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Effects of developmental alcohol and valproic acid exposure on play behavior of ferrets

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Abstract

Exposure to alcohol and valproic acid (VPA) during pregnancy can lead to fetal alcohol spectrum disorders and fetal valproate syndrome, respectively. Altered social behavior is a hallmark of both these conditions and there is ample evidence showing that developmental exposure to alcohol and VPA affect social behavior in rodents. However, results from rodent models are somewhat difficult to translate to humans owing to the substantial differences in brain development, morphology, and connectivity. Since the cortex folding pattern is closely related to its specialization and that social behavior is strongly influenced by cortical structures, here we studied the effects of developmental alcohol and VPA exposure on the play behavior of the ferret, a gyrencephalic animal known for its playful nature. Animals were injected with alcohol (3.5 g/kg, i.p.), VPA (200 mg/kg, i.p.) or saline (i.p.) every other day during the brain growth spurt period, between postnatal days 10 and 30. The play behavior of pairs of the same experimental group was evaluated 3 weeks later. Both treatments induced significant behavioral differences compared to controls. Alcohol and VPA exposed ferrets played less than saline treated ones, but while animals from the alcohol group displayed a delay in start playing with each other, VPA treated ones spent most of the time close to one another without playing. These findings not only extend previous results on the effects of developmental exposure to alcohol and VPA on social behavior, but make the ferret a great model to study the underlying mechanisms of social interaction.

INTRODUCTION

Fetal alcohol spectrum disorder (FASD) and fetal valproate syndrome are neurodevelopmental disorders (Lindhout and Schmidt, 1986; May et al., 2014; Ornoy, 2009; Rihtman et al., 2013; Riley et al., 2011) characterized by behavioral and physiological abnormalities that include sensory processing deficits, which in turn are associated with deficits in social interaction and autistic behavior (Carr et al., 2010; Nanson, 1992; Rihtman et al., 2013; Stevens et al., 2013; Williams et al., 2001). While there is extensive evidence that

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developmental exposure to alcohol or valproic acid (VPA) can remarkably affect social interactions in rodents (Meyer and Riley, 1986; Kelly et al., 2000; Varlinskaya and Mooney, 2014; Burenkova et al., 2014; Yochum et al., 2008; Charles Lawrence et al., 2008), much less is known about the effects of these teratogenic drugs in the social behavior of gyrencephalic species. A more complex cortex leads to more sophisticated behavior, which might be even more susceptible to the effects of teratogens. Therefore, studies of social behavior in gyrencephalic species would be a good addition to the insightful results already demonstrated in the rodent model. Particularly, the ferret is a good candidate because of their social and playful traits (Stockman et al., 1986; Rabe et al., 1985) and because ferrets share elements of cortical organization that are more common in humans than in rodents, such as the presence of ocular dominance and orientation selectivity columns in the visual cortex (Medina et al., 2003; Medina et al., 2005; Krahe et al., 2005), clear tonotopic maps in the auditory cortex (Bizley et al., 2005), and a somatosensory cortex that is less dominated by vibrissae (Juliano et al., 1996; Keniston et al., 2009; Foxworthy and Meredith, 2011).

We have demonstrated that ferrets exposed to ethanol during the “brain growth spurt”, a period of increased synaptogenesis that occurs during the first postnatal weeks in rodents, and during the third trimester of gestation in humans, present severe and long-lasting deficits in sensory function (Krahe et al., 2009; Medina et al., 2003; Medina et al., 2005). Some of these deficits, such as the disruption of orientation selectivity, were also observed after exposure to VPA during the same developmental period (Pohl-Guimaraes et al., 2011). It is not known, however, whether these animals also exhibit social interaction deficits. To examine this possibility, here we investigated the social behavior of juvenile ferrets exposed to alcohol or VPA early during development. Specifically, we hypothesize that ferrets exposed to alcohol or VPA during the brain growth spurt will play less with their peers than controls.

