

Research report

Prenatal exposure to valproic acid reduces social responses and alters mRNA levels of opioid receptor and pre-pro-peptide in discrete brain regions of adolescent and adult male rats

Edel M. Hughes^{a,c}, Patricia Calcagno^{a,b}, Morgane Clarke^{a,b}, Connie Sanchez^d, Karen Smith^d, John P. Kelly^{b,c}, David P. Finn^{b,c}, Michelle Roche^{a,c,*}

^a Physiology, School of Medicine, National University of Ireland, Galway, University Road, Galway, Ireland

^b Pharmacology and Therapeutics, School of Medicine, National University of Ireland, Galway, University Road, Galway, Ireland

^c Centre for Pain Research and Galway Neuroscience Centre, National University of Ireland, Galway, Ireland

^d Alkermes Inc., Waltham, MA, USA

HIGHLIGHTS

- Adolescent and adult rats prenatally exposed to VPA display reduced social responding.
- VPA-exposed adolescent and adult rats exhibited reduced *OPRK1* and *PDYN* in discrete brain regions.
- Reduced *OPRK1-PDYN* expression may underlie the deficits in social responding in VPA rat model.

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ABSTRACT

Altered social behaviours are a hallmark of several psychiatric and developmental disorders. Clinical and pre-clinical data have demonstrated that prenatal exposure to valproic acid (VPA), an anti-epileptic and mood stabiliser, is associated with impaired social responses, and thus provides a useful model for the evaluation of neurobiological mechanisms underlying altered social behaviours. The opioid system is widely recognised to regulate and modulate social behaviours, however few studies have examined if the endogenous opioid system is altered in animal models of social impairment. The present study examined social behavioural responses of adolescent and adult male rats prenatally exposed to VPA, and the expression of mRNA encoding opioid receptors and pre-pro-peptides in discrete brain regions. Adolescent and adult rats prenatally exposed to VPA spent less time engaging in social behaviours in the direct social interaction test and exhibited reduced sociability and social novelty preference in the 3-chamber sociability test, compared to saline-treated counterparts. The VPA-exposed adolescent rats exhibited significantly reduced kappa opioid receptor (*oprk1*) and pre-pro-dynorphin (*pdyn*) mRNA expression in the cerebral cortex, and reduced *oprk1* and nociceptin/orphanin FQ (*oprl1*) mRNA expression in the hypothalamus. Adult rats prenatally exposed to VPA exhibited decreased mRNA expression of *oprk1* and *pdyn* in hypothalamus, reduced pro-opiomelanocortin (*pomc*) in the striatum and an increase in delta opioid receptor (*oprd1*) mRNA in the amygdaloid cortex, when compared to saline-treated counterparts. Mu opioid receptor (*oprm1*) mRNA expression did not differ between saline and VPA-exposed rats in any region examined. The data demonstrate that impaired social behaviours in adolescent and adult rats prenatally exposed to VPA is accompanied by altered mRNA expression of opioid receptors and pre-pro-peptides in a region specific manner. In particular, both adolescent and adult VPA-exposed rats exhibit reduced *oprk1-pdyn* mRNA expression in several brain regions, which are associated with deficits in social behavioural responding in the model.

Abbreviations: DOP, delta-opioid receptor; *oprd1*, delta opioid receptor gene; DYN, dynorphin; ENK, enkephalin; KOP, kappa-opioid receptor; *oprk1*, kappa opioid receptor gene; MOP, mu-opioid receptor; *oprm1*, mu opioid receptor gene; N/OFQ, nociceptin; *oprl1*, nociceptin/orphanin FQ gene; *pdyn*, pre-pro-dynorphin gene; *pomc*, pro-opiomelanocortin gene; VPA, valproic acid

* Corresponding author at: Physiology, School of Medicine, National University of Ireland, Galway, University Road, Galway, Ireland.

E-mail address: Michelle.roche@nuigalway.ie (M. Roche).

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1. Introduction

Increasing evidence indicates that exposure to environmental factors during early life confers a significant risk for the development of psychiatric disorders such as depression, anxiety and autism spectrum disorders. For example, ingestion of the anti-epileptic and mood stabiliser valproic acid (VPA) during pregnancy is associated with impaired neural tube closure, anatomical and molecular changes in the offspring resulting in altered behavioural responses including social anhedonia, repetitive behaviour and communication deficits (Bromley et al., 2010, 2016; Ornoy, 2009). Replication of these findings in rodents prenatally exposed to VPA has been proposed as a useful model to study the neurobiology associated with impaired social responses (reviewed in Nicolini and Fahnstock (2018)). A wealth of studies over the past 5 years has seen social behaviours being assessed, with consistent reporting of deficits in social play, approach/avoidance, social motivation and cognition in rodents prenatally exposed to VPA (Campolongo et al., 2018; Du et al., 2017; Melancia et al., 2018; Qin et al., 2016). However, to our knowledge, only one study has directly compared the effect of prenatal VPA exposure on social responses in multiple social paradigms at different developmental stages. Servadio et al. (2016) demonstrated that rats prenatally exposed to VPA exhibit impaired sociability in the 3-chamber test in both adolescence and adulthood, and reduced social play behaviour in adolescence. While this study demonstrated a deficit in social behaviours across both developmental time points, the authors did not examine social novelty preference behaviour, a measure of social cognition, or assess direct social interactive behaviour of rats during adulthood.

