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Adverse maternal environment alters *Oprl1* variant expression in mouse hippocampus

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Abstract

An adverse maternal environment (AME) and Western diet (WD) in early life predispose offspring toward cognitive impairment in humans and mice. Cognitive impairment associates with hippocampal dysfunction. An important regulator of hippocampal function is the hippocampal Nociceptin/Orphanin FQ (N/OFQ) system. Previous studies find links between dysregulation of hippocampal N/OFQ receptor (NOP) expression and impaired cognitive function. NOP is encoded by the opioid receptor-like 1 (*Oprl1*) gene that contains multiple mRNA variants and isoforms. Regulation of *Oprl1* expression includes histone modifications within the promoter. We tested the hypothesis that an AME and a postweaning WD increase the expression of hippocampal *Oprl1* and select variants concurrent with altered histone code in the promoter. We created an AME-WD model combining maternal WD and prenatal environmental stress plus postweaning WD in the mouse. We analyzed the hippocampal expression of *Oprl1*, *Oprl1* variants, and histone modifications in the *Oprl1* promoter in offspring at postnatal day (P) 21 and P100. An AME and an AME-WD significantly increased the total hippocampal expression of *Oprl1* and variant V4 concurrently with an increased accumulation of active histone marks in the promoter of male offspring. We concluded that an AME and an AME-WD alter hippocampal *Oprl1* expression in offspring through an epigenetic mechanism in a variant-specific and sex-specific manner. Altered hippocampal *Oprl1* expression may contribute to cognitive impairment seen in adult males in this model. Epigenetic regulation of *Oprl1* is a potential mechanism by which an AME and a WD may contribute to neurocognitive impairment in male offspring.

KEYWORDS
adverse maternal environment, hippocampus, histone modifications, *Oprl1* variants

1 | INTRODUCTION

The maternal environment mediates the long-term health of offspring in both humans and animals (Alastalo et al., 2013; Arcego et al., 2016; Barker et al., 1993; Ke et al., 2020; Tozuka et al., 2010). An adverse maternal environment (AME) appears to increase the likelihood of multiple later life pathophysiologies. For example, maternal poverty predisposes offspring toward later life obesity in the United States (Levine, 2011). More than 14% of total US population and 3.4 million more children are currently living in poverty (Parolin et al., 2022). Additionally, a Western diet (WD) has been a significant contributor to the growing rate of obesity over the last several decades (Rakhra et al., 2020).

An AME and the consumption of a WD in early postnatal life increase the risk for cognitive impairment later in life in humans (Alastalo et al., 2013; Barker et al., 1993; Cordner et al., 2019) and animal models (Arcego et al., 2016; Tozuka et al., 2010; Weaver et al., 2004). We previously demonstrated that AME together with postweaning WD impair learning and memory function in adult male mice (Ke et al., 2020). Moreover, AME male offspring demonstrate reduced hippocampal neurogenesis (Ke, Huang, et al., 2021). However, the pathogenesis underlying these changes remain poorly understood. Cognitive functions involve many signaling pathways including the Nociceptin/Orphanin FQ (N/OFQ) system in the hippocampus (Andero, 2015; Sardari et al., 2015; Zaveri, 2003). The N/OFQ system in the hippocampus plays an essential role in cognitive functions in adult humans and animals (Andero, 2015; Zaveri, 2003). While several signaling pathways have been found to be programmed by AME (Criado-Marrero et al., 2020; Lemche, 2018; Pillai et al., 2018; Wang et al., 2020), the effects of AME on N/OFQ system in developing hippocampus remain unknown.

N/OFQ participates in numerous physiological functions though binding to the nociceptin opioid peptide receptor (NOP) (Lambert, 2008). The opioid receptor-like 1 (*Oprl1*) gene encodes NOP, which exists as a member of the opioid subfamily of G protein-coupled receptors (Mollereau et al., 1994). N/OFQ and NOP are widely express in the central nervous system and peripheral organs and participate in many processes including learning and memory (Andero, 2015; Bodnar, 2013; Mallimo & Kusnecov, 2013). NOP activates Gi/o proteins, a family of heterotrimeric G protein alpha subunits that primarily inhibit the cAMP dependent pathway and thus inhibits neuronal activity (Mouledous, 2019). NOP agonism impairs learning and memory whereas NOP antagonists have been shown to block this effect (Redrobe et al., 2000; Sardari et al., 2015). Moreover, studies in transgenic Pnoc/*Oprl1* knockout mice correlate with

pharmacological studies, indicating that decreased activation of NOP is associated with enhanced memory (Andero, 2015). In contrast, NOP activation impairs memory (Andero, 2015). Importantly, N/OFQ-NOP receptor memory functions appear vulnerable to physiological stress (Mallimo & Kusnecov, 2013).

Most species demonstrate significant conservation of the *Oprl1* gene, which produces multiple mRNA variants due to alternative splicing (Curro et al., 2001; Pan et al., 1998). The mouse *Oprl1* gene contains 4 exons. Alternative splicing of mouse *Oprl1* gene generates 12 mRNA variants (V) and multiple isoforms. V1–4, 8–9, and 12 are protein coding transcripts. Transcripts V5–7 undergo nonsense mediated decay and V10–11 are noncoding transcripts (ENSMUSG00000027584). Five of the *Oprl1* splicing variants are differentially expressed in various mouse brain regions (Pan et al., 1998). Given that different variants and isoforms display different biological functions and pharmacological effects (Pan et al., 1998), delving into the expression pattern of *Oprl1* splicing variants in the hippocampus exposed to AME and AME-WD will likely be more informative than looking solely at the total expression.

Regulation of *Oprl1* expression and alternative splicing involves histone covalent modifications, including histone acetylation (ac) and methylation (me) (Caputi et al., 2014, 2016). For example, histone H3 lysine (K) 9 acetylation (ac), H3K14ac, and H3K4 trimethylation (me3) in the promoter region lead to gene activation while H3K36me3 marks actively transcribed regions (Black et al., 2012; Graff & Tsai, 2013). In contrast, H3K9me3 and H3K27me3 in the promoter region lead to gene silencing (Black et al., 2012). All of these histone marks display vulnerability to perinatal insults (Ke et al., 2010, 2011).

Despite this previous work elucidating the impact of AME on neurodevelopmental issues such as memory and learning as well as hippocampal neurogenesis, the effects of AME and AME-WD on the expression of *Oprl1* and its variant composition in the hippocampus are unknown. We hypothesized that AME and AME-WD would alter variant expression of *Oprl1* and *Oprl1* promoter histone code.

