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Evidence from Pyrosequencing Indicates that Natural Variation in Animal

Personality is Associated with DRD4 DNA Methylation

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Abstract

Personality traits are heritable and respond to natural selection, but are at the same time influenced by the ontogenetic environment. Epigenetic effects, such as DNA methylation, have been proposed as a key mechanism to control personality variation. However, to date little is known about the contribution of epigenetic effects to natural variation in behaviour. Here we show that great tit (*Parus major*) lines artificially selected for divergent exploratory behaviour for four generations differ in their DNA methylation levels at the Dopamine Receptor D4 (DRD4) gene. This D4 receptor is statistically associated with personality traits in both humans and non-human animals, including the great tit. Previous work in this songbird failed to detect functional genetic polymorphisms within DRD4 that could account for the gene-trait association. However, our observation supports the idea that DRD4 is functionally involved in exploratory behaviour but that its effects are mediated by DNA methylation. While the exact mechanism underlying the transgenerational consistency of DRD4 methylation remains to be elucidated, this study shows that epigenetic mechanisms are involved in shaping natural variation in personality traits. We outline how this first finding provides a basis for investigating the epigenetic contribution to personality traits in natural systems and its subsequent role for understanding the ecology and evolution of behavioural consistency.

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Introduction

Personality – the general tendency of individuals to differ in patterns of behaviour that are consistent across time and over contexts - is important in explaining individual differences in health and fitness in both humans and non-human animals (Clark & Ehlinger 1987; John *et al.* 2010; Réale *et al.* 2007). A significant heritable component underlies part of the variation in personality traits (van Oers *et al.* 2005; van Oers & Mueller 2010), however, attempts to identify genetic polymorphisms consistently associated with personality traits have thus far met with limited success (Balestri *et al.* 2014; van Oers & Mueller 2010). Moreover, recent evidence shows that environmental experiences during early development can be just as important in explaining both variation and consistency in personality traits (Curley & Branchi 2013; Stamps & Groothuis 2010). It remains unclear, both for humans (Bouchard & McGue 2003) and non-human animals (van Oers & Mueller 2010), what proportion of personality results are fixed and what proportion is variable and plastic during an individual's lifetime. Therefore, it is important to understand the molecular mechanisms that link the heritable variation in animal personality traits to environmental developmental plasticity (Youngson & Whitelaw 2008).

Epigenetic mechanisms - the collective chemical and physical processes that program the genome to express its genes in a time-, cell- and environment-dependent manner through non-mutagenic means (Jablonka & Raz 2009) - are good candidates to play an important role in explaining personality variation (Ledon-Rettig *et al.* 2013). These epigenetic modifications can be induced by rapid changes in the environment, and are found to explain long-lasting developmental effects (Bossdorf *et al.* 2008) that may even pass across generations (Curley & Branchi 2013; Groothuis & Trillmich 2011; van Oers & Mueller 2010). Several molecular

mechanisms have been discovered that are responsible for epigenetic influences on genome function, among which DNA methylation is one of the best-studied (Bender 2004; Jaenisch & Bird 2003). Methylation of cytosines in CpG dinucleotide contexts, particularly within CpG islands (CGIs), can affect gene expression both in promotor regions (Bender 2004) and in the gene body (Ball *et al.* 2009).

An epigenetic basis for personality-related disorders has been recently suggested in humans (Kaminsky *et al.* 2008; Kumsta *et al.* 2013; Paquette & Marsit 2014) but in other animals only a handful of studies have investigated the link between variation in DNA methylation and behaviour, and these were mainly focused on domestic rodents in a laboratory setting (Champagne & Curley 2009; Dias & Ressler 2014; Herb *et al.* 2012; Massart *et al.* 2012; Szyf *et al.* 2005; Weaver *et al.* 2004). These studies on lab-bred animals have been critical for exposing the association of DNA methylation variation of candidate genes and the expression of these genes. For example in rats, variation in stress resilience was attributed to an epigenetically controlled transcription of the BDNF gene, resulting in differential expression. While low novelty-seeking rats were found to up-regulate the expression of the BDNF gene following social defeat, no such reaction was present in high novelty seeking rats (Duclot & Kabbaj 2013). Another study demonstrated that rats receiving more grooming as pups had lower stress responses and showed different methylation patterns of genes associated with the glucocorticoid stress response compared to rats that received less grooming (Szyf *et al.* 2005; Weaver *et al.* 2004). Experimental methyl supplementation altered both the epigenetic markings and the stress phenotype (Weaver 2005), indicating a causal epigenetic control of the plastic stress response. However, very little is known about an epigenetic involvement in explaining behavioural variation in behavioural traits in natural

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systems, and no information exists on the role of epigenetics in natural variation in personality (Ledon-Rettig *et al.* 2013). Studying the underlying epigenetic mechanisms, such as DNA methylation in these ecological systems is essential to answer questions on its ecological and evolutionary significance.

In many studies on reward-seeking behaviours related to impulsivity, aggression, exploration and novelty seeking in humans and non-human animals, Dopamine Receptor D4 (DRD4) emerges as a major candidate gene explaining genetic variation (Dulawa *et al.* 1999; Ebstein *et al.* 1996; Flisikowski *et al.* 2009; Frieling *et al.* 2010; Munafó *et al.* 2008; Szekely *et al.* 2004). In great tits (*Parus major*), allelic variants in exon three of the DRD4 gene are statistically associated with exploratory behaviour in experimental populations that were selected for divergent levels of exploratory behaviour and in hand-reared wild birds (Fidler *et al.* 2007) and also in some (but not all) natural populations (Korsten *et al.* 2010; Mueller *et al.* 2013). However, the observed association could not be linked to any functional (non-synonymous) single nucleotide polymorphism (SNP) or deletion site within the DRD4 gene or its flanking regions, although there were signs of selection for this polymorphism (Korsten *et al.* 2010; Mueller *et al.* 2013). Thus, DRD4 seems to play a role in heritable variation in great tit exploratory behaviour but the underlying molecular mechanism remains to be demonstrated.

Here, we studied variation in methylation levels of the DRD4 gene in two great tit lines that were selected for high and low early exploratory behaviour for four generations and that showed heritable differences in exploratory behaviour. By investigating levels of DNA methylation in the DRD4 gene in brain and blood tissue of selection line birds, we showed

that methylation variation at a CGI overlapping the DRD4 transcription start site is associated with heritable variation in exploratory behaviour. This suggests that heritable divergence in this behavioural trait may involve epigenetic modification of DRD4. This finding also suggests that to understand natural variation and evolutionary significance of personality variation in this ecological model system, future work should include efforts to understand the causes and consequences of DNA methylation variation at this candidate gene.

