Autism-like behaviours with transient histone hyperacetylation in mice treated prenatally with valproic acid

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Abstract

Maternal use of valproic acid (VPA) during pregnancy has been implicated in the aetiology of autism spectrum disorders in children, and rodents prenatally exposed to VPA showed behavioural alterations similar to those observed in humans with autism. However, the exact mechanism for VPA-induced behavioural alterations is not known. To study this point, we examined the effects of prenatal exposure to VPA and valpromide, a VPA analog lacking histone deacetylase inhibition activity, on behaviours, cortical pathology and histone acetylation levels in mice. Mice exposed to VPA at embryonic day 12.5 (E12.5), but not at E9 and E14.5, displayed social interaction deficits, anxiety-like behaviour and memory deficits at age 4–8 wk. In contrast to male mice, the social interaction deficits (a decrease in sniffing behaviour) were not observed in female mice at age 8 wk. The exposure to VPA at E12.5 decreased the number of Nissl-positive cells in the middle and lower layers of the prefrontal cortex and in the lower layers of the somatosensory cortex at age 8 wk. Furthermore, VPA exposure caused a transient increase in acetylated histone levels in the embryonic brain, followed by an increase in apoptotic cell death in the neocortex and a decrease in cell proliferation in the ganglionic eminence. In contrast, prenatal exposure to valpromide at E12.5 did not affect the behavioural, biochemical and histological parameters. Furthermore, these findings suggest that VPA-induced histone hyperacetylation plays a key role in cortical pathology and abnormal autism-like behaviours in mice.

Introduction

Autism spectrum disorders (ASD) are a range of complex neurodevelopmental disorders characterized by severe impairments in reciprocal social interaction, language, and communication and by restricted, repetitive and stereotyped behaviour patterns (Geschwind & Levitt, 2007; Persico & Bourgeron, 2006). Although genetics have been believed to play a prominent role in the cause of ASD, to date, no specific genes or combination of genes have been consistently associated with the conditions. In contrast, the last decade of epidemiological studies suggest the importance of environmental factors during early life including the fetal period, in the aetiology of ASD (Ronald & Hoekstra, 2011). In addition, previous post-mortem and neuroanatomical imaging studies of patients with autism have highlighted abnormal structures of the cerebellum, amygdala, brainstem and limbic system (Courchesne, 1997). Furthermore, functional brain-imaging studies have recently revealed that symptoms of ASD may be related to micro-anatomical abnormalities, especially dysfunction in the cortical regions (Stigler *et al.* 2011; van Kooten *et al.* 2008). However, the precise mechanism for the pathology of ASD is not known.

Valproic acid (VPA) has been widely used as a first-line antiepileptic drug and for the therapy of bipolar disorders for several years (Henry, 2003). The

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mechanism of action is not fully understood, but it may be related, at least in part, to reduced neuronal activity by blocking sodium and calcium channels, and by enhancing the function of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) as a GABA transaminase inhibitor in human brain (Gould *et al.* 2004; Kwan *et al.* 2001). VPA is also known as a human teratogen (Eadie, 2008). Maternal ingestion of medication 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 a 'fetal valproate syndrome', similar to the features of ASD (Williams & Hersh, 1997; Williams *et al.* 2001). VPA indirectly inhibits histone deacetylase (HDAC) (Phiel *et al.* 2001), and is classified as a broad-spectrum inhibitor against class I (HDAC1, HDAC 2, HDAC 3, HDAC 8) and class IIb (HDAC6, HDAC10) HDAC families (Kazantsev & Thompson, 2008). Phiel *et al.* (2001) proposed that inhibition of HDAC might be involved in VPA-induced birth defects and the efficacy of VPA in the treatment of bipolar disorder. Rodents, prenatally exposed to VPA, that exhibit some behavioural impairments similar to the features of autism are considered to be an animal model of autism (Roullet *et al.* 2010; Schneider & Przewłocki, 2005; Wagner *et al.* 2006). In these studies, the impairment in motor activity, attention and social interaction are well shown, but the precise mechanism for VPA-induced behavioural alterations is not known.

In the present study, we examined the embryonic stage-dependent effects of VPA on social interaction, anxiety behaviour, cognitive function and brain morphological changes of cortical regions during postnatal development, and then measured acetylated levels of the histones H3 and H4 after VPA exposure. We further examined whether prenatal exposure to valpromide (VPD), a VPA analog lacking HDAC inhibition activity, induces behavioural alterations, morphological changes and hyperacetylated levels of the histones H3 and H4 in order to clarify the role of HDAC inhibition in the VPA-induced effects.

Methods

Chemicals and antibodies

Reagents were obtained from the following sources: 2-propylpentanoic acid (valproic acid; VPA) sodium salt (Sigma-Aldrich Co. USA); VPD (Wako Pure Chemical Industries Ltd, Japan); goat anti-rat IgG (H+L) (Biotin), rat anti-BrdU IgG$_{2a}$ [clone BU1/75 (ICR1)] (Abcam plc, UK); 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI), *In Situ* Cell Death Detection kit (Fluorescein) (Roche Diagnostics GmbH, Germany); rabbit anti-histone H3 IgG, rabbit anti-histone H4 IgG (Cell Signaling Technology Inc., USA); rabbit anti-acetyl-histone H3 IgG, rabbit anti-acetyl-histone H4 IgG (Millipore Co., USA), Restore™ Plus Western Blot Stripping Buffer (Thermo Fisher Scientific, USA); rat anti-Ki-67 IgG$_{1k}$ (clone TEC-3), Fluorescent Mounting Medium (DakoCytomation Inc., USA); horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (KPL, USA). All other chemicals used were of the highest purity commercially available.

Mice and drug administration

Eighty-four female ICR (CD1) mice were purchased from Japan SLC Inc. (Japan) at 8 d gestation and housed individually in plastic cages under a standard 12-h light/dark cycle (lights on 08:00 hours) at a constant temperature of $22 \pm 1\ °C$. The animals had *ad libitum* access to food and water, and were handled in accordance with the guidelines established by the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences, Osaka University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

The pregnant mice were injected with either 500 mg/kg VPA (i.p.) (Markram *et al.* 2008), 150 mg/kg VPD (s.c.) (Eikel *et al.* 2006; Nishikawa & Scatton, 1985) or vehicle at 9, 12.5 and 14.5 d gestation. VPA and VPD were dissolved in isotonic 0.9% NaCl solution and distilled water containing 10% DMSO and 20% Tween-20, respectively, and the volume of injection was 10 ml/kg. All animals were returned to their home cages immediately after the injection and left undisturbed until weaning of the offspring. On average, 12–14 pups were obtained from VPA- VPD- and vehicle-treated mothers. Malformed pups were born at a rate of 30–40% from mothers treated with VPA at 9 d gestation. The offspring without malformed pups were weaned, sexed and caged in groups of 5–6 mice of the same sex at postnatal day (PD) 21, and were then subjected to behavioural and biochemical analyses.

