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Paternal valproic acid exposure in mice triggers behavioral alterations in offspring

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ABSTRACT

Sodium valproate (VPA) is the most widely used antiepileptic drug and is increasingly also being used for several non-epileptic indications including migraines and bipolar disorder. It is known that maternal VPA exposure during pregnancy increases the risk of autism spectrum disorder (ASD) in children. Animal model studies have shown that maternal treatment with VPA in rodents conveys an increased risk for ASD-like phenotypes at the molecular, cellular, and behavioral levels. In contrast, the effect of paternal VPA exposure on behaviors in offspring is unknown. This study seeks to investigate whether paternal VPA exposure in rodents triggers behavioral and epigenetic alterations in offspring. The results show that paternal VPA exposure impairs object cognitive memory, suppresses the hyperactivity evoked by an NMDA receptor antagonist in male and female offspring, and disturbs sensorimotor gating in only females. In addition, since VPA is well known as an inhibitor of histone deacetylases, we examined the levels of acetylated histone H3 in the frontal cortex and hippocampus in the offspring of VPA-exposed sires. Interestingly, paternal VPA exposure down-regulates the levels of acetylated histone H3 in the brain in offspring even though VPA exposure increased acetylated histone H3 levels in the testes of sires. Collectively, these findings suggest that paternal VPA exposure may disturb the histone acetylation balance in the brain of offspring through changes in the germline epigenome, leading to behavioral alterations in offspring.

1. Introduction

Sodium valproate (VPA) is the most widely used antiepileptic drug with a broad spectrum of activity both in partial and generalized seizures (Davis et al., 1994). It is also being increasingly used in other medical conditions, such as mood disorders (Nasrallah et al., 2006) and migraine treatment (Krymchantowski et al., 2002). VPA operates via multiple mechanisms of action. It acts to elevate GABA levels in the brain by increasing the availability of α-ketoglutarate precursors or by inactivation of α-ketoglutarate dehydrogenase. These actions increase the activity of glutamate decarboxylase (GAD), which is the enzyme responsible for GABA synthesis (Monti et al., 2009). On the other hand, VPA also inhibits GABA catabolism by decreasing the degradation mediated by GABA transaminase and succinate semialdehyde dehydrogenase (Monti et al., 2009). Together, these lead to increased GABAergic activities, which contributes to the therapeutic effect of VPA. Additionally, VPA has been reported to inhibit histone deacetylases (HDACs) (Phiel et al., 2001), and is classified as a broad-spectrum inhibitor of class I (HDAC1, 2, 3, 8) and class IIb (HDAC6, 10) HDAC families (Kazantsev and Thompson, 2008). This inhibition may also contribute to the therapeutic effect of VPA on neurological diseases, given that evidence has shown that histone modifications play a role in the neuropathology of both epilepsy and mood disorders (Machado-Vieira et al., 2011; Reddy et al., 2018).

The therapeutic application of VPA is somewhat limited by its potential adverse effects on the gastrointestinal, neurological, hematological, and reproductive systems (Perucca, 2002). Most notably, VPA is known to be a human teratogen (Eadie, 2008). Maternal ingestion of VPA during pregnancy is associated with an approximately 3-fold increase in the rate of major malformations (Ornoy, 2009). In addition, clinical studies of children suggest that exposure to VPA in utero may result in fetal valproate syndrome, which exhibits features similar to those of autism spectrum disorder (ASD) (Williams et al., 2001; Williams and Hersh, 1997). Correspondingly, such prenatal and postnatal defects have been found in rodents prenatally exposed to VPA (Roullet et al., 2010; Schneider and Przewlocki, 2005; Wagner et al., 2006). Phiel et al. (Phiel et al., 2001) have proposed that such VPA-induced birth defects as well as ASD-like phenotypes in offspring are

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attributed to the inhibition of HDACs by VPA, rather than GABAergic activation.

There are numerous reports of experience-driven heritable changes in the epigenome involving maternal or paternal behavior, diet, exposure to drugs, and endocrine disruption (Heard and Martienssen, 2014), which can propagate through the germline (Lim and Brunet, 2013) without changes in the DNA sequence, leading to phenotypic changes in subsequent generations. Paternal, not maternal, transmission should be examined when studying experience-driven heritable changes in the epigenome as this serves to remove the influence of confounding factors such as in utero substances and stress exposure of dams (Bolte et al., 2019; Roullet et al., 2013). As well, it is known that the intergenerational transmission of paternal effects causes epigenetic changes in the sperm in response to environmental exposure to substances and stressors. In fact, children show an increased risk for asthma if their fathers, but not mothers, smoked prior to conception, in particular at early puberty, which is a critical and vulnerable period of sperm development (Bohacek et al., 2013). This suggests that the descendants may be better prepared to face this environment through epigenetic inheritance/transmission if the sires are experiencing an adverse environment. In this context, chronic medication use in the male parent is significant as it is possible that most chronic medications have at least some effect on the germline epigenome (Jarred et al., 2018) and could cause alteration of gene expression in offspring. Therefore, if we can accurately determine epigenetic modifications, the transgenerational epigenome may then be available as a new therapeutic modality to predict, prevent, and treat negative epigenetic consequences on offspring. This goal motivated our group to investigate the effects of paternal experience, specifically exposure to chronic VPA, on the brain epigenome and behaviors in offspring. To do so we utilized a mouse model and examined the intergenerational transmission of the paternal environment and experience in the offspring of VPA-exposed sires.

2. Materials and methods

2.1. Animals

Male and female C57BL/6 J mice used as parents were obtained from Japan SLC Inc. (7–9 weeks old, 25–41 g; Hamamatsu, Japan). The mice were kept in a regulated environment (24 ± 1 °C, 55 ± 5% humidity) under a 12-h light/dark cycle (lights on at 7:45 a.m.) and given food and tap water ad libitum. The experimental protocols concerning the use of laboratory animals were approved by the Animal Ethics Board of Meijo University and followed the guidelines of the Japanese Pharmacological Society (Folia Pharmacol. Japan, 1992, 99: 35A); the Interministerial Decree of May 25th, 1987 (Ministry of Education, Japan); and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No.8023, revised 1978). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. VPA exposure and breeding

VPA was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in sterile sodium chloride (0.9% saline). Male C57BL/6 J mice were randomly divided into four groups: Group 1 (vehicle-treated control, $n = 15$), Group 2 (VPA at 30 mg/kg, $n = 2$), Group 3 (VPA at 100 mg/kg, $n = 17$), and Group 4 (VPA at 300 mg/kg, n = 2). At 8 weeks of age, male C57BL/6 J mice were injected daily with VPA (30, 100, or 300 mg/kg) intraperitoneally (IP) for 4 weeks. During the last week of VPA injection, we placed a VPA-exposed male mouse in a cage with two naïve females, and they remained co-housed for a week for natural mating (Fig. 1A). Males continued to receive daily VPA treatment during the breeding period to avoid the attenuating effects of VPA withdrawal on male germ cells.

