Pentyl-4-yn-VPA, a histone deacetylase inhibitor, ameliorates deficits in social behavior and cognition in a rodent model of autism spectrum disorders.

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ABSTRACT

In utero exposure of rodents to valproic acid (VPA) has been proposed to induce an adult phenotype with behavioural characteristics reminiscent of those observed in autism spectrum disorder (ASD). Our previous studies have demonstrated the social cognition deficits observed in this model, a major core symptom of ASD, to be ameliorated following chronic administration of histone deacetylase (HDAC) inhibitors. Using this model, we now demonstrate pentyl-4-yn-VPA, an analogue of valproate and HDAC inhibitor, to significantly ameliorate deficits in social cognition as measured using the social approach avoidance paradigm as an indicator of social reciprocity and spatial learning to interrogate dorsal stream cognitive processing. The effects obtained with pentyl-4-yn-VPA were found to be similar to those obtained with SAHA, a pan-specific HDAC inhibitor. Histones isolated from the cerebellar cortex and immunoblotted with antibodies recognising lysine-specific modification revealed SAHA and pentyl-4-yn-VPA to enhance the acetylation status of H4K8. Additionally, the action of pentyl-4-yn-VPA, could be differentiated from that of SAHA by its ability to decrease H3K9 acetylation and enhance H3K14 acetylation. The histone modifications mediated by pentyl-4-yn-VPA are suggested to act cooperatively through differential acetylation of the promoter and transcription regions of active genes.

KEY WORDS

Social cognition; SAHA; VPA; H3K9ac.

1. INTRODUCTION

Autism spectrum disorder (ASD) is a pervasive developmental disorder that arises in approximately 1% of the world-wide population during the first three years of life. ASD comprises a heterogenous group of conditions characterised by impairments in reciprocal social interactions and the presence of restricted and repetitive and stereotyped behaviours (Fombonne, 2009; DSM-V; American Psychiatric Association, 2013). Individuals with ASD vary greatly in cognitive development and the precise nature of this phenotype remains to be defined (Charman et al., 2011).

Although a heritable condition (Rosenberg et al., 2009), the precise etiology of ASD remains poorly defined. Genetic loss of function of the gene encoding methyl CpG binding protein 2 (*MECP2*) gene, which is observed in Rett's syndrome, modifies transcriptional control by preventing its interaction with methylated DNA and histone deacetylases (HDACs), specifically HDAC1 and HDAC2 (Amir et al., 1999). Environmental factors, such as teratogen exposure in first trimester, including exposure to the valproate HDAC inhibitor (Eikel et al., 2006), have also been associated with a significant risk of developing ASD (Rasalam et al., 2005). These gene x environment interactions have been suggested to give rise to abnormal synapse remodelling and the developmental emergence of ASD, in part, through impaired cell adhesion molecule function (Zoghbi, 2003; Wang et al., 2009; Foley et al., 2012). Thus the resulting neuroanatomical malformations (Courchesne et al., 2011) inexorably lead to a spectrum of malfunctions, sometimes generally referred to as developmental disconnection syndromes (Geschwind and Levitt, 2007; Shi et al., 2013).

Previously, we have demonstrated chronic HDAC inhibition, with either pan-specific or HDAC1-3 isoform-specific inhibitors, to significantly ameliorate deficits in both social interaction and cognition in Wistar rats with prior *in utero* exposure to the valproate (VPA) teratogen (Foley et al., 2012). Further, in this rodent model of ASD, HDAC inhibition restored the ability of the neural cell adhesion molecule (NCAM) to increase its polysialylation (PSA) state, an event believed necessary for the synapse restructuring involved in the effective consolidation of spatial learning (Murphy et al., 1996; Seymour et al., 2008).

Surprisingly, this study failed to demonstrate any ameliorative effect of VPA on the social interaction and cognition deficits in this ASD model despite its well-established HDAC inhibitory actions and, as a consequence, we determined the effect of its analogue of 2-*n*-pentyl-4-pentynoic acid (pentyl-4-yn-VPA) on ameliorating the social interaction and spatial learning deficits observed in rats with prior *in utero* exposure to VPA. The pentyl-4-yn-VPA analogue was specifically selected as it is a HDAC inhibitor (Eikel et al., 2006) known to enhance both spatial learning and delayed match-to-position paradigms (Murphy et al., 2001; O'Loinsigh et al., 2004).

2. MATERIALS AND METHODS

2.1. Animal maintenance

Experimentally naïve male Wistar rats were acquired from Harlan UK and maintained in the Biomedical Facility at University College Dublin (UCD). The animals were housed in groups of 3-4 in holding cages supplemented with a cardboard tube shelter. All animals received food and water *ad libitum* and were maintained at 22-24 ºC on a 12 hour-light/dark cycle. Animals were allowed one week to acclimatise to the experimental rooms before any procedures were performed. All animals were examined and weighed daily. The experimental procedures performed were approved by the UCD Animal Research Ethics Committee, conformed to EU Council Directive 86–609–EEC, and were carried out by individuals who held the appropriate license issued by the Department of Health.