Behavioural analyses

In behavioural analyses, animals were divided to two experimental groups. Mice in group 1 were subjected to the open-field test (day 1), the elevated plus-maze test (day 2) and the social interaction test (day 3)

with a 1-d interval between each test. Mice in group 2 were subjected to only the water maze test (days 1–14). The open-field, elevated plus-maze, social interaction and water maze tests were performed at 08:00–20:00, 16:00–20:00, 10:00–14:00 and 10:00–16:00 hours, respectively. None of the mice were subjected to same behavioural test again, so the behaviours at ages 4 wk and 8 wk were evaluated using different animals.

For a full description of methods of behavioural analyses, see Supplementary Methods (available online).

Nissl staining

Nissl staining was performed as previously reported (Takuma *et al.* 2007). Briefly, after completion of the behavioural analyses, mice were deeply anaesthetized with pentobarbital and perfused intracardially with 4% paraformaldehyde (PFA) in 0.2 M phosphate-buffered solution (PBS). The brains were removed, post-fixed with the same fixative and cryoprotected with 15% sucrose-containing PBS. Sections (20 μm) containing somatosensory cortex, prefrontal cortex, striatum, amygdala and hippocampus were obtained using a cryostat (CM1510; Leica Microsystems GmbH, Germany), and mounted on slides. The sections were air-dried, stained with 0.1% Cresyl Violet solution for 5–10 min, and protected with a coverslip. Digitized images of the Nissl-stained sections were obtained with a fluoro phase-contrast microscope system (Biorevo BZ-9000, Keyence Co., Japan) using a 10 × magnification lens. Nissl-positive neuronal cell numbers were manually and rigidly counted within the prefrontal cortex (layers II/III and V), somatosensory cortex (layers II/III and IV–V), striatum amygdala and hippocampus of the scanned digital images. The total cell counts were averaged from at least three sections per animal.

BrdU birthdating and morphometric quantitation

Pregnant females received a single i.p. injection of BrdU (50 mg/kg) at embryonic day 12.5 (E12.5), E13.5 or E14.5. Offspring were anaesthetized and perfusion-fixed with 4% PFA on PD7. Cryosections (20 μm) containing prefrontal cortex and somatosensory cortex were treated with 2 N HCl for 30 min to retrieve epitopes, and immunostained with rat anti-BrdU (1:100), followed by biotin-conjugated goat anti-rat IgG (1:250) for 30 min at room temperature. Biotinylated secondary antibodies were detected using the ABC immunoperoxidase kit (Vector Laboratories Inc., USA). Digitized images of anatomically matched sections from each mouse were obtained using a Biorevo BZ-9000 microscope system (10 × magnification). BrdU-positive cells were counted within 500-μm-wide radial strips, subdivided into 10 bins of equal depth, of the somatosensory cortex from the pial surface. Histograms were expressed as average numbers ($\pm$ S.E.M.) of BrdU-labelled cells in each bin.

Measurement of apoptosis and proliferation in the embryonic brains

Pregnant mice were sacrificed at 12 or 24 h after a single drug injection, and embryos were removed from the uterus. For TUNEL staining as a marker of apoptosis, each embryo was embedded in a Tissue-Tek® Cryomold 1 (Miles Inc., USA) using an OCT Compound (Sakura Finetek Japan Co. Ltd, Japan), and snap-frozen on dry ice. Ultrathin cryosections (12 μm) containing the ganglionic eminence were obtained using a cryostat (CM1510), mounted on slides, fixed with 1% PFA at 20 °C for 30 min and treated with 1% Triton-X in 1% sodium citrate. The sections were incubated with TUNEL reaction mixture according to the manufacturer's instructions of the *In Situ* Cell Death Detection kit (Fluorescein), subsequently stained with 1 μg/ml DAPI solution, and mounted using a Fluorescent Mounting Medium (Dako). Digitized images were obtained using a Biorevo BZ-9000 microscope system (10 × magnification). Numbers of TUNEL-positive cells were summed throughout the neocortex of each embryo, and expressed as the means $\pm$ S.E.M. For Ki67 immunostaining as a marker of proliferation, the head of each embryo was fixed with 4% PFA and embedded using an OCT Compound. Ultrathin cryosections (12 μm) containing the ganglionic eminence were microwaved in 10 mM citrate buffer (pH 6.0) for 10 min to retrieve antigens, and then preincubated for 1 h in 5% goat serum in 0.3% Triton X-100 in 0.1 M PBS to block non-specific binding by antibodies. The sections were then incubated at 4 °C overnight with rat anti-Ki67 (1:50), followed by biotin-conjugated goat anti-rat IgG (1:250) for 30 min at room temperature. Biotinylated secondary antibodies were detected using the ABC immunoperoxidase kit (Vector Laboratories). Digitized images were obtained using a Biorevo BZ-9000 microscope system (4 × magnification).

Histone extraction and evaluation of histone acetylation

Histone extraction was performed according to the method reported previously (Chwang *et al.* 2007;

Levenson *et al.* 2004). Tissues were homogenized for 12 strokes using a Teflon glass homogenizer, and centrifuged at 7800 *g* for 1 min. The pellets containing the nuclear fraction were suspended in 1 ml of 0.4 N H_2SO_4, kept on ice for 30 min to extract histones, and centrifuged at 14 000 *g* for 10 min. The supernatants were mixed with 250 μl of TCA containing 4 mg/ml deoxycholic acid, kept on ice for 30 min, and centrifuged at 14 000 *g* for 30 min. The pellets were washed twice with 1 ml of acidified acetone (0.1% HCl) for 5 min and then pure acetone for 5 min. The final resulting protein pellets were suspended in 10 mM Tris (pH 8.0). Protein concentrations were determined using BCA™ protein assay reagents (Thermo Fisher Scientific). Adequate amounts of protein were separated by SDS–polyacrylamide gel electrophoresis (proteins, gel H3 analysis: 3 μg/lane, 15%; H4 analysis: 1 μg/lane, 12%) and then transferred electrophoretically to a hydrophobic polyvinylidene fluoride (PVDF) membrane. The blotted membranes were blocked for 2 h in 5% non-fat skim milk/TBS-T (20 mM Tris–HCl, 137 mM NaCl, and 0.1% Tween-20; pH 7.6), and incubated overnight at 4 °C with primary antibodies against anti-histone H3 (1:2000) or anti-histone H4 (1:1000), followed by HRP-conjugated anti-rabbit IgG (1:20 000). The immune complexes were visualized using Amersham™ ECL™ Plus Western Blotting Detection Reagents (GE Healthcare Bio-Sciences). Then, the membranes were stripped for 30 min at room temperature in Restore™ Plus Western Blot Stripping Buffer (Thermo Fisher Scientific) and re-probed with the primary antibodies against the respective acetylated histones, anti-acetyl-histone H3 (1:100 000) and anti-acetyl-histone H4 (1:90 000), overnight, followed by HRP-conjugated anti-rabbit IgG (1:20 000). The immune complexes were visualized using Amersham ECL Western Blotting Detection Reagents (GE Healthcare Bio-Sciences) and quantified using a light-capture cooled CCD camera system for bio/chemiluminescence detection (AE-6981; Atto Co., Japan).

