

Embryonic Exposure to Valproic Acid Affects Social Predispositions for Dynamic Cues of Animate Motion in Newly-Hatched Chicks

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

P.S., E.V., O.R.-S. and G.V. conceived and designed the experiments; E.L. and A.P. conducted the experiments; P.S., E.V. and O.R.-S. developed the behavioural paradigms; E.L., A.P., P.S., O.R.-S. and E.V. analyzed the data; E.L. and P.S. drafted the manuscript; E.L., A. P., O.R.-S., E.V., P.S. and G.V. wrote the manuscript. All the authors gave final approval for publication.

Keywords

valproic acid (VPA), Social predispositions, Newly-hatched chicks, Autism spectrum disorder (ASD), animacy, Gallus gallus

Abstract

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Early predispositions to preferentially orient towards cues associated with social partners have been documented in several vertebrate species including human neonates and domestic chicks. Human newborns at high familiar risk of Autism Spectrum Disorder (ASD) show differences in their attention toward these predisposed stimuli, suggesting potential impairments in these social-orienting mechanisms in ASD. Using embryonic exposure to valproic acid (VPA) we modelled ASD behavioural deficits in domestic chicks. To investigate social predispositions towards animate motion in domestic chicks, we focused on self-propulsion, using two video-animations representing a simple red circle moving at constant speed (speed-constant) or one that was changing its speed (accelerating and decelerating; speed-change). Using a six minutes spontaneous choice test for the two stimuli, we compared unlearned preferences for stimuli that autonomously change speed between VPA- and vehicle-injected chicks. We found that the preference for speed changes was abolished in VPA-injected chicks compared to vehicle-injected controls. These results add to previous findings indicating similar impairments for static social stimuli and suggest a specific effect of VPA on the development of mechanisms that enhance orienting towards animate stimuli. These findings strengthen the hypothesis of an early impairment of predispositions in the early development of ASD. Hence, early predispositions are a potentially useful tool to detect early ASD symptoms in human neonates and to investigate the molecular and neurobiological mechanisms underlying the onset of this neurodevelopmental disorder.

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Ethics statements

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This study was carried out in accordance with the recommendations of the Italian and European Community laws for the ethical treatment of animals'. The protocol was approved by the Ethical Committee of the University of Trento and licensed by the Italian Health Ministry (permit number 986/2016-PR).

Data availability statement

Generated Statement: All datasets generated for this study are included in the manuscript and the supplementary files.

In review

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2 **Dynamic Cues of Animate Motion in Newly-Hatched Chicks**

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26 **Keywords: valproic acid (VPA), social predispositions, newly-hatched chick, Autism Spectrum**
27 **Disorder (ASD), animacy, *Gallus gallus***

28 **Abstract**

29 Early predispositions to preferentially orient towards cues associated with social partners have been
30 documented in several vertebrate species including human neonates and domestic chicks. Human
31 newborns at high familial risk of Autism Spectrum Disorder (ASD) show differences in their
32 attention toward these predisposed stimuli, suggesting potential impairments in these social-orienting
33 mechanisms in ASD. Using embryonic exposure to valproic acid (VPA) we modelled ASD
34 behavioural deficits in domestic chicks. To investigate social predispositions towards animate motion
35 in domestic chicks, we focused on self-propulsion, using two video-animations representing a simple
36 red circle moving at constant speed (speed-constant) or one that was changing its speed (accelerating
37 and decelerating; speed-change). Using a six minutes spontaneous choice test for the two stimuli, we
38 compared unlearned preferences for stimuli that autonomously change speed between VPA- and
39 vehicle-injected chicks. We found that the preference for speed changes was abolished in VPA-
40 injected chicks compared to vehicle-injected controls. These results add to previous findings
41 indicating similar impairments for static social stimuli and suggest a specific effect of VPA on the
42 development of mechanisms that enhance orienting towards animate stimuli. These findings
43 strengthen the hypothesis of an early impairment of predispositions in the early development of ASD.
44 Hence, early predispositions are a potentially useful tool to detect early ASD symptoms in human
45 neonates and to investigate the molecular and neurobiological mechanisms underlying the onset of
46 this neurodevelopmental disorder.