MATERIALS AND METHODS

Animals

Lactating ferrets with litters were bought from Marshall Farms (New Rose, NY) and housed in a 12:12 light-dark cycle. Food and water were available ad libitum. Kits received a single i.p. injection of alcohol (ALCOH; 3.5 g/kg alcohol, i.p., n = 8), valproic acid (VPA; 200 mg/kg, i.p., n = 10), or an equivalent volume of saline (SAL; i.p., n = 12) every other day starting on P10 and ending on P30. Regarding cortical development, this period is roughly similar to P4-P10 in rodents and do the third trimester of human gestation (Clancy et al., 2001; Medina et al., 2003; Medina et al., 2005), and encompasses the time when several intracortical, thalamocortical and corticothalamic connections are being formed (Bock et al., 2012; Callaway and Borrell, 2011; Dalva, 2010; McLaughlin and Juliano, 2003). Following a prolonged alcohol- and VPA-free period (21 ± 1.6 days) and well before sexual maturation (30–50 weeks of age) (Ryan and Robinson, 1987), the play behavior of 30 ferrets (6 males and 24 females from 12 litters) was evaluated. Experiments were conducted at Virginia Commonwealth University and all procedures were performed in compliance with the Institutional Animal Care and Use Committee.

Play behavior

The play behavior of juvenile ferrets (7.2 ± 0.2 weeks) was video recorded for 10 min in a glass box ($70 \times 40 \times 45$ cm). Animals were kept isolated from each other for 18 h before testing and for the evaluation of the play behavior they were paired with counterparts of the same experimental group (i.e. ALCOH vs. ALCOH, VPA vs. VPA, and SAL vs. SAL). Animal pairs could be from the same or the opposite sex, however they were always from different litters. Before each testing session the glass box was placed in the sink, thoroughly flushed with tap water and dried. We avoided using an alcohol cleaning solution since it could differentially affect animals early exposed to alcohol (Youngentob and Glendinning, 2009).

An observer, who was blind to the treatment, later scored the time animals spent: 1) playing (Play behavior, Fig. 1A), 2) in contact with each other, but without playing (Cold contact, Fig. 1B), and 3) distant from each other (No contact, Fig. 1C). Play behavior was considered as any playful interaction between animals such as biting, jumping and rolling over, pushing, or galloping toward and away from their counterpart. Cold contact, on the other hand, was scored when animals touched or leaned over each other, but without any apparent playfulness. Finally, No contact comprised behaviors made when animals were apart from each other such as walking around, sniffing, grooming, or exploring the testing chamber. Behavioral analysis was conducted within 1 min intervals for the total testing session (10 min). There were no significant effects of sex (univariate ANOVAs, Play behavior, $F = 4.31$, $P = 0.07$; Cold contact, $F = 0.22$, $P = 0.81$; No contact, $F = 2.60$, $P = 0.15$) or litter (univariate ANOVAs, Play behavior, $F = 0.17$, $P = 0.70$; Cold contact, $F = 0.33$, $P = 0.59$; No contact, $F = 1.12$, $P = 0.33$). Moreover, no significant differences between pairs of the same or opposite sex were observed regarding the total time animals interacted with each other (Same sex, 420.6 ± 22.66 s; Opposite sex, 404.12 ± 44.99 s; T-test, $p = 0.72$).

Statistical analysis

Data were submitted to univariate or repeated-measures ANOVAs followed by post hoc comparisons to ascertain group differences. Differences between experimental groups were also determined using Wilcoxon Mann-Whitney U tests. For all statistical tests, significance was set at $P < 0.05$ (2-tailed). Data is shown as mean and $\pm$ SEM.

RESULTS

Table 1 shows offspring weight gain during alcohol and valproic acid treatments. While the mean litter weight increased significantly from P10 to P30 for all experimental groups [age: $F(2.7,84.1) = 490$; $P < .001$], ALCOH and VPA exhibited a slower growth than SAL [$F(2.7,84.1) = 8.8$, $P < .001$]. At P30, the mean offspring weight of ALCOH and VPA treated animals was, respectively, $22.4 \pm 4.6\%$ smaller than that of SAL group. There was no sex effect or interaction between sex and treatment. At the age of behavioral testing (~P50) ALCOH and VALP animals still weighted less than SAL ones (data not shown)