Statistics

Statistical analysis of the experimental data was performed using Prism 5 for Mac OS X (GraphPad Software, USA). The significance of differences was determined by one-way ANOVA, followed by Dunnett's *post-hoc* test, and two-way repeated-measures ANOVA for multifactorial comparisons. Unpaired *t* test was used for two-group comparisons. The criterion for statistical significance was $p < 0.05$.

Results

Effect of prenatal exposure to VPA or VPD on affective-like behaviours of offspring

Prenatal exposure to VPA is teratogenic (Hrubec *et al.* 2006) and is also associated with neurodevelopmental deficits, relevant to autism and mental retardation, in laboratory animals (Markram *et al.* 2008). In this study, we examined whether mice prenatally exposed to VPA show autistic-like behaviours. When VPA was injected to pregnant mice at E9, a number of deformed pups were observed and the other normal remnants never displayed behavioural abnormalities until age 8 wk (S. Kataoka, unpublished observations). In contrast, pregnant mice that received a VPA injection at E12.5 and E14.5 did not have deformed pups. The present study demonstrated that male offspring exposed to VPA at E12.5 (Fig. 1), but not E14.5 (Supplementary Fig. S1, online), displayed different performances in the open-field, social interaction, and elevated plus-maze tests, compared to saline-treated controls, at adolescence. Open-field test revealed that E12.5 exposure to VPA reduced locomotor activity (Fig. 1*a*), rearing (Fig. 1*b*) and number of entries into the centre field (Fig. 1*c*) in mice aged 4 and 8 wk. In addition, decrease in the time spent in the open arms (Fig. 1*d*), the numbers of open-arm entries (Supplementary Fig. S2*a*), the number of closed-arm entries (Supplementary Fig. S2*b*), the ratio of open-arm entries (Supplementary Fig. S2*c*), and increase in the time spent in closed arms (Fig. 1*e*) were observed in 8-wk-old mice exposed to VPA at E12.5 in the elevated plus-maze test. There was no difference in locomotor activity during the initial 10 min at age 8 wk [distance travelled, saline: 38.14 ± 2.79 m ($n = 15$), VPA: 33.14 ± 1.63 m ($n = 19$), $t = 1.622$, d.f. $= 32$, $p = 0.1145$, by unpaired *t* test]. In the social interaction test, E12.5 exposure to VPA reduced duration of sniffing (Fig. 1*f*) at ages 4–8 wk without affecting allogrooming and aggression (data not shown). Furthermore, male offspring exposed to VPD, a VPA analog lacking HDAC inhibitory activity, at E12.5 did not display abnormal affective-like behaviours (Fig. 1*a–f*) as seen in animals exposed to VPA.

A sex difference in the incidence of autism as large as four males to one female has been reported (Coleman, 1978; Wing & wing, 1976). Thus, in order to specify whether there is a sex difference in the behavioural effect of prenatal exposure to VPA, we analysed the performance of 8-wk-old female offspring exposed to VPA at E12.5 in the open-field, elevated plus-maze and social interaction tests (Supplementary Fig. S3). Female offspring exposed to VPA at E12.5 displayed
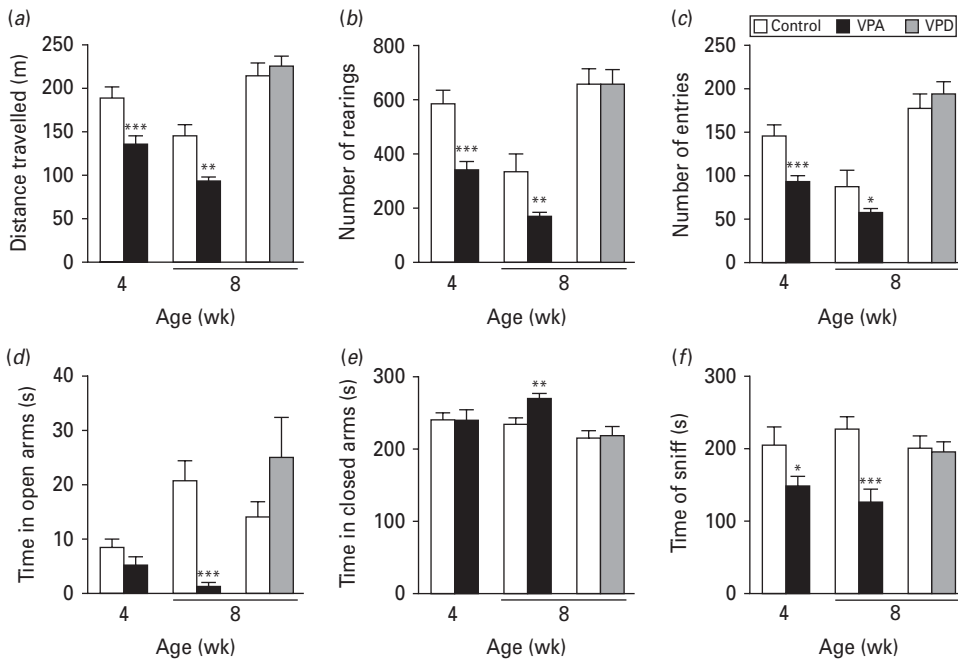
Fig. 1. Prenatal exposure to valproic acid (VPA), but not valpromide (VPD), at embryonic day 12.5 (E12.5) causes abnormal performance of male mice in the social interaction, elevated plus-maze and open-field tests. Male offspring born to mothers treated with VPA (500 mg/kg i.p.), VPD (150 mg/kg s.c.), saline or vehicle at E12.5 were subjected to behavioural analysis at ages 4 and 8 wk. (*a–c*) Open-field test. VPA-exposed offspring displayed significantly less travelling (*a*), rearing (*b*) and entries into the centre field (*c*) than saline-treated controls during a 90-min test session. (*d, e*) Elevated plus-maze test. VPA-exposed offspring spent significantly less time in the open arms (*d*), and more time in the closed arms (*e*) than saline-treated controls during a 5-min test session at age 8 wk. (*f*) Social interaction test. VPA-exposed offspring displayed significantly less time face sniffing to an age-matched intruder mouse than saline-treated controls during a 20-min test session. Values represent means $\pm$ s.e.m. [(*a–c*) 4 wk, control, $n=15$; VPA, $n=19$; 8 wk, control, $n=15$; VPA, $n=19$; (*d, e*) 4 wk, control, $n=9$; VPA, $n=11$; 8 wk, control, $n=14$; VPA, $n=18$; (*f*) 4 wk, control, $n=8$; VPA, $n=10$; 8 wk, control, $n=9$; VPA, $n=8$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs.* control (unpaired *t* test).

similar behavioural changes to males in both open-field (Supplementary Fig. S3*a–c*) and elevated plus-maze tests (Supplementary Fig. S3*d, e*), while there was an increase in duration of sniffing in female offspring in the social interaction test (Supplementary Fig. S3*f*).