Materials and Methods

Subjects

Birds originated from the fourth generation of two lines artificially selected for four generations for low (SE) and high (FE) levels of Early Exploratory Behaviour, and were all born in 1997. Early Exploratory Behaviour is a combination of the fast or slow reaction towards a novel environment (exploratory behaviour) and a novel object (boldness), measured right after independence (for test protocol see Drent *et al.* 2003). These personality traits have been validated in great tits and associated with several physiological, behavioural and life-history traits, and have also been found to influence fitness (van Oers & Naguib 2013). For the selection experiment, pairs of great tits were selected on high and low levels of exploratory behaviour and housed in half-open aviaries (2 x 2.5 x 4m) to breed at the NIOO-KNAW. Eggs that were laid were transferred to a natural nest of foster parents when clutches were full. In the field freshly hatched chicks were cross-fostered in such a way that each foster parent-pair raised half a brood of FE and half a brood of SE chicks. When chicks were 10 days old, they were transferred back to the aviary facilities at the NIOO-KNAW and hand reared until independence as described in Drent *et al.* (2003). From these

offspring we selected the most extreme birds for the next generation, avoiding inbreeding and multiple offspring per family. A heritability was found of 54% after four generation of selection (for test protocol see Drent *et al.* 2003)

We used both blood and brain samples from male and female great tits which died from natural causes in our aviaries (ages 5-8 years) and which were stored at -20 °C. Blood samples were collected while the birds were still alive in the period 2002-2005. For a sample overview, see Table S1. Avian blood contains nucleated red blood cells, so more than 90% of the DNA isolated from avian blood samples originates from erythrocytes. We aimed to create a balanced sample set, but one blood sample failed in all tests, therefore 12 SE birds (8 males and 4 females from 5 different families) and 11 FE birds (6 males and 5 females from 4 families) were used to determine the DRD4 methylation levels in blood (Table S1). Brain tissue was available for a subset of birds for which we had blood samples: 10 SE (7 males and 3 females from 5 families) and 8 FE birds (5 males and 2 females from 4 families; see Table S1).

DNA extraction

Blood samples consisted of either 10 µl whole blood in 1 ml Cell Lysis Solution (Gentra Puregene Kit, Qiagen, USA), or 10 µl of whole blood stored in Queens buffer (Seutin *et al.* 1991). All samples were stored at room temperature. Total DNA was prepared by using 250 µl of the stored blood samples with 750 µl Cell Lysis Solution (Gentra Puregene Kit, Qiagen, USA) incubated with proteinase K at 55°C overnight, followed by DNA extraction following the manufacturer's protocol. DNA was stored in DNA Hydration Solution (Qiagen, USA).

Brain samples were collected from the same individual birds as used for the blood sample analysis (Table S1). Brains were dissected out of frozen birds (-20 °C) and hypothalamus- and hippocampus-enriched regions were isolated as described in (Lindqvist *et al.* 2007). To ensure that we isolated DNA from the same area for each individual, we made landmarks on a cutting board to consistently cut the same part of the brains using a razor blade. These regions were then incubated overnight at 55 °C in 750 µl Cell Lysis Solution (Gentra Puregene Kit, Qiagen, USA) with 20 µl proteinase K. 250 µl of this lysed tissue was added to 250 µl Cell Lysis Solution. To remove excess of fat and proteins, 500 µl 24:1 chloroform:isoamylalcohol was added and mixed until homogeneous, followed by 10 min centrifugation at 12.000x g after which the upper layer was collected. Cell Lysis Solution was added to this upper layer until 500 µl of sample liquid was obtained and total DNA was extracted according to the manufacturer's protocol. DNA was stored in DNA Hydration Solution (Qiagen, USA), and the concentration was determined with a Nanodrop 2000 (Thermo Scientific, USA).

CpG position selection in the DRD4 gene for pyrosequence assay design

A CGI motif was searched in the DRD4 genomic region (GenBank accession DQ006802) using CpGfinder (Softberry, USA) with the base pair numbering set to 1 on the transcription start site. Within the DRD4 gene, four regions were chosen that contained high densities of CpG dinucleotides within a short range and had enough surrounding sequence variety for primer design (Fig. 1). The DRD4 5' upstream region was too dense with CpGs and primer design proved impossible. The PyroMark Assay Design SW 2.0 (Qiagen, USA) was used to design the pyrosequence assays, which contain two PCR primers of which one is labelled with Biotin and one sequence primer opposite of the Biotinylated primer. The selection lines are still highly variable and single nucleotide polymorphisms or deletions/insertions could lead to

out-of-frame sequencing and were avoided as much as possible when developing the assays. If this proved impossible the SNPs were added manually to the reference sequence. Assay A is located in exon 1, within the CGI, 241 bp downstream of the transcription start site, and contains 8 CpG sites within 73 bp of sequence length. This assay was only used for assessing the global methylation level in exon 1, because many samples were excluded due to unreliable methylation scores, resulting in a too low sample size to be analysed for sex and personality differences. Assay B is located in intron 1, within the CGI, 515 bp downstream of the transcription start site, and contains 12 CpG sites within 71 bp of sequence length. Assay C is located in exon 3, 7918 bp downstream of the transcription start site, and contains 6 CpG sites within 55 bp of sequence length. Assay D is located in exon 3, 8423 bp downstream of the transcription start site, and contains 8 CpG sites within 47 bp of sequence length. See table S2 for an overview of the primer sequences.

