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The evolution of *Homo sapiens denisova* and *Homo sapiens neanderthalensis* miRNA targeting genes in the prenatal and postnatal brain

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Abstract

Background: As the evolution of miRNA genes has been found to be one of the important factors in formation of the modern type of man, we performed a comparative analysis of the evolution of miRNA genes in two archaic hominines, *Homo sapiens neanderthalensis* and *Homo sapiens denisova*, and elucidated the expression of their target mRNAs in bain.

Results: A comparative analysis of the genomes of primates, including species in the genus *Homo*, identified a group of miRNA genes having fixed substitutions with important implications for the evolution of *Homo sapiens neanderthalensis* and *Homo sapiens denisova*. The mRNAs targeted by miRNAs with mutations specific for *Homo sapiens denisova* exhibited enhanced expression during postnatal brain development in modern humans. By contrast, the expression of mRNAs targeted by miRNAs bearing variations specific for *Homo sapiens neanderthalensis* was shown to be enhanced in prenatal brain development.

Conclusions: Our results highlight the importance of changes in miRNA gene sequences in the course of *Homo sapiens denisova* and *Homo sapiens neanderthalensis* evolution. The genetic alterations of miRNAs regulating the spatiotemporal expression of multiple genes in the prenatal and postnatal brain may contribute to the progressive evolution of brain function, which is consistent with the observations of fine technical and typological properties of tools and decorative items reported from archaeological Denisovan sites. The data also suggest that differential spatial-temporal regulation of gene products promoted by the subspecies-specific mutations in the miRNA genes might have occurred in the brains of *Homo sapiens denisova* and *Homo sapiens neanderthalensis*, potentially contributing to the cultural differences between these two archaic hominines.

Background

Early in the 21st century, fossils of an ancient man who lived 50-45 thousand years (ky) ago were found in the Denisova cave in the Altai mountains [1]. The *Homo sapiens denisova* (*H. s. d.*) nuclear genome was first sequenced in 2010 [2] and then re-sequenced in 2012 [3]. The *Homo sapiens neanderthalensis* (*H. s. n.*) nuclear

genome was first sequenced in 2010 [4] and for a second time in 2014 [5]. A comparative analysis of the archaic hominine (*H. s. d., H. s. n.)* and *Homo sapiens sapiens* (*H. s. s.*) genomes assessed the genetic contribution of *H. s. d.* and *H. s. n* to the genetic and biological profile of the modern *H. s. s.* populations [6]. It was shown that the *H. s. n.* lineage is a sister lineage to *H. s. d.* [2]. The population split of Neanderthals and Denisovans from modern humans was estimated to have occurred 550-765 ky ago and the split time of Neanderthals and Denisovans 380-473 ky ago [5]. Considering that the evolution of miRNA genes could be one of the key factors in the

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formation of the modern type of man [7-10], we analyzed the evolution of miRNA genes in *H. s. n.* and *H. s. d.* and the expression of their target mRNAs. Using improved versions of the *H. s. d.* and *H. s. n.* genomes, we performed computer-assisted comparisons of the *H. s. n., H. s. d.* and *H. s. s.* genomes to reveal the structural and functional organization of microRNAs (miRNAs) as well as the mRNAs targeted by these miRNAs. In both the Neanderthal and Denisovan genomes, we found miRNA genes with fixed substitutions in mature miRNAs and multiple substitutions in pre-miRNA regions involved in pre-miRNA processing.

Our analysis of spatiotemporal gene expression in human tissues demonstrated that the miRNAs bearing new genetic variants fixed in the Denisovan genome regulated target mRNAs with the highest levels of expression in the postnatal human brain.

Results and discussion

To identify miRNA genes that evolved divergently in the genus Homo, we first used the map and pre-miRNA gene sequence data for 1595 experimentally confirmed *H. s. s.* miRNAs from the miRBase database rel. 19 [11]. We selected the *H. s. d.* and *H. s. n.* pre-miRNAs with highly confident sequences that are orthologous to *H. s. s.* pre-miRNAs. Using the selection procedure described in the Methods section (Selection of miRNA genes in *H. s. d.* and *H. s. n.* with no match in *H. s. s.*), we identified 1298 *H. s. d.* and 1329 *H. s. n.* pre-miRNAs perfectly matched to *H. s. s.* pre-miRNA gene sequences. In addition, we identified and selected for further study 106 *H. s. d.* and 102 *H. s. n.* diverged genes for pre-miRNA sequences, which were different from *H. s. s.* pre-miRNA genes by at least a single nucleotide. The other 191 *H. s. d.* and 164 *H. s. n.* pre-miRNAs did not pass quality sequence control in the selection procedure

Further, we explored the alignment of the genomes of six primates available in Ensembl rel. 69 [12]. Each of the selected *H. s. d.* and *H. s. n.* pre-miRNA genes non-identical to *H. s. s.* orthologs had at least one ortholog in another primate. This result indicated that these pre-miRNA coding genes were present in the genome of the common ancestor of *H. s. s.*, *H. s. d.* and *H. s. n.* long before the evolutionary split between these hominines. In following up this analysis, we confirmed the existence of orthologs for all selected *H. s. d.* and *H. s. n.* miRNA genes except one *H. s. d.* miRNA gene.

Next, we analyzed all substitutions found in the archaic hominine miRNA genes selected as described above. First, we excluded a few doubtful nucleotide substitutions observed in the sequencing data for the pre-miRNA genes of *H. s. d.* and *H. s. n.* (see Methods section). Then, we mapped the remaining genetic variants found in the pre-miRNA genes of *H. s. d.* and *H. s. n.* onto the secondary

structures of the corresponding pre-miRNAs in *H. s. s.* available at the miRBase rel. 19 [11]. We selected the *H. s. d.* and *H. s. n.* pre-miRNAs with (i) nucleotide substitutions (deletions, insertions) in the regions corresponding to the sequences of mature miRNAs (and/or miRNAs*) responsible for binding to target mRNAs or (ii) multiple (two or more) densely spaced substitutions within a pre-miRNA region involved in pre-miRNA processing. From these pre-miRNA groups, we excluded the pre-miRNAs that occur in the *H. s. s.* genome in more than one copy. The aim of this exclusion was to select pre-miRNAs with unique functions. The selection yielded *H. s. d.* and *H. s. n.* pre-miRNAs with nucleotide substitutions that might contribute to significant functional differences from the *H. s. s.* pre-miRNAs.

The functional annotation of the diverged pre-miRNA genes in archaic hominines was performed as follows. First, we selected the miRNAs expressed in the central nervous system (CNS) using the miRGator 3.0 [13] and ChIP-seq data for H3K4me3 (histone H3 trimethyl K4) modified histones marking active promoters in primate cortical neurons [14]. Second, the potential target mRNAs of these central nervous system (CNS)-active miRNAs were identified using data from miRGator 3.0 [13]. miRGator 3.0 contains experimental miRNA/target-mRNAs co-expression data obtained under various conditions. Therefore, we extracted from miRGator 3.0 genes with co-expression correlation coefficient with miRNA $r \leq -0.9$ [13]. We used the co-expression evidence to identify putative interactions between miRNAs and their target mRNAs instead of the CLIP-seq experiments results because the latter technology requires cell lysis, which facilitates the interaction of components that are usually segregated by cellular compartments [15], and many miRNA/mRNAs interactions identified by CLIP-seq are non-canonical, which in turn does not mediate repression [16]. However, co-expression data indicate pathways (not genes) targeted by miRNA but do not allow the identification of direct miRNA/mRNA interactions. The latter has a side advantage because, in this approach, we selected against target genes with limited effect on tissue functioning [17]. Third, to identify the human brain structures (topologically different brain regions) showing increased levels of the target mRNAs (henceforth referred to as the target brain structures), we performed a randomization test using the Human Allen Brain Atlas [18] and the BrainSpan Atlas [19]. We employed the randomization test for this purpose because the standard analytic annotation enrichment techniques are inapplicable for miRNA functional enrichment analysis [20].

The characteristics of the nucleotide substitutions in each of the CNS-active pre-miRNA genes diverged in Denisovan and Neanderthal are shown in Tables 1 and 2.