In review

47 **1 Introduction**

48 Neonates of some vertebrate species orient their first approach responses towards objects that exhibit
49 features present in social partners and caregivers: face-like configuration, biological motion and self-
50 propulsion. Comparative research on human infants and newly-hatched domestic chicks (*Gallus*
51 *gallus*) found striking similarities in the static and dynamic visual cues that attract attention of these
52 different species soon after birth (Di Giorgio et al., 2017a). Among dynamic cues, point-light
53 displays depicting biological motion are preferred by neonates of both species to the same
54 configuration of dots rigidly rotating or moving randomly (Simion et al., 2008; Vallortigara and
55 Regolin, 2006). Chicks also seem to have a spontaneous preference for objects autonomously starting
56 to move over objects set in motion after a collision (Mascalzoni et al., 2010) and for objects
57 autonomously changing their speed over constant moving ones (Rosa-Salva et al., 2016). Similarly,
58 human neonates exhibit a looking preference for self-propelled objects autonomously starting from
59 rest (Di Giorgio et al., 2017b).

60 Alterations in social predispositions appear to be linked to Autistic Spectrum Disorders (ASD) a
61 complex group of neurodevelopmental disabilities characterised by important deficits in the domain
62 of social cognition (Sacrey et al., 2015). Impairments in face discrimination and recognition have
63 been widely observed in ASD individuals (Dawson et al., 2005). Young children with ASD show
64 altered processing of stimuli depicting biological motion (Freitag et al., 2008; Klin et al., 2009) and
65 difficulties in spontaneous categorization of self-propelled motion as animate (Rutherford et al.,
66 2006). Neonates at high familiar risk of ASD show significant differences compared to low-risk
67 neonates in the preference for a face-like stimulus and for biological motion, suggesting an
68 impairment in the development of the predisposed mechanisms for detecting animate beings (Di
69 Giorgio et al., 2016). Observing the same impairment for both static and dynamic stimuli in a
70 different species would argue in favour of a common developmental origin of these predispositions.
71 Valproic acid (VPA) is an anticonvulsant and a mood stabilizer, widely used to treat epilepsy,
72 migraine and bipolar disorder (Johannessen and Johannessen, 2003). In humans, prenatal exposure to
73 VPA has been shown to increase the risk of developing ASD (Christensen et al., 2013). Embryonic
74 exposure to VPA has been widely used to model the ASD syndrome in rodents (see for a review
75 Nicolini and Fahnestock, 2018). Embryonic exposure to VPA has been shown to induce impairments
76 in chicks' aggregative behaviour (Nishigori et al., 2013) and in their early predisposition for static
77 stimuli (Sgadò et al., 2018).

78 To further study the effect of VPA on early predispositions, and to investigate whether the
79 impairment for static cues is accompanied by impairment in predispositions for dynamic cues, we
80 compared the spontaneous preference for self-propelled stimuli in VPA- and vehicle-injected chicks.

81 **2 Manuscript text**

82 **2.1 Materials and Methods**

83 *Ethical statement.* All experiments comply with the current Italian and European Community laws
84 for the ethical treatment of animals. The experimental procedures were approved by the Ethical
85 Committee of the University of Trento and licensed by the Italian Health Ministry (permit number
86 986/2016-PR).

87 *Embryonic injections.* Fertilized eggs of domestic chicks (*Gallus gallus*), of the Ross 308 (Aviagen)
88 strain, were obtained from a local commercial hatchery (Agricola Berica, Montegalda (VI), Italy) and
89 incubated at 37.7 °C and 60% of relative humidity in the darkness. The first day of incubation was
90 considered embryonic day 0 (E0). At E14, fertilized eggs were selected by candling before injection.