2.2. Prenatal exposure of pregnant Wistar rats to valproic acid

Time-mated female Wistar rats were obtained from Harlan UK, delivered to the UCD Biomedical Facility at gestational day 5, and housed individually under standard laboratory conditions, as described above. The *in utero* VPA rodent model of ASD employed has been previously described (Foley et al., 2012) and is based on the model of Schneider and Przewłocki (2005). Briefly, pregnant Wistar rats received a single intra-peritoneal dose of 600 mg/kg VPA (Sigma, UK) (VPA/VEH) in a 1 ml/kg volume of dH_2O on gestational day 12.5 (E12.5). The control dams received a single similar volume injection of dH_2O at the same gestational time-point (VEH/VEH). All animals exposed to VPA during gestation developed a characteristic ‘kink’ in the tail and this was used to confirm successful exposure to the teratogenic actions of VPA. Litters were standardised to n=8 per litter (maximising the number of males) and then

left undisturbed until the time of weaning on postnatal day (P) 25 when the male offspring were housed in groups of 3-4, as described above, until behavioural testing commenced on P72.

2.3. Drug administration and behavioural analysis

Prior to analysis of social interaction and spatial learning ability, the vehicle- and VPA-treated animals were transferred on P 67 to the experimental rooms, housed for a period of 5 days and drug-treated according to the schedule outlined in Fig. 1.

Pentyl-4-yn-VPA was custom synthesized (Celtic Catalysts, IRL) and administered as the sodium salt by the intraperitoneal route (i.p.) at 84 mg/kg in a 1 ml/kg dose volume of 0.9% vehicle, a dose previously demonstrated to have significant cognition-enhancing actions (Murphy et al., 2001; O'Loinsigh et al., 2004). Suberoylanilide hydroxamic acid (SAHA) was purchased from Axxora, UK, and administered i.p. at 5 mg/kg in a 2 ml/kg dose volume of using 2-hydroxypropyl-beta-cyclodextrin as vehicle (9 g/L). This dose of SAHA, when administered chronically, was previously demonstrated to reverse the social interaction and spatial learning deficits induced by *in utero* VPA exposure (Foley et al., 2012). As such, SAHA also served as an appropriate drug to compare with the actions of pentyl-4yn-VPA. The drugs were administered for 8 days prior to and on each day during behavioural analysis (administered 1 hour pre-training), a total of 18 days drug exposure.

2.3.1. Social approach-avoidance paradigm

The social-approach avoidance paradigm employed has been previously described (Foley et al., 2012) and was based on the model of Brodkin and colleagues (2004). The paradigm assessed the propensity of the test Wistar rat to approach and associate

with an unfamiliar, experimentally naive stimulus rat of the same strain, equal size (weight) and age, and unrelated to the animals of the experimental cohorts. This behavioural response was evaluated in a rectangular box containing two identical chambers at either end which contained the stimulus animal. These were separated from a central area by clear Perspex sheets with multiple, small, evenly spaced holes over the entire wall to permit auditory, visual and olfactory interactions. The test animal was placed in the central portion of the apparatus between two black Perspex baffles that created three equally sized and interconnected areas and allowed to interact with the stimulus animal (randomly placed in either the left or right chamber). In the experimental protocol the animal was initially allowed to explore and acclimatize to the apparatus for a five min period in the absence of the stimulus animal. Following a five min rest period in the home cage the test animal was returned to the apparatus and permitted to interact with the stimulus animal for a further five min period. In all cases the stimulus animal was a drug-naïve and the test animal had been exposed to vehicle *in utero* (VEH/VEH), VPA *in utero* (VPA/VEH), or exposed to VPA *in utero* and later treated with HDAC inhibitors (VPA/SAHA or VPA/pentyl-4-yn-VPA). A different naïve stimulus animal was paired with every test animal.

Exploratory behaviour was monitored using an overhead video camera linked to Ethovision XT (Noldus, UK) tracking software. Total time spent in each chamber of the apparatus was calculated, expressed as the mean ± S.E.M. (n=5-6), and significant differences between drug treatment and the amount of time animals spent in a specific chamber were determined by two-way ANOVA followed by a Bonferroni post-hoc test. In addition, the sum of time engaged in social interactive behaviours at the separating baffle was recorded for each treatment group and analysed by a Student's

t-test. This was employed to distinguish non-social effects confounding time spent in the social chamber, for example sedation, from efforts to directly engage in social behaviour with the stimulus animal. Scoring was only conducted in the test animal. These behaviours included nose-poking (any entry of nose into the holes in the Perspex baffle separating the test and stimulus animals), licking (all passing of tongue over or across the holes in/and the Perspex baffle separating the test and stimulus animals), chewing (efforts to bite through/around the holes and edges of the clear Perspex baffle separating the test and stimulus animal), gnawing (persistent chewing for greater than 5 s), digging (all efforts to dig under the Perspex baffle separating the test and stimulus animals, as opposed to non-specific digging in the substrate material in any other area of the apparatus) and head-following (the time spent by the test animal moving to track the head position of the stimulus animal) and were assessed manually by an observer who was blind to the treatment. Behaviours such as rearing, head weaving and grooming were not included as they were not directed to the stimulus rat.

