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Article

The Role of Serotonergic Gene Methylation in Regulating Anxiety-Related Personality Traits in Chimpanzees

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Simple Summary: Serotonin is a neurotransmitter known to regulate psychological health (depression, anxiety) and personality in humans. Environmental conditions like early life stress or medication use can impact the serotonin system, for example, through epigenetic modification of the genes that code for its different components. While human studies are currently exploring the effects of serotonergic methylation, an example of epigenetic modification, primate studies are largely lacking. In this study we investigate to what extent environmental effects impact personality traits in captive chimpanzees through methylation of two genes that play a major role in serotonin transmission: the gene coding for its receptor subtype 1A (*HTR1A*) and the gene coding for its transporter (*SLC6A4*). Using a methylation array identical to human studies, we show that methylation has an impact on four chimpanzee personality traits and overall seems to reduce anxiety and aggression while promoting more prosocial and explorative personality traits. Different from human studies, early atypical social rearing conditions had only a minor impact on methylation. The results from this study highlight evolutionarily conserved methylation sites that can be targeted in future hypothesis driven studies across species.

Abstract: While low serotonergic activity is often associated with psychological disorders like depression, anxiety, mood and personality disorders, variations in serotonin also contribute to normal personality differences. Here we investigate the role of blood DNA methylation levels at individual CpG sites of two key serotonergic genes (serotonin receptor gene 1A, *HTR1A*; serotonin transporter gene, *SLC6A4*) in predicting personality of captive chimpanzees. We find associations between methylation at 9/48 CpG sites with four personality dimensions: Dominance, Reactivity/Dependability, Agreeableness and Openness. Directionality of effects were CpG location-dependent and confirmed a role of serotonergic methylation in reducing anxiety (Dominance) and aggression related personality (Reactivity/Undependability) while simultaneously promoting prosocial (Agreeableness) and exploratory personality (Openness). While early-life adversity has been shown to impact serotonergic methylation patterns in other species, here, atypical early social rearing experiences only had a modest impact on CpG methylation levels in this chimpanzee sample. While the precise environmental factors impacting serotonergic methylation in chimpanzees thus remain to be identified, our study suggests a role in shaping natural variation in animal personality. The results of this study offer a basis for future hypothesis driven testing in additional populations and species to better understand the impact of ecology and evolution on complex behavioral traits.

Keywords: Illumina Infinium Methylation EPIC array; rearing; behavioral style; primate

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1. Introduction

Serotonin (5-hydroxythalamine or 5-HT) is a chemical produced by nerve cells that has an impact on almost every physiological process in the body including the gastrointestinal, cardiovascular, and central nervous systems. Reduced serotonin activity plays a major role in the development of a number of psychiatric disorders that are highly prevalent in modern human populations including depression, anxiety, and mood and personality disorders. The levels of 5-HT that are available for neural signaling and regulate its activity are dependent on several factors such as the availability of its rate limiting enzyme tryptophan hydroxylase-2 (TPH2), the number of serotonin reuptake transporters (5-HTT), and the number of available serotonin receptors. For example, patients with depression typically show an increased expression levels of serotonin receptor subtype 1A (HTR1A), and treatment of depression most often involves the use of selective serotonin reuptake inhibitors (SSRIs) which block the 5-HTT, thereby increasing the amount of available serotonin for neural signaling.

Serotonin functioning also accounts for normal variation in temperament or personality [1–4]. Links have been reported in humans between variation in serotonergic signaling and scores on personality traits with high loadings for personality items related to impulsivity, aggression, and stress/anxiety [1–4]. For example, low serotonin activity typically relates to higher scores on Neuroticism, a personality dimension measured by the Revised NEO Personality Inventory (or NEO-PI-R) with six underlying facets labeled anxiety, angry hostility, depression, self-consciousness, impulsiveness, and vulnerability [1,5,6]. Other studies report a link with Harm Avoidance, a personality dimension that strongly correlates with Neuroticism [7], but is measured using a different personality assay, the Tridimensional Personality Questionnaire (TPQ) [8]. Individuals scoring high on Harm Avoidance tend to worry more, fear uncertainty more, be shyer with strangers and more susceptible to fatigue.

