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RESEARCH PAPER

Sex-specific autistic endophenotypes induced by prenatal exposure to valproic acid involve anandamide signalling

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BACKGROUND AND PURPOSE

Autism spectrum disorder (ASD) is more commonly diagnosed in males than in females. Prenatal exposure to the antiepileptic drug valproic acid (VPA) is an environmental risk factor of ASD. Male rats prenatally exposed to VPA show socio-emotional autistic-like dysfunctions that have been related to changes in the activity of the endocannabinoid anandamide. Here, we have investigated if prenatal VPA induced sex-specific autistic endophenotypes involving anandamide signalling.

EXPERIMENTAL APPROACH

We studied sex-specific differences in the ASD-like socio-emotional, cognitive and repetitive symptoms displayed during development of Wistar rats of both sexes, prenatally exposed to VPA. The involvement of anandamide was followed by Western blotting of cannabinoid CB_1 receptors and by inhibiting its metabolism.

KEY RESULTS

Female rats were less vulnerable to the deleterious effects of prenatal VPA exposure on social communication, emotional reactivity and cognitive performance than male rats. Conversely, as observed in male rats, prenatal VPA exposure induced selective deficits in social play behaviour and stereotypies in the female rat offspring. At the neurochemical level, prenatal VPA exposure altered phosphorylation of CB_1 receptors in a sex-specific, age-specific and tissue-specific manner. Enhancing anandamide signalling through inhibition of its degradation reversed the behavioural deficits displayed by VPA-exposed animals of both sexes.

CONCLUSIONS AND IMPLICATIONS

These findings highlight sexually dimorphic consequences of prenatal VPA exposure that may be related to sex-specific effects of VPA on endocannabinoid neurotransmission in the course of development and introduce a new therapeutic target for reversing autistic-like symptoms in both sexes.

Abbreviations

% OE, percentage of open-arm entries; % TO, percentage of time spent in the open arms; ASDs, autism spectrum disorders; GD, gestational day; PND, postnatal day; USVs, isolation-induced ultrasonic vocalizations; VPA, valproic acid

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Introduction

The term autism spectrum disorder (ASD) refers to a group of pervasive developmental psychiatric disorders, emerging in early life and characterized by impairments in social interaction, restricted communication abilities and stereotyped/repetitive behaviours (American Psychiatric Association, 2013). Common co-morbid features include anxiety and intellectual disability (Lai *et al*., 2014). The aetiology of ASD is still controversial, involving both environmental and genetic factors (Kim and Leventhal, 2015; Karimi *et al*., 2017).

One of the most striking but consistent findings in ASD epidemiology is the higher rate of diagnosis in males than in females, with a recently suggested 3:1 male-to-female ratio (Loomes *et al*., 2017). Sex differences in the phenotypic presentation of the disease might lead to missed or delayed diagnosis in females (Rivet and Matson, 2011a,b; Lai and Baron-Cohen, 2015; Bargiela *et al*., 2016). Compared with females, males with ASD show more aggression, hyperactivity, reduced prosocial behaviour and increased repetitive/restricted behaviours (Werling and Geschwind, 2013). Conversely, females with ASD often camouflage the autistic core deficits with better language and social competences (Lehnhardt *et al*., 2016; Hull *et al*., 2017), and they often have lower average intellectual abilities than males (Banach *et al*., 2009).

As the male predominance in ASD has been long documented, most clinical and epidemiological studies have been conducted on the male population (Rivet and Matson, 2011a, b). The lack of research focused on gender differences in ASD is mirrored at the preclinical level, where animal models of ASD have been mainly developed and validated in males (Beery and Zucker, 2011; Kokras and Dalla, 2014). Nevertheless, it is essential that preclinical research is performed on animals of both sexes, to enhance the validity of animal models and contribute to gender-oriented prevention, diagnosis and treatment of psychiatric disorders (Hughes, 2007).

Valproic acid (**VPA**) is a widely used and effective antiepileptic and mood stabilizer drug. However, VPA is also a known teratogen and, when given during pregnancy, it can induce various congenital malformations (Kozma, 2001; Kini, 2006), including autistic-like features in the exposed children, such as impaired communication, reduced sociability and stereotyped behaviours. For this reason, prenatal VPA exposure is a recognized environmental risk factor for ASD (Williams and Hersh, 1997; Williams *et al*., 2001).

Based on thsee clinical observations, prenatal VPA exposure in rodents is a widely used environmental preclinical model of ASD with face and construct validity (Williams *et al*., 2001; Schneider and Przewlocki, 2005; Wagner *et al*., 2006; Roullet *et al*., 2013; Sabers *et al*., 2014; Servadio *et al*., 2015, 2016). This model, however, has been mainly validated and used in male rodents.

Here, we have performed a longitudinal study from birth to adulthood to evaluate potential sexually dimorphic effects induced in rodents by prenatal VPA exposure on core and comorbid behavioural traits that are frequently impaired, often in a sex-specific manner, in ASD patients: (i) social communication, (ii) social behaviour, (iii) social discrimination, (iv) stereotypies, (v) anxiety and (vi) cognitive performance. Furthermore, because the endocannabinoid system plays a key role in brain development (Maccarrone *et al*., 2014), it has been recently involved in ASD (Siniscalco *et al*., 2013; Chakrabarti *et al*., 2015; Zamberletti *et al*., 2017) and it is modulated by sex hormones during critical developmental ages (Viveros *et al*., 2011). Here, we have investigated its role in the sexually dimorphic behavioural consequences of prenatal VPA exposure.

Methods

Animals

All animal care and experimental procedures complied with the guidelines of the Italian Ministry of Health (D.L. 26/14) and the European Community Directive 2010/63/EU), and were approved by the Italian Ministry of Health (Rome, Italy). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny *et al*., 2010; McGrath and Lilley, 2015).

Primiparous female Wistar rats (Charles River, Calco (Lecco), Italy), weighing 250 ± 15 g, were mated overnight. The morning when spermatozoa were found was designated as gestational day (GD) 1. Pregnant rats were singly housed in Macrolon cages (40 × 26 × 20 cm; $l \times w \times h$), under controlled conditions (temperature 20–21°C, 55–65% relative humidity, 12/12 h light cycle with lights on at 07:00 h, standard bedding (Charles River), enriched environmental conditions [wooden toys and irradiated certified diamond twists (Envigo, Italy)] and food and water *ad libitum*). On GD 12.5, dams received a single i.p. injection of either sodium valproate (VPA, n = 54) or saline (SAL, n = 53). On postnatal day (PND) 1, litters were culled to four males and four females. On PND 21, pups were weaned and housed in groups of three. The experiments were carried out on the male and female offspring during infancy (PNDs 5, 9 and 13), adolescence (PND 35) and adulthood (PND 90) (Figure 1). One pup per litter from different litters per treatment group (SAL or VPA) was randomly used in each experiment. Sample size (n) was based on our previous experiments and power analysis with the software GPower. Potential outliers within each data set were calculated using the GraphPad software. Sample size is indicated in the figure legends. All behavioural tests were assessed by a trained observer who was unaware of the treatments.

To collect brain samples for the Western blot experiments, at PNDs 35 and 90, rats were rapidly decapitated, as it is known that other killing methods such as anaesthetic overdose or CO_2 inhalation can alter brain neurochemistry (Woodbury *et al*., 1958; Karmarkar *et al*., 2010; Pierozan *et al*., 2017), thus affecting the results of the biochemical experiments described here. At the end of the behavioural experiments, rats were killed by CO_2 inhalation.