Social responses are mediated and modulated by numerous neurotransmitter and neuropeptide systems, with the endogenous opioid system well recognised to play an important role (for review see Pellissier et al. (2017)). The endogenous opioid system is comprised of four main types of receptors; Mu (MOP), Kappa (KOP), Delta (DOP) and

nociceptin/orphaninFQ (NOP), which are encoded by distinct genes and activated by the endogenous opioid ligands β -endorphin, dynorphin (DYN), enkephalin (ENK) and nociception (N/OFQ). Opioid receptors and peptides are highly expressed in key brain regions such as the prefrontal cortex, amygdala, hippocampus and nucleus accumbens which govern social motivation, cognition and reward (Felix-Ortiz and Tye, 2014; Gunaydin et al., 2014; Ko, 2017; Mansour et al., 1994). Multiple studies have demonstrated that pharmacological modulation of opioid receptors alters social responses in rodents, effects dependant on the opioid modulator, context and condition under examination (Losey et al., 2014; Smith et al., 2017). Despite the wealth of research in this area, there are few studies have examined changes in opioid receptor expression in preclinical models which exhibit social impairments. Early life stress and pain represent a significant risk factor for the development of social anhedonia and both have been demonstrated to elicit significant changes in opioid receptor and peptide mRNA in key brain regions that modulate social responses (Chang et al., 2019; Victoria et al., 2013). Only two studies to date have examined changes in the endogenous opioid system in rodents prenatally exposed to VPA. Schneider et al. (2007) demonstrated reduced *penk* mRNA levels in the dorsal striatum and nucleus accumbens of adult rats prenatally exposed to VPA. These alterations were accompanied by a diminished response to the opioid antagonist naloxone in the conditioned place avoidance task (Schneider et al., 2007), indicating altered opioid functionality and reward processing in adult rats prenatally exposed to VPA. More recently, MOP-positive striosomes were found to be reduced in the caudal and rostral striatum of 14-day old mice prenatally exposed to VPA (Kuo and Liu, 2017). However, it is unknown if prenatal exposure to VPA alters the expression of other opioid receptors or peptides, and whether such effects are present across developmental ages.

The aim of the present study was to directly compare the effect of prenatal VPA exposure on social reward, motivation and cognition in rats during both adolescence and adulthood. In addition, the

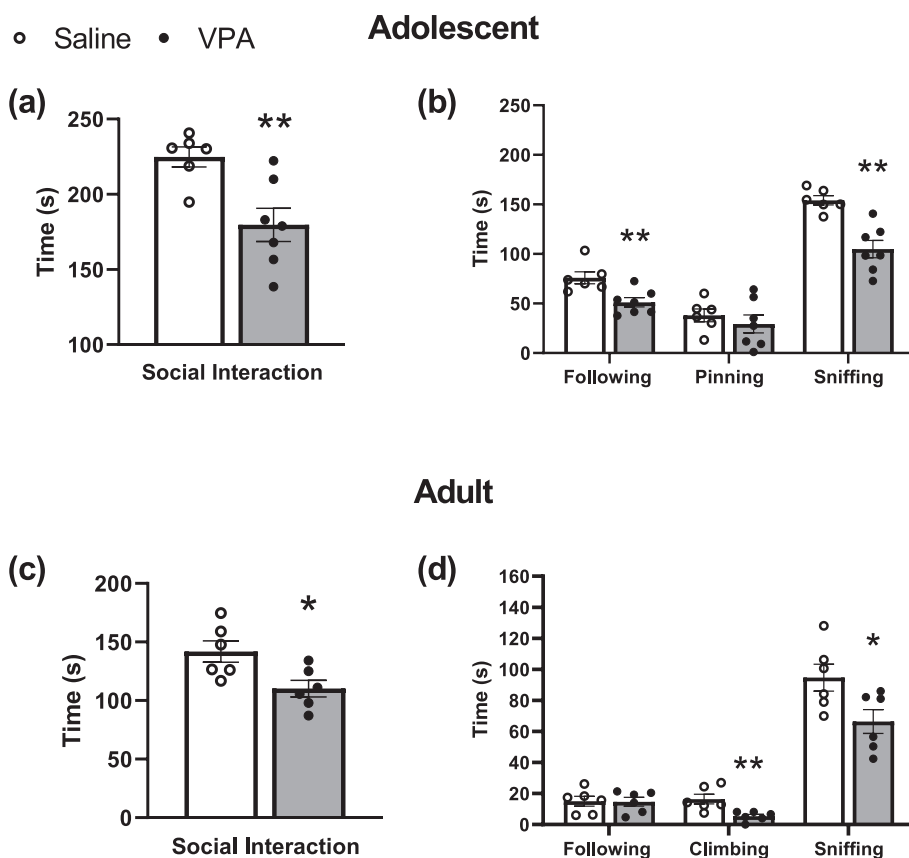
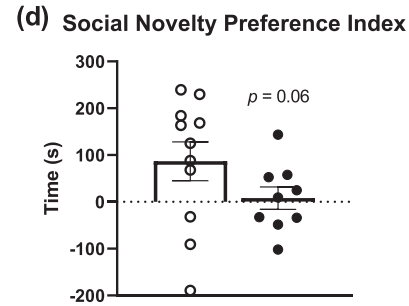
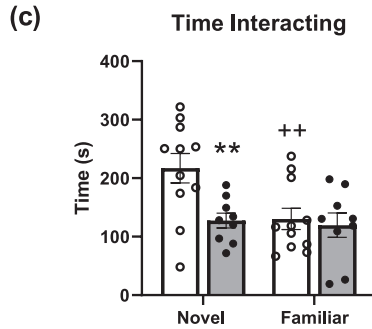
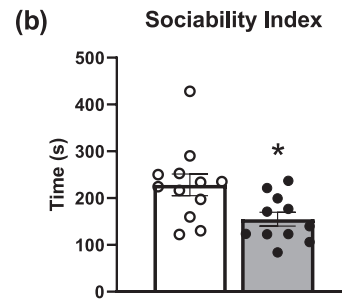
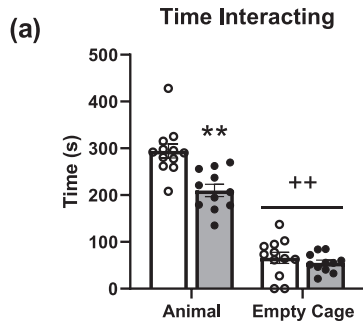


Fig. 1. The effect of prenatal exposure to VPA or saline on the duration of time spent (s) (a) directly interacting, and (b) following, pinning and sniffing by pairs of adolescent male rats or (c) directly interacting, and (d) following, climbing over and under and sniffing by pairs of adult male rats over a 5-minute period. Data presented as individual data points and mean \pm SEM. N = 6–7 pairs/group. * $p < 0.05$ vs saline-exposed counterpart.