Several lines of evidence confirm the association between serotonin and anxiety related personality dimensions. Serotonergic drugs treat psychopathological disorders with symptoms like depression and anxiety that reflect high levels of Neurotic personality and SSRIs are shown to decrease Neuroticism, which mediates the effect of SSRIs on depressive symptoms [9–11]. Neurological evidence from PET (positron emission tomography) scans also shows that Neuroticism can be predicted based on binding activity of serotonin to its receptor and/or transporter [1,12,13]. While Neuroticism is also estimated to have a significant heritable basis (15%) in humans [14], studies investigating the molecular genetic basis of the association between serotonin and Neuroticism are inconclusive and report rather small genetic effects [15]. While specific variants in the genes coding for the transporter and receptor have been shown to alter gene transcription, protein structure and/or function, making individuals more prone to developing specific personality phenotypes [7,16–19], environmental factors, like early life stress or trauma have been shown to mediate these effects by influencing epigenetic processes like DNA methylation [20]. In humans, epigenetic modifications of serotonergic genes contribute substantially to the pathogenicity of major depression and anxiety disorders [21,22]. For example, increased levels of methylation in the proximal promoter region of 5-HTT are associated with lower transporter expression and higher threat-related responsiveness of the amygdala [22], whereas lower methylation results in better stress-adaptive reaction [21].

The role of epigenetic effects on the development of personality is gaining increasing interest but remains understudied in nonhuman primates [but see 23]. Chimpanzees offer an interesting comparative framework as they are, together with bonobos, genetically most closely related to humans and thus show considerable overlap in the neural architecture supporting personality processes and physiology [24–27]. In chimpanzees, personality dimensions have also been quantified using identical methods to human personality research, yielding similar multidimensional personality models [28]. Comparative work in the two species can thus yield interesting novel information on the generalizability and functioning of serotonergic personality regulation across species. In the current study, we

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investigate the link between blood CpG methylation levels of two key serotonergic genes (the gene coding for the serotonin transporter: *SLC6A4* and *HTR1A*) and personality scores of captive chimpanzees. Chimpanzee personality data were assessed in a previous study using questionnaire data [28]. This approach yielded six personality components: Reactivity/Undependability, Dominance, Openness, Extraversion, Agreeableness and Methodical. Different from the human Neuroticism dimension, trait items related to anxiety, depression and impulsive behavior do not fall on one dimension in chimpanzees, but are scattered among three dimensions: Reactivity, Dominance, and Extraversion (Table 1 for item loadings on each dimension) [28]. We thus expect to find epigenetic serotonergic effects to target primarily those three dimensions.

Table 1: Adjectives loading onto varimax-rotated chimpanzee personality traits.

Trait	Adjectives / behaviors
Reactivity/ Undependability	+ Irritable + Temp./moody + Deceptive + Impulsive + Defiant + Mischievous + Jealous + Manipulative + Stingy + Bullying + Aggressive + Eccentric + Socially inept + Excitable + Autistic - Calm
Dominance	+ Bold + Relaxed + Dominant - Fearful - Timid - Cautious - Dependent - Anxious
Extraversion	+ Active + Playful + Sexual + Affiliative- Solitary - Depressed
Openness	+ Human oriented + Inquisitive/curious + Inventive + Intelligent + Affectionate/ Friendly + Persistent
Agreeableness	+ Protective + Considerate
Methodical	+ Self-caring + Methodical

Chimpanzee Dominance and Extraversion also showed significant heritability estimates ($h^2 = 0.195$ and $h^2 = 0.381$, respectively), which were dependent on early social rearing conditions, with mother-reared individuals showing higher heritability rates for both dimensions compared to chimpanzees reared by humans in a nursery setting [29]. Given that stress has been shown to impact serotonergic methylation in other species, with early life stress [20] or mild life stress [30] causing higher *SLC6A4* or *HTR1A* methylation levels in humans or rodents, respectively, we predicted that nursery reared individuals might have higher serotonergic methylation levels compared to mother reared individuals. Early social rearing conditions have previously been shown to mediate the association between dopaminergic methylation patterns and Extraversion in chimpanzees, but effects were modest [23].

2. Materials and Methods

2.1. Subjects

All chimpanzees were housed at the National Center for Chimpanzee Care, which is part of the Michale E. Keeling Center for Comparative Medicine and Research (KCCMR), UT MD Anderson Cancer Center, Bastrop, TX. The guidelines from the American Psychological Association for the ethical treatment of animals in research were followed throughout this project.

2.2. Personality

A chimpanzee-specific questionnaire was developed to determine chimpanzee personality traits in previous work and validated using behavioral observations (Freeman et al. 2013). Personality data were obtained for 99 chimpanzees, 50 of which were included in this study as blood samples were available to determine corresponding methylation profiles (21 females, 29 males, mean age: 25 years, age range at time of personality sampling: 12-51 years). A total of 41 personality items were rated on a Likert scale from 1 to 7 (least to most descriptive) by a total of 17 staff members that were familiar with the