Drug administration

VPA was dissolved in saline and administered at a dose (500 mg·kg^{-1} on GD 12.5) that induces autistic-like behavioural changes in male rat offspring (Servadio *et al*., 2016). The anandamide hydrolysis inhibitor **URB597** was dissolved in 5% Tween 80/5% polyethylene glycol/saline and administered i.p. at a dose of 0.050 mg·kg^{-1}: URB597 was administered 2 h before each behavioural test (Servadio *et al*., 2016), as *in vivo* experiments have shown that, 2 h after URB597

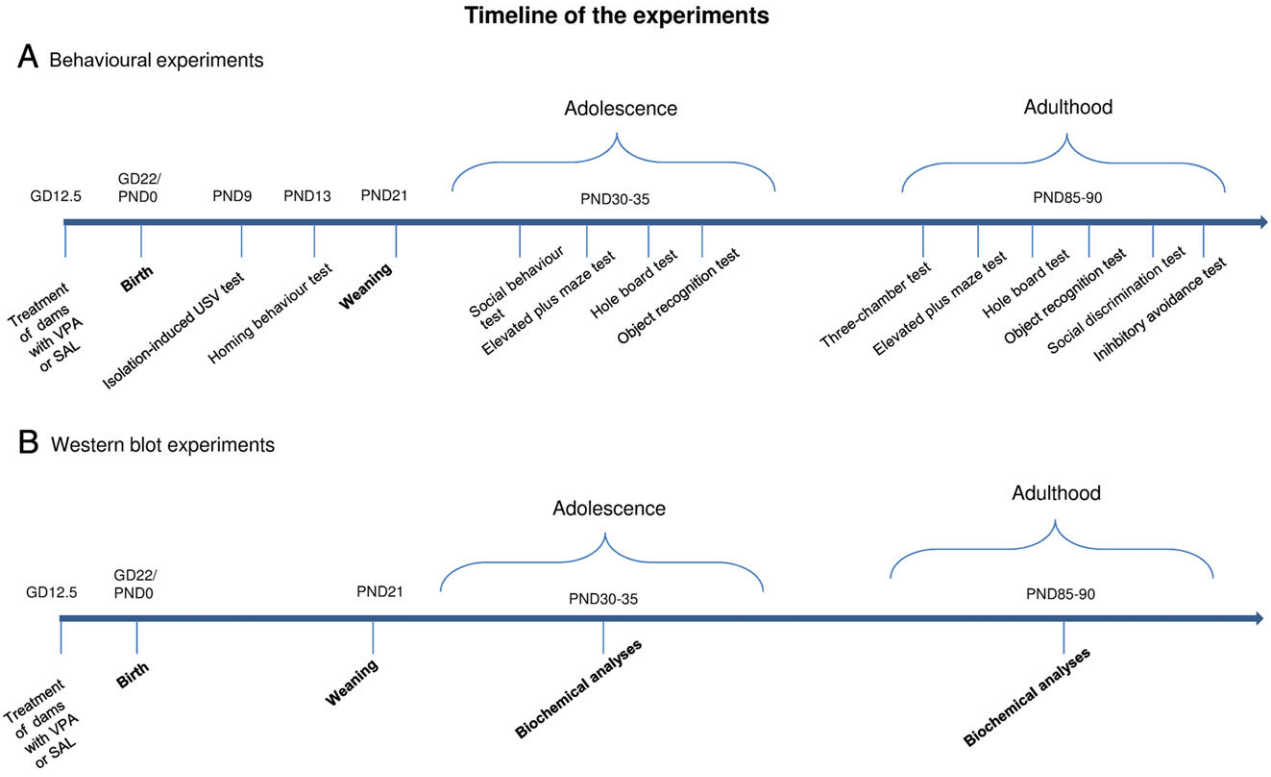

Figure 1
Timeline of the behavioural (A) and biochemical (B) experiments.

injection, the enzyme hydrolysing anandamide, **fatty acid amide hydrolase**, is maximally inhibited and **anandamide** levels are significantly increased (Gobbi *et al.*, 2005; Kathuria *et al.*, 2003). In the inhibitory avoidance test only, URB597 was administered immediately after the acquisition trial, in order to exclude any drug-induced variability in the training phase (e.g. pain sensitivity, motivation and locomotion). Solutions were administered in a volume of 2 mL·kg^{-1} at adolescence and 1 mL·kg^{-1} at adulthood.

Determination of the oestrous cycle

To rule out the possibility that any change observed in the female offspring could be due to cycle fluctuations, we monitored the oestrous cycle in both VPA-exposed and control females, to ensure that they would always be tested at the same phase of the oestrous cycle.

Western blot analysis of phosphorylated and total CB_1 receptors

Rats were rapidly decapitated, and their brains were removed and cut into coronal slices on a cold plate. The prefrontal cortex, dorsal striatum, nucleus accumbens, hippocampus, amygdala and cerebellum were dissected by hand under microscopic control within 2 min. Tissues were stored at −80°C until use. Lysates and protein separation were performed as previously described (Servadio *et al.*, 2016). Immunoblots were incubated with primary antibodies against CB_1 receptors and against phosphorylated CB_1 receptors (1:500) (Santa Cruz Biotechnology, Dallas (Texas), USA), followed by secondary peroxidase-conjugated antibodies (1:3000) (Santa Cruz Biotechnology). Immunoreactivity was detected by enhanced chemiluminescence (GE Healthcare, UK). Sample loading was normalized with anti-tubulin (Sigma-Aldrich, Milano, Italy) antibody. Bound antibodies to proteins on nitrocellulose were visualized by using enhanced chemoluminescence detection (GE Healthcare) and exposure to ChemiDoc Imaging System (Bio-Rad, Milano, Italy). Images were analysed with ImageJ (National Institutes of Health, MD, USA). Western blot experiments were performed in duplicate.

Behavioural tests

Isolation-induced ultrasonic vocalizations. On PND 9, the isolation-induced ultrasonic vocalizations (USVs) emitted by each pup removed from the nest and placed into a Plexiglas arena were detected for 3 min by an ultrasound microphone (Avisoft Bioacoustics, Germany) sensitive to frequencies between 10 and 200 kHz. The USVs were analysed quantitatively using Avisoft Recorder software (version 5.1).

Homing behaviour. On PND 13, following 30 min of isolation, each pup was placed for 4 min into a box whose floor was covered for 1/3 with bedding from the pup's home cage and for 2/3 with clean bedding. The following parameters were scored using the Observer 3.0 software (Noldus, The Netherlands): latency (s) to reach the home cage bedding and total time (s) spent in the nest bedding area (Scattoni *et al.*, 2008).

Social play behaviour. The test was performed in a sound-attenuated chamber under dim light conditions, as previously described (Trezza and Vanderschuren, 2008; Trezza and Vanderschuren, 2009). The 35-day-old rats were individually habituated to the test cage for 10 min on the two days before testing. On the test day, the animals were isolated for 3 h before testing. The test consisted of placing VPA-exposed or SAL-exposed rats together with an untreated animal for 15 min.

The following parameters were scored for each animal of a pair using the Observer 3.0 software (Noldus) (Trezza *et al.*, 2010):

- Pinning: the most characteristic posture of social play in rats that occurs when one animal is solicited to play by its test partner and rotates to its dorsal surface with the other animal standing over it.
- Pouncing: one animal is soliciting the other to play, by attempting to nose or rub the nape of the neck of the partner.
- Partial rotation: upon nape contact, the recipient animal rotates along its longitudinal axis but then stops and keeps one or both hind feet firmly planted on the ground.
- Evasion: upon solicitation, the recipient animal avoids nape contact by leaping, running or turning away from the partner.
- Social exploration: sniffing any part of the body of the test partner.
- Play responsiveness: the percentage of response to play solicitation, calculated as the probability of an animal of being pinned in response to play solicitation (pouncing) by the stimulus partner.

