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Effects of *in ovo* injection of serotonin on behavior and hypothalamic genes

expression in post hatch-chicks

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ABSTRACT

Serotonin (5-HT) is essential for neuronal development and behavioral regulation. Serotonin, upon *in ovo* administration, has been previously reported to modulate aggressive behavior in avian species, however it still remains unknown if maternal serotonin affects chick behavior through hypothalamic gene expression. We injected an equal volume of saline (control) and either a 5μg (low) or a 15μg (high) dose of 5-HT into embryos on embryonic day 11 (E11). The blood concentration of 5-HT was determined in chicks on post-hatching D3 (Day 3) and D45. The behavioral fear and aggression were analyzed at D10 and D52. In isolation test, the latency to vocalize and walk, the duration of vocalizations and walking were recorded. Hypothalamic expression of genes related to the serotonin pathway and the methylation status of hypothalamic 5-HT 1A receptor (5-HTR1A) were measured at D56. Chicks treated with embryonic 5-HT exhibited a certain decrease in the degree of fear as determined by the duration of vocalizations ($p < 0.05$) in an isolation test. They also exhibited a significantly less aggressive behavior ($p < 0.05$) as compared to chicks given saline control. We also noticed that the higher dosage was consequently associated with elevated concentration of 5-HT in blood. Exposure to high dosage of 5-HT significantly down-regulated Monoamine oxidase A (MAO-A) and B (MAO-B). Alternatively, the mRNA expression of Hypothalamic tryptophan hydroxylase 2 (TPH2) was significantly up-regulated ($p < 0.05$). However, the mRNA levels of Tryptophan hydroxylase 1 (TPH1) and the transporter for 5-HT (5-HTT) remained unchanged. Interestingly, 5-HTR1A was found to be significantly increased in the high-dosage

42 group (p < 0.05). The promoter region of 5-HTR1A gene was significantly

43 hypomethylated (p < 0.05) which correlated negatively with the mRNA expression of

44 5-HTR1A genes (r=-0.682, p=0.005). These results indicate that *in ovo* serotonin

45 injection affects aggressive and fearful behaviors by modifying the expression of genes

46 involved in the serotonergic pathway, through DNA methylation mediated epigenetic

47 mechanisms.

48 **Key words:** serotonin, behavior, gene expression, DNA methylation

49 **1. Introduction**

50 Aggressive behavior in animals is attested as the fight for living space, food, social

51 status, copulation, fear and other factors that determine individual's survival (Li, *et al*.,

52 2016). In poultry industry, aggressions in chick population could cause an increase in

53 social stress and skin injuries, that along with the increased mortality could seriously

54 affect animal welfare in general and economic interests of farmers in particular

55 (Cunningham and van Tienhoven, 1984; Millman, *et al*., 2000). Several studies looking

56 for a new target for the treatment of aggressive behavior in chicken, point towards

57 serotonin (5-hydroytryptamine, 5-HT) as a significant option. Previous studies have

58 shown that exogenous supplementation of 5-HT precursor could alleviate anxiety and

59 aggressive behaviors in laying hens (Bello, et al., 2018; van Hierden, *et al*., 2004).

60 Furthermore, serotonin is recognized as a positive indicator for welfare and a significant

61 negative correlation has been found between the level of 5-HT in the peripheral blood

62 and brain and the frequency of aggressive behaviors including feather pecking in laying

63   hens (Dennis, et al., 2008; Kops, et al., 2017; van der Eijk, *et al*., 2019). However, the

64   biological mechanisms by which serotonin affects behavior in laying hens is still being

65   explored.

66   A vast amount of scientific evidence shows that dysregulation of central 5-HT activity

67   is key to the psychological condition and aggressive behavioral regulation (Bortolato,

68   et al., 2013; Dennis and Cheng, 2014; Kästner, et al., 2019). Determined by the under

69   combined action of central 5-HT synthesis, reuptake and metabolism, deficiency in

70   central 5-HT activity can lead to an increase in aggression (Fineberg, et al., 2010) and

71   fearfulness (Marcinkiewcz, et al., 2016). In the pathway leading to 5-HT synthesis,

72   Tryptophan is first hydroxylated to 5-hydroxytryptophan (5-HTP) by tryptophan

73   hydroxylase (TPH), which is also the rate-limiting enzyme in the biosynthesis of the

74   central serotonin system (Matthes, et al., 2019). The free 5-HT then gets released into

75   the synaptic cleft across presynaptic plasma membrane by the serotonin transporter (5-

76   HTT) or gets inactivated by MAO (Popova, 2006). Ultimately, the central serotonergic

77   system serves its functions in activating different serotonin receptor subtypes largely

78   through a range of downstream signaling molecules (Olivier, 2015).