2 | METHODS

2.1 | Animals

All experiments were conducted in accordance with the Public Health Services Policy on Human Care and Use of Laboratory Animals and all procedures were approved by the Medical College of Wisconsin Institutional Animal Care

and Use Committee (American Physiological Society & World Medical Association General Assembly, 2002). In this study, we used the mouse model of the AME that has been previously described (Ke et al., 2020). In brief, the AME was induced in mice by exposing 6-week-old C57/Bl6 female mice randomly to either a control diet (CD) or a WD (Ke et al., 2020). Dams in the CD group experienced a normal environment throughout pregnancy and are designated as Control (Con). Dams fed a WD experienced a "stressed" environment the last third of pregnancy. The combination of chronic WD and gestational stress is designated as AME (Ke et al., 2020). Dams from both Con and AME groups delivered spontaneously, and litters were culled to six, with three female and three male pups in each litter. At postnatal day 21 (P21), pups from both Con and AME groups were either sacrificed for studying the immediate effect of AME or weaned and permanently placed on either a CD or a WD, creating four experimental groups: Con-CD, Con-WD, AME-CD, AME-WD for a later time point study (Ke et al., 2020). At P100, when the mice were clearly adults at this stage and post the changes that occur in adolescent as well as are not in senescence, pups from four experimental groups were anesthetized, sacrificed, and the hippocampi (HP) were dissected for molecular studies. For immunohistochemistry (IHC) studies, animals were individually fixed via intra-cardiac perfusion with 4% paraformaldehyde in PBS, brains were removed and prepared for paraffin sectioning as previously described (Ke et al., 2022; Ke, Huang, et al., 2021). A total of 40 pregnant female mice were used in the study (Ke et al., 2020). One male and one female pup from the same litter were used and counted as $N = 1$. To have a N of six, six males and six females from six different litters were used for each experiment with $N = 6$ L/group. HP from control mice at P7, P21 and P100 were also harvested for the determination of the *Oprl1* alternative splicing variant expression pattern developmentally.

2.2 | RNA isolation and real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA extraction and cDNA syntheses were performed as previously described (Cohen et al., 2016). mRNA levels of *Oprl1* total (Mm.PT.58.5266522.g, Integrated DNA Technologies) and its splicing variants were calculated relative to hypoxanthine phosphoribosyltransferase 1 (HPRT1, Mm.PT.39a.22214828, Integrated DNA Technologies), which was used as an internal control. Primer and probe sequences for *Oprl1* variants were designed based on the sequences obtained from Ensembl.org (ENSMUSG00000027584) and are listed in Table 1. A unique primer set was designed for each variant. Either a forward or reverse primer crossed the exon junction for

all variants except for V8–9 and V12. The primer sets for V8–9 and V12 crossed the coding and noncoding junctions. All primer sets were confirmed by sequencing. The corresponding locations are shown in the schematic representation of *Oprl1* splicing variants in Figure 4b.

2.3 | Protein isolation and immunoblot

Hippocampal tissue proteins' isolation and immunoblots were performed as previously described (Cohen et al., 2016). Antibody against *Oprl1* (ThermoFisher Scientific, Cat#PA5-70443) at 1:50 dilution was used to determine protein abundance and Vinculin (Cell Signaling, Cat #13901) at 1:10000 dilution was used as a loading control.

2.4 | Immunohistochemistry

IHC was used to localize *Oprl1* expression in the hippocampus at P21 and P100. IHC was performed by the Histology Core Lab in the Department of Pathology Medical College of Wisconsin using a Leica Bond Rx automated staining platform as previously described (Ke et al., 2022). A rabbit anti-*Oprl1* (Cat# bs-0181R, Bioss Antibodies Inc.) 1:100 was used to determine *Oprl1* protein abundance in three hippocampal subregions (CA1, CA3, and denate gyrus [DG]). The percentage of *Oprl1* positive cell density of each hippocampal subregion area was quantified by imageJ.

2.5 | Chromatin isolation and chromatin immunoprecipitation assay

Hippocampal chromatin isolations and chromatin immunoprecipitation assays with antibodies against histone H3 lysine (K) 4 trimethylation (H3K4me3, #9751, Cell Signaling Technology), H3K9me3 (#13969, Cell Signaling Technology), H3K27me3 (#9733, Cell Signaling Technology), H3K36me3 (#4909, Cell Signaling Technology), H3K9 acetylation (H3K9ac, #9649, Cell Signaling Technology), and H3K14ac (#7627, Cell Signaling Technology) were performed as previously described (Ke et al., 2022; Ke, Huang, et al., 2021). Real-time PCR was used to quantitate the amount of DNA from the *Oprl1* promoter with primers and probe listed in Table 1.

2.6 | Statistics

GraphPad Prism 6 (GraphPad Software, San Diego, CA) was used to perform all analyses. All data presented are

TABLE 1 Primers for real time RT-PCR and ChIP assays

	Forward	Reverse	Probe
Real-time RT-PCR			
Oprl1			
V1	5′ CTGTTGGGAGGAACTGTACTGAG	5′ GAGACAGGTTCCCTTGAAAGT	5′ TTCTGGGAGGTCTTGTATGGCAGC
V2	5′ CCCTAATATGAGGAACTGTACTGAG	5′ GAGACAGGTTCCCTTGAAAGT	5′ TTCTGGGAGGTCTTGTATGGCAGC
V3	5′ TTGGACTCAAGGTCACCATC	5′ CTTGGTGTGCATGACGAG	5′ CTCTACTTGGCTGTGTGCATCGGG
V4	5′ GGGCTCTACTTGGCTGT	5′ CTCCCAGCTGAGGATGA	5′ CATACATGACGAGGCAGTTCCC
V5	5′ CTTGGACTCAAGGTCACCATC	5′ CAATGCCCTCCCAGATGAC	5′ CTCTACTTGGCTGTGTGCATCGGG
V6	5′ CTCTGAGAGGAGTCTTAAGAGAGA	5′ TGGTGTGCCTGAATGTGAA	5′ AGCATCTCTCTCTCTGATTCCTTCCACA
V7	5′ TGTATGTCATCCTCAGACAACATT	5′ TCAGTCTCTTAAGACTCCTCTC	5′ TGAACTTTACAGGCAGTGCCCTGA
V8	5′ AGTGGAGGATGAAGGTCAGT	5′ ACCAGGCACTCGATCTCT	5′ AGTCCTCCTCCCTGACCAATCAGT
V9	5′ TCATGTGCCTGTTAGTGTAGTT	5′ CTGGAACAGGACACAGAAAGA	5′ CTCAGTGGACTGTGTCCAGCACTG
V10	5′ TTGGAGAGATCGAGTGCCT	5′ GCAGACAGAGATGATCAGAACC	5′ AGATGCAGATGGCAAATACAGGGC
V12	5′ ATGACTAGGCGTGGACCT	5′ AGTTCAGGGTCAACCTAGAGAG	5′ CCACAGAGCCCATCTACACCCAAC
ChIP			
Oprl1 promoter	5′ TGTGTCTTGTGTGAGCCGATTT	5′ TTTGGCTTCCTTCTCCAACCTGCG	5′ CAGTGCGGCAGCCAGAA