Three-chamber test. The test was performed as previously described (Servadio *et al.*, 2016). The apparatus was a rectangular three-chamber box, with two lateral chambers (30 × 35 × 35 cm; $l \times w \times h$) connected to a central chamber (15 × 35 × 35 cm; $l \times w \times h$). Each lateral chamber contained a small Plexiglas cylindrical cage. At PND 90, each experimental rat was individually allowed to explore a three-chamber apparatus for 10 min and then confined in the central compartment. An unfamiliar stimulus animal was confined in a cage located in one chamber of the apparatus, while the cage in the other chamber was left empty. Both doors to the side chambers were then opened, allowing the experimental animal to explore the apparatus for 10 min. The per cent of time spent in social approach (sniffing the stimulus animal) and the percentage of time spent exploring the empty chamber were scored using the Observer 3.0 software (Noldus).

Hole board test. The test was performed in a sound-attenuated chamber under dim light conditions, as previously described (Makanjuola *et al.*, 1977; Servadio *et al.*, 2016). The apparatus consisted of a grey square metal table (40 × 40 × 10 cm; $l \times w \times h$) with 16 evenly spaced holes (4 cm in diameter), inserted in a Plexiglas arena (40 × 40 × 60 cm; $l \times w \times h$). Each rat was individually placed in the apparatus for 5 min. Each session was recorded with a camera positioned above the apparatus for subsequent behavioural analysis performed using the Observer 3.0 software (Noldus). Dipping behaviour was scored by the number of times an animal inserted its head into a hole at least up to the eye level.

Elevated plus maze. The elevated plus maze apparatus comprised two open (50 × 10 × 40 cm^3; $l \times w \times h$) and two closed arms (50 × 10 × 40 cm^3; $l \times w \times h$) that extended from a common central platform (10 × 10 cm^2). Rats were individually placed on the central platform for 5 min and allowed to explore the apparatus. The following parameters were scored using the Observer 3.0 software (Noldus):

- % time spent in the open arms (% TO): (seconds spent on the open arms of the maze/seconds spent on the open + closed arms) × 100;
- % open arm entries (% OE): (number of entries into the open arms of the maze/number of entries into open + closed arms) × 100.

Social discrimination. The test was performed at PND 90. Briefly, animals were isolated for 7 days before testing. The test consisted of a learning trial and a retrieval trial, which were separated by a 30 min intertrial interval. During the learning trial, a juvenile (30 days old), unfamiliar rat was introduced into the home cage of the experimental rat for 5 min. The time spent by the experimental rat investigating (sniffing, allogrooming and following) the juvenile was measured. Thirty minutes after, the juvenile used in the learning trial was returned to the same adult's cage together with a novel juvenile. The time spent by the adult exploring the novel and the familiar juveniles was monitored for 5 min. The discrimination index was calculated as the difference in time exploring the novel and the familiar animal, expressed as the percentage ratio of the total time spent exploring both animals (Campolongo *et al.*, 2007).

Novel object recognition. On the training trial, each rat was individually placed into an open-field arena containing two identical objects (A1 and A2), equidistant from each other, and allowed to explore the objects for 5 min. Thirty minutes later, one copy of the familiar object (A3) and a new object (B) were placed in the same location as during the training trial. Each rat was placed in the apparatus for 5 min, and the time spent exploring each object was recorded. The discrimination index was calculated as the difference in time exploring the novel and the familiar objects, expressed as the percentage ratio of the total time spent exploring both objects (Campolongo *et al.*, 2013).

Inhibitory avoidance. On the first day, 90-day-old rats were individually placed in the illuminated compartment of an inhibitory avoidance apparatus (Ugo Basile, Gemonio (Varese), Italy). After 10 s, the sliding door was opened, and the time taken by the animal to enter into the dark compartment was measured (latency). Once the animal entered the dark compartment, the sliding door was closed, and a mild shock (0.6 mA) was delivered through the floor for 2 s. Twenty-four hours later, the animal was placed in the lit compartment, and the latency to re-enter (retention

latency) the dark compartment was recorded (Campolongo *et al.*, 2007).

Locomotor activity. To determine whether the behavioural effects induced by prenatal VPA exposure were secondary to any change in locomotor activity, at PND 35 and PND 90, rats from both experimental groups were individually placed into an open-field arena, and their locomotor activity was scored for 5 min as follows: a grid, dividing the arena into equally sized squares, was projected over the recordings, and the number of line crossings made by the animal was recorded using the Observer 3.0 software (Noldus).

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2018). Data are expressed as mean ± SEM. To assess the effects of the prenatal treatments (VPA or SAL) in the male and female offspring, the behavioural and biochemical data were analysed by two-way ANOVA, with treatment and sex as factors. Two-way ANOVA was also used to assess the effects of prenatal (VPA or SAL) and postnatal (URB597 or vehicle) treatments.

The accepted value for significance was $P < 0.05$. If main or interaction effects were significant, the Student–Newman–Keuls *post hoc* test was used for individual group comparisons. The software Sigma Plot (13.0; Systat Software, Inc, USA) was used for statistical analysis of the data.

Materials

The compounds used in these studies were supplied as follows: VPA was supplied by Cayman (Michigan, USA) and URB597 by Sigma.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b).

Results

Reproduction data

No differences in body weight gains were observed between VPA-treated and SAL-treated dams. Prenatal VPA exposure did not affect pregnancy length, litter size at birth, male/female ratio, pup weight gain and postnatal vitality (Supporting Information Table S1).

Locomotor activity

Although there was a significant effect of sex on locomotor activity (two-way ANOVA), no differences between VPA-exposed and control animals of both sexes were found in locomotor activity both at adolescence and adulthood (data not shown).

Sex-specific effects of prenatal VPA exposure on social communication and social discrimination in the infant rat offspring

Prenatal VPA exposure differentially affected the USVs emitted by male and female pups separated from the nest on PND 9, with no effect of sex or of prenatal treatment but a significant interaction of (sex × prenatal treatment). Indeed, VPA-exposed male but not female pups vocalized significantly less (Figure 2A) compared with SAL-exposed pups. As

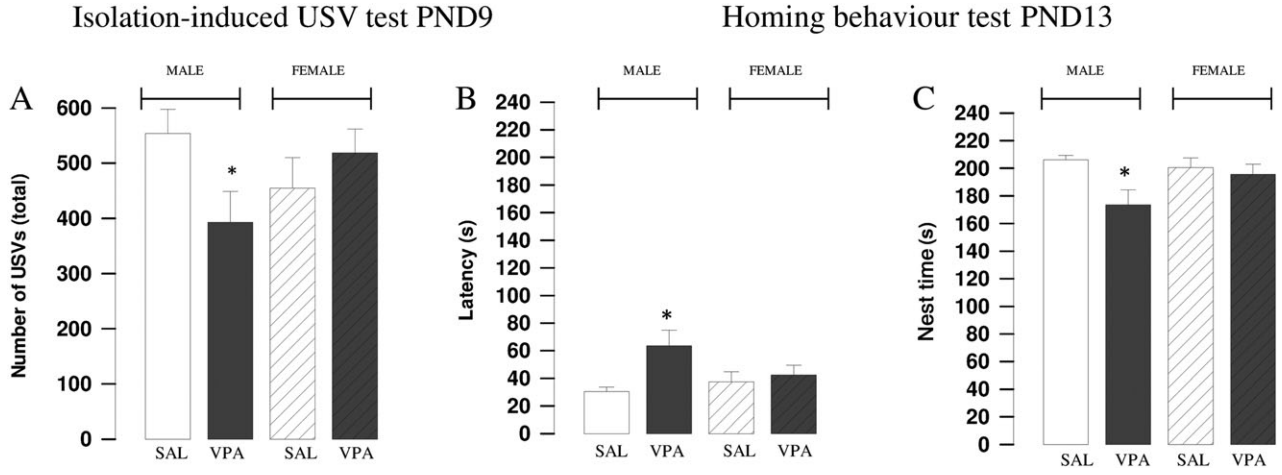

Figure 2

Sex-specific effects of prenatal VPA exposure on social communication and social discrimination in the infant rat offspring. (A) VPA-exposed male but not female pups vocalized significantly less compared with SAL-exposed pups (male: SAL, $n = 13$ and VPA, $n = 17$; female: SAL, $n = 13$ and VPA, $n = 16$). When tested in the homing behaviour test, the male but not the female offspring prenatally exposed to VPA displayed a lower latency to reach the home cage bedding (B) and spent less time in the nest area (C) compared with SAL-exposed male animals (male: SAL, $n = 24$ and VPA, $n = 26$; female: SAL, $n = 10$ and VPA, $n = 14$). Data are means ± SEM. *$P < 0.05$, significantly different from SAL group; two-way ANOVA with Student–Newman–Keuls *post hoc* test.