Histograms depicting the distribution of the time animals spent playing (Fig. 1D, G and J), in close contact to each other but not playing (Fig. 1E, H and K), and distant from one

another (Fig. 1F, I and L) are shown in Figure 1 for ALCOH (Fig. 1D–F), VPA (Fig. 1G–I) and SAL (Fig. 1J–L) groups. While no differences were found for the distributions regarding the time animals spent without interacting with each other (Fig. 1F, I and L; SAL vs. ALCOH, $U = 49,000$, $P = 0.97$; Saline vs. VPA, $U = 44,500$, $P = 0.68$; ALCOH vs. VPA, $U = 47,000$, $P = 0.85$; Mann-Whitney U tests), animals from the VPA group displayed significantly more time in “cold” contact to one another along the testing session than animals treated with alcohol and saline (Fig. 1E, H and K; SAL vs. VPA, $U = 13,000$, $P < 0.01$; ALCOH vs. VPA, $U = 3,500$, $P < 0.001$; Mann-Whitney U tests). No differences were observed between SAL and ALCOH groups (Fig. 1E and H; $U = 41,000$, $P = 0.53$; Mann-Whitney U test). Regarding play behavior, the distribution of the time saline treated animals spent playing was significantly different from that exhibited by the VPA group (Fig 1D and J; SAL vs. VPA, $U = 12,000$, $P < 0.01$; Mann-Whitney U test), with VPA treated animals showing less time playing as well as a delay to start to do so compared to the SAL group. Similarly, although the distributions of alcohol and saline treated animals were not statically different (Fig. 1D and G; $U = 45,000$, $P = 0.74$; Mann-Whitney U test), ALCOH animals seemed to engage in playful behaviors later than SAL ones.

To further explore this scenario, we investigated the behavior patterns displayed by alcohol, VPA, and saline treated animals during the first and last 5 minutes of the testing period. Figure 2 illustrates the average time animals spent playing (Fig. 2A–C), in cold contact (Fig. 2D–F), and apart from one another (Fig. 2G–I) for the total time (Fig. 2A, D and G), the first (Fig. 2B, E and H), and the last 5 minutes (Fig. 2C, F and I) of the testing session. Indeed, animals in the ALCOH and VPA groups play significantly less than saline treated animals during the first 5 minutes of the testing session (Fig. 2B; univariate ANOVA, $F = 8.96$; $P < 0.01$), with ALCOH animals showing an increase in play behavior by the end of the testing period (Fig. 2C). This could explain why for the total time no differences were observed between ALCOH and SAL groups (Fig 2A). VPA treated animals on the other hand, spent significantly less time playing than the other groups (Fig. 2A–C). That does not mean they completely ignored each other, only that they rather stayed in close proximity to one another without playing (Fig. 2D–F; First 5 min, $F = 7.71$; $P < 0.01$; Last 5 min, $F = 3.99$; $P < 0.05$; Total time, $F = 6.44$; $P < 0.05$; univariate ANOVAs). This is further supported by the fact that the amount of time the animals spent apart from each other was similar for all groups (Fig 2G–I; First 5 min, $F = 2.80$; $P = 0.10$, Last 5 min, $F = 1.05$, $P = 0.40$, Total time, $F = 0.04$, $P = 0.96$; univariate ANOVAs).

We next compared the percent of time animals spent interacting (*Play behavior* and *Cold contact*) or not with each other (*No contact*) as well as the number of times animals engaged in *Play behavior* and *Cold contact* during the testing session. Figure 3 shows the average percent time saline, alcohol and VPA treated animals spent interacting or not. As expected, the percent of time spent in either condition was similar for all groups (univariate ANOVA, $F = 0.00$; $P = 1.00$). However, there was a marked difference between groups regarding the number of times animals displayed *Play behavior* or *Cold contact* (Fig. 3; ALCOH vs. SAL, $\chi^2 = 3.46$, $P = 0.06$, VPA vs. SAL, $\chi^2 = 10.56$, $P < 0.01$; Chi-square tests). While almost half of the interactions observed between saline treated pairs were playful, ALCOH and VPA groups showed only 37% and 29% of such kind of interactions, respectively.

DISCUSSION

Here we show that exposure to alcohol and VPA during a period roughly similar to the last months of gestation in humans alters the way juvenile ferrets interact. Both treatments induced significant behavioral differences compared to those observed in control animals, and confirm the long-term negative effects of early developmental exposure to these drugs. Interestingly, alcohol and VPA treatments differently affected the way animals interacted with one another. In general, alcohol treated animals spent the same time playing compared to controls, but took longer to start playing with each other, whereas the effects of prenatal treatment with VPA were more severe. VPA treated animals clearly played less than the other two groups and preferred to stay close without playing. Our findings show that while VPA treated animals do not spend much time playing, ethanol treated ones have difficulty to start to get involved in this type of social interaction.

