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PhD Thesis

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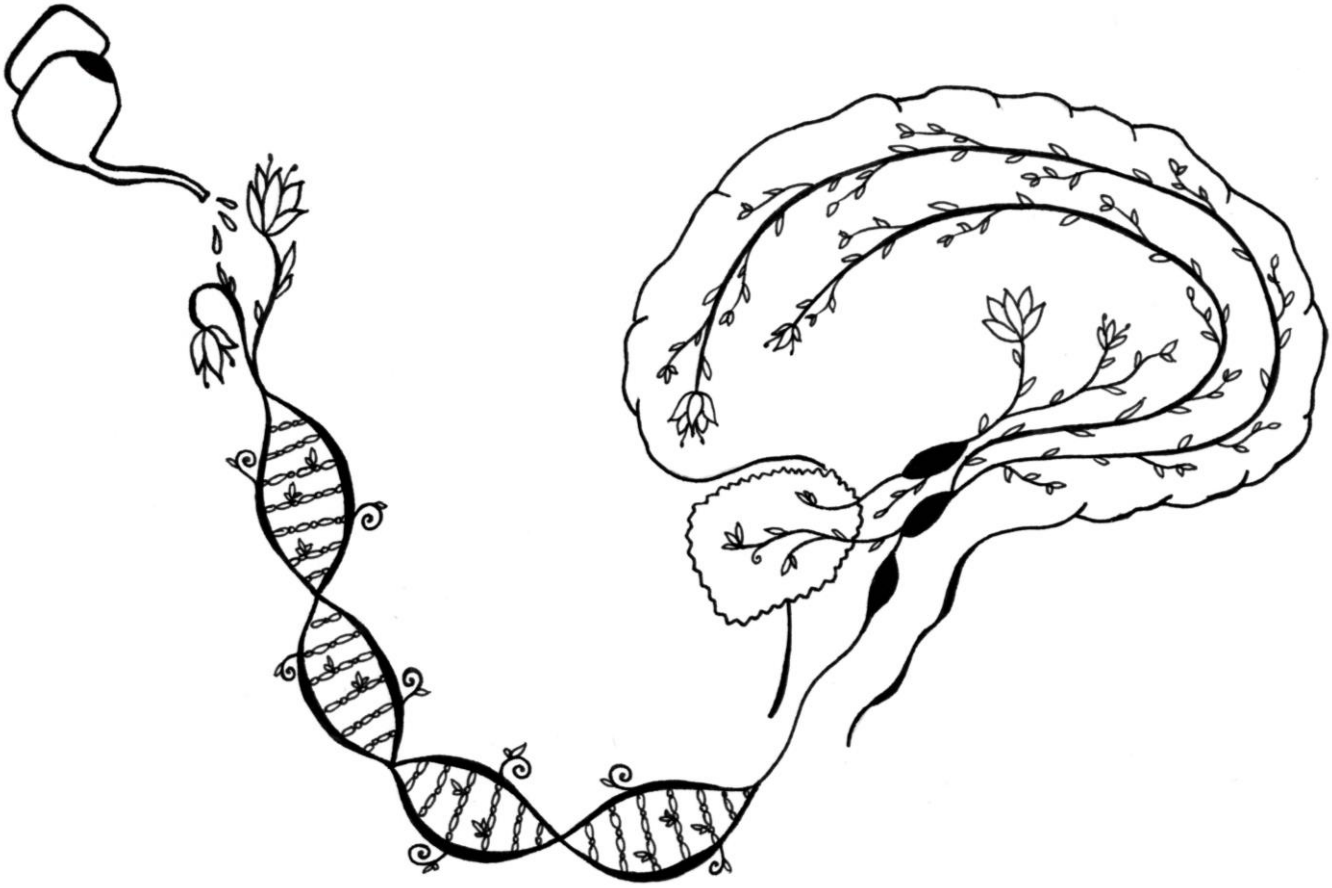

Genetic and Epigenetic Determinants of Serotonin Neurotransmission: Mapping Predictive Risk- and Treatment Markers for Depressive Episodes

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Acknowledgements

The first thing that I have learned about PhD theses is that, when reading someone else's thesis, everyone always looks for the acknowledgements section first. So yes, I have felt a particular pressure in writing this section. All joking aside, I cannot see how I could have possibly carried out this PhD project without the contribution of the many wonderful and bright people that accompanied me in this journey.

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List of Manuscripts

This thesis is based on the following manuscripts:

I. **Bruzzone, S.E.P.**, Nasser, A., Aripaka, S.S., Spies, M., Ozenne, B., Jensen, P.S., Knudsen, G.M., Frokjaer, V.G. and Fisher, P.M. *Genetic contributions to brain serotonin transporter levels in healthy adults*. 2023. Scientific Reports, 13(1), 16426.

II. **Bruzzone, S.E.P.**, Ozenne, B., Fisher, P. M., Ortega, G., Jensen, P. S., Dam, V. H., Svarer, C., Knudsen, G. M., Lesch, K.P., Frokjaer, V. G. *No association between peripheral, serotonin-gene related DNA methylation and brain serotonin neurotransmission in the healthy and depressed state*. 2024. Clinical Epigenetics.

III. **Bruzzone, S.E.P.**, Ozenne, B., Fisher, P. M., Ortega, Knudsen, G. M., Lesch, K.P., Frokjaer, V. G. *Evaluating DNA methylation status of serotonin relevant genes as biomarkers for clinical outcomes of antidepressant treatment*. 2024. Progress in Neuropsychopharmacology and Biological Psychiatry.

IV. **Bruzzone, S.E.P.**, van den Oord, E. J. C. G., Aberg, K., van der Wee, N., Penninx, B. W. J. H., Frokjaer, V. G., Jansen, R. *DNA methylation of serotonin-related genes in depression and depression chronicity: a large-scale replication study*. (in final prep.).

Other related articles that are not included in this thesis:

1. Jensen, K.H.R., McCulloch, D.E.W., Olsen, A.S., **Bruzzone, S.E.P.**, Larsen, S.V., Fisher, P.M. and Frokjaer, V.G., 2022. *Effects of an oral contraceptive on dynamic brain states and network modularity in a serial single-subject study*. Frontiers in Neuroscience, 16, 855582.

2. **Bruzzone, S.E.P.**, Garre, V.F.S., Høgh, S.; O'Donnell, K. J., Frokjaer, V.G., Eid, R. S. *A scoping review of functional genomics in perinatal depression*. (submitted)

Summary

Serotonin transmission crucially regulates a variety of brain functions, including neurodevelopment, cognition, mood and stress responses. The serotonin system is also the main target of antidepressant medications and alterations in serotonin function have been reported in relation to psychopathology e.g. depression, although the link between serotonin (dys)function and depression is still unclear.

Genetic and epigenetic variation (e.g. DNA methylation) within the serotonin system has been suggested to be relevant for depression risk. However, it is unknown to what extent this variation affects human serotonin neurotransmission, in the healthy and in the pathological (e.g. depressed) state.

Specifically, DNA methylation of serotonin-relevant genes (e.g. serotonin transporter [*SLC6A4*], tryptophan hydroxylase 2 [*TPH2*]) has been proposed as a promising biomarker of gene-environment interactions, that could be used to reflect adversities experienced in early life, predict individual risk for depression and likelihood to respond to antidepressant treatment as well as to inform on underlying mechanisms. However, findings are mixed and replication of previous observations is strongly needed to fully uncover the biomarker potential and clinical implications of this epigenetic modification.

In addition, DNA methylation, which is a tissue-specific modification, is generally measured in DNA from peripheral blood cells. Nonetheless, the link between peripheral DNA methylation of serotonergic genes and brain proxies for serotonin neurotransmission measured in-vivo (e.g. serotonin transporter, 5-HTT; serotonin 4 receptor, 5-HT_4) is unexplored.

The main goals of this thesis were to characterize how genetic and epigenetic variation within genes relevant for serotonin function can shape in-vivo serotonergic neurotransmission, both in the healthy and in the depressed state, and to gain a better understanding of how DNA methylation of serotonin-related genes can be used as a biomarker in the context of depression and antidepressant treatment.

In **Study I**, we 1) examined the association between a set of genetic variants within five serotonin-relevant genes and brain 5-HTT levels in healthy adult participants and 2) evaluated whether genetic variation per se could predict brain 5-HTT levels. In **Study II**, we evaluated whether peripheral epigenetic variation within the *SLC6A4* and *TPH2* genes was associated with 1) 5-HTT or 5-HT_4 brain levels in healthy adults or 5-HT_4 in patients with depression and/or 2) with measures of early life and recent stress, depressive and anxiety state traits in healthy participants and patients with depression. In **Study III**, we evaluated whether *SLC6A4* and/or

TPH2 methylation 1) predicted clinical outcomes following antidepressant treatment and/or 2) changed following antidepressant treatment. In **Study IV**, we tried to replicate the link between *SLC6A4* and/or *TPH2* and depression status and childhood trauma that was described in literature using data from four large datasets (three based on blood and one on postmortem brain samples). Next, we moved beyond these two genes by examining whether DNA methylation at 27 centrally-relevant genes for serotonin function is enriched in depression or childhood trauma and, in one of the cohorts, if it is associated with: 1) depressive symptoms, 2) childhood trauma or 3) depression chronicity.

In **Study I**, we found that individuals carrying the T-allele of the rs1137070 variant in the monoamine oxidase A gene (*MAOA*) had increased 5-HTT binding but, despite this association, genetic information was not sufficient to predict 5-HTT brain levels. In **Study II**, we found no link between *SLC6A4/TPH2* methylation measured in blood and brain 5-HTT or 5-HT_4 levels, nor with measures of environmental stress, depressive or anxiety state symptoms. In **Study III**, we found that patients with higher baseline *TPH2* methylation levels were more likely to respond to treatment after 8 weeks of treatment with SSRI. However, neither *SLC6A4* nor *TPH2* methylation could predict clinical outcomes following antidepressant treatment and only marginal changes in their methylation levels were observed over 12 weeks of treatment. In **Study IV**, we found no evidence for an association between DNA methylation of neither gene and depression status, childhood trauma or depression chronicity in four independent datasets.

The findings from these studies suggest that genetic and epigenetic variation within the serotonin system as captured in peripheral blood might have limited impact on in-vivo serotonin neurotransmission, both in healthy participants and in patients with depression. In addition, peripheral DNA methylation of serotonin-relevant genes is unlikely to be used as a biomarker for neither depression risk nor antidepressant treatment outcomes, and their DNA methylation levels are not associated with depression status or childhood trauma when measured peripherally nor with depression status in postmortem brain tissue.

Taken together, this thesis shows valuable insights into the interpretation of genetic variation and peripheral DNA methylation in serotonin-relevant genes in relation to serotonin neurotransmission and suggests that DNA methylation of serotonin-relevant genes unlikely provides critical insights into mechanisms underlying depression or clinical outcomes after antidepressant treatment or represents a clinically useful biomarker of depression status or early life adversities.

Dansk Resume

Serotonin er et signalstof i hjernen, der spiller en central rolle for mange forskellige processer bl.a. ved at regulere udviklingen af nervesystemet, tænkning, humør og stressreaktivitet. Serotoninsystemet er også det primære mål for antidepressiv medicin, og ændringer i serotoninaktiviteten er blevet forbundet med psykiske lidelser som depression. Dog er forbindelsen mellem serotonin og depression stadig uklar.

Genetiske og epigenetiske forskelle (som f.eks. DNA-methylering) i serotoninsystemet kan have betydning for risikoen for depression. Men det er stadig uklart, hvor meget disse forskelle påvirker serotoninfunktionen i hjernen – både hos raske og hos personer med depression.

Særligt er DNA-methylering af serotonin-relaterede gener som SLC6A4 (serotonintransporteren) og TPH2 (et enzym, der er vigtigt for serotoninproduktionen) blevet foreslået som mulige biomarkører, der kan vise, hvordan gener og miljø interagerer. Disse markører kan potentielt bruges til at forudsige risikoen for depression og effekten af antidepressiv behandling. Dog er resultaterne fra tidligere studier blandede, og der er brug for flere undersøgelser for at forstå, om denne metode virkelig har klinisk potentiale.

Desuden bliver DNA-methylering, som er en vævsspecifik ændring, normalt målt i DNA fra blodprøver. Men sammenhængen mellem DNA-methylering i blodet på serotonin-relaterede gener og hjernefunktioner relateret til serotonin (f.eks. serotonintransporteren, 5-HTT; og serotonin 4-receptoren, 5-HT4) er endnu ikke undersøgt.

Formålet med denne afhandling var at undersøge, hvordan genetiske og epigenetiske variationer i serotonin-relaterede gener påvirker serotoninaktiviteten i hjernen hos både raske individer og deprimerede patienter. Derudover undersøges det også, om DNA-methylering af serotonin- gener kan bruges som en biomarkør for depression og for antidepressiv behandlingseffekt.

I Studie I undersøgte vi sammenhængen mellem variationer i fem serotonin-relaterede gener og niveauet af 5-HTT i hjernen hos raske individer, samt om disse genvariationer statistisk kunne forudsige niveauet af 5-HTT i hjernen. I Studie II undersøgte vi om epigenetiske variationer i SLC6A4 og TPH2 generne var forbundet med 1) 5-HTT eller 5-HT4 niveauer i hjernen hos raske individer og 5-HT4 niveauer i hjernen hos patienter med depression og 2) mål for stress oplevet tidligt i livet eller nyligt stress samt symptomer på depression og angst hos både raske individer og patienter med depression. I Studie III undersøgte vi, om SLC6A4 og TPH2 genmethylering 1) kunne forudsige behandlingseffekt af antidepressiv medicin og/eller 2) om de ændrede sig efter behandling med antidepressiv medicin. I Studie IV forsøgte vi at replikere tidligere fund, der har

vist en forbindelse mellem methylering af SLC6A4 og TPH2 generne og depression og barndomstraumer. Dette gjorde vi vha. af fire store datasæt (tre baseret på blodprøver og ét baseret på post mortem hjernevævsprøver). Dernæst undersøgte vi også, om methylering af 27 andre serotonin-relaterede gener var øget i individer med depression eller barndomstraumer. Til sidst undersøgte vi også i ét af de fire datasæt om methyleringen af disse 27 gener var forbundet med 1) depressive symptomer, 2) barndomstraumer og 3) varigheden af depression.

