Mol Neurobiol (2016) 53:6355–6361
DOI 10.1007/s12035-015-9534-2

Left-Right Axis Differentiation and Functional Lateralization: a Haplotype in the Methyltransferase Encoding Gene *SETDB2* Might Mediate Handedness in Healthy Adults

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Received: 8 September 2015 / Accepted: 8 November 2015 / Published online: 16 November 2015

Abstract Handedness is a multifactorial trait, and genes contributing to the differentiation of the left-right axis during embryogenesis have been identified as a major gene group associated with this trait. The methyltransferase SETDB2 (SET domain, bifurcated 2) has been shown to regulate structural left-right asymmetry in the vertebrate central nervous system by suppressing *fgf8* expression. Here, we investigated the relation of genetic variation in *SETDB2*—and its paralogue *SETDB1*—with different handedness phenotypes in 950 healthy adult participants. We identified a haplotype on *SETDB2* for which homozygous individuals showed a significantly lower lateralization quotient for handedness than the rest of the cohort after correction for multiple comparisons. Moreover, direction of handedness was significantly associated with genetic variation in this haplotype. This effect was mainly, but not exclusively, driven by the sequence variation rs4942830, as individuals homozygous for the A allele of this single nucleotide polymorphism had a significantly lower lateralization quotient than individuals with at least one T allele. These findings further confirm a role of genetic pathways relevant for structural left-right axis differentiation for functional lateralization. Moreover, as the protein encoded by *SETDB2* regulates gene expression epigenetically by histone H3 methylation, our findings highlight the importance of investigating the role of epigenetic modulations of gene expression in relation to handedness.

Keywords Genetic association study · SNP · Laterality · Lateralization · Direction of lateralization · Cerebral asymmetries · Ontogenesis

Introduction

Left-right preferences in the usage of forelimbs has been reported in dozens of vertebrate species [1], with human handedness being one of the strongest and certainly the most investigated of these preferences [2–6]. Twin and adoption studies [7, 8] suggest that handedness is at least partly heritable. While handedness was initially thought to be determined by a single gene [9], it is now generally accepted that it is a multifactorial trait, involving complex polygenic influences [10–14]. One of the major gene groups contributing to individual left- or right-handedness are genes involved in the formation of the left-right body symmetry [15–17]. Left-right axis formation is a critical step in embryonic development. During early embryogenesis the basic organization of the vertebrate body plan is established and the antero-posterior, dorso-ventral and left-right axes are formed [18]. Out of three axes, the left-right axis forms last, in a process which involves four distinct steps, and which is controlled for by the Nodal

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signaling pathway [19]. The central components in this pathway include *Nodal*, *Lefty1*, *Lefty2*, and *Pitx2* [20].

In addition to Nodal signaling, establishment of neuroanatomical asymmetries in the CNS also depends on the fibroblast growth factor 8 gene *fgf8*. Regan et al. [21] showed that zebrafish embryos, in which FGF signaling was blocked pharmacologically, did not develop the typical neuroanatomical asymmetries in the epithalamus. Similar results were also obtained in *fgf8* mutant zebrafish. However, provision of exogenous Fgf8 successfully induced asymmetrical CNS development. This Fgf8-mediated asymmetry induction in zebrafish is modulated by Setdb2. *SETDB2* (SET domain, bifurcated 2), located on chromosome 13q14 in humans, encodes a SET domain containing protein that modulates gene expression epigenetically through histone H3 and that likely acts as a histone H3 methyltransferase [22]. Histone H3 methyltransferase activity is known to be important for epigenetic landscaping during human embryonic stem cell differentiation to neural cells and controlling neural precursor cell fate during development [23, 24]. Interestingly, Xu et al. [25] proposed that Setdb2 regulates left-right asymmetry in the vertebrate CNS by suppressing *fgf8* expression. These authors provide evidence that zebrafish embryos lacking the Setdb2 protein showed left-right randomized expression of *southpaw*, *pitx2*, and *lefty2* and left-right randomization of structural left-right asymmetry in the diencephalon.