91 Embryo injection was performed according to previous reports (Nishigori et al., 2013; Sgadò et al.,
92 2018). Briefly, a small hole was made on the eggshell above the air sac, and 35 μ moles of VPA
93 (Sodium Valproate, Sigma Aldrich) dissolved in double distilled injectable water were administered
94 to each fertilized egg, in a volume of 200 μ l. Age-matched control eggs were injected using the same
95 procedure with 200 μ l of vehicle (double distilled injectable water). After sealing the hole with paper
96 tape, eggs were placed back in the incubator (FIEM srl, Italy). Previous reports have analysed the
97 effect of different doses and time of administration of VPA on embryonic development in different
98 vertebrate species (see for a review Ranger and Ellenbroek, 2016; Roullet et al., 2013). The typical
99 dose and time of administration in rodents is 200-500mg/kg in acute, single dose administration
100 between E12 and E14. In domestic chicks, administration of 35 μ moles/egg (corresponding to 100
101 mg/kg) has been tested between E10 and E14 with differential effects on hatching rate, showing a
102 dramatic decrease of hatchings at E10 and a significant decrease of hatchings at E12 but no
103 significant effect at E14 (Nishigori et al., 2013). Administration of 35 μ moles/egg at E14 induced
104 social deficits without affecting hatchability, motor behaviour and imprinting abilities (Nishigori et
105 al., 2013; Sgadò et al., 2018).

106 During incubation and hatching, eggs and chicks were maintained in complete darkness, preventing
107 any visual experience prior to the test. Controlling the visual experience during pre- and post-natal
108 development enable to exclude any interference of visual stimuli in the expression predispositions
109 towards animacy cues, and to demonstrate the innate nature of these mechanisms. Each chick was
110 tested only once.

111 *Apparatus, stimuli and test.* We used the same procedure previously described to assess chicks'
112 predispositions for speed-change. Briefly, carefully avoiding any other visual experience, the day of
113 hatching chicks were individually placed in the centre of the test apparatus, a corridor (85x30x30
114 cm), open at the two ends where two video screens were displaying the experimental stimuli. The
115 corridor was divided in three sectors: a central sector (45 cm long) delimited by two steps, that the
116 animals had to climb to enter the two choice sectors (each 20 cm long) immediately adjacent to the
117 two screens. Stimuli were two video-animations representing the movement of a simple red circle. In
118 one video the object was moving at constant speed (speed-constant) and in the other one it was
119 changing its speed (accelerating and decelerating; speed-change). A spontaneous choice test of six
120 minutes was performed for the two stimuli. Chicks' preference for the speed-change stimulus was
121 measured by the ratio of time (in seconds) spent in the choice sector near the speed-change stimulus
122 divided by the cumulative time spent in either of the choice sectors (preference score). Chicks
123 remaining in the central sector were not included in the analyses. Values of this ratio could range
124 from 0 (full choice for the speed-constant), to 1 (full choice for the speed-change), whereas 0.5
125 represented no preference. For more detailed information on the procedure, see Rosa-Salva et al.,
126 (2016). Chicks' level of motility was measured by evaluating the latency (in seconds) to first
127 approach irrespective of the stimulus approached. The tests were performed manually and scored
128 online. To evaluate reliability of scoring and potential biases, 10% of all subjects were scored again
129 offline by a second experimenter blind to the treatment group and right/left position of the two
130 stimuli. Overall, we blindly coded videos of 10 animals randomly chosen from both treatment
131 groups. We obtained a Pearson's correlation of 1.000, $p < 0.001$ between the preference scores
132 calculated using our original data and the blind coding. For the present study 51 VPA-injected
133 (males=27) and 52 vehicle-injected (males=26) chicks were tested.

134
135 *Data analysis.* Effects of Treatment (VPA and vehicle injection) and Sex (male, female) on the
136 preference for the speed-change stimulus were assessed by a multifactorial analysis of variance
137 (ANOVA) on the dependent variable preference score. One-sample two-tailed *t*-tests were run to test
138 significant departures from chance level (0.5) of the preference score, separately for the two groups.
139 The number of chicks that first approached the speed-change or the speed-constant stimulus in the

140 two treatment groups was compared using the chi-square test of independence. Effects of Treatment
 141 and Sex on latency to first approach were assessed by an ANOVA on the latency to first approach
 142 one of the stimuli. All statistical analyses were performed with IBM SPSS Statistic for Windows
 143 (*RRID:SCR_002865*). Alpha was set to 0.05 for all the tests.