I Studie I fandt vi, at personer med T-allelen af rs1137070-varianten i genet for monoaminoxidase A (MAOA) havde øget 5-HTT-binding. Men på trods af denne sammenhæng kunne genetisk information ikke bruges til at forudsige 5-HTT-niveauerne i hjernen. I Studie II fandt vi ingen sammenhæng mellem SLC6A4/TPH2-methylering målt i blodet og hhv. 5-HTT- eller 5-HT4-niveauer i hjernen. Vi fandt heller ingen sammenhæng med stress eller symptomer på depression og angst. I Studie III fandt vi, at patienter med højere TPH2-methylering før behandling klarede sig bedre efter 8 ugers behandling med antidepressiv medicin. Dog kunne denne sammenhæng ikke bruges til at forudsige, hvilke patienter der ville blive symptomfrie, og samtidig så vi kun små ændringer i methylering efter 12 ugers behandling. Til sidst fandt vi i Studie IV ingen sammenhæng i nogen af de fire datasæt mellem de 27 undersøgte gener og hhv. depressive symptomer, barndomstraumer eller varigheden af depression.

Resultaterne fra disse studier tyder på, at genetiske og epigenetiske variationer i serotoninsystemet, som kan måles i blodet, sandsynligvis har en begrænset betydning for serotoninaktiviteten i hjernen hos både raske personer og personer med depression. Derudover ser det ud til, at DNA-methylering af serotonin-relaterede gener i blodet ikke kan bruges som en markør for hverken risikoen for depression eller effekten af antidepressiv behandling. Methyleringsniveauerne af disse gener hænger heller ikke sammen med depression, hverken når de måles i blodet eller i post mortem hjernevæv.

Samlet set giver denne afhandling vigtig ny indsigt i genetisk variation og DNA-methylering i serotonin-relaterede gener, og hvordan de relaterer sig til serotoninsystemet og dets funktion i nervesystemet. Desuden viser vi her, at DNA-methylering i de undersøgte gener sandsynligvis ikke er klinisk relevant som biomarkør for hverken depression eller tidlige barndomstraumer.

Abbreviations

5-HT	Serotonin
5-HT_{1A}	Serotonin 1A receptor
5-HT_{2A}	Serotonin 2A receptor
5-HT_{4}	Serotonin 4 receptor
5-HTT	Serotonin Transporter (protein)
5-HTTLPR	Serotonin-Transporter-Linked Promoter Region
BDI	Beck's Depression Inventory
BDNF	Brain-Derived Neurotrophic Factor
BMI	Body Mass Index
bp	(DNA) base-pair
CATS	Childhood Abuse Trauma Scale
CpG	Cytosine—phosphate—Guanine
CpG_{LV}	Latent variable based on CpG values
$CpG_{LV+cells}$	CpG_{LV} adjusted for cell proportions
DNA	Deoxyribonucleic Acid
GAD10	Generalized Anxiety Disorder 10-item
GWAS	Genome-Wide Association Study
HAMD	Hamilton Depressive Rating Scale
$HAMD_{17}$	17-item HAMD
$HAMD_{6}$	6-item HAMD
HC	Healthy Control
HTR1A	Serotonin Receptor 1A gene
HTR2A	Serotonin Receptor 2A gene
kb	(DNA) kilo-bases
LV	Latent Variable
LVM	Latent Variable Model
MAOA	Monoamine Oxidase A
MBD-seq	Methyl-Binding Domain sequencing
MDD	Major Depressive Disorder
MRI	Magnetic Resonance Imaging
MRTM/MRTM2	Multilinear Reference Tissue Model

MWAS	Methylome-Wide Association Study
PBI	Parental Bonding Inventory
PET	Positron Emission Tomography
PSS	Perceived Stress Scale
ROI	Region Of Interest
SLC6A4	Serotonin Transporter gene
SLE	Stressful Life Events
SNRI	Serotonin-Norepinephrine Reuptake Inhibitor
SSRI	Selective Serotonin Reuptake Inhibitor
TPH2, *TPH2*	Tryptophan Hydroxylase 2 (protein, *gene*)

“There’s no gene for fate.”

(GATTACA. Niccol, 1997)

BACKGROUND

1. The serotonin system

The serotonin system regulates a variety of physiological processes, spanning from mood, cognition, neuroplasticity, sleep, stress responses and appetite regulation to cardiovascular, inflammatory and metabolic functions (Jonnakuty & Gragnoli, 2008). Serotonin signaling is also fundamental for neurodevelopment, during which it drives neuronal differentiation, maturation and migration (Gaspar et al., 2003). However, little is known on the exact mechanisms underlying most of the serotonin-related functions, likely due to the complexity of the serotonin system and its constituents.

Serotonin, or 5-hydroxytryptamine (5-HT), is synthesized starting from the amino acid L-tryptophan, which is first hydroxylated into hydroxy-L-tryptophan by the enzyme tryptophan hydroxylase (TPH), in the rate-limiting step in serotonin biosynthesis. Next, hydroxy-L-tryptophan is converted into serotonin by the aromatic amino acid decarboxylase (AADC) (Grahame-Smith, 1964; Walther & Bader, 2003) (**Figure 1A**). There are two isoforms of TPH: TPH1, almost exclusively expressed in the periphery (specifically gut, spleen and thymus) and in the pineal gland and TPH2, which is only expressed in the brain, i.e. the raphe nuclei where serotonergic neuronal cell bodies are located. (Walther & Bader, 2003). Notably, although serotonin is normally thought of as a neurotransmitter relevant for brain function, 95% total 5-HT is produced in the periphery, where it acts as a hormone, while only 5% of 5-HT is synthesized in in the brain, where it acts as a neurotransmitter (Jacobs & Azmitia, 1992; Jonnakuty & Gragnoli, 2008).

In this thesis, we will primarily focus on the effects of this 5% of total serotonin on serotonergic neurotransmission.

Brain serotonin is synthesized in the nuclei of serotonergic neurons that are located within the brainstem raphe nuclei (Dahlström, and Fuxe, 1964). Serotonergic neurons represent only a small fraction of the total neurons of the human brain (~1/1,000,000). Nonetheless, projections of the raphe nuclei innervate the entire forebrain, cerebellum and medulla oblongata, making it the most widespread neurotransmitter in the brain (Dahlström and Fuxe, 1964) (**Figure 1B**). Serotonin exerts its action via approximately 14 different receptor subtypes, which are coded by 17 genes and one pseudogene. Of these genes, 12 encode for metabotropic receptors, each of which can activate different downstream signaling pathways, 5 encode for the subunits of 5-HT_3, the only ionotropic serotonin receptor. The pseudogene encodes for 5-ht_{5b}, which is not

translated into a full protein and whose function is unknown (Sharp & Barnes, 2020). The main genes of the serotonin system and their roles in serotonin transmission are listed in **Table 1**.

Serotonin neurotransmission is closely regulated by serotonin transporters, membrane proteins that reuptake the excess serotonin within the extracellular space back into the pre-synaptic neuron. The transporter with the highest affinity for serotonin is the serotonin transporter (SERT or 5-HTT), which is also the main target of selective-serotonin reuptake inhibitors (SSRI), a widely used class of medications for a variety of psychiatric conditions (Artigas et al., 2002). For this reason, and for its essential role in regulating serotonin levels, genetic and epigenetic variation within SERT gene (*SLC6A4*) has been investigated by a large body of research.

Upon reuptake, serotonin is either packed back into high-density vesicles by vesicular monoamine transporters (VMATs), ready to be released again, or it is degraded by monoamine oxidase A (MAOA) (Wimalasena, 2011) (**Figure 1C**).

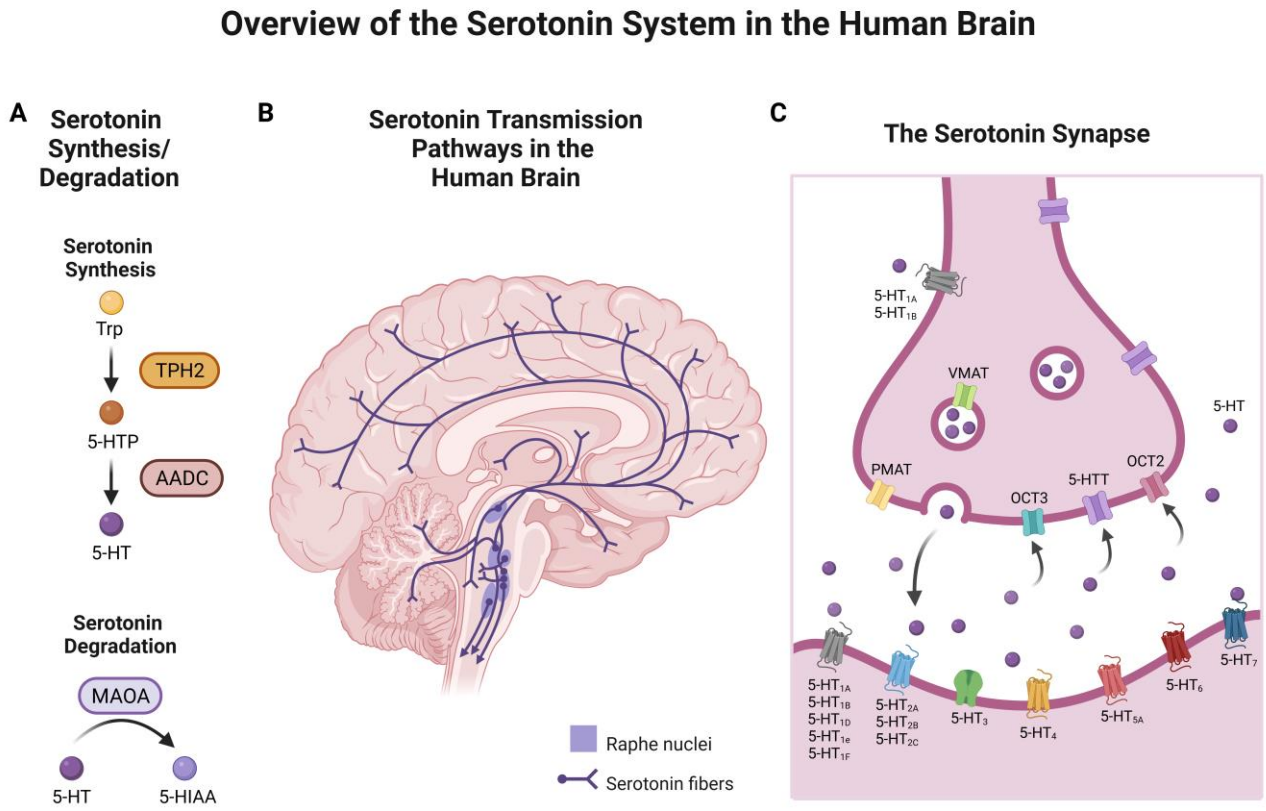

Figure 1. Overview of the serotonin system in the human brain: Brain serotonin synthesis and degradation pathways (A); brain serotonergic projections (B), the main serotonin receptors and transporters at the serotonin synapse (C). Trp: tryptophan; 5-HTTP: 5-hydroxytryptophan; 5-HT: serotonin; TPH2: tryptophan hydroxylase 2; AADC: aromatic decarboxylase; MAOA: monoamine oxidase A. Created with BioRender.com by S. E. P. Bruzzone.

The serotonin system has been widely investigated by genetic, pharmacological interventions, behavioral and imaging studies in both animal models and humans. Nonetheless, we still know very little about the effects of serotonin function and dysfunction on complex phenotypes such as behavior or neuropsychiatric disorders.

Gene Name	Gene Product	Gene Function
HTR1A	Serotonin receptor 1A (5-HT_{1A})	Serotonin receptors
HTR1B	Serotonin receptor 1B (5-HT_{1B})	
HTR1D	Serotonin receptor 1D (5-HT_{1D})	
HTR1E	Serotonin receptor 1e (5-HT_{1e})	
HTR1F	Serotonin receptor 1F (5-HT_{1F})	
HTR2A	Serotonin receptor 2A (5-HT_{2A})	
HTR2B	Serotonin receptor 2B (5-HT_{2B})	
HTR3A	Serotonin receptor 3A (5-HT_{3A})	
HTR3B	Serotonin receptor 3B (5-HT_{3B})	
HTR3C	Serotonin receptor 3C (5-HT_{3C})	
HTR3D	Serotonin receptor 3D (5-HT_{3D})	
HTR3E	Serotonin receptor 3E (5-HT_{3E})	
HTR4	Serotonin receptor 4 (5-HT_{4})	
HTR5A	Serotonin receptor 5A (5-HT_{5A})	
HTR5B	Serotonin receptor 5b (5-HT_{5b})	
HTR6	Serotonin receptor 6 (5-HT_{6})	
HTR7	Serotonin receptor 7 (5-HT_{7})	
SLC6A4	Serotonin Transporter (5-HTT; SERT)	Serotonin transporters
SLC22A1	Organic Cation Transporter 1 (OCT1)	
SLC22A2	Organic Cation Transporter 2 (OCT2)	
SLC22A3	Organic Cation Transporter 3 (OCT3)	
SLC18A1	Vesicular Monoamine Transporter 1 (VMAT1)	
SLC18A2	Vesicular Monoamine Transporter 2 (VMAT2)	
SLC29A4	Plasma Membrane Monoamine Transporter (PMAT)	
TPH1	Tryptophan hydroxylase 1 (TPH1)	Essential enzymes for serotonin synthesis
TPH2	Tryptophan hydroxylase 2 (TPH2)	
AADC	Aromatic L-amino Acid Decarboxylase	
MAOA	Monoamine Oxidase A	Essential enzymes for serotonin degradation

Table 1. List of the genes that are centrally relevant for serotonin neurotransmission.