2.3.2. Water maze spatial learning paradigm

As the biological motion deficits associated with ASD have been suggested to reflect a more widespread dysfunction within the dorsal stream of cognitive processing (Spencer et al., 2000; Milne et al., 2005), we determined if animals with prior in utero exposure to VPA were impaired in spatial learning as this function is dependent on the dorsal aspect of the hippocampus (Moser et al., 1993; Maguire et al., 2000). The water-maze paradigm consisted of both acquisition and recall components. During acquisition, each trial started with the rat placed facing the wall of the maze at one of three designated locations. Animals were then allowed to freely explore the maze and the time taken to find the hidden platform within a 90-s criterion period was defined

as the escape latency time. On the first trial only, rats failing to locate the platform within the 90-s period were guided to it and placed on it for 10 s. Escape latencies were measured over 5 trials in each daily session and an inter-trial rest interval of 300 s was allowed. Animals were trained in daily sessions over 4 days. Swim behaviour in the water-maze paradigm was monitored using Ethovision XT (Noldus, UK). Recall of platform position was assessed by a probe test at 24 h (P 84) and 72 h (P 86) following the final training session. During the probe test the platform was removed and animals were allowed to explore the maze for 30 s and the time spent in each quadrant was used to compare recall of the original position of the platform. All data were calculated as mean ± S.E.M. (n=6) for all trials of all sessions and the presence of significant difference between treatments was determined by two-way ANOVA and a Bonferroni post-hoc test of the data set.

2.4. Immunoblot analysis of core histone acetylation status

2.4.1. Histone isolation and sample preparation

Core histone proteins were isolated from the cerebellum as the neuroanatomical anomalies observed in autism are recapitulated in this region brain in rats with prior *in utero* exposure to VPA (Ingram et al., 2000) but also because it provided the tissue volume necessary for these studies, as adapted from a previously published procedure (Levenson et al., 2004). Briefly, animals were sacrificed by cervical dislocation, the brain regions of interest quickly dissected, frozen in liquid nitrogen and stored at -80^{o}C until use. When required, the samples were thawed and hand-homogenised in 500 µl of ice-cold isolation buffer (10 mM HEPES, pH 7.4, containing 2 mM EDTA, 0.32 M sucrose, 0.1% protease and phosphatase buffers), centrifuged at 7,700 g for 10 min at 4^{o}C. The supernatant was discarded and the remaining pellet containing the

nuclear material was dissolved in 1 ml of 0.4N H_2SO_4 (Sigma-Aldrich, UK) for 20 hrs on a nutator at 4^{o}C. Following over-night incubation samples were spun at 14,000 g for 10 min to pellet any undissolved material and the supernatant was removed and transferred to a fresh tube. The precipitated core histone proteins proteins were dissolved by the slow addition of 250 µl of ice-cold 100% trichloro-acetic acid (TCA) to the solution which was allowed to incubate overnight at 4^{o}C. The precipitated protein samples were centrifuged at 14,000 g for 30 min, the supernatant removed and the remaining pellet subjected to two sequential 5 min washes in ice-cold acetic acid by centrifugation (14,000 g) to remove excess TCA. Isolated histone samples were allowed to air-dry on the bench for 10 min and were subsequently solubilised in 100 µl of 10mM Tris buffer, pH 7.4.

2.4.2. SDS-PAGE electrophoresis and immunoblot analyis of isolated histone proteins

Core histone protein concentrations were determined by the BCA™ assay (Pierce, Rockford, IL) and samples of equal protein concentration were boiled for 10 min in 70 mM Tris-HCl, pH 6.8, containing 33 mM NaCl, 1 mM EDTA, 2% (w/v) SDS, 0.01 % (w/v) bromophenol blue, 10% glycerol and 3% (v/v) dithiothreitol reducing agent. The reduced and solubilised proteins were separated using pre-prepared 15% polyacrylamide gels and transferred to nitrocellulose membranes (Biorad, UK). Pre-stained molecular weight markers were co-electrophoresed with the protein samples (Sigma, UK). Remaining reactive groups on the nitrocellulose sheet were then inactivated using Tris buffered saline solution (TBS-T) blocking buffer (10 mM Tris–HCl, pH 7.4, containing 150 mM NaCl, 0.05% v/v Tween-20, and 5% w/v non-fat milk powder or bovine serum albumin) and the membrane subsequently incubated overnight at 4 °C in blocking buffer (5% v/v) containing a mouse monoclonal

antibody directed to specific acetylated (ac) lysine (K) residues on histone H3 or H4 (acH3K9, acH3K14 and acH4K8; Cell Signal®, USA; 1:20,000 dilution). Following overnight incubation, the nitrocellulose membrane was washed three times in TBS-T and incubated for 1 h in blocking buffer containing a horseradish peroxidase-conjugated anti-mouse IgG monoclonal antibody (Novagen, UK; 1:20,000 dilution). After incubation, the nitrocellulose sheet was washed three times in washing buffer, exposed for 5 min to a chemiluminescent peroxidase substrate (Pierce Rockford, IL) washed and exposed to X-ray film (Fuji, UK) until optimal resolution of the protein bands was achieved. To control for equal loading of core histone H3 and H4 proteins, membranes were incubated in a reblot solution (Millipore, UK) for 7 min, washed 3 times for 10 min in TBS-T and re-probed as detailed above using antibodies directed against acH3K9, acH3K14 or acH4K8. The X-ray films were scanned, converted into a digital format and the immunostained band density analysed using ImageJ software. The naphthol black-stained cellulose sheet was also scanned and digitised and used to confirm the presence of immunostained bands corresponding to the 5 core histone proteins present in the nucleosome.