Table 1. *Homo sapiens denisova* miRNAs that differ from *Homo sapiens sapiens* miRNAs

#	miRNA*	Ancestral state (h: human, d: Denisovan)	Difference (human/ Denisovan) **	Genome position (hg 19)			DB SNP ID	Allele frequency (1000 genomes [28])	African population frequency (1000 genomes [28]or dbSNP 141 [29])
				Chromosome	Position	Strand			
*Homo sapiens denisova* miRNA mutations common to previously observed human miRNA polymorphisms								
1	{hsa-mir-1178}	d	{A/G}	12	120151493	-	rs7311975	C:0.1382	0.4191
2	{hsa-mir-1252}	d	{A/G}	12	79813049	+	rs115256251	G:0.0032	0.0098
3	{hsa-mir-1269a}	d	{G/A}	4	67142620	+	rs73239138	A: 0.3942	0.7277
4	{hsa-mir-146a}	d	{C/G}	5	159912418	+	rs2910164	C:0.3815	0.475(YRI)
5	{hsa-mir-2682 (3p)}	d	{G/A}	1	98510847	-	rs74904371	T:0.0208	0.0053
6	{hsa-mir-3161}	d	{-/A}	11	48118347	+	rs11382316	A:0.7599	+(BUSHMAN)
7	{hsa-mir-4804 (5p)}	d	{C/G}	5	72174432	+	rs266435	G: 0.8001	0.7269
8	{hsa-mir-608}	d	{C/G}	10	102734778	+	rs4919510	G:0.3638	0.4402
		h	A/G	10	102734813	+	-	-	-
9	hsa-mir-3124 (3p)	d	C/A	1	249120631	+	rs115160731	A:0.0128	0.0469
10	hsa-mir-4514	h	T/C	15	81289798	-	rs116034786	G:0.0136	0.0371
11	hsa-mir-1269b	h	C/(G|C)	17	12820646	-	rs7210937	C:0.3520	0.5976
12	hsa-mir-6085	h	A/-	15	62635322	+	rs372168584	-:0.0006	0.0023
13	hsa-mir-378e	d	T/C	5	169455502	+	rs367764573	C:0.0026	0.0008
		h	T/C	5	169455551	+	rs376752141	C:0.0026	0.0008
14	hsa-mir-662	h	T/A	16	820215	+	rs74656628	A:0.1374	0.3971
		h	G/A	16	820249	+	rs9745376	A:0.0543	0.1921
15	hsa-mir-4463	d	-/AG	6	76138146	+	rs5877455	AG:0.7079	0.9198
16	{hsa-mir-532 (3p, 5p)}	d	{A/G}	X	49767832	+	rs456615	G:1	0.7587
17		d	{A/G}	X	49767835	+	rs456617	G:1	0.7587
18	{hsa-mir-943}	h	A/G	4	1988176	-	rs368905227	T: 0.0004	0
		d	{-/AG}	4	1988188	-	rs3034718	CT: 0.2766	0.4629
		d	{A/G}	4	1988193	-	rs1077020	C: 0.2504	0.3865
*Homo sapiens denisova*-specific mutations								
1	hsa-mir-1321	h	G/T	X	85090839	+	-	-	
2	hsa-mir-1909	h	G/C	19	1816168	-	-	-	
3	hsa-mir-3143	h	T/C	6	27115417	+	-	-	
4	hsa-mir-3152 (5p)	h	A/-	9	18573332	+	-	-	
5	hsa-mir-3185	h	G/A	17	46801808	-	-	-	
6	hsa-mir-4478	h	T/A	9	124882437	-	-	-	
7	hsa-mir-4700 (3p)	h	C/T	12	121161049	+	-	-	
8	hsa-mir-4710	h	T/C	14	105144064	-	-	-	
9	hsa-mir-5687	h	C/T	5	54804702	-	rs545080149	A:0.0002	0.0008
10	hsa-mir-609	h	G/A	10	105978625	-	-	-	
11	{hsa-mir-671 (3p)}	h	{C/T}	7	150935593	+	-	-	

* bold - expression in the central neural system (miRGator 3.0 data [13] and data from [14]), underline - common between *H. s. d.* and *H. s. n.*

** bold - location in mature miRNA, {} common between *H. s. d.* and *H. s. n.*, italic - location in canonical seed (nucleotides 2-7) region, based on [40]

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Table 2. *Homo sapiens neanderthalensis* miRNAs that differ from *Homo sapiens sapiens* miRNAs

#	miRNA*	Ancestral state (h: human, n: Neanderthal; c: chimpanzee)	Mutation (human/ Neanderthal) **	Genome position (hg 19)			DB SNP ID	Allele frequency (1000 genomes [28])	African population frequency (1000 genomes [28]or dbSNP 141 [29])
				Chromosome	Position	Strand			
	Homo sapiens neanderthalensis miRNA mutations common to previously observed human miRNA polymorphisms								
1	{hsa-mir-1178}	n	*{A/G}*	12	120151493	-	rs7311975	C:0.1382	0.4191
2	{hsa-mir-1252}	n	*{A/G}*	12	79813049	+	rs115256251	G:0.0032	0.0098
3	{hsa-mir-1269a}	n	*{G/A}*	4	67142620	+	rs73239138	A:0.3942	0.7277
4	{hsa-mir-146a}	n	*{C/G}*	5	159912418	+	rs2910164	C:0.3815	0.475(YRI)
5	{hsa-mir-2682 (3p)}	n	*{G/A}*	1	98510847	-	rs74904371	T:0.0208	0.0053
6	{hsa-mir-3161}	n	*{-/A}*	11	48118347	+	rs11382316	A:0.7599	+(BUSHMAN)
7	{hsa-mir-4804 (5p)}	n	*{C/G}*	5	72174432	+	rs266435	G:0.8001	0.7269
8	{hsa-mir-608}	n	*{C/G}*	10	102734778	+	rs4919510	G:0.3638	0.4402
9	hsa-mir-1343	n	**T/C**	11	34963416	+	rs2986407	C:0.7668	0.792
10	hsa-mir-3129 (5p)	h	**G/C**	2	189997816	-	rs192364638	C:1	1
11	hsa-mir-4274	n	**-/CAC**	4	7461827	+	rs35245133	CAC:0.858	+(BUSHMAN)
12	hsa-mir-4293	h	*C/G*	10	14425221	-	rs12220909	G:0.9619	0.9992
13	hsa-mir-5189	n	G/C	16	88535400	+	rs80296158	C:0.0507	0.1831
14	hsa-mir-149 (3p, 5p)	n	A/G	2	241395500	+	rs71428439	G:0.1440	0.1112
15		n	T/C	2	241395503	+	rs2292832	C:0.3866	0.2716
16	hsa-mir-1908	n	A/G	11	61582708	-	rs174561	C:0.2796	0.0197
		h	G/A	11	61582709	-	-	-	-
17	hsa-mir-3938	n	TT/-	3	55886573	-	rs10575780	-:0.2426	0.4667
18	hsa-mir-4463	n	-/AG	6	76138146	+	rs398110299	AG:0.7079	0.9198
19	hsa-mir-4719	n	T/C	16	76902847	+	rs7500280	C:0.3293	0,2171
		n	G/A	16	76902850	+	rs7499278	A:0.7528	0.9455
20	{hsa-mir-532} {(3p, 5p)}	n	{A/G}	X	49767832	+	rs456615	G:1	0.7587
21		n	{A/G}	X	49767835	+	rs456617	G:1	0.7587
22	{hsa-mir-943}	n	{-/GA}	4	1988188	-	rs3034718	CT:0.2766	0.4629
		n	{A/G}	4	1988193	-	rs1077020	C:0.2504	0.3865
	Homo sapiens neanderthalensis-specific mutations								
1	hsa-mir-1208	h	**T/C**	8	129162377	+	-	-	-
2	hsa-mir-4532	h	**C/T**	20	56470458	+	-	-	-
3	hsa-mir-4718	h	**C/T**	16	12814185	+	-	-	-
4	hsa-mir-615	h	**C/T**	12	54427811	+	-	-	-
5	hsa-mir-639	h	**T/C**	19	14640435	+	rs561305115	C:0.0064	0.004
		h	G/C	19	14640399	+	rs372602559	C:0.0062	0.004
6	{hsa-mir-671 (3p)}	h	*{C/T}*	7	150935593	+	-	-	
7	hsa-mir-6715b (3p)	h	**G/T**	10	114059393	-	rs182337914	A:0.0012	0
8	hsa-mir-4749 (3p)	h	*C/(C|T)*	19	50357891	+	rs372882504	-	1(C)
9	hsa-mir-3939	h(A)/c(G)	A/(G|A)	6	167411298	-	rs80032204	-	0.915(A)

Table 2. *Homo sapiens neanderthalensis* miRNAs that differ from *Homo sapiens sapiens* miRNAs (Continued)

h(A)/c(G)	A/(G|A)	6	167411300	-	rs77840042	-	0.912(A)
h(C)/c(T)	C/(C|T)	6	167411301	-	rs75692943	-	0.912(C)
h	*G/(G|A)*	6	167411334	-	rs75823810	-	0.954(G)
h	*C/(C|T)*	6	167411337	-	rs73024232	-	0.957(C)
h	C/(C|T)	6	167411362	-	rs77072520	-	0.972(C)