Figure 2 shows the effect of prenatal VPA exposure on learning ability in mice using the Morris water-maze task. Both groups (mice prenatally exposed to VPA or saline) showed a progressive decline in escape latency during training trials on days 1–6 (Fig. 2*a*, two-way repeated-measures ANOVA: $F_{5,145}=10.22$, $p<0.0001$). Two-way repeated-measures ANOVA also revealed a significant overall effect of VPA treatment on escape latency ($F_{1,29}=7.62$, $p<0.01$), whereas the interaction between drug treatment and training day was not significant ($F_{5,145}=1.95$, $p=0.0903$). In contrast, differences were not observed between groups on any swim parameter, such as swim speed and distance swum to platform (Supplementary Table S1). On day 7, the animals were subjected to the probe test and

then to the visible test. In the probe test (Fig. 2*b*), saline-exposed mice swam preferentially in the T quadrant, and there was a significant difference in time spent between four quadrants (one-way ANOVA: $F_{3,48}=13.60$, $p<0.0001$). In contrast, there was no significant difference in time spent between four quadrants in the VPA-exposed mice (one-way ANOVA: $F_{3,68}=6.842$, $p=0.3851$). In the visible test performed after the probe test, there was no significant difference in the escape latency between mice pre-natally exposed to VPA and saline (Supplementary Table S1). In the probe and visible test, differences were not observed between groups on swim speed and distance swum (Supplementary Table S1).

To determine that the decrease in number of target location crossings in mice exposed to VPA at E12.5 during the probe trial was due to leaning disability or perseveration to specific place, we reversed the location of the platform and retrained the same cohort of mice (so-called reversal training). Similar to normal training, mice prenatally exposed to VPA and saline
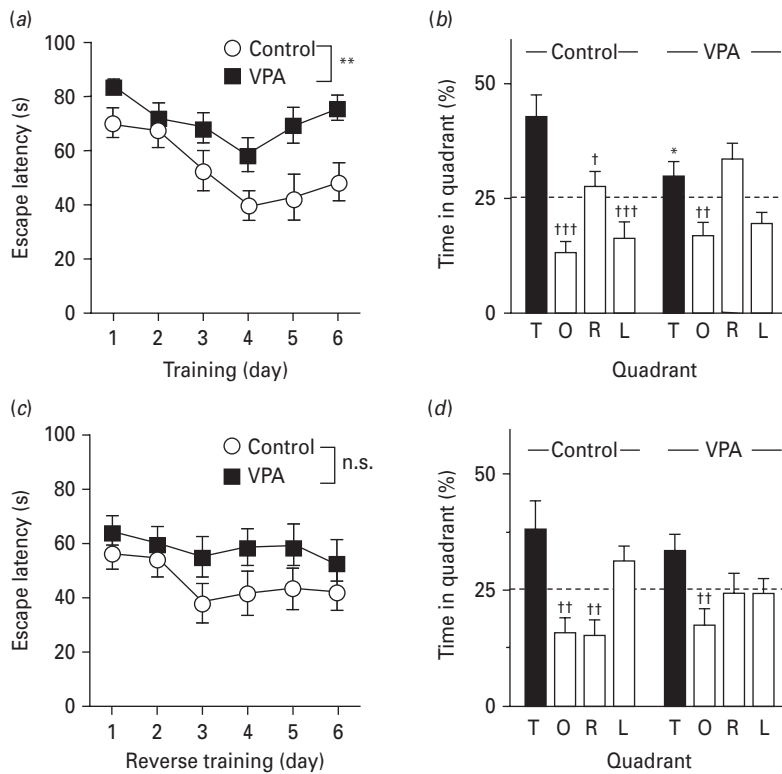
Fig. 2. Prenatal exposure to valproic acid (VPA) at embryonic day 12.5 (E12.5) causes spatial learning and memory deficits in the water maze test. Offspring born to mothers treated i.p. with VPA (500 mg/kg) or saline at E12.5 were subjected to behavioural analysis at age 8 wk. (*a*) Acquisition profiles showing escape latency during training trials (days 1–6). Mice were subjected for 6 d to three swim sessions per day, with a 10-min inter-trial time interval. Data are shown in terms of median duration of three swim sessions to find the hidden platform. (*b*) Probe test performances. The platform was removed and animals were submitted to a 60-s swim 24 h after the final training trial. Time spent in each quadrant was measured. (*c*) Acquisition profiles showing escape latency during reverse training trial (days 8–13). Mice were first subjected to 6 d training followed by the probe test on day 7, as described above. On day 8, the platform was moved to the opposite quadrant, and the mice were subjected for 6 d to three swim sessions per day, with a 10-min inter-trial time interval, in order to learn the new location of the platform. Data are shown in terms of median duration of three swim sessions to find the hidden platform. (*d*) Probe test performances after reverse training. The platform was removed and animals were subjected to a 60-s swim 24 h after the final training trial. Time spent in each quadrant was measured. Quadrants: T, training; L, adjacent left; O, opposite; R, adjacent right. Values represent means ± S.E.M. (control: $n = 13$; VPA: $n = 18$). ** $p < 0.01$ *vs.* control (*a*: two-way repeated-measures ANOVA). * $p < 0.05$ *vs.* control (*b*: unpaired *t* test), [†] $p < 0.05$, [††] $p < 0.01$, [†††] $p < 0.001$ *vs.* training quadrant of each group (*b*: Dunnett's multiple comparison test).

showed a progressive decline in escape latency during reverse training trials on days 8–13 (Fig. 2*c*, two-way repeated-measures ANOVA: $F_{5,145} = 3.89$, $p < 0.01$). However, there was no difference in the effect of drug treatment and the interaction between drug treatment and training day (two-way repeated-measures ANOVA, drug: $F_{1,29} = 1.83$, $p = 0.1865$; drug × day: $F_{5,145} = 0.79$, $p = 0.5611$). In the probe test on day 7 (Fig. 2*d*), both saline- and VPA-exposed mice swam preferentially in the T quadrant, and there was a significant difference in time spent between the four quadrants (one-way ANOVA, saline: $F_{3,48} = 7.124$, $p < 0.001$; VPA: $F_{3,68} = 3.366$, $p < 0.05$).

Effect of prenatal exposure to VPA at E12.5 on the number of neuronal cells in the neocortex