The values were expressed as the mean$\pm$S.E.M. (n=3) and significant differences ($P<0.05$) were determined using an unpaired Student's *t*-test.

3. RESULTS

All animals exposed to VPA *in utero* exhibited a 'tail kink' and this deformity was used to confirm successful exposure to the teratogen, as we have described previously (Foley et al., 2012). No other gross developmental abnormalities were observed in VPA-exposed animals. Litter size and male to female sex ratio was unaltered and weight gain was unimpaired over the time period examined.

3.1. Social approach avoidance paradigm

Deficits in social reciprocation were examined in rats with prior *in utero* exposure to VPA using a social approach-avoidance paradigm. During the acclimatisation phase of the paradigm both vehicle-treated and VPA-exposed cohorts exhibited no preference for any chamber and spent equal amounts of time exploring all areas of the apparatus ($F[2,17]=0.01$ and 0.84, $P=0.99$ and 0.46, vehicle and VPA-treated, respectively). The introduction of a stimulus rat into either the left or right chamber resulted in the vehicle-treated animal showing a significantly increased tendency for social interaction ($F[2,17]=67.57$; $P<0.0001$) (Fig. 2A). In contrast, the VPA-treated animals displayed a significant reduction in interaction with the stimulus rat ($F[2,17]=0.94$; $P=0.41$), spending approximately 30% less time in the social chamber as compared to the vehicle-treated rat (Fig. 2A). Comparison by 2-way ANOVA (treatment x chamber) again demonstrated a significant effect of chamber ($F[2,54]=99.5$; $P<0.0001$). Analysis by a Bonferroni post-hoc test confirmed a significant difference between VEH/VEH and VPA/VEH groups for the time spent in the social chamber of the apparatus ($P<0.05$). The marked asociality exhibited by VPA-treated animals was further evidenced by the significant reduction in time

engaged in social behaviours (P=0.02; Student's t-test), as compared to the vehicle-treated cohorts (Fig. 2B).

The social cognition deficits observed in the VPA-treated animals could be reversed by treatment with HDAC inhibitors. VPA-exposed animals given the pan-specific HDAC SAHA inhibitor had no effect on the time spent exploring the individual areas of the apparatus during the acclimatisation phase of the paradigm (data not shown). However, treatment of VPA-exposed cohorts with SAHA resulted in a significant preference for the social chamber of the apparatus following the introduction of a stimulus rat ($F[2,14]=64.4$; $P<0.0001$) (Fig. 2A). Analysis between treatment groups (VPA/SAHA vs VPA/VEH) confirmed a significant enhancement of social preference seen as increased time in the social chamber ($P<0.05$; a Bonferroni post-hoc test). SAHA treatment also resulted in a significant increase in total time actively engaged in social behaviour (P=0.002; Student's t-test) (Fig. 2B). Thus, as demonstrated previously (Foley et al., 2012), the effects of SAHA on social cognition are direct as they are observed in vehicle-treated animals.

A similar amelioration of social cognition deficits in VPA-treated animals was observed following treatment with the pentyl-4-yn-VPA HDAC inhibitor. VPA-exposed animals administered pentyl-4-yn-VPA spent similar times exploring the individual areas of the apparatus during the acclimatisation phase of the paradigm (data not shown). However, following introduction of the stimulus rat, VPA-exposed cohorts treated with pentyl-4-yn-VPA resulted in a significant preference for the social chamber of the apparatus ($F[2,17]=67.1$; $P<0.0001$) (Fig. 2A). Comparison by two-way ANOVA and a Bonferroni post-hoc test indicated pentyl-4yn-VPA to significantly improve social preference as compared to VEH/VEH animals with increased time being spent in social and decreased time in the non-social chambers

(P<0.05; a Bonferroni post-hoc test). Pentyl-4-yn-VPA treatment also resulted a significant increase in total time actively engaged in social behaviour (P=0.0001; Student's t-test) (Fig. 2B). Pentyl-4-yn-VPA administration to animals treated with vehicle in utero (VEH/pentyl-4-yn-VPA) did not result in any significant alteration in social preference in the social approach avoidance task as compared to VEH/VEH controls (212.9±15.4 vs 180.5±11.2, respectively, time (s) spent in social chamber of the apparatus, F[1,28]=0.9; P=0.34 two-way ANOVA).