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chimpanzees for at least 6 months and felt confident to produce accurate ratings (caregivers, trainer, enrichment technician, behavioral research coordinator and colony manager). Each expert was asked to rate as many chimpanzees as they felt confident rating, leading to each of the 17 raters scoring on average 72 chimpanzees (range 9 to 99). Interrater reliabilities were high for all adjectives save "predictable", which was therefore excluded from further analysis. Six personality domains were revealed using principal component analysis on the means across raters: Reactivity/Undependability, Dominance, Extraversion, Openness, Agreeableness, and Methodical (Table 1 for items loading on each factor and supplementary Table S1 for detailed adjective scores). The validity of the structure of these six factors was then further evaluated by experts in chimpanzee behavior and examined for convergent and predictive validity [28]. Five out of six dimensions show strong evidence for convergent and predictive validity, meaning they are consistent among raters, across time, among studies, and show expected correlations with independently collected quantitative behavioral observations [28,31–33]. The sixth dimension, Methodical, tended to differ between studies and given that it showed poor construct validity we excluded it here.

2.3. DNA extraction and methylation

Blood samples were used to extract DNA for methylation analysis for 49 chimpanzees with available personality profiles and known early social rearing conditions. Blood sampling was done when chimpanzees were between 12 and 51 years old and the time between blood sampling and collection of personality ratings was on average 2.11 years (SD= 3.96). Extractions of genomic DNA were performed with the automated QiaCube (Qiagen) using the QIAampDNA Mini Kit for 200 µl of whole blood. Extracted DNA samples were brought to a concentration of ~70 ng/µL and concentrations were confirmed with a Nanodrop 2000 (Thermo-Fisher Scientific) spectrophotometer. Next, samples underwent bisulfite conversion and were then analyzed using the Illumina Infinium Methylation EPIC array at the Yale Center for Genome Analysis. This array was designed for measuring methylation levels at over 850,000 CpG sites in the human genome. Therefore, for the purpose of using it in chimpanzees, we limited our dataset to CpG sites targeted by probes that map to the chimpanzee (panTro2.1.4) genome with 0–2 mismatch, with no mismatches within 5 bp of the target CpG, and with no SNPs known in chimpanzees following [34]. We also filtered out probes with spectral intensities not significantly different from background levels, that do not target CpG dinucleotides, that are on the sex chromosomes, and for which fewer than 3 beads were counted for 5% or more of the samples with the ChAMP v2.18.3 R package [35]. Because blood tissue is a composite of multiple cell types with proportions that vary over time and across individuals, we corrected the methylation data for variation in cell type composition using the refbase function in the ChAMP package [35].

2.4. Statistical analysis

2.4.1. Methylation vs personality

All statistical analyses were performed in R (www.r-project.org, version 3.3.2). To test for *HTR1A* and *SLC6A4* methylation effects on personality, we ran linear models using the lm function in the lme4 package in R [36]. Each personality factor was tested as an outcome variable in a separate model. As fixed effects we included sex and methylation scores at individual CpG sites for each gene [20]. The age at which blood was collected and individual relatedness coefficients were entered as covariates. Relatedness coefficients were calculated using the degree of relatedness of each individual to all other individuals in the colony and extracted based on the entire pedigree using the kinship2 package in R (https://cran.r-project.org/package=kinship2). Model selection was done using the Akaike Information Criterion (AIC). We examined diagnostic plots (residuals vs. leverage, QQ plots, etc.) and Shapiro-Wilk tests to confirm the assumptions of linear models. As we included individual CpG sites, we employed a p-value correction using a false

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discovery rate (FDR) correction of 5% for each personality dimension [37] to account for multiple testing. To allow for cross-study comparison of our results, we also included a principal component approach sometimes used in literature that reduces model complexity by clustering the CpGs that are present for each gene (N_{HTR1A} = 22, N_{SLC6A4} = 26) in fewer overarching dimensions (Supplementary Table S2-S3). None of the associations between personality dimensions and PCA scores were significant after FDR correction (Supplementary Table S4).

2.4.2. Rearing vs methylation

Finally, differences in methylation scores were tested between individuals with different early social rearing backgrounds for those CpGs that showed a significant association with personality. Mother-reared individuals were tested against individuals that were raised by human care staff in a nursery setting. These models were run separately from the models above, given that we excluded three wild-born individuals from this analysis. This was done because a sample size of three does not allow for meaningful statistical comparison to the other two rearing categories, and because often the background of wild-born individuals is complex as they were partially mother-and human-reared, depending on how old they were when they entered into captivity. For the remaining 13 nursery-reared and 34 mother-reared chimpanzees in our sample, we treated individual CpG methylation scores as outcome variables and rearing background was entered as fixed effects while a correction for age, sex and relatedness remains in the model, as similarly described for the linear models above. Both mother- and nursery-reared chimpanzees had access to outdoor enclosures and showed minimal variance in environmental factors outside of rearing conditions.