The adult mammalian cerebral cortex has a six-layered laminar structure, which is built by migration of successively produced projection neurons in an 'inside-out' fashion, such that early-born neurons form in the deep cortical layer and late-born neurons migrate into the superficial layer (Molyneaux *et al.* 2007). In addition, the birthdate-labelling study using adenovirus vectors indicates that neurons born on E12.5 are distributed mainly around layer V in the mouse somatosensory cortex (Hashimoto & Mikoshiba 2004;
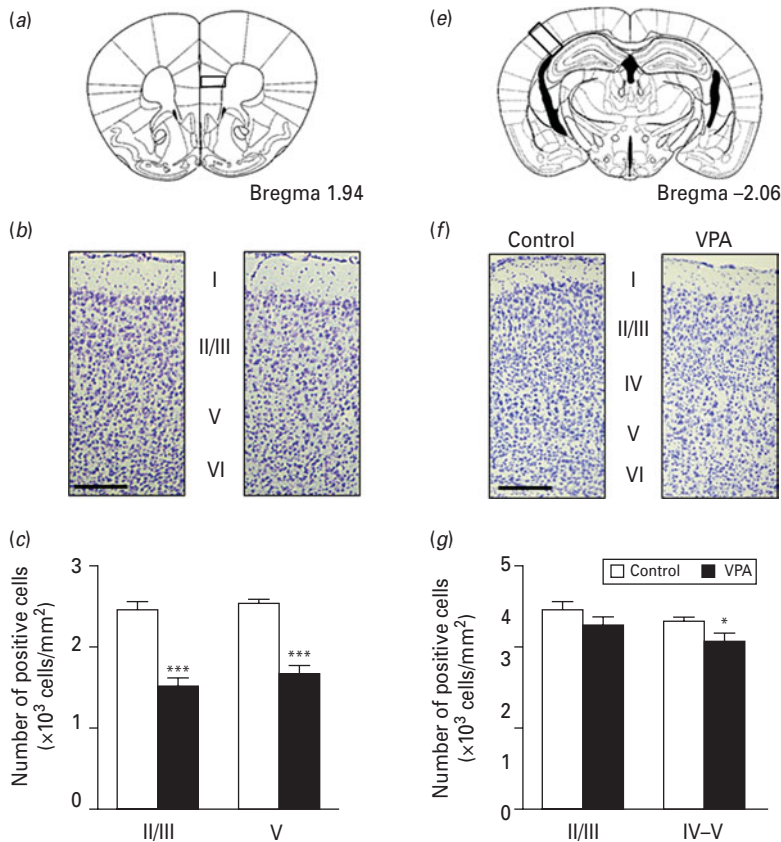
Fig. 3. Prenatal exposure to valproic acid (VPA) at embryonic day 12.5 (E12.5) causes histological changes in the prefrontal cortex and somatosensory cortex of mice. Offspring born to mothers treated i.p. with VPA (500 mg/kg) or saline at E12.5 were subjected to histochemical analysis at age 8 wk. Nissl-positive cells were visualized by Cresyl Violet staining. (*a*, *d*) Schematic diagrams of brain sections adapted from the mouse brain atlas (Franklin & Paxinos, 2001) (*b*, *e*) Typical microscopic images of the Nissl-stained prefrontal cortex (*b*) and somatosensory cortex (*e*) from VPA-exposed (*right*) and saline-treated (*left*) offspring. Scale bar, 200 μm. (*c*, *f*) Numbers of Nissl-positive cells in layers II/III and IV–V of the prefrontal cortex and somatosensory cortex (layers II–III and V of the prefrontal cortex, and layers II/III and IV–V of the somatosensory cortex). Values represent means $\pm$ S.E.M. ($n = 4$). * $p < 0.05$, *** $p < 0.001$ *vs.* saline (unpaired *t* test).

Molyneaux *et al.* 2007). The present study examined whether prenatal VPA exposure could induce morphological changes in the neocortex by a Nissl-staining method (Fig. 3). Microscopic images of the prefrontal cortex (Fig. 3*b*) and somatosensory cortex (Fig. 3*e*) showed that prenatal VPA exposure at E12.5 caused neuronal cell loss in the middle and lower layers of the prefrontal cortex, and in the lower layer of the somatosensory cortex without affecting the thickness of each layer at age 8 wk. Figure 3(*c*, *f*) shows the number of Nissl-positive cells in layers II–III and V of the prefrontal cortex and II/III and IV–V of the somatosensory cortex, respectively. Early prenatal VPA exposure (E12.5) significantly decreased the number of Nissl-positive cells in layers II–III and V of the prefrontal cortex (Fig. 3*c*) and in layers IV–V of the somatosensory cortex, but not in layers II/III (Fig. 3*f*).

In contrast, early prenatal VPA exposure (E12.5) did not affect the number of Nissl-positive cells in the striatum, amygdala and hippocampus (Supplementary Fig. S5). Furthermore, we found that late prenatal VPA exposure (E14.5) had no effect on the number of Nissl-positive cells in the somatosensory cortex (Supplementary Fig. S4). Figures 4 and 5 show the effect of early prenatal VPA exposure (E12.5) on the distribution of newborn cells generated from E12.5 to E14.5 in the prefrontal cortex (Fig. 4) and somatosensory cortex (Fig. 5) of 7-d-old mice. The BrdU birthdate-labelling method showed a successful 'inside-out' distribution of BrdU birthdate-labelled cells in 10-bin-sectioned immunostaining images from both saline- and VPA-exposed mice (Figs 4*a*–*c*, 5*a*–*c*). Prenatal VPA exposure (E12.5) significantly decreased the number of BrdU-positive cells
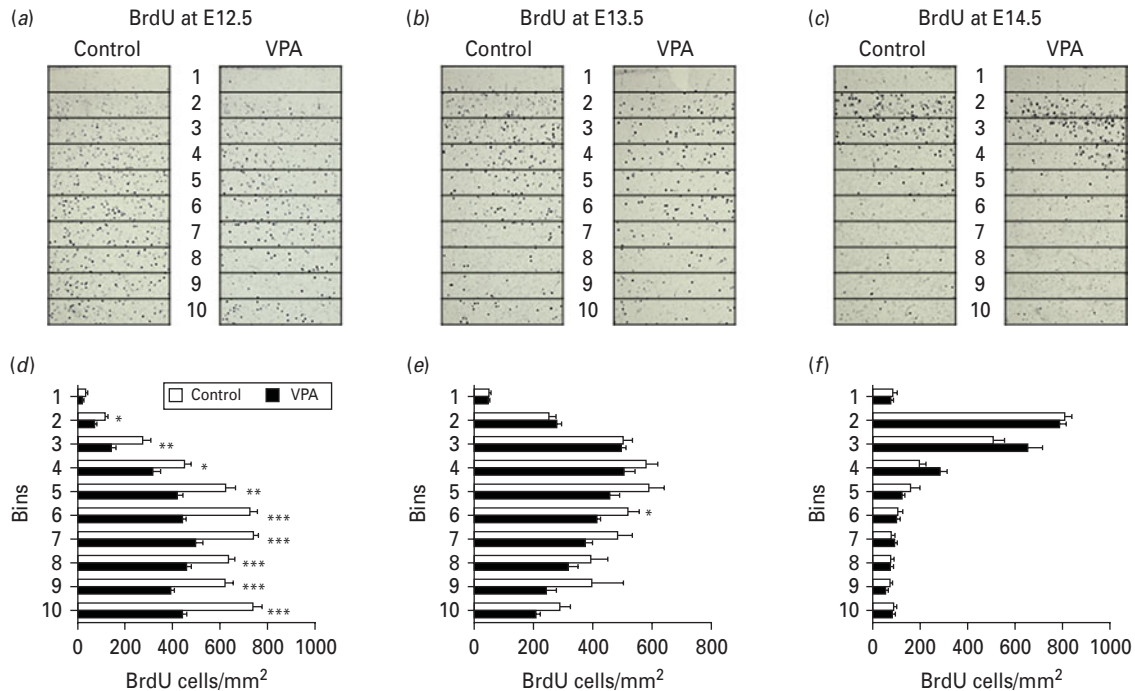
Fig. 4. Prenatal exposure to valproic acid (VPA) at embryonic day 12.5 (E12.5) impairs migration into the prefrontal cortex of cells born between E12.5 and E13.5 in mice. Offspring born to mothers treated i.p. with VPA (500 mg/kg) or saline at E12.5 were subjected to immunohistochemical analysis at age 7 d. Newborn cells were labelled by injecting BrdU into pregnant mice at E12.5 (*a, d*), E13.5 (*b, e*) and E14.5 (*c, f*), and traced by an anti-BrdU ABC immunoperoxidase method. (*a–c*) Typical microscopic images of BrdU-positive cells in the somatosensory cortex (500 μm wide) from VPA-exposed (*right*) and saline-treated (*left*) offspring. Numbers 1–10 indicate bins of equal depth spanning the thickness of the cortex. (*d–f*) Numbers of BrdU-positive cells born in each bin of the prefrontal cortex. Values represent means $\pm$ s.e.m. [(*d*) $n = 7$, (*e*) $n = 7$, (*f*) $n = 7$]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* saline (unpaired t test).