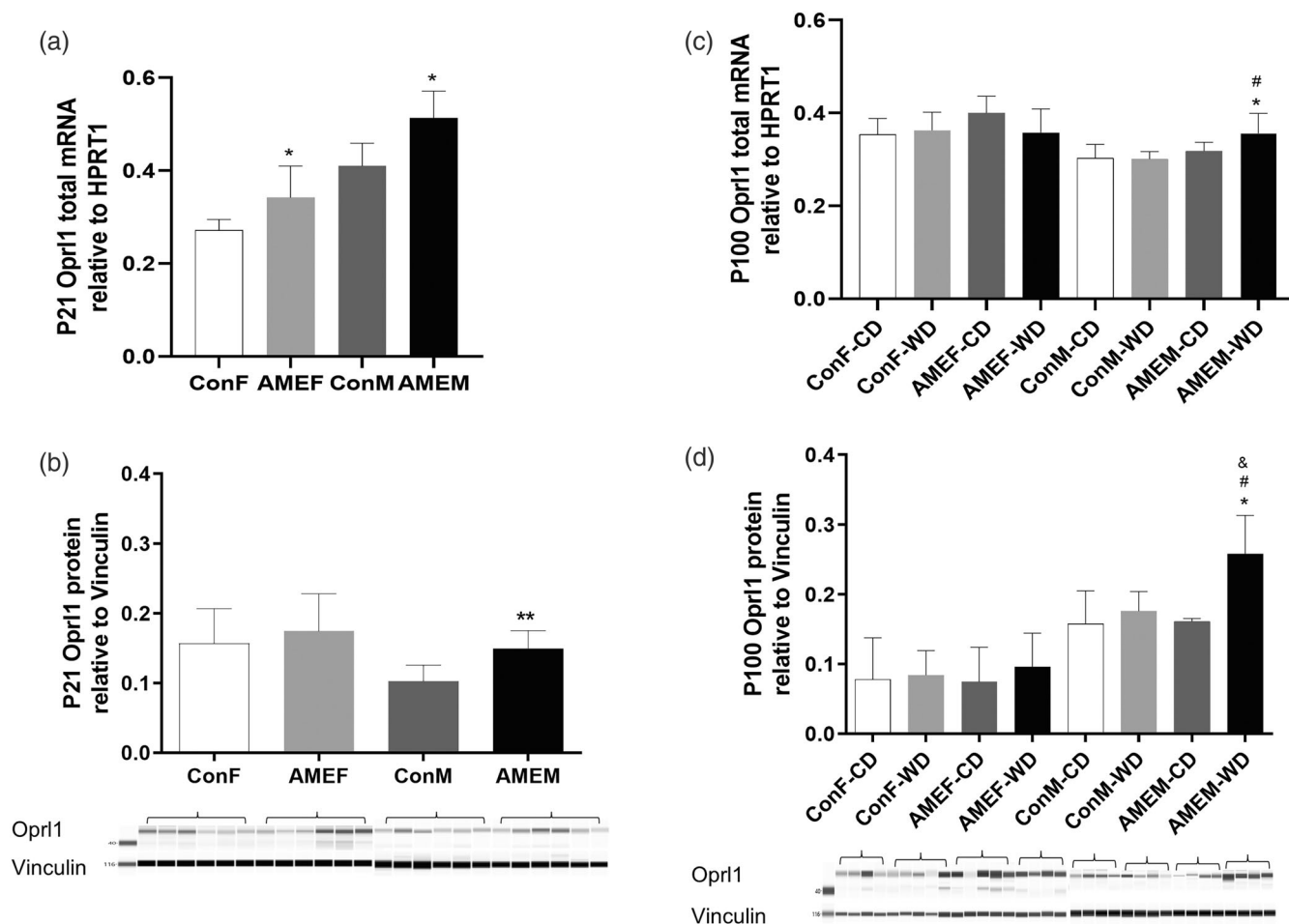Abbreviation: ChIP, chromatin immunoprecipitation.

FIGURE 1 Hippocampal *Oprl1* total mRNA and protein levels in P21 and P100 offspring. Data were presented as mean ± SD. (a) Hippocampal *Oprl1* total mRNA levels in P21. (b) Hippocampal *Oprl1* protein levels in P21. *$p < .05$, **$p < .01$ when compared to sex-matched control. (c) Hippocampal *Oprl1* total mRNA levels in P100. (d) Hippocampal *Oprl1* protein levels in P100. $N = 6$ L/group. *$p < .05$ when compared to sex-matched con-CD, #$p < .05$ when compared to sex-matched con-WD, &$p < .05$ when compared to sec-matched AME-CD. AME, adverse maternal environment; CD, control diet; WD, Western diet

expressed as mean ± SD. Adequate technical and biological replicates were used for all experiments. Four-group comparisons were analyzed by ANOVA followed by post-hoc Tukey's multiple comparisons test. Two-group comparisons were analyzed by Mann–Whitney (nonparametric) test. Significance was set as $p < .05$.

3 | RESULTS

3.1 | AME and AME-WD increased hippocampal *Oprl1* expression in males at P21 and P100

We first determined the effects of AME on hippocampal *Oprl1* expression at P21, immediately following exposure to maternal insults. In P21 males (M), AME significantly increased hippocampal *Oprl1* total mRNA and protein levels compared to controls (Con) (Figure 1a,b). In P21 females (F), AME also significantly increased hippocampal *Oprl1* total mRNA levels compared to the controls (Figure 1a). However, AME did not affect hippocampal *Oprl1* protein levels in females compared to the controls (Figure 1b).