○ Saline ● VPA

Adolescent



Adult

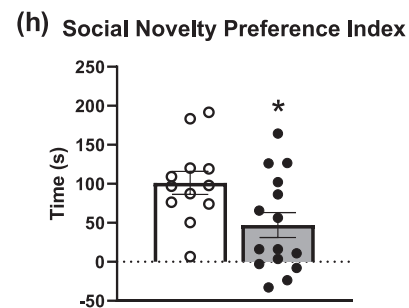
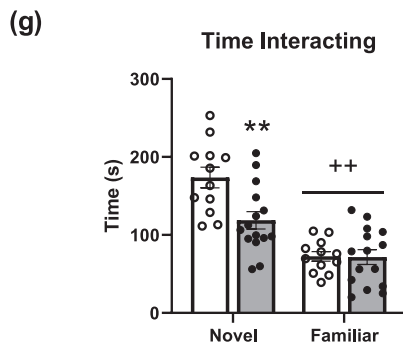
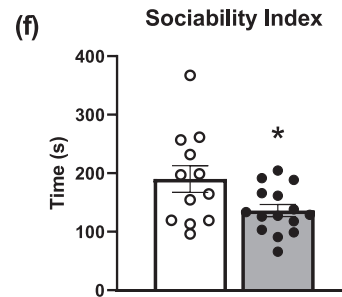
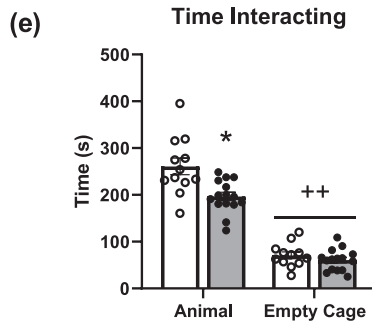


Fig. 2. The effect of prenatal exposure to saline or VPA on sociability and social novelty preference of male adolescent (a–d) or adult (e–h) rats in the 3-chamber test. Data presented as individual data points and mean ± SEM. N = 11–12/group. ** $p < 0.01$, * $p < 0.05$ vs saline-exposed counterparts, ++ $p < 0.01$, + $p < 0.05$ vs corresponding animal/novel group.

developmental consequences of prenatal exposure to VPA on opioid receptor and pre-pro-peptide mRNA expression was assessed in brain regions known to mediate and modulate social responses.

2. Results

2.1. The effect of prenatal exposure to VPA on direct social interaction behaviour of male rats during adolescence and adulthood

Social play and reciprocal interaction in rodents are inherently rewarding and as such the direct social interaction test is a key tool in assessing the functionality of the social reward circuitry.