* bold - expression in the central neural system (MiRGator 3.0 data [13] and data from [14])

** bold - location in mature miRNA, {} - common between *H. s. d.* and for *H. s. n.*, italic - location in canonical seed (nucleotides 2-7) region, based on [40]

These miRNA genes were stratified into four groups. The first group consists of 9 miRNAs with fixed mutations unique to the *H. s. d.* lineage. The second group includes 5 miRNAs with fixed mutations unique to the *H. s. n.* lineage. Two other miRNA gene groups were compiled of 14 *H. s. d.* and 18 *H. s. n.* miRNA genes bearing variants that match to one of the polymorphic alleles in *H. s. s.* Most polymorphisms in these miRNAs are SNPs (single nucleotide polymorphisms). Several substitutions are observed in the seed region (based on Table 1 and 2) in six out of 14 *H. s. d.* and five out of 18 *H. s. n.* pre-miRNAs common to human polymorphisms. Only one indel in the seed region was observed in hsa-mir-3161 in both *H. s. n.* and *H. s. d.* The minority of miRNAs with polymorphisms in the seed region is not sufficient to claim that the general capacity of these miRNA pools have altered binding specificity to target mRNAs. This result allowed us to use the third and fourth pools as a control to compare the target specificities with miRNA bearing fixed variants specific for *H. s. n.* and *H. s. d.*

Next, we searched for the brain regions and CNS development stage showing the most abundant expression of mRNAs targeted by the miRNAs that diverged in archaic hominines (see Methods section). For this purpose, we used data in (1) the BrainSpan Exon micro-array (BrainSpan) [19] and (2) the Allen Human Brain Atlas as updated March 7, 2013 (AHBA) [18].

The results of the analysis of BrainSpan data [19] demonstrated that mRNAs targeted by miRNAs bearing fixed mutations specific for *H. s. d.* are enriched in the postnatal stage of brain development, whereas mRNAs targeted by miRNAs bearing fixed mutations specific for *H. s. n.* are most abundant in the prenatal brain (Figure 1). In contrast, no development stage-dependent expression was found for mRNAs targeted by miRNAs matched to polymorphic variants in *H. s. s.* (Figure 2). Thus, we can anticipate the differential effect of genetic variations found in archaic hominines on brain function and development in Denisovans and Neanderthals. The effects are not likely to be due to bias in the number of mRNA targets. The number of targeted genes is similar between miRNAs bearing fixed mutations specific for *H. s. n.* and *H. s. d.* (Ensembl gene IDs): 1260 target genes for *H. s. n.* and 1547 for *H. s. d.* (excluding targets of hsa-mir-671-3p miRNA that possess specific variants common for *H. s. d.* and *H. s. n.*). In addition, the number of targeted genes is similar between *H. s. n.* and *H. s. d.* miRNAs matched to polymorphic variants in *H. s. s.* (Ensembl gene IDs): 4072
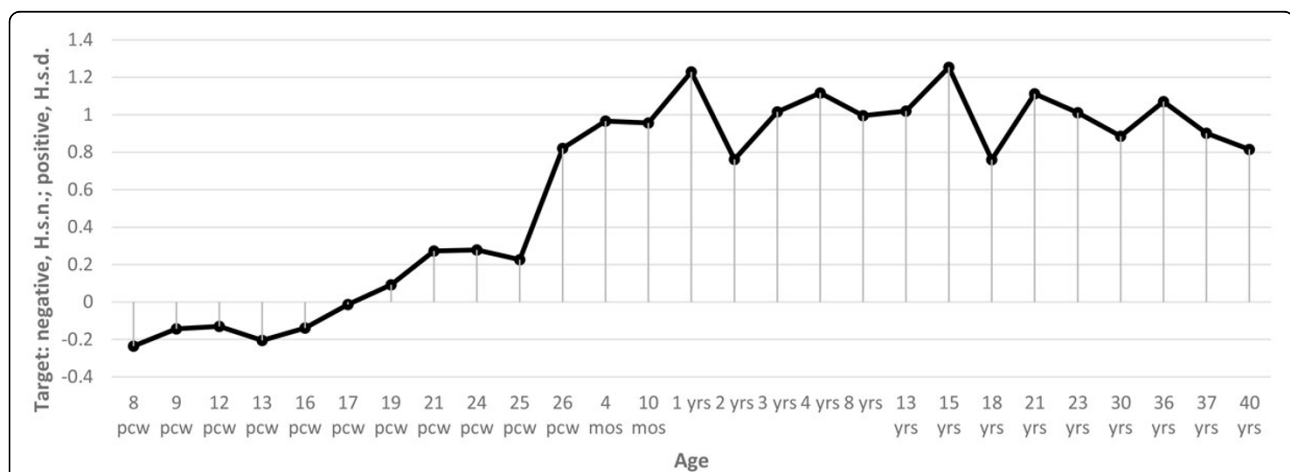

Figure 1 Target stages of human brain development for *H. s. d.* and *H. s. n.* miRNA pools expressed in neural tissues and having unique mutations. Y-axis, comparisons of Cohen's *d* values of the regulatory potential for selected *H. s. d.* (positive values) and *H. s. n.* (negative values) miRNA pools; X- axis, age: pcw - post conception weeks, m - postnatal months, y - postnatal years.
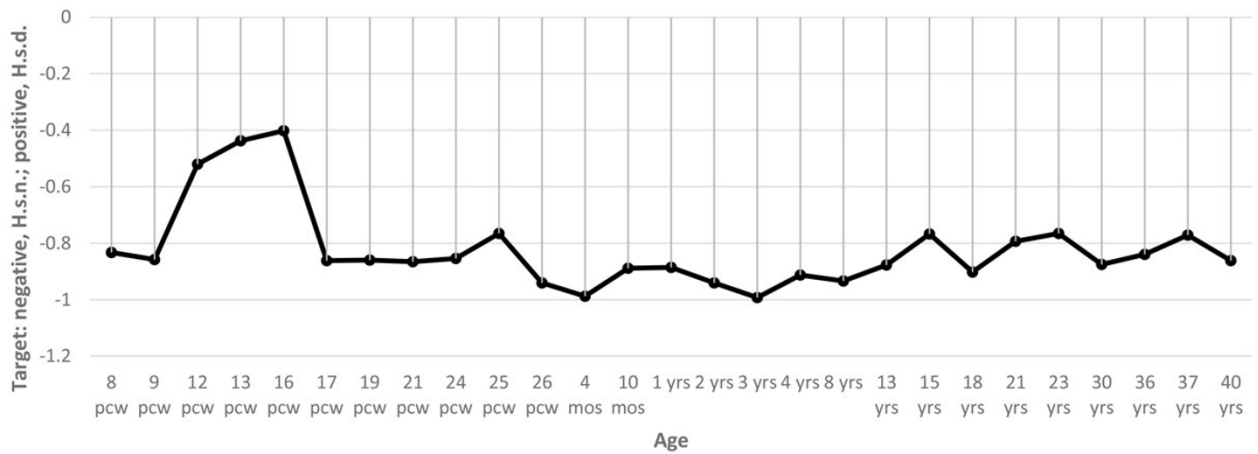
Figure 2 Target stages of human brain development for *H. s. d.* and *H. s. n.* miRNA pools expressed in neural tissues and having mutations shared with known human polymorphisms. Y-axis, comparisons of Cohen's *d* values of the regulatory potential for selected *H. s. d.* (positive values) and *H. s. n.* (negative values) miRNA pools; X-axis, age: pcw - post conception weeks, m - postnatal months, y - postnatal years.

target genes for *H. s. n.* and 3653 for *H. s. d.* It is interesting that the highest expression of transcripts targeted by miRNAs with polymorphisms unique for *H. s. d.* is confined to the broad thalamus regions and the prefrontal cortex (Figure 3). Nevertheless, there are multiple forebrain regions known to be important for speech that exhibit higher expression of miRNA targets from the set with polymorphisms unique for *H. s. d.* (Figure 3): the

inferiolateral temporal cortex, medial and ventrolateral prefrontal cortex. It is of interest that the thalamus plays a significant role in the shaping of the human language-ready brain [21].