compared to saline-treated controls in bin nos. 2–10 (a BrdU pulse at E12.5) (Fig. 4*d*) and bin no. 6 (a BrdU pulse at E13.5) (Fig. 4*e*) of the prefrontal cortex, and in bin nos. 8–10 (a BrdU pulse at E12.5) (Fig. 5*d*) and bin nos. 4, 5 and 8 (a BrdU pulse at E13.5) (Fig. 5*e*) of the somatosensory cortex. In contrast, prenatal VPA at E12.5 did not affect the distribution of E14.5 birthdate-labelled cells in either of the cortical areas (Figs 4*f*, 5*f*).

Effect of prenatal exposure to VPA or VPD at E12.5 on apoptotic-like cell death in the neocortex and neuronal proliferation in the ganglionic eminence

With respect to prenatal VPA exposure (E12.5)-mediated neuronal loss, we examined whether exposure to VPA stimulates cell death or inhibits cell proliferation. TUNEL staining showed that a pre-eminent expression of apoptotic-like cell death was observed in VPA-treated mice, whereas few apoptotic-featured cells were seen in saline-treated controls (Fig. 6*a–e*, Supplementary Fig. S6*a–d*). The effect of prenatal VPA exposure (E12.5) on the expression of TUNEL-positive

cells was more evident at 12 h than at 24 h, suggesting a transient effect. The studies on cell proliferation by Ki67-immunostained histochemical analysis are shown in Fig. 6(*f, g*), and in Supplementary Fig. S6(*e, f*). Although strong signals of Ki67 were detected in the neocortex and ganglionic eminence of embryonic brains prepared at 12 h after saline and VPA injections, the signal intensity of the VPA-exposed group was weaker in the ganglionic eminence (Fig. 6*g*) than that of the saline-treated controls (Fig. 6*f*). Such a difference between saline- and VPA-exposed embryonic brains disappeared at 24 h after drug injection (Supplementary Fig. S6*e, f*). In addition, we found that late prenatal VPA exposure (E14.5) did not affect TUNEL-positive cell numbers of apoptosis in the neocortex (Supplementary Fig. S5*d, h*) and immunostaining intensity against Ki67 in the ganglionic eminence (Supplementary Fig. S6*j, l*). In contrast to VPA, VPD exposure could neither induce apoptotic-like cell death in the neocortex (Supplementary Fig. S8*d, h*) nor decrease neuronal proliferation in the ganglionic eminence (Supplementary Fig. S8*j, l*).
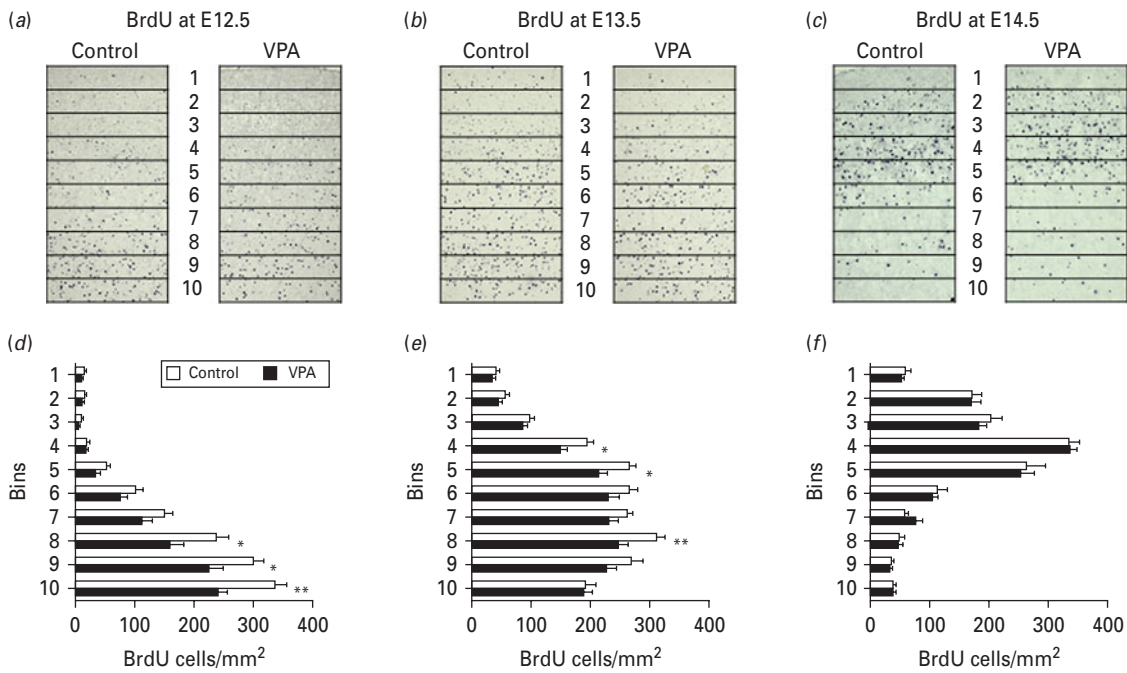
Fig. 5. Prenatal exposure to valproic acid (VPA) at embryonic day 12.5 (E12.5) impairs migration into the somatosensory cortex of cells born between E12.5 and E13.5 in mice. Offspring born to mothers treated i.p. with VPA (500 mg/kg) or saline at E12.5 were subjected to immunohistochemical analysis at age 7 d. Newborn cells were labelled by injecting BrdU into pregnant mice at E12.5 (*a, d*), E13.5 (*b, e*) and E14.5 (*c, f*), and traced by an anti-BrdU ABC immunoperoxidase method. (*a–c*) Typical microscopic images of BrdU-positive cells in the somatosensory cortex (500 μm wide) from VPA-exposed (*right*) and saline-treated (*left*) offspring. Numbers 1–10 indicate bins of equal depth spanning the thickness of the cortex. (*d–f*) Numbers of BrdU-positive cells born in each bin of the somatosensory cortex. Values represent means $\pm$ S.E.M. [(*d*) control: $n = 7$; VPA: $n = 9$; (*e*) control: $n = 8$; VPA: $n = 9$; (*f*) control: $n = 7$; VPA: $n = 9$]. * $p < 0.05$, ** $p < 0.01$ *vs.* saline (unpaired *t* test).