We next determined the effects of an AME-WD on hippocampal *Oprl1* expression at P100 when the adult males displayed learning and memory deficits. In P100 males, similarly, the AME-WD significantly increased hippocampal *Oprl1* total mRNA levels when compared to either Con male CD group (ConM-CD) or ConM WD group (ConM-WD) (Figure 1c). AME-WD also significantly increased hippocampal protein levels when compared to either ConM-CD or ConM-WD or AMEM-CD (Figure 1d). In P100 females, however, neither AME
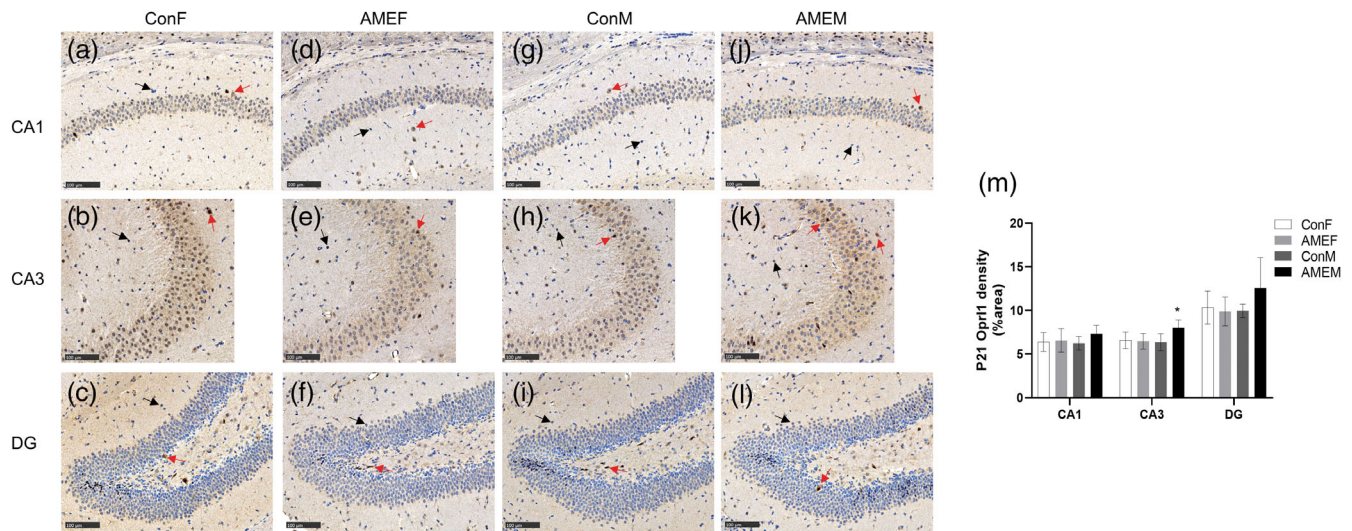
FIGURE 2 *Oprl1* protein abundance in P21 hippocampal subregions. (a–f) *Oprl1* abundance in P21 female hippocampal CA1 (a and d), CA3 (b and e), and DG (c and f) in Con (a–c) versus AME (d–f). (g–l) *Oprl1* abundance in P21 male hippocampal CA1 (g and j), CA3 (h and k), and DG (i and l) in Con (g–i) versus AME (j–l). Red arrows pointed cells represent *Oprl1* positive cells while black arrows pointed cells represent *Oprl1* negative cells. (m) Quantifications of *Oprl1* positive cell density. Data were presented as mean $\pm$ SD. $N = 4$ L/group. Scale bar = 100 μm, *$p < .05$ when compared to sex-matched control. AME, adverse maternal environment; DG, dentate gyrus
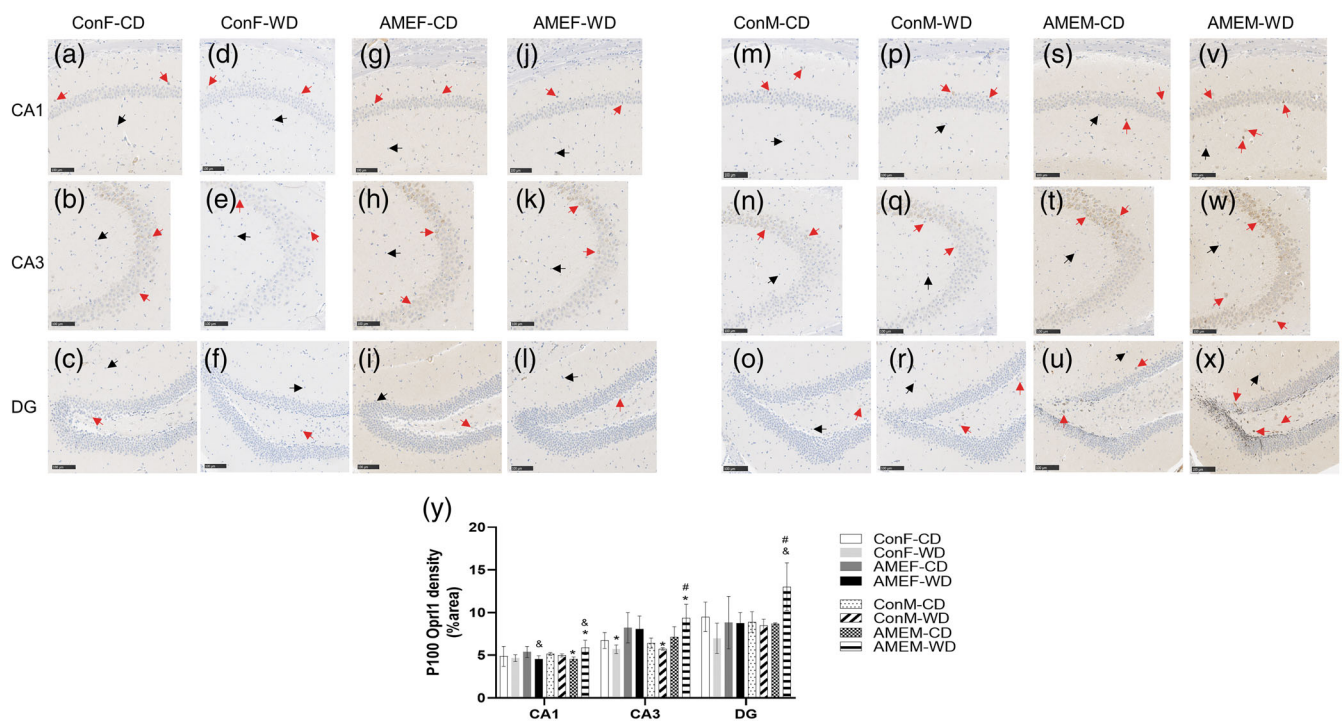

FIGURE 3 *Oprl1* protein abundance in P100 hippocampal subregions. (a–l) *Oprl1* abundance in P100 female hippocampal CA1 (a, d, g, and j), CA3 (b, e, h, and k), and DG (c, f, i, and l) in Con-CD (a–c), Con-WD (d–f), AME-CD (g–i), and AME-WD (j–l). (m–x): *Oprl1* abundance in P100 male hippocampal CA1 (m, p, s, and v), CA3 (n, q, t, and w), and DG (o, r, u, and x) in Con-CD (m–o), Con-WD (p–r), AME-CD (s–u), and AME-WD (v–x). Red arrows pointed cells represent *Oprl1* positive cells while black arrows pointed cells represent *Oprl1* negative cells. (y) Quantifications of *Oprl1* positive cell density. Data were presented as mean $\pm$ SD. Scale bar = 100 μm, $N = 4$ L/group. *$p < .05$ when compared to sex-matched Con-CD, #$p < .05$ when compared to sex-matched Con-WD, &$p < .05$ when compared to sec-matched AME-CD. AME, adverse maternal environment; CD, control diet; DG, dentate gyrus; WD, Western diet

alone, WD alone nor the combination of the two altered hippocampal *Oprl1* expression in either mRNA or protein levels (Figure 1c,d).