2. Genetic variation within the serotonin system

2.1. Evidence from animal models

Research on animal models (rodents) provided important insights into the consequences of genetically altered serotonin levels on behavior, cognition and stress coping behavior. Animal studies also allow us to gain a deeper understanding of the behavioral effects of altered serotonin signaling from embryonic development until adulthood. For instance, both 5-HTT knock-out (5-HTT$^{-/-}$) rats and mice, which, by lacking 5-HTT, are exposed to increased extracellular serotonin levels from fetal life, exhibit increased anxiety-like behavior and decreased social behavior, along with somatosensory alterations (Kalueff et al., 2010). Interestingly, heterozygous knock-outs for 5-HTT (5-HTT$^{+/-}$), do not show any neurochemical or behavioral alteration compared to wildtypes (5-HTT$^{+/+}$). However, when exposed to stressors (e.g. early maternal separation), they manifest anxiety- and depressive-like behaviors (Houwing et al., 2017), suggesting that genetic variants that do not affect baseline behavior might affect individual resilience to psychopathology when in combination with environmental stressors.

TPH2$^{-/-}$ rodents instead, who lack brain serotonin from postnatal development, are characterized by increased aggression, high rates of postnatal lethality and poor maternal care (Pratelli & Pasqualetti, 2019). In addition, some studies show that, upon environmental stress exposure, TPH2$^{-/-}$ mice display blunted glucocorticoid stress response (Brivio et al., 2018), decreased prosocial behavior (Weidner et al., 2019) and increased depressive-like behavior (Brivio et al., 2018) compared to TPH2$^{+/+}$, although the latter has not been confirmed by all studies (Pratelli & Pasqualetti, 2019).

Taken together, these phenotypes suggest that genetic alterations in serotonin neurotransmission may play a role in psychopathology, especially when individuals with impaired serotonin function experience environmental stressors.

2.2. Evidence from studies on human participants

Genetic variation within the serotonin system in humans has been broadly researched, especially in relation to emotional processing, mood disorders and antidepressant treatment response.

The most commonly studied gene of the serotonin system is *SLC6A4*, which encodes for 5-HTT, although genes encoding for TPH2 (*TPH2*), MAOA (*MAOA*) and several serotonin receptors, e.g. 5-HT1A, 5-HT2A (*HTR1A*, *HTR2A*) have also been extensively characterized (Albert et al., 2019; Fan et al., 2010; Kulikova & Kulikov, 2019; Lin et al., 2014; Munafò et al., 2003; Ottenhof et al., 2018; Spies et al., 2020; Huai_Neng Wu et al., 2024).

Specifically, variation within the serotonin-transporter-linked promoter region (5-HTTLPR), occurring in the promoter region of *SLC6A4*, has been widely investigated. 5-HTTLPR has two common forms: the short (s) allele, found in 43% of the population with Caucasian ethnicity, and the long (L) allele, found in about 57% of the population (Lesch, 1996). Pioneering studies based on in-vitro functional assays showed that the s-allele is characterized by lower gene expression and serotonin reuptake compared to the L allele (Heils et al., 1996; Lesch, 1996) and linked the s-allele to anxiety-related personality traits (Lesch, 1996). The 5-HTTLPR genotype is normally analyzed in combination with the rs25531 genotype, an A/G single nucleotide polymorphism (SNP) occurring in the L-allele, which affects *SLC6A4* expression such that L-carriers who also carry the rs25531 G allele present gene expression comparable to that of s-carriers (Nakamura et al., 2000).

5-HTTLPR was linked for the first time to the gene-by-environment term when Caspi and colleagues (Caspi et al., 2003) showed that individuals carrying the s-allele were more likely to develop depressive symptoms when they had a history of early life stress. This gave rise to a multitude of studies aiming to link 5-HTTLPR to brain activity related to e.g. emotional processing and cognition, such as fear-induced amygdala reactivity. However, large-scale genetic studies failed to confirm earlier findings about a link between 5-HTTLPR/rs25531 and depression in presence of environmental stress (Border et al., 2019; Culverhouse et al., 2018). Most of the studies evaluating the link between 5-HTTLPR and affective processing via functional magnetic resonance imaging (fMRI) provided contrasting findings too (Raab et al., 2016). Similarly, common SNPs within the *TPH2, MAOA, HTR1A and HTR2A* genes were repeatedly linked to depression vulnerability (Kato & Serretti, 2010; Ottenhof et al., 2018) but genome-wide association studies (GWAS) failed to confirm their link with depression (Levey et al., 2021).

2.3. Genetic variation and in-vivo molecular imaging

Contrasting fMRI findings relating 5-HTTLPR to brain activity might be due to the fact that a single transcriptional variant is unlikely to affect a brain function which, instead, is plausibly the result of myriad underlying processes. However, discrepancies in the literature might also be due to inherent limitations of the method. Indeed, fMRI provides an indirect measurement of brain function (e.g. blood-oxygen-level-dependent or BOLD signal, which provides an estimate of brain activity based on blood oxygen levels)(Logothetis & Wandell, 2004). Instead, positron emission tomography (PET) imaging allows to directly estimate brain protein levels in a highly reproducible way, giving PET a major advantage over fMRI when it comes to relating genetic variation and brain architecture.

Notably, a study showed that 5-HTTLPR S-carriers were more likely to experience seasonal depression and that 5-HTT binding in their putamen was negatively associated with daytime duration (Kalbitzer et al., 2013). In addition, genetic variants in other serotonin-relevant genes e.g. rs7333412 in the serotonin 2A receptor gene (*HTR2A),* were found to affect brain 5-HTT binding in patients with depression and bipolar disorder, such that individuals with AA genotype showed lower thalamic 5-HTT binding compared to G- carriers (Laje et al., 2010), suggesting a link between genetic variation, brain serotonergic architecture and pathological conditions.

Genetic variation within the serotonin system of healthy participants has been less extensively characterized and less is known about how genetic variants shape the healthy serotonin architecture. Evidence from PET imaging suggests that S-carriers have 9% lower serotonin 4 receptor (5-HT_4) binding in the neocortex (Fisher et al., 2012; Fisher, et al., 2015) and show increased amygdala reactivity to fearful faces (Fisher et al., 2015b) compared to individuals with LL genotype, suggesting that genetic variation might affect emotional processing and the in-vivo serotonin architecture even in absence of signs of psychopathology. Nonetheless, the relation between 5-HTTLPR and brain 5-HTT binding in healthy participants appears limited (Fisher et al., 2017; Murthy et al., 2010; Praschak-Rieder et al., 2007), calling for more replication studies in larger cohorts. Furthermore, it is unknown whether genetic variation within other genes that are relevant for serotonin neurotransmission (e.g. *HTR1A*, *HTR2A*, *MAOA*) can affect brain 5-HTT binding and possibly be relevant in the context of disease.

3. Selective Serotonin Reuptake Inhibitors

Selective serotonin reuptake inhibitors (SSRI) are the most widely used antidepressant medications. In Denmark, ~426,000 people use SSRI (Sundhedsdatastyrelsen, 2021) to treat major depressive disorders but also other neuropsychiatric disorders such as anxiety, post-traumatic stress syndrome and obsessive-compulsive disorder.

The popularity of SSRIs is mostly due to their high efficacy levels, combined with a good tolerability profile (Cipriani et al., 2018). Nonetheless, SSRIs are not effective for all patients: only about 50-60% patients respond to SSRI treatment (Berton & Nestler, 2006; Trivedi et al., 2006). In addition, positive treatment effects only appear after about two weeks. As it is not known why only some patients respond to treatment, and what treatment is optimal for each individual patient, the current strategy in clinical practice consists in proceeding by trials and errors. This has consequences not only on possible side effects due to the administration of a medication that is not suitable for a specific patient, but also on treatment onset, that gets further delayed, possibly resulting in symptoms worsening.

Our limited understanding on individual variability in response to antidepressant treatment is likely influenced by the fact that mechanisms underlying SSRI effectiveness are still not completely understood. SSRIs primarily target 5-HTT, which they block, inducing an increase in extracellular serotonin levels. Higher serotonin levels within the extracellular space interact with all the available serotonin receptors, enhancing serotonin neurotransmission. However, as treatment effects are only experienced after two weeks, it is unlikely that the SSRI-induced increase in serotonin levels is sufficient to generate an antidepressant effect. Instead, cumulative evidence suggests that a series of complex neuroadaptive changes is required to produce this effect. One of these changes might be through desensitization of serotonin 1A (5-HT_{1A}) receptors, which inhibit serotonergic neurons and their serotonin release. Thus, an initial increase in serotonin levels might be followed by a decrease in serotonin levels and serotonin signaling. Another mechanism that was suggested more recently implies that brain-derived neurotrophic factor (BDNF) and it receptor TrkB are the main mediators of the neuroadaptive changes underlying antidepressant effects (Casarotto et al., 2022).

As SSRIs do not work equally well for everyone, biomarkers that could predict what patient will respond to what specific antidepressant treatment would be immensely beneficial in a personalized psychiatry perspective. Such biomarkers could guide clinicians' treatment choices and avoid unnecessary side effects as well as allow prompt and effective interventions. Genetic variation within genes relevant for serotonin function was substantially investigated by pharmacogenetics studies focusing on multiple candidate genes (Kato & Serretti, 2010). However, similarly to the link with psychopathology, the association with antidepressant treatment response was mostly inconsistent across these studies (Fabbri & Serretti, 2020).

4. Major depressive disorder

Major depressive disorder (MDD or depression) affects about 4.4% of the global population. Its incidence is rapidly increasing (GBD 2019 Mental Disorder Collaborators, 2022; World Health Organization, 2011) and it is anticipated to become "the leading cause of disease burden" in the world by 2030 (World Health Organization, 2011).

Individuals affected by depression present a variety of symptoms, divided into core and secondary symptoms, according to the International Classification of Diseases 10th Edition (ICD-10). Depressive symptoms include (but are not limited to) persistent depressed mood, loss of interest in previously enjoyed activities (anhedonia), fatigue and concentration difficulties (ICD-10, 2019); see **Table 2** for a complete list of the depressive symptoms.

Depressive symptoms can present in heterogeneous combinations across different patients, which complicates depression diagnosis. Thus, depression diagnosis is based on symptoms clusters. Specifically, according to the ICD-10, patients must have experienced two of the core symptoms and at least two out of the seven secondary symptoms for at least two weeks, and all symptoms must have been experienced throughout the two weeks(American Psychiatric Association, 2013). Importantly, current diagnostic criteria are not based on the aetiology of depression.

Depressive symptoms according to ICD-10	
Core symptoms	**Secondary symptoms**
• Depressed mood • Loss of interest or pleasure • Low energy/fatigue	• Reduced self-esteem/self-confidence • Sense of guilt and worthlessness • Recurrent thoughts of self-harm or suicide • Diminished ability to think/concentrate • Psychomotor agitation or retardation • Sleep disturbances • Significantly reduced or increased appetite

Table 2. Depressive symptoms according to the ICD-10.

4.1. The monoamine hypothesis of depression

MDD was first linked to serotonin following the discovery of serotonin-acting antidepressants (e.g. imipramine), which increase the levels of serotonin, noradrenaline and dopamine (Kuhn, 1958). This led to the formulation of the so-called “monoamine theory of depression”, according to which low serotonin levels would cause depression (Bremshey et al., 2024). However, the pathophysiology of depression is likely much more complex and current evidence does not allow neither to confirm nor to reject this hypothesis (Jauhar, Cowen, et al., 2023; Sharp & Collins, 2023).

Notably, recent PET studies described reduced 5-HT_4 brain binding in patients with depression (Köhler-Forsberg et al., 2023b) and impaired serotonin release in a subgroup of patients with depression, suggesting that serotonergic-depression might be a distinct depression subtype (Erritzoe et al., 2023). Nonetheless, these findings need to be confirmed in larger cohorts and, importantly, it is unclear whether the observed alterations in serotonin function reflect underlying mechanisms of disease or the consequences of depression.

4.2. Depression, genes and environment

Although the findings presenting 5-HTTLPR as a gene-by-environment marker were not confirmed by later studies, compelling evidence suggests that MDD arises from the contribution of both environmental and genetic factors (Kwong et al., 2019). For instance, experiencing traumatic events (e.g. parental neglect, sexual or physical abuse) especially early in life, chronic physical illness or having a low socio-economic status can increase the risk of developing depression (Buckman et al., 2022; Katon, 2011; Mandelli et al., 2015). Nonetheless, not all individuals exposed to such circumstances develop MDD, suggesting that some might be more genetically predisposed than others to develop depression. Studies including first-degree family members and twins suggest that depression heritability is between 30% and 50% (Kendall et al., 2021). Importantly, MDD is a complex trait, which is likely to arise from small contributions of many gene variants. Thus, focusing on one or few candidate genes is unlikely to completely explain genetic variation underlying this condition. However, it is important to note that also most of genome-wide studies reported contrasting findings, with little or no overlap of the variants detected by different studies (Kendall et al., 2021).