Bisulfite conversion, methylation specific PCR and pyrosequencing

One or 2 µg of total genomic DNA was bisulfite-converted using the EpiTect bisulfite conversion kit (Qiagen, USA) and eluted from the supplied washing column with 20 µl of supplied elution buffer. One µl of this eluate was used in a 25 µl PCR reaction using the PyroMark PCR Kit (Qiagen, USA) according to the manufacturer's protocol but without Q-solution, 100 nM or 200 nM forward primer and 100 nM or 200 nM reverse biotinylated primer (see Table S2 for primer overview). PCR conditions were as follows: 94 °C for 15 min. followed by 45 cycles of 94 °C for 30 sec., 61 °C for 30 sec., 72 °C for 30 sec.; ending with 72 °C for 7 min. The PCR product was purified using streptavidin Sepharose HP beads (GE Healthcare Europe, The Netherlands) followed by hybridization of the sequencing primer with the biotinylated PCR product as described in the Pyromark Q24 vacuum workstation

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guide (Qiagen, USA). The methylation percentage per CpG position was determined using the Pyromark Q24 Advance software (Qiagen, USA). In short, pyrosequencing produces quantitative measures of DNA methylation based on a sequencing-by-synthesis method (Tost & Gut 2007). The DNA methylation percentage is assessed by the ratio of real-time incorporated C and T nucleotides, through the conversion of released pyrophosphate into a light signal. In assay B, C and D we included a control step to determine the completeness of bisulfite conversion (Table S2). We sequenced each individual multiple times for each assay. The Pyromark Q24 Advance software checked reliability of the results, and samples containing CpG sites marked as unreliable methylation results by the software were re-run. CpG sites that were again marked as unreliable after re-running were excluded. After this quality control we averaged the methylation level for each site per individual for all reliable samples (range 1-4) for further analysis. The intra-assay coefficient of variation (CV) was 1.8% and the inter-assay CV was 5.4%.

Verification of pyrosequence results with bisulfite sequencing

To verify that no mutation or single nucleotide polymorphisms could interfere with the pyrosequencing of assay B, which showed the most interesting variation in our study, the PCR products were cloned and sequenced (Macrogen, The Netherlands). The PCR products were generated with primer set AssayB_Fw and AssayB_Rv (without biotin label) from each individual sample and were pooled prior to sequencing. This resulted in sequences from thirty-three clones containing the PCR fragment from assay B (S1 Fig.). These sequences indicated that no previously unknown SNP or other mutation could have interfered with the pyrosequencing and that the results in our study are not due to out of frame sequencing. In addition no parental imprinting was observed as this would have been observed as

fragments with none to low methylation and fully methylated fragments (S2 Fig.).

Statistical analysis

To investigate possible sex- and personality-dependent differences in patterns of DNA methylation in blood and brain samples, a joint analysis including all samples of both tissue types was done using a general linear mixed model (GLMM) with the percentage of methylation as dependent variable and personality type (line; FE or SE), Tissue (brain or blood), Site (CpG site) and Sex (male, female) and their two- and three-way interactions as fixed factors. Family (nested within personality type) and individual (nested within family and within personality type) were included in the model as random factors to account for the fact that individuals within families were not independent measures due to the hierarchical structure in our data.

To assess associations between methylation levels and sex and personality in the two tissues separately, we also performed GLMM models per tissue type (blood and brain), including site, sex and personality type and their two and three way interactions. We conducted a backwards elimination method and provide F and P values for the factors before removing them from the model. For significant interactions, we conducted pairwise post-hoc comparisons (with F-tests and DF between 14.5 and 16.0) between the estimated marginal means with Bonferroni adjustments for multiple comparisons using the Pairwise Comparisons options in the Linear Mixed Models analysis.

In some instances no reliable pyrosequence result were obtained for a particular sample and those samples were thus excluded from the analysis (see above). Therefore, the number of blood tissue samples analysed for site 1-12 (Assay B) were: 11 SE and 9 FE; for site 13-18

(Assay C): 7 SE and 10 FE; for site 19-26 (Assay D): 8SE and 8 FE. Number of brain tissue samples analysed for site 1-12 (Assay B): 9 SE and 8 FE; for site 13-18 (Assay C): 8SE, 6FE; for site 19-26 (Assay D): 10 SE and 8 FE. For all models the residuals were checked for normality. We used IBM SPSS Statistics 22 for Windows for all statistical analyses.

Results

DRD4 methylation pattern

We found one CGI ranging from -118bp to 632bp, overlapping part of the putative promotor region, the transcription start site, the translation initiation site and the 5' part of intron 1 (Fig. 1). This CGI contained a CG percentage of 73.5% and an observed to expected CpG ratio of 0.895. In mammals and chicken roughly 50–65% of transcription starts sites (TSS) overlap with CGI predictions (Hu *et al.* 2013; Li *et al.* 2011; Long *et al.* 2013), which is seen here for DRD4 too. However, the CGI in the great tit DRD4 shows features of both a TSS CGI but also gene body CGI as it overlaps TSS, exon 1 and part of intron 1.

The methylation levels in the DRD4 gene were assayed in four regions of roughly 60-80 base pairs that contained a high density of CpG dinucleotides. We first assessed mean methylation levels per assay in blood samples for both personality groups together and observed that DRD4 methylation levels are low in assay A (7.4% methylation) located in exon 1, which corroborates observations in mammals and chicken that most CGIs overlap with regions of low methylation (non-methylated islands) (Long *et al.* 2013). The methylation levels increase sharply in assay C (89.6%) and D (90.8%), located in the 5' and 3' region of exon 3 respectively, while assay B (35.2%) located in intron 1 shows intermediate

methylation levels (see Fig. 2). This increase of DNA methylation towards the 3' end of the gene is consistent with patterns of gene body methylation in mammals, where DNA methylation level of the first exon is tightly linked to transcriptional silencing (Brenet *et al.* 2011). The low methylation in Assay A may suggest that DRD4 is (highly) expressed in great tits. The strong increase of DRD4 methylation towards 3' could be an additional indication for high expression level (but see Ball *et al.* 2009; Brenet *et al.* 2011).

Methylation levels associate with personality type

Next, we determined methylation levels for every CpG site within assays B, C, and D for each sample individually. In an initial analyses we found that differences in methylation levels per CpG site between personality types were tissue dependent (Table 1; Ptype*Site*Tissue, $F_{25,749.9} = 1.56$, $P = 0.04$). We therefore analysed results for both tissues separately, even though in general we found a high correlation ($r_p = 0.97$, $P < 0.0001$) between blood and brain methylation levels.

CpG methylation levels in blood tissue differed significantly between FE and SE individuals, with FE showing higher methylation levels in some but not all sites (Fig. 3A and Table 2; Ptype*Site, $F_{25,371.0} = 8.78$, $P < 0.0001$). A post-hoc analysis revealed that these significant differences were restricted to CpG sites 1-11 and showed a tendency for position 12 (Assay B; $P = 0.059$; Fig. 3A) while for position 13-26 (Assay C and D) methylation levels did not differ significantly between individuals originating from the two selection lines. Similarly, the CpG methylation levels in brain tissue were different between FE and SE and this effect differed between sites as well (Table 2; Ptype*Site, $F_{25,364.1} = 2.70$, $P < 0.0001$). A post-hoc analysis testing the difference between the lines at each site separately revealed that also in brain tissue FE individuals had higher CpG methylation, and that this was caused by

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differences at CpG positions 1-6, 8 and 9 (Assay B), while FE and SE individuals did not differ in CpG methylation for other positions (Table 2 and Fig. 3B).