Using IHC, we found that in P21, increased *Oprl1* abundance was mainly in CA3 region of hippocampus in AME males (AMEM) when compared to sex-matched
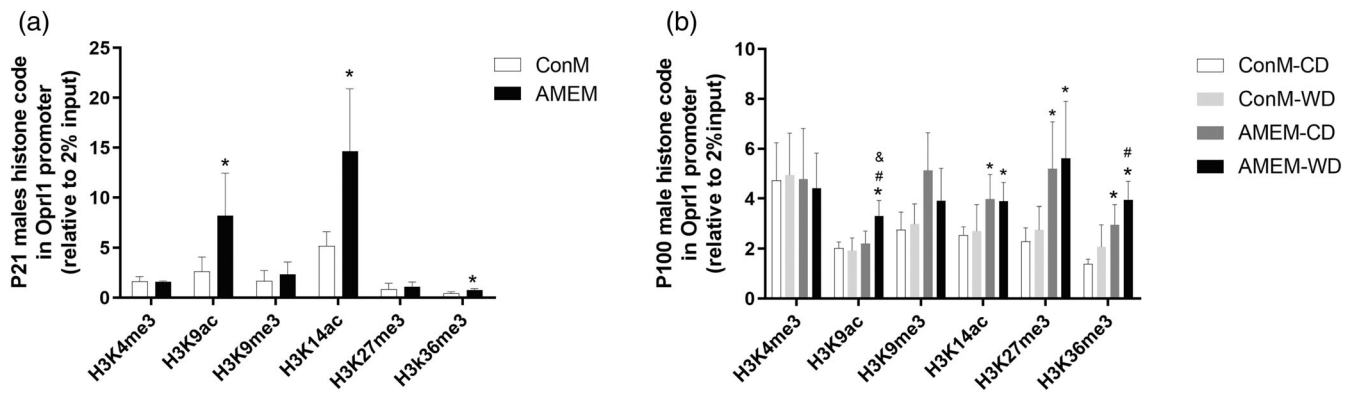
FIGURE 4 Histone code in *Oprl1* promoter region in male hippocampi at P21 and P100. Data were presented as mean ± SD. (a) Histone code in P21 male hippocampi. *$p < .05$ when compared to ConM. (b) Histone code in P100 male hippocampi. $N = 6$ L/group. *$p < .05$ when compared ConM-CD, #$p < .05$ when compared to ConM-WD, &$p < .05$ when compared to AMEM-CD. AME, adverse maternal environment; CD, control diet; WD, Western diet;

controls with no difference in the *Oprl1* expression in any hippocampal subregions in females between ConF and AMEF groups (Figure 2a–m). In P100 males, increased *Oprl1* expression was predominant in CA1 and CA3 regions in AMEM-WD when compared to ConM-CD. Furthermore, increased *Oprl1* expression was noted in CA1 and DG regions in AMEM-WD when compared to AMEM-CD indicating a diet effect. Meanwhile, increased *Oprl1* expression was also observed in CA3 region in AMEM-WD when compared to ConM-WD indicating an AME effect (Figure 3a–y). In P100 females, interestingly, decreased *Oprl1* expression was noted in CA3 region in ConF-WD compared to ConF-CD. Additionally, decreased *Oprl1* expression was also noted in CA1 region in AMEF-WD compared to AMEF-CD. The data in P100 females indicate a diet effect. Together, our data suggest that an AME and an AME-WD affect hippocampal *Oprl1* expression in a sex-specific manner.

3.2 | AME and AME-WD increased hippocampal densities of active histone marks in the *Oprl1* promoter in males at P21 and P100

Given that AME and AME-WD display consistent effects upon the expression of *Oprl1* in male HP and *Oprl1* expression is known to be regulated in part by histone modifications in the promoter region, we examined the effect of AME on histone modifications in *Oprl1* promoter in male HP at P21 and P100. In P21 males, AMEM significantly increased hippocampal densities of active histone marks H3K9ac, H3K14ac, and H3K36me3 in the *Oprl1* promoter region when compared to the controls (Figure 4a). In P100 males, AMEM-WD significantly increased hippocampal H3K9ac densities in *Oprl1*

promoter when compared to either ConM-CD or ConM-WD or AMEM-CD. AMEM-WD also significantly increased H3K36me3 in the promoter region when compared to either ConM-CD or ConM-WD (Figure 4b). Additionally, both AMEM-CD and AMEM-WD significantly increased H3K14ac densities in *Oprl1* promoter region when compared to ConM-CD, indicating an AME effect. Surprisingly, AMEM had similar effect on H3K27me3 densities in both CD and WD groups.

3.3 | Developmental expression of *Oprl1* alternative splicing mRNA variants in control mouse HP

Alternative splicing of *Oprl1* generates multiple mRNA variants (Figure 5a,b). Different variants and isoforms display different biological functions and pharmacological effects (Pan et al., 1998). Thus, it is important to examine the expression pattern of *Oprl1* splicing variants in the hippocampus developmentally.