Since a number of differences in structural brain asymmetries have been reported between left- and right-handers [26], the involvement of *SETDB2* in CNS asymmetry formation makes it an interesting candidate gene for functional asymmetries such as handedness. Interestingly, sequence variations in *SETDB2* have been shown to be relevant for a behavioral trait in non-human animals. Using homozygosity mapping and interval resequencing in hunting and herding dogs, Akkad et al. [27] could show that a polymorphism in *SETDB2* was associated with so-called pointing behavior, as defined by a prolonged halt of movement to indicate the position of the hiding animal. While the relation between pointing behavior and handedness is rather vague, this finding indicates that variation in *SETDB2* can be relevant for mammalian motor behavior.

One important issue in genetics studies on handedness is how to define the phenotype [28]. Recently, Lien et al. [29] showed that degree or strength of handedness (e.g., how consistently a person favors to use one hand over the other) has a higher heritability (0.67), than a continuous handedness index (0.52), or the direction of handedness (0.39). Moreover, Arning et al. [30] showed that different handedness phenotypes (lateralization quotient, handedness direction, and handedness strength) were differentially related to genetic variation in the androgen receptor gene *AR*. This finding exemplifies that genetic association studies aimed at investigating handedness can benefit from the inclusion of more than one handedness phenotype.

This is especially the case when investigating the role of genes involved in the left-right body differentiation. In zebrafish, Concha et al. [31] demonstrated that when Nodal pathway genes were not expressed, the fish still exhibited neuroanatomical asymmetries, and only their direction was randomized. Thus, Nodal signaling seems to regulate the direction of structural asymmetries, while their strength is likely to be controlled for by a different signaling pathway.

The aim of the present study was to investigate the role of *SETDB2* and its paralog *SETDB1* for functional lateralization in humans. To this end, we performed a genetic association study using single nucleotide polymorphisms (SNPs) in the *SETDB1* and *SETDB2* genes as well as their haplotypes in relation to different handedness phenotypes.

Methods

Cohort

The cohort consisted of 950 healthy German adults (403 men and 547 women), without any history of psychiatric or neurological disease (mean age 26.08 years, SD=9.45). The participants were genetically unrelated and of Caucasian descent for at least two generations. No forced right-handers were included in the study. All participants gave written informed consent and were treated in accordance with the declaration of Helsinki. The study was approved by the ethics committee, Ruhr-University Bochum, Germany.

Genotyping and Haplotype Analysis

DNA samples for genotyping analyses were obtained via isolation from buccal swaps using Qiagen DNA isolation kits (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. DNA amount and quality was determined using a NanoDrop Spectrophotometer. SNPs investigated were selected based on their tagging potential—as indicated by Haploview software (https://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview)—in order to characterize the complete gene or at least the promoter and coding regions. SNPs were further selected based on their minor allele frequency for robust statistical analysis with a cutoff at 10 %. Genotyping was performed via polymerase chain reaction (PCR) with subsequent restriction fragment length polymorphism (RFLP) analysis (see Table 1).

Hardy-Weinberg equilibrium was evaluated using Pearson's goodness-of-fit chi-square test (degree of freedom=1). All SNPs passed testing of HWE as shown in Table 2 (adjusted for multiple testing, $n=7$). Haplotype analyses were performed using Phase 2.0 (http://www.bioinf.man.ac.uk/phase/). The

Table 1 List of oligonucleotides for *SETDB1* and *SETDB2* SNPs

Gene	Chr	Rs#	Sequence 5′-3′	Restriction enzyme	MM Ref.	Location
SETDB1 NM_001145415	1q21.3	rs72704685	F - agtctcgctcggtcacttag	*HaeIII*		5′
			R -ttgttttccgttctcagccc			
		rs11204744	F - aaagagtggaattgccagga	*SatI*		Intron 3
			R -aaggcaagtggatcacaagg			
		rs11204747	F - ggccagttaggtcccaacata	*MwoI*		Intron 14
			R -ggaagcctctttcactgcac			
SETDB2 NM_031915	13q14.2	rs41284778	F - ccactagcccatttcacagg	*RsaI*		Intron 1
			R -gaatgccataccgtaagtggta		C	
		rs4942830	F - tgaggccaaggagaggagta	*BsaAI*		Intron 1
			R -cctctcggctccttacgttt			
		rs7998427	F - tgaataatttattttaacagaacaaccg	*HpaII*	A	Exon 7
			R -cgtggagtgctgaagaatga			
		rs2057413	F - ttcattgtagaatgtgtgggttc	*TaaI*		Exon 10
			R -ctctgggttcctcagctgtt			

Chr chromosomal position of the corresponding gene; *Rs#* identifier as indicated by dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/), the sequences of primers are shown for 5′ to 3′; *F* forward primer; *R* reverse primer; underlined nucleotides indicate deviation from the reference sequence which were exchanged in order to create a restriction site; *MM Ref.* reference nucleotide at mismatch position; *Location* position of the investigated SNPs according to position transcript variant NM_001145415 for *SETDB1* and NM_031915 for *SETDB2*

distribution of corresponding *SETDB1* and *SETDB2* haplotypes are shown in Table 3.