Sex specific differences in methylation levels

In addition to the observed differences in personality type, sexes also differed in methylation levels. Although sex differences in CpG methylation levels were not tissue dependent (Sex*Tissue, $F_{25,724.1} = 0.99$, $P = 0.48$), the sex effect could only be shown in blood ($F_{25,371.1} = 2.54$, $P < 0.001$; Table 2) and not in brain tissue ($F_{25,339.3} = 0.26$, $P = 0.16$; Table 2). In blood, methylation levels were significantly higher for females compared to males for CpG sites 1-6, and the difference showed a trend for CpG sites 8 and 9 (Table 2). This effect of sex was the same for both selection lines (Sex*Ptype, $F_{25,346.1} = 0.78$, $P = 0.77$).

Discussion

To explore a possible epigenetic contribution to personality trait variation, we set out to study the levels of DNA methylation at the DRD4 gene in great tits artificially selected for contrasting levels of Early Exploratory Behaviour (FE vs SE) during four generations. We found significant methylation differences between FE and SE exploring birds in the downstream part of a CGI that partly overlaps the gene's first intron and transcription start site. This suggests that epigenetic variation at DRD4 is involved in functional, heritable divergence in great tit exploratory behaviour. Ideally, we would have associated DRD4 methylation levels in blood and brain tissue with the expression of DRD4 in these tissues. Unfortunately, we were unable to study the brain expression levels of DRD4 in the samples studies, because these selection line birds were not sacrificed for the purpose of gene

expression analysis and died of natural causes. Thus the functional link between DRD4 methylation and expression remains to be demonstrated in future gene expression studies.

DRD4 has been implicated in personality trait variation in different species before. A study on repetitive variants in DRD4 exon III in humans showed a positive correlation between DRD4 genotypes and novelty seeking (Ebstein *et al.* 1996), and a more recent meta-analysis showed that this association reflects a more general association between DRD4 and novelty seeking behaviour (Munafó *et al.* 2008). Similarly, in birds, high levels of genomic variation are present in functional regions of the DRD4 gene (Abe *et al.* 2011). Previous studies in great tits have also found genetic associations of DRD4 and personality differences, however, pinpointing functional genetic DRD4 variation has remained elusive as statistically significant DRD4-personality associations were based on a synonymous (and thus non-causal) SNP (Fidler *et al.* 2007; Korsten *et al.* 2010). One possible explanation for the presence of a statistical association but the lack of local functional genetic variation is allele-specific epigenetic differences that are associated with SNP variation in DRD4, as has been observed previously in humans (Docherty *et al.* 2012). Alternatively, functional epigenetic variation that is not associated with SNP variants may account for missing heritability (Maher 2008), and might be partly responsible for recent failure in a genome-wide-association-study to detect a significant association with personality traits for several great tit populations (Mueller *et al.* 2013).

Although the link between DNA methylation and gene expression is not always straightforward (Jones 2012), we hypothesize that differential methylation at the DRD4 gene affects gene activity and ultimately personality variation. Indirect evidence for a functional

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interpretation is provided by a recent great tit study using whole-genome bisulfite and RNAseq data. This study showed that across all genes, higher CG methylation at transcription start sites and within gene-bodies is associated with lower gene expression in the great tit (Figure 4b, Laine *et al.* 2016). This indirectly supports the interpretation that higher DNA methylation in the 5' region of a gene (as we observe for DRD4) is associated with reduced gene expression.

We also point out that the pattern of DRD4 methylation that we observed is consistent with a functional role: the methylation difference between selection lines was not constant across the entire length of DRD4 but was restricted to an area near the transcription start site, whereas no significant methylation differences were found in two highly methylated regions in exon 3 (Assay C and D). The methylation levels of around 35% observed in the differentially methylated region of Assay B correspond to low-methylated regions (LMRs) observed in mice (Stadler *et al.* 2011). These LMRs are often associated with enhancer regions and show dynamic methylation, owing either to competing methylation and demethylation in time or to inaccurate maintenance of methylation during cell division (Jones 2012; Stadler *et al.* 2011). Only the first intron (Assay B) showed high variation in methylation levels indicating that methylation in this region might be in a dynamic state, which suggests the presence of putative DRD4 enhancers in this region. Differential methylation at intronic enhancers is known to affect gene expression (Hoivik *et al.* 2011; Unoki & Nakamura 2003) and DRD4 activity may thus be affected, contributing to observed variation in early exploratory behaviour.

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One limitation of our study is that we have screened DNA methylation in DRD4 only and not in other genes. Although DNA methylation levels can co-vary considerably across the genome causing consistent differences in methylation levels between samples at many loci (Shabalin *et al.* 2015), individual differences in methylation levels are likely to vary among genes. It is therefore important in future work to demonstrate if the observed methylation differentiation between selection lines is specific to DRD4 or if it is a present over larger genomic scales. In our study we did not screen separate control genes for this purpose, however we used two 3' regions within the DRD4 gene that serve as suitable controls (Assay C and D). These control regions are towards the 3' end of the gene and show no difference in methylation between the selection lines. Instead, we only find methylation differences between the lines in assay B, which is towards the 5' end of the gene. Thus, while validating more genes and genomic loci would be desirable, it is highly unlikely that the observed methylation differences between selection lines merely reflect consistent differences in methylation levels over large genomic regions.

The observed methylation difference between fast and slow exploring birds provides supporting evidence for the involvement of DRD4 in determining variation in Early Exploratory Behaviour. Moreover, the results suggest that in order to understand the underlying mechanisms of natural variation and evolutionary divergence in great tit personality traits it may be important to further explore the causes and consequences of DRD4 methylation. Recently, epigenetic variation in DRD4 and serotonin transporter (SERT) were found to be higher in urban compared to forest dwelling great tits (Riyahi *et al.* 2015). No association between DNA methylation and exploratory behaviour was found in that study for DRD4 or SERT. To our knowledge our study therefore presents the first empirical

evidence for the involvement of epigenetic mechanisms in personality trait divergence in a wild species.

