Supplementary Information 2

Functional and molecular enrichment analysis

Functional analysis

There are 249 probes showing suggestive evidence of association (P<1E-05) annotated to 182 unique genes.

Several GO-terms and KEGG pathways were nominally enriched among ADHD-suggestive CpGs. Three of them are of interest: The nicotinate and nicotinamide metabolism pathway (KEGG) (P-value=0.009, FDR=0.899), the riboflavin metabolism pathway (KEGG) (P-value=0.021, FDR=0.896) and the retrograde endocannabinoid signaling pathway (KEGG) (P-value=0.022, FDR=0.896).

Nicotinate (niacin) and nicotinamide are precursors of the coenzymes nicotinamide-adenine dinucleotide (NAD+) and nicotinamide-adenine dinucleotide phosphate (NADP+). These coenzymes, NAD+ and NADP+, are crucial for many metabolic pathways including glycolysis, TCA cycle, pentose phosphate cycle, fatty acid biosynthesis. When NAD+ and NADP+ are interchanged in a reaction with their reduced forms, NADH and NADPH respectively, they are important cofactors in several hundred redox reactions (Magni et al. 2004). Mechanisms related to fatty acid oxidation have been previously related to ADHD (Walton et al. 2017; Wilmot et al. 2016).

Riboflavin (vitamin B2, E101) is an essential component for the cofactors FAD (flavin-adenine dinucleotide) and FMN (flavin mononucleotide). Together with NAD+ and NADP+, FAD and FMN are important hydrogen carriers and take part in more than 100 redox reactions involved in energy metabolism (Rivlin 1970). Low levels of vitamin B2 were associated with ADHD diagnosis in adults (Landaas et al. 2016).

Endocannabinoids modulate synaptic function. By activating cannabinoid receptors expressed in the central nervous system, these lipid messengers can regulate several neural functions and behaviors. As experimental tools advance, the repertoire of known endocannabinoid-mediated effects at the synapse, and their underlying mechanism, continues to expand. Retrograde signaling is the principal mode by which endocannabinoids mediate short- and long-term forms of plasticity at both excitatory and inhibitory synapses (Castillo et al. 2012).

GO collection: Seven-hundred fifteen pathways were nominally associated with suggestive probes but none remained significant following FDR-correction.

	Term	Ont	N	DE	P.DE	FDR
GO:0010256	endomembrane system organization	BP	413	14	5,32E-05	0,63202644
GO:0030953	astral microtubule organization	BP	7	3	5,67E-05	0,63202644
GO:0001968	fibronectin binding	MF	26	4	9,65E-05	0,68993315
GO:0006999	nuclear pore organization	BP	15	3	0,000258202	0,68993315
GO:0018995	host	CC	68	5	0,000299251	0,68993315
GO:0043657	host cell	CC	68	5	0,000299251	0,68993315
GO:0022411	cellular component disassembly	BP	549	15	0,000335307	0,68993315
GO:0044446	intracellular organelle part	CC	8568	105	0,000367984	0,68993315
GO:0044422	organelle part	CC	8793	107	0,000408591	0,68993315
GO:0044215	other organism	CC	74	5	0,000409846	0,68993315

KEGG collection: Seven pathways were nominally associated with suggestive probes but none remained significant following FDR-correction.

	Pathway	**N**	**DE**	**P.DE**	**FDR**
path:hsa00770	Pantothenate and CoA biosynthesis	19	6	0,00678533	0,89591107
path:hsa00760	Nicotinate and nicotinamide metabolism	29	7	0,008778	0,89591107
path:hsa04621	NOD-like receptor signaling pathway	153	21	0,00987479	0,89591107
path:hsa04130	SNARE interactions in vesicular transport	33	7	0,01436319	0,89591107
path:hsa00740	Riboflavin metabolism	8	3	0,02099477	0,89591107
path:hsa04723	Retrograde endocannabinoid signaling	133	21	0,02190592	0,89591107
path:hsa05161	Hepatitis B	131	21	0,02246899	0,89591107
path:hsa05206	MicroRNAs in cancer	279	34	0,02372962	0,89591107
path:hsa04115	p53 signaling pathway	72	13	0,02443394	0,89591107
path:hsa00780	Biotin metabolism	3	2	0,02730377	0,90102448

Molecular enrichment analysis

Summary

ADHD-FDR CpGs were enriched for 3'UTR and body regions and depleted for Tss1500, Tss200 and first exon regions. They were also enriched in open sea positions and N- and S-shelfs, and depleted in N- and S-shores and CpG islands. The same pattern was observed for hypomethylated FDR CpGs, and the inverse for hypermethylated FDR CpGs. Regarding blood 15-chormatine states, FDR hypomethylated CpGs showed enrichment for transcription (TxWk, Tx), enhancers (EnhG, Enh), and ZNF genes and repeats and quiescent positions, while depletion for transcription start sites (TssA, TssAFlnk, TxFlnk), bivalent (TssBiv, BivFlnk, EnhBiv) and repressor (ReprPC) positions. The pattern for hypermehylated FDR CpGs was inverted. Overall, this indicates that ADHD-associated hypomethylation tends to happen in enhancers, transcribed regions, and quiescent regions, while hypermethylation tends to happen in transcription start sites and bivalent state regions.
Similar enrichment/depletion patters were observed with suggestive CpGs.
See below the detailed findings from the molecular enrichment analysis.