Phenotyping

Handedness assessment was conducted using the Edinburgh Handedness Inventory [32]. In this 10-item questionnaire, participants have to indicate their individual hand preferences for different activities such as writing or striking a match. Based on the participants' answers, three different handedness phenotypes were determined. First, a laterality quotient (LQ) was determined using the equation LQ=[(R-L)/(R+L)]*100, with "R" indicating the number of right sided preferences and "L" indicating the number of left-sided preferences. The LQ ranges from −100 to 100, with negative values indicating a

Table 2 Genotype distributions for *SETDB1* and *SETDB2* SNPs as well as the corresponding corrected *p* values for HWE testing

Gene	SNP ID	Alleles		Genotypes			Percent (%)	HWE *p*
		1	2	11	12	22		
SETDB1	rs72704685	C	G	397	380	117	94	0.60
	rs11204744	A	G	444	259	36	78	1.00
	rs11204747	A	G	125	382	349	90	0.28
SETDB2	rs41284778	C	G	850	75	0	97	1.00
	rs4942830	A	T	275	491	157	97	0.09
	rs7998427	A	G	75	395	456	97	1.00
	rs2057413	A	G	472	383	60	96	0.91

The variable "%" indicates the percentage of successfully genotyped participants for the corresponding SNP. Please note that amplification of the corresponding loci was not always possible, resulting in varying sample sizes for different SNPs (and different degrees of freedom in the statistical analyses)

Table 3 Haplotypes generated via Phase 2.0 for the three genotyped SNPs in *SETDB1* and four SNPs in *SETDB2*, respectively

Gene	ID	Haplotype	E(freq)	S.E.	O(freq) A1	O(freq) A2
SETDB1	1	CAG	0.393	0.00395	0.682	0.139
	2	CAA	0.032	0.00179	0.034	0.027
	3	CGG	0.228	0.00391	0.151	0.275
	4	CGA	0.003	0.00101	0.000	0.000
	5	GAG	0.011	0.00140	0.008	0.010
	6	GAA	0.325	0.00132	0.124	0.537
	7	GGG	0.008	0.00118	0.001	0.012
SETDB2	1	CAAG	0.010	0.00068	0.013	0.000
	2	CAGA	0.548	0.00129	0.813	0.289
	3	CAGG	0.008	0.00095	0.003	0.013
	4	CTAA	0.030	0.00075	0.023	0.039
	5	CTAG	0.259	0.00092	0.124	0.391
	6	CTGA	0.099	0.00135	0.024	0.176
	7	CTGG	0.007	0.00090	0.000	0.013
	8	GAGA	0.002	0.00034	0.000	0.003
	9	GTGA	0.037	0.00048	0.000	0.077

The SNPs included in each haplotype are depicted from left to right: *SETDB1* rs72704685, rs11204744 and rs11204747. *SETDB2*: rs41284778, rs4942830, rs7998427 and rs2057413. *E(freq)* estimated frequency of the corresponding haplotype, *S.E.* standard error, *O(freq)* observed frequency of the corresponding haplotype, *A1* allele 1, *A2* allele 2