This first finding, in a species that has become an ecological model species for natural behavioural studies (van Oers & Naguib 2013), can help to focus new lines of investigation on the ecological and evolutionary epigenetics of animal behaviour. For instance, one intriguing question concerns the heritability of DRD4 methylation variation. The observed DRD4 methylation differences between selection lines, suggests transgenerational consistency of DRD4 methylation that is associated with exploratory behaviour. This is in contrast with a study comparing DRD4 methylation patterns in human monozygotic and dizygotic twins, where no heritable effect but only familiar effects were found (Wong *et al.* 2010). Unlike plants, in which meiotic stability of cytosine methylation is well-established (Cortijo *et al.* 2014), transgenerational consistency of methylation patterns in vertebrates may involve (1) repeated *de novo* establishment of the same methylation pattern in each new generation after widespread DNA methylation resetting between generations (Feng *et al.* 2010) controlled by underlying genetic differences, (2) transmission of meiotically stable epi-alleles to the next generation without resetting (Manikkam *et al.* 2012) or (3) parent-offspring behavioural interactions that are perpetuated each generation (Weaver *et al.* 2004). Our experimental design, in which individuals from both selection lines are cross-fostered and reared in similar environments, rules out parent-offspring interaction and environmental induction as a cause of consistent methylation differences between the selection lines and we assume a widespread DNA methylation resetting between the generations that is under genetic control. As no cis-genetic variation at DRD4 was observed (Docherty *et al.* 2012) the establishment of line-specific DRD4 methylation is likely under

trans-genetic control. Identifying such trans-acting loci that control DRD4 methylation, for instance using QTL approaches based on between-line crosses, may be an important step in understanding the mechanistic basis of divergence and adaptation in personality traits.

Another important question is to what extent environmental effects can modify DRD4 methylation. Personality traits typically show long-term consistency within individuals, still there is adaptive significance in a considerable degree of plasticity in response to early developmental conditions (Groothuis & Trillmich 2011). A popular idea is that through environmental effects on DNA methylation, behavioural phenotypes can be controlled in concert with environmental demands (Jensen 2013; Kappeler & Meaney 2010), and may be effectuated via maternal effects, via for example maternal hormones, during embryonic development. The case of DRD4 in great tits, whose methylation may be partly under trans-genetic control, may offer an opportunity to investigate if and how genetic and environmental control of DRD4 methylation may be balanced to jointly determine the behavioural phenotype.

In this study we show that epigenetic patterns in DRD4 are associated with personality difference in great tits, indicating a functional role for DRD4 in explaining personality variation. In addition, the epigenetic expression regulation is partly heritable, indicating that genetic factors may play a role in the observed methylation variation. This might explain the dynamic regulation of personality, since the observed epigenetic variation makes the system flexible in a consistent but plastic way.

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References

- Abe H, Ito Si, Inoue-Murayama M (2011) Polymorphisms in the Extracellular Region of Dopamine Receptor D4 Within and Among Avian Orders. *Journal of Molecular Evolution* **72**, 253-264.
- Balestri M, Calati R, Serretti A, De Ronchi D (2014) Genetic modulation of personality traits: a systematic review of the literature. *International Clinical Psychopharmacology* **29**, 1-15.
- Ball MP, Li JB, Gao Y, *et al.* (2009) Targeted and genome-scale strategies reveal gene-body methylation signatures in human cells. *Nature Biotechnology* **27**, 361-368.
- Bender J (2004) DNA methylation and epigenetics. *Annual Review of Plant Biology* **55**, 41-68.
- Bossdorf O, Richards CL, Pigliucci M (2008) Epigenetics for ecologists. *Ecology Letters* **11**, 106-115.
- Bouchard TJ, McGue M (2003) Genetic and environmental influences on human psychological differences. *Journal of Neurobiology* **54**, 4-45.
- Brenet F, Moh M, Funk P, *et al.* (2011) DNA Methylation of the First Exon Is Tightly Linked to Transcriptional Silencing. *PLoS ONE* **6**, e14524.
- Champagne FA, Curley JP (2009) Epigenetic mechanisms mediating the long-term effects of maternal care on development. *Neuroscience & Biobehavioral Reviews* **33**, 593-600.
- Clark AB, Ehlinger TJ (1987) Pattern and adaptation in individual behavioral differences. In: *Perspectives in Ethology* (eds. Bateson P, Klopfer P), pp. 1-47. Plenum Press, New York.
- Cortijo S, Wardenaar R, Colome-Tatche M, *et al.* (2014) Mapping the Epigenetic Basis of Complex Traits. *Science* **343**, 1145-1148.
- Curley JP, Branchi I (2013) Ontogeny of stable individual differences. In: *Animal Personalities: Behavior, Physiology, and Evolution* (eds. Carere C, Maestripieri D). The University of Chicago Press, Chicago and London.
- Dias BG, Ressler KJ (2014) Parental olfactory experience influences behavior and neural structure in subsequent generations. *Nature Neuroscience* **17**, 89-96.
- Docherty SJ, Davis OS, Haworth CM, *et al.* (2012) A genetic association study of DNA methylation levels in the DRD4 gene region finds associations with nearby SNPs. *Behavioral and Brain Functions* **8**, 31.
- Drent PJ, van Oers K, van Noordwijk AJ (2003) Realized heritability of personalities in the great tit (*Parus major*). *Proceedings of the Royal Society of London Series B-Biological Sciences* **270**, 45-51.
- Duclot F, Kabbaj M (2013) Individual Differences in Novelty Seeking Predict Subsequent Vulnerability to Social Defeat through a Differential Epigenetic Regulation of Brain-Derived Neurotrophic Factor Expression. *Journal of Neuroscience* **33**, 11048-11060.
- Dulawa SC, Grandy DK, Low MJ, Paulus MP, Geyer MA (1999) Dopamine D4 receptor-knock-out mice exhibit reduced exploration of novel stimuli. *Journal of Neuroscience* **19**, 9550-9556.
- Ebstein RP, Novick O, Umansky R, *et al.* (1996) Dopamine D4 receptor (D4DR) exon III polymorphism associated with the human personality trait of novelty seeking. *Nature Genetics* **12**, 78-80.
- Feng S, Jacobsen SE, Reik W (2010) Epigenetic Reprogramming in Plant and Animal Development. *Science* **330**,