In contrast, when we consider miRNAs of ancient humans expressed in the central nervous system and having variants shared with known human polymorphisms, the picture changes drastically (Figure 4): all brain
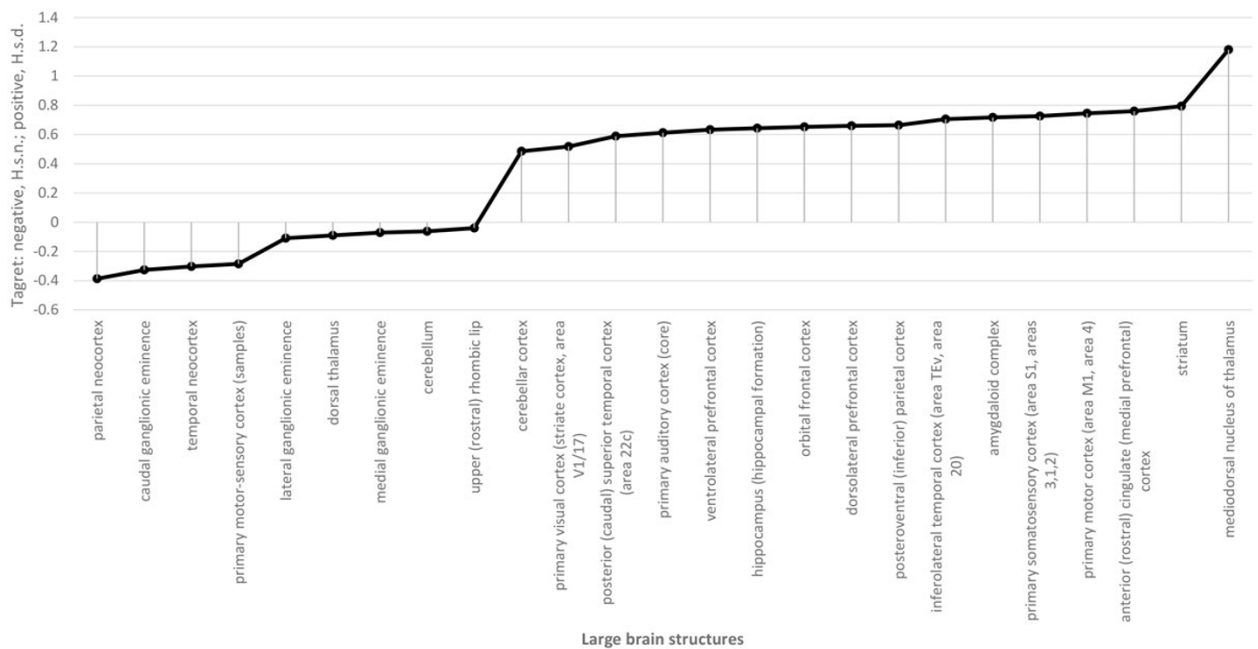

Figure 3 Target structures of human brain for tested and control *H. s. d.* and *H. s. n.* miRNA pools expressed in neural tissues and having unique mutations. Y-axis, comparisons of Cohen's *d* values of the regulatory potential for selected *H. s. d.* (positive values) and *H. s. n.* (negative values) miRNA pools; X- axis, large brain structures.

Figure 4 Target structures of human brain for tested and control *H. s. d.* and *H. s. n.* miRNA pools expressed in neural tissues and having mutations shared with known human polymorphisms. *Y*-axis, comparisons of Cohen's *d* values of the regulatory potential for selected *H. s. d.* (positive values) and *H. s. n.* (negative values) miRNA pools; *X*- axis, large brain structures.
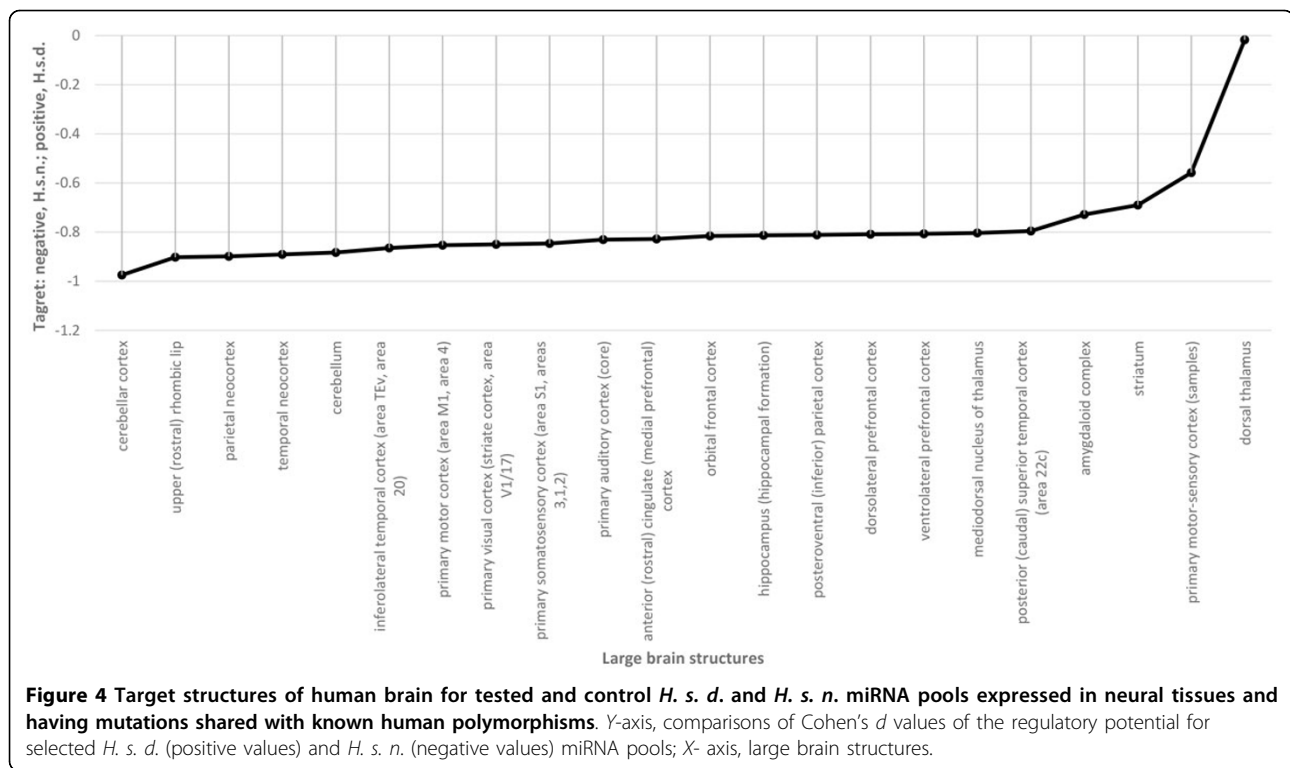
structures are targeted more by polymorphic *H. s. n.* miRNAs than by polymorphic *H. s. d.* miRNAs. The larger signal in *H. s. n.*, shown in Figures 2 and 4, is likely due to a higher number of miRNAs expressed in the central nervous system and sharing variation with the human population for *H. s. n.* (18, see Table 2) than for *H. s. d.* (14, see Table 1). Considering common miRNAs sharing human polymorphisms in these organisms (10 miRNAs), the specific numbers are 4 and 8, respectively (351 *H. s. d.*-specific targets versus 972 *H. s. n.*-specific targets (Ensembl gene IDs)). Given that the largest number of gene targets was found for the polymorphic hsa-mir-149 (5p) miRNA (Table 3) in *H. s. n.*, this difference between the two hominine subspecies can be attributed to this particular miRNA.

The fine target structures identified using AHBA data for gene expression in the left hemisphere of the brain [18] are presented in Tables 4 and 5. For identification of the fine target brain structures, we used the difference in regulatory potential between the *H. s. d.* and *H. s. n.* samples (Table 4, 5) as a marker (see Methods section). Importantly (Table 4), the difference between the target structures of unique *H. s. d.* and *H. s. n.* miR-NAs expressed in the central nervous system tissues is not significant (effect size measured as the absolute value of difference between Cohen's *d* statistics<0.5) for all fine brain structures except two: the fusiform gyrus for *H. s. d.* miRNAs and the precentral gyrus for *H. s. n.*

miRNAs. The picture obtained based on the target mRNAs for miRNAs expressed in the central nervous system tissues with known human polymorphisms (Table 4) is the same (compare Table 4 and 5): for the vast majority of fine brain structures, the difference between targeting by *H. s. d.* and *H. s. n.* is not significant, except for two thalamus structures and the lateral orbital gyrus, targeted by *H. s. d.* miRNAs. It is important that the fusiform and lateral orbital gyri are responsible for face perception and socialization, respectively [22,23]. Taken together, these findings indicate an important role of unique *H. s. d.* miRNAs in the development of specific brain structures in Denisovans, which likely enabled them to reach an unparalleled level of craftsmanship [1].