Our findings are consistent with prior evidence from clinical and rodent studies. Exposure to VPA in rats and mice at the time of closure of neuronal tube, as well as during the first two postnatal weeks leads to behavioral and neurological deficits that resemble autism. Among such effects, treated animals often exhibit decreased exploratory activity, and reduced social behaviors (Schneider and Przewlocki, 2005; Schneider et al., 2007; Wagner et al., 2006; Yochum et al., 2008). Clinical studies of children exposed to VPA in utero also show deficits in social interactions and autistic-like behaviors (Christensen et al., 2013; Moore et al., 2000; Williams et al., 2001), a condition known as fetal valproate syndrome. Similarly, several studies have consistently reported deficits in social behavior of both children and rodents exposed to alcohol during brain development across a wide range of exposure durations and doses (Bishop et al., 2007; Boschen et al., 2014; Harris et al., 1995; Kelly et al., 2000; Stevens et al., 2013; Varlinskaya and Mooney, 2014). Interestingly, however, the types of social impairments or “autistic behaviors” observed in FASD are different than those of children with autism and valproate syndrome. Children with FASD are generally less impaired on social interactions (Bishop et al., 2007). They desire to interact with other kids but often do so in an “improper manner”, such as taking longer time to initiate playing or withdrawing prematurely (Kully-Martens et al., 2012). Likewise, our results showed that alcohol treated ferrets took longer to interact with each other and VPA treated animals displayed a remarkable decrease in playing time compared to controls.

Dysfunction of the appropriate balance between excitation and inhibition in the corticolimbic system underlies at least part the neurobiology of several psychiatric disorders, including FASD and valproic acid syndrome (Gogolla et al., 2009; Fukuchi et al., 2009; Diaz et al., 2014; Baculis et al., 2015). Several studies have demonstrated that exposure to both VPA or alcohol during the third trimester equivalent of human gestation can suppress NMDA receptor function and enhance GABAergic receptor stimulation (Hsiao et al., 1999; Lovinger et al., 1989; Sanderson et al., 2009; Savage et al., 1992). Moreover, VPA actions on sodium voltage-dependent channels may cause an overall reduction in neural activity (Vreugdenhil and Wadman, 1999). These actions during the brain growth spurt period, even if transitory, may disturb the establishment of neuronal circuits, leading to a long lasting imbalance between excitatory/inhibitory neurotransmission. Thus, considering the effects of alcohol and VPA developmental exposure on cortical maturation as well as the

importance of cortical neural circuitry in the regulation of social behaviors (Rankin et al., 2006; Rankin et al., 2004; Kipps et al., 2009; Rosen et al., 2002; Tucker et al., 1995), it is comprehensible why exposure to VPA and alcohol during the third trimester equivalent of human gestation would disrupt social behavior in ferrets. Neuronal death is another factor that may play a role in the disruption of play behavior by early exposure to these drugs. It has been extensively demonstrated that during the third trimester of development neurons are particularly susceptible to apoptosis triggered by drugs that suppress synaptic neurotransmission, such as alcohol, pentobarbital, isofluorene and VPA (Bittigau et al., 2002; Brambrink et al., 2010; Ikonomidou et al., 2000). Therefore, it is possible that an increased cell death during a period where synapses are being formed and circuits are being refined, contributes to the behavioral differences observed here.

One should note that in the present study, in spite of the fact that we used very simple behavioral classifications (playing, cold contact and no interaction), we were able to see clear differences between the experimental groups. We believe that this is because ferrets are extremely playful (Fagen, 1981), which make them particularly useful as an experimental model for investigating the teratogenicity of environmental effects on social behavior. Another reason in favor of this animal model is that the brain growth spurt period, which in humans occurs during the third trimester of gestation, takes place during the first postnatal month of life in ferrets. Such postnatal development allows, for example, molecular manipulations and teratogenic exposures targeted to a particular area of the neocortex during a restricted period of time (Foxworthy and Medina, 2015; Krahe and Medina, 2010; Paul et al., 2010). In contrast, neurodevelopmental events of the third trimester equivalent of human gestation, such as the increased synaptogenesis (Clancy et al., 2001) and sulcal infolding (Sawada and Watanabe, 2012), occur prenatally in cats and most primates, making complex intrauterine surgeries a requirement for some experimental manipulations. On the other hand, the use of ferrets is also advantageous if compared to rodents since they have a highly developed cortex. For instance, ferrets have ocular dominance and orientation columns in the visual cortex (Krahe et al., 2009; Ruthazer et al., 1999; White et al., 2001), clear tonotopic maps in the auditory cortex (Bizley et al., 2005), and a somatosensory cortex that is less dominated by vibrissae (Foxworthy and Meredith, 2011; Juliano et al., 1996; Keniston et al., 2009). Moreover, the gyrification processes of ferrets is homologous with those of human and non-human primates (Sawada and Watanabe, 2012).