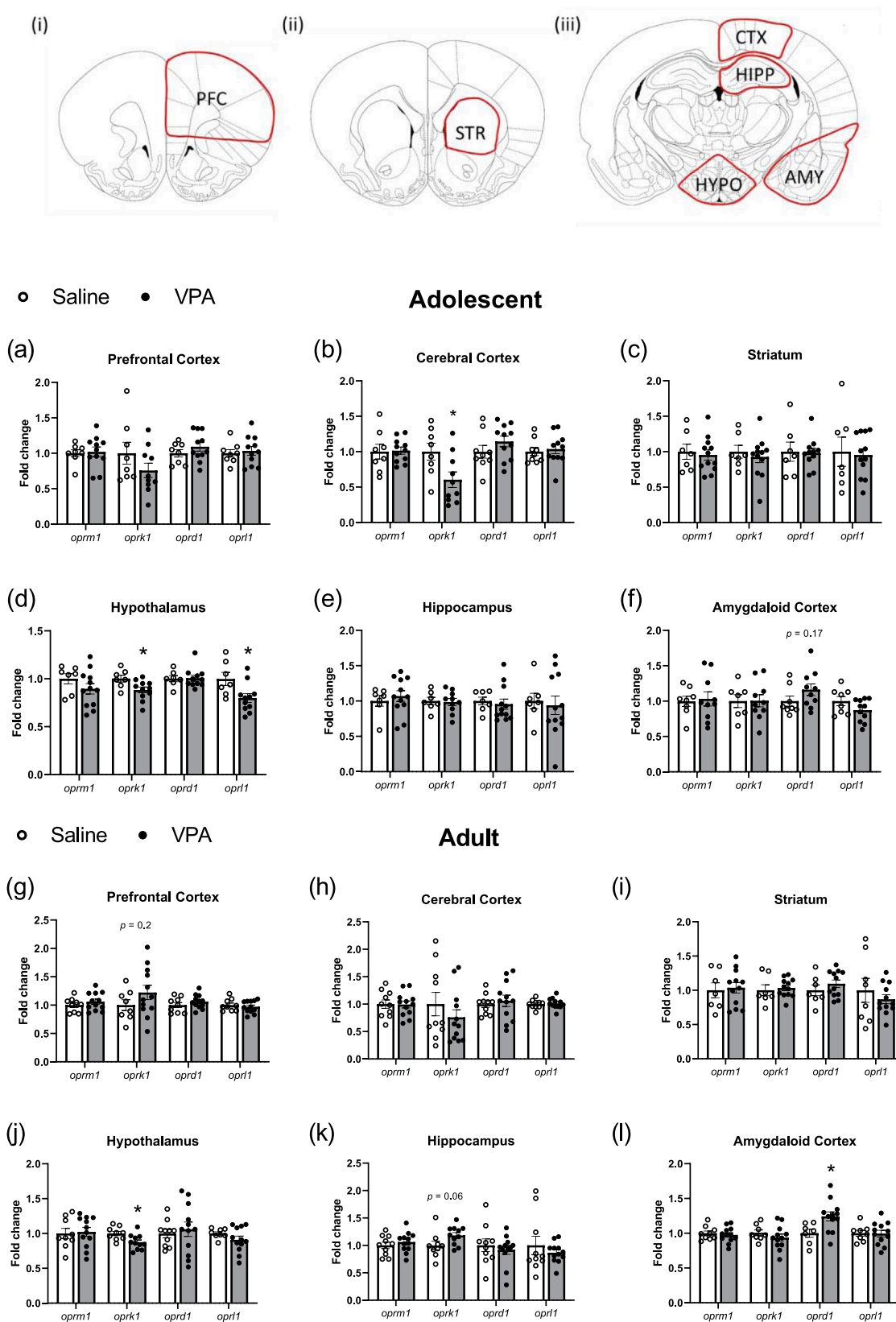


Fig. 3. Schematic representation of the brain regions dissected for qRT-PCR analysis (i-iii). PFC, prefrontal cortex; STR, striatum (dorsal); CTX, cortex; HIPP, hippocampus (dorsal); HYPO, hypothalamus; AMY, amygdaloid cortex (Paxinos, 2006). mRNA expression of endogenous opioid receptors in discrete brain regions of adolescent (a–f) and adult (g–l) rats prenatally exposed to saline or VPA. Data presented as fold change from average of saline exposed group. Individual data and mean \pm SEM presented. N = 10–12/group. * $p < 0.05$ vs saline-exposed counterparts.

Pairs of adolescent rats prenatally exposed to VPA spent significantly less time (s) interacting when compared to pairs of saline-treated rats in the first 5 min of the trail ($t(11) = 3.4, p = 0.006$) of (Fig. 2a). Analysis of the three most prominent social interactive behaviours during this test, following, pinning and sniffing, revealed that pairs of VPA-exposed animals exhibited less time following ($t(11) = 3.3, p = 0.007$) and sniffing ($t(11) = 4.7, p = 0.001$) when compared to saline-exposed counterparts (Fig. 1b). There was no effect of prenatal VPA exposure on duration of pinning during the trial. There was no significant effect of VPA on social interactive behaviour in the 5–10 min period of the trial (data not shown).

Pairs of adult rats prenatally exposed to VPA spent significantly less time interacting when compared to pairs of saline-treated rats in first 5 min ($t(10) = 2.7, p = 0.02$) of the 10-minute testing period (Fig. 1c). Analysis of specific behavioural traits revealed that rats prenatally exposed to VPA spent significantly less time (s) climbing ($t(10) = 3.3, p = 0.008$; Fig. 2i) and sniffing ($t(10) = 2.4, p = 0.04$ Fig. 1d) when compared to saline-exposed counterparts. There was no effect of prenatal VPA exposure on social interactive behaviours during the 5–10 min period of the trail (data not shown).

2.2. Prenatal exposure to VPA results in reduced sociability and social novelty preference of adolescent and adult male rats

The 3-chamber test is designed such that the test animal is free to choose whether to engage with the social stimulus or not. As such this test is used to assess social motivation (sociability) and social cognition (novelty preference).

Two way ANOVA revealed a significant effect of stimulus ($F(1,42) = 94.6, p < 0.001$) and VPA ($F(1,42) = 11.1, p = 0.002$) on time spent interacting with the animal vs novel cage. *Post hoc* analysis revealed that all animals exhibited a preference for interacting with the animal over the novel empty cage ($p < 0.001$). However, VPA-exposed rats spent significantly less time interacting with the animal when compared to saline-exposed counterparts ($p = 0.006$) (Fig. 2a). From the duration of time interacting, a sociability index was calculated by subtracting the duration of time spent interacting with the novel object from the time interacting with the animal. Analysis revealed that adolescent VPA-exposed rats exhibited a reduced sociability index ($t(21) = 2.6, p = 0.02$) when compared to saline-exposed counterparts (Fig. 2b).

Analysis of the social novelty preference trial revealed a significant effect of VPA ($F(1,36) = 6.02, p = 0.02$) and stimulus ($F(1,36) = 5.4, p = 0.03$) on the duration of time spent interacting with the novel or familiar animal. *Post hoc* analysis revealed that saline-, but not VPA-, exposed rats exhibited a preference for the novel vs familiar animal ($p = 0.003$), and VPA-exposed rats spent significantly less time interacting with the novel animal when compared to saline-exposed counterparts ($p = 0.004$; Fig. 2c). Analysis of the social novelty preference index revealed that this measure was lower in VPA-exposed rats, but just failed to reach statistical significance ($p = 0.06$) (Fig. 2d).

Analysis of responding of adult rats during the sociability phase of the trial, revealed a significant effect of VPA ($F(1,50) = 6.4, p = 0.015$) and stimulus ($F(1,50) = 220.6, p < 0.001$) on the duration of time (s) spent interacting with either the animal or novel empty cage (Fig. 2e). *Post hoc* analysis revealed that all animals exhibited a preference for interacting with the animal over the novel empty cage ($p < 0.001$), however, VPA-exposed adult rats spent significantly less time directly interacting with the animal when compared to saline-exposed counterparts ($p = 0.03$). VPA-exposed adult rats exhibit a lower sociability index when compared to saline-exposed counterparts ($t(25) = 2.3, p = 0.03$) (Fig. 2f).