In addition to the identification of target brain structures for *H. s. d.* and *H. s. n.* miRNA activity, we found highly enriched functional categories of target gene annotations. We used the DAVID 6.7 functional annotation system [24] and the SP_PIR_KEYWORDS data class, with genes expressed in the human brain as background (16767 genes from BrainSpan). The results of the functional enrichment test are shown in Table 6. The most important difference between the target genes for unique *H. s. d.* and *H. s. n.* miRNAs expressed in the central nervous system tissues is the transport category enriched in target genes of *H. s. d.* miRNAs. Similar observations can be made considering miRNAs

Table 3 Number of target genes for each miRNA shown in Tables 1 and 2, based on MiRGator 3.0 data [13].

miRNA	Number of target genes (Ensembl gene IDs)*
hsa-mir-1178	701
hsa-mir-1208	878
hsa-mir-1252	1228
hsa-mir-1269a	639
hsa-mir-1269b	43
hsa-mir-1321	1159
hsa-mir-1343	128
hsa-mir-146a	140
hsa-mir-149 (3p)	181
hsa-mir-149 (5p)	495
hsa-mir-1908	25
hsa-mir-1909	33
hsa-mir-2682 (3p)	15
hsa-mir-3124 (3p)	151
hsa-mir-3129 (5p)	101
hsa-mir-3143	121
hsa-mir-3152 (5p)	33
hsa-mir-3161	62
hsa-mir-3185	102
hsa-mir-378e	49
hsa-mir-3938	65
hsa-mir-3939	1
hsa-mir-4274	42
hsa-mir-4293	0
hsa-mir-4463	40
hsa-mir-4478	76
hsa-mir-4514	134
hsa-mir-4532	10
hsa-mir-4700 (3p)	76
hsa-mir-4710	52
hsa-mir-4718	42
hsa-mir-4719	73
hsa-mir-4749 (3p)	32
hsa-mir-4804 (5p)	1
hsa-mir-5189	0
hsa-mir-532 (3p)	598
hsa-mir-532 (5p)	454
hsa-mir-5687	0
hsa-mir-608	1039
hsa-mir-6085	-**
hsa-mir-609	538
hsa-mir-615	200
hsa-mir-639	625
hsa-mir-662	256
hsa-mir-671 (3p)	168
hsa-mir-6715b (3p)	-**

Table 3 Number of target genes for each miRNA shown in Tables 1 and 2, based on MiRGator 3.0 data [13]. (Continued)

hsa-mir-943	512

* genes with Pearson correlation coefficient of co-expression ≤-0.9.

** these miRNAs were not annotated in mirGator 3.0

expressed in central nervous system tissues with substitutions shared with known human polymorphisms: "functional response" categories (such as activator, transcription regulation, synapse) enriched in target genes of *H. s. d.* and the developmental category enriched in target genes of such *H. s. n.* miRNAs. It would be reasonable to speculate that that the evolutionary changes in miRNA-transregulators might contribute to alterations in functional activities in specific brain regions in Denisovans, whereas in Neanderthals, the mutations in miRNAs could promote alterations in brain development and structure.

Conclusions

In this work, we identified miRNA genes of archaic humans bearing sequence variations in comparison with the modern human genome sequence: 29 genes for *H. s. d.* and 31 genes for *H. s. n.* Almost one-third of those genes contain variations specific for archaic humans (11 genes for *H. s. d.* and 9 for *H. s. n.*). The analysis of human gene expression data resulted in 9 *H. s. d.* and 5 *H. s. n.* genes with specific archaic variations and the expression of their human orthologs in the central nervous system.

The detailed analysis of the human brain gene expression data demonstrated that the brain regions with the most abundant expression of mRNAs targeted by the *H. s. d.* miRNAs with fixed mutations are confined to the thalamus and the prefrontal cortex (especially the fusiform gyrus), no large brain regions are targeted by *H. s. n.* miRNAs with specific mutations. The only small brain regions targeted by *H. s. n.* miRNAs with such mutations are the superior lateral aspect and the central sulcus of the precentral gyrus.

We identified differences in gene expression pattern during human brain development for the targets of human orthologs for the selected miRNAs. Targets for miRNA genes with mutations specific for *H. s. d.* were expressed predominantly in the later prenatal and early postnatal development stages. Targets for miRNAs with mutations specific for *H. s. n.* were expressed predominantly in early prenatal brain development stages. These results may reflect a potential association between the changes in the Denisovan miRNA genes reported in this study and the brain development of *H. s. d.*, which likely allowed them to reach their high level of craftsmanship.

Table 4 Results of the identification of brain structures of the left hemisphere characterized by the difference in regulatory potential for *H.s. d.* and *H. s. n.* miRNAs expressed in neural tissues and having unique substitutions.

AHBA Struct. ID *	Structure description. ID **	p, H. s. d.	p, H. s. n.	Cohen's d, H. s. d.	Cohen's d, H. s. n.	(Cohen's d, H. s. d.) - (Cohen's d, H. s. n.)
4158	fusiform g., b. of the its	0.00226	0.00662	5.94882	5.26565	0.68317
4273	short insular gyri	0.0041	0.0062	5.65202	5.40279	0.24923
4045	inferior frontal g., orbital pt.	0.00676	0.00884	5.37587	5.16541	0.21046
4023	superior frontal g., medial b.	0.00552	0.00626	5.46649	5.30947	0.15702
4090	postcentral g., b. of the posterior central sulcus	0.00764	0.00988	5.21272	5.07402	0.1387
4120	precuneus, superior lateral b.	0.00702	0.00788	5.30175	5.16909	0.13266
4060	lateral orbital g.	0.00804	0.00972	5.19963	5.07262	0.12701
4143	middle temporal g., inferior b.	0.00816	0.00936	5.1509	5.04074	0.11016
4088	postcentral g., superior lateral aspect	0.00856	0.00978	5.15144	5.04957	0.10187
4039	inferior frontal g., triangular pt.	0.00786	0.00906	5.20687	5.11107	0.0958
4215	superior occipital g., inferior b.	0.0076	0.00796	5.23815	5.16956	0.06859
4251	subiculum	0.00644	0.0066	5.35415	5.28977	0.06438
4074	paracentral lobule, anterior pt., inferior b.	0.00912	0.0091	5.15364	5.09767	0.05597
4223	cingulate g., frontal pt., superior b.	0.00404	0.0042	5.62605	5.58632	0.03973
4288	putamen	0.00728	0.00834	5.23485	5.19766	0.03719
4160	fusiform g., b. of cos	0.00712	0.00692	5.30752	5.27444	0.03308
4245	parahippocampal g., b. of cos	0.00762	0.00692	5.24375	5.22006	0.02369
4014	precentral g., inferior lateral aspect	0.0085	0.00936	5.11266	5.09452	0.01814
4136	superior temporal g., inferior b.	0.00764	0.00728	5.20265	5.23345	-0.0308
4051	medial orbital g.	0.0067	0.00554	5.35583	5.43806	-0.08223
4224	cingulate g., frontal pt., inferior b.	0.00664	0.00608	5.26935	5.35976	-0.09041
4087	postcentral g., b. of the central sulcus	0.0096	0.00756	5.12513	5.23082	-0.10569
4098	supraparietal lobule, superior b.	0.00742	0.00572	5.23545	5.3476	-0.11215
4151	inferior temporal g., b. of mts	0.00888	0.00732	5.11307	5.22869	-0.11562
4030	middle frontal g., superior b.	0.00984	0.00766	5.08151	5.20719	-0.12568
4201	occipito-temporal g., inferior b.	0.00948	0.00782	5.05788	5.20134	-0.14346
4031	middle frontal g., inferior b.	0.00548	0.00426	5.39941	5.54788	-0.14847
4024	superior frontal g., lateral b.	0.00308	0.00302	5.72827	5.89251	-0.16424
4142	middle temporal g., superior b.	0.00948	0.00736	5.08104	5.25797	-0.17693
4150	inferior temporal g., lateral b.	0.00704	0.0054	5.30094	5.50188	-0.20094
4149	inferior temporal g., b. of the its.	0.0095	0.00712	5.09672	5.32253	-0.22581
4159	fusiform g., lateral b.	0.00944	0.00742	5.03024	5.25885	-0.22861
4214	superior occipital g., superior b.	0.00612	0.0047	5.31198	5.55676	-0.24478
4258	dentate g.	0.0073	0.00506	5.29347	5.56439	-0.27092
4193	lingual g., peristriate	0.00976	0.00616	5.08997	5.4184	-0.32843
4013	precentral g., superior lateral aspect	0.00968	0.00596	5.05046	5.38028	-0.32982
4015	precentral g., b. of the central sulcus	0.00898	0.00342	5.10895	5.70299	-0.59404

* - For each target brain structure, the following data are provided: (1) the AHBA ID and (2) its description; (3) and (4) *p*, the probability of observing $L_{krand} \geq L_{krand}^{Q}$ by random chance using unique *H. s. d.* and *H. s. n.* miRNAs expressed in central nervous system tissues; (5) and (6) the Cohen's *d* statistic of difference between L_{krand} and L_{krand}^{Q} value distributions for unique *H. s. d.* and *H. s. n.* miRNAs expressed in the central nervous system tissues; (7) difference between these Cohen's *d* statistics.