We measured mRNA levels of *Oprl1* variants in the hippocampus of control mice at P7, P21, and P100. P7 represents the first rapid stage of brain development postnatally in rodents (Gottlieb et al., 1977). We first measured total *Oprl1* mRNA at the three time points examined to determine if there is a sex difference. P7 mice expressed the highest mRNA levels of total *Oprl1* while P21 and P100 mice expressed similar mRNA levels of total *Oprl1* in both sexes with higher expression levels in males at P7 and P21 (Figure 5c). Among 12 variants (V), V1–10 and 12 were detected (Figure 5d). In general, the younger the mice, the lower levels of V1, 3, 5, and 8 were detected in the hippocampus. While the younger the mice, the higher levels of V2, 4, 6–7, and 9–10 were detected at the same time in the hippocampus. V12 was
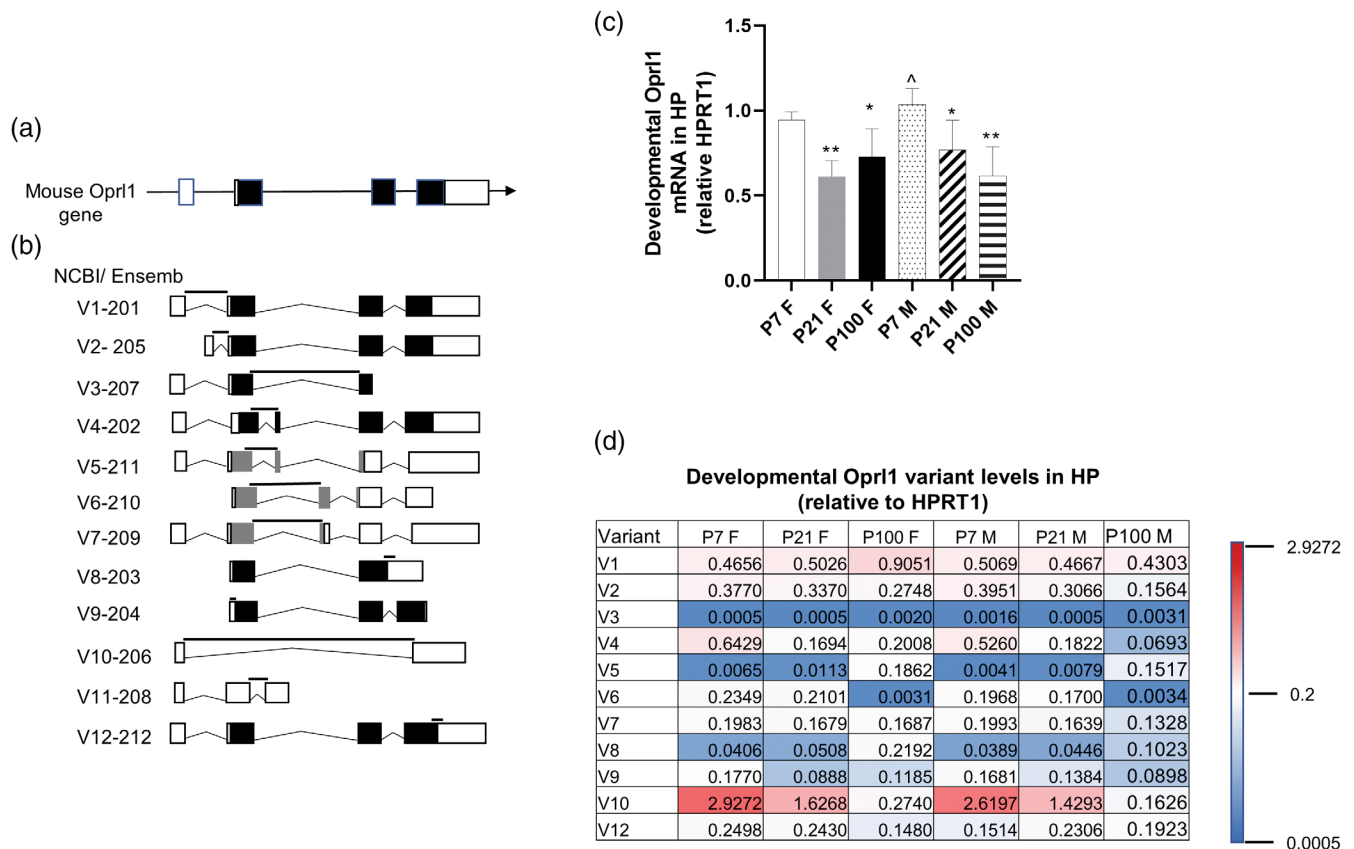
FIGURE 5 Developmental expression pattern of *Oprl1* total and its variants in the hippocampus. Data were presented as mean ± SD. (a) A schematic of mouse *Oprl1* gene. White boxes represent noncoding exons while black boxes represent coding exons. Gray boxes represent nonsense mediated decay exons. (b) A schematic representation of *Oprl1* splicing variants. Numbers on the left represent the designations of the variants according to the information from NCBI and Ensembl. The horizontal line above exon(s) of each variant represents the location of the primers for the variant. (c) Total *Oprl1* mRNA expression in the hippocampus at three different time points. (d) Heat map represents the developmental expression levels of V1-10 and V12 in the hippocampus at three different time points. The numbers in the heat map represent the means of the variants at each time point. The gradient color bar represents different expression levels with blue represents low expression levels while red represents high expression levels. $N = 3$ L/group. $*p < .05$ compared to P7, $**p < 0.01$ compared to P7, $^p < .05$ compared to females

expressed at similar levels cross three different time points. V10 was predominantly expressed while V3 was the least expressed in the hippocampus. A sex difference was noted at P7 for V3, 4, and 12 and at P100 for V1. We then examined the effects of AME and AME-WD on the expression of *Oprl1* coding variants (V1–9 and 12) in the hippocampus at P21 and P100.

3.4 | AME-WD increased hippocampal *Oprl1* V4 levels in males at P21 and P100

In P21 males, AME significantly increased hippocampal V4 but decreased V1 levels when compared to the controls (Figure 6a). In P21 females, AME significantly increased hippocampal V4 and V7 levels compared to the controls (Figure 6b).

In P100 males, AMEM-WD also significantly increased hippocampal V4 levels compared to either ConM-CD or ConM-WD or AMEM-CD. Moreover, AMEM-WD significantly increased hippocampal V1–2 levels when compared to either ConM-WD or AMEM-CD (Figure 6c). Yet, in P100 females, hippocampal *Oprl1* variant levels were differentially affected in different experimental groups. In summary, AMEF-WD significantly decreased hippocampal V4, V6, and V7 levels when compared to ConF-CD. AMEF-CD also significantly increased hippocampal V2 and V9 levels when compared to ConF-CD (Figure 6d).