3. Results

3.1. HTR1A and SLC6A4 methylation sites

We identified 21 *HTR1A*-specific CpG sites and 27 *SLC6A4*-specific CpG sites and confirmed their presence in the chimpanzee genome. Coordinates and IDs of individual CpG sites in the human and chimpanzee genome are shown in Table 2.

3.2. Associations between CpG methylation scores and personality

Methylation levels of nine CpG sites (three for *HTR1A* and six for *SLC6A4*) were significantly associated with four personality traits (Dominance, Agreeableness, Reactivity and Openness) after FDR correction for multiple testing (Table 3, Figure 1). Higher methylation of SLC6A4 probe cg10901968 predicted lower Dominance (p_{adj} = 0.005), Agreeableness (p_{adj} = 0.003) and Openness (p_{adj} = 0.022) scores. Higher methylation of *HTR1A* probe cg23448729 similarly resulted in lower Dominance scores (p_{adj} = 0.021) while higher scores on *HTR1A* probe cg27615388 predicted lower scores on Reactivity (p_{adj} = 0.001) and Openness (p_{adj} = 0.038). Conversely, higher scores on *HTR1A* probe cg02266732 predicted higher scores on Openness (p_{adj} = 0.038). Finally, Agreeableness scores were further associated significantly with scores on five more *SLC6A4* probes, four of which showed positive associations (cg14312898: padj = 0.006; cg22584138: p_{adj} = 0.038; cg05016953: p_{adj} = 0.003; cg26741280: p_{adj} = 0.012) and one showed a negative association (cg26741280: p_{adj} = 0.034). For overview of remaining associations between individual CpGs and personality domains see Supplementary Tables S5-S9.

Table 2: 48 Chimpanzee serotonergic CpG sites identified and included in this study: 21 in the serotonin receptor gene 1A (*HTR1A*) and 27 in the serotonin transporter gene (*SLC6A4*).

Gene	CpG ID	Functional region	Location	CHR	Coordinate hg19
HTR1A	cg11432303	TSS1500	shore	5	63259058
	cg20598238	TSS1500	shore	5	63258783
	cg08764163	TSS1500	shore	5	63258768

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	cg27420687	TSS1500	shore	5	63258592
	cg16807523	TSS1500	shore	5	63258434
	cg10198270	TSS200	shore	5	63258311
	cg13666507	TSS1500	shore	5	63257941
	cg07839533	TSS1500	island	5	63257885
	cg15092168	TSS1500	island	5	63257873
	cg11615755	TSS1500	island	5	63257867
	cg09698471	TSS1500	island	5	63257847
	cg08259925	TSS1500	island	5	63257813
	cg16280141	TSS1500	island	5	63257753
	cg02266732	**TSS200**	**island**	**5**	**63257710**
	cg04694812	TSS200	island	5	63257554
	cg04427003	1stExon	island	5	63257499
	cg17386123	1stExon	island	5	63257353
	cg27615388	**1stExon**	**island**	**5**	**63257092**
	cg04799838	1stExon	island	5	63256926
	cg10588470	1stExon	island	5	63256619
	cg23448729	**1stExon**	**shore**	**5**	**63256285**
SLC6A4	cg12074493	TSS1500	shore	17	28564117
	cg06841846	TSS1500	shore	17	28564094
	cg14312898	**TSS1500**	**shore**	**17**	**28563979**
	cg03829016	TSS1500	shore	17	28563859
	cg18584905	TSS1500	shore	17	28563300
	cg10901968	**TSS200**	**island**	**17**	**28563108**
	cg26741280	**TSS200**	**island**	**17**	**28563089**
	cg25725890	TSS200	island	17	28563054
	cg05016953	**1stExon**	**island**	**17**	**28562813**
	cg06373684	1stExon	island	17	28562751
	cg26438554	1stExon	island	17	28562733
	cg14692377	1stExon	island	17	28562685
	cg03363743	**5'UTR**	**island**	**17**	**28562474**
	cg22584138	**5'UTR**	**shore**	**17**	**28562220**
	cg05951817	5'UTR	shore	17	28562142
	cg00386645	5'UTR	shore	17	28560965
	cg10241426	5'UTR	shelf	17	28558934
	cg16647683	5'UTR	opensea	17	28558098
	cg01991100	5'UTR	opensea	17	28555935
	cg09921370	5'UTR	opensea	17	28555315
	cg01330016	5'UTR	opensea	17	28549806
	cg10146136	Gene body	opensea	17	28547550
	cg27427014	Gene body	opensea	17	28539980
	cg08743901	Gene body	opensea	17	28535556
	cg06961290	Gene body	opensea	17	28535040
	cg20209182	Gene body	opensea	17	28530849
	cg20592995	3'UTR	opensea	17	28524160

CHR = Chromosome number, 5′ UTR = 5 end untranslated region, TSS1500 = region 1500bp upstream of transcription site, TSS200 = region 200bp upstream of transcription site, 3′ UTR = 3 end untranslated region. Boldface indicates CpGs significantly associated with chimpanzee personality in this study.