** - g., gyrus; b., bank; pt., part.

Table 5 Results of the identification of brain structures of the left hemisphere characterized by the difference between regulatory potential for *H.s. d.* and *H. s. n.* miRNAs expressed in neural tissues and having substitutions shared with known human polymorphisms.

AHBA Struct. ID *	Structure description. ID **	p, H. s. d.	p, H. s. n.	Cohen's d, H. s. d.	Cohen's d, H. s. n.	(Cohen's d, H. s. d.) - (Cohen's d, H. s. n.)
4060	lateral orbital g.	0.00194	0.00816	5.92943	5.18602	0.74341
4288	putamen	0.0018	0.00634	6.03981	5.29693	0.74288
4258	dentate g.	0.00156	0.0045	6.18038	5.55627	0.62411
4901	inferior rostral g.	0.0045	0.0084	5.59337	5.12814	0.46523
4193	lingual g., peristriate	0.00268	0.00618	5.78555	5.32347	0.46208
4187	cuneus, striate	0.00474	0.00956	5.50591	5.04395	0.46196
4151	inferior temporal g., b. of mts	0.0042	0.00632	5.60432	5.29836	0.30596
4194	lingual g., striate	0.00714	0.00878	5.28597	5.12021	0.16576
4160	fusiform g., b. of cos	0.00622	0.00794	5.32299	5.17916	0.14383
4417	lateral group of nuclei, ventral division	0.00708	0.00514	5.27528	5.39697	-0.12169
4256	CA3 field	0.00958	0.00798	5.06621	5.22156	-0.15535
4169	transverse gyri	0.00936	0.00686	5.12785	5.29476	-0.16691
4282	body of caudate nucleus	0.00672	0.00534	5.25194	5.42113	-0.16919
4012	precentral g., b. of the precentral sulcus	0.0064	0.00446	5.29517	5.51095	-0.21578
4244	parahippocampal g., lateral b.	0.00688	0.00454	5.31264	5.53652	-0.22388
4186	cuneus, peristriate	0.00386	0.00274	5.64365	5.88129	-0.23764
4150	inferior temporal g., lateral b.	0.00776	0.00522	5.21501	5.45297	-0.23796
4106	supramarginal g., superior b.	0.00518	0.00342	5.46435	5.70277	-0.23842
4088	postcentral g., superior lateral aspect	0.0024	0.0015	5.93668	6.17562	-0.23894
4121	precuneus, inferior lateral b.	0.004	0.0023	5.65682	5.89645	-0.23963
4166	Heschl's g.	0.00858	0.00516	5.19857	5.44275	-0.24418
4013	precentral g., superior lateral aspect	0.00452	0.00298	5.55536	5.80945	-0.25409
4015	precentral g., b. of the central sulcus	0.00814	0.00564	5.16805	5.42259	-0.25454
4120	precuneus, superior lateral b.	0.00246	0.00182	5.89955	6.15638	-0.25683
4245	parahippocampal g., b. of the cos	0.00672	0.00426	5.31989	5.58364	-0.26375
4223	cingulate g., frontal pt., superior b.	0.0052	0.00352	5.41984	5.68604	-0.2662
4048	g. rectus	0.00998	0.00646	5.01861	5.28996	-0.27135
4074	paracentral lobule, anterior pt., inferior b.	0.0042	0.00242	5.61742	5.89182	-0.2744
4113	angular g., superior b.	0.00912	0.00522	5.10417	5.38524	-0.28107
4280	head of caudate nucleus	0.00482	0.00302	5.49717	5.78461	-0.28744
4114	angular g., inferior b.	0.00686	0.00394	5.33423	5.6244	-0.29017
4087	postcentral g., b. of the central sulcus	0.00414	0.00222	5.64523	5.93594	-0.29071
4030	middle frontal g., superior b.	0.0032	0.00166	5.79657	6.08899	-0.29242
4270	long insular gyri	0.0097	0.00618	5.07045	5.36942	-0.29897
4014	precentral g., inferior lateral aspect	0.00318	0.00174	5.80203	6.10501	-0.30298
4200	occipito-temporal g., superior b.	0.00542	0.00314	5.44127	5.74428	-0.30301
4255	CA2 field	0.00338	0.0016	5.78178	6.08596	-0.30418
4273	short insular gyri	0.00502	0.0029	5.47924	5.79333	-0.31409
4099	supraparietal lobule, inferior b.	0.00482	0.00234	5.56715	5.88285	-0.3157
4045	inferior frontal g., orbital pt.	0.00272	0.00138	5.81445	6.1365	-0.32205
4039	Inferior frontal g., triangular pt.	0.00808	0.00528	5.16319	5.49488	-0.33169
4230	cingulate g., parietal pt., superior b.	0.00918	0.00546	5.13338	5.46665	-0.33327
4159	fusiform g., lateral b.	0.00336	0.00222	5.69632	6.03013	-0.33381
4023	superior frontal g., medial b.	0.00146	0.0007	6.19956	6.53647	-0.33691

Table 5 Results of the identification of brain structures of the left hemisphere characterized by the difference between regulatory potential for *H.s. d.* and *H. s. n.* miRNAs expressed in neural tissues and having substitutions shared with known human polymorphisms. *(Continued)*

4136	superior temporal g., inferior b.	0.00402	0.0018	5.66885	6.0067	-0.33785
4031	middle frontal g., inferior b.	0.00106	0.00058	6.29966	6.63933	-0.33967
4208	inferior occipital g., inferior b.	0.0017	0.00084	6.10349	6.44887	-0.34538
4251	subiculum	0.00486	0.00248	5.51224	5.8579	-0.34566
4073	paracentral lobule, anterior pt., superior b.	0.00522	0.0031	5.46017	5.81365	-0.35348
4079	frontal operculum	0.00502	0.0028	5.49085	5.84766	-0.35681
4149	inferior temporal g., b. of the its	0.00308	0.00176	5.82221	6.1795	-0.35729
4257	CA4 field	0.00612	0.00322	5.37575	5.73712	-0.36137
4215	superior occipital g., inferior b.	0.00876	0.00438	5.16436	5.5294	-0.36504
4063	subcallosal g.	0.00762	0.00424	5.24311	5.61246	-0.36935
4214	superior occipital g., superior b.	0.0035	0.00216	5.64522	6.01907	-0.37385
4143	middle temporal g., inferior b.	0.00444	0.00228	5.57522	5.95362	-0.3784
4207	inferior occipital g., superior b.	0.00632	0.00364	5.26928	5.65709	-0.38781
4051	medial orbital g.	0.00214	0.00096	5.9903	6.39314	-0.40284
4898	superior rostral g.	0.00612	0.00272	5.39109	5.80014	-0.40905
4098	supraparietal lobule, superior b.	0.00442	0.00198	5.63525	6.04788	-0.41263
4089	postcentral g., inferior lateral aspect	0.00762	0.00398	5.25268	5.67386	-0.42118
4107	supramarginal g., inferior b.	0.00668	0.00342	5.34353	5.7746	-0.43107
4178	planum polare	0.0065	0.00232	5.33903	5.77072	-0.43169
4224	cingulate g., frontal pt., inferior b.	0.00554	0.00254	5.42489	5.86867	-0.44378
4090	postcentral g., b. of the posterior central sulcus	0.00486	0.00206	5.53829	5.99888	-0.46059
4024	superior frontal g., lateral b.	0.00074	0.00022	6.59581	7.06305	-0.46724
4158	fusiform g., b. of the its	0.00142	0.00038	6.22982	6.69803	-0.46821
4201	occipito-temporal g., inferior b.	0.00284	0.00124	5.76999	6.2488	-0.47881
4142	middle temporal g., superior b.	0.00698	0.00336	5.26624	5.76073	-0.49449

* - For each target brain structure, the following data are provided: (1) the AHBA ID and (2) its description; (3) and (4) *p*, the probability of observing $L_{krand} \geq L_{krand}^{Q}$ by random chance using *H. s. d.* and *H. s. n.* miRNA with known human polymorphisms expressed in central nervous system tissues; (5) and (6) the Cohen's *d* statistic of the difference between the L_{krand} and L_{krand}^{Q} value distributions for *H. s. d.* and *H. s. n.* miRNAs with known human polymorphisms expressed in central nervous system tissues; (7) the difference between these Cohen's *d* statistics.