Two-way ANOVA revealed a significant effect of VPA ($F(1,50) = 7.1, p = 0.01$), stimulus ($F(1,50) = 50, p < 0.001$) and VPA \times stimulus interaction effect ($F(1,50) = 6.6, p = 0.013$) on duration of time spent interacting with either a novel or familiar animal

by adult animals over a 10 min period in the social novelty preference phase of the test (Fig. 2g). *Post hoc* analysis revealed that while all animals exhibited a preference for interacting with the novel over the familiar social stimulus, VPA-exposed animals spent significantly less time interacting with the novel animal when compared to saline-exposed counterparts ($p = 0.001$). Additionally, the social novelty preference index of adult VPA-exposed rats was significantly lower than saline-exposed counterparts ($t(25) = 2.4, p = 0.02$; Fig. 2h).

2.3. mRNA expression of endogenous opioid receptors in brain regions of adolescent and adult male rats prenatally exposed to either saline or VPA

The mRNA expression of opioid receptor and pre-pro-peptides were measured in discrete brain regions of adolescent and adult rats to investigate the developmental effects of prenatal VPA exposure on the opioid system. Analysis revealed that *oprk1* mRNA expression was reduced in the cerebral cortex ($t(16) = 2.439, p = 0.03$), while *oprk1* ($t(16) = 2.4, p = 0.03$) and *opr11* mRNA expression ($t(16) = 2.5, p = 0.02$) was reduced in the hypothalamus, of adolescent rats prenatally exposed to VPA when compared to saline-exposed counterparts (Fig. 3a–f). There was no difference in the expression of *oprm1* or *opr11* between saline- and VPA-exposed rats in any of the brain regions examined.

In adult rats prenatally exposed to VPA, RT-qPCR analysis revealed that *oprk1* mRNA expression was reduced in the hypothalamus ($t(18) = 2.5, p = 0.02$) and *opr11* was increased in the amygdaloid cortex ($t(17) = -2.5, p = 0.02$), when compared to saline-exposed counterparts (Fig. 3g–l). There was a trend for an increase in *oprk1* expression in the hippocampus of adult rats prenatally exposed to VPA vs saline-exposed counterparts, however this failed to reach statistical significance ($p = 0.06$). There was no difference in the expression of *oprm1* or *opr11* between saline and VPA-exposed rats in any of the brain regions examined.

2.4. mRNA expression of opioid pre-pro-peptides in brain regions of male adolescent and adult rats prenatally exposed to either saline or VPA

RT-qPCR analysis of opioid pre-pro-peptide expression in brain regions of adolescent rats revealed that *pdyn* expression was reduced ($t(17) = 2.4, p = 0.03$) in the cerebral cortex of VPA-exposed rats when compared to saline-exposed counterparts (Fig. 4b). Although, *pomc* expression tended to be increased in the cerebral cortex ($t(16) = 1.8, p = 0.09$) and *penk* expression tended to be decreased in the hypothalamus ($t(16) = 2.53, p = 0.06$) of adolescent VPA- vs saline-exposed rats, these changes failed to reach statistical significance (Fig. 4a–f).

In adult rats, RT-qPCR analysis of the expression of opioid pre-pro-peptides revealed that *pomc* was reduced in the striatum ($t(16) = 2.6, p = 0.018$) and *pdyn* was reduced in the hypothalamus ($t(19) = 2.5, p = 0.023$) of VPA-exposed rats when compared to saline-exposed counterparts (Fig. 4). There was no significant effect of prenatal VPA exposure on expression of *penk* in any of the brain regions examined (Fig. 4g–i).

3. Discussion

Although prenatal VPA exposure has been shown by several groups to be associated with social impairments, there has been a lack of studies evaluating behaviour using multiple paradigms and over different developmental periods. The present study demonstrated that prenatal exposure to VPA is associated with reduced social behaviours in both the direct social interaction test and 3-chamber test, at both adolescent and adult ages. Thus, this study confirms and expands on the existing literature demonstrating that prenatal VPA exposure is associated with lasting deficits in social reward, motivation and cognition through to adulthood. Furthermore, this study examined and revealed alterations