5. Epigenetics

Although it is commonly recognized that MDD arises from a combination of genetic and environmental factors, how genes and environment interact at the biological level is still unclear.

In this framework, epigenetic modifications might reflect such gene-environment interactions at the molecular level, possibly allowing us to understand more about the pathophysiology of depression (Penner-Goeke & Binder, 2019) (**Figure 2**).

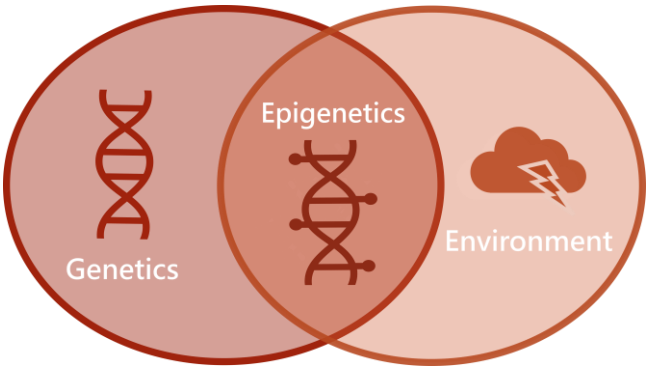

Figure 2. Epigenetics as a molecular link between genetics and environment.

Epigenetics consists of a set of modifications that can induce stable changes in gene expression and, consequently, cellular phenotypes, without modifying the underlying DNA sequence (Jaenisch & Bird, 2003). Epigenetic modifications are involved in common biological processes such as development, during which they drive tissue differentiation, but can also be induced in response to environmental challenges (e.g. stressors, pharmacological agents, pollutants, temperature, diet), allowing organisms to adapt to the ever-changing environment (Gibney &

Nolan, 2010). Epigenetic modifications are also sensitive to genetic variation (Villicaña & Bell, 2021), indicating their intermediate role in the complex interplay between genetics and environment.

Notably, the epigenome seems especially sensitive to environmental stressors during early developmental stages (Murgatroyd et al., 2009; O'Donnell & Meaney, 2020). Indeed, changes in gene expression occurred during one of these phases are more likely to endure throughout life and affect future disease vulnerability (Seckl & Holmes, 2007). Chronic stress can also affect the epigenome also in adulthood, possibly increasing individual predisposition to developing e.g. depression (Park et al., 2019; Stankiewicz et al., 2013). This is supported by a large body of literature linking early life adversities or chronic stress later in life and depression to alterations across multiple epigenetic modifications, both in preclinical and clinical studies (Alasaari et al., 2012; Alyamani et al., 2022; Argentieri et al., 2017; Gladish et al., 2022; Lam et al., 2012; O'Donnell & Meaney, 2020; Palma-Gudiel & Fañanás, 2017; Penner-Goeke & Binder, 2019).

Importantly, the term "epigenetics" refers to a variety of mechanisms that can regulate gene expression at different levels. These include, e.g., DNA methylation, histone modifications, chromatin remodeling and small non-coding RNAs (e.g. microRNAs) (Gibney & Nolan, 2010). In this thesis, we focused on DNA methylation.

5.1. DNA methylation

DNA methylation is the most stable and broadly studied epigenetic mechanism. It involves the covalent addition of a methyl group (-CH_3) to the 5th carbon of cytosines located next to guanines (5'—C—phosphate—G—3'), which are usually referred to as CpG sites, where p stands for the phosphate group linking the two nucleosides. Although DNA methylation can also occur at other nucleosides, CpG sites are the most commonly methylated sites and the effects of their methylation on gene expression are the best understood compared to those of other epigenetic modifications.

There are approximately 28 million CpG sites in the human genome, most of which are grouped in ~30,000 CpG islands, areas of the genome in which CpG sites are densely packed (Ehrlich et al., 1982). About half of the CpG islands are located in the promoter region of genes, while the other half is located in the gene body or in intergenic regions.

DNA methylation is mediated by a class of enzymes referred to as DNA methyltransferases (DNMT), each of which plays a specific role in establishing DNA methylation patterns. For instance, DNMT1 is involved in tissue differentiation and allows maintenance of cell-type specific

DNA methylation marks throughout cell proliferations. In contrast, DNMT3A and DNMT3B mediate de-novo methylation and are particularly responsive to environmental factors (Elliott et al., 2016; Radford, 2018). Thus, although DNA methylation is a dynamic modification that can be directly impacted by the environment, DNA methylation patterns can also be maintained throughout different cell generations.

The exact roles of DNA methylation in gene regulation are yet to be completely understood. However, it is clear that DNA methylation plays an essential role in gene suppression. This is done in different ways. For example, differentially methylated CpG sites are recognized by specialized transcription factors, which can promote or suppress gene expression via recruitment of other protein complexes (Zhu et al., 2016). Usually, methylated CpG sites are not recognized by transcription factors, limiting the accessibility of DNA to gene transcription (**Figure 3**). DNA methylation is also essential to maintain a state of closed chromatin, in which DNA is tightly packed and is not accessible for gene transcription (e.g. X-linked inactivation, in which

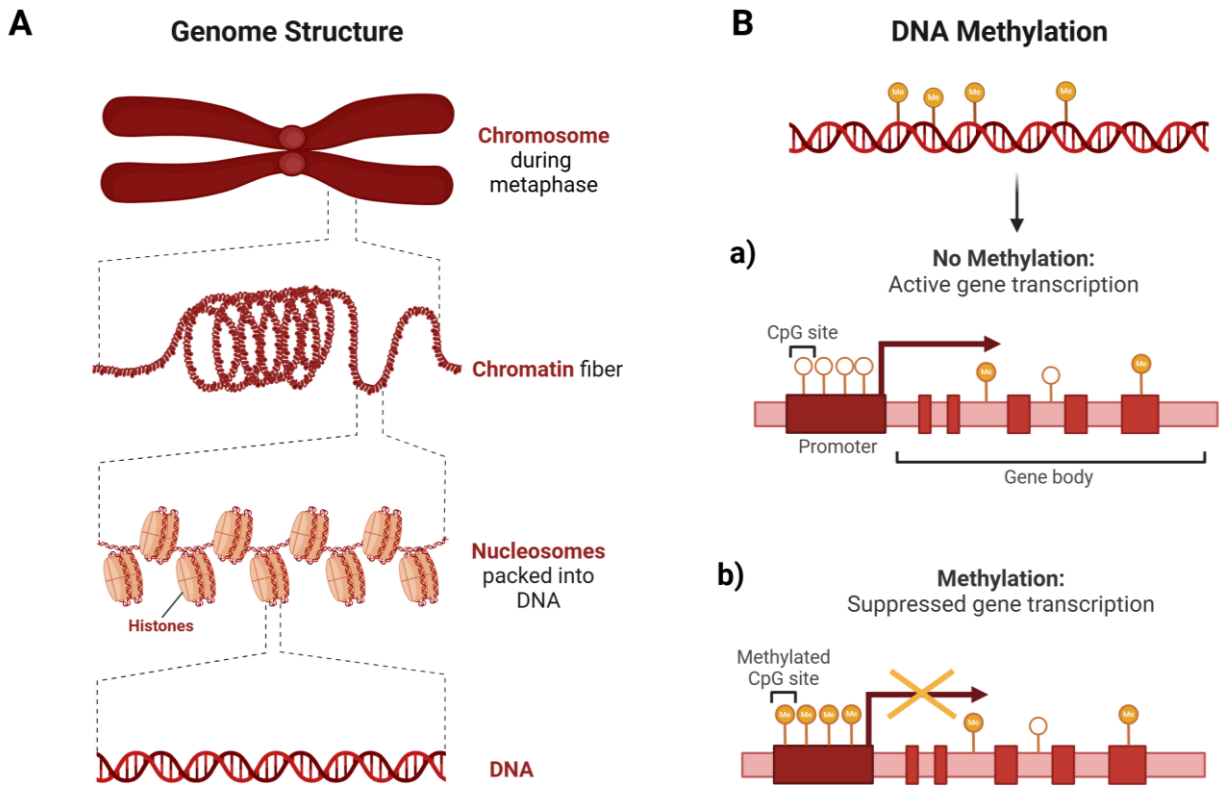

Figure 3. Overview of the chromosomic structure (A) and of DNA methylation in regulating gene expression (B). Figure created with BioRender.com by S. E. P. Bruzzone

large regions of one of the X chromosomes are silenced to maintain a balanced amount of gene expression between XX and XY individuals) (Radford, 2018).

While DNA methylation in the proximity of promoter regions is usually linked to transcriptional repression, the role of DNA methylation within gene bodies is more complex and less clearly understood (Maunakea et al., 2010; O'Donnell & Meaney, 2020). Interestingly, methylation of CpG sites within promoter regions seems to be more conserved across tissues compared to methylation of CpG sites within the gene body (Maunakea et al., 2010).

6. DNA methylation as a risk, diagnostic and treatment marker

Biomarkers for depression risk, diagnosis and treatment are strongly needed, as: 1) being able to predict individual risk would allow us to direct prevention strategies that could limit the consequences of more severe forms of depression, which are also more difficult to treat (Kornstein & Schneider, 2001); 2) depression is a highly heterogeneous disorder and depressive symptoms can manifest also as consequences of other e.g. somatic conditions. Having a tool that allows to define depressive status would provide an objective measurement of depressive status and aid psychiatric diagnosis; 3) antidepressant medications (e.g. SSRIs) are effective only in a subgroup of patients and it is unknown what medication is best for what patient. Instead, the current approach consists in prescribing medications based on trials and errors, causing delays in treatment onset, along with treatment-related side effects.

In this context, DNA methylation holds unique potential as a biomarker. First, adverse life events (especially if experienced in childhood) are major predictors of depression later in life (Liu, 2017). Thus, if information of environmental stress is embedded in the epigenome under the form of DNA methylation, this could represent an important marker for depression risk. Second, pre-existing alterations in DNA methylation (also independently of early life stress) might interfere with treatment mechanisms and affect individual predisposition to respond to antidepressant medications. Thus, identifying these alterations would allow to develop biomarkers to direct treatment choices, avoiding to proceed by trial and error. Third, as DNA methylation is a dynamic modification, longitudinal intervention studies involving antidepressants medications might inform on novel mechanisms underlying treatment outcomes and treatment resistance (**Figure 4**).

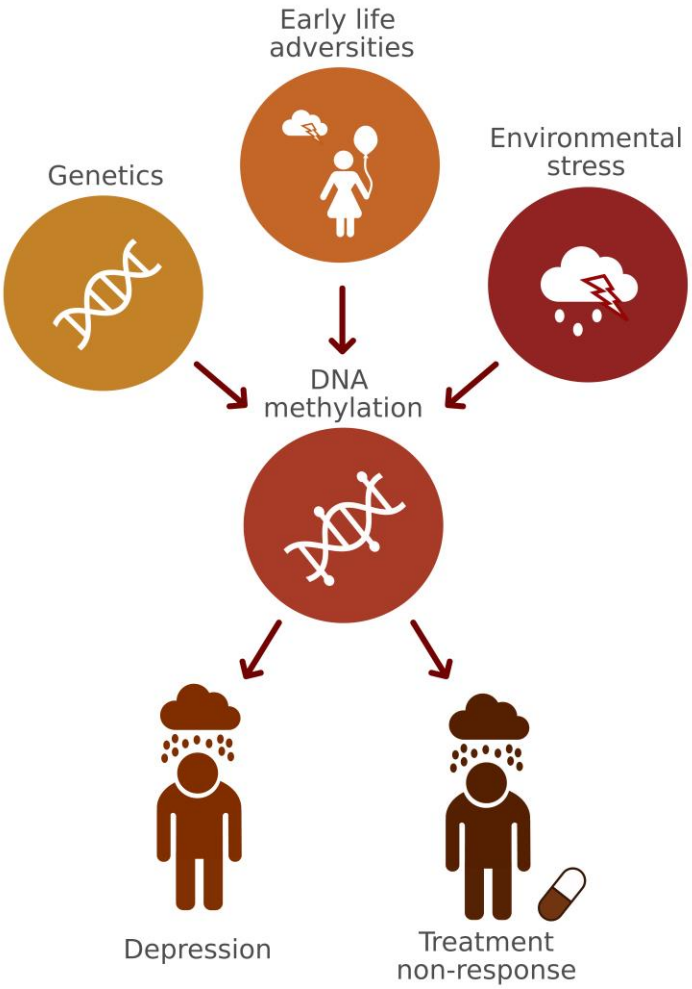

Figure 4. Epigenetics as a mediator between genetics and environmental stress (experienced both recently and in early life) in the development of depression or affecting antidepressant treatment outcomes.

6.1. DNA methylation of serotonin-relevant genes as biomarkers

Given the crucial role of the serotonin system in neurodevelopment, antidepressant medications, stress and stress-related psychopathology, and the fact that DNA methylation may represent gene-environment interactions at the biological level, DNA methylation levels of genes relevant to serotonin neurotransmission have been proposed as promising biomarkers of depression risk and antidepressant treatment outcome (Palma-Gudiel & Fañanás, 2017; Shen et al., 2020a; Ziegler & Domschke, 2018).

The serotonin transporter gene (*SLC6A4*) is the most extensively studied gene (Palma-Gudiel & Fañanás, 2017), although similar findings were also reported for *TPH2* (Shen et al., 2020a; Zhang et al., 2015) and *MAOA* (Ziegler & Domschke, 2018). Notably, *SLC6A4* methylation levels have been repeatedly linked to childhood adversities (Palma-Gudiel & Fañanás, 2017) and, to a minor extent, to recent stress (Alasaari et al., 2012), depressive and panic disorder symptoms (Leibold et al., 2020; Palma-Gudiel & Fañanás, 2017). In addition, pre-treatment *SLC6A4* methylation levels have been linked to clinical outcomes after antidepressant treatment by several studies (Domschke et al., 2014; Kang et al., 2013; Schiele et al., 2021).