Table 3: Model statistics for *HTR1A* and *SLC6A4* individual CpG scores and their significant associations with personality dimensions in chimpanzees.

Personality	Gene	Cg probe	Est	SE	t	p	p_{adj}	sign.
Dominance	*HTR1A*	cg23448729	-6.129	1.652	-3.710	0.001	0.021	*
	SLC6A4	cg10901968	-59.575	14.500	-4.109	0.000	0.005	**
Agreeableness	*SLC6A4*	cg14312898	7.521	2.051	3.667	0.001	0.006	**
		cg22584138	2.375	0.859	2.766	0.008	0.038	*
		cg05016953	14.287	3.562	4.011	0.000	0.003	**
		cg03363743	-4.568	1.587	-2.879	0.006	0.034	*

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		cg10901968	-37.428	9.303	-4.023	0.000	0.003	**
		cg26741280	15.793	4.740	3.331	0.002	0.012	*
Reactivity	*HTR1A*	cg27615388	-7.345	1.646	-4.463	0.000	0.001	**
Openness	*HTR1A*	cg02266732	8.661	2.820	3.072	0.004	0.038	*
		cg27615388	-5.184	1.627	-3.187	0.003	0.038	*
	SLC6A4	cg10901968	-38.955	10.859	-3.587	0.001	0.022	*

* indicates p<0.05, ** indicates p<0.01 (after FDR correction for multiple testing).

3.3. Influence of rearing on methylation

Methylation levels of only one out of the nine individual CpG probes that showed significant associations with personality domains were significantly influenced by early rearing history (Table 4), where methylation levels of mother reared individuals were higher for *SLC6A4* probe cg26741280 than for nursery reared ones (t = -7.345, df = 45, p_{adj} = 0.001; Figure 2).

4. Discussion

This study investigated the role of epigenetic modification of two key serotonergic genes (*HTR1A* and *SLC6A4*) on the expression of personality in captive chimpanzees. Methylation levels at different CpG sites in both genes showed significant associations with four personality dimensions: Dominance, Reactivity, Agreeableness and Openness.

While 48 CpG sites were present in both genes combined, only nine showed significant associations with personality, three of which were located on *HTR1A* and the remaining six on *SLC6A4*. For *HTR1A*, these three CpGs showed associations with three different personality dimensions: Dominance, Reactivity and Openness. Higher methylation of cg02266732, a CpG located in the CpG island in the 200bp region upstream of the transcription start site, was associated with higher scores on Openness, indicating that those chimpanzees were rated higher on items like Human oriented, Inquisitive/Curious, Inventive, Intelligent, Affectionate/Friendly, and Persistent. Given that in general DNA methylation of CpGs located in or near the promoter region result in reduced expression of the gene [38], this indicates that in individuals with higher methylation levels at this site, fewer HTR1A receptors are available. As HTR1A typically have an inhibitory effect on serotonin production [39], having fewer of them would result in increased serotonergic signaling and explains higher levels of explorative and social personality profiles in these chimpanzees through serotonin induced anxiety relief. For the other two CpG sites, the association between methylation and behavior was reversed. Higher methylation scores of cg23448729, located in the *HTR1A* exon, were associated with lower Dominance scores. This means that individuals would be scored as more anxious and cautious, while scoring lower on bold and independent. Similarly, higher methylation of cg27615388, also located in exon 1, predicted lower Dominance scores, but also lower Reactivity and Openness scores, meaning individuals would behave more irritable, aggressive or socially inept and less explorative and affectionate. For both CpGs, these results are in line with reduced serotonergic activity and thus likely higher levels of inhibitory *HTR1A* transcription. The fact that the directionality of the association between serotonergic methylation and behavior changes dependent on the location of the significant CpG loci, is in line with previous findings where positive associations are typically found between methylation and gene expression in probes located in the gene body, whereas negative ones are found for probes closer to the transcription start site [40].