for the parameters measured in the homing behaviour test, analysis with two-way ANOVA showed significant effects only for prenatal treatment. *Post hoc* analysis revealed that VPA-exposed male but not female pups showed longer latency to reach the home cage bedding (Figure 2B) and spent less time in the nest area (Figure 2C) compared with SAL-exposed pups.

Sex-specific effects of prenatal VPA exposure on core and secondary autistic-like features in the adolescent rat offspring

A two-way ANOVA analysis performed on the parameters measured in the social play behaviour test gave the following results. For frequency of pinning (male: SAL = 15.6 ± 1.9, VPA = 13.7 ± 1.9; female: SAL = 3.8 ± 1.9, VPA = 2.7 ± 1.8) and for frequency of pouncing (male: SAL = 38.1 ± 3.4, VPA = 44.5 ± 3.4; female: SAL = 15.8 ± 3.4, VPA = 21.2 ± 3.1), there were significant effects of sex only. For the frequency of partial rotation there were significant effects of sex and prenatal treatment, for frequency of evasion there were significant effects of prenatal treatment and for play responsiveness (male: SAL = 49.9 ± 4.8, VPA = 32.8 ± 4.6; female: SAL 23.0 ± 4.3, VPA 9.4 ± 4.3) there were significant effects of sex and prenatal treatment. *Post hoc* analysis revealed that, when solicited to play by the test partner, VPA-exposed male and female rats responded with an increased frequency of evasion (Figure 3A) and partial rotation (Figure 3B) than SAL-exposed rats. However, general social exploration, although showing significant effects of sex differing between sexes, was not affected by prenatal exposure to VPA in both males and females (data not shown).

Prenatal VPA exposure induced sex-specific effects in the offspring tested in the hole board test with head dipping being affected significantly by sex, prenatal treatment and the interaction. In the elevated plus maze test, values for %

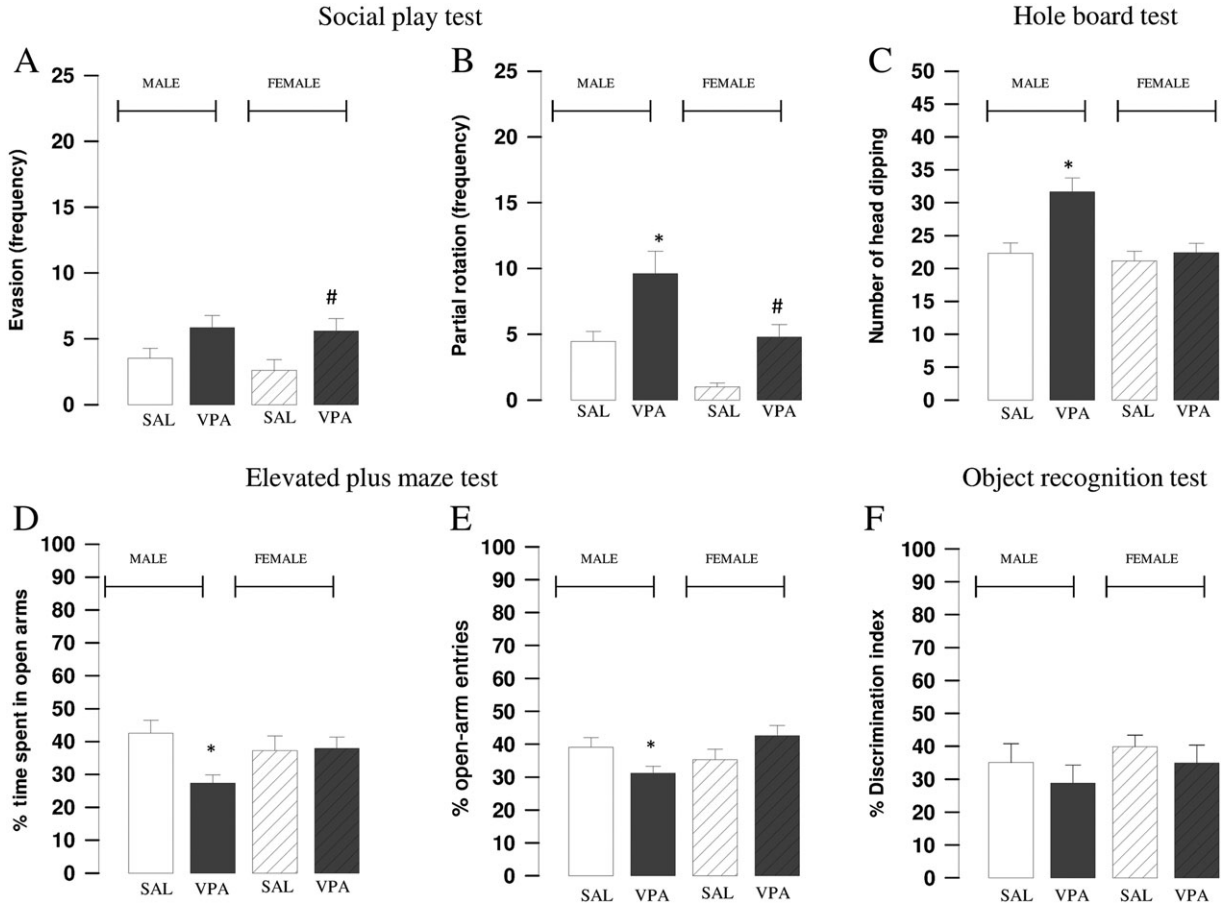

Figure 3

Effects of prenatal VPA exposure on core and secondary autistic-like features in the male and female rat offspring at PND 35. VPA-exposed male and female rats responded to play solicitation with an increased frequency of evasion (A) and partial rotation (B) compared with SAL-exposed animals (male: SAL, $n = 13$ and VPA, $n = 13$; female: SAL, $n = 13$ and VPA, $n = 15$). VPA-exposed males but not females showed stereotypic behaviours in the hole board test (C) compared with SAL-exposed male animals (male: SAL, $n = 12$ and VPA, $n = 12$; female: SAL, $n = 15$ and VPA, $n = 15$). VPA-exposed males but not females spent less time in the open arms (D) and made less open entries (E) in the elevated plus maze test compared with SAL-exposed male animals (male: SAL, $n = 23$ and VPA, $n = 21$; female: SAL, $n = 13$ and VPA, $n = 13$). No differences among groups were found in the object recognition test (F) (male: SAL, $n = 7$ and VPA, $n = 11$; female: SAL, $n = 10$ and VPA, $n = 11$). Data are means ± SEM. $^{*}P < 0.05$, significantly different from SAL group; $^{\#}P < 0.05$, significantly different from SAL group; two-way ANOVA with Student–Newman–Keuls *post hoc* test.