3.2. Spatial learning paradigm

The cognitive deficits associated with *in utero* VPA exposure and the cognition-enhancing effects of SAHA and pentyl-4-yn-VPA were explored using the water maze paradigm. All experimental groups employed readily acquired the position of the hidden platform during the training phase of the water-maze paradigm and a significant effect of training session was observed (2-way ANOVA: treatment x training session; F[3,76]=20.3; P<0.0001). However, cohorts administered VPA *in utero* were significantly slower at reaching the target platform than vehicle-treated controls across all four training sessions (F[3,76]=3.0; P=0.04) (Fig. 3A). Furthermore, retention of platform position was significantly disrupted in VPA-exposed cohorts as in the 24 hour probe trial, animals spent approximately 30% of time in the target quadrant, performing just above chance level (Fig. 3B). This is in stark contrast to control cohorts which spent a significantly longer period of time in the target quadrant (49.6%; P=0.026; Student's t-test). Recall of the platform position was also poor in the 72 hour post-training probe trial during which the VPA-treated animals spent less than ~20% of time in the target quadrant compared to ~40% by vehicle controls (P=0.004; Student's t-test) (Fig. 3B).

Treatment of VPA-exposed cohorts with SAHA significantly reversed the deficits in both acquisition and recall of the water-maze task. During the acquisition phase VPA-treated cohorts treated with SAHA displayed significantly reduced escape latencies over the 4 training sessions compared to VPA-treated controls (a Bonferroni post-hoc test; $P<0.05$) (Fig. 3A). Similarly, those treated with pentyl-4-yn-VPA also displayed significantly reduced escape latencies in the training sessions as compared to VPA-treated controls (a Bonferroni post-hoc test; $P<0.05$) (Fig. 3A) and this effect was indistinguishable from that obtained with SAHA. Moreover, task consolidation was also significantly improved as recall of the platform position during recall probe trials as animals treated with SAHA and pentyl-4-yn-VPA spent approximately 60% and 50% of time searching the target quadrant 24 hours post-training and this level of retention was maintained at ~40% in both treatment groups during the 72 hour post-training trial (Fig. 3B).

3.3 Influence of SAHA and pentyl-4-yn-VPA on histone acetylation status

In order to determine if altered behavioural phenotype was correlated with drug-induced change in histone acetylation status, we carried out an immunoblot analysis of cerebellar tissue using antibodies that recognised specific acetylated lysine residues on histone proteins H3 and H4. We specifically determined change in H3K9, H3K14 and H4K8 acetylation status as modification of these lysine residues have been associated with change in behavioural plasticity (Guan et al., 2009). Immunoblots, developed using antibodies to specific acetylated lysine residues, reliably detected a single band that corresponded to the molecular weights of both histone proteins H3 and H4 (Fig. 4A-C). Semi-quantitative analysis of these immunoblots further revealed a significant increase in H4K8 acetylation status following chronic administration of either SAHA or pentyl-4-yn-VPA (~30 and 50% in respect of vehicle-treated animals;

$P<0.05$; unpaired Student's t-test) (Fig. 4A). Although SAHA was found to have no effect on the acetylation status of H3K9 or H3K14 (Fig. 4B and C), pentyl-4-yn-VPA showed small (~8% in respect of vehicle-treated animals) but significant ($P<0.05$; unpaired Student's t-test) change in acetylation status of H3K9 (decrease; Fig. 4B) and H3K14 (increase; Fig. 4C). These alterations did not relate to change in protein load as all values were normalised to histone protein expression.

4. DISCUSSION

The association of *in utero* exposure to teratogens and an increased incidence of autism (Rasalam et al., 2005) forms the basis for using offspring of Wistar rats with prior VPA exposure as a model for ASD (Rodier et al., 1996; Schneider and Przewłocki, 2005). Administration of VPA at embryonic day 12 follows neural tube closure and coincides with the formation of the brainstem nuclei which, as a consequence, result in a reduction in the size of these nuclei, the complement of cerebellar neurons, and serotoninergic function, all of which are the most prominent features of the autistic brain (Rodier et al., 1996; Miyazaki et al., 2005; Tashiro et al., 2011). The presence of a tail kink in the offspring of the VPA-exposed dams suggests that all animals used in this study had impaired neuralation (Vorhees, 1987).

The significant social deficits observed in this study, using the social approach avoidance paradigm as a measure of affiliative behaviour and the spatial learning deficits as an indicator of disrupted cognition, previously have been reported in animals with prior *in utero* exposure to VPA (Rodier et al., 1996; Schneider and Przewłocki, 2005; Foley et al., 2012). The social cognitive domain has been argued to be an important target for linking phenotype to cognitive process to brain structure in autism (Losh et al., 2009) and this is consistent with the most clinically profound deficit in ASD which is the processing of perceptual stimuli (Annaz et al., 2011) and/or their abnormal interpretation (Rutherford et al., 2012). Thus, one of the most significant findings in this study is that SAHA and pentyl-4-yn-VPA ameliorated the social and cognitive deficits in the VPA model of ASD and it is of particular interest to note that pentyl-4-yn-VPA exerted a similar action to SAHA as a previous study failed to obtain similar effects with VPA (150 mg/kg) in either the spatial learning or social approach avoidance paradigms (Foley et al., 2012). This specific effect of the

pentyl-4-yn-VPA analogue on spatial learning and the social approach-avoidance paradigm may, in part, be related to its greater effectiveness in inhibiting HDAC as compared to VPA (Eikel et al., 2006) and that it most significantly augments water maze spatial learning ability (Murphy et al., 2001) and the delayed-match-to-position task (O'Loinsigh et al., 2004)