In conclusion, our results show that the ferret can be a great model to study the effects of teratogens on social interactions. It demonstrates that this can be achieved with a relative small sample of animals and simple behavioral assessments. Furthermore, the innate “social brain” of ferrets can perhaps help us understand the complexity of human social behavior.

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Figure 1.

A–C, schematic of the measured conditions (see Methods) depicting the outlines of ferrets playing (A, *Play behavior*), in contact with one another but without playing (B, *Cold contact*), and distant from each other (C, *No contact*). D–L, histograms illustrate the duration of *Play behavior* (D, G and J), *Cold contact* (E, H and K) and *No contact* (F, I and L) throughout the testing session (1 min intervals) for Saline (D–F), ALCOH (G–I) and VPA (J–L) groups. Note that saline treated animals engage in *Play behavior* sooner than the animals from the other groups (D, G and J) and that ferrets that received VPA display more *Cold contact* compared to alcohol and saline treated animals (E, H and K). Error bars represent $\pm$ SEM. SAL, n = 12 animals from 8 litters; ALCOH, n = 8 animals from 6 litters; VPA, n = 10 from 5 litters.

Figure 2.

A–G, bar graphs showing the averaged time spent in *Play behavior* (A–C), *Cold contact* (D–F), and *No contact* (G–I) for SAL, ALCOH, and VPA groups. The left column (A, D and G) depicts the time spent in each condition for the whole testing session (10 min), whereas the middle (B, E and H) and right (C, F and I) columns the averages for the first and last five-minutes of the testing session, respectively. Note that: (1) animals from the SAL group spent significantly more time playing during the first five minutes of the testing session compared to the ALCOH and VPA groups (B), and (2) ferrets from the VPA group display significantly greater amounts of time in *Cold contact* than the other experimental groups (D–F). Error bars represent $\pm$ SEM. SAL, n = 12 animals from 8 litters; ALCOH, n = 8 animals

from 6 litters; VPA, n = 10 from 5 litters. Univariate ANOVAs followed by LSD tests; *, $P < 0.05$; **, $P < 0.01$.

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Figure 3.

A–C, composite bar graphs displaying the averaged percent of time that the animals were interacting or not with each other during the testing session for Saline (A), ALCOH (B), and VPA (C) groups. Pie charts show the percentage of the number of times animals engaged in *Play behavior* and *Cold contact* when they were interacting with each other. Numbers in parentheses indicate the number of times a particular condition was observed. Error bars represent $\pm$ SEM. SAL, n = 12 animals from 8 litters; ALCOH, n = 8 animals from 6 litters; VPA, n = 10 from 5 litters.

Table

Body mass (g)												
Group	Age in days											
	10	12	14	16	18	20	22	24	26	28	30	
SAL	43 ± 2	54 ± 3	59 ± 4	68 ± 5	76 ± 6	90 ± 6	108 ± 6	127 ± 6	142 ± 6	156 ± 6	171 ± 6	
ALCOH	46 ± 2	51 ± 2	56 ± 3	67 ± 3	77 ± 3	83 ± 4	98 ± 6	107 ± 5 *	120 ± 6 *	125 ± 6 *	132 ± 7 *	
VPA	47 ± 2	54 ± 3	61 ± 4	67 ± 5	75 ± 5	85 ± 6	98 ± 6	109 ± 6 *	120 ± 6 *	130 ± 6 *	142 ± 8 *	

Data are presented as mean ± SE. Numbers in parentheses represent number of litters. Repeated-measures ANOVA followed by LSD tests; ALCOH vs. SAL and VPA vs. SAL;

* P < 0.05,

** P < 0.01.