hippocampus.

Drug addiction considered as life-long behavioral abnormalities that was caused by repeated exposure to drug abuse [32-34]. Increasing evidence suggested that long-lasting gene expression changes on the brain mediate the addiction phenotype, and work over the past decade had demonstrated chromatin modifications involved in these gene transcriptional regulations [16, 33-37]. Among these chromatin remodeling researches of drug abuse models, histone acetylation is the most studied by far. Histone acetylation changes had been observed at several addiction-related genes in the nucleus accumbens (NAc), a key reward region of the brain. For instance, H4 acetylation was upregluated on the *c-Fos* promoter acutely, but not chronically. By contrast, H3 acetylation elevations were only after chronic cocaine on the *BDNF*, *Cdk5* and *CaMKII*a [18, 20, 21, 38]. Moreover, genome-wide studies using ChIP-chip had displayed a comprehensive map of genes transcription by histone acetylation after chronic cocaine [34]. Our research showed that chronic pethidine use caused a robust increase in H3 acetylation at promoters *BDNF*, *FosB*, and *CaMKII*a, demonstrating that chronic-pethidine-induced gene transcriptional activation was occurred the subiculum of hippocampus and modulated by H3 acetylation.

## Conclusion

Chronic pethidine experience induced H3 acetylation elevation in the subiculum of hippocampus, which modulated gene transcriptional activation and may be involved in neural plasticity changes in opioid abuse.

## Acknowledgements

**Disclosure of conflict of interest**

None.

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