mainly left-sided preference. Positive values, on the other hand, indicate a mainly right-sided preference. Based on the ,LQ we determined a dichotomous variable LR indicating whether an individual was left or right handed by assigning all participants with a negative LQ value to group 1 (left-handers) and all participants with a positive LQ value to group 2 (right-handers). This was done in order to generate a measure for the direction of the behavioral preference, independent of its strength. Moreover, the absolute value of the LQ was used to gain a measure for the strength of the behavioral preference (ST), independent of its direction.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 20. Analysis of individual SNPs was performed using dominant models to ensure adequate statistical power even for comparisons with a low frequency of the rare homozygous genotype. Handedness LQ and ST were analyzed parametrically using univariate ANOVAS with the respective phenotype as dependent variable. Since handedness is a sex-dependent trait [33] and we encountered sex-dependent association between genetic variation in candidate genes and handedness in a previous study [30], we also included sex as a second fixed factor into the model in order to be aware of such effects. Since LR was not interval-scaled, this dependent variable was analyzed non-parametrically using Mann-Whitney *U* tests. Statistical significance was assumed to be $p<0.0071$ ($p<0.05$, Bonferroni-corrected for the number of tested SNPs). This is likely to be an overly conservative correction since at least some of the individual genotypes correlated between different SNPs. In addition, haplotypes generated using all SNPs of each gene were analyzed comparably to single SNPs. Haplotype analyses with LQ, LR, and ST were performed using models with individuals homozygous for the haplotype with the highest estimated frequency (haplotype 1 for *SETDB1* and haplotype 2 for *SETDB2*; see Table 2) compared to all other individuals.

Results

The average LQ (see Fig. 1 for the distribution of this variable) was 73.33 (SD=48.18) and average ST (see Fig. 2 for the distribution of this variable) was 85.62 (SD=19.16). Nine percent of participants were classified as left-handers and 91 % as right-handers. To determine to what extent the phenotypes were correlated, two-sided Spearman correlation coefficients were calculated. All phenotypes showed significant positive correlations with each other (LQ-ST: $\rho=0.89$, $p<0.01$; LQ-LR: $\rho=0.52$, $p<0.01$; ST-LR: $\rho=0.20$, $p<0.01$).

The *p* values for the analysis of individual SNPs are indicated in Table 4 and the LQs for the different genotypes in Table 5. None of the main effects or interactions with the factor sex reached significance (all *p* values>0.26). For *SETDB2*, effects were only observed for rs4942830. For this SNP, individuals homozygous for the A allele (66.12±2.94) had a significantly lower LQ than individuals with at least one T allele (75.90±1.40) ($F_{(1,916)}=7.79$; $p=0.005$). Testing an intermediate model with three genotypes lowered the F-value ($F_{(2,914)}=4.30$; $p=0.01$), hinting towards a recessive model. Also, an effect significant on the nominal but not the corrected significance level was observed for LR ($U=84645.00$; $Z=-2.23$; $p=0.026$). Here, individuals homozygous for the A allele showed a trend towards a higher incidence of left-handedness (left-handers 12 %, right-handers 88 %) than individuals with at least one T allele (left-handers 7.5 %, right-handers 92.5 %).

For *SETDB1*, no effect reached significance on the corrected significance level, but a nominally significant trend was observed for rs72704685. This trend indicated that individuals homozygous for the C allele had a higher LQ (76.85±2.46) than individuals with at least one G allele (70.04±2.19; $F_{(1,887)}=4.28$; $p=0.04$).

The observed frequencies for the haplotypes are shown in Table 3 (in the last two columns). For both genes, the haplotypes with the highest estimated frequency also had the highest observed frequency (haplotype 1 for *SETDB1* and haplotype 2 for *SETDB2).* For the haplotype analyses, all main effects of sex and all interactions with sex failed to reach significance (all *p* values>0.30). For *SETDB1*, the analysis comparing individuals homozygous for haplotype 1 against all other individuals revealed no significant effects for LQ ($p=0.92$), LR ($p=0.66$), and ST ($p=0.46$). For *SETDB2*, the haplotype comparisons for a recessive model, in which individuals homozygous for haplotype 2 (carrying the A allele) were compared against all other individuals, revealed a significant effect for LQ ($F_{(1,925)}=10.30$; $p=0.001$). This effect indicated that individuals homozygous for haplotype 2 on average had lower LQs (64.99±3.00) than the rest of the cohort (76.31±1.86). In an intermediate model, this effect failed to reach significance ($p=0.53$). Moreover, the effect for LR reached significance ($U=82659.00$; $Z=-2.62$; $p=0.009$), indicating that the incidence of left-handedness was higher in individuals homozygous for haplotype 2 (left-handers 12.6 %, right-handers 87.4 %) than in the rest of the cohort (left-handers 7.2 %, right-handers 92.8 %). In contrast, the analysis for ST failed to reach significance ($p=0.58$).