A second curious feature of pentyl-4-yn-VPA relates to its ability to correct cognitive deficits in the adult that were induced by the *in utero* administration of the parent molecule VPA, both being HDAC inhibitors (Eikel et al., 2006). The teratogenic action of VPA, however, is believed to be mediated through its anti-proliferative potential (Regan, 1985; Courage-Maguire et al., 1997) and this appears to involve a HDAC1 mechanism that regulates for transcriptional control of stem cell differentiation (Phiel et al., 2001; Eikel et al., 2006; Dovey et al., 2010). The ameliorative actions of pentyl-4-yn-VPA on the social cognition deficits in the adult, in contrast, most likely arise from its greater potency as compared to VPA, allowing it to influence signaling events regulated by HDAC2, an isoform more specifically associated with cognition (Eikel et al., 2006; Guan et al., 2009).

The second most important finding in these studies is the observation that both pentyl-4-yn-VPA and SAHA significantly increase histone protein H4 acetylation. There is evidence to indicate increased histone acetylation, particularly on H3 and H4, histones, is necessary for synaptic plasticity and memory formation (Guan et al., 2009; Bousiges et al., 2010). Such an action would facilitate access of regulatory proteins to DNA (Lee et al., 1993) and altered translation leading to the synaptic and behavioural aberrations associated with autism (Santini et al., 2013). The modest actions of pentyl-4-yn-VPA and SAHA in modulating H3 histone protein acetylation require further investigation. It is worth noting, however, that these histone

modifications may act cooperatively as, for example, H3K9ac has been shown to be located in the regions surrounding the transcription start sites and the H4K8ac are in the promoter and transcribed regions of active genes (Agalioti et al., 2002; Wang et al., 2008).

ACKNOWLEDGEMENT

Berand Neuropharmacology provided financial support for the conduct of the research and in the preparation of the article.

DISCLOSURE/CONFLICT OF INTEREST

AGF and CMR are members of the Board of Directors for Berand Neuropharmacology. AGF is an employee and AWC a former employee of Berand Neuropharmacology.

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FIGURE LEGENDS

Fig. 1: Experimental design employed in establishing the effect of HDAC inhibitors SAHA and pentyl-4-yn-VPA on the social cognition and water maze spatial learning paradigms in the VPA rodent model of autism. E: Embryonic day; P: Postnatal day.

Fig. 2: Influence of SAHA and pentyl-4-yn-VPA on the social approach-avoidance deficits in the VPA rodent model of autism. The time spent by the experimental animal in the social, centre and non-social areas is shown in Panel A and the time engaged in social behaviours is illustrated in Panel B. All values are the mean$\pm$S.E.M. (n=5-6) and values significantly different (Student's t-test, $P<0.05$) from the control animal (VEH/VEH) are indicated with an asterisk and those significantly different from the animals with prior *in utero* exposure (VPA/VEH) are indicated with a cross.

Fig. 3: Influence of SAHA and pentyl-4-yn-VPA on spatial learning deficits in the VPA rodent model of autism. Panel A compares task acquisition rates between control and VPA-treated animals and between VPA-treated animals following administration of SAHA and pentyl-4-n-VPA (Panel A). The influence of the drugs on recall of platform position is shown in Panel B. All values are the mean$\pm$S.E.M. (n=6) and values significantly different (Student's t-test, $P<0.05$) from the control animal (VEH/VEH) are indicated with an asterisk.

Fig. 4: Influence of SAHA and pentyl-4-yn-VPA on H3K9, H3K14 and H4K8 acetylation status in the adult cerebellum of rats with prior exposure to VPA. Immunoblots of the major protein product identified by each antibody and the histone bands identified in the corresponding naphthol-stained tissue extracts and their semi-quantitative densitometric analysis are illustrated. The values are expressed as the

mean±S.E.M. and significant differences ($P<0.05$; unpaired Student's *t*-test) between the vehicle and drug-treated animals are indicated by an asterisk.