Nonetheless, findings are extremely heterogeneous and replications of former studies is strongly needed.

Specifically, major limitations in existing literature involve: 1) unclear direction of the findings, with studies reporting that both hyper- and hypomethylation of serotonin-relevant genes is associated with e.g. greater childhood trauma, increased depressive symptoms and worse treatment outcome; 2) lack of correction for blood cell type proportions: given that DNA methylation can be a tissue-specific mechanisms and that blood is a heterogeneous tissue, different proportions of blood immune cell types across individuals might have biased previous results and possibly explain the heterogeneity of current findings; 3) different CpG sites being examined in different studies, making comparison across findings challenging; 4) differential results depending on whether relevant genotypes (e.g. 5-HTTLPR) for gene expression were included or not in the analyses; 5) small sample sizes, with many studies being based on N<100 and most of studies based on N<200 participants; 6) heterogeneous methodology, e.g. in the methods used for DNA methylation determination or in the CpG sites examined.

6.2. Strengths and limitations of studying DNA methylation in human participants

Thanks to its extreme stability, DNA methylation is particularly suitable to study epigenetic hallmarks of disease in clinical samples. For example, it can be easily examined in frozen tissues

and DNA methylation levels remain unchanged for years upon collection (Li et al., 2018). In human participants, DNA methylation is most commonly investigated in easily accessible tissues, such as blood or saliva. While this can present several advantages e.g. for the development of clinical tools, it also poses some limitations to the interpretation of research findings.

Major strengths include: 1) blood can be easily collected and with extremely low risk for the patients; 2) as blood comes in contact with all body compartments, including the brain, it might indirectly reflect pathological processes occurring in the brain (Aberg et al., 2013); 3) blood-derived DNA methylation signatures might also inform on (dys)function of the immune system in the pathological status, providing novel insights into disease mechanisms; 4) some studies report covariation between methylation of some CpG sites measured in blood and in post-mortem brains (Aberg, et al., 2020a; Hannon et al., 2015). Nonetheless, DNA methylation is tissue-specific and CpG sites that are relevant for disease might lie in the 20-30% of CpG sites that are not correlated between the two tissues. In addition, molecular signatures measured in post-mortem brains can depend on factors related to how the tissue is handled, such as the time between tissue collection and processing, resulting in relevant differences between in-vivo and post-mortem conditions (Rhein et al., 2015). Thus, more research is needed to understand the relation between DNA methylation measured peripherally and in-vivo brain architecture, especially when the genes of interest are involved in functions that are relevant for neurotransmission (e.g. *SLC6A4* or *TPH2*).

Another issue is the complexity of the environmental factors (exposome) that humans are exposed to. DNA methylation can be induced by a plethora of factors, e.g. temperature, medications, stress, physical activity, diet, which could confound the DNA methylation findings. While in animal experiments the environmental conditions are maintained as stable as possible (thus, environmental effects on DNA methylation should be limited and homogeneous across animals reared in the same conditions), it is nearly impossible to map all the factors that could affect human participants' methylome. Thus, to disentangle the effects of interest from other confounding factors, deep phenotyping of the participants is really needed, along with replication studies, solid study designs involving longitudinal, randomized controlled trials and direct interventions.

AIMS AND HYPOTHESES

The overarching aim of this thesis was to gain a better understanding of the effects of genetic and epigenetic variation within serotonin-related genes on serotonin neurotransmission, both in healthy and disease (depression) conditions and its relevance in reflecting depression status or early life stress as well as guiding antidepressant treatment choices.

Study I

The aim of this study was to determine the association between common genetic variants within genes related to serotonin function (*SLC6A4*, *HTR1A*, *HTR2A*, *MAOA*, *BDNF*) and brain 5-HTT levels in a cohort of healthy participants and to predict brain 5-HTT levels based on genetic variation. We hypothesized that variants that can affect serotonin or 5-HTT protein levels would affect brain 5-HTT binding.

Study II

The aim of this study was to investigate whether DNA methylation measured peripherally (i.e. blood) in genes key for serotonin neurotransmission (*SLC6A4*, *TPH2*) are linked to brain proxies of serotonin neurotransmission (5-HTT, 5-HT_4) binding in both healthy controls and patients with depression. In addition, we evaluated whether early life and recent stress, depressive and anxiety symptoms were associated with *SLC6A4/TPH2* methylation. We hypothesized that peripheral DNA methylation of *SLC6A4* and *TPH2* is associated with 5-HTT and/or 5-HT_4 binding and early life/recent stress and depressive and/or anxiety symptoms, making of peripherally-measured *SLC6A4* and/or *TPH2* promising biomarkers reflecting gene-by-environment interactions that may influence brain serotonin neurotransmission.

Study III

The aim of this study was to evaluated whether *SLC6A4* and/or *TPH2* methylation can be used as biomarkers to predict clinical outcomes after antidepressant treatment. We also aimed to replicate previous findings specifically linking two *SLC6A4* CpG sites (chr17: 30,236,071; chr17: 30,236,083) and clinical outcomes following antidepressant treatment. Based on previous literature, we hypothesized that greater baseline *SLC6A4* methylation, supposedly corresponding to lower 5-HTT levels, and therefore increased 5-HT levels, would be associated with better clinical response after SSRI treatment. Similarly, we hypothesized that *TPH2* hypomethylation, supposedly corresponding to greater TPH2 levels and therefore higher 5-HT levels, would be associated with better clinical outcomes.

Study IV

The aim of this study was to replicate the previously reported associations between DNA methylation of *SLC6A4* and *TPH2* and depression status or childhood trauma in four large datasets from methylome-wide association studies (MWAS). We also aimed to evaluate whether centrally relevant serotonin-related genes are enriched in the association between DNA methylation and depression and childhood trauma. Finally, we examined whether DNA methylation of a set of 27 centrally-relevant serotonin genes was associated with depressive symptoms and long-term depression outcomes (i.e. 2 years), especially in those patients that were taking serotonergic-acting antidepressant medications, in one large cohort with longitudinal data. We hypothesized that serotonin-related genes would be enriched in the link between depression and childhood trauma and that we would detect patterns pointing to decreased serotonin neurotransmission that are associated with greater depressive symptoms and depression chronicity at 2-year follow-up, especially in patients treated with antidepressant medications.

METHODS

7. Study Cohorts

All studies were carried out in compliance with the declaration of Helsinki. A general overview of the demographic characteristics of the participants included in the studies is provided in **Table 3**. More detailed information can be found in the articles attached in the Appendix.

Study	Healthy Participants	Patients with MDD	Age	Sex (F/M)
Study I	140	-	26.7 ± 7.2	84/56
Study II	297	90	29.6 ± 12.4	273/150
Study III	-	89	26.7 ± 7.7	63/26
Study IV	14078	2215	50.3± 15.4	7352/8941

Table 3. Basic demographics of the cohorts included in Study I-IV.

Cimbi database (Study I and II)

Healthy participants were selected from the **Cimbi** database (Knudsen et al., 2016) based on availability of the following information: 1) [^{11}C]DASB (Study I and II) or [^{11}C]SB207145 PET scans (Study II); 2) blood samples for genotyping (Study I & II) or evaluation of DNA methylation (Study II); 3) *SLC6A4* 5-HTTLPR and rs23351 and *BDNF* rs6265 (val66met) polymorphisms. To evaluate associations between *SLC6A4* or *TPH2* methylation and measures of early life stress, depressive or anxiety symptoms or currently perceived stress (Study II), we included data of healthy participants based on the availability of: 1) blood samples and 2) measurements of environmental stress, anxiety and depressive symptoms.

Participants were included only if they: 1) were younger than or were 60 years of age; 2) did not have a diagnosis of either a primary psychiatric disease, a severe systemic or a neurological disease; 3) had no history or sign of drug abuse; 4) self-identified with European ancestry. In addition, in Study II we only included participants for whom blood samples and PET scans were collected no more than one week apart.

We identified N=140 healthy participants with [^{11}C]DASB PET scans for Study I and N=142 for Study II. We identified N=112 healthy participants with [^{11}C]SB207145 PET scans and N=297 healthy participants with measures of environmental stress or depressive or anxiety symptoms for Study II.

For detailed demographics of the healthy participants included in the studies, see Table 1 of Study I (Bruzzone et al., 2023) or Table 2 and S1 of Study II (Bruzzone et al., 2024).

NeuroPharm 1 cohort (Study II and III)

MDD patients were part of the NeuroPharm-1 (**NP1**) trial, which is an open-label, non-randomized longitudinal study (Köhler-Forsberg et al., 2020). Patients were included in the studies based on the availability of: 1) blood samples (Study II & III); 2) [^{11}C]SB207145 PET scans. Detailed inclusion and exclusion criteria for the trial are listed in (Köhler-Forsberg et al., 2020).

The trial involved administering the SSRI escitalopram to previously unmedicated patients and monitoring them over a 12-week period. In case patients did not respond to escitalopram by trial week 4, they were switched to the serotonin and norepinephrine reuptake inhibitor (SNRI) duloxetine.

In Study I, we only included the unmedicated patients that were recruited at baseline. In study III we included data from patients at baseline, trial weeks 8 and 12. We selected N=90 patients for whom the data of interest were available at baseline. Data for N=76 and N=72 patients were available at weeks 8 and 12 respectively. Detailed demographics are provided in Table 1 of Study II (Bruzzone et al., 2024) and Table 1 of Study III (Bruzzone et al., 2025).

Study cohorts for replication (Study IV)

We used data from four established cohorts. Specifically, we used data from:

- N=1132 participants (n=812 MDD patients, n=320 healthy controls) from the Netherlands Study of Depression and Anxiety (NESDA) (Penninx et al., 2008).
- 1034 samples from N=560 participants of the Great Smoky Mountains Study (GSMS) (Costello et al., 2016).
- N=14443 participants (n=1290 MDD patients, n=13153 healthy controls) that were provided by TruDiagnostics (https://www.trudiagnostic.com/) and will be referred to as TD.
- N= 206 donors (n=113 MDD, n=93 without MDD) from the MDDbrain cohort (Aberg, et al., 2020b).

8. Measures of environmental stress and psychiatric state traits (Study II, IV)

In the healthy cohort from the Cimbi database, we used the following as measures of environmental stress:

- The stressful life events (SLE) questionnaire, which we used as a measure of both recent and lifetime stress. Statistical analyses were performed on recent and total SLE scores separately.
- The parental bonding inventory (PBI), which we interpreted as a measure of early life environment and therefore a proxy for early life stress. For both mother and father, PBI

provides measurements of the "overprotection" and "care" subscales. For statistical analyses, a combined score of both parents was used for care and overprotection.

In the NP1 cohort, we used:

- PBI
- Perceived stress state (PSS), which we used to evaluate recent subjective stress.
- Childhood abuse trauma scale (CATS), which indicated the presence of severe traumas experienced throughout childhood.
- Generalized anxiety disorder 10-item (GAD10), to evaluate presence of comorbid anxiety symptoms.
- Beck's Depression Inventory (BDI), which described recent depressive symptoms.

In Study IV, childhood trauma was estimated using the Childhood Trauma Inventory (CTI) (De Graaf et al., 2004) in the NESDA cohort and using scores from the Child and Adolescents Psychiatric Assessment (CAPA/YAPA) (Angold & Jane Costello, 2000) in the GSMS cohort.

9. Clinical Assessments and Outcomes (Study II, III, IV)

For patients of the NeuroPharm trial (Study II & III), MDD was diagnosed by a trained clinician based on a face-to-face diagnostic interview supported by the Mini International Neuropsychiatric Interview (MINI) and confirmed by a certified psychiatrist.

The 6- and 17-item Hamilton depressive rating scale (HAMD) were used as an estimate of depressive symptoms severity (Hamilton, 1967). All MDD patients had moderate to severe depression ($HAMD_{17}$>17). More details regarding diagnostic, inclusion and exclusion criteria are described in the original protocol article (Köhler-Forsberg et al., 2020).

In Study III, we defined a categorical and a continuous clinical outcome for statistical analyses based on the HAMD scores. These outcomes were used to get an estimate of which patients were more likely to show an improvement in symptoms after taking SSRIs, although the lack of a placebo-control group does not allow to draw causal conclusions. The categorical outcome dichotomized MDD patients between responders and non-responders: responders showed a ≥ 50% decrease in $HAMD_{17}$ scores at trial week 8; non-responders showed a < 50% decrease. The continuous outcome consisted in continuous values of percent change of $HAMD_6$ scores at trial week 8 vs $HAMD_6$ score at baseline: (week 8 $HAMD_6$ – baseline $HAMD_6$/baseline $HAMD_6$)*100.

For patients of Study IV, MDD status was defined differently for each cohort. In NESDA, patients were diagnosed by clinicians using the Composite International Diagnostic Interview (CIDI)

interview. Severity of depressive symptoms was measured with the Inventory of Depressive Symptomatology (IDS). In GSMS, with scores from the CAPA/YAPA. In TD, depression status was based on subjective reports (yes/no answer) to the question "were you ever diagnosed with depression?". In MDDbrain, depression was defined according to family reports.

10. DNA purification (Study I, II, III)

DNA was purified from whole blood (Study I, II & III) or buffy coat (Study I & II) samples. Before processing, the samples were stored at either -20°C or -80°C. DNA was isolated according to the manufacturer's protocol with either the QIAamp DNA Blood Mini Kit (Qiagen,Valencia, CA) or of the FlexiGene Kit (Qiagen, Hilden, Germany) (Study II & III). DNA concentration and purity levels were evaluated using an UV-Vis spectrophotometer (Nanodrop 2000, Thermo Scientific or the NanoPhotometer N60, Implen).