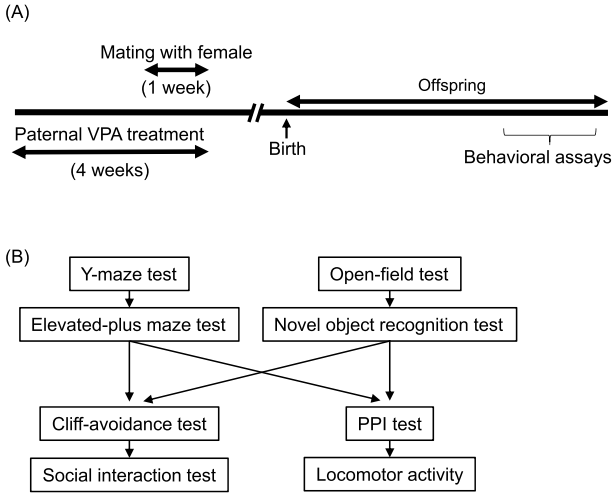

Fig. 1. Experimental schedule
To develop a model for the intergenerational influence of chronic paternal VPA administration on behaviors and histone modification in offspring, male C57BL/6 J mice were treated daily with VPA (30, 100, and 300 mg/kg) for 4 weeks. In the last week of VPA injection, VPA-exposed male mice were placed into a cage with naïve females for breeding, and they remained co-housed for a week. Males continued receiving daily VPA treatment during the breeding period. Offspring obtained from breeding were weaned at postnatal day 28 and group-housed. When the offspring reached postnatal day 56, behavioral analyses on the offspring of VPA-exposed sires were carried out (A). When the same mouse received multiple tests, an inter-test interval of a few days was used and the order in which each test was performed is shown in Fig. 1B.

2.3. Behavioral analysis

Offspring of VPA-exposed sires were weaned and group-housed at postnatal day 28. When the offspring reached postnatal day 56, behavioral analyses were carried out (Fig. 1A). When the same mouse received multiple tests, an inter-test interval of a few days was used and the order in which each test was performed is shown in Fig. 1B.

2.3.1. Open-field test

Mice were placed in the center of the arena and were allowed to explore the open-field (square: 50 × 50 cm, height: 35 cm) for 10 min under moderate light conditions (80 lx), while their activity was measured automatically using the Ethovision automated tracking program (Noldus Information Technology, Sterling, VA) (Ibi et al., 2009; Udagawa et al., 2015). The open-field was divided into four square areas (inner: 12.5 × 12.5 cm; mid-inner: 25 × 25 cm; mid-outer: 37.5 × 37.5 cm), and an outer arena (50 × 50 cm) on the outmost square in the open-field. The movement of mice was measured via a camera mounted above the open-field. Measurements included distance and time spent in each of the sections.

2.3.2. Y-maze test

The Y-maze test was carried out as described previously (Hiramatsu et al., 2010; Ibi et al., 2010). In this test, each arm is 40 cm long, 12 cm high, 3 cm wide at the bottom, and 10 cm wide at the top. The arms converge in an equilateral triangular central area that is 4 cm at its longest axis. Each mouse is placed individually at the center of the apparatus and allowed to move freely through the maze during an 8-min session. The series of arm entries is recorded visually. Alternation is defined as successive entries into the three arms, on overlapping triplet sets. The percent alternation is calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus 2) multiplied by 100. Spontaneous alternation (%) is defined as successive entries into the three arms on overlapping triplet sets and is associated with the capacity of short-term memory.

2.3.3. Novel object recognition test

A novel object recognition test was carried out, as described previously (Ibi et al., 2017; Ibi et al., 2013). The male and female mice were individually habituated to an open box (30 × 30 × 35 cm high) for 3 days. During the training session, two novel objects were placed in the open field and the animals were allowed to explore the objects for 10 min under moderate light conditions (20 lx). The time spent exploring each object was recorded. During the retention sessions, the animals were placed in the same box 24 h after the training session, and one of the familiar objects used during training was replaced by a novel object which the mice were allowed to explore freely for 5 min. The preference index in the retention session, i.e., the ratio of the time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as the ratio of time spent exploring the object that was replaced by a novel object in the retention session to the total exploration time. The explorative behavior in both sessions was recorded on video for subsequent blind scoring.

2.3.4. Elevated-plus maze test

The elevated-plus maze consisted of two open (25 × 8 cm) and two closed (25 × 8 × 20 cm) arms emanating from a common central platform (8 × 8 cm) to form a plus sign (Udagawa et al., 2015). The entire apparatus was elevated to 50 cm above floor level under moderately bright conditions (170 lx). The test began by placing a mouse on the central platform of the maze facing an open arm. An arm entry was defined as all four paws having entered into the arm. The duration of time spent in an arm and number of arm entries was measured for 5 min.

2.3.5. Cliff-avoidance test

The cliff avoidance test was conducted as reported previously (Kuroda et al., 2011). Cliff-avoidance and jumping were evaluated using a round platform (an inverted glass container with a diameter of 13 cm and a height of 20 cm); mice were placed on the platform, and their behavior was video recorded for 10 min.

2.3.6. Social interaction (resident-intruder) test

We used the experimental paradigm described in a previous study (Ibi et al., 2008) to measure social behavior (e.g., social interaction). The offspring of control or VPA-exposed sires were individually housed in a home cage (29 × 18 × 12 cm) for 2 days before the trial. We used 10–15-week-old male or female C57BL/6 J mice as intruders which were the same sex to the resident mice and had not shown aggressive behavior. In the first trial (5 min duration), an intruder mouse was introduced into the resident's home cage under bright light conditions (75 lx). The duration of social interaction (close following, inspection, anogenital sniffing, and other social body contacts excepting aggressive behavior) was analyzed. Four trials, with an inter-trial interval of 30 min, were used to analyze social behavior using the same intruder mouse.

2.3.7. Prepulse inhibition test

Prepulse inhibition (PPI) of the acoustic startle response was measured using an SR-LAB System (San Diego Instruments, San Diego, CA). The stimulus consisted of a 20-ms prepulse, a 100-ms delay, and then a 40-ms startle pulse. The intensity of the prepulse (PP) was 4, 8, or 16-dB above the 70-dB background noise. The amount of PPI was calculated as a percentage of the 120-dB acoustic startle response: 100 − [(startle reactivity on prepulse + startle pulse)/startle reactivity on startle pulse] × 100.