Discussion

Functional hemispheric asymmetries have been reported for many cognitive domains, including language [34, 35], processing of emotions [36], face processing [37], visuo-spatial processing [38], and body image [39]. Despite their high

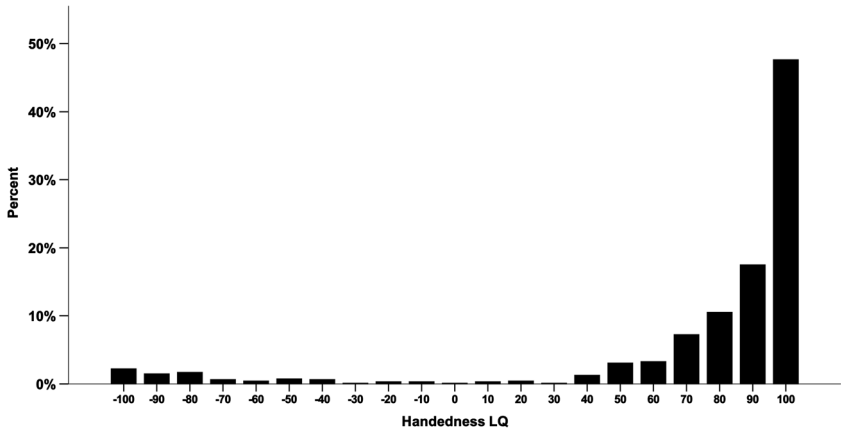

Fig. 1 Distribution of LQ for all 950 participants in percent. Participants were assigned to one of 21 groups, based on their individual LQ (e.g., the group "10" includes all participants with an LQ between 1 and 10)

relevance for functional brain organization, we only begin to understand their genetic background. Most progress in this regard has been made in relation to handedness. Genes implicated in the ontogenesis of human hand preferences include *PCSK6* [15–17], *AR* [30, 40, 41], and *LRRTM1* [42, 43]. A recent genetic linkage study in an isolated Dutch population [44] did not observe genome-wide evidence for linkage for handedness, but found suggestive linkage for left-handedness in the 22q13 region (rs728592 and rs932497). Most authors agree that handedness is a trait that involves complex polygenic influences. Based on GWAS results, McManus et al. [11] estimated the number of involved genes to be at least around 30–40. The present study adds to this literature by providing evidence for an association of genetic variation in *SETDB2* and handedness in healthy adults, as participants homozygous for *SETDB2* haplotype 2 had a significantly lower LQ (lateralization quotient) than the rest of the cohort. Our data show that the genetic variations identified have significant but rather subtle associations with handedness. This might explain why the associations found in the present study based on a hypothesis driven candidate gene approach have not been reported by previous handedness GWAS [45], as these studies typically have been underpowered to detect a multitude of small genetic contributions to the phenotype which might also be regulated epigenetically [11].

On the SNP level, only rs4942830 revealed a significant association with LQ and a trend for LR (direction of handedness) using a recessive model. Rs4942830 is located in intron 1 of the *SETDB2* gene and lies within a region of high H3K27ac activity as indicated by ENCODE (https://genome.ucsc.edu/ENCODE/). Sequence analysis revealed that rs4942830 might alter the motif of a so-called enhancer box which is mostly found in promoter regions of eukaryotes and acts as a regulator of gene expression, i.e., in neurons and muscles [46]. In case of a T>A exchange for this SNP, the transcription factor BHLHE40 might lose or lower its binding capacity. Using haplotype analyses, we observed an increase of significance for a lower LQ in individuals homozygous for the *SETDB2* haplotype 2 (including the homozygous A allele

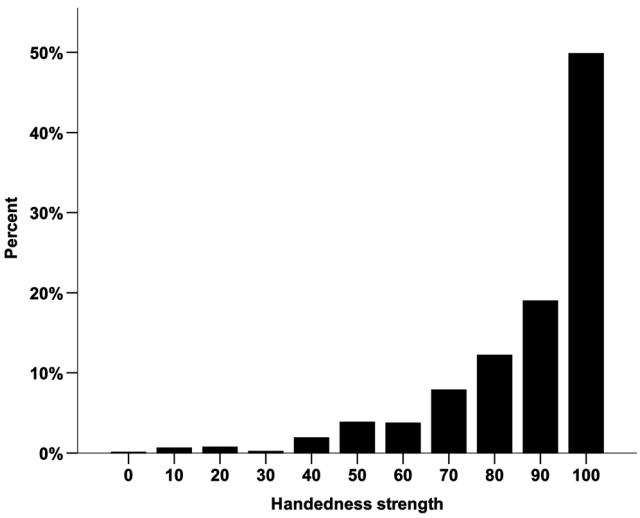

Fig. 2 Distribution of ST for all 950 participants in percent. Participants were assigned to one of 11 groups, based on their individual ST (e.g., the group "10" includes all participants with an ST between 1 and 10)