144 2.2 Results

145 The average egg hatchability was 75%. Results of the ANOVA on the preference for the speed-
 146 change stimulus showed a significant effect of Treatment ($F_{(1,99)}=4.296$, $p=0.041$; Fig. 1A), and no
 147 significant effect of Sex ($F_{(1,99)}=0.0001$, $p=0.992$) nor any significant interaction (Treatment \times Sex:
 148 $F_{(1,99)}=0.151$, $p=0.698$). In the control group (vehicle-injected), the preference for approaching the
 149 speed-change stimulus was similar to what previously observed, and the preference scores were
 150 significantly higher than chance level ($t_{(51)}=2.365$, $p=0.011$; $M=0.673$, $SEM=0.066$, Fig. 1A). On the
 151 contrary, VPA exposure significantly reduced the preference for the speed-change stimulus: the
 152 preference scores for approaching the speed change stimulus did not differ from chance level
 153 ($t_{(50)}=-0.406$, $p=0.686$; $M=0.472$, $SEM=0.696$, Fig. 1A). A significant difference between the two
 154 groups was found also in the number of chicks that first approached the speed-change stimulus
 155 ($\chi^2=4.314$, $p=0.047$). While in the vehicle-injected group a significantly higher number of chicks first
 156 approached the speed-change stimulus ($\chi^2=6.231$, $p=0.018$; speed-change $N=35$, speed-constant
 157 $N=17$), in the VPA-treated group no significant difference was found in the number of chicks that
 158 approached the two stimuli ($\chi^2=0.176$, $p=0.78$; speed-change $N=24$, speed-constant $N=27$).
 159 To evaluate motility, we measured the latency to the first approach, independent of the stimulus, and
 160 found no significant effects of Treatment ($F_{(1,99)}=2.672$, $p=0.105$; Fig. 1B), Sex ($F_{(1,99)}=1.124$,
 161 $p=0.292$), nor any interaction ($F_{(1,99)}=0.000$, $p=0.99$).

162 2.3 Discussion

163 We investigated unlearned predispositions to orient towards animate motion cues in VPA-injected
 164 chicks compared to vehicle-injected controls, using a choice preference test between a speed-change
 165 and a constant moving stimulus. We showed a detrimental effect of VPA on the typical unlearned
 166 preference for the speed-change stimuli conveying animacy cues (Rosa-Salva et al., 2016). These
 167 results are in line with previous studies [investigating static cues to animacy \(such as the head and](#)
 168 [neck region of the mother hen, Sgadò et al., 2018\)](#) and our hypothesis of a disruption of unlearned
 169 predispositions in animal models of ASD.

171 In phylogenetically distant species of vertebrates, such as domestic chicks and humans, similar
 172 mechanisms have been described to drive early approach responses towards static and dynamic cues
 173 typically associated with animate figures. The adaptive function of early predispositions has been
 174 hypothesized to be in directing attention toward highly important animate stimuli, enabling future
 175 learning through experience and enhancing social interactions (Johnson et al., 2015; Di Giorgio et al.,
 176 2017a; Powell et al., 2018). In chicks, predispositions are likely to orient the young animal toward
 177 the mother hen (or other brood mates), directing subsequent filial imprinting responses towards
 178 animate stimuli (Miura and Matsushima, 2016). In human newborns, subcortical fast and automatic
 179 mechanisms have been hypothesized to underlie these social predispositions, directing attention
 180 toward animate entities to create an early social bond with the caretakers and social companions
 181 (Tomalski et al., 2009; Johnson et al., 2015; Di Giorgio et al., 2017a). Subsequently, experience may
 182 modulate and specialize more sophisticated mechanisms devoted to the processing of social stimuli
 183 (Johnson et al., 2015; Versace et al., 2016).

184 Several accounts suggest that abnormalities in this early social-orienting system may lead to deficits
 185 in social stimuli processing, limiting attention to salient social stimuli, decreasing their reward value
 186 and resulting in the atypical social behaviour associated with ASD.