4 | DISCUSSION

An AME and the consumption of a WD impair offspring learning and memory functions later in life. Activation of
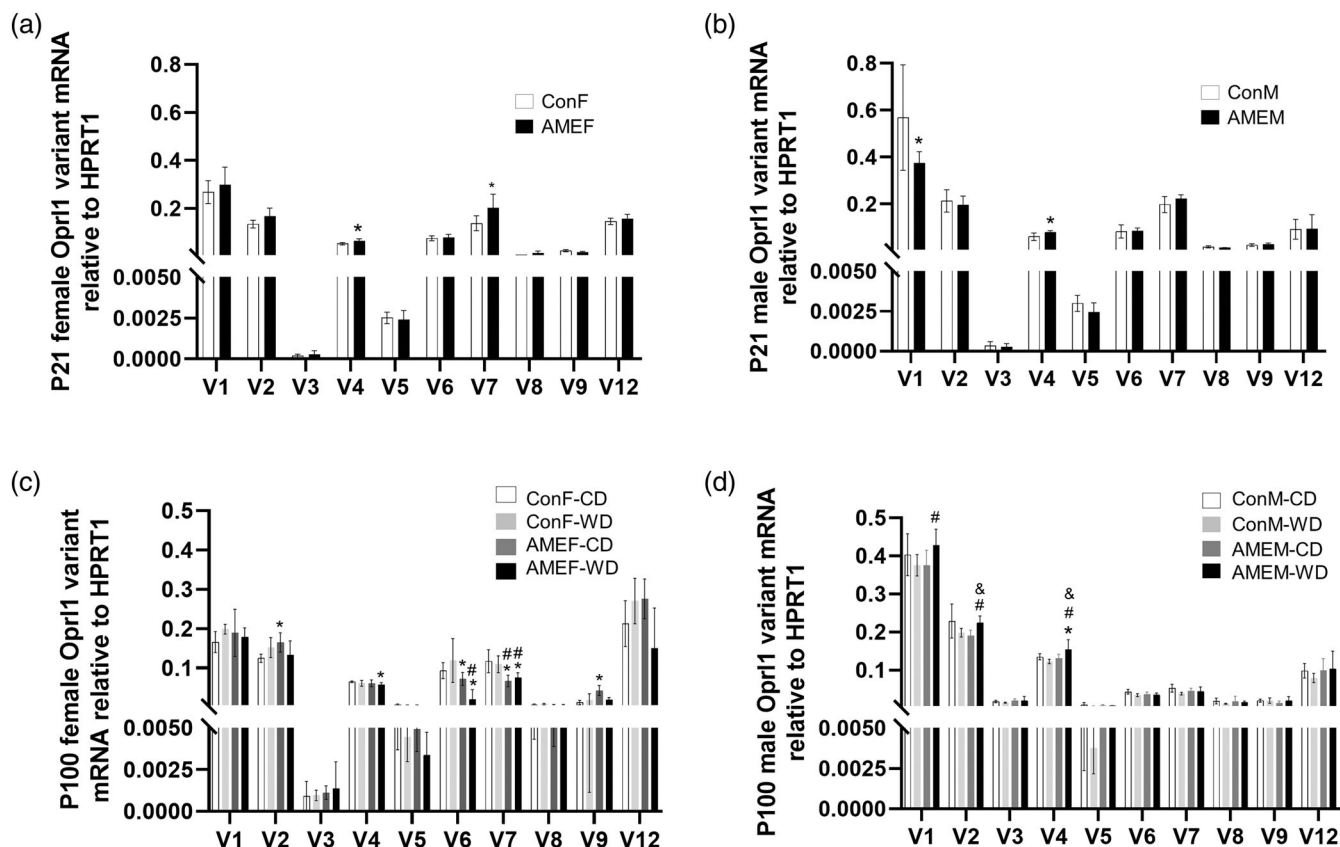
FIGURE 6 Hippocampal expression of *Oprl1* coding variants in P21 and P100 offspring. Data were presented as mean ± SD. (a) Hippocampal expression of *Oprl1* variants in P21 females. (b) Hippocampal expression of *Oprl1* variants in P21 males. *$p < .05$ when compared to sex-matched controls. (c) Hippocampal expression of *Oprl1* variants in P100 females. (d) Hippocampal expression of *Oprl1* variants in P100 males. $N = 6$ L/group. *$p < .05$ when compared to sex-matched Con-CD, #$p < .05$ when compared to sex-matched Con-WD, &$p < .05$ when compared to sec-matched AME-CD. AME, adverse maternal environment; CD, control diet; WD, Western diet

N/OPQ signaling through its cognate receptor NOP also impairs memory (Ke et al., 2020; Redrobe et al., 2000; Sardari et al., 2015). We therefore hypothesized that AME and postweaning WD alter variant expression of *Oprl1* and *Oprl1* promoter histone code in an established model of AME and postweaning WD characterized by male offspring memory impairment. We found that AME increased the expression of *Oprl1* and variant V4 concurrently with the accumulation of activating histone mark H3K9ac, H3K14ac, and H3K36me3 in the promoter region in juvenile male mouse hippocampus. Importantly, these changes persist into adulthood in male AME-WD mice. These findings suggest that early life stress and diet endured by the mother affect *Oprl1* expression via an epigenetic mechanism in the hippocampus. Moreover, a postweaning WD sustains an altered epigenetic *Oprl1* profile into adulthood, which in turn may contribute to cognitive impairment later in life. To our knowledge, this is the first evidence that an AME and an AME-WD affect the expression of *Oprl1* and its

splice variant concurrent with altered histone code in the promoter in offspring hippocampus.

AME and postweaning WD impair cognitive function in adult males in our model. In our previous study, we found that male AME-WD mice took significantly more time finding the platform compared to male control mice (Con-CD) on Days 1 and 5 of Morris Water Maze testing, a hippocampus-dependent functional test, indicating that both nonspatial and spatial learning and memory are impaired (Ke et al., 2020). Our findings revealing AME and AME-WD induced an upregulation of *Oprl1* expression in male offspring HP in this study suggest that upregulation of *Oprl1* may play a role in impaired cognitive function in this model. Our data are consistent with studies showing that dexamethasone exposure in the neonatal period increases *Oprl1* mRNA levels in adult male rats with subsequent decreased cognitive function (Chang, 2014; Neal Jr et al., 2003). Additionally, increasing *Oprl1* activity through a pharmacological approach (*Oprl1* agonists' administration) impairs spatial learning and memory while

this effect is attenuated via *Oprl1* antagonists' administration in adult rats (Redrobe et al., 2000; Sardari et al., 2015). Furthermore, transgenic studies reveal that deletion of *Oprl1* in mice improves performance in memory tests that are highly dependent on hippocampal functioning (Andero, 2015; Kuzmin et al., 2009; Taverna et al., 2005). Taken together, these observations suggest that *Oprl1* may regulate hippocampal dependent memory functions.

Our observation that an AME-WD increases hippocampal *Oprl1* expression in juvenile males provides further relevant context to previous findings. An AME impairs neurogenesis in juvenile male HP by decreasing cell proliferation (Ki67+ cells), neuronal differentiation (NeuroD1+ cells), and the numbers of mature neurons (NeuN+ cells) in the dentate gyrus region (Ke, Huang, et al., 2021). Similarly, pharmacological blockade of *Oprl1* increases the number of immature neurons (DXC+ cells), thereby restoring chronic mild stress-induced neurogenesis impairment in adult male rat hippocampus (Vitale et al., 2017). While we establish an association between upregulated hippocampal *Oprl1* expression and impaired cognitive function, as well as decreased neurogenesis in our study, the underline mechanisms require further investigations.

Although hippocampal *Oprl1* mRNA levels are also increased in juvenile AME females, the protein levels are not affected in this study, suggesting that post-transcriptional regulations may be involved in the female HP. Despite WD decreased *Oprl1* abundance in CA1 and CA3 region in both ConF-WD and AMEF-WD, overall hippocampal *Oprl1* expression is not affected at either mRNA levels or protein levels in adult females in any experimental groups, which is consistent with nonaffected learning and memory functions seen in this model. These results imply that an AME affects *Oprl1* expression in a sex-specific manner. Future studies are warranted to investigate underline mechanisms.