Table 4 *p* values for the different association tests (LQ and ST have been analyzed using ANOVAs and LR has been analyzed using Mann-Whitney *U* tests)

Gene	Rs#	LQ	LR	ST
SETDB1	rs72704685	0.04*	0.055	0.37
	rs11204744	0.13	0.40	0.08
	rs11204747	0.19	0.30	0.46
SETDB2	rs41284778	0.39	0.52	0.98
	rs4942830	*0.005***	0.026*	0.73
	rs7998427	0.20	0.25	0.31
	rs2057413	0.33	0.54	0.56

Effects significant at the $p<0.007$ level (the adjusted p value for multiple comparisons) are given in italics (numbers)

*$p<0.05$; **$p>0.01$; nominally significant effects are indicated by asterisks

Table 5 LQs for the different genotypes for the different SNPs

Gene	Rs#	Genotype	LQ	Difference
SETDB1	rs72704685	CC	76.85	6.81
		CG/GG	70.04	
	rs11204744	AA	69.70	5.67
		AG/GG	75.37	
	rs11204747	AG/AA	71.38	4.51
		GG	75.89	
SETDB2	rs41284778	CC	72.88	5.09
		CG/GG	77.97	
	rs4942830	AA	66.12	9.78
		AT/TT	75.90	
	rs7998427	AG/AA	75.05	4.08
		GG	70.97	
	rs2057413	AA	71.53	3.12
		AG/GG	74.65	

of rs4942830) compared to the analysis considering the SNP alone. This is in line with the results for rs4942830 itself, but also directly indicates that there might be other minor regulating elements in *cis* based on the increased effect.

Taken together, our study is in line with previous work [15–17] showing that genes contributing to the formation of the left-right body symmetry can also be relevant for functional lateralization. Particularly interesting in this regard is the finding that the effect found for LQ, a composite score including both strength and direction of handedness in one number, was mainly driven by the direction of handedness as the analysis for LR reached significance, while the analysis for ST failed to do so. About 12.6 % of the individuals homozygous for haplotype 2 were left-handers, while only 7.2 % of the rest of the cohort were left-handers. The idea that *SETDB2* is mainly associated with handedness direction is in line with the findings of Xu et al. [25] on structural CNS asymmetries in zebrafish. These authors described that knockdown of zebrafish *Setdb2* randomized the direction of visceral and diencephalic asymmetry in the fish, suggesting a role of this gene for asymmetry direction. Thus, our data tentatively support the idea that strength and direction of human handedness are controlled for by two different ontogenetic pathways, as has been suggested for structural asymmetries in the zebrafish brain [31].

As always for candidate gene studies, independent replication in larger cohorts is necessary before any final conclusions can be drawn. Replication studies would potentially benefit from the inclusion of a behavioral handedness measure such as the peg board task used by Scerri and colleagues in their *PCSK6* study [17]. While use of the Edinburgh Handedness Inventory is standard in the field, the use of questionnaires to assess handedness possesses inherent limitations, as answering a questionnaire is highly subjective [47]. Apart from that, this research has also several other implications for future studies. In a twin study, Medland et al. [48] observed that additive genetic effects accounted for only about 24 % of variance in handedness data, implicating non-genetic processes might have a high relevance for the ontogenesis of handedness. Since the protein encoded by *SETDB2* regulates gene expression epigenetically by histone H3 methylation [22], our findings highlight the importance of investigating the role of epigenetic modulations of gene expression in relation to handedness as previously suggested by Ocklenburg et al. [49]. On the molecular lever, future studies are warranted for investigating more markers around rs4942830, possible changes in transcription factor binding capacity to the corresponding rs4942830 allele (via EMSA) as well as the analysis of single nucleotide exchanges (via CRISPR/Cas9) on the epigenetic changes during development.

Compliance with Ethical Standards

Ethical Approval All participants gave written informed consent and were treated in accordance with the declaration of Helsinki. The study was approved by the ethics committee, Ruhr-University Bochum, Germany.

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