- 622-627.
- Fidler AE, van Oers K, Drent PJ, *et al.* (2007) Drd4 gene polymorphisms are associated with personality variation in a passerine bird. *Proceedings of the Royal Society B: Biological Sciences* **274**, 1685-1691.
- Flisikowski K, Schwarzenbacher H, Wysocki M, *et al.* (2009) Variation in neighbouring genes of the dopaminergic and serotonergic systems affects feather pecking behaviour of laying hens. *Animal Genetics* **40**, 192-199.
- Frieling H, Romer KD, Scholz S, *et al.* (2010) Epigenetic Dysregulation of Dopaminergic Genes in Eating Disorders. *International Journal of Eating Disorders* **43**, 577-583.
- Groothuis TGG, Trillmich F (2011) Unfolding personalities: the importance of studying ontogeny. *Developmental Psychobiology* **53**, 641-655.
- Herb BR, Wolschin F, Hansen KD, *et al.* (2012) Reversible switching between epigenetic states in honeybee behavioral subcastes. *Nature Neuroscience* **15**, 1371-1373.
- Hoivik EA, Bjanesoy TE, Mai O, *et al.* (2011) DNA Methylation of Intronic Enhancers Directs Tissue-Specific Expression of Steroidogenic Factor 1/Adrenal 4 Binding Protein (SF-1/Ad4BP). *Endocrinology* **152**, 2100-2112.
- Hu Y, Xu H, Li Z, *et al.* (2013) Comparison of the Genome-Wide DNA Methylation Profiles between Fast-Growing and Slow-Growing Broilers. *PLoS ONE* **8**, e56411.
- Jablonka E, Raz G (2009) Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. *Quarterly Review of Biology* **84**, 131-176.
- Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics* **33**, 245-254.
- Jensen P (2013) Transgenerational epigenetic effects on animal behaviour. *Progress in Biophysics and Molecular Biology* **113**, 447.
- John OP, Robins RW, Pervin LA (2010) *Handbook of Personality: Theory and Research* Guilford Press, New York, NY.
- Jones PA (2012) Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics* **13**, 484-492.
- Kaminsky Z, Petronis A, Wang S-C, *et al.* (2008) Epigenetics of personality traits: an illustrative study of identical twins discordant for risk-taking behavior. *Twin Research and Human Genetics* **11**, 1-11.
- Kappeler L, Meaney MJ (2010) Epigenetics and parental effects. *BioEssays* **32**, 818-827.
- Korsten P, Mueller JC, Hermannstadter C, *et al.* (2010) Association between DRD4 gene polymorphism and personality variation in great tits: a test across four wild populations. *Molecular Ecology* **19**, 832-843.
- Kumsta R, Hummel E, Chen FS, Heinrichs M (2013) Epigenetic regulation of the oxytocin receptor gene: implications for behavioral neuroscience. *Frontiers in Neuroscience* **7**.
- Ledon-Rettig CC, Richards CL, Martin LB (2013) Epigenetics for behavioral ecologists. *Behavioral Ecology* **24**, 311-324.
- Laine VN, Gossmann TI, Schachtschneider KM, *et al.* (2016) Evolutionary signals of selection on cognition from the great tit genome and methylome. *Nature Communications* **In Press**.
- Li QH, Li N, Hu XX, *et al.* (2011) Genome-Wide Mapping of DNA Methylation in Chicken. *PLoS ONE* **6**.
- Lindqvist C, Janczak AM, Natt D, *et al.* (2007) Transmission of stress-induced learning impairment and associated brain gene expression from parents to offspring in chickens. *PLoS ONE* **2**, e364.
- Long HK, Sims D, Heger A, *et al.* (2013) Epigenetic conservation at gene regulatory elements revealed by non-methylated DNA profiling in seven vertebrates. *eLife* **2**.
- Maher B (2008) Personal genomes: The case of the missing heritability. *Nature* **456**.
- Manikkam M, Guerrero-Bosagna C, Tracey R, Haque MM, Skinner MK (2012) Transgenerational actions of environmental compounds on reproductive disease and identification of epigenetic biomarkers of ancestral exposures. *PLoS ONE* **7**, e31901.
- Massart R, Mongeau R, Lanfumey L (2012) Beyond the monoaminergic hypothesis: neuroplasticity and epigenetic changes in a transgenic mouse model of depression. *Philosophical Transactions of the Royal Society B: Biological Sciences* **367**, 2485-2494.
- Mueller JC, Korsten P, Hermannstadter C, *et al.* (2013) Haplotype structure, adaptive history and associations with exploratory behaviour of the DRD4 gene region in four great tit (*Parus major*) populations. *Molecular Ecology* **22**, 2797-2809.
- Munafó MR, Yalcin B, Willis-Owen SA, Flint J (2008) Association of the dopamine D4 receptor (DRD4) gene and approach-related personality traits: Meta-analysis and new data. *Biological Psychiatry* **63**, 197-206.
- Paquette AG, Marsit CJ (2014) The Developmental Basis of Epigenetic Regulation of HTR2A and Psychiatric Outcomes. *Journal of Cellular Biochemistry*, n/a-n/a.