This study also demonstrates regional variation in terms of the vulnerability of the developing hippocampus relative to the insults used in this study. AME predominantly affected hippocampal CA3 region through increased *Oprl1* expression in juvenile males while AME-WD increased *Oprl1* abundance mainly in CA1 and CA3 regions in adult males when compared to the controls. Hippocampal CA1 and CA3 are both required for contextual encoding of extinction (Ji & Maren, 2008). The CA1 region is essential for context-dependent retrieval whereas the CA3 region contributes to acquiring and encoding spatial information and plays a critical role in long-term spatial memory (Cherubini & Miles, 2015; Gilbert & Brushfield, 2009; Holahan & Routtenberg, 2011; Ji & Maren, 2008). Our findings may be a byproduct of the normal developmental pattern of hippocampal *Oprl1* expression with early life

expression being confined predominantly to the CA3 region by spreading to CA1 region later in life thereby being affected by the postweaning WD. Our findings of increased *Oprl1* expression in CA1 and DG regions in AMEM-WD when compared to AMEM-CD that indicates a diet effect. Furthermore, our data of increased *Oprl1* expression in the CA3 region in AMEM-WD when compared to ConM-WD indicates an AME effect. Collectively, our data suggest that an AME plus a second hit by a postweaning WD are necessary for the upregulation of *Oprl1* expression in male adult hippocampus seen in this model.

Regulation of *Oprl1* expression involves histone modifications in the promoter (Caputi et al., 2014, 2016). Previous studies add relevance to our findings. For example, decreased H3K9ac occupancy at *Oprl1* promoter reduces *Oprl1* expression levels after acute and repeated 3,4-methylenedioxy-methamphetamine (MDMA) exposure (Caputi et al., 2016). Respective changes in the activating H3K4me3 and repressive mark H3K27me3 occupancy led to the anticipated changes in the *Oprl1* promoter consistent after cocaine exposure (Caputi et al., 2014). Similarly, we demonstrated that increased occupancy of activating histone marks H3K9ac, H3K14ac, and H3K36me3 at hippocampal *Oprl1* promoter occurs concurrently with upregulated *Oprl1* mRNA levels in male juvenile AME HP. Increased densities of these three active marks at hippocampal *Oprl1* promoter persist into adulthood as do the changes in *Oprl1* hippocampal expression from AME-WD males. Finally, H3K36me3 occupancy enriches the body of active genes and appears to be associated with transcriptional elongation (Mikkelsen et al., 2007; Wang et al., 2018). H3K36me3 also plays a role in transcriptional initiation (Wang et al., 2018; Zhang et al., 2014). Indeed, increased H3K36me occupancy in the IGF1 promoter region occurs concurrently with upregulated IGF1 expression induced by CCI in juvenile rat HP (Schober et al., 2012). Together, our data suggest that enriched activating histone code at *Oprl1* promoter contribute to upregulation of *Oprl1* expression in male HP at both time points.

Our findings of increased repressive mark H3K27me3 densities in the *Oprl1* promoter region in both AMEM-CD and AMEM-WD groups indicate an AME effect and the existing of bivalent histone modifications. Bivalent histone modifications that are characterized by being marked with opposing histone modifications correlate with both a "repressive" and "active" gene expression and are often seen in embryonic stem cells (Tomomi Tsubouchi, 2013). The presence of "bivalent" histone modifications at the promoters of lineage-specifying genes has been implicated in establishing a chromatin context in which multiple lineage options are primed in

readiness for subsequent developmental cues (Tomomi Tsubouchi, 2013). We have previously shown that bivalent histone modifications also exist in both promoter and the body of IGF1 gene in the hippocampus of preterm lambs exposed to noninvasive ventilation at P21 (Ke, Xing, et al., 2021). Yet, the role of AME-induced bivalent histone modifications seen in the adult mice in this study is unknown.

Alternative splicing of *Oprl1* gene generates multiple variants (Curro et al., 2001; Pan et al., 1998). Five different splice variants with differential regional expressions occur in the mouse brain (Pan et al., 1998). We found 11 *Oprl1* mRNA variants with differential expression pattern in the hippocampus. The expression levels of V1, 3, 5, and 8 trend lower in younger mouse hippocampus with V2, 4, 6–7, and 9–10 trending higher. Our findings imply that different *Oprl1* variants may play a role at different stages during hippocampal development. V1 exists as a canonical transcript and encodes *Oprl1* protein KOR-3 (UniProtP35377). V3–4 and 6–8 encode KOR-3d, KOR-3a, KOR-3b, KOR-3c, and KOR-3e isoforms, respectively (Pan et al., 1998). Different pharmacological responses to agonists characterize these different variants and isoforms (Pan et al., 1998). Our findings of sex differences in *Oprl1* total mRNA and a few variants suggest that endogenous sex steroids may play a role in these changes.

Our findings of AME-WD leading to increased hippocampal *Oprl1* V4 mRNA levels in adulthood suggest that increased V4 levels may account for elevated total *Oprl1* levels seen in this model. V4 contains an additional internal exon, which results in translation initiation from an alternate start codon compared to V1. The encoded isoform KOR-3a is shorter with a distinct N-terminus compared to isoform KOR-3 (Acc# NM_001318922.1). This truncated isoform may affect *Oprl1* biological functions that require further investigations. Furthermore, hippocampal V4 and V7 levels are increased in AME juvenile females. However, hippocampal V4 and V7 levels are decreased in AME-WD adult females that are in accord with nonaffected cognitive function. While the *Oprl1* genetic variants have been shown to be altered in neuropsychiatric disorders (Andero et al., 2013; Briant et al., 2010), our data demonstrate that an AME and an AME-WD affect *Oprl1* expression in a variant-specific and sex-specific manner. We speculate that aberrant expression levels of V4 may contribute to upregulated *Oprl1* expression in affected HP and subsequently lead to learning and memory impairment seen in males in our model.

All molecular analyses performed in this study utilized whole homogenized hippocampus, thus limiting our ability to understand the contribution of hippocampal subfields but allowed for a comprehensive hippocampal investigation. Future studies include FISH probes for RNA may delineate region specificity. Another limitation is that this study did not directly link *Oprl1* expression after an AME-WD and neurogenesis that may be a mechanism for hippocampal dysfunction. Future studies are warranted to investigate the mechanisms underline using an *Oprl1* agonist in vivo and siRNA knock down in vitro.

In summary, we demonstrate that AME increases *Oprl1* total and V4 expression in association with altered histone code at *Oprl1* promoter in male juvenile hippocampus. Furthermore, an AME plus a second hit by a postweaning WD shows the similar effects on gene expression and histone code in male adult hippocampus. These results advance the field by providing the first report of altered *Oprl1* mRNA variant expression and histone code in the promoter in the hippocampus of male mice exposed to AME and second hit.

CONFLICT OF INTEREST

All authors declare that no conflict of interest exists.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article. Raw data the support the findings of this study are available from the corresponding author, upon reasonable request.

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