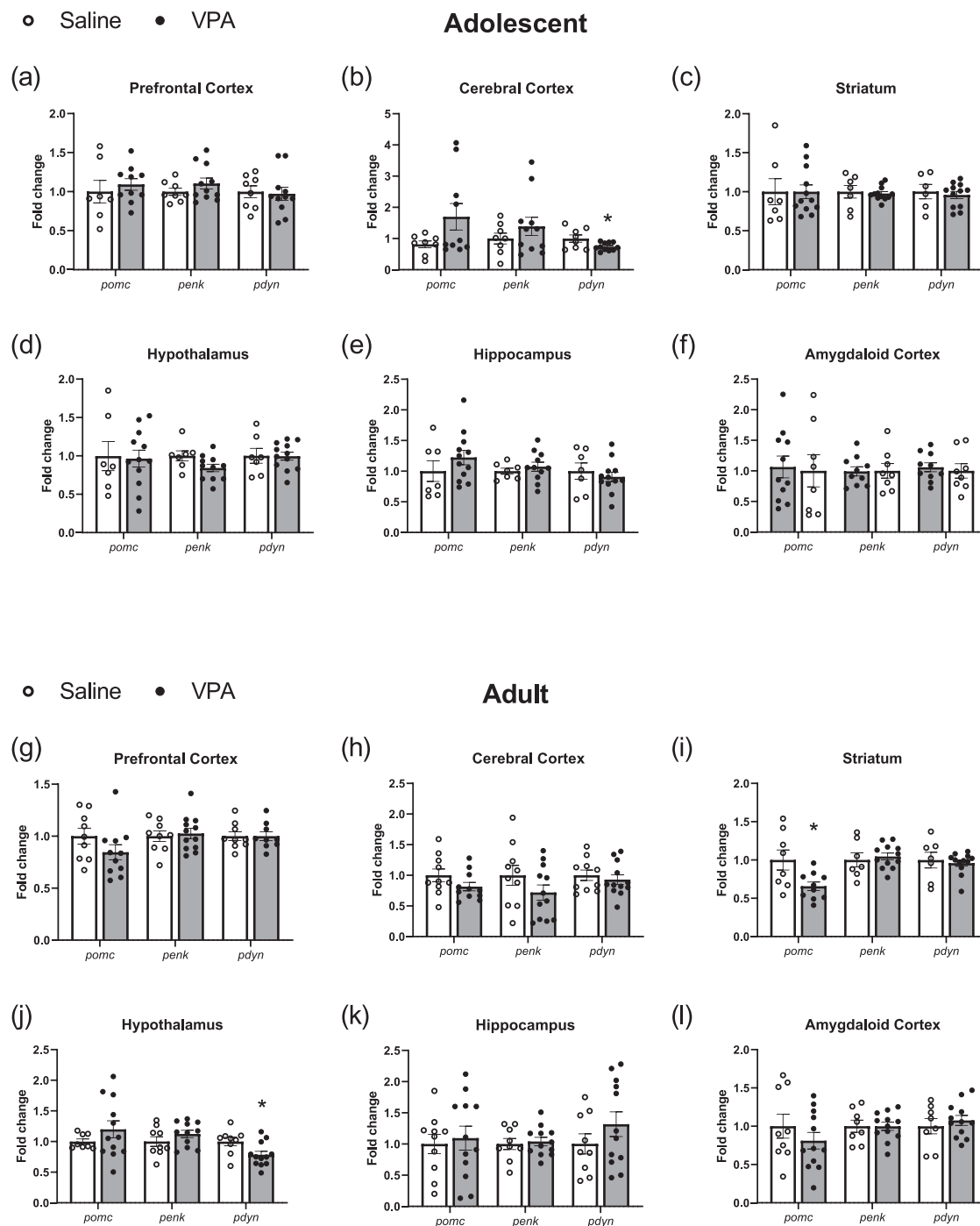


Fig. 4. mRNA expression of opioid pre-pro-peptides in discrete brain regions of adolescent (a–f) and adult (g–l) rats prenatally exposed to saline or VPA. Data presented as fold change from average of saline exposed group. Individual data and mean \pm SEM presented N = 10–12/group. * $p < 0.05$ vs saline-exposed.

in the expression of receptors and pre-pro-peptides of the endogenous opioid system in several brain regions known to play key roles in social responding. Thus, alterations in expression, and possibly functionality, of the endogenous opioid system is associated with social impairments observed in rats prenatally exposed to VPA.

3.1. Prenatal exposure to VPA results in long term impairments in social responding

The current study builds on the existing literature demonstrating impaired social responding of adolescent and adult rats prenatally

exposed to VPA in two behavioural paradigms designed to assess multiple aspects of social responses including social reward, motivation, approach and cognition. Behavioural interactions between conspecifics during the direct social interaction test is inherently rewarding, engaging the mesolimbic reward system (Manduca et al., 2016; Trezza et al., 2011). The present study demonstrates that pairs of adolescent animals prenatally exposed to VPA spent less time engaged in direct social contact and in particular in following and sniffing behaviours, but not pinning behaviour, compared to saline-exposed counterparts. While the decrease in social interaction behaviour has previously been reported in rats prenatally exposed to VPA, in contrast

to our data, these earlier studies had demonstrated a reduction in play-related pinning behaviour (Du et al., 2017; Markram et al., 2008; Schneider and Przewlocki, 2005). Methodological differences such as duration of social isolation prior to testing, different size arenas and lighting conditions between studies may account for the discrepancies between studies. In line previous published data (Schneider and Przewlocki, 2005; Schneider et al., 2008; Servadio et al., 2018), our results also indicate that adult rats prenatally exposed to VPA exhibit a decrease in social interactive behaviour. Analysis of discrete behaviour during this test revealed a selective decrease in the duration of time spent sniffing and climbing over-under one another by rats prenatally exposed to VPA vs saline counterparts. Similar findings were reported by Markram et al. (2008) who describe reduced sniffing in 3-month old Wistar Han rats, and Cuevas-Olguin et al demonstrating decreased sniffing and crawling behaviour in Sprague-Dawley rats prenatally exposed to VPA at a similar age to those used in this present study (Cuevas-Olguin et al., 2017). Taken together the data demonstrate that prenatal VPA exposure results in deficits in direct social interactive and investigative behaviour.

The 3-chamber test differs from the direct social interaction test in that social interactions are initiated by one animal, preventing reciprocal social contact. This paradigm can be used to assess social motivation by measuring social approach and interaction in the sociability trial, and social cognition by measuring the preference for social novelty over familiarity in the social novelty preference trial. In accordance with previously published work from our lab (Kerr et al., 2013, 2016) and others (Al-Amin et al., 2015; Kim et al., 2014a; Kumar and Sharma, 2016; Kumar et al., 2015; Win-Shwe et al., 2018), the current study demonstrated that rats prenatally exposed to VPA spent less time interacting with a novel conspecific animal during adolescence and adulthood. Furthermore, we have confirmed that prenatal VPA exposure also impairs social novelty preference in rats in both adolescence and adulthood, which is in line with previous published research (Bambini-Junior et al., 2014; Cai et al., 2019; Castro et al., 2017; Khalaj et al., 2018; Kim et al., 2011, 2013, 2014a,b, 2017; Kumar and Sharma, 2016; Kumar et al., 2015; Mirza and Sharma, 2019; Qin et al., 2016; Win-Shwe et al., 2018). Thus, these data add to the existing literature indicating that prenatal VPA exposure in rodents results in deficits in social motivation, approach/avoidance and cognition that persist into adulthood.