11. Genotyping

In this thesis, two different techniques were used. The first technique involves three main steps: 1) DNA amplification of the DNA sequence of interest via polymerase chain reaction (PCR); 2) digestion of the amplified fragments with restriction enzymes, which cuts different genotypes into DNA fragments of different lengths and 3) electrophoresis on agarose gel followed by reading and interpretation of the bands appearing on the gel. The genotype is established based on the molecular weight and number of bands observed on the gel. The second technique involves using Taqman Genotyping Assays (T. Fisher, n.d.), which simply consists in 1) amplifying DNA using a heat-resistant polymerase enzyme (TaqMan polymerase) and primers (probes) including a fluorescent dye, using a real-time PCR (RT-PCR) system and 2) reading the results through the RT-PCR system software, which will show different colors for different genotypes (e.g., in the case of a A>G mutation, green for AA, blue for AG and red for GG). While the first technique is cheaper and more advantageous when it comes to genotyping highly redundant (e.g. promoter regions with CpG islands) and polymorphic DNA regions (e.g. HTTLPR in *SLC6A4*), as well as genetic variants other than single-nucleotide polymorphisms (SNPs), e.g. copy-number variants, it is also more time consuming and laborious. In addition, as the genotype call is made by the experimenter based on the gel image, it can be more error-prone. Taqman genotyping assays instead, provide a cost-effective tool which is less time consuming and less error-prone, although it can only be used for SNPs which are not located in highly polymorphic or repetitive regions.

Genotyping (Study I, II, III)

HT1AR rs6295, *HT2AR* rs7333412 (Study I), *BDNF* rs6265 (val66met), and MAOA rs1137070 (Study I & II) single-nucleotide polymorphisms (SNP) were genotyped using TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA) with genotype-specific probes (*BDNF*: C_11592758_10, *HTR1A*: C__11904666_10, *HTR2A*: C__29235757, *MAOA*: C___8878813_20). Allelic discrimination was carried out using the LightCycler 480 RT-PCR System (Roche Diagnostics, IN). Participants dichotomization based on genotype was performed as follows: *HT1AR:* CC vs G*; HT2AR:* AA vs G.*; BDNF:* val/val (G) vs met (A); *MAOA*: CC vs T-.

The L and S alleles of the 5-HTTLPR variant (Study I, II & III) were determined using PCR amplification followed by gel electrophoresis on a 2% agarose gel. Forward primer sequence was 5'-TAATGTCCCTACTGCAGCCC-3'; reverse primer sequence was: 5'-GGGACTGAGCTGGACAACC-3'. The G/A mutation in the 5-HTTLPR region (rs25531) was detected by digesting the PCR product with the restriction enzyme MspI and then running gel electrophoresis for allelic discrimination. Genotypes of 5-HTTLPR combined both the L/S variant and the rs25531 SNP. For statistical analyses, participants were divided into L_AL_A vs S-carriers. Detailed protocols have been previously described (Fisher et al., 2017; Fisher, et al., 2015a; Madsen et al., 2015). Similarly, *TPH2* rs4570625 (G-703T) (Study II & III) was determined using PCR amplification with specific primers (forward: 5'-tttccatgatttccagtagagag-3'; reverse: 5'-aagctttttctgacttgacaaat-3') followed by enzymatic digestion with APOI and gel electrophoresis on 3% agarose gel for allelic discrimination. Detailed protocol was previously described by Gutknecht et al. (2007).

Hardy-Weinberg equilibrium (Study I, II, III)

Allelic frequencies were in Hardy-Weinberg equilibrium ($p>0.1$) for all genotypes in all cohorts, except for 5-HTLPR in the cohort including imaging data of 5-HTT ($p=0.01$; Study I, II) and *TPH2* rs4570625 in the healthy cohort including 5-HT_4 imaging data ($p=0.01$; Study II). In the first case, it was expected due to *SLC6A4* 5-HTTLPR genotyping information being an inclusion criteria to obtain equally-sized groups in previous studies involving 5-HTT imaging (Frokjaer et al., 2015; Mc Mahon et al., 2016). In the second case, it happened by chance, as the genotyping was performed on all cohorts included in Study II in an unbiased manner and it was in equilibrium on the whole cohort but not in this specific subgroup.

12. DNA Methylation Assays

There are various methods to determine DNA methylation levels. In this thesis we used three main methods, depending on the research questions and the available data.

The first method is bisulfite conversion followed by pyrosequencing. It is the method that provides estimates at the single-base resolution with the highest precision and is currently considered as the "gold standard" to estimate DNA methylation levels (Tost, 2018). This method was used in Study II and III. However, the elevated costs along with intrinsic technical limitations (e.g. only short – 300-500bp - genomic sequences can be efficiently sequenced), do not allow to use pyrosequencing to perform large-scale studies on clinical cohorts. Instead, methods such as Methyl-CpG-Binding Domain Sequencing (MBD-seq) and the Infinium Human Methylation EPIC (Illumina) arrays, which cover a much larger large portion of the genome (the first ~28,000,000 CpG sites and the latter ~850,000 CpG sites), are preferrable when the focus is having an overview of the human methylome. These methods were used in Study IV.

12.1. Bisulfite conversion and pyrosequencing

This method involves four main steps (which are illustrated in **Figure 5**):

1. Bisulfite conversion: treating DNA with sodium bisulfite induces a conversion of all the non-methylated cytosines into uracil molecules. This way, methylated cytosines within CpG sites remain cytosines while all the other cytosines in the genome are converted into uracil molecules.

2. DNA amplification: DNA is amplified with polymerase chain reaction (PCR). During PCR amplification, uracil is replaced with thymine by the DNA polymerase. The reverse primer is conjugated to a biotin molecule, which will be incorporated in the amplicon and will allow the subsequent DNA isolation.

3. Isolation of the DNA template: DNA is first denaturated at 90°C. Then, using streptavidin-coated beads, only the amplicons containing biotin are isolated. Streptavidin binds to biotin in a covalent and highly specific manner, allowing to efficiently purify molecules of interest. The isolated DNA molecules represent the template DNA that will be pyrosequenced.

4. Pyrosequencing: It involves a series of consecutive reactions, involving the use of the single-strand DNA amplicons, the deoxynucleotides triphosphates (dNTP) for each nucleotide, adenosine 5' phosphosulfate (APS) and the enzymes luciferase, DNA polymerase and sulfurylase. Pyrosequencing is a "sequencing by synthesis" method, meaning that the sequences of interest are determined by synthesizing the DNA sequence of interest. Thus, at each cycle, one dNTP is released by the pyrosequencer. If the dNTP corresponds to the base complementary to the one on the DNA molecule of interest (e.g. if the first base on the sequence is A and T is added), the DNA polymerase incorporates the dNTP. When incorporating the dNTP, a pyrophosphate (PPi) molecule is released. PPi is used by the sulphurylase to convert APS into

ATP. Then, the luciferase uses ATP to oxidize luciferin into oxyluciferin and release light, which is read by a charge coupled device camera. This generates a peak whose height is proportional to the number of nucleotides incorporated. The graph depicting the peaks is referred to as pyrogram. Finally, the apyrase degrades the dNTPs and ATP that were not used and the reaction restarts (Qiagen, n.d.).

Although a CpG site can only be methylated or non-methylated, methylation estimates are provided in terms of percentages. These values represent the percentage of DNA molecules containing a methylated CpG site vs those that contained a non-methylated CpG site.

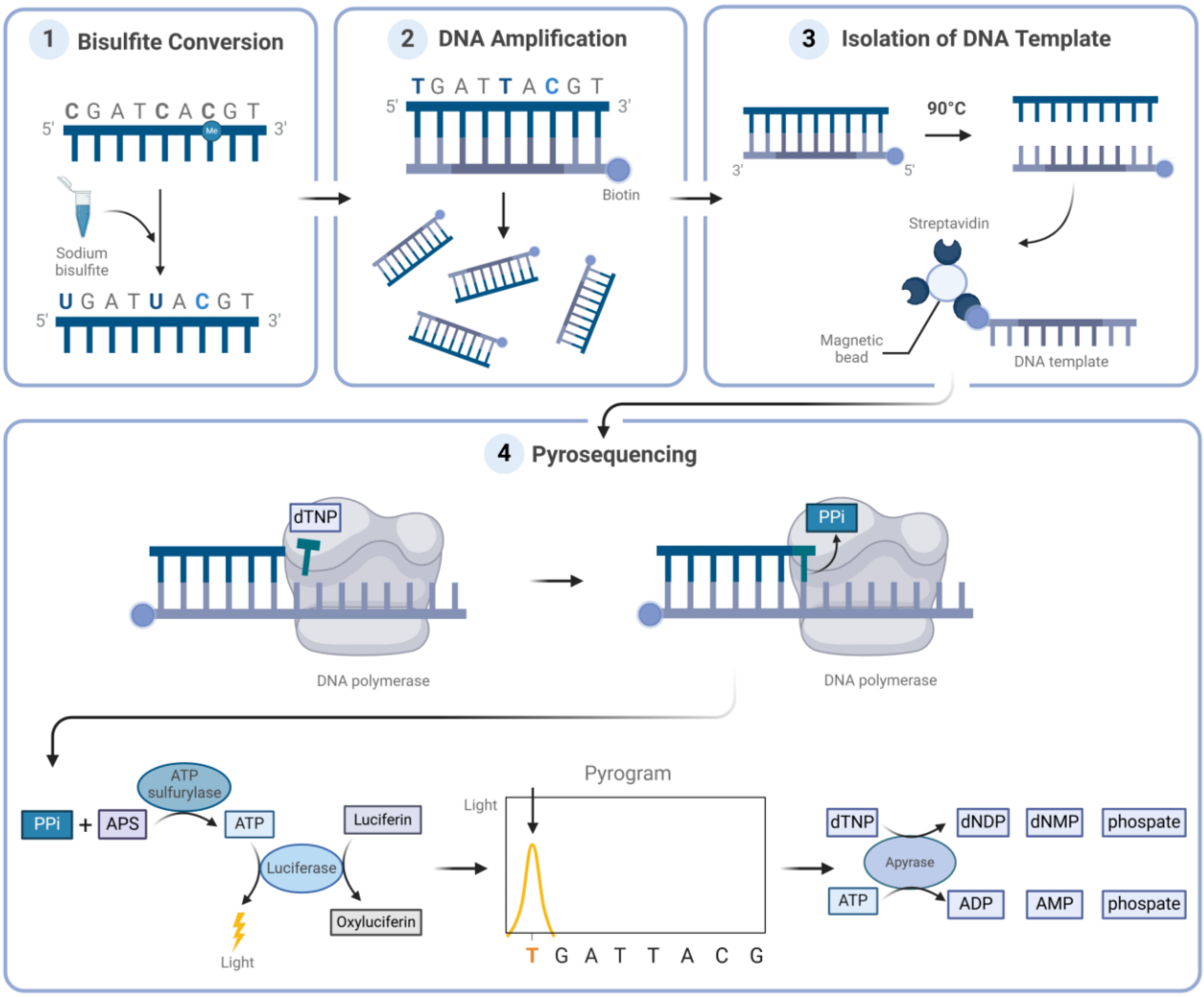

Figure 5. *Main steps of bisulfite conversion and pyrosequencing. A: adenine; C: cytosine; G: guanine; T: thymine; PPi: pyrophosphate; APS: adenosine 5' phosphosulfate; ATP: adenosine triphosphate; ADP: adenosine diphosphate; dNTP: deoxynucleotides triphosphates; dNDP: deoxynucleotides diphosphates; AMP: adenosine monophosphate. Created with BioRender.com by S. E. P. Bruzzone.*

SLC6A4 and TPH2 methylation in Cimbi and NP1 cohorts (Study II and III)

In Study II and III, SLC6A4 methylation was measured at four CpG sites within the CpG island in its promoter region (chr17:30236071-30236090), while *TPH2* methylation was estimated at six CpG sites at 5' UTR of the gene (chr12:71938979-71938877). The CpG sites were selected based on previous studies linking them to gene expression (Iga et al., 2016; Philibert et al., 2007; Zhang et al., 2015), symptoms of psychiatric disorders such as MDD (Iga et al., 2016; Kang et al., 2013; Kim et al., 2013), panic disorder (Leibold et al., 2020), attention deficit disorder (Akhrif et al., 2023) or antidepressant treatment outcome (Domschke et al., 2014; Kang et al., 2013; Schiele et al., 2021; Shen et al., 2020b; Tan et al., 2022) as well as to current (Alasaari et al., 2012) or early life (Kang et al., 2013; Shen et al., 2020b) stress.

DNA samples were first bisulfite-converted with the EpiTect 96 Bisulfite Kit (Qiagen) and then amplified using PCR. Gel electrophoresis was used to quality-check the PCR outcome. Then, the amplicons were pyrosequenced using the PyroMark Q96 ID (Qiagen) system. Fully methylated (100%), non-methylated (0%), partially methylated (50%) samples (Epitect PCR Control DNA Set, Qiagen) and DNAse free H_2O were used as controls. Analyses of each sample were run in duplicates. The average between two duplicates was used as input for statistical analyses. The PyroMark software (Qiagen) provided methylation percentages, pyrograms and quality reports. The outputs of the pyrosequencing were quality-checked by visual inspection of the pyrograms and evaluation of the quality reports. More details on the protocol as well as primer sequences are reported in the method section and Table S3 of Study II (Bruzzone et al., 2024).