Effect of prenatal exposure to VPA or VPD at E12.5 on histone hyperacetylation

In view of the recent observation that VPA can induce tumour cell death via HDAC inhibition (Hrebackova *et al.* 2010), we investigated the effect of prenatal VPA exposure on acetylated histone levels in whole tissue lysates of embryonic brains prepared from pregnant mice with a drug injection at E12.5 (Fig. 7*a, b*). Prenatal VPA exposure increased the acetylated levels of both histones H3 and H4 immediately after VPA exposure for up to 6 h, although it did not affect the total levels. In contrast, VPD did not affect the acetylated levels of histones H3/4 at 2 h after drug exposure (Fig. 7*c*).

Discussion

The present study demonstrates that male mice prenatally exposed to VPA at E12.5, but not at E9 and E14.5, express abnormal behaviours at adolescence and may be useful as an animal model of autism. The mice exhibited behavioural alterations and histochemical changes in the middle and lower layers of the prefrontal cortex and in the lower layers of the somatosensory cortex. They showed a transient increase in acetylated levels of histones H3 and H4, an increase in TUNEL-positive cell numbers and a decrease in cell proliferation in the embryonic brain. In contrast to VPA, prenatal exposure to VPD, a VPA analog lacking HDAC inhibition activity, did not cause any behavioural, histological and biochemical changes.

VPA dosing during pregnancy results in an increased risk of ASD (Moore *et al.* 2000), and rodents prenatally exposed to VPA showed behavioural alterations similar to those in human with autism (Ingram *et al.* 2000). In this study, we used 500 mg/kg VPA to induce autistic-like behaviours in mice, referring to a previous study of the autistic rat model (Markram *et al.* 2008). Similar to the rat model (Kim *et al.* 2011), we found that mouse offspring prenatally exposed to VPA at E12.5, but not at E9 and E14.5, displayed social interaction deficits and anxiety-like behaviour at age 4–8 weeks. The present observation that prenatal VPA exposure impaired acquisition of mice without affecting motor activity and visual performance in the Morris water-maze task is in agreement
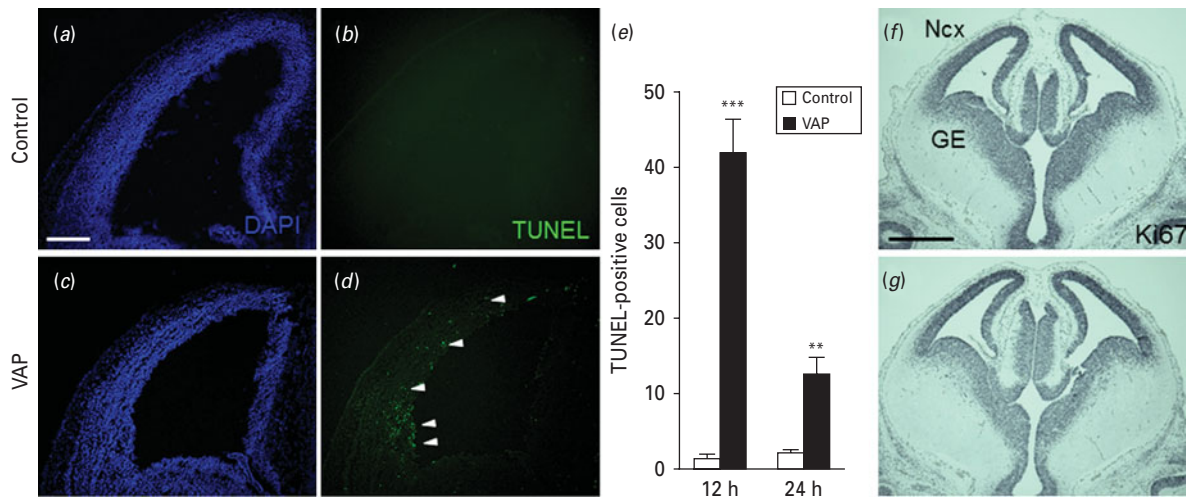
Fig. 6. Prenatal exposure to valproic acid (VPA) at embryonic day 12.5 (E12.5) increases apoptosis and reduces cell proliferation in the mouse embryonic brain. Embryos obtained from mothers treated i.p. with VPA (500 mg/kg) or saline at E12.5 were subjected to immunohistochemical analysis at 12 and 24 h after drug treatment. (*a–d*) Typical fluoromicroscopic images stained by DAPI (blue) and TUNEL reaction mixtures (green) of brain sections including the neocortex from VPA-exposed (*c, d*) and saline-treated (*a, b*) embryos at 12 h after drug exposure. Scale bar, 200 μm. (*e*) Numbers of TUNEL-positive cells in the neocortex. Values represent means $\pm$ S.E.M. (12 h, control, $n=3$; VPA, $n=4$; 24 h, control, $n=4$; VPA, $n=4$). ** $p<0.01$, *** $p<0.001$ *vs.* saline (unpaired *t* test). (*f, g*) Typical microscopic stain by anti-Ki67 of coronal brain sections from VPA-exposed (*g*) and saline-treated (*f*) embryos at 12 h after drug exposure. Ncx, Neocortex; GE, ganglionic eminence. Scale bar, 500 μm.

with the previous finding in BALB/c mice (Wagner *et al.* 2006), although there is a discrepant report in rats that that prenatal VPA exposure did not affect the spatial learning performance (Markram *et al.* 2008). As regards the memory deficits, we have also observed cognitive impairment in mice exposed to VPA at E12.5 in a novel object recognition test (S. Kataoka, unpublished observations). Furthermore, this study shows that there is a sex difference in the effect of prenatal VPA exposure: VPA causes a decrease in sniffing behaviour in male, but not in female, mice in the social interaction test. This finding may be in agreement with the apparent significant higher risk of ASD in males. Taken together, it is likely that mouse offspring prenatally exposed to VPA at E12.5 display autistic-like behavioural phenotypes, which are observed in genetic mouse models of the disease (Moy *et al.* 2006; Silverman *et al.* 2010). However, mice prenatally exposed to VPA at E12.5 exhibit decreased locomotor activity, in contrast to previous rat studies (Schneider & Przewłocki, 2005; Schneider *et al.* 2006).

In mice, neurogenesis begins around E12.5 (Shinozaki *et al.* 2002) and neurons born on E12.5 mainly migrate into layer V in the neocortex (Hashimoto & Mikoshiba, 2004; Molyneaux *et al.* 2007). The present study demonstrates that prenatal VPA exposure at E12.5, but not at E14.5, significantly decreased the neuronal cell numbers in layers II–III

and V of the prefrontal cortex and in layers IV–V of the somatosensory cortex at age 8 wk. This effect was specific to these brain regions, since VPA exposure did not affect the number of Nissl-positive cells in the striatum, amygdala and hippocampus. Furthermore, we revealed that VPA exposure at E12.5 caused less localization of BrdU-labelled cells born on E12.5 and E13.5 in the lower and middle layers, respectively, at PD7. These observations suggest that VPA exposure at E12.5 reduces migration of newborn neurons generated between E12.5 and E13.5 and results in the neuronal loss in layers IV–V of the somatosensory cortex. Since the prefrontal cortex and somatosensory cortex are highly implicated in social behaviours (Adolphs, 2001; Bechara *et al.* 2000), it is likely that the alteration may lead to behavioural alterations in mice prenatally exposed to VPA at E12.5, but not at E9 and E14.5.