** - g., gyrus; b., bank; pt., part.

Methods

Selection of miRNA genes in *H. s. d.* and *H. s. n.* with no match in *H. s. s.*

We used data on the chromosomal localization of 1595 experimentally confirmed *H. s. s.* pre-miRNAs in the miRBase database rel. 19 [11] and selected the best-sequenced *H. s. d.* and *H. s. n.* pre-miRNAs orthologous to *H. s. s.* pre-miRNA genes. The *H. s. d.* and *H. s. n.* genomes appear as short nucleotide sequences (reads) mapped onto the human genome [3]. To ensure that only the best-sequenced *H. s. d.* and *H. s. n.* pre-miR-NAs would remain, we used filtering methods.

(I) The consensus sequences of *H. s. d.* and *H. s. n.* pre-miRNAs were combined from reads with a quality of mapping onto *H. s. s.* pre-miRNA genes not less than 15 [3].

(II) The consensus sequences of *H. s. d.* and *H. s. n.* pre-miRNAs included only those read nucleotides (a) for which the Phred sequence quality [25] was not less than 30 [2] and (b) that were located in the middle part of the reads: the first and last three read nucleotides were discarded according to [3].

(III) Any position in the consensus with coverage of less than 5 was assumed to be undetermined [3].

(IV) The consensus sequences of *H. s. d.* and *H. s. n.* pre-miRNAs with undetermined positions were discarded.

(V) The consensus sequences of *H. s. d.* and *H. s. n.* pre-miRNAs that (a) had nucleotide substitutions that *H. s. s.* lacked and (b) were combined from reads mapped onto the human genome with a quality of less than 30 were discarded [2,3].

Table 6 Annotation enrichment of genes targeted by *H. s. d.* and *H. s. n.* miRNAs expressed in neural tissues, based on DAVID 6.7 [24]; BrainSpan gene list (16767 genes expressed in human brain) used as a background.

SP_PIR_KEYWORDS category *	# of genes	Corrected *p*-value	
		Bonferroni	Benjamini
Target genes of unique *H. s. d.* miRNAs			
transport	176	0.04072	0.04072
Target genes of unique *H. s. n.* miRNAs			
-			
Target genes of *H. s. d.* miRNAs having substitutions shared with known human polymorphisms			
phosphoprotein	1018	1.56E-22	1.56E-22
alternative splicing	1013	4.70E-19	2.35E-19
transcription	298	3.72E-06	1.24E-06
transcription regulation	292	3.86E-06	9.64E-07
metal-binding	403	2.56E-05	5.13E-06
chromosomal rearrangement	63	7.14E-05	1.19E-05
zinc	302	8.79E-05	1.26E-05
activator	98	2.27E-04	2.84E-05
cell junction	76	0.00179	1.99E-04
zinc-finger	231	0.00455	4.56E-04
synapse	45	0.01100	0.00100
DNA-binding	246	0.02287	0.00193
Target genes of *H. s. n.* miRNAs having substitutions shared with known human polymorphisms			
phosphoprotein	565	3.62E-04	3.62E-04
alternative splicing	574	0.00226	0.00113
developmental protein	84	0.00665	0.00222
cytoplasm	279	0.00715	0.00179

* bold - common between target genes of *H. s. n.* and *H. s. d.* miRNAs having substitutions shared with known human polymorphisms.

(VI) If there were two or more polymorphic states at a consensus position in *H. s. d.* and *H. s. n.* pre-miRNAs, only those alternative states that (a) had comparable frequencies (the difference between the frequencies would not exceed 1.3) and (b) passed filtering stages I-V were considered.

(VII) Additionally, we manually analyzed all mapped reads of selected *H. s. d.* and *H. s. n.* pre-miRNAs for the presence of PCR artifacts. We did not find any PCR artifacts in the positions of selected *H. s. d.* and *H. s. n.* pre-miRNAs.

To select pre-miRNA coding genes that were present in the genome of the common ancestor of *H. s. s.*, *H. s. d.* and *H. s. n.* we analyzed the multiple genome alignment of six primates from Ensembl rel. 69 [12]. The minimum length of any ortholog (excluding unreadable and polymorphic nucleotides) was considered to be not less than 70% of the length of the corresponding *H. s. s.* pre-miRNA.

We mapped all the nucleotide substitutions found by comparing the pre-miRNA orthologs in the *H. s. s.*/*H. s. d.* and *H. s. s.*/*H. s. n.* pairs onto the secondary structures of the corresponding pre-miRNAs in *H. s. s.* contained in the miRBase database rel. 19 [11].

We selected *H. s. d.* and *H. s. n.* pre-miRNA genes with (i) nucleotide substitutions (deletions, insertions) in the regions corresponding to the sequences of mature miRNAs (and/or miRNAs*) or (ii) multiple substitutions within a region not larger than 1/20 of the pre-miRNA length. In the first case (i), the altered mature miRNAs are of special interest because they change the pattern of complementary interactions between miRNAs and their target mRNAs in *H. s. d.* or *H. s. n.* and make it different from *H. s. s.* In the second case (ii), the probability of observing multiple changes within a small region of *H. s. d.* or *H. s. n.* pre-miRNA by random chance is extremely low. For instance, the estimate of the total number of differences between *H. s. d.* and *H. s. s.* is approximately 1,650,000 [3]. Under the assumption of a uniform distribution of mutation fixation events in a 6400 bp long sequence (1/20 of the length of 1600 pre-miRNA genes, each gene being approximately 80 bp in length), the estimate of the probability of two mutations being fixed simultaneously is less than 0.002.

To ensure that the mutations in our selection of miR-NAs were not DNA sequencing errors, we analyzed the frequencies of all polymorphic consensuses of *H. s. d.* and *H. s. n.* DNA (see steps V and VI of the filtering protocol), considering their tetranucleotide context. Thus, we used data in the UCSC Genome Browser phyloP46way to select evolutionarily conserved regions of the human genome (phyloP conservation score > 0.1; regions were ≥7 bp in length, which eventually totaled 454775413 positions or ~15% of the human genome). The occurrence of the polymorphic variants of the consensuses in the *H. s. d.* and *H. s. n.* genomes was then assessed considering their tetranucleotide context. We selected pre-miRNAs with mutations that either do not occur in the 454,775,413-strong selection of evolutionary conservative positions analyzed ($p \leq 2 \cdot 10^{-9}$) or are single occurrences ($p = 2 \cdot 10^{-9}$).

To ensure that the mutations in the set of selected miRNAs were not a result of PCR amplification in DNA sequencing (see step VII of the filtering protocol), we performed a manual analysis of *H. s. d.* and *H. s. n.* short reads alignment on a reference *H. s. s.* genome (hg19). We found no traces of the PCR amplification of short reads in miRNA genome positions: any single read in these positions started and stopped at unique genome locations.

Using the blastn program in BLAST 2.2.26+ [26] on the pre-miRNAs containing substitutions/polymorphisms in mature miRNAs at the last filtering stage, we discarded pre-miRNAs that occurred in more than one copy in the *H. s. s.* genome (E-value cut-off: $1 \cdot 10^{-6}$; blastn default settings).

Identification of genes targeted by H. s. s. miRNAs orthologous to H. s. d. and H. s. n. miRNAs

For each orthologous *H. s. s.* miRNA, we identified the genes encoding its target mRNAs. This identification was performed using miRGator 3.0 [13] (we used only those target mRNAs whose expression correlated with microRNA expression with $r < -0.9$) and the ChIP-seq data for H3K4me3 histones marking active promoters in primate cortical neurons [14]. We used the ID converter from bioDBnet [27] to convert the HUGO name list of target mRNA IDs (from miRGator 3.0 prediction) into a list of Ensembl gene IDs and a list of Refseq IDs.