12.2. MBD-seq

MBD-seq is an affinity-based capture method. The method involves using methyl-CpG-binding domain (MBD) proteins, which can recognize and specifically bind to methylated CpG sites (Du et al., 2015). Shortly, DNA is first fragmented into ~150bp fragments, which are incubated with MBD-coated beads. This step allows to only select the methylated fragments, that will be used to create a sequencing library and will be then sequenced using next generation sequencing. Reads are subsequently aligned and data are quality controlled. Finally, after filtering and normalizing the data, statistical analyses are performed (Aberg et al., 2018). Contrary to pyrosequencing, which provides methylation estimates as percentages for each CpG site, MBD-seq output consists of the sum of the total amount of methylation at a locus, where the locus can be equal to or smaller than the sequenced fragments (~150bp). Consequently, the methylation status of neighboring CpG sites tends to be highly correlated (Aberg et al., 2020).

The main advantages of MBD-seq consist in: 1) high coverage of the human genome (almost complete, ~28 million CpG sites); 2) cost-effective and 3) comparable results to those obtained with other "gold standard" methods (e.g. whole-genome bisulfite sequencing or Illumina Methylation Arrays). Main limitations include: a) no single-base resolution; b) lack of coverage for hydroxymethylated CpGs or methylated cytosines that are not followed by guanines, c) lack of absolute quantitative methylation levels, d) lower or no representation of hypo- and unmethylated CpG sites respectively and e) data analysis is more challenging due to the larger amount of data (Aberg, et al., 2020).

MBD-seq in the NESDA, GSMS and MDDbrain cohorts (Study IV)

DNA methylation in blood and postmortem brain samples was estimated using an optimized protocol for MBD-seq (Chan et al., 2017) whose specifics have been previously described in details (Aberg, et al., 2020a; Clark et al., 2020). Briefly, DNA was fragmented into ~150bp (blood samples) or ~50bp (brain samples) sequences using ultrasonication and the methylated regions were isolated using MethylMiner™ (Invitrogen). The isolated fragments were used to prepare a library which was sequenced with NextSeq500 (Illumina) (blood samples) or SOLiD 5500xl Wildfire (Life Technologies). Then, reads were aligned using Bowtie2 (Langmead & Salzberg, 2012) and data were quality-controlled and processed using RaMWAS, a Bioconductor package (Shabalin et al., 2018). After quality control, the total number of CpG sites available for data analyses was 21,869,561 for the NESDA cohort, 22,670,747 for the GSMS cohort and 22.0 million in brain samples.

12.3. Illumina Infinium Methylation EPIC BeadChips arrays

In contrast with MBD-seq, Illumina Infinium Methylation EPIC BeadChips provide high-resolution quantitative, site-specific DNA methylation estimates. However, the number of CpG sites analyzed is predetermined and limited by the number of probes (~850,000), which usually target specific regions (e.g. CpG islands, promoters). The method is based on different principles from MBD-seq, as bisulfite-treated and fragmented (150-300bp) DNA is hybridized to CpG-specific probes that are immobilized on a BeadChip. Methylated vs non-methylated status is defined based on the incorporation of a complementary nucleotide, which is labelled with one of two fluorescent molecules. The fluorescent signal is detected by a scanner, that defines the probe as methylated or non-methylated based on the detected fluorescent signal. Data are then background-corrected, quality controlled, normalized and filtered. DNA methylation estimates (β) are derived from the ratio between methylated and non-methylated signal according to the

formula: β=methylated signal/unmethylated signal + methylated signal +100. Finally, statistical analyses are performed (Illumina, n.d.; Pidsley et al., 2016).

Illumina EPIC BeadChips arrays in the TD cohort (Study IV)

500ng of bisulfite-converted DNA were placed in a well of the MethylationEPIC BeadChip and imaged with the Illumina iScan SQ (Illumina) for each sample. Data was first normalized using the *minfi* package (Teschendorff et al., 2013) and then quality-controlled to discard faulty probes or samples, according to a workflow that was previously reported (Chan et al., 2020). After quality control, 595,218 CpG sites were available for statistical analyses.

12.4. Selection of serotonin-related genes (Study IV)

Serotonin-related genes were identified by using the KEGG Atlas (Okuda et al., 2008) and literature (Daws, 2021; Sharp & Barnes, 2020). We initially included 29 genes encoding for all serotonin receptors, transporters, serotonin biosynthesis and degradation. However, as *MAOA* and the gene encoding for serotonin receptor 2C (*HTR2C*) are located on the X chromosome, they were excluded from analyses, resulting in a total of 27 genes tested. For each gene, we included CpG sites within the entire gene of interest (GRCh37/hg19 from UCSC Genome Browser, https://genome.ucsc.edu/), as well as a 10kb region upstream to gene start, indicating the putative promoter region.

13. Positron Emission Tomography

Positron emission tomography (PET) is a molecular imaging method that allows us to quantify proteins of interest in the living human body and brain. This is extremely useful when it comes to studying in-vivo pharmacological interactions or the structure of complex molecular systems such as the serotonin system (e.g. via receptor or transporter binding), which could otherwise be studied only in post-mortem brains. Specifically, in the studies included in this thesis, PET was used to estimate 5-HTT and 5-HT_4 receptor binding.

With PET, targets of interest can be visualized via a radiotracer, which specifically binds to the target protein (e.g. a receptor), and which is administered to participants (typically via intravenous bolus injection) shortly before the scan. Radiotracers are labelled with a radioactive isotope, which gradually decays, emitting a positron. After travelling for a short distance (0.2-2mm), the positron collides with an electron and the two particles annihilate, releasing two gamma rays that are emitted at an angle of about 180° from each other. The gamma rays are then detected by the PET camera, which is made of a ring of detectors (**Figure 6**).

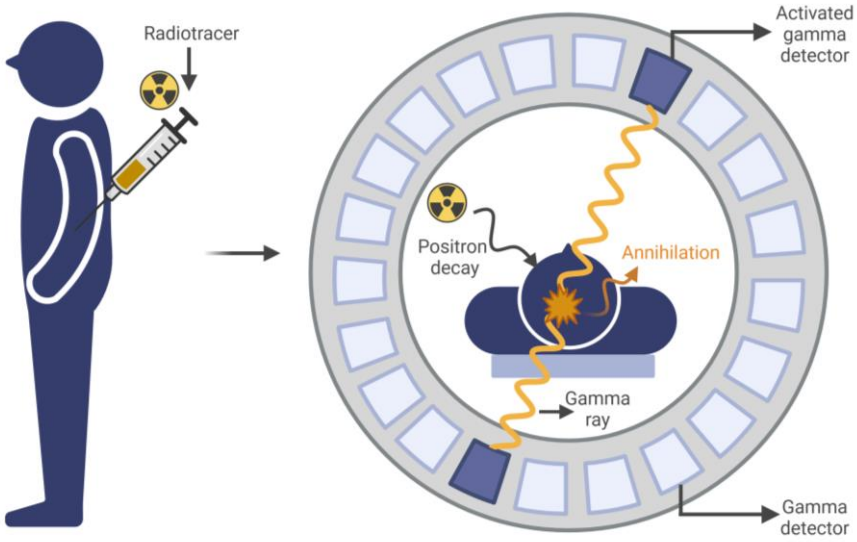

Figure 6. *Overview of the basic principles of positron emission tomography (PET) signal. Participants are injected with a radiotracer, which emits a positron while decaying. When the positron collides with an electron, it annihilates, releasing gamma rays. These gamma rays are detected by the gamma detectors in the PET camera. Created with BioRender.com by S.E.P. Bruzzone.*

Following the scan, which lasts about 60-120 minutes, data are pre-processed. This involves motion correction, to eliminate potential artefacts due to minimal brain movements occurred during the acquisition, and co-registration with a T1-weighted structural magnetic resonance (MR) image, which allows to reconstruct the PET signal into brain regions of interest.

Finally, receptor binding (corresponding to the density of receptors that are not bound to their endogenous ligands and are therefore available) is estimated using kinetic modelling (e.g. the simplified or multilinear reference tissue model), which provides the non-displaceable binding potential (BP_{ND}). BP_{ND} describes the amount of radiotracer that is specifically bound to its target (e.g. a receptor or transporter) relative to free or non-specific binding. The non-specific binding is described based on a reference tissue, which is an area that is known to have low target binding.

The following section describes the PET imaging methods that were used in Study I and II to image 5-HTT and 5-HT_4 receptor in healthy participants and MDD patients.

[^{11}C]DASB and [^{11}C]SB207145 PET scans (Study I and II)

5-HTT and 5-HT_4 binding of each healthy participant and MDD patient was estimated using PET and kinetic modelling.

Briefly, participants first received a bolus injection of the specific radiotracer ([^{11}C]DASB for 5-HTT or [^{11}C]SB207145 for 5-HT$_4$) and then underwent a 90-min (for [^{11}C]DASB) or a 120-min (for [^{11}C]SB207145) dynamic scan. In-plane resolution of the 3D images was either 2mm or 6mm, depending on the scanner used (Siemens High-resolution Research Tomography [HRRT] [CTI/Siemens] or an 18-ring GE-Advance PET scanner [General Electric, Milwaukee, USA], respectively). Participants were also scanned with a 3-Tesla MR to get T1-weighted MPRage structural images. MPRage scans were taken using either a Trio, a Verio or a Prisma scanner. MR and PET scans were co-registered using either automatic image registration (GE-Advance) or SPM (Tzourio-Mazoyer et al., 2002) (HRRT). Next, PVElab was used to automatically outline brain regions of interest. PMOD (Zurich,Switzerland) was used to define regional 5-HTT or 5-HT$_4$ BP_{ND} by kinetic modelling. Multilinear reference tissue model (MRTM/MRTM2) ([^{11}C]DASB scans) or the Simplified Reference Tissue Model ([^{11}C]SB207145 scans) were used to compute mean time-activity curves for average grey matter voxels. This was done for each hemisphere. For both [^{11}C]DASB and [^{11}C]SB207145 scans, cerebellum was used as a reference region (due to low 5-HTT and 5-HT$_4$ binding in this region).

A more detailed description of the scans is provided in the methods sections of Study I and II (Bruzzone et al., 2024; Bruzzone et al., 2023).

14. Statistical Analyses

All statistical analyses were carried out in R v4.1.2 (Study I-II-III) or v4.4.1 (https://cran.r-project.org/) using relevant packages.

14.1. Study I

First, we evaluated whether either of the five serotonin-related genotypes (rs6265 [or val/met], 5-HTTLPR/rs25531, rs6295, rs7333412, rs1137070 within *BDNF*, *SLC6A4*, *HTR1A*, *HTR2A*, and *MAOA* genes respectively) was associated with 5-HTT binding. To do so, we used a latent variable model (LVM) similar to the one used by (Fisher et al., 2017). Briefly, after modeling the shared correlations between 5-HTT binding across our regions of interest (amygdala, caudate, hippocampus, midbrain, neocortex, putamen and thalamus) into a latent variable (5-HTT$_{LV}$), we modeled the association between genotypes and covariates (age, sex, MR- and PET scanner) and 5-HTT$_{LV}$. Caudate was used as a reference region.

Next, we examined whether our genotypes of interest predicted brain 5-HTT binding. To this end, for each brain region, we used a random forest model with 500 trees per forest, in which each tree had p/3 sampled features (p indicates the number of input variables). The random forest

models were embedded in a 5-fold cross-validation framework, in which residuals were permuted 10,000 times and each permutation was resampled 10 times. The root mean-squared error (RMSE) of test data over all folds was used to: 1) determine model performance; 2) compare models with vs without genotype information in terms of RMSE percent change. We defined statistical significance based on the null distribution obtained from the 10,000 permutations and corrected the p-values from each of the models (n=7) for multiple comparisons using Bonferroni correction.

14.2. Study II

As primary analyses, we determined the association between *SLC6A4/TPH2* methylation and 5-HTT or 5-HT_4 binding using LVMs, similarly to study I. We modelled regional 5-HTT or 5-HT_4 binding into latent variables (5-HTT_{LV} and 5-HT_{4LV} respectively). 5-HTT_{LV} was modelled based on the same regions of interest as those used in study I; 5-HT_{4LV} was based on 5-HT_4 binding in caudate, hippocampus, neocortex and putamen. These brain areas were determined according to 5-HTT or 5-HT_4 brain distribution. Separate models were run for *SLC6A4* and *TPH2* methylation, for a total of four LVMs (Figure 2 and 3 in (Bruzzone et al., 2024)). Age, sex PET-, MR scanner and genotypes that could affect *SLC6A4* (5-HTTLPR/rs25531) and *TPH2* gene expression (rs4570625) were included as covariates, along with genotypes that were previously associated with 5-HTT or 5-HT_4 binding (Bruzzone et al., 2023; Fisher et al., 2017; Fisher et al., 2015).

We conducted two sets of sensitivity analyses, as some information was only available for a subset of participants. The first set involved running multiple linear regression models evaluating the association between *SLC6A4/TPH2* methylation and measures of environmental stress (SLE, PBI, CATS) or psychiatric state traits (GAD10, PSS, BDI, HAMD). Results were adjusted for multiple comparisons using Bonferroni correction for the number of CpG sites (4 for models with *SLC6A4* and 6 for models with *TPH2*). The second set involved accounting for blood cells proportions in all the statistical analyses. Blood cells proportions were derived from measured blood cell counts (granulocyte precursors, lymphocytes, monocytes and neutrophils) over the total number of leukocytes. First, we modelled *SLC6A4/TPH2* methylation into latent variables (CpG_{LV}). Age, sex and cell proportions were regressed out of CpG_{LV} ($CpG_{LV+cells}$). Next, we evaluated the covariance between $CpG_{LV+cells}$ and 5-HTT_{LV}, 5-HT_{4LV} or measures of environmental stress or psychiatric state traits. Likelihood ratio tests were used to determine differences in models accounting for cells proportions ($CpG_{LV+cells}$) vs models that did not (CpG_{LV}). An overview of the statistical analyses is provided in Figure 1 from Study I (Bruzzone et al., 2024).