3.2. Prenatal exposure to VPA alters levels of mRNA encoding opioid receptors and pre-pro-peptides

The endogenous opioid system plays a key role in mediating and modulating social motivation, cognition and reward processing. Deficits in social behaviours observed following prenatal exposure to VPA have been proposed to result from altered opioid functionality, reduced hedonic tone and deficits in reward processing (Kuo and Liu, 2017; Schneider et al., 2007). However, a comprehensive characterisation of endogenous opioid receptors and pre-pro-peptides expression in various brain regions known to play key roles in social responses in rats prenatally exposed to VPA across different developmental ages has not been undertaken until now. The present study revealed alterations in the mRNA expression of *oprk1*, *opr1* and *pdyn* in several brain regions. In particular, prenatal VPA exposure resulted in a decrease in *oprk1* mRNA expression in the cerebral cortex and hypothalamus, decreased *pdyn* in the cerebral cortex, and decreased *opr1* in the hypothalamus during adolescence. There was a similar effect of prenatal VPA exposure on opioid mRNA expression in adulthood, where *oprk1* mRNA expression was decreased in the cerebral cortex and hypothalamus, and a trend for an increase in *oprk1* in the prefrontal cortex and hippocampus. Although the receptor and pre-pro-peptide expression slightly varies between adolescence and adulthood, the effects of prenatal VPA-exposure in reducing the expression of *oprk1* and *pdyn* in the cerebral cortex and hypothalamus remains consistent at both ages. Previous

studies have demonstrated lower levels of *oprk1* mRNA in mice that experienced social victories, but not defeats, during agonistic interactions (Goloshchapov et al., 2005). Furthermore, a recent PET study carried out in humans also found that KOP levels in were negatively correlated with social status, and this relationship was particularly evident in regions of the social brain such as the amygdala, anterior cingulate cortex and hippocampus (Matuskey et al., 2019). Furthermore, KOP activation has been demonstrated to reduce social responses (Robles et al., 2014; Vanderschuren et al., 1995). While this previously published data may seem at odds with the current findings demonstrating a decrease in *oprk1* and *pdyn* expression in discrete brain regions in a model demonstrated to exhibit social impairments, further studies will determine if mRNA changes observed herein translate into protein changes and the functional state of KOP in rats prenatally exposed to VPA.

In addition to changes in *oprk1*-*pdyn* expression, *opr1* expression was found to be increased in the amygdaloid cortex of adult rats prenatally exposed to VPA, with a similar trend observed in adolescent rats. Early life maternal stress has also been reported to increase DOP receptor density in the basomedial amygdala of rats (Ploj and Nylander, 2003). Thus, it remains to be determined if the increased *opr1* expression in the amygdala is a maladaptive or adaptive consequences of altered neuronal function following maternal pharmacological or environmental stress/intervention. Schneider and colleagues previously reported that prenatal VPA exposure was associated with reduced striatal *penk* mRNA levels in adult rats (Schneider et al. (2007)). The data in the current study did not reveal any change in *penk* mRNA expression in the striatum of adult or adolescent rats prenatally exposed to VPA, but rather a slight but non-significant decrease was noted in the hypothalamus of adolescent rats. Discrepancies between these studies are most likely due to methodological issues such as specific area of the striatum examined. Furthermore, given that the hypothalamus is composed of a variety of subnuclei with varying functions, it is possible that *penk* expression may be altered in discrete hypothalamic areas, an effect diluted (and thus non-significant) when whole hypothalamic tissue is assessed for expression changes. Thus, taking the current and published data together, prenatal VPA exposure is associated with alterations in *opr1*-*penk* mRNA expression in brain regions that modulate social responding.

An abundance of literature has implicated MOP in social processing in both humans and rodents (for review see (Pellissier et al., 2017)) and thus it is possible that the deficits in social responses in rats prenatally exposed to VPA may result from altered *oprm1* expression. In line with this theory, *oprm1* knockout mice have been demonstrated to exhibit profound impairments in social responses (Cinque et al., 2012; Moles et al., 2004). However, the data in this study did not reveal any effect of prenatal VPA exposure on *oprm1* expression in any of the brain regions, during either adolescence or adulthood. In comparison, Kuo and Liu, reported that adolescent rats prenatally exposed to VPA exhibit a decrease in the area of MOP-positive striosomes (Kuo and Liu, 2017). Although the current study demonstrated that *oprm1* levels are not altered in the brain regions of rats prenatally exposed to VPA examined in the current study, we cannot rule out that changes may exist in more discrete subnuclei or that MOP protein levels (as reported by Kuo and Liu) or functionality of the receptors may be altered in the model and in turn underlie, at least in part, some of social behavioural deficits observed.

3.3. Conclusion

The present data demonstrate that prenatal exposure to VPA results in impaired social reward, motivation and cognition during both adolescence and adulthood. These behavioural effects are accompanied by several changes in the endogenous opioid system in key brain regions that underlie social responses. Further studies will determine if alterations in the opioid system, and in particular the KOP system, mediate or

modulate the social impairments observed following prenatal VPA exposure.

4. Experimental procedures

4.1. Breeding and animal husbandry

Male and female Sprague-Dawley (200–340 g; Charles River Laboratories, UK) rats were housed in groups of 3 under controlled conditions (temperature 20–24 °C, humidity 40–50% relative humidity and 12/12 h light cycle with lights on at 7am). Animals were left undisturbed for 7 days to acclimatise before mating. Food and water were available *ad libitum*. Females were mated overnight and the presence of spermatozoa via vaginal smear the next morning deemed gestational day (GD) 0.5 after which they were singly housed. On GD12.5, pregnant dams received a subcutaneous injection of either VPA (600 mg/kg) or saline (0.89%) at a volume of 2 ml/kg. Females were left undisturbed to raise their own pups until weaning on post-natal day (PND) 21, after which male rats were group housed (3–6 per cage). Male offspring were behaviourally tested during adolescence (PND38–43) or adulthood (PND65–67). The experimental protocol was carried out in accordance with the guidelines of the Animal Care and Research Ethics Committee, National University of Ireland Galway under licence from the Irish Health Products Regulatory Authority and in compliance with the ARRIVE guidelines and the European Communities Council directive 2010/63/EU.