TO were affected only by the interaction, as were the values for % OE, measured at PND 35. Indeed, VPA-exposed males but not females showed increased head-dipping behaviour in the hole board test (Figure 3C), spent less time in the open arms of the elevated plus maze (Figure 3D) and made less open-arm entries (Figure 3E) compared with SAL-exposed animals. VPA-exposed male and female animals did not show impaired novel object recognition at PND 35 (Figure 3F).

Sex-specific effects of prenatal VPA exposure on core and secondary autistic-like features in the adult rat offspring

A two-way ANOVA analysis performed on the percentage of time spent by the experimental rat sniffing the stimulus animal in the three-chamber test showed there were significant effects of sex only. *Post hoc* analysis revealed that 90-day-old male, but not female, rats prenatally exposed to VPA showed decreased sociability, as they spent less time sniffing the stimulus animal (Figure 4A) compared with SAL-exposed rats. No difference between VPA-exposed and SAL-exposed animals of both sexes was found in the percentage of time spent exploring the empty chamber.

Prenatal VPA exposure induced stereotypic behaviour in the adult offspring of both sexes with frequency of head dipping showing there were significant effects of sex and of prenatal treatment. Compared with SAL-exposed animals, both VPA-exposed adult males (Figure 4B) and females (Figure 4B) showed an increased number of head dippings.

Prenatal VPA induced sex-specific effects in the adult offspring tested in the elevated plus maze test with % TO values showing there were significant effects of sex and the interaction (sex x prenatal treatment), whereas for the % OE values there were significant effects of sex, prenatal treatment and the interaction. Indeed, VPA-exposed males but not females spent less time in the open arms (Figure 4C) and made less open-arm entries (Figure 4D) compared with SAL-exposed animals.

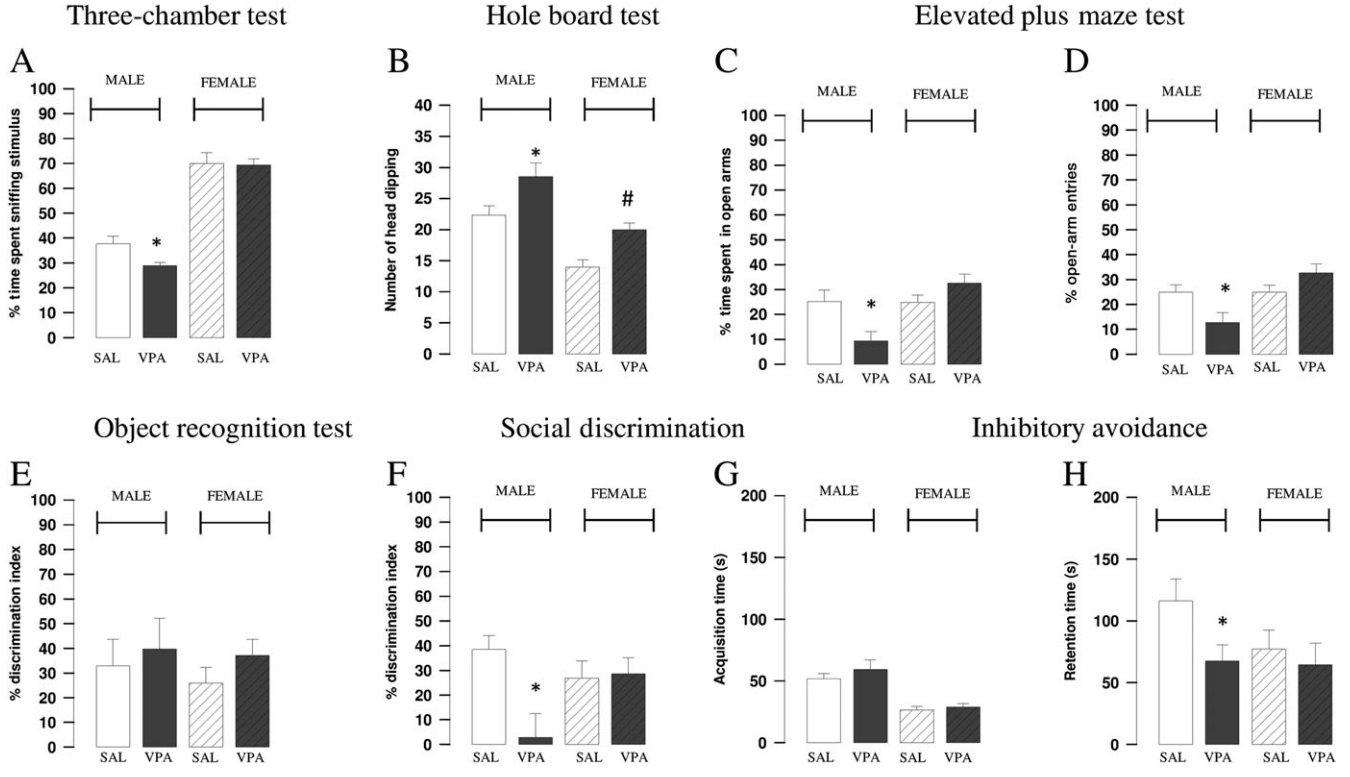

Figure 4

Effects of prenatal VPA exposure on core and secondary autistic-like features in the male and female rat offspring at PND 90. Prenatal VPA exposure reduced sociability of male but not female rats in the three-chamber test (A) (male: SAL, $n = 9$ and VPA, $n = 9$; female: SAL, $n = 8$ and VPA, $n = 7$) while it induced stereotypic behaviour in the hole board test both in male and female animals (B) (male: SAL, $n = 12$ and VPA, $n = 12$; female: SAL, $n = 15$ and VPA, $n = 15$). VPA-exposed male but not female rats spent less time in the open arms (C) and made less open-arm entries (D) in the elevated plus maze test (male: SAL, $n = 13$ and VPA, $n = 10$; female: SAL, $n = 16$ and VPA, $n = 16$). No differences among groups were found in the object recognition test (E) (male: SAL, $n = 12$ and VPA, $n = 10$; female: SAL, $n = 15$ and VPA, $n = 16$). Male but not female rats prenatally exposed to VPA showed impaired social discrimination (F) (male: SAL, $n = 11$ and VPA, $n = 11$; female: SAL, $n = 10$ and VPA, $n = 10$). No differences among groups were found in the acquisition trial of the inhibitory avoidance test (G). However, male but not female rats prenatally exposed to VPA showed impaired memory consolidation during the retention session (H) (male: SAL, $n = 15$ and VPA, $n = 15$; female: SAL, $n = 12$ and VPA, $n = 14$). Data are means ± SEM. $^{*}P < 0.05$, significantly different from SAL group; $^{\#}P < 0.05$, significantly different from SAL group; two-way ANOVA with Student–Newman–Keuls *post hoc* test.

Similar to the test results obtained on PND 35, VPA-exposed male and female animals did not show impaired novel object recognition at PND 90 (Figure 3E). However, VPA exposure did induce sex-specific deficits in social discrimination, by prenatal treatment and for the interaction. VPA-exposed adult males but not females showed a lower discrimination index (Figure 4F) compared with SAL-exposed animals.

Approach latencies during the first trial of the inhibitory avoidance test were similar among groups, although there were significant effects of sex (Figure 4G). However, after 24 h, prenatal VPA exposure induced sex-specific effects on memory retention . Males, but not females, prenatally exposed to VPA had retention latencies significantly shorter than SAL-exposed rats (Figure 4H), indicating a decreased ability of VPA-exposed males to consolidate aversive memories.