187 To investigate the contribution of these social-orienting mechanisms in atypical social behaviour
 188 related to ASD, we modelled ASD-like social impairments in domestic chicks using embryonic
 189 exposure to VPA. We then measured preference responses to different social stimuli, either stationary
 190 (the face-like configuration visible in a stuffed hen, Sgadò et al., 2018) or dynamic (speed-changes,
 191 this work), in visually-naïve VPA- injected and vehicle-injected domestic chicks.

192 In this study, we have investigated social predispositions towards animate motion, focusing on the
 193 predisposition to approach objects that appear self-propelled due to an “internal energy source” that
 194 produces changes of speed. Using behavioural responses to visual stimuli, we have documented the
 195 absence of the typical predisposed preferences for animacy stimuli in domestic chicks, as a
 196 consequence of embryonic VPA exposure. This drug has been used to model ASD core deficits in
 197 other vertebrate species (Ranger and Ellenbroek, 2016) although chicks are the first precocial species
 198 in which its effect on social behaviour has been investigated (Nishigori et al., 2013; Sgadò et al.,
 199 2018). Precocial species, like domestic chicks, are characterized by the early maturation of the motor
 200 and sensory system, that allows to perform behavioural tests soon after birth, before gaining any
 201 social experience. Our findings, hence, open new possibilities to tackle the early onset of
 202 predispositions relevant for social life, focusing on dynamic cues.

203 Moreover, these findings extend previous literature reporting impairments in the preference response
 204 for static, face-like configurations of the stuffed hen stimulus (Sgadò et al., 2018). The observation of
 205 a parallel impairment in social predispositions for both static and dynamic cues in different species,
 206 suggests a common developmental origin of this social-orienting system. Since the neuroanatomical
 207 substrates of predispositions for approaching static and dynamic stimuli are at least partially different
 208 (Mayer et al., 2017, Lorenzi et al., 2017), observing here the impairment of both classes of
 209 predispositions suggests the existence of a common mechanism.

210 Our work on VPA-mediated impairment of early predispositions, together with the deficits
 211 documented in human neonates at high risk of ASD (Di Giorgio et al., 2016), supports the hypothesis
 212 of early social orienting mechanisms shared across species whose impairment or delay might have a
 213 pivotal role in the pathogenesis of autism.

214 Future studies should capitalize on these findings to investigate the molecular and neurobiological
 215 mechanisms underlying those ASD early symptoms that are associated with predisposed orienting
 216 mechanisms towards social stimuli.

217 2.4 Figures

218 *Figure 1. (A)* Social preference responses for the speed-change stimulus shown as the ratio of time
 219 (in seconds) spent in the choice sector near the speed-change stimulus divided by the cumulative time
 220 spent in either of sectors (see Methods for details). Analysis of variance of social preference scores
 221 using Treatment and Sex as between-subjects factors, revealed a significant main effect of Treatment
 222 (line with asterisks), with no other main effects or interactions. Preference scores were significantly
 223 different from chance level for vehicle-injected chicks (control group), but not for VPA-treated
 224 chicks. Asterisks on top of bars indicate significant departures from chance level, marked by the red
 225 line at 0.5. *(B)* Latency to first approach assessed as a measure of motility. Analysis of variance on
 226 number of rotations using Treatment and Sex as between-subject factors, showing no significant
 227 main effects of Treatment, Sex or interaction Treatment × Sex. Data represent mean ± SEM, *p <
 228 0.05.

229 3 Conflict of Interest

230 The authors declare that the research was conducted in the absence of any commercial or financial
231 relationships that could be construed as a potential conflict of interest.

232 4 Author Contributions

233 P.S., E.V., O.R.-S. and G.V. conceived and designed the experiments; E.L. and A.P. conducted the
234 experiments; P.S., E.V. and O.R.-S. developed the behavioural paradigms; E.L., A.P., P.S., O.R.-S.
235 and E.V. analysed the data; E.L. and P.S. drafted the manuscript; E.L., A. P., O.R.-S., E.V., P.S. and
236 G.V. wrote the manuscript. All the authors gave final approval for publication.

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317 **8 Data Availability Statement**

318 The dataset generated for this study is available as Supplementary Material.

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Figure 1.TIFF