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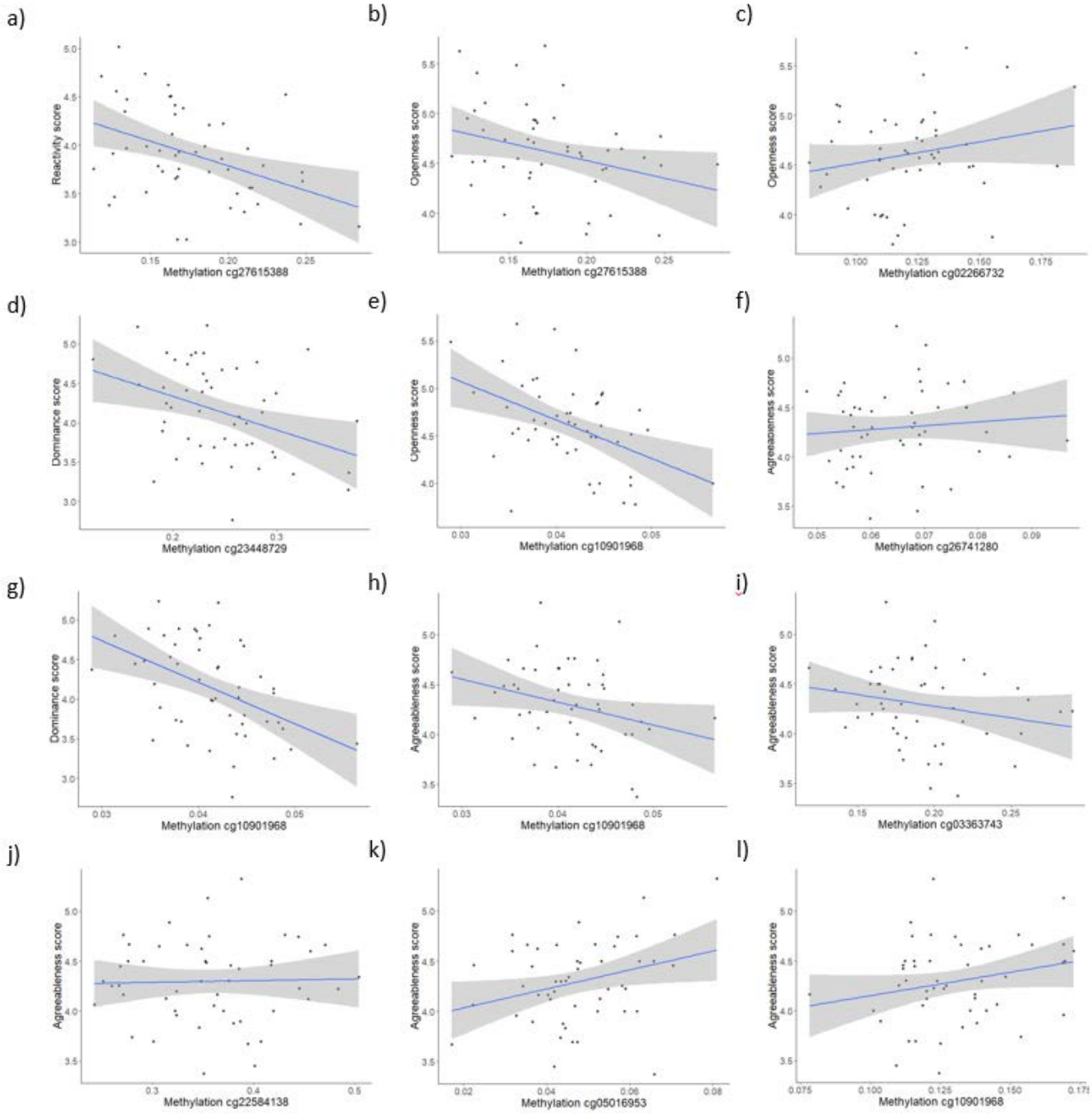

Figure 1. Significant associations between individual CpG methylation scores and personality domains for *HTR1A* (a-d) and *SLC6A4* (e-l).

Table 4: Model statistics for rearing effects on methylation rates of personality related CpG sites

Gene	Cg probe	Est	SE	t	*p*	Sign.
HTR1A	cg23448729	-0.018	0.017	-1.103	0.276	
	cg27615388	-0.006	0.013	-0.473	0.639	
	cg02266732	0.006	0.008	0.715	0.479	
SLC6A4	cg14312898	0.007	0.007	0.932	0.357	
	cg22584138	0.004	0.023	0.160	0.874	
	cg05016953	0.004	0.004	0.999	0.323	

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cg03363743	0.009	0.012	0.787	0.436	
cg10901968	0.000	0.002	-0.256	0.799	
cg26741280	-0.008	0.003	-2.870	0.006	**

Estimates are for nursery reared individuals in comparison to mother reared chimpanzees. ** indicates p<0.01 .