79   Among a variety of identified serotonin receptors, 5-HTR1A attracts particular

80   attention due to its pivotal role in the mechanisms related to aggressive behavior

81   (Popova and Naumenko, 2013). Some studies provide converging lines of evidence that

82   5-HTR1A also contributes towards the modulation of aggressive behavior. In a similar

83   study, rats lacking 5-HTR1A gene expression in the brain showed an augmented

84   aggressive behavior (Naumenko, et al., 2013). 5-HTR1A polymorphism generates

85   impairments in emotional and cognitive processing, causing inability to cope with

86   stressful situations, thus resulting in an increased aggressive behavior (Beste, et al.,

87   2010a; Beste, et al., 2010b). Additionally, several studies describe new data on the

88   unique role of the 5-HTR1A in epigenetic modulation. Specifically, increased DNA

89   methylation of HTR1A promoter in hypothalamus has been reported to function in

90   unpredictable chronic mild stress (François, et al., 2015). Hypomethylation of the

91   HTR1A promoter in lymphocytes from lupus erythematosus patients correlates with

92   increased 5-HT1A expression (Xu, et al., 2011). More recently, it was reported that

93   exposure to serotonin during embryonic development can affect mood and aggressive

94   behavior in chicken offspring, probably through involvement of epigenetically

95   regulated of 5-HTR1A (Dennis, et al., 2013a).

96   The embryonic development period is critical for epigenetic reprogramming (Liu,

97   et al., 2019) and the changes induced by DNA methylation during this period have been

98   reported to greatly impact aggressive behavior (Ahmed, et al., 2014). Although many

99   studies have highlighted the influence of 5-HT on aggressive behavior, the mechanistic

100   details remain unclear. In this study we propose a role for *in ovo* injected serotonin in

101   regulating the aggressive behaviors in chick by affecting the 5-HT pathway through

102   regulation of gene expression and identify the possible underlying epigenetic

103   mechanisms. We find evidence that link these changes in the chick behavior to

104   perturbations in the expression of genes related to the central serotonergic system in

105   particular through the regulation of CpG methylation of 5-HTR1A gene promoter

106   during embryonic life.

2. Materials and Methods

2.1 Ethics

All animals used in this study were approved by the Animal Ethics Committee of Hebei Agricultural University (University Identification Number: HB/2019/03). Every effort was made to minimize animal pain, suffering, and distress throughout the experiment.

2.2 Experimental Design

Three hundred fertilized chicken eggs were selected from a breeding company（DAWU, Baoding, China）and randomly divided into three groups each bearing 100 eggs. All the eggs were incubated at an average egg shell temperature and relative humidity of 37.3 °C and 65.0% respectively. The injection conditions were set as described previously (Ahmed, et al., 2013). Briefly, the eggs were removed from the incubator and injected with either saline (control), 5μg (low) or 15μg (high) dose of serotonin (Sigma, St. Louis, MO, USA) on embryonic day 11 (E11). Before injection the solutions were sterilized by heating at 180℃ for 30 minutes. A small amount of melted wax was placed on the damaged area to limit the excessive air exchange through the eggshell that may lead to egg contamination. After all the eggs were treated, they were carefully placed in the incubator until hatched. After hatching, the chicks in each group were reared in separate cages (10 birds/cage), keeping the conditions for temperature, humidity and light cycle same for all the three groups. The chicks had unlimited access to water and feed. The experiment procedure is shown in Fig.1A. The body weight was recorded from day of hatching (D1) to 8th week. On D3 and D45, blood samples were

collected for 5-HT measurements. Behavior tests were evaluated on D10 and D52 of the trial (one week after the blood test). At the end of the experimental timeline, all the birds were sacrificed by cervical dislocation. Hypothalamic samples were collected under sterile conditions and stored at -80 °C for further analysis.

2.3 Blood 5-HT assay

Five birds from each treatment group were sampled (1mL blood approx.) on D3 and D45 for 5-HT assay. ==The blood 5-HT concentrations (ng/mL) were measured with a commercially available enzyme immunoassay kit (Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions.==

2.4 Behavior test

The isolation test was conducted on the birds on D10 as described in a previous study (Kops, et al., 2017). Five randomly chosen chicks from each treatment group were placed in a cylinder (diameter 28 cm), outside the home pen, but in the same room. The latency to vocalize and walk, the duration of vocalizations and walking were recorded by using a video camera and microphone that was hung above the cage during the test period.