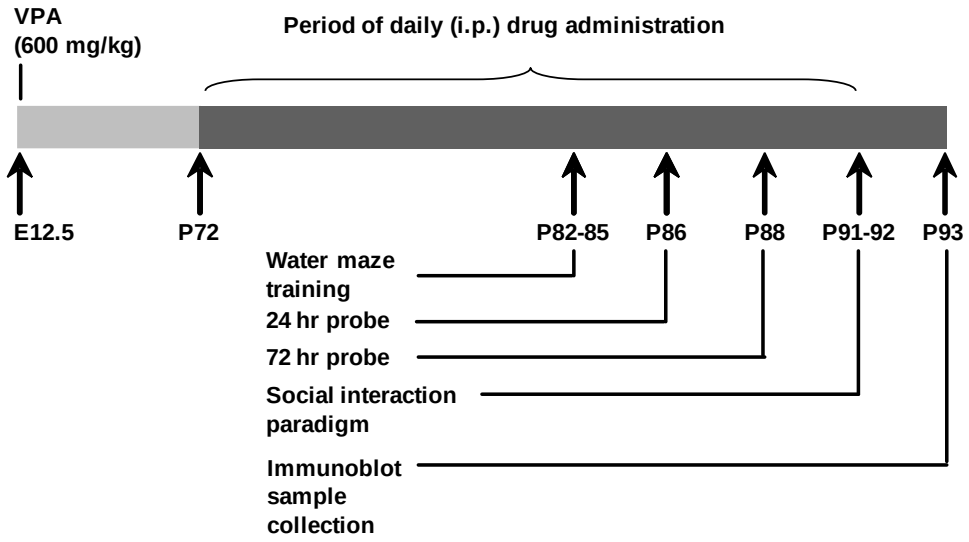

Fig. 1 Foley et al.

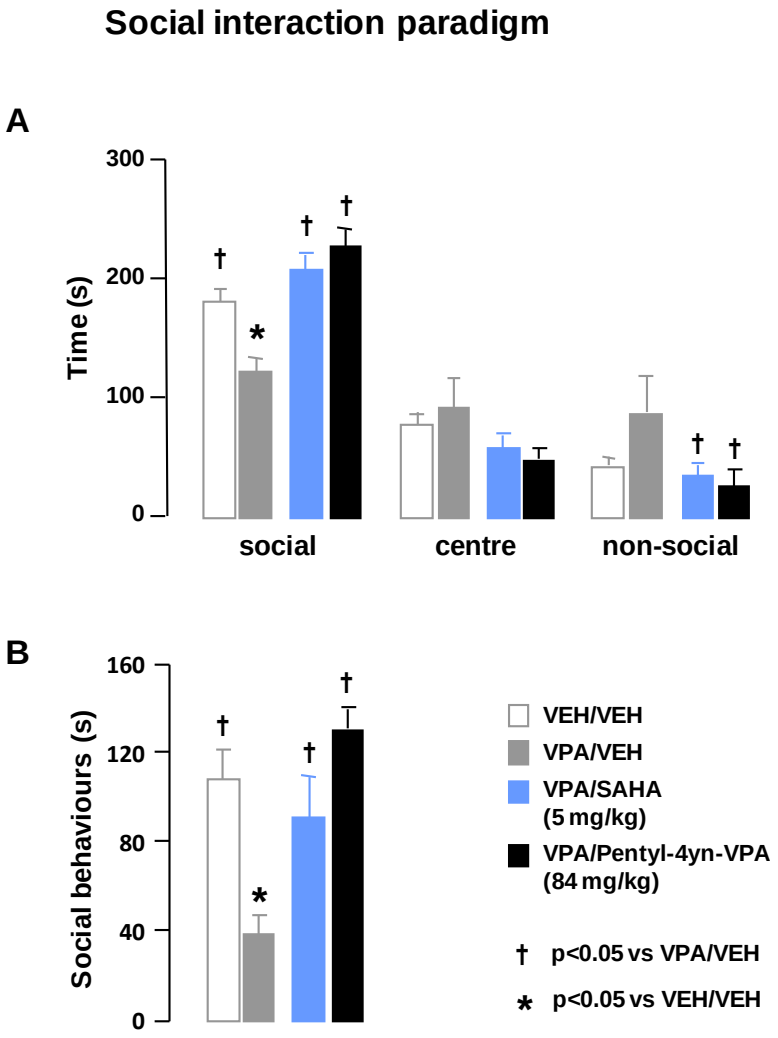

Fig. 2 Foley et al.