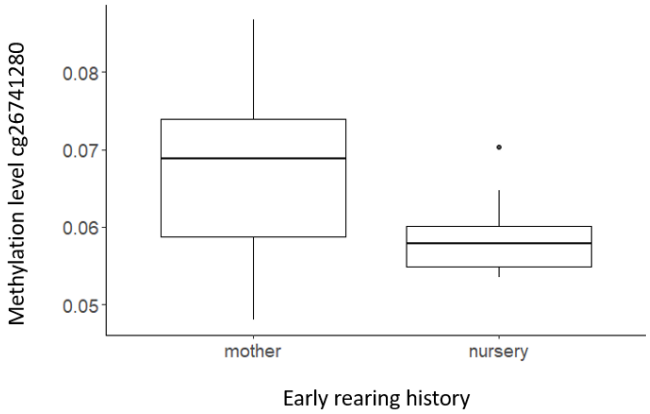

Figure 2: Impact of early rearing history on methylation scores of cg26741280.

For the serotonin transporter gene, methylation scores on six CpGs were found to be associated with three different personality dimensions. Higher levels of methylation of cg10901968, a CpG site located within the CpG island in the 200bp region upstream of the transcription start site of the gene, resulted in lower Dominance, Agreeableness and Openness scores, indicating that these individuals would overall behave more anxious and cautious, less considerate, and protective of other chimpanzees, and less explorative and affectionate. These results are in line with our expectations based on literature where in general, higher levels of *SLC6A4* methylation result in lower *SLC6A4* expression, which results in higher risk for depression, anxiety and reduced behavioral stress reactivity. Higher methylation levels of the remaining five *SLC6A4* CpGs were associated with either higher (cg14312898, cg26741280, cg05016953, cg22584138) or lower Agreeableness scores (cg03363743), with no clear pattern for the impact of the location of the CpG inside or outside of the CpG island or gene body.

The Agreeableness dimension only has two item loadings, protective and considerate, and chimpanzees scoring high are considered individuals that show higher levels of concern for other chimpanzees, intervene more often to prevent harm or annoyance coming to them, and consoling them when in distress to provide reassurance. While this dimension might reflect empathetic capacity and/or prosociality in chimpanzees, this requires confirmation with additional behavioral testing. Data from coded behavioral observations do show that individuals scoring high on Agreeableness show more affiliative behaviors and were less likely to displace others and solicit other chimpanzees for assistance during fights [28]. While the socio-negative effects of low serotonin are more often emphasized in literature due to its role in anxiety disorders and depression, its reversed associations with socio-positive behaviors are not unexpected. Although reports are scarcer, studies in both animals and humans have documented links between higher serotonin activity and higher levels of prosocial and affiliative behaviors and suggest that serotonin might actually function to promote an individual's potential for self-control of emotional reactivity and increase its aversiveness to the harming of others [41]. Previously we showed that in chimpanzees, a genetic mutation causing an amino acid change in *HTR1A* (Pro248Gln) is associated with both higher levels of socio-negative behaviors (anxiety and aggression), and lower levels of two socio-positive behaviors (grooming, proximity) [42].

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Interestingly, no significant associations were found between the *HTR1A* polymorphism and personality dimension scores in this larger cohort of chimpanzees [42], confirming the claims that serotonergic genetic effects on personality are modest [15]. Epigenetic effects thus appear to have a bigger impact on chimpanzee personality than genetic ones, but this requires further testing in additional chimpanzee populations. Previous work did report potential genotype effects of a famous repeat polymorphism in the promoter region of *SLC6A4* known as 5-HTTLPR (serotonin-transporter-linked promoter region) on methylation levels of specific CpG sites [cg18584905, cg10901968, cg25725890, cg22584138, cg05951817; see 43,44]. Of these CpGs, cg22584138 and cg10901968 showed significant positive associations with chimpanzee personality in our study, which could thus be mediated through underlying 5-HTTLPR genotype differences. While the investigation of genotype by methylation interaction effects on behavioral profiles would offer promising avenues for future work, a larger dataset would be needed to obtain sufficient power to disentangle these effects. Developing large-scale studies in great apes is challenging as they require both blood and/or post-mortem tissue samples, which are collected opportunistically, and matching behavioral profiles.

Despite sample size limitations, our study yields important clues regarding the reproducibility and generalizability of human findings to other species. Especially for *SLC6A4*, where methods used across studies are less heterogenous [45], comparisons can be made for CpG site-specific phenotype associations. These show that at least for two of the CpGs associated with chimpanzee Agreeableness (cg05016953 and cg22584138), functional comparative validity with human studies is present. Human studies report links for these CpGs with differential 5-HTT expression patterns [44] and with responsiveness to antidepressant treatment (cg05016953) [46]. This indicates that methylation levels of these CpG sites have robust, evolutionarily conserved effects on 5-HTT functioning. Unfortunately, studies investigating links between individual serotonergic methylation and personality so far tend to focus on personality disorders (antisocial personality disorder, borderline personality disorder) or rather report links with behavioral tendencies (aggressiveness, antisocial behavior) instead of personality dimensions that rely on a similar construct as ours (cf. the Big Five) [for a review see 47]. More studies are thus needed to confirm the cross-species validity of CpG effects on personality.