2.3.8. Locomotor activity

Each mouse was placed in a standard transparent rectangular rodent cage (25 × 30 × 18 cm) under moderate light conditions (15 lx). Locomotor activity was then measured using digital counters with infrared sensors (Scanet SV-10; Melquest Co. Ltd., Japan). This was done by measuring the mouse's sensitivity to the NMDA antagonist dizocilpine, also known as (+)-MK801 (Sigma-Aldrich, St. Louis, MO). Each mouse was allowed a 90-min habituation period before (+)-MK801 (0.3 mg/kg, IP) treatment. Locomotor activity was then measured for 120 min immediately after (+)-MK801 treatment.

2.4. Seizure observation and kindling procedure

We used the PTZ-induced kindling model described in previous reports (Schroder et al., 1993; Schroeder et al., 1998). 8-week-old male C57BL/6 J mice were intraperitoneally injected with PTZ (35 mg/kg) once every 48 h, and mice showing more than two consecutive Stage 4 seizures were defined as kindled mice. Control animals were injected with saline. We examined seizure events during a 20 min observation period after each stimulation. The seizure intensity was scored as follows (Mizoguchi et al., 2011; Schroder et al., 1993; Schroeder et al., 1998): Stage 0, no response; Stage 1, ear and facial twitching; Stage 2, convulsive twitching axially through the body; Stage 3, myoclonic jerks and rearing; Stage 4, turning over onto the side, wild running, and wild jumping; Stage 5, generalized tonic-clonic seizures; and Stage 6, death.

2.5. Immunohistochemistry

Mice that had not previously been subjected to behavioral analysis were deeply anesthetized with ethyl carbamate (1.5 g/kg IP, Katayama Chemical, Osaka, Japan) and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Their brains and testes were removed, post-fixed in the same fixative, and then cryoprotected. Twenty micrometer-thick coronal brain sections and testes were cut on a cryostat; subsequently, the free-floating sections were transferred to 24-well dishes containing phosphate-buffered saline (PBS). After blocking with 10% goat serum/PBS for 60 min, rabbit anti-acetyl histone H3 (Lys9) (AcH3; Merck-Millipore 17–658, 1:200), mouse anti-glial fibrillary acidic protein (GFAP; Merck-Millipore MAB3402, 1:1000), and mouse anti-NeuN (Abcam, Cambridge, UK, 1:1000) diluted in 10% goat serum/PBS were applied to the sections, which were then incubated overnight at 4 °C. After washing in PBS, goat anti-mouse Alexa Fluor 568 and anti-rabbit Alexa Fluor 488 antibodies (1:3000; Invitrogen, Eugene, OR) were added to the sections for 2 h at room temperature. The samples were observed using a confocal-laser scanning microscope (LSM 800; Zeiss, Jene, Germany). Counterstaining with 4′,6-diamidino-2-phenylindole (DAPI) allowed the determination of brain areas and laminar borders. In addition, phalloidin was used to stain the testes as phalloidin binds with high affinity to filamentous (F)-actin, which is particularly abundant in the peritubular myoid cells in the testis (Losinno et al., 2012). For the quantification of AcH3 immunoreactivity, the signal intensity of the immunoreactivity in each cell of the brain and testes was measured with NIH ImageJ v1.62 software by an experimenter blinded to the treatment.

2.6. Statistical analysis

Statistical analysis was performed and figures were produced using Prism 7 (GraphPad Software, Inc., San Diego, CA). It was not possible to assume that the behavioral data had a Gaussian distribution; therefore, the data were expressed as median and interquartile range values. Significance was evaluated using the Mann–Whitney *U* test for comparisons between two groups, and Kruskal-Wallis non-parametric one-way or two-way ANOVA followed by Bonferroni's test was used for multiple comparisons.

The quantifications of immunohistochemistry are shown as means ± SEM. An unpaired *t*-test was used to compare two groups, and one-way ANOVA followed by Dunnett's test was used for multiple comparisons. The criterion for significance was $p < .05$.

3. Results

3.1. General appearance in offspring of VPA-exposed sires

8-week-old male C57BL/6 J mice were injected daily with VPA (30, 100, and 300 mg/kg, IP) for 4 weeks. During the last week of VPA injection, we placed VPA-exposed male mice into a cage with naïve females. The animals remained co-housed for a week. Males continued receiving daily VPA treatment during the breeding period.

A previous study demonstrated that daily treatment with VPA (IP) for 4 weeks at doses of 100, 200, and 400 mg/kg/day produced sperm toxicity in a dose-dependent manner. Notably, a decrease in the number of sperm, weight of the testes and epididymis, as well as sperm head abnormalities and DNA damage were found in male mice exposed to high doses of VPA (i.e., 200 and 400 mg/kg) (Khan et al., 2011). In the present study, female mating with sires chronically administered 300 mg/kg VPA showed infertility (data not shown), which was attributed to sperm toxicity (Khan et al., 2011; Ourique et al., 2016a; Ourique et al., 2016b).

Accordingly, in this study we investigated the offspring phenotypes of sires treated with VPA at doses of 30 and 100 mg/kg, in which offspring were viable and did not display any gross histological abnormalities in the fontal cortex and hippocampus (data not shown). Offspring of sires with daily treatment of VPA at a dose of 100 mg/kg were used unless otherwise indicated. Additionally, no difference was found in the body weight of adult offspring of VPA-treated sires (100 mg/kg) and the control group (data not shown), suggesting that paternal VPA treatment did not affect the growth of the offspring.

3.2. Cognitive function in offspring of VPA-exposed sires

Novel object recognition, a non-force driving and spontaneous memory test, is based on curiosity about a novel object (Leger et al., 2013). During the training session, there was no biased exploratory preference in offspring of both control and VPA-exposed sires (Fig. 2A). In the retention session, which was carried out 24 h after the training session, the control offspring displayed significantly higher exploratory preference for the novel object than that in the training session, while the offspring of the VPA-exposed sires showed nearly equal exploratory preference between training and retention sessions (Fig. 2A). There was no difference between the sexes. [median (interquartile range) exploratory preference % (p-value, training vs retention), male vehicle: training session 51.23 (50.34–52.15), retention session 58.90 (57.62–61.20) ($p < .001$); male VPA: training session 56.52 (52.00–56.76), retention session 69.20 (55.15–70.00) ($p = .54$); female vehicle: training session: 50.78 (49.5–53.7), retention session 61.10 (56.3–74.3) ($p < .01$); female VPA: training session 53.29 (46.7–55.4), retention session 55.89 (54.5–68.6) ($p = .29$)]. Total exploration time in the training or retention sessions did not differ between the two groups (Fig. 2B), suggesting that the control offspring normally recognized the novel object, but the offspring of the VPA-exposed sires did not.