It is generally thought that neuronal loss is due to stimulation of cell death or inhibition of cell proliferation (Mantamadiotis *et al.* 2002; Wang *et al.* 2003). Previous studies have showed both pro-apoptotic and anti-proliferative properties of VPA in various cells (Blaheta & Cinatl, 2002; Shabbeer *et al.* 2007). In cells of the CNS, VPA has been reported to reduce the proliferation of neuroblastoma cells (Rocchi *et al.* 2005) and neural progenitor cells (Jung *et al.* 2008), and induce apoptosis in microglial cells (Dragunow *et al.*
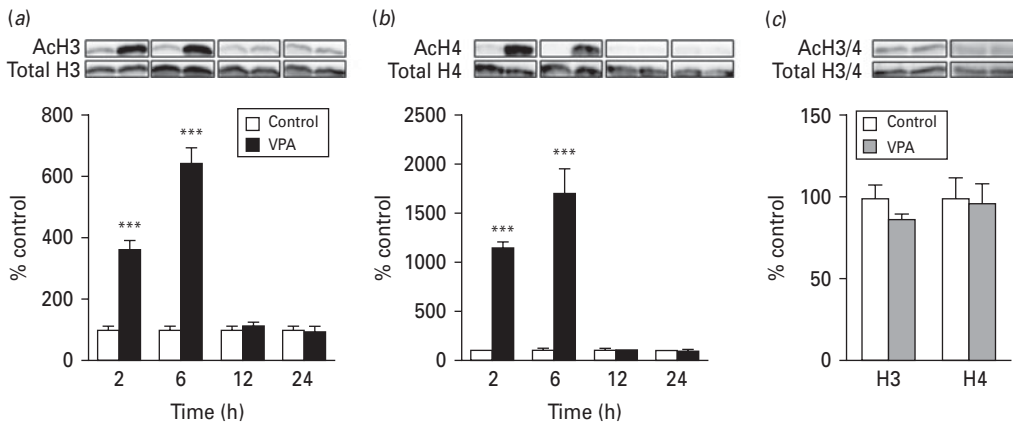
Fig. 7. Prenatal exposure to valproic acid (VPA) at embryonic day 12.5 (E12.5) rapidly increases acetylated levels of histones H3 and H4 in the mouse embryonic brain. Embryos obtained from mothers treated with VPA (500 mg/kg i.p.) (*a*, *b*), valpromide (VPD) (150 mg/kg s.c.) (*c*), saline (i.p.) (*a*, *b*) or vehicle (s.c) (*c*) at E12.5 were subjected to biochemical analysis at the indicated times (*a*, *b*) or 6 h (*c*) after drug treatment. Whole brains from embryos exposed to the indicated drugs were lysed and subjected to SDS–PAGE. Each panel consists of typical immunoblot images detected by antibodies against acetylated (upper) and total histones (middle), and quantification of acetylated levels of histones H3 and H4, which were normalized by total levels of the respective histones and expressed as % of saline- or vehicle-treated control. (*a*) Change in acetylated levels of histone H3 after VPA exposure. (*b*) Change in acetylated levels of histone H4 after VPA exposure. (*c*) Change in acetylated levels of histones H3 and H4 after VPD exposure. Values represent means ± S.E.M. ($n = 4$). *** $p < 0.001$ *vs.* saline (unpaired *t* test).

2006). The present study revealed that VPA exposure remarkably induced apoptotic-like cell death in the embryonic neocortex at the peak level within 24 h after drug administration to pregnant mice. In addition to the VPA-mediated apoptotic phenomenon, we observed that VPA exposure also immediately reduced cell proliferation in the ganglionic eminence of the embryonic brain. In contrast, VPA exposure at E14.5 had no effect on cell death and proliferation. Since neurons generated in the neocortex and ganglionic eminence finally differentiate into excitatory and inhibitory neurons, respectively, and migrate to cortical cell layers (Lee *et al.* 2011; Marin & Rubenstein, 2001), it is likely that the VPA-induced neuronal loss in the lower layers of the somatosensory cortex is due to both apoptosis and reduced proliferation of newly generating neuronal cells during early embryonic brain development, especially around E12.5. A variety of processes are known to be involved in apoptosis and cell proliferation. Therefore, further studies are required to address how VPA administration can activate apoptotic cell death pathways or inhibit proliferation signals.

Hrebackova *et al.* (2010) have suggested that VPA-induced histone hyperacetylation may be a possible mechanism for tumour cell death. In addition, several studies have proposed that the HDAC inhibitory action of VPA is closely related to teratogenesis (Downing *et al.* 2010; Tung & Winn, 2010). However,

to date, there is no study about the involvement of HDAC inhibition in VPA-induced ASD. The present study indicates that the transient hyperacetylation of histones H3 and H4, which is considered as a consequence of HDAC inhibition, occurred in the embryonic brain immediately after VPA exposure. To better clarify the role of HDAC inhibition in VPA-induced autistic-like behaviours in mice, we examined the effect of prenatal exposure to VPD (150 mg/kg s.c.), which exerts an equivalent pharmacological effect to VPA without affecting HDAC activity (Eikel *et al.* 2006; Nishikawa & Scatton, 1985), on behavioural performance of offspring. We found that prenatal exposure to VPD at E12.5 failed to exert such hyperacetylation of histones. We also found that prenatal VPD exposure did not cause any change, such as behavioural alterations and embryonic brain developmental impairment, observed in mice prenatally exposed to VPA. These observations suggest that the transient HDAC inhibition during the critical period of embryonic brain development plays a fundamental role in the pathogenesis of ASD in offspring exposed to VPA. On the other hand, Lagger *et al.* (2002) found that the homologous class I HDACs (HDAC1, HDAC2, HDAC3) are highly expressed from mid to late gestation, at the peak around E12.5. The finding may give us a reasonable mechanistic explanation of why the expression of autistic-like behaviours is limited in mice prenatally exposed to VPA at E12.5, and it

supports our idea that HDAC inhibition during the critical period of embryonic brain development plays a key role in the aetiology of ASD.

In conclusion, this study demonstrates that in mice, prenatal VPA exposure at E12.5 causes social interaction deficits, anxiety-like behaviour, learning deficits and decreases in neocortical neuron density, similar to those observed in humans with autism. Furthermore, it is likely that these changes may be mediated by inhibition of class I and/or IIb HDACs in a specific embryonic period. This suggestion is supported by the comparative study with VPA and VPD. However, the effect of HDAC activity was transient, while the behavioural phenotypes and some of the histological finding were postnatal. Although further studies are required to clarify how HDAC inhibition causes anatomical and behavioural changes, the present study implies that dysfunction of HDAC during pregnancy may be responsible for a higher risk of ASD in off-spring.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org/pnp).

Statement of Interest

None.

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