- Réale D, Reader SM, Sol D, McDougall PT, Dingemanse NJ (2007) Integrating temperament in ecology and evolutionary biology. *Biological Reviews* **82**, 291-318.
- Riyahi S, Sanchez-Delgado M, Calafell F, Monk D, Senar JC (2015) Combined epigenetic and intraspecific variation of the DRD4 and SERT genes influence novelty seeking behavior in great tit *Parus major*. *Epigenetics* **10**, 516-525.
- Seutin G, White BN, Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology* **69**, 82-90.
- Shabalin AA, Aberg KA, van den Oord E (2015) Candidate gene methylation studies are at high risk of erroneous conclusions. *Epigenomics* **7**, 13-15.
- Stadler MB, Murr R, Burger L, *et al.* (2011) DNA-binding factors shape the mouse methylome at distal regulatory regions. *Nature* **480**, 490-495.
- Stamps JA, Groothuis TGG (2010) Ontogeny of animal personality: Relevance, concepts and perspectives. *Biological Reviews* **85**, 301-325.
- Szekely A, Ronai Z, Nemoda Z, *et al.* (2004) Human personality dimensions of persistence and harm avoidance associated with DRD4 and 5-HTTLPR polymorphisms. *American Journal of Medical Genetics* **126B**, 106-110.
- Szyf M, Weaver ICG, Champagne FA, Diorio J, Meaney MJ (2005) Maternal programming of steroid receptor expression and phenotype through DNA methylation in the rat. *Frontiers in Neuroendocrinology* **26**, 139-162.
- Tost J, Gut IG (2007) DNA methylation analysis by pyrosequencing. **2**, 2265-2275.
- Unoki M, Nakamura Y (2003) Methylation at CpG islands in intron 1 of EGR2 confers enhancer-like activity. *FEBS Letters* **554**, 67-72.
- van Oers K, De Jong G, van Noordwijk AJ, Kempenaers B, Drent PJ (2005) Contribution of genetics to the study of animal personalities: a review of case studies. *Behaviour* **142**, 1185-1206. DOI: 1110.1163/156853905774539364.
- van Oers K, Mueller JC (2010) Evolutionary genomics of animal personality. *Philosophical Transactions of the Royal Society of London, Series B* **365**, 3991-4000.
- van Oers K, Naguib M (2013) Avian personality. In: *Animal Personalities: Behavior, Physiology, and Evolution* (eds. Carere C, Maestripieri D), pp. 66-95. The University of Chicago Press, Chicago and London.
- Weaver ICG (2005) Reversal of Maternal Programming of Stress Responses in Adult Offspring through Methyl Supplementation: Altering Epigenetic Marking Later in Life. *Journal of Neuroscience* **25**, 11045-11054.
- Weaver ICG, Cervoni N, Champagne FA, *et al.* (2004) Epigenetic programming by maternal behavior. *Nat Neurosci* **7**, 847-854.
- Wong CCY, Caspi A, Williams B, *et al.* (2010) A longitudinal study of epigenetic variation in twins. *Epigenetics* **5**.
- Youngson NA, Whitelaw E (2008) Transgenerational epigenetic effects. In: *Annual Review of Genomics and Human Genetics*, pp. 233-257.

Author contributions

ECV, ACM and MVZ performed the research. ECV analysed data. SPC dissected the brains. KJFV and KvO designed the research and analysed data. KvO provided the bird samples. ECV, KJFV and KvO wrote the manuscript. All authors provided input during the writing of the manuscript.

Data Accessibility

Genotype and phenotype data were deposited in the Dryad repository:
doi:10.5061/dryad.fj662

Supporting Information

Table S1. Samples of birds used in this study.

Table S2. Primer and target sequences of the four pyrosequence assays.

Figure S1. Alignment of assay 5 PCR fragments showing the absence of unknown SNPs.

Figure S2. Diagram showing the CpG methylation of the complete region amplified by the assay B primer set.

Figure 1. Overview of the pyrosequence assay positions in the DRD4 gene. A) CpG density in the DRD4 gene. B) Structure of the DRD4 gene with the pyrosequence assay positions (black box with white letters), exons (dark grey arrows) and CpG island (light grey box). The numbers below the axis denote base pairs starting from the transcription start site. C) Target sequence of the four different assays with in red the CpG sites, which are numbered consecutively across assay B, C and D.

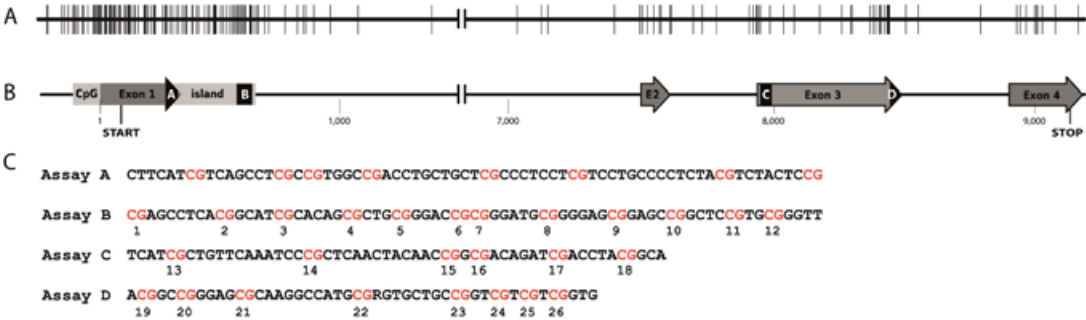


Figure 2. The mean methylation levels in blood tissue. The increase in methylation towards the 3' end of DRD4 is shown with the position of the assay in the gene on the x-axis. The four pyrosequence assay positions (A, B, C & D; black box with white letters), exons (dark grey arrows) and CpG island (light grey box) are shown on the x-axis with the numbers denoting base pairs starting from the transcription start site.

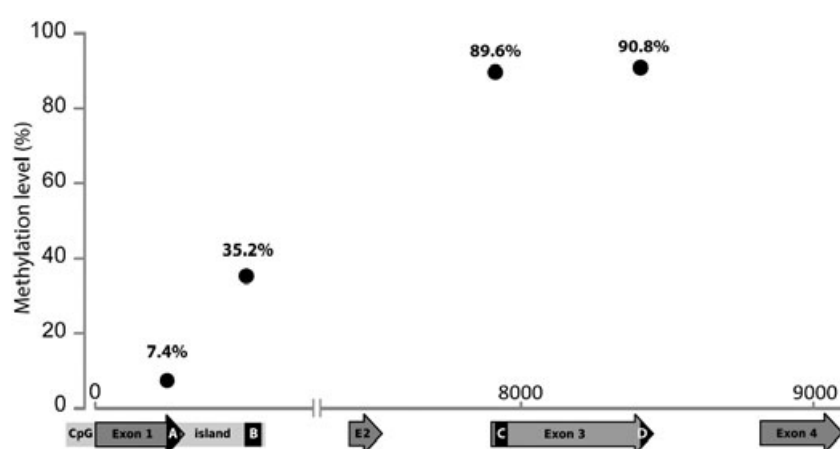


Figure 3. DNA methylation levels in blood and brain tissue. Methylation levels in (A) blood tissue and (B) brain tissue for assay B, C and D, per selection line and sex, where triangles indicate females and circles indicate males. Black markers indicate slow selection line (SE), red markers indicate fast selection line (FE). Error bars represent S.E.M.