PPI tests were carried out to examine sensorimotor gating in offspring (Ibi et al., 2010), in which PPI deficits were observed only in females (Table 1), without affecting startle response (Fig. 2C, D).

Conversely, in the Y-maze test to evaluate spatial short-term memory (Ibi et al., 2010), both male and female offspring of VPA-exposed sires did not show any changes in spontaneous alternation and the number of arm entries in comparison with control offspring (Table 1).

Together, these results show that paternal VPA exposure attenuates object cognitive memory in both male and female offspring, but sensorimotor gating deficits were observed only in female offspring without affecting spatial short-term memory.

In addition to cognitive behaviors, locomotor activity was also tested before and after the administration of an NMDA antagonist, dizocilpine ((+)-MK801), as the behavioral response to (+)-MK801 reflects the glutamatergic function and sensitivity of NMDA receptors, and the hyperactivity evoked by an NMDA receptor antagonist resembles certain aspects of psychosis (Ibi et al., 2017; Ibi et al., 2010). Interestingly, hyperactivity evoked by (+)-MK801 treatment in offspring of VPA-exposed sires was significantly lower than in the control offspring of VPA-exposed sires. Sex-based differences were not observed, although paternal VPA treatment did not affect the basal locomotor activity before (+)-MK801 treatment (Fig. 2E). Given the changes in the response to an NMDA antagonist, paternal VPA treatment may affect glutamatergic function and structure in the brain of offspring even though it does not seem to trigger the development of psychosis in offspring.

3.3. Emotional behaviors in offspring of VPA-treated sires

To investigate the effect of paternal VPA exposure on the emotional behavior of offspring in adulthood, the open-field, elevated-plus maze, cliff-avoidance, and social interaction tests were carried out. Paternal VPA exposure did not affect the distance moved and the spent time by offspring in each section of the open-field test (Table 1). In the elevated-plus maze test, there was no difference between the offspring of control and VPA-exposed sires in the time spent on the open arm (Table 1) and the number of open arm entries (Table 1). For the cliff-avoidance test, there was no difference in the jumping latency and number between the two groups (Table 1), suggesting that paternal VPA exposure does not affect anxiety and impulsivity-related behaviors in offspring. There were no sex-based differences in these tests.

In the social interaction test, paternal VPA exposure decreased social interaction behaviors in male (Fig. 2F), but not female, offspring although aggressive behaviors in either sex were not influenced by paternal VPA exposure (male $F_{1,92} = 2.88$, $p = .093$; female $F_{1,48} = 1.00$, $p = .322$). Together, paternal VPA exposure partially and sex-dependently attenuated social interaction, but other emotional behaviors in offspring were not influenced.

3.4. Histone acetylation in the brains of offspring of VPA-treated sires

As shown in Fig. 2, VPA, a class I and II HDAC inhibitor (Jarred et al., 2018), affected cognitive and emotional behaviors in the offspring of VPA-treated sires, raising the possibility that paternal VPA exposure may affect histone acetylation in the offsprings' brains, leading to behavioral alterations. To test this, we investigated the levels of AcH3 in the neurons of the prefrontal cortex (PFC) and hippocampus. The immunohistochemical assay showed the signal of AcH3 in the DAPI-positive nucleus (Figs. 3A and 4A–C), in which AcH3 levels were significantly down-regulated in the NeuN-positive neuronal nucleus in the PFC (Fig. 3) and hippocampal CA1 region (Fig. 4A,D) in female offspring, but not male offspring of VPA-exposed sires [relative level (average) ± SE; PFC: vehicle 1.00 ± 0.17, VPA 1.13 ± 0.03, $p = .54$; CA1: vehicle 1.00 ± 0.08, VPA 0.99 ± 0.13, $p = .93$; CA3: vehicle 1.00 ± 0.16, VPA 0.78 ± 0.06, $p = .28$; DG: vehicle 1.00 ± 0.13, VPA 0.85 ± 0.05, $p = .41$]. In addition, two-way ANOVA revealed that paternal VPA treatment significantly decreased the AcH3 levels in the hippocampus of female offspring ($F_{1,48} = 11.80$, $p = .0012$).

Conversely, paternal VPA exposure did not affect AcH3 levels in GFAP-positive astroglial cells of the PFC, in which there was no sex difference [average ± SE (relative level); vehicle 1.00 ± 0.046, VPA 0.94 ± 0.099; $p = .55$] and hippocampus (CA1: vehicle 1.00 ± 0.046, VPA 0.79 ± 0.13; $p = .12$). Together, paternal VPA exposure down-regulated the levels of acetylated histone in the neurons, but not astroglial cells, of the offsprings' brains.