Sex-specific, age-specific and tissue-specific changes in phosphorylation of CB_1 receptors

Phosphorylated CB_1 receptors, which may reflect the activation of this receptor (Garcia *et al.*, 1998; Daigle *et al.*, 2008), are abundant in brain areas such as the nucleus accumbens and the amygdala (Orio *et al.*, 2009), which are also involved in anandamide modulation of social reward (Trezza *et al.*, 2012). To investigate whether VPA prenatal exposure induced changes in the activity of male and female brain CB receptors, we measured phosphorylated and total CB_1 receptor protein expression in prefrontal cortex, dorsal striatum, hippocampus, amygdala, nucleus accumbens and cerebellum of male and female rats at PNDs 35 and 90. The results of the two-way ANOVA analyses performed for each brain region at both ages are shown in Supporting Information Table S2 (ratio between phosphorylated and total CB_1 receptors and Supporting Information Table S3 (total content of CB_1 receptors), while the original Western blots for each data set are shown in Figure 5. *Post hoc* analyses revealed that, compared with SAL-exposed animals, VPA-exposed males but not females displayed increased phosphorylation of CB_1 receptors at PNDs 35 and 90 in the dorsal striatum (Figure 5A, E), reduced phosphorylation of CB_1 receptors in the hippocampus at both ages (Figure 5C, G) and only at PND 90 in the amygdala (Figure 5H). Conversely, VPA-exposed females displayed increased phosphorylation of CB_1 receptors in the prefrontal cortex only at PND 35 (Figure 5B).

Pharmacological blockade of anandamide hydrolysis corrects the partial ASD-like symptoms displayed by VPA-exposed female rats

Systemic administration of the anandamide hydrolysis inhibitor URB597 has been found to rescue the communicative deficits, the socio-emotional alterations and the stereotypies displayed by VPA-exposed male rats (Servadio *et al.*, 2016). Here, we showed that URB597 also mitigated the altered social play patterns displayed by VPA-exposed female rats at PND 35. For frequency of partial rotation, there were significant effects of prenatal treatment (VPA), of treatment (URB597) and of the interaction (prenatal treatment x treatment). URB597 was also able to mitigate the stereotypic behaviour displayed by female rats at PND 90, as significant effects of prenatal treatment and the interaction were found. Indeed, *post hoc* analyses showed that at PND 35, VPA-exposed females responded to play solicitation with a higher frequency of partial rotation (Figure 6A) and evasion (Figure 6B) compared with SAL-exposed rats. However, when they were treated with URB597, the frequency of partial rotation was normalized to the level of SAL-exposed rats, while the frequency of evasion was attenuated. Furthermore, VPA-exposed females displayed stereotypies at PND 90 (Figure 6C). However, when they were treated with URB597, this parameter was normalized to the level of SAL-exposed rats.

Pharmacological blockade of anandamide hydrolysis corrects the cognitive deficits displayed by VPA-exposed male rats

Systemic administration of URB597 counteracted the deficits displayed by VPA-exposed adult males in social discrimination (significant effects of treatment and the interaction). For the inhibitory avoidance task, the acquisition time showed no significant effects and retention time was significantly affected only by the interaction (prenatal treatment x treatment). *Post hoc* analysis revealed that, compared with SAL-exposed rats, VPA-exposed animals showed impaired social discrimination (Figure 6D) and memory retention (Figure 6F), which were normalized when VPA-exposed animals were treated with URB597.

Discussion

We found that prenatal exposure to VPA, an environmental risk factor for ASD, induced sex-specific autistic-like features in the rat offspring. While VPA induced a wide range of socio-emotional and cognitive deficits in the male offspring in the course of development, the female offspring was only partly affected. Despite these sex-specific endophenotypes, we identified a pharmacological target to correct the behavioural deficits displayed by VPA-exposed rats of both sexes.

Persistent deficits in social communication and social interaction are key features of ASD, and it has been suggested that the delayed or missed diagnosis of ASD in girls may be related to marked sex differences in the phenotypic presentation of these symptoms (Rivet and Matson, 2011b; Lai and Baron-Cohen, 2015; Bargiela *et al.*, 2016). Consistent with this possibility, at infancy, male but not female pups prenatally exposed to VPA showed communicative deficits, since they emitted less USVs than control pups when isolated from the dam and siblings. In rodents, these USVs play a fundamental role in mother–offspring interactions, as they induce maternal retrieval and elicit care-giving behaviours in the dam (Servadio *et al.*, 2015).

Because olfaction and, in particular, the learned association between maternal odours and maternal stimulation, is essential for the proper development of social behaviour and social recognition (Melo *et al.*, 2006), we next analysed the ability of male and female pups to discriminate between a neutral odour and their own nest odour in the homing behaviour test. While male pups prenatally exposed to VPA showed early deficits in social discrimination in this test, VPA-exposed female pups showed intact discriminative