The results do clearly support a role of serotonin methylation in chimpanzee personality regulation that differs from that of dopamine. In the same sample of chimpanzees, methylation levels of the dopamine receptor type D2 (*DRD2*) previously showed strong associations with Extraversion and Openness, two personality dimensions that reflect a tendency to actively explore or engage with novelty [23]. While a modest link with Openness was present in our study for three out of 48 serotonergic CpG sites, no associations were found with Extraversion, while scores on Extraversion were significantly associated with methylation levels of 13 out of 16 *DRD2* CpG sites. While the serotonin system is more involved in self-regulation and emotional stability and targets the limbic system of the brain, dopamine stimulates exploration-related personality traits through reward-center activation [48]. Both neurotransmitters thus have closely intertwined functions, but methylation patterns in both systems regulate personality in rather distinct ways.

Finally, we investigated whether atypical early social conditions in nursery reared individuals accounted for some of the variation in serotonergic methylation patterns in our population of chimpanzees. For those 9 CpGs that showed associations with chimpanzee personality, only one was differentially methylated between nursery reared versus mother reared chimpanzees. This CpG site, cg26741280, was located in a CpG island, close to the transcription start site of the serotonin transporter gene (*SLC6A4*), where nursery-reared individuals showed lower methylation levels than mother-reared individuals. These results are in contrast to the general consensus that childhood adversity leads to overall higher levels of *SLC6A4* methylation in humans [for review see 20] and rhesus macaques [49] but align with findings where promoter-specific CpG sites were undermethylated in individuals with high-stress environments [50]. The contrast between these studies is likely explained by the heterogeneity in methods that were used. As evidenced

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by our results above, the location of the CpG sites is important in determining the direction of the effect, something that average methylation scores across genes do not take into account [40]. It is thus possible that nursery-reared individuals experience reduced long-term stress-resistance compared to mother-reared ones, which is associated with decreased methylation of promoter CpG cg26741280, higher *SLC6A4* transcription and results in lower Agreeableness. The effects of rearing are nonetheless modest, as evidenced by only one CpG being affected by rearing background and functional assays are needed to investigate to what extent cg26741280 methylation levels impact *SLC6A4* transcription in chimpanzees. It thus appears that either the differences between nursery reared and mother reared chimpanzees are modest and thus did not have a major impact on epigenetic modification of the serotonergic system as shown for human children with a trauma background, or factors other than rearing conditions play a more prominent role in determining adult serotonergic methylation patterns. In humans, for example, links have been documented with prenatal infections, childhood exposure to greenness in the environment and medicine use [51,52].

5. Conclusions

In conclusion, our results confirmed an evolutionarily stable role of serotonergic methylation in simultaneously reducing anxiety (Dominance) and aggression (Reactivity/Undependability) related personality aspects while promoting prosocial (Agreeableness) and exploratory (Openness) personality. They also show that methylation effects on chimpanzee personality were CpG-specific and heavily dependent on the location of the CpG [38,40]. This highlights the importance of investigating methylation patterns at the CpG site-level level as opposed to using average gene methylation scores or technologies like mass spectrometry that do not allow CpG-specific methylation resolution [20]. These results offer an important basis for future hypothesis driven work that can target specific CpGs and investigate their role in regulating neuroanatomical and/or behavioral phenotypes in other populations and species.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Supplementary Table S1 : Factor loadings of chimpanzee personality traits on six varimax-rotated factors; Supplementary Table S2: *HTR1A* CpG factor item loadings on varimax-rotated factors; Supplementary Table S3: *SLC6A4* CpG factor item loadings on varimax-rotated factors; Supplementary Table S4: Model statistics for *HTR1A* and *SLC6A4* CpG composite measure scores and their association with personality dimensions in chimpanzees; Supplementary Table S5: Model statistics for *HTR1A* and *SLC6A4* CpG individual CpG methylation scores and their association with Dominance scores in chimpanzees; Supplementary Table S6: Model statistics for *HTR1A* and *SLC6A4* individual CpG methylation scores and their association with Agreeableness scores in chimpanzees; Supplementary Table S7: Model statistics for *HTR1A* and *SLC6A4* CpG individual CpG scores and their association with Openness scores in chimpanzees; Supplementary Table S8: Model statistics for *HTR1A* and *SLC6A4* CpG individual CpG scores and their association with Reactivity scores in chimpanzees; Supplementary Table S9: Model statistics for *HTR1A* and *SLC6A4* CpG individual CpG scores and their association with Extraversion scores in chimpanzees

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