3.5. Histone acetylation in the testes of VPA-exposed male mice

To investigate the effect of chronic exposure of VPA at a dose of

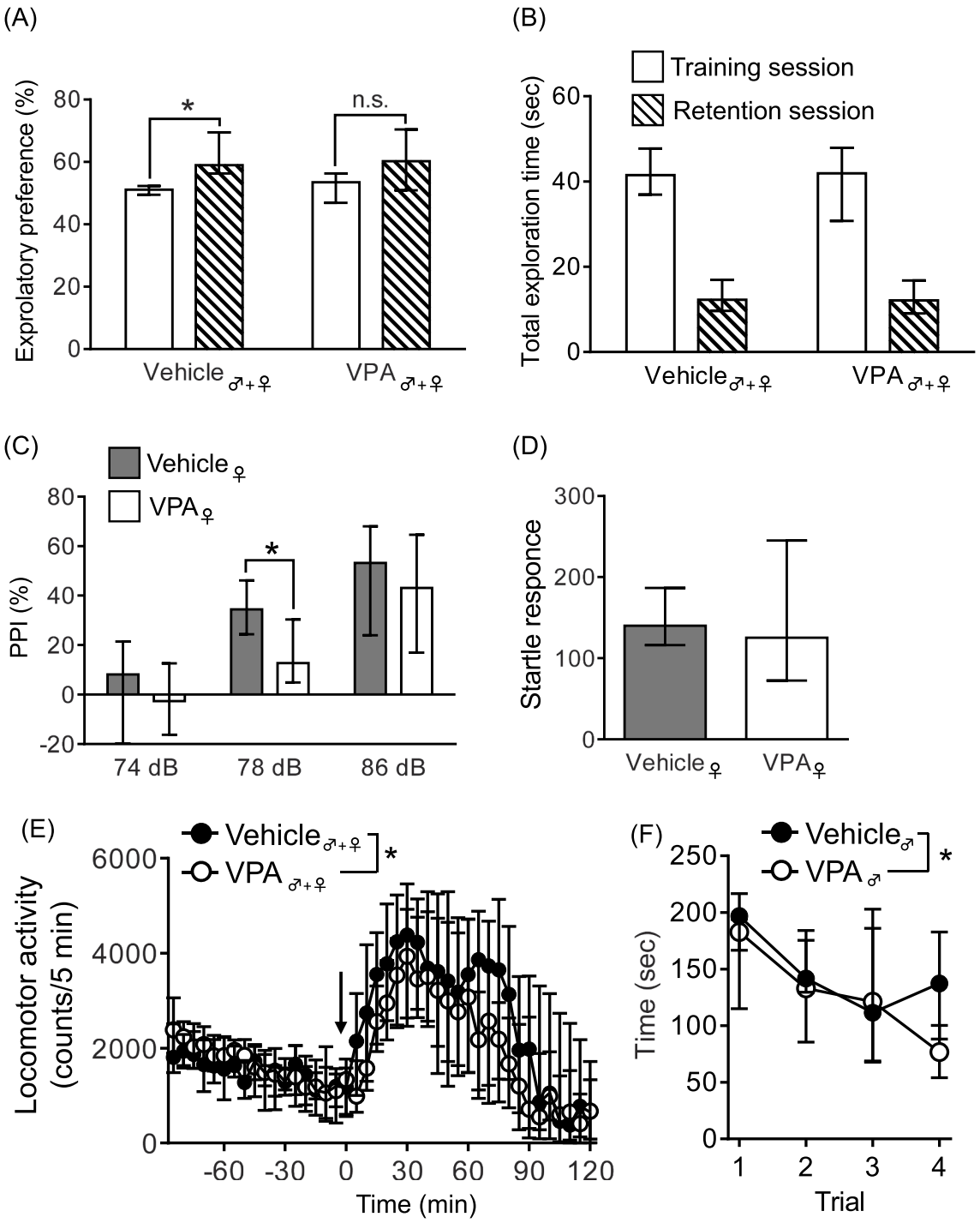

Fig. 2. Behavioral alternations in offspring of VPA-exposed sires

Exploratory preference (A) and total exploration time (B) in the novel object recognition test. The retention session was carried out 24 h after the training session. n = 12–21 (vehicle: 10 males and 11 females; VPA: 3 males and 9 females). (C) A PPI test was performed in the offspring of VPA-treated sires. PPI (%) at three different prepulse intensities (74, 78, and 86 dB). (D) Acoustic startle amplitude as measured in trials without prepulse. n = 10–11 (vehicle: 11 females; VPA: 10 females). Locomotor activity shown in 5-min blocks for 90 and 120 min before and after (+)-MK801 (0.3 mg/kg, IP) treatment, respectively. Time of injection is indicated by arrow. (E) n = 24–32 (vehicle: 12 males and 20 females; VPA: 11 males and 13 females). (F) Social interaction test in which 4 trials, with an inter-trial interval of 30 min, were used to analyze social behavior using the same intruder mouse. n = 12–13 (vehicle: 13 males; VPA: 12 males).

Values represent the median and interquartile range. Mann–Whitney U test (A, B, C, D); Bonferroni's test (E, F). *p < .05 vs. vehicle-treated control group; n.s., not significant.

100 mg/kg on histone acetylation in the germline of male mice, sires' testes were dissected immediately after a week of mating, and we subsequently measured the levels of AcH3 in the testes. Chronic VPA treatment increased the levels of AcH3 in the testes (Fig. 5A, B), which was attributed to HDAC inhibition of VPA.

3.6. Anti-epileptic effect of VPA in a mouse model of epilepsy

Next, to determine whether chronic VPA treatment under this experimental condition achieved full therapeutic effect, we tested the antiepileptic effect of VPA in a mouse kindling model of epilepsy, which

Table 1
Summary of behavioral phenotypes in offspring of VPA-exposed sires.

Test	Index	Measurement item		♂		*P* value	♀		P value
				Vehicle-offspring	VPA-offspring		Vehicle-offspring	VPA-offspring	
PPI test	PPI	74 dB		10.56 (−11.5–13.4)	25.00 (−12.06–35.33)	$p = .37$	8.13 (−10.23–21.22)	−2.66 (−13.57–9.48)	$p = .41$
		78 dB		8.86 (4.4–23.0)	18.76 (12.10–44.02)	$p = .23$	34.42 (24.61–42.07)	12.75 (5.11–22.53)	$p < .05$
		86 dB		33.93 (14.6–56.7)	45.04 (24.13–65.70)	$p = .60$	53.19 (32.39–66.58)	43.11 (22.36–60.74)	$p = .28$
Y-maze test	Short term memory	Total arm entry (count)		35.50 (29.75–39.25)	23.00 (21.50–27.50)	$p = .18$	35.00 (30.75–40.00)	41.00 (33.75–45.25)	$p = .29$
		Alternation (%)		60.26 (58.40–64.44)	67.67 (60.79–75.00)	$p = .18$	59.63 (57.16–62.53)	54.03 (50.97–59.77)	$p = .25$
Social interaction test	Sosial behaviors	Social interaction (*sec*)	Trial 1	196.75 (172.53–215.85)	182.72 (123.40–188.28)	$p < .05$	167.91 (153.40–200.14)	166.04 (157.01–201.40)	$p = .99$
			Trial 2	141.75 (131.88–174.36)	132.95 (96.51–171.55)		115.94 (104.16–123.44)	92.38 (80.93–121.60)	
			Trial 3	111.40 (69.92–186.51)	121.22 (70.63–175.21)		78.28 (41.44–114.20)	77.91 (66.90–109.03)	
			Trial 4	137.30 (95.47–178.05)	76.63 (57.73–99.52)		33.57 (24.51–92.50)	47.04 (40.53–62.00)	
Open field test	Exploratry activity	Distance moved	Inner	256.12 (206.33–357.31)	293.39 (209.46–315.46)	$p = .95$	307.14 (253.61–349.96)	267.16 (228.17–390.91)	$p = .84$
		(cm/10 min)	Mid inner	638.92 (477.45–891.852)	726.61 (693.81–774.14)	$p = .88$	861.71 (736.15–948.29)	947.03 (647.81–1001.63)	$p = .96$
			Mid outer	2098.22 (1066.38–2850.22)	1257.15 (1234.68–2257.42)	$p = .45$	2613.37 (1555.76–2969.93)	1705.28 (1342.36–2951.34)	$p = .57$
			Outer	2683.64 (2334.46–2980.50)	2505.98 (2497.05–2775.98)	$p = .94$	3340.12 (2865.66–3725.68)	2772.38 (2617.48–2911.81)	$p = .06$
	Anxiety-like behavior	Time spent	Inner	23.36 (19.08–29.31)	30.50 (22.22–31.30)	$p = .88$	20.29 (18.27–25.84)	24.64 (15.42–28.37)	$p = .81$
		(sec/10 min)	Mid inner	53.69 (39.62–62.53)	67.00 (47.95–73.17)	$p = .46$	53.92 (47.63–66.50)	67.27 (47.42–77.13)	$p = .63$
			Mid outer	192.36 (100.81–239.57)	127.29 (123.02–192.37)	$p = .61$	176.71 (126.54–203.26)	139.92 (102.38–223.96)	$p = .83$
			Outer	402.74 (357.55–441.40)	376.60 (373.47–405.70)	$p = .87$	394.52 (386.08–411.09)	386.76 (361.87–424.79)	$p = .77$
Cliff avoidance test	Impulsivity	Jumping latency (sec)		600 (338.01–600.00)	399.22 (48.86–600.00)	$p = .25$	600 (401.69–600.00)	600.00 (354.50–600.00)	$p = .99$
		Jumping count (count)		0.00 (0.00–1.00)	1.00 (0.00–2.00)	$p = .45$	0.00 (0.00–1.00)	0.00 (0.00–1.00)	$p = .79$
Elevated-plus maze test	Anxiety-like behavior	Duration of open arm (sec)		14.26 (0.00–23.69)	1.04 (0.00–8.44)	$p = .24$	9.75 (3.99–19.60)	16.27 (13.96–20.97)	$p = .25$
		Entry of open arm (count)		2.00 (0.00–3.50)	1.00 (0.00–1.00)	$p = .72$	3.00 (0.50–3.50)	3.50 (3.00–4.00)	$p = .37$