Genic vs. intergenic probes

Suggestive CpGs, specially the hypomethylated, were enriched in intergenic regions.

SUGGESTIVE	Genic (N=355420)	Intergenic (N=117397)
Sig SUGG (N=249)	170 (0.05%)	79 (0.075%)
No sig SUGG (N=472568)	355250 (99.95%)	117318 (99.93%)

X-squared = 5.9862, df = 1, p-value = 0.01442
Odds Ratio: 0.8904

	Genic (N=355420)	Intergenic (N=117397)
Hyper SUGG (N=39)	28	11
Rest (N=472778)	355392	117386

X-squared = 0.091614, df = 1, p-value = 0.7621
Odds Ratio: 1.1893

	Genic (N=355420)	Intergenic (N=117397)
Hypo SUGG (N=210)	142	68
Rest (N= 472607)	355278	117329

X-squared = 6.0209, df = 1, **p-value = 0.01414**
Odds Ratio: 0.6896305

Relative gene position

Suggestive CpGs were enriched for 3'UTR regions and depleted for TSS200 and first exon regions. The same is observed for hypomethylated.

	Suggestive	
	Yes	**No**
Hyper	39	239939
Hypo	210	232629

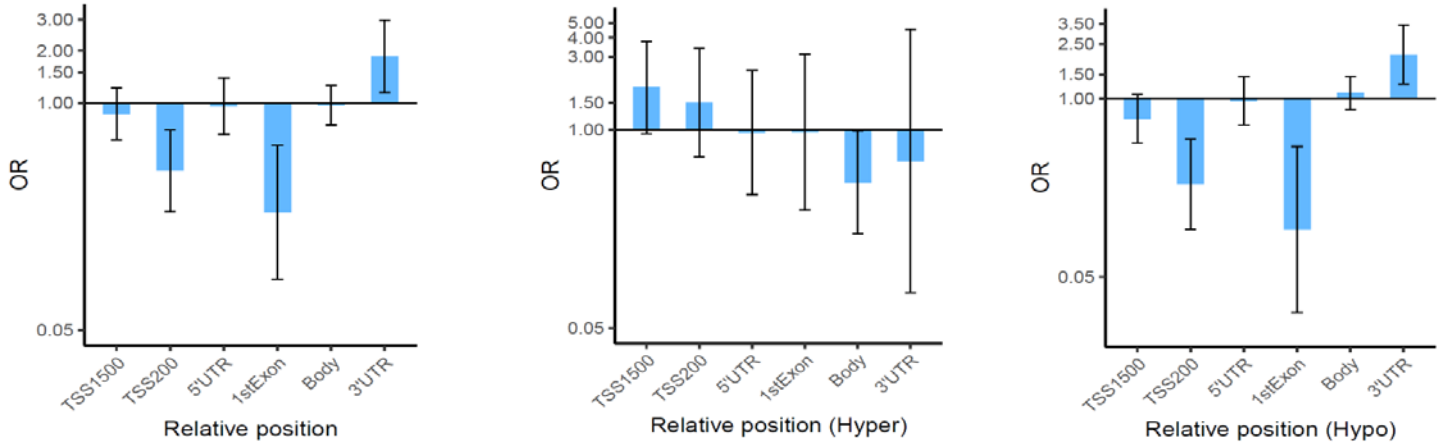

Figure 1. Bar plots depicting OR and 95% CIs for suggestive significant (left), hypermethylated suggestive (center) and hypomethylated suggestive probes (right) compared to no suggestive significant probes in relation to genic position.

Relation to CpG Island

Suggestive CpGs were enriched for open sea, north shelf and south shelf regions and depleted for south shore and islands. This pattern is manteined for hypomethylated CpGs.

Suggestive	Island	North Shelf	North Shore	Open Sea	South Shelf	South Shore
No sig sugg	145756 (30.8%)	24110 (5.1%)	61104 (12.9%)	172193 (36.4%)	21629 (4.6%)	47776 (10.1%)
Sig sugg	10 (4.0%)	25 (10.0%)	22 (8.8%)	161 (64.7%)	21 (8.4%)	10 (4.0%)