4.2. Social interaction test

The direct social interaction test was performed in a novel home cage arena (adolescent: 45 × 28 × 22 cm, adult: 56 × 38 × 22 cm) containing grey 3Rs bedding, under low lighting conditions (LUX 5). Animals were singly housed for 24 h prior to testing as this isolation has been shown to increase levels of sociability throughout the test (Niesink and Van Ree, 1989). On the experiment day, a pair of animals from the same treatment group, but not siblings or cage mates, were placed in the novel testing cage for a 10 min period. Animals were paired by weight, with differences in weight between a pair of animals no more than 10 g in order to prevent aggressive or dominance behaviour. A camera was placed above the arena and the test was recorded for subsequent analysis. The duration of time spent in social interaction (physical contact, sniffing, following, pinning or climbing over-under) was manually assessed by an experimenter blind to treatment group and data collected with the aid of Ethovision XT v11.5, over a 10 min period.

4.3. The 3-chamber test

The sociability test was conducted in a novel 3-chamber apparatus which allows for the measurement of social motivation, approach and preference (Crawley, 2004) and carried out as previously described (Kerr et al., 2013, 2016). In brief, animals were placed into a novel arena (30 cm × 75.5 cm adolescence and 90.5 cm × 46 cm adulthood) composed of three communicating chambers separated by Perspex walls with central openings allowing access to all chambers. The lighting in the arena was set to LUX 20. Animals were single-housed for 24 h prior to testing in the 3-chamber apparatus. The test animal was placed in the centre and allowed to explore each chamber of the empty apparatus for a period of 10 min (habituation period). The test animal was then allowed to explore the arena with a confined novel stimulus animal or novel cage placed in each of the outer arenas for a further 10 min (sociability phase). The 3rd phase of this test involved replacing the novel cage with a novel animal such that the stimulus animal in the sociability phase remained and acted as the familiar animal. The test animal was then allowed to explore the arena for a further 10 min (social novelty preference phase). All behaviour was recorded and later

manually scored and analysed by an experimenter blind to treatment group and data collected with the aid of Ethovision XT 11.5. Behaviours analysed included the time spent (s) directly interacting (sniffing, climbing, approaching) with the animal or novel cage during the sociability phase or time spent directly interacting with the novel and familiar animal during the social novelty preference phase.

4.4. mRNA expression of opioid receptors and pre-pro-peptides in discrete brain regions

Animals were sacrificed by decapitation, brains removed and discrete brain regions (pre-frontal cortex (Bregma 4.7 to 3.2 mm), dorsal striatum (Bregma 1.7 to 0.48 mm), hypothalamus (Bregma –1.80 to –3.60), dorsal hippocampus (Bregma –2.3 mm to –3.6 mm), cerebral cortex (Bregma –2.3 mm to –3.6 mm) and amygdaloid cortex (Bregma –2.3 mm to –3.6 mm) were dissected out on an ice cold plate and stored at –80 °C until analysis. Dissections were carried out as previously described (Burke et al., 2010, 2019) (Fig. 3) and every care was taken to ensure that similar regions and amounts of tissues were taken for each dissection. The regions were chosen as they are known to play key roles in social motivation, cognition and reward (Felix-Ortiz and Tye, 2014; Gunaydin et al., 2014; Ko, 2017; Mansour et al., 1994). The cerebral cortex included the cingulate cortex, motor and somatosensory cortices and thus is a key collective region in the cognitive, sensory and motor responses required during social interactive engagement. RT-qPCR was carried out as previously described (Flannery et al., 2018; Henry et al., 2014; Kerr et al., 2013). In brief, RNA was extracted from tissues using Nucleospin® RNA II total isolation kit (Macherey-Nagel, Germany) and reverse transcribed into cDNA using a high capacity cDNA kit (Applied Biosystems, Warrington, UK). Taqman gene expression assays were used to assess expression of *oprm1* (Rn01430371_m1), *oprk1* (Rn00567737_m1), *oprd1* (Rn00561699_m1), *opr11* (Rn00440563_m1), *pomc* (Rn00595020_m1), *penk* (Rn00567566_m1) and *pdyn* (Rn00571351_m1) using an ABI Step One Plus qPCR machine (Applied Biosystems, Warrington, UK). β -actin gene expression was used as an endogenous control. Expression was analysed using the $\Delta\Delta$ CT method and expressed as fold change from saline-exposed counterparts.

4.5. Statistical analysis

SPSS (IBM, New York, USA) statistical package was used to analyse all data. Normality and homogeneity of variance were confirmed using Shapiro–Wilk and Levene test respectively, where $p > 0.05$. When normality was rejected, three transformation were applied, in this order: square root of the data values, log of the data values, and ranking of the data values. Also, it was checked if the highest standard deviation was less than or equal to 2 times the smallest standard deviation for the particular data set being analysed. All data was normally distributed either before or following transformations. Data were analysed using unpaired *t*-test or Two-way ANOVA followed by Fisher's LSD *post-hoc* analysis where appropriate and considered significant when $p < 0.05$. All graphs representing data were constructed using GraphPad Prism 8.0 and results expressed as individual data points and group means \pm standard error of the mean.

Declarations of Competing Interest

None.

CRediT authorship contribution statement

Edel M. Hughes: Investigation, Formal analysis, Writing - original draft. **Patricia Calcagno:** Investigation. **Morgane Clarke:** Investigation. **Connie Sanchez:** Conceptualization. **Karen Smith:** Conceptualization. **John P. Kelly:** Supervision. **David P. Finn:**

Supervision. **Michelle Roche**: Conceptualization, Supervision, Writing - review & editing.

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