All data indicate “median and interquartile range (Q1-Q3)”.

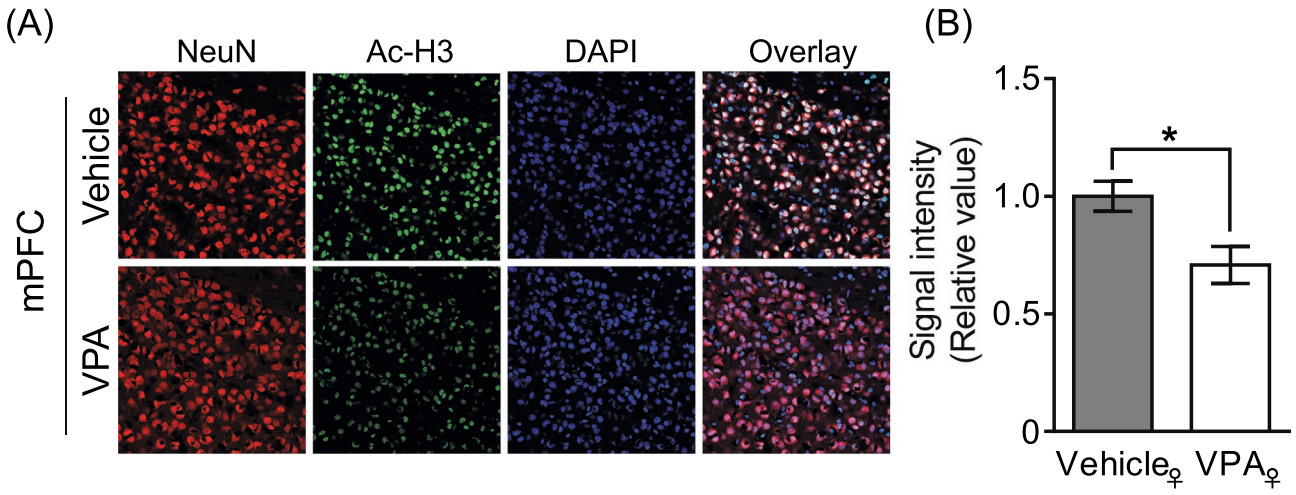

Fig. 3. Acetylated histone H3 in offspring PFC of VPA-treated sires
(A) Representative immunohistochemical images of AcH3 in the nucleus of neurons in PFC from offspring of vehicle- or VPA-exposed sires. (B) Quantification assessment of the relative levels of signal intensity showed a significant decrease in AcH3 staining (intensity/cell) in the neuronal nucleus of female offspring of VPA-exposed sires, expressed as relative average level ± SEM (n = 7–8 females). Student t-test. *$p < .05$ vs. vehicle-treated control group.

is the most frequently used model of epileptogenesis, whereby repeated administration of an initially subconvulsive electrical or chemical stimulation eventually leads to the development of generalized motor seizures (Zheng et al., 1998). For this experiment, mice were administered VPA at 100 or 300 mg/kg 30 min prior to each PTZ dose. VPA at 100 mg/kg partially suppressed kindling seizures, but significantly, a dose of 300 mg/kg completely suppressed seizures (Fig. 5C). This suggests that daily treatment of VPA has a therapeutic effect, given that its treatment every 48 h showed antiepileptic effects to some extent.

Together, chronic VPA treatment to achieve full therapeutic effect would up-regulate the levels of acetylated histone in male germline cells, possibly leading to an effect on behaviors and brain histone acetylation in offspring.

4. Discussion

Histone modifications affect various biological processes such as transcriptional activation and inactivation, chromosome packaging, and DNA damage and repair. Histone H3 is acetylated at lysines 9, 14, 18, 23, and 56, methylated at arginine 2 and lysines 4, 9, 27, 36, and 79, and phosphorylated at serine 10, serine 28, threonine 3, and threonine 11. Histone H4 is primarily acetylated at lysines 5, 8, 12, and 16, methylated at arginine 3 and lysine 20, and phosphorylated at serine 1 (Nestler et al., 2016). We examined the levels of acetylation of lysine 9 on histone H3 as some studies have reported that VPA treatment up-regulates the levels of acetylation of lysine 9 on histone H3 (Gates et al., 2017; Hezroni et al., 2011). Moreover, it is possible that this modification of lysine 9 in germline cells including testes and sperm is affected by paternal experiences (Rollo et al., 2017; Vassoler et al., 2013). This motivated us to investigate the levels of lysine 9 acetylation on histone H3 in the sires' testes and the brains of their offspring; however, the role of epigenetic modification of other amino acids of histone in paternal experience transmission to offspring remains unknown.