The aggressive behavior of the birds was observed on D52 as described in a previous study (Kitaysky, et al., 2003). Briefly, birds (2 males and 3 females) were randomly selected from each treatment group (control, low, and high treatment groups, i.e., three birds/cage; n = five/treatment) and were observed for aggressive behavior in a cage (90 × 80 × 70 cm, L × W × H respectively) besides their home pens. For visual identification,

149 each bird was marked with a differently colored ring (yellow, green and blue

150 respectively) on right leg. The behaviors were recorded using a digital video recording

151 system and analyzed for aggressive behavior variables such as pecking and kicking.

152 Pecking is defined by fight where a bird hits another with its beak or pecks quickly on

153 the other bird's head and neck. In kicking, one bird flies towards another striking it with

154 its foot.

155 **2.5 RNA extraction and real-time PCR analysis**

156 The hypothalamic tissue was retrieved from -80 °C and total RNA was extracted using

157 the total RNA extraction kit (Invitrogen, 12183-555, USA) in accordance with the

158 manufacturer's instructions. The quality of RNA was verified using a nucleic acid

159 quantification analyzer (Smart Spec Plus BIO-RAD). Total RNA was reverse-

160 transcribed into cDNA using Super Script™ III First-Strand Synthesis (Invitrogen,

161 11752-050, USA) according to the manufacturer's instructions. The resulting cDNA

162 was stored in -80°C until further analysis. Successful cDNA synthesis was confirmed

163 by amplifying the house-keeping β-actin mRNA via PCR. The qRT-PCR primer

164 sequences for 5-HTR1A, TPH1, TPH2, 5-HTT, MAO-A and MAO-B genes are listed

165 in Table 1. The relative expression level of each gene was calculated in triplicate for

166 each sample using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). β-actin was

167 employed as an internal control for the normalization of gene expression levels.

168 **2.6 DNA methylation assay**

169 DNA was extracted from 1g hypothalamic tissue using the Tissue DNA extraction kit

170 (BioTeKe Corpration). The quality and concentration of extracted DNA were

171 determined by agarose gel electrophoresis and nucleic acid quantification. To perform

172 bisulfite conversion of the target sequence, the Epitect Bisulfite Kit (Zymo) was used

173 according to the manufacturer's protocol. The following PCR conditions were used for

174 amplification of the bisulfite-treated genomic DNA: one cycle, 94℃ for 4 min; 45

175 cycles, 94℃ for 20 sec; 56℃ for 30 sec; 72℃ for 1 min; and one cycle, 72℃ for 3

176 min. After PCR amplification, 3μl of the PCR product was mixed with 1 μL of 6X

177 loading buffer and run on a 1.5% agarose gel. Unincorporated dinucleotide

178 triphosphates (dNTPs) were removed by shrimp alkaline phosphatase (SAP; Agena, Inc)

179 treatment. In the same step, RNase-A (Agena, Inc) was added to cleave the *in vitro*

180 transcripts (T-cleavage assay). Conditioning of the phosphate backbone was achieved

181 by adding 6 MG of Clean Resin (Agena, Inc) before performing MALDI-TOF MS

182 analysis. Finally, the RNase-A treated product was robotically dispensed onto silicon

183 matrix preloaded chips (Spectro CHIP; Agena, Inc). Th e mass spectra were collected

184 using a Mass ARRAY Compact MALDI-TOF (Agena, Inc), and spectra's methylation

185 ratios were obtained using Epi TYPER™ software (Agena, Inc). We designed the

186 primers for the 5-HTR1A genes to cover the regions with the most CpG sites. The

187 sequences of Meth Primer are listed in Table 1.

188 **2.7 Statistical analysis**

189 Data were tested for normal distribution and homogeneity of variance. One-way

190 ANOVA was used for statistical analysis with SPSS version 21.0 for Windows software

191 (SPSS Inc., Chicago, IL, USA). The results were expressed as mean ± SD and a value

192   of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Growth performance

195   *In ovo* injection with serotonin did not affect the post hatch growth rate of the newly

196   hatched chicks. However, the body weight of the chicks in the high-dose treatment was

197   higher than the chicks in the control group, as measured in the 8th week ($p < 0.05$,

198   Fig.1B).

3.2 Behavior

200   In isolation test, the duration of vocalizations in high-dose treatment group was shorter

201   than that of the control group ($p < 0.05$, Fig.3A) whereas, the chicks in the low-dose

202   treatment group showed no such change. However, latency to vocalize and walk and

203   the duration of walking in high- and low-dose treatment chicks were similar to those of

204   control chicks ($p > 0.05$, Fig.3A).