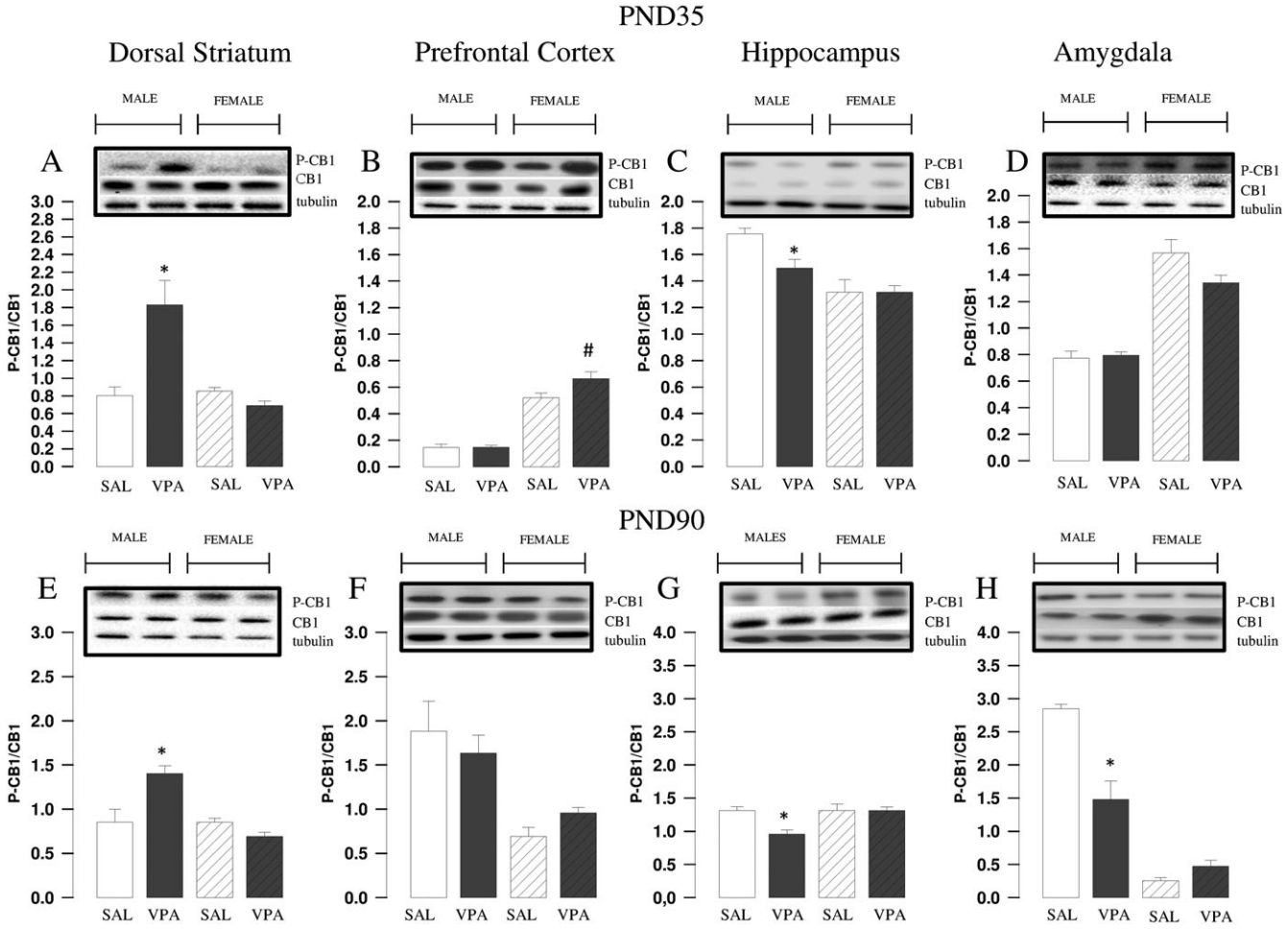

Figure 5

Sex-specific, age-specific and tissue-specific changes in phosphorylation of CB_1 receptors induced by prenatal VPA exposure. VPA-exposed male but not female rats displayed altered phosphorylation of CB_1 receptors in dorsal striatum [PND 35 (A), male: SAL, $n = 5$ and VPA, $n = 5$; female: SAL, $n = 5$ and VPA, $n = 6$; PND 90 (E), male: SAL, $n = 5$ and VPA, $n = 5$; female: SAL, $n = 5$ and VPA, $n = 6$] and hippocampus [PND 35 (C), male: SAL, $n = 10$ and VPA, $n = 8$; female: SAL, $n = 5$ and VPA, $n = 6$; PND 90 (G), male: SAL, $n = 8$ and VPA, $n = 9$; female: SAL, $n = 5$ and VPA, $n = 6$]. VPA-exposed male but not female rats displayed reduced phosphorylation of CB_1 receptors at PND 90 in the amygdala (H) (male: SAL, $n = 6$ and VPA, $n = 10$; female: SAL, $n = 5$ and VPA, $n = 6$). Conversely, VPA-exposed female rats displayed increased phosphorylation of CB_1 receptors in the prefrontal cortex only at PND 35 (B) (male: SAL, $n = 6$ and VPA, $n = 6$; female: SAL, $n = 4$ and VPA, $n = 5$). Data are means ± SEM. $^*P < 0.05$, significantly different from SAL group; $^{\#}P < 0.05$, significantly different from SAL group; two-way ANOVA with Student–Newman–Keuls *post hoc* test.

abilities. The sex-related differences observed in the homing behaviour test may be due to a sex-specific alteration of the olfactory system in the course of development. Furthermore, given the role of the endocannabinoid system in olfactory processes (Soria-Gomez *et al.*, 2014), changes in endocannabinoid activity may also be involved.

Together, these results show that, at infancy, female rat pups are less vulnerable than males to the deleterious effects induced by prenatal VPA exposure on social communication and social discrimination.

In rats, play behaviour peaks during adolescence, and it is considered the first form of non-maternal-oriented social behaviour, whose practice is crucial for social, cognitive, emotional and sensorimotor development (Vanderschuren *et al.*, 2016). At adolescence, both VPA-exposed male and female rats showed atypical patterns of social play behaviour. They responded to play solicitation mainly by partial rotation and evasion, rather than reciprocating the playful interaction. However, only the male but not the female VPA-exposed offspring showed enduring social deficits when tested at adulthood in the three-chamber test. A possible explanation of this finding is that prenatal VPA exposure induces in male rats a wide range of social impairments in the course of development, ranging from social play deficits at adolescence to altered sociability at adulthood. Conversely, the social deficits displayed by female rats prenatally exposed to VPA may be restricted to play-related behaviours at adolescence. To support this possibility, 4-week-old male but not female rats prenatally exposed to VPA showed altered sociability in the three-chamber test (Kerr *et al.*, 2016).

These findings are in line with the observation that female children, adolescents and adults with ASD demonstrate fewer socio-communicative symptoms compared with their male counterparts (Lai *et al.*, 2011; Dworzynski *et al.*, 2012;