Khan and colleagues demonstrated that daily treatment with VPA at high doses (200 and 400 mg/kg) for 21 days damages spermatogonia, spermatocytes, and spermatids in male mice, leading to male infertility, but a moderate dose of VPA (100 mg/kg) does not have this affect (Khan et al., 2011). Likewise, we found that dams crossed with sires that had been chronically treated with VPA at 300 mg/kg did not conceive (data not shown), which also supports the thought that chronic treatment with high doses of VPA sterilizes sires. Conversely, the sperm and testes of sires chronically treated with VPA at 100 mg/kg were reportedly normal in every aspect (Khan et al., 2011), which is consistent with the results of our study that showed that daily injection of VPA at 30 and 100 mg/kg for 21 days did not adversely affect male fertility in mice (5–10 pups delivered per dam). As shown in Fig. 2, chronic paternal treatment of VPA at 100 mg/kg affects emotional and cognitive behaviors in offspring; however, chronic treatment of VPA at 30 mg/kg slightly and partially changed offspring behaviors, but this change was not statistically significant (data not shown). These results suggest that the adverse effects of chronic VPA treatment on male parent germ cells and offspring behavioral phenotypes develop in a dose-dependent manner. Regarding the dose of VPA required to achieve an antiepileptic effect, pre-treatment of VPA at 300 mg/kg almost entirely suppressed PTZ-induced kindling seizures in mice (Fig. 5C). In contrast, VPA at 100 mg/kg partially suppressed these seizures (Fig. 5C). Therefore, the dose of VPA required to be clinically effective in at least partially suppressing seizures will likely affect the germline cells in males, and may lead to fertility issues in the male parent or behavioral alterations in their offspring.

To our knowledge, no studies have been done to clarify the association of paternal VPA treatment with the mental condition of their offspring. This study is the first report to demonstrate behavioral and epigenetic alterations in the offspring of male parents receiving chronic VPA treatment. While extensive literature exists on use of drugs in pregnancy by females, these results suggest that it is important to consider medication usage by the father of the offspring as well (Alexander et al., 2016). For instance, we have already reported that chronic treatment with antipsychotics up-regulates HDAC2 expression levels in mouse and human brain (Ibi et al., 2017; Kurita et al., 2012), but there is no report investigating histone acetylation in the germline cells of males receiving chronic antipsychotic treatment. Our findings emphasize that attention should be paid to epigenetic modulators such as VPA and antipsychotics in males whose female partner is attempting to conceive.

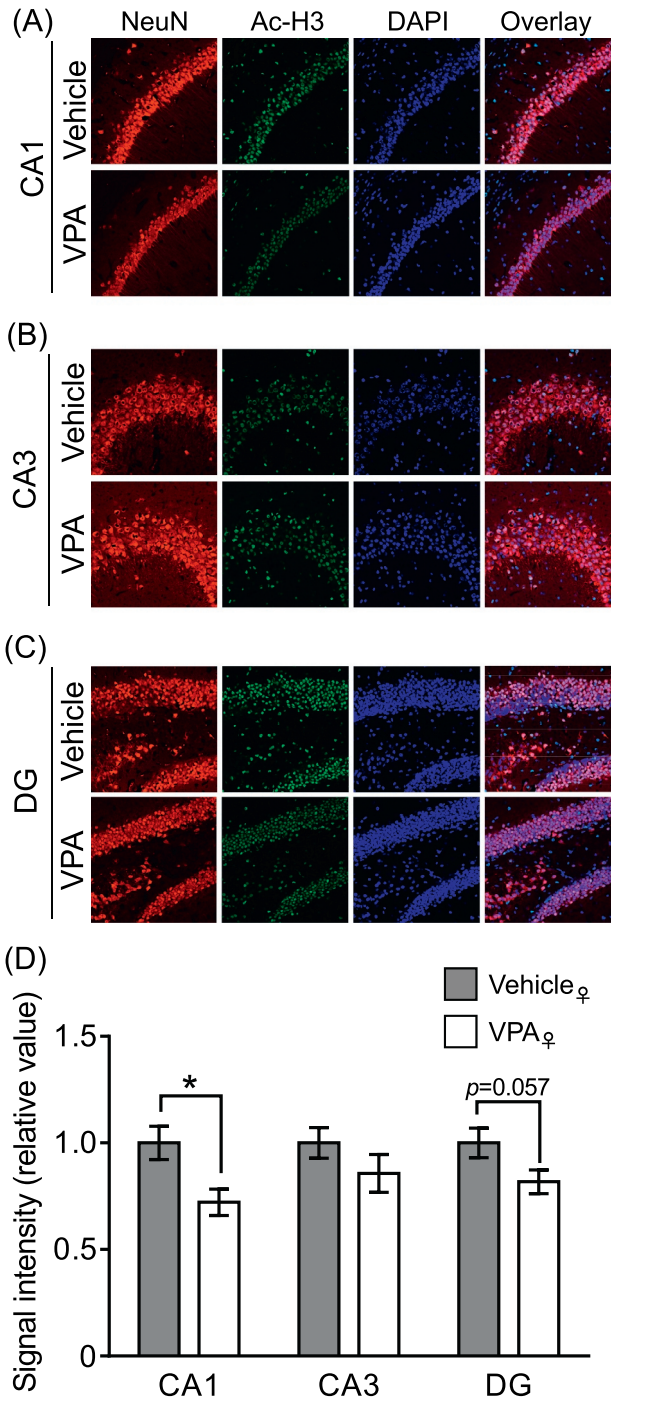

Fig. 4. Acetylated histone H3 in the hippocampal subregions of offspring of VPA-sired mice

Representative immunohistochemical images of AcH3 in the nucleus of neurons in hippocampal (A) CA1, (B) CA3, and (C) DG from offspring of vehicle- or VPA-exposed sires. (D) Quantification of the relative levels of signal intensity showed a significant decrease in AcH3 staining (intensity/cell) in the hippocampal CA1 region of female offspring of vehicle- or VPA-exposed sires, expressed as relative average level ± SEM (n = 9 females). Student t-test. *p < .05 vs. vehicle-treated control group.

In the present study, we found that paternal VPA treatment induced sex-based differences in behavior alteration in offspring. Notably, PPI deficit was observed in only the female offspring of VPA-exposed sires, but social behavior impairment occurred in only male offspring (Fig. 2C, F). Both of these behaviors are reportedly affected by various sex steroid hormones (Bell, 2018; Gogos, 2013), which may contribute to the sex-based differences in behavior alteration.

On the other hand, object cognitive memory deficits and suppression of (+)-MK801-induced hyperactivity were present in both sexes (Fig. 2A, E), raising the possibility that the regions of the brain involved in these behaviors may be less influenced by sex hormone system modifications. Given these commonalities in both the male and female offspring and that the glutamatergic system in the brain is intimately involved with both object cognitive memory and (+)-MK801-induced hyperactivity (Nilsson et al., 1997; Thomas et al., 2017), further investigation of the effects of paternal VPA treatment on histone acetylation and the genes involved in the glutamatergic system is needed. In addition to behaviors, the present study demonstrated that the down-regulation of acetylated histone levels in the offspring brain of VPA-exposed sires was observed only in females (Figs. 3 and 4). Along with this sex-based difference in the expression/activity of HDACs in the brain (Elsner et al., 2018; Gilbert et al., 2019; Pujol Lopez et al., 2016), there were understandably sex-based changes of histone acetylation in the brain. Previous reports have revealed that sex steroid hormones and their receptors, in conjunction with histone acetyltransferase (HAT) or HDAC, orchestrate orderly patterns of histone acetylation (McCarthy et al., 2009), leading us to consider that sex hormones may contribute to sex-based differences in histone modification in the brain and result in behavioral differences between male and female offspring. Additionally, the underlying mechanism associated with the up-regulation of histone acetylation in the testes of VPA-treated sires with down-regulated histone acetylation in the brains of female offspring also remains unclear. Further study is necessary to unlock these mysteries.