205   In high-dose treatment chicks, the frequency of aggressive pecking was less than that

206   of the control chicks ($p < 0.05$, Fig.3B) as measured for the aggressive test. Again, there

207   was no significant difference between the low-dose treatment and the control group

208   chicks. However, birds from either of the serotonin-treated group had any difference in

209   kicking, compared with the control birds ($p > 0.05$, Fig.3B).

3.3 Blood 5-HT and hypothalamic expression of serotonergic genes

211   Compared with the low-dose and control group, 5-HT concentration in blood was

212   higher in the high-dose treatment group at D3 post-hatch ($p < 0.05$, Fig.2). At the later

213   timepoint of D45, chicks from high-dose treatment still had significantly higher 5-HT

214   concentration compared to controls, but no significant differences were observed in

215   low-dose treatment ($p > 0.05$, Fig.2). Based on the above data, we consider that chicks

216   treated with the high-dose of serotonin maintained an increased blood 5-HT

217   concentration at a significant level in the whole experimental period.

218   The mRNA expression of 5-HTT, TPH1, TPH2, MAO-A and MAO-B in the chicken

219   hypothalamus upon *in ovo* injection with serotonin are shown in Figure 4 and Figure 5.

220   The mRNA of the rate-limiting 5-HT biosynthesis enzyme TPH2, which mediates 5-

221   HT synthesis from catalytic tryptophan, was significantly up-regulated ($p < 0.05$,

222   Fig.4A) in serotonin-treated chicks. Conversely, the relative mRNA expression of

223   MAO-B, which converts 5-HT to 5-hydroxyindoleacetic acid performing the catabolic

224   reaction for the serotonin pathway, was significantly down-regulated in serotonin-

225   treated chicks ($p < 0.05$, Fig.4B). The decrease in MAO-A mRNA expression was

226   observed only in the high-dose treatment group chicks ($p < 0.05$, Fig.4B). However, no

227   significant alterations were detected for other synthesis and transport genes, including

228   TPH1 and 5-HTT ($p > 0.05$, Fig.4C).

229   **3.4 Hypothalamic 5-HTR1A gene expression and promoter methylation**

230   *In ovo* injection with serotonin significantly up-regulated the expression of 5-HTR1A

231   in the chicken hypothalamus ($p < 0.05$, Fig.5A), which is reported to modulate mood

232   and aggressive behavior regulation. The structure of the chicken 5-HTR1A gene

233   promoter indicating its methylation status, as seen in the MassARRAY analysis, is

234   shown schematically in Fig.5B. Hypomethylation of the 5-HTR1A promoter region was

235   observed in serotonin-treated chicks (Fig.5C, $p < 0.05$), which was found to be

236   negatively correlated with the mRNA expression of 5-HTR1A genes (r=-0.682,

237   p=0.005, Fig.5D). The number and distribution of all the twenty-nine CpG islands and

238   their methylation levels in the 5-HTR1A promoter regions of the hypothalamus were

239   analyzed but significant differences were observed only at a few CpG sites (Figure 5E).

240   **4. Discussion**

241   In the current study, we provide an evidence that lower aggression and fear are

242   associated with the increased body weight in the chicken, prenatally treated with

243   serotonin. 5-HT is a well-known central neurotransmitter that affects neuroendocrine

244   function and physiological status thus playing a role in mood control, anxiety, food

245   intake, aggression and social behaviors (Gibson, 2018; Maney and Goodson, 2011;

246   Walther and Bader, 2003). Alterations in serotonergic system during embryonic

247   development have the potential to greatly impact behavior (Grieb and Ragan, 2019;

248   Maciag, et al., 2006), but the type and extent of behavioral changes are not fully

249   understood. Here, we found that a high dose 5-HT on day 11 of embryonic development

250   significantly decreases the frequency of aggressive behavior in post-hatch chicks. Our

251   findings corroborate to a similar finding by Dennis, et al., 2013a, where they show that

252   serotonin, if provided early in incubation can reduce aggressiveness of chicks at 9

253   weeks. Furthermore, some studies also relate aggression to high fearfulness and anxiety

254   (Bolhuis, et al., 2009; Uitdehaag, et al., 2008). The isolation test examines a bird's fear

255 of social isolation. In another study the longer latency time and the lesser duration for

256 vocalizations indicated that laying hens were emotionally stable and that was related to

257 a lower degree of fear (Dennis, et al., 2013b; Kops, et al., 2017). In our study chicks

258 exposed to high dose serotonin had a shorter duration of vocalizations. This active

259 behavior possibly suggests that injecting the embryo with 5-HT could increase positive

260 emotions in chicks, reduce the degree of fear in them and make them less aggressive,

261 ultimately causing them to gain significantly higher body weight.