For functional annotation of the mRNAs targeted by the selected miRNAs, we chose two independent sources of experimental data that contained the most complete quantitative information on mRNA expression in human central nervous system tissues. One of these sources was BrainSpan [19] (Exon microarray, 16767 genes), which integrates normalized quantitative data on gene expression provided by expression time series experiments in broad brain regions. The other source of data was the Allen Human Brain Atlas, as updated March 2013 [18] (20791 genes), which contains normalized quantitative data on the expression of mRNAs in tiny brain regions. The expression of these mRNAs was analyzed in six brains: H0351.1009, H0351.1012, H0351.1015, H0351.1016, H0351.2001 and H0351.2002. If the microarray contained more than one probe for measuring the expression of the same mRNA, then only the probe producing the maximum value that significantly differed from background values was taken for analysis. In addition, to improve the robustness of our results, we used only data that were consistent for at least 4 out of 6 brains.

Identification of tissues with increased levels of mRNA expression regulated by the pool of miRNAs

In our work, we are interested in the tissues and brain structures most likely affected by the pool of miRNAs with structural changes between ancient and modern humans. Unfortunately, the data on miRNA expression in the vast majority of human tissues and brain structures are currently incomplete both because the number of functional miRNAs is incomplete [30] and because of the number of functional miRNA/target-mRNA interactions under ongoing debates regarding the usage of various factors in miRNA/target-mRNA binding [31,32]. Therefore, we used an indirect approach to identify tissues subject to dynamical changes in miRNA expression. Because miRNAs are negative post-transcriptional regulators of gene expression, we suppose that changes in their expression levels will largely affect the tissues with high abundance of their mRNA targets. Based on this assumption, likely candidate tissues are the ones with high expression of the target mRNAs for the set of miRNAs under consideration. To express quantitatively the degree of post-transcriptional regulation by specific miRNAs in different tissues, we introduced the miRNAs' regulatory potential.

Let $W(k)$ denote the set of human mRNAs expressed in the k-th tissue and targeted by all known human miRNAs. It should be noted that the overwhelming majority of genes in the human genome (~20000) [33] can be targeted by various miRNAs [13,30,34]. Therefore, the set of all genes in the genome is a good approximation of the miRNA targetome. Thus, we approximated $W(k)$ as the set of all human mRNAs expressed in the k-th tissue. $W(k_Q)$ denotes the subset of target mRNAs regulated in the k-th tissue by the miRNA pool, Q, and $J(k_Q)$ denotes the size of this subset.

The regulatory potential, L_k^Q, exerted by the miRNA pool, Q, on mRNAs with expression in the k-th tissue was estimated using the following formula:

$$L_k^Q = \sum_{j \in W(k_Q)} E_j^k \tag{1}$$

Here, E_j^k is the level of expression of the j-th target mRNA in the subset $W(k_Q)$ in the k-th tissue, and E_j^k values were obtained using data from BrainSpan [18] or AHBA[19]. The L_k^Q value obtained in this manner corresponds to the number of mRNA molecules that can be regulated in the k-th tissue (or brain structure) by mature miRNAs in the pool Q and characterizes its post-transcriptional regulatory potential in that tissue.

In search of the tissues targeted by the miRNAs from the pool Q, we selected the ones with the highest regulatory potential L_k^Q in comparison with the background level of expression of all miRNA targets expressed in the k-th tissue, $W(k)$. To estimate the significance of the difference between the pool's Q regulatory potential and the background, we used a resampling test (see Figure 5).

First, we performed random sampling without replacement of $J(k_Q)/2$ mRNAs from the set $W(k_Q)$ of human mRNAs targeted by miRNAs in the pool Q (the $W(k_{Qrand})$ subset). For the $W(k_{Qrand})$ subset, we calculated L_{krand}^Q using Formula (1). The sampling procedure was repeated 10^5 times. We estimated the mean and standard deviation of the L_{krand}^Q values. The selection of half the number of mRNAs in the $W(k_Q)$ subset allowed us
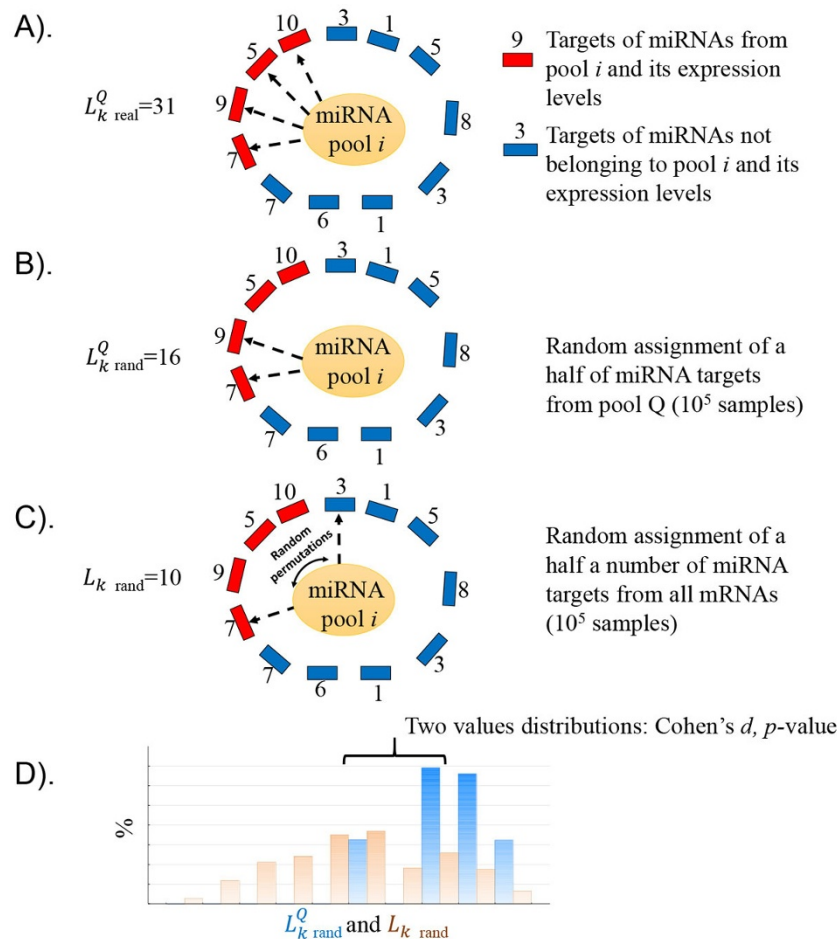
Figure 5 A toy example of the resampling test to identify tissues with increased levels of mRNAs expression regulated by the pool Q of the miRNAs in tissue k. A). Calculation of the regulatory potential for the miRNA pool Q, L_k^Q. B). Calculation of the regulatory potential L_{krand}^Q for the resampled miRNA pool Q in $W(k_{Qrand})$ subset. C). Calculation of the regulatory potential L_{krand} in the $W(k_{rand})$ subset. D). Distribution of L_{krand}^Q (blue) and L_{krand} (brown) values in 10^5 resampling experiments and their comparison by Cohen's d statistics.

to account for uncertainties in miRNA annotation (for example, the possibility that the miRNA dataset under consideration is incomplete and has incomplete annotation of target mRNAs).

Second, we performed sampling of mRNAs from the background distribution, using the $W(k)$ set of all miRNA targets expressed in the k-th tissue. For this purpose, we selected $J(k_Q)/2$ mRNAs from the $W(k)$ randomly without replacement. We termed this subset $W(k_{rand})$. We calculated

$$L_{krand} = \sum_{j \in W(k_{rand})} E_j^k, \qquad (2)$$

as the estimate of the background miRNA regulatory potential; here, E_j^k is the expression level of the j-th target mRNA in the subset $W(k_{rand})$. This procedure was repeated 10^5 times and allowed us to estimate the mean and standard deviation of L_{krand}.

We calculated the difference between the L_{krand} and L_{krand}^Q value distributions using Cohen's d statistic (effect size value) [35,36]. The p-value of $L_{krand} \geq L_{krand}^Q$ was calculated using Student's t-statistic. The difference between the L_{krand} and L_{krand}^Q value distributions was considered significant at p-value less or equal to 0.01, implying that the k-th tissue was targeted by the miRNAs from the pool Q. It is important to note that the data resampling approach described above does not rely on distributional assumptions and simultaneously allowed us to control Type I error, and corrects for any hidden data correlation [37-39]. Therefore, we did not need to perform multiple testing corrections (e.g., Bonferroni correction) of the significance level [36-39] and therefore could use the selected p-value threshold directly.

Competing interests
The authors have declared that no competing interests exist.

Authors' contributions
K.V.G., A.D.A. and E.I.R. conceived the project. K.V.G. performed all data analysis. N.A.K., E.I.R. and A.P.D. coordinated the project. All authors contributed to the final manuscript preparation, discussed the results and their implications, and have read and approved the final manuscript.

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