In mammalian spermiogenesis, male germ cells differentiate from haploid spermatids to motile sperm (Steger, 1999), during which the majority of histones are replaced by transition proteins and subsequently protamines to facilitate chromatin hyper-compaction. The content of protamine is indispensable for the final phase maturation of the spermatozoa nucleus (Bao and Bedford, 2016). Meanwhile, it has been proposed that the remaining histones carry essential marks for the establishment of epigenetic information in the offspring (Steilmann et al., 2011). For instance, lysine 9 acetylation on histone H3 (H3K9ac) was reportedly detectable in the spermatogonia, spermatocytes, elongating spermatids, and ejaculated spermatozoa of men (Steilmann et al., 2011). Together with this current study, the association and balance between H3K9ac and protamine in the sperm of VPA-treated sires should be further analyzed to investigate the mechanism underlying the transgenerational effect of paternal VPA exposure on behavioral changes in offspring. Additionally, further study is required to identify the common epigenetic changes in the germline of VPA-treated sires and the brains of their offspring. This could lead to the identification of a transgenerational epigenomic marker.

In conclusion, we found that paternal chronic VPA treatment affected acetylated histone levels in the brains of offspring and caused sex-dependent behavioral changes in offspring through modifications of the germline cell epigenome in sires. This indicates the importance of considering the potential adverse effects to germline cells and offspring development and behaviors that may occur in males chronically exposed to epigenetic modulators such as VPA.

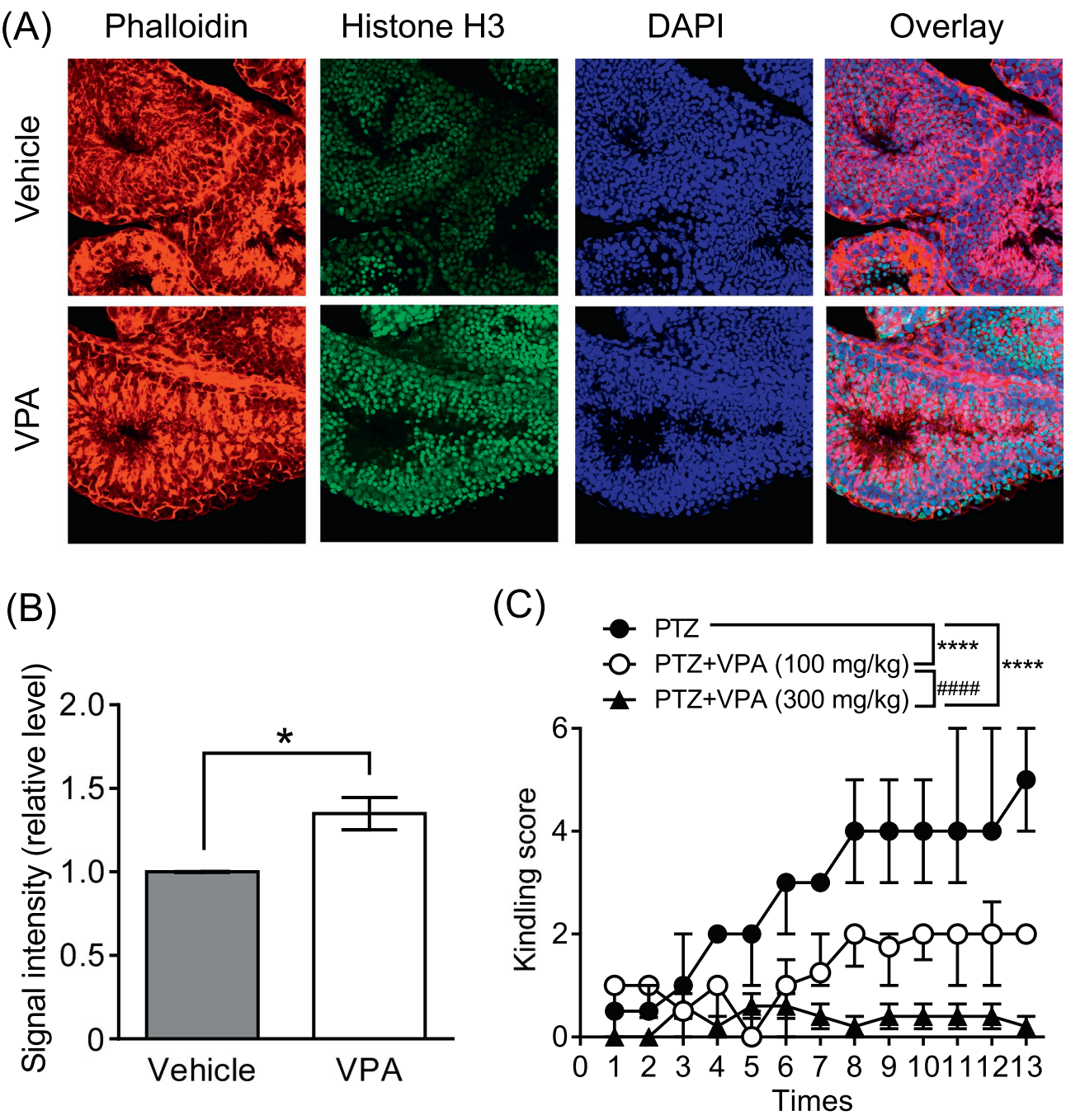

Fig. 5. Acetylated histone H3 in the testes of VPA-exposed sires
(A) Representative immunohistochemical images of AcH3 in the tubules of vehicle- or VPA-exposed sires. (B) Quantification of the relative levels of signal intensity showed a significant increase in AcH3 staining (intensity/cell) in the testicular tubules of VPA-exposed sires, expressed as relative average level ± SEM ($n = 3$). Student t-test. $^{*}p < .05$ vs. vehicle-treated control group. (C) Treatment of VPA at 100 and 300 mg/kg significantly suppressed PTZ-induced epileptic seizures in the sires. Values represent the median and interquartile range. Bonferroni's test (C). $^{****}p < .0001$ vs. PTZ alone, $^{\#\#\#\#}p < .0001$ vs. treatment of PTZ with VPA (100 mg/kg) ($n = 5$–14 males).

Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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