262 High blood 5-HT levels were previously associated with less fear-related and

263 aggressive behavior in chickens (Bolhuis, et al., 2009) as well as in dogs (Rosado, et

264 al., 2011). In this study we also substantiate that high dose treatment group with a

265 continually elevated 5-HT in the blood is accompanied by less fear-related behavior

266 and aggression in newly hatched chicks. Previously, a positive correlation has been

267 drawn between blood 5-HT and central 5-HT concentrations in the brain (Uitdehaag, et

268 al., 2011; Yubero-Lahoz, et al., 2014). Animals exhibiting relatively low central 5-HT

269 concentrations or reduced cerebrospinal fluid concentrations of 5-hydroxyindoleacetic

270 acid exhibited fear-related behaviors or aggressiveness (Ferrari, et al., 2005; Higley and

271 Linnoila, 1997). However, as it is impossible to determine serotonin metabolism in

272 brain in a cell-specific manner, we measured serotonergic genes expression in the

273 hypothalamus of chicken at the molecular level. The up-regulation of TPH2 and 5-

274 HTR1A suggest an upregulation of 5-HT synthesis and release, whereas the down-

275 regulation of MAO-A and MAO-B point to an inhibited 5-HT metabolism and hence

276 suppressed turnover. The combined effects of enhanced 5-HT biosynthesis and

277 suppressed 5-HT catabolism may contribute to higher 5-HT accumulation in

278 hypothalamic regions of the high dose treatment chicks. But we did not find any change

279 in TPH1 expression. This discrepancy could be explained by the fact that TPH1 is

280 mainly expressed in the gastrointestinal tract and the pineal gland (Swami and Weber,

281 2018). Surprisingly, our findings controvert a previously done study that claimed that

282 5-HTT gene expression could be a cause for a lower degree of fear in mammals

283 (Bocchio, et al., 2015). This could mean that mammals and birds may be differed in the

284 correlation between 5-HTT expression and fear-related behaviors, an area that needs

285 more exploration.

286 The implication of 5-HT1A receptors in aggression is very well supported by numerous

287 lines of evidence. Rodents genetically selected for high aggression exhibit distinct

288 alterations of expression and sensitivity of 5-HT1A receptor (Popova, et al., 2005), and

289 an up-regulated hypothalamic 5-HTR1A expression suppresses aggressive behavior in

290 chickens (Idriss, et al., 2017). It is well established that epigenetic changes affecting 5-

291 HTR1A expression could impact responsiveness towards 5-HT, consequently

292 impacting aggressive response (Dennis, et al., 2013b). Furthermore, epigenetic

293 regulation of 5-HTR1A through DNA methylation and histone modifications, can be

294 altered by embryonic exposure to serotonin, which may lead to a plasticity in serotonin

295 system (Dennis, et al., 2013a). In the present study, we show that the high dose of 5-

296 HT decreases the CpG methylation status of 5-HTR1A gene promoter, which is

297 conversely correlated with the mRNA abundance of 5-HTR1A (r=-0.682, p=0.005).

298 This decrease is accompanied by a lower aggressive behavior in the high-dose treatment

group chicks. Nevertheless, transcriptional regulation of gene expression is a complex mechanism, and the levels of mRNA do not always match the methylation level of the promoter. For instance, 5-HTR1A promoter in low-dose treatment group chicks was significantly hypomethylated, whereas no alteration was detected in its mRNA expression.

In conclusion, our study provides evidence that *in ovo* injection leading to embryonic exposure to serotonin may modulate a chicken's aggressive and fearful behavior through modulations of hypothalamic 5-HT gene expression. We have reasons to believe that the DNA methylation of the 5-HTR1A gene promoter may contribute, at least in part, towards the regulation of such behavior as caused by the embryonic exposure to serotonin. Our results provide potential mechanisms for methylation of the 5-HTR1A promoter which works in close agreement with the embryonic exposure to 5-HT influencing growth and behavior in birds upon development.

Acknowledgments

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