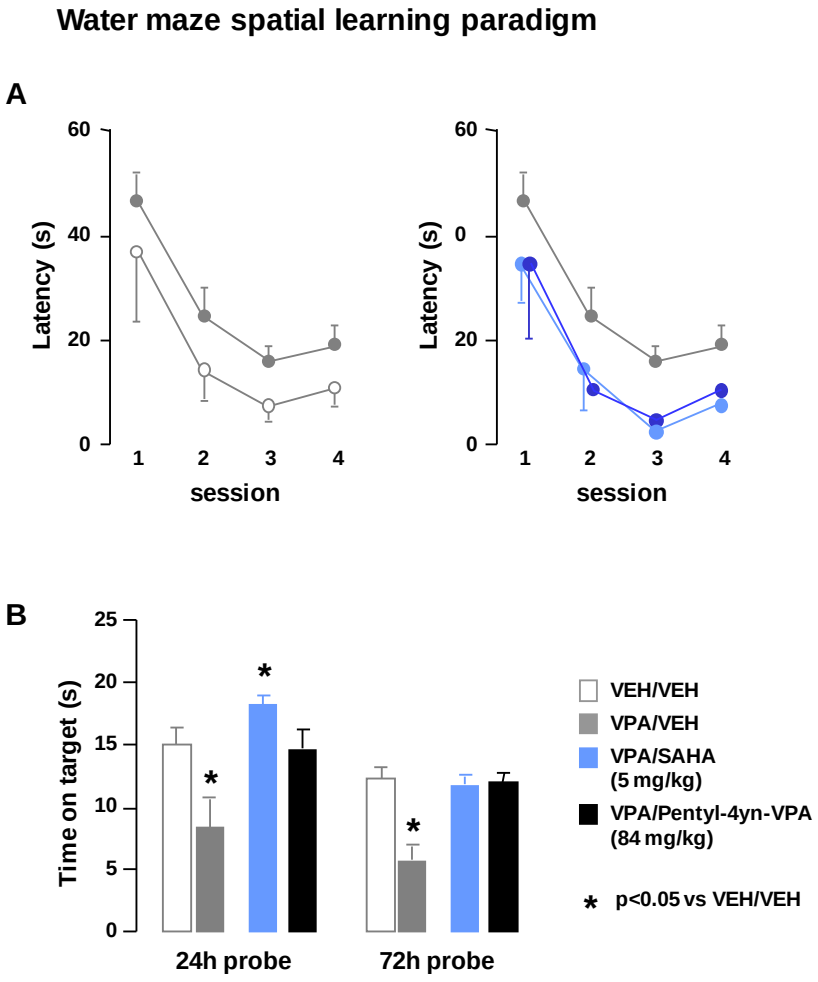

Fig. 3 Foley et al.

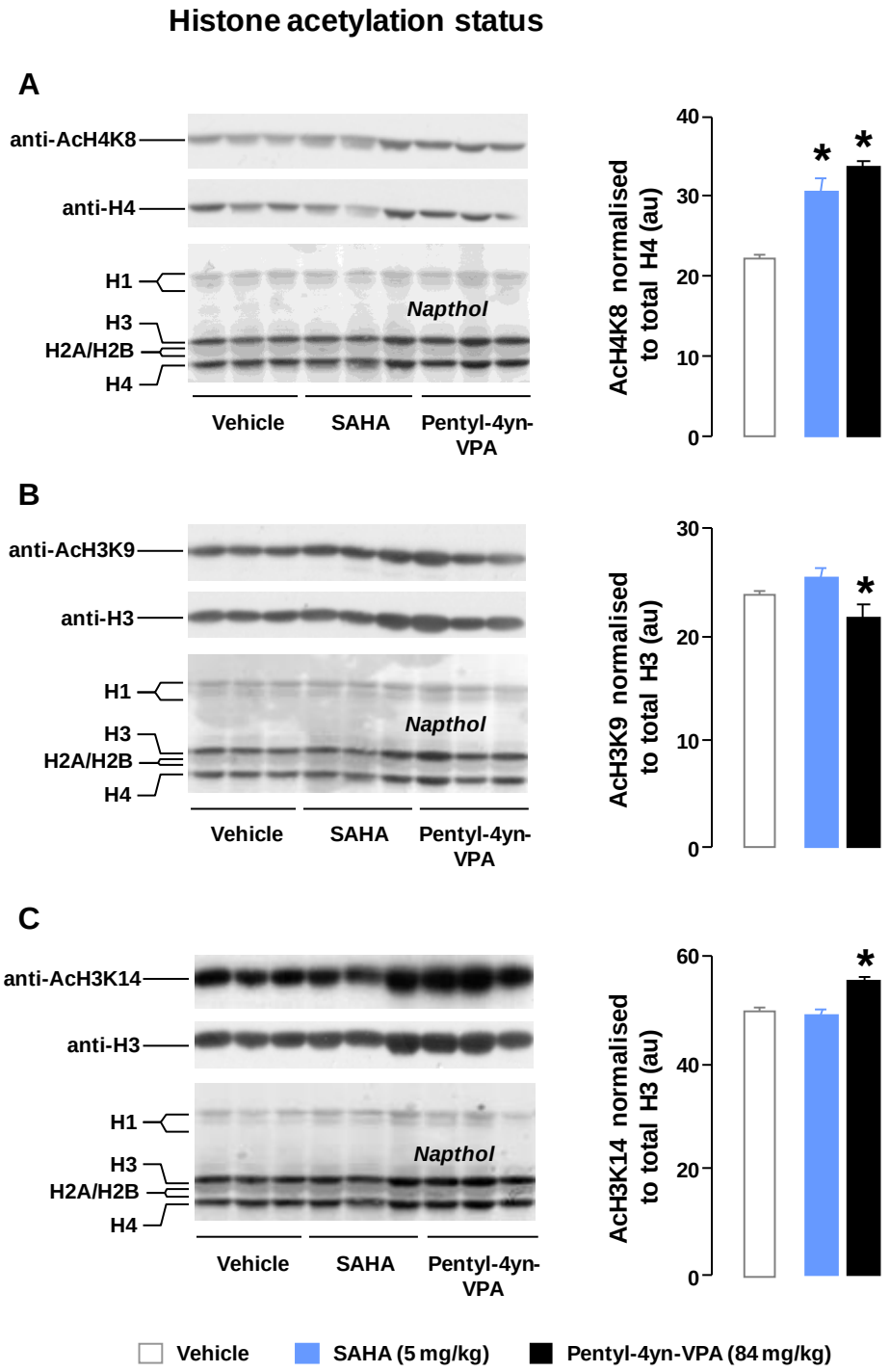

Fig. 4 Foley et al.