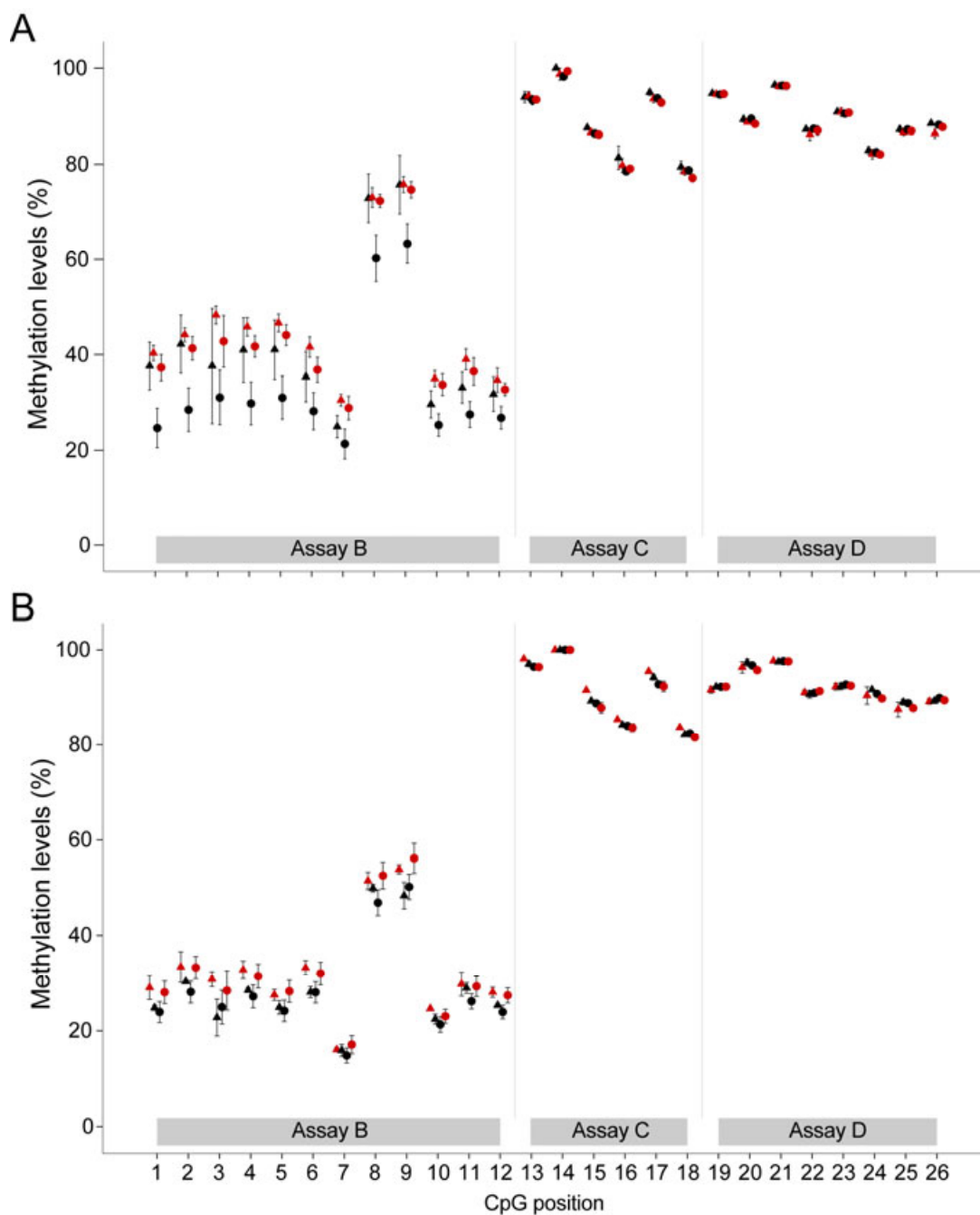


Table 1. Model output from a combined brain and blood analysis on methylation levels.

Factor	F	P
Ptype * Site * Sex	$F_{25,699.3} = 0.60$	0.94
Tissue * Site * Sex	$F_{25,724.1} = 0.99$	0.48
Ptype * Tissue * Site	$F_{25,749.9} = 1.56$	0.04

A general linear mixed effect model (GLMM) was used to test sex- and personality-related differences in methylation levels between the blood and brain tissue. Personality type (PTYPE), Sex, CpG position (Site) and Tissue (blood or brain) were included as fixed factors. Family (nested within personality type) and individual (nested within family and within personality type) were included in the model as random factors to account for the hierarchical structure in our data. Only the three way interactions are presented, but they were tested with the two-way interactions and main effects in the model (not shown).

Table 2. Model output for separate analysis on blood and brain tissues

	Factor	F	P
Blood	Ptype x Site x Sex	$F_{25,346.1} = 0.78$	0.77
	Ptype x Sex	$F_{1,14.2} = 0.85$	0.37
	Site x Sex	$F_{25,371.1} = 2.54$	<0.0001
	Post hoc analysis	Site 8	0.052
		Site 9	0.059
		Site 10-26	>0.10
	Ptype x Site	$F_{25,371.0} = 8.78$	<0.0001
	Post hoc analysis	Site 1-11	<0.05
		Site 12	0.059
		Site 13-26	>0.10
Brain	Ptype x Site x Sex	$F_{25,314.23} = 0.21$	1.00
	Ptype x Sex	$F_{1,11.5} = 2.28$	0.16
	Site x Sex	$F_{25,339.3} = 0.26$	1.00
	Ptype x Site	$F_{25,364.1} = 2.70$	<0.0001***
	Post hoc analysis	Site 1-6,8,9	<0.05
		Site 12	0.09
		Site 7,13-26	>0.15
	Sex	$F_{1,11.2} = 0.14$	0.72

Separate GLMM models per tissue type (blood and brain) were performed to assess associations between methylation levels and sex and personality over the different CpG positions. Personality type (PTYPE), Sex, CpG position (Site) and Tissue (blood or brain), their two-way interactions and the three-way interaction between personality type, sex and CpG position were included as fixed factors. Family (nested within personality type) and individual (nested within family and within personality type) were included in the model as random factors to account for the hierarchical structure in our data. We conducted a backwards elimination method and provide F and P values for the factors before removing them from the model. We conducted pairwise post-hoc comparisons with Bonferroni adjustments for multiple comparisons of the significant interactions site*sex and Ptype*site in blood and Ptype*site in brain. The main effect of sex in blood is not shown, due to the

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site*sex interaction. Sample sizes for blood were: Site 1-12 (11SE, 9FE) – Site 13-18 (7SE, 10FE) – Site 19-26 (8SE, 8FE) and for brain: Site1-12 (9SE, 8FE) for Site13-18 (8SE, 6FE) and for Site19-26 (10SE, 8FE).