X-squared = 144.82, df = 5, p-value < 2.2e-16

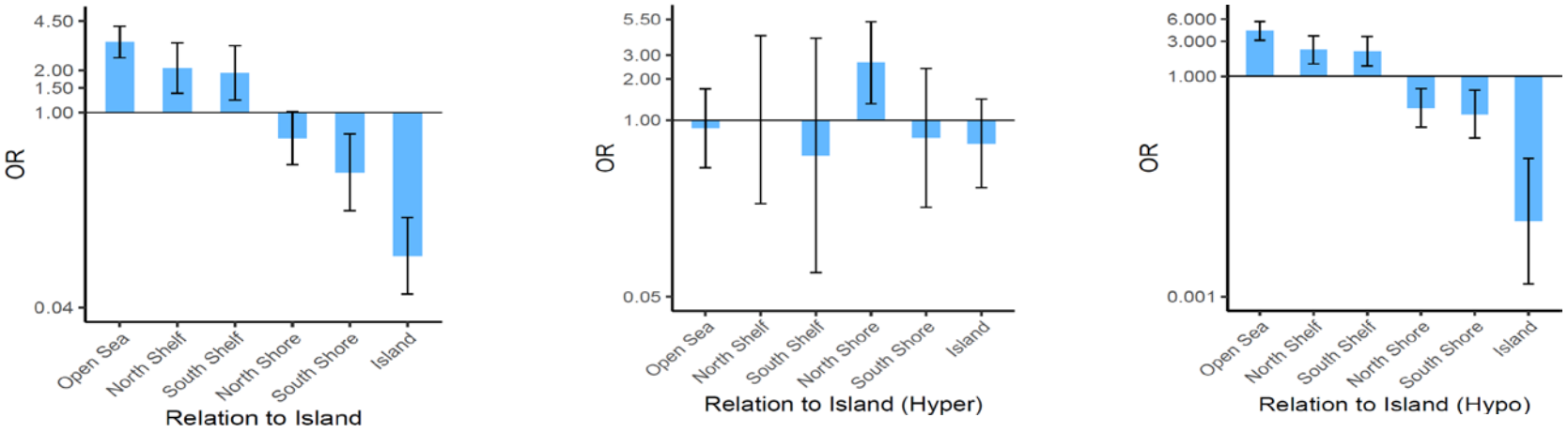

Figure 2. Bar plots depicting OR and 95% CIs for suggestive significant (left), hypermethylated suggestive (center) and hypomethylated suggestive CpGs (right) compared to no suggestive significant CpGs in relation to CpG island.

Chromatin states

Both suggestive CpGs and hypomethylated suggestive CpGs showed the same pattern: enrichment for transcription (Tx and TxWk) and quiescent positions and depletion for transcription start site positions (TSSA, TxFlnk, TxFlnk) and bivalent (EnhBiv) and repressor (ReprPC) positions. Overall, hypermethylated suggestive CpgS showed an opposite pattern of results compared to hypomethylated CpGs. The states are described in Page 2.

	Suggestive	
	Yes	**No**
Hyper	39	239939
Hypo	210	232629

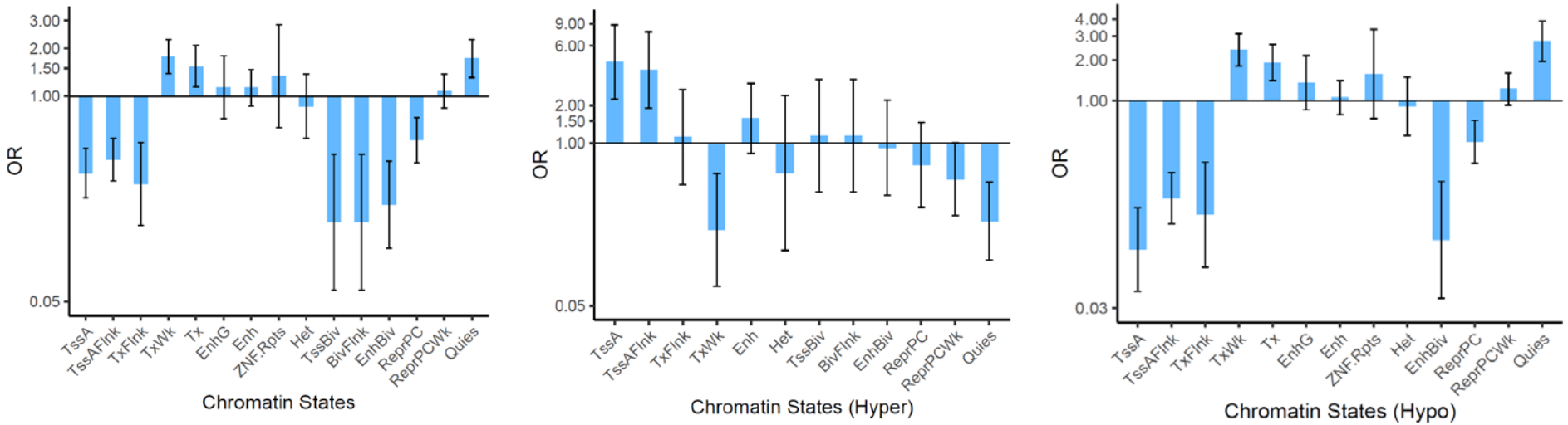

Figure 3. Bar plots depicting OR and 95% CIs for suggestive significant (left), hypermethylated suggestive (center) and hypomethylated suggestive probes (right) compared to no suggestive significant probes for the different chromatin states.

References

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Wilmot B, Fry R, Smeester L, Musser ED, Mill J, Nigg JT. 2016. Methylomic analysis of salivary DNA in childhood ADHD identifies altered DNA methylation in *VIPR2*. J. Child Psychol. Psychiatry 57:152–160; doi:10.1111/jcpp.12457.