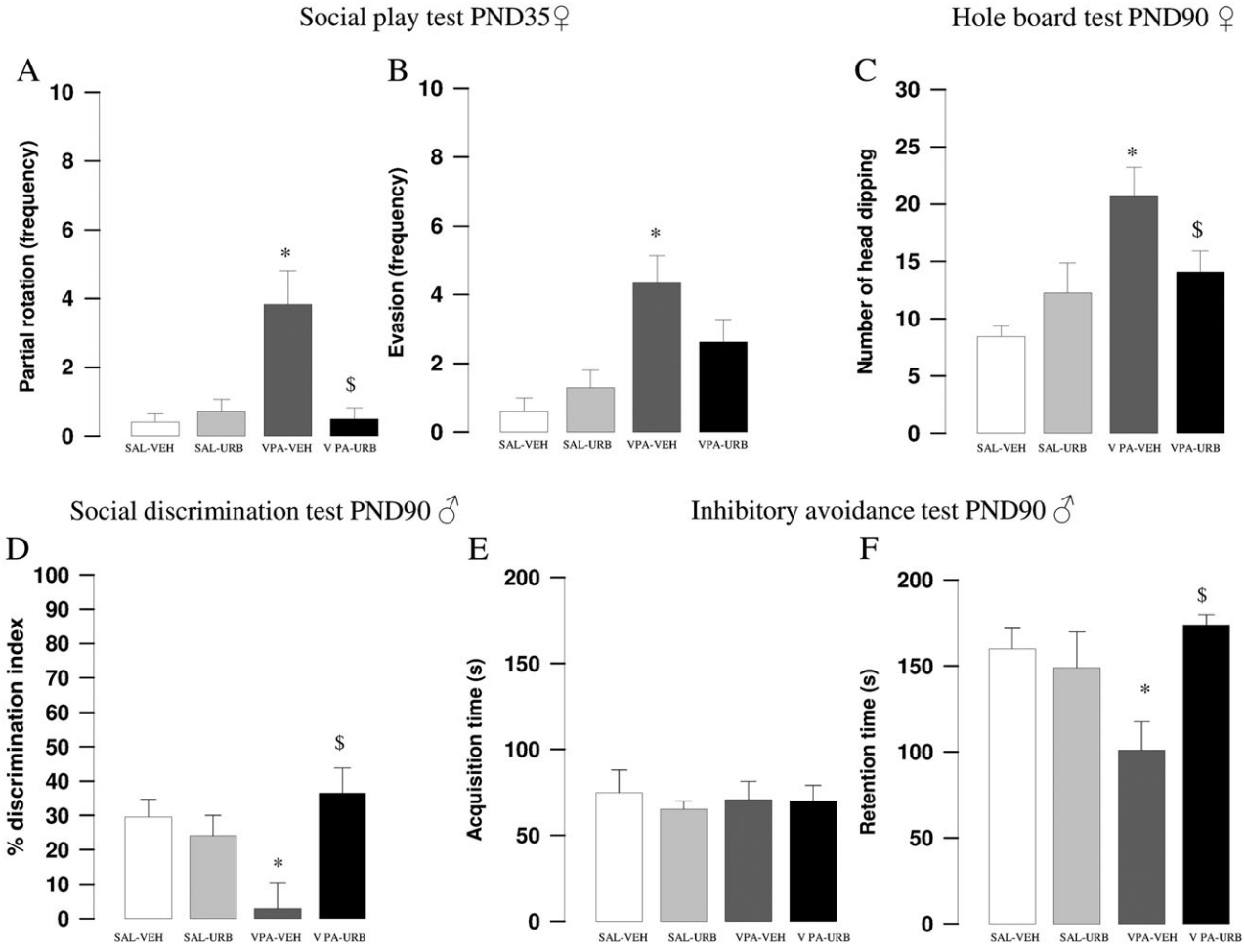

Figure 6

Pharmacological interference with anandamide hydrolysis corrects the partial behavioural alterations displayed by VPA-exposed female rats and the cognitive deficit found in VPA-exposed male rats. The administration of URB597 normalized the altered pattern of social play behaviour (A, B) displayed by VPA-exposed female rats at PND 35 [SAL-vehicle (VEH), $n = 5$; SAL-URB, $n = 7$; VPA-VEH, $n = 6$; and VPA-URB, $n = 8$] and their stereotypic behaviour at PND 90 (C) (SAL-VEH, $n = 7$; SAL-URB, $n = 8$; VPA-VEH, $n = 9$; and VPA-URB, $n = 10$). URB597 also reversed the deficits displayed by VPA-exposed male rats in the social discrimination (D) (SAL-VEH, $n = 8$; VPA-VEH, $n = 9$; SAL-URB, $n = 8$; and VPA-URB, $n = 8$) and inhibitory avoidance (E, F) (SAL-VEH, $n = 14$; VPA-VEH, $n = 12$; SAL-URB, $n = 8$; and VPA-URB, $n = 7$) tests. Data are means ± SEM. $^{*}P < 0.05$, significantly different from SAL-VEH group; $^{\$}P < 0.05$, significantly different from VPA-VEH group; two-way ANOVA with Student–Newman–Keuls *post hoc* test.

Head *et al.*, 2014), making it difficult to recognize ASD in female subjects on the basis of their social and communicative competences.

Stereotypies and/or restricted interests are the second core symptoms of ASD and seem to be more evident in boys rather than in girls with ASD (Hartley and Sikora, 2009; Supekar and Menon, 2015). Accordingly, VPA-exposed male rats showed marked stereotypies both at adolescence and adulthood. Conversely, as previously reported (Schneider *et al.*, 2008), VPA-exposed female rats showed stereotypic behaviours only at adulthood. Interestingly, behavioural differences were found between male and female rats in baseline condition, with control females spending more time sniffing the stimulus cage of the three-chamber apparatus and making less head dips in the hole board test compared with control males. These sex-specific differences may be due to a more explorative behaviour found in females rather than males, as previously reported (Lynn and Brown, 2009).

Anxiety is a common co-morbid feature displayed by autistic patients (Lai *et al.*, 2014). We found that VPA-exposed male, but not female, rats showed an anxious-like phenotype both in adolescence, as previously reported (Schneider *et al.*, 2008), and in adulthood.

ASD patients frequently display atypical cognitive performance, such as impaired social cognition (Lai *et al.*, 2014), while object recognition is often intact (Dawson *et al.*, 2002). Interestingly, female patients demonstrate better access to emotionally salient memories than males (Goddard *et al.*, 2014). Consistent with these findings, we found that male, but not female, rats prenatally exposed to VPA showed deficits in social discrimination from infancy till adulthood. Furthermore, VPA-exposed male but not female adult rats showed impaired emotional memory in the inhibitory avoidance task. Both VPA-exposed male and female rats, however, showed intact object recognition, as previously reported in male animals only (Schneider *et al.*, 2007; Banerjee *et al.*, 2014).

The attenuated ASD-like phenotype found in VPA-exposed female rats mirrors the sex differences in the symptoms displayed by ASD patients. It is unlikely that these sex differences are due to interference by sex hormones, with the teratogenic potential of VPA. Indeed, equal morphological changes are observed in the brain of male and female rats exposed to the same dose of VPA used in the present study (Favre *et al.*, 2013). Moreover, oestrogen receptors are not detected in the rat brain before GD 16 (Miranda and Toran-Allerand, 1992). It is more likely that the ASD-like attenuated phenotype found in VPA-exposed female rats arise in the course of development. Indeed, sexual dimorphism exists in the expression of genes related to ASD, with males being more susceptible than females to perturbations in genes involved in synaptic plasticity. Furthermore, sexually dimorphic neural pathways are involved in synaptic structure, function and plasticity. Thus, a higher male-to-female ratio in autism may arise because males have a lower threshold than females for aberrant changes in synaptic dynamics during development following a genetic or environmental insult (Mottron *et al.*, 2015). In line with this possibility, a perturbed synaptic maturation due to an altered glutamatergic neuronal differentiation was found in VPA-exposed male but not female rats (Kim *et al.*, 2013).

Endocannabinoids play a key role in brain development and synaptic plasticity (Maccarrone *et al.*, 2014), and they have been recently involved in ASD. Indeed, a consistent number of studies indicated that endocannabinoids modulate several behaviours and processes that are compromised in ASD. Furthermore, an impaired endocannabinoid activity has been observed in preclinical models of ASD (Kerr *et al.*, 2013; Zamberletti *et al.*, 2017) and in ASD patients (Siniscalco *et al.*, 2013). Sex hormones have been found to modulate brain endocannabinoid activity, thus influencing cannabinoid-mediated physiopathological processes (Fattore and Fratta, 2010; Viveros *et al.*, 2011). Thus, an alternative explanation to the sexually dimorphic behavioural consequences of prenatal VPA exposure may involve a sex-specific effect on endocannabinoid neurotransmission in the course of development.

In line with this possibility, we here report that male rats prenatally exposed to VPA display altered phosphorylation of CB_1 receptors in the dorsal striatum and hippocampus both at adolescence and adulthood and in the amygdala at adulthood only. Conversely, VPA-exposed female rats display altered activation of CB_1 receptors only in the prefrontal cortex at adolescence. The altered activation of CB_1 receptors found in the dorsal striatum, hippocampus and amygdala of male rats prenatally exposed to VPA may underlie their profound behavioural deficits in the socio-emotional and cognitive domains, given the well-known role of endocannabinoid neurotransmission within these brain areas in the control of emotional and cognitive states (Freund *et al.*, 2003).

Enhancement of anandamide activity by inhibiting its degradation ameliorates the socio-emotional and communicative deficits and the stereotypies displayed by VPA-exposed male rats (Kerr *et al.*, 2016; Servadio *et al.*, 2016). Here, we have extended these findings by showing that pharmacological interference with anandamide metabolism ameliorated (i) the deficits in social discrimination and emotional memory displayed by VPA-exposed adult male rat and (ii) the atypical social phenotype and the stereotypies displayed by VPA-exposed female rats.

Overall, two main conclusions can be drawn from our results. First, sex-specific changes in endocannabinoid neurotransmission may underlie the deleterious effects of environmental risk factors on ASD-relevant behaviours. Second, although more studies are needed to test the consequences of chronic inhibition of anandamide hydrolysis, our study points to an important role of this endocannabinoid in the autistic-like traits displayed by male and female VPA-exposed rats. Thus, the endocannabinoid system may be a therapeutic target for the core and associated symptoms displayed by autistic patients of both sexes.

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Author contributions

S.S., F.M. and M.S. performed, analysed and contributed to the design of the behavioural experiments. P.C., V.P. and M.P. contributed to the design of the experiments and edited the manuscript. V.C. performed, analysed and contributed to the design of the biochemical experiments. S.S. and F.M. wrote the manuscript. V.T. supervised the project, designed the experiments and wrote the manuscript.

Conflict of interest

The authors declare that, except for income received from their primary employers, no financial support or compensation has been received from any individual or corporate entity over the past 5 years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Table S1 Reproduction parameters.
Table S2 Two-way ANOVA analyses performed on the Western blot data (ratio between phosphorylated and total CB1 cannabinoid receptor) for each brain region at PNDs 35 and 90.
Table S3 Two-way ANOVA analyses performed on the Western blot data (total CB1 cannabinoid receptor) for each brain region at PNDs 35 and 90.