14.3. Study III

As primary analyses, we first determined the association between *SLC6A4/TPH2* methylation and categorical or continuous clinical outcomes using LVMs. This involved modeling *SLC6A4/TPH2* methylation into a latent variable ($SLC6A4_{LV}$ or $TPH2_{LV}$) that was adjusted for age, sex, baseline depressive symptoms and cell proportions and then regressed against clinical outcomes, for a total of four LVMs. Next, we investigated whether *SLC6A4/TPH2* methylation would predict clinical outcomes, using one receiving operating characteristics (ROC) curve per gene and quantifying their area under the curve (AUC). Finally, we examined changes of *SLC6A4/TPH2* methylation over 12 weeks of trial using linear mixed models including age, sex, week (0, 8 or 12) and genotype as covariates. For a subset of participants (n=69), we also conducted linear mixed model analysis including escitalopram plasma concentration as a covariate.

As secondary analyses, we used multiple linear regression and logistic regression models to evaluate association between single CpG sites within *SLC6A4* and *TPH2* that might have been unnoticed by modelling all CpG sites within one latent variable. Finally, we used multiple linear regressions and Pearson's correlation to confirm previous findings linking *SLC6A4* CpG1 and CpG2 to clinical outcomes (Domschke et al., 2014; Kang et al., 2013; Schiele et al., 2021). We used the same statistical models, timing (8 or 12 weeks) and type of clinical outcomes (clinical response, clinical remission, change in $HAMD_{17}$) and covariates (where available) as those used in the previous studies. An overview of the statistical analyses is provided in Figure 1 of Study III (Bruzzone et al., 2025).

14.4. Study IV

To replicate previous findings involving *SLC6A4/TPH2*, we used data from MWASs evaluating the association between DNA methylation and depression status or early life adversities using multiple linear regression models. Analyses were conducted in the RaMWAS package in R (Shabalin et al., 2018). The following covariates were used: demographics, such as sex, age, ethnicity; lifestyle variables such as smoking and alcohol use; cell-type proportions, that were estimated using either the EpiDISH package in R (Zheng et al., 2018) or reference methylomes (Aberg, et al., 2020b); assay-related artifacts, such as well position in Illumina arrays for EPIC data, batch number, peak location for MBD-seq (Aberg, et al., 2020b; Clark et al., 2020). Finally, residual variance was accounted for by regressing out the first principal component, that was identified by applying principal component analysis (PCA) and then identified using a scree test.

We used CpG sites that were previously linked to depression or early life adversities that were described by 22 studies, which are reported in Table S2 and S3 of Study IV in the appendix, along with the exact locations of the CpG sites included. Not all CpG sites that were described by previous literature were present in our datasets. Thus, in case a CpG site was not present in our datasets, we would examine the CpG site that was located the closest to the one reported by literature. Significance threshold was set to p=0.05.

Enrichment tests were conducted using the shiftR package (https://cran.r-project.org/web/packages/shiftR/index.html). Tests were conducted separately in each cohort. Top MWAS p-values were cross-classified against a gene being or not (yes/no) a serotonin-related gene. The null-hypothesis (the enrichment odds ratio equals one) was tested using Cramer's V and circular permutations, as previously described (Aberg, et al., 2020b; Chan et al., 2020).

Finally, using the NESDA cohort, we expanded our focus beyond *SLC6A4/TPH2* and examined the relation between 27 centrally relevant serotonin-related genes (13,967 CpG sites in total) and: i) depressive symptoms (IDS scores); ii) childhood trauma (CTQ scores); iii) depression chronicity after 2-year follow-up, both in the full sample (N=812) and in subgroups including iiia) patients taking TCA or SSRI medications (n=280) and iiib) patients taking SSRI medications (n=244). The motivation behind research questions iiia) and iiib) came from the fact that DNA methylation of serotonin-related genes was repeatedly linked to clinical effects following antidepressant treatment (Bruzzone et al., 2025; Domschke et al., 2014; Iga et al., 2016; Kang et al., 2013; Schiele et al., 2021). We assumed that, if participants that were taking antidepressants at baseline were still classified as "depressed" after two years, we could interpret it as a lack of response to antidepressant treatment. To address these questions, we used raw methylation data (after quality control and normalization steps). Multiple linear regression models were used to evaluate the association between DNA methylation data and outcomes i), ii), iii), iiia) and iiib). Covariates included the top principal components (as identified via PCA), technical covariates, age, sex, BMI and cell proportions. Corrections for multiple comparisons were conducted with false-discovery rate (FDR) and significance threshold was set at FDR-corrected p-value=0.1.

RESULTS

In this section, we report the main results from the analyses described in the methods section. More detailed results are described in the manuscripts that can be found in the **Appendix.**

15. Study I

In this study, we aimed to determine whether a set of genetic variants relevant to serotonin function 1) is associated with brain 5-HTT binding and 2) can predict 5-HTT binding.

Association between 5-HT-related genotypes and 5-HTT brain binding

We found that *MAOA* rs1137070 T-carriers had ~2-11% greater 5-HTT binding across different regions vs CC participants (p = 0.039), with the highest binding in caudate (~11%) and putamen (~9%) and the lowest binding in amygdala (~2%). We also observed, as previously reported (Fisher et al., 2017), that *BDNF* rs6265 (or val/met) met-carriers had 2-6% greater 5-HTT$_{LV}$ in

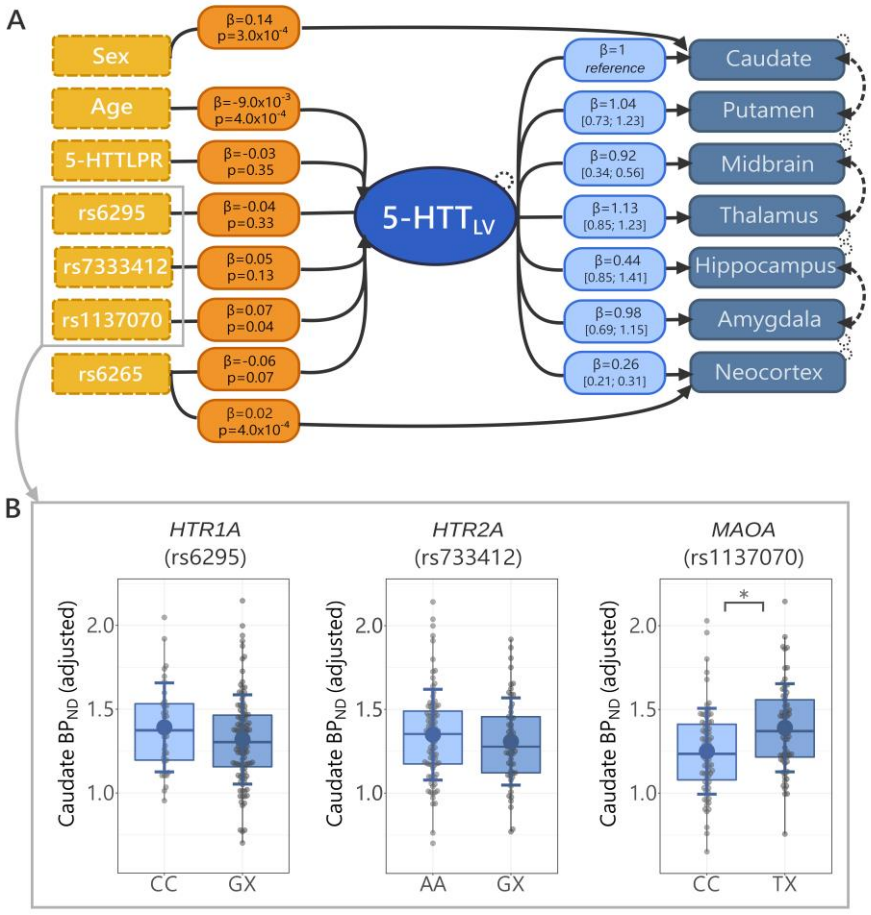

Figure 7. Statistical model and results from the latent variable model linking serotonin-related genetic variants and brain serotonin transporter (5-HTT) binding (A). Boxplots depicting 5-HTT binding for different genotypes within genetic variants within the serotonin receptor 1A (*HTR1A*), the serotonin receptor 2A (*HTR2A*) and monoamine oxidase A (*MAOA*). 5-HTT$_{LV}$:latent variable modeling 5-HTT binding in the regions of interest; β: parameter estimates. Hatched circles depict error estimates. Hatched arrows depict shared correlations across regions.

subcortical areas; greater 5-HTT_{LV} in caudate for males compared to females and a decrease in 5-HTT_{LV} with increasing age.

We observed no association between any of the other genotypes (*HTR1A* rs6295, *HTR2A* rs7333412 and *SLC6A4* 5-HTTLPR) and 5-HTT_{LV}. Results from the LVM are reported in **Figure 7**.

Prediction of brain 5-HTT binding based on 5-HT-related genotypes

Results from the prediction analyses are reported in **Figure 8**. We observed that including genotype information marginally increased prediction of 5-HTT binding before correcting for multiple comparisons (uncorrected p-value, p_{unc}=0.036). However, the statistical significance did not withstand correction for multiple comparisons. We observed no prediction improvement for any of the other examined regions (ΔRMSE = 1.6%; p_{unc}>0.05).

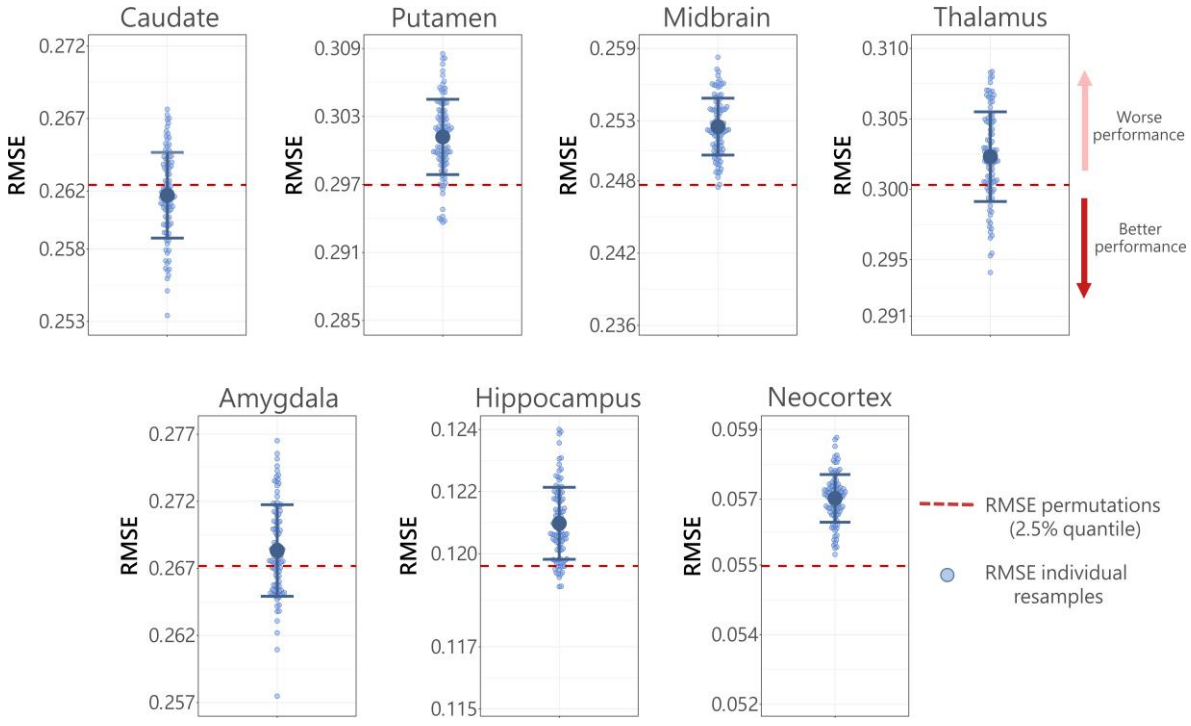

Figure 8. Plots describing random forest model performance. The dark blue dots of the error plots that are below the red hatched line (e.g. Caudate) indicate that the model including genotype information performed better than chance (with p<0.05). Dark blue error plots represent the distribution of root mean squared error (RMSE) values obtained from resampling models accounting for genotype information. Light blue dots indicate individual RMSE from these models. The red hatched line represents the 2.5% quantile of the average RMSE values obtained from models that did not account for genotype information. Reprinted from Study I, Bruzzone et al., Scientific Reports, 2023. DOI:https://doi.org/10.1038/s41598-023-43690-x

16. Study II

In this study, we aimed to determine: 1) the association between peripheral *SLC6A4/TPH2* methylation and brain binding of markers of serotonin neurotransmission (5-HTT and 5-HT$_4$); 2) the association between peripheral *SLC6A4/TPH2* methylation and measures of environmental stress or psychiatric state traits; 3) whether our correcting for blood cell proportions affected results from aims 1) and 2).

Association between peripheral SLC6A4/TPH2 methylation and brain 5-HTT or 5-HT$_4$ brain binding

Results from LVMs are reported in **Figure 9** and **Figure 10** (Bruzzone et al., 2024).

We observed no statistically significant association between *SLC6A4* or *TPH2* methylation and brain 5-HTT or 5-HT$_4$ latent variables, neither in the healthy nor in the MDD cohort. Unadjusted p-values comprised values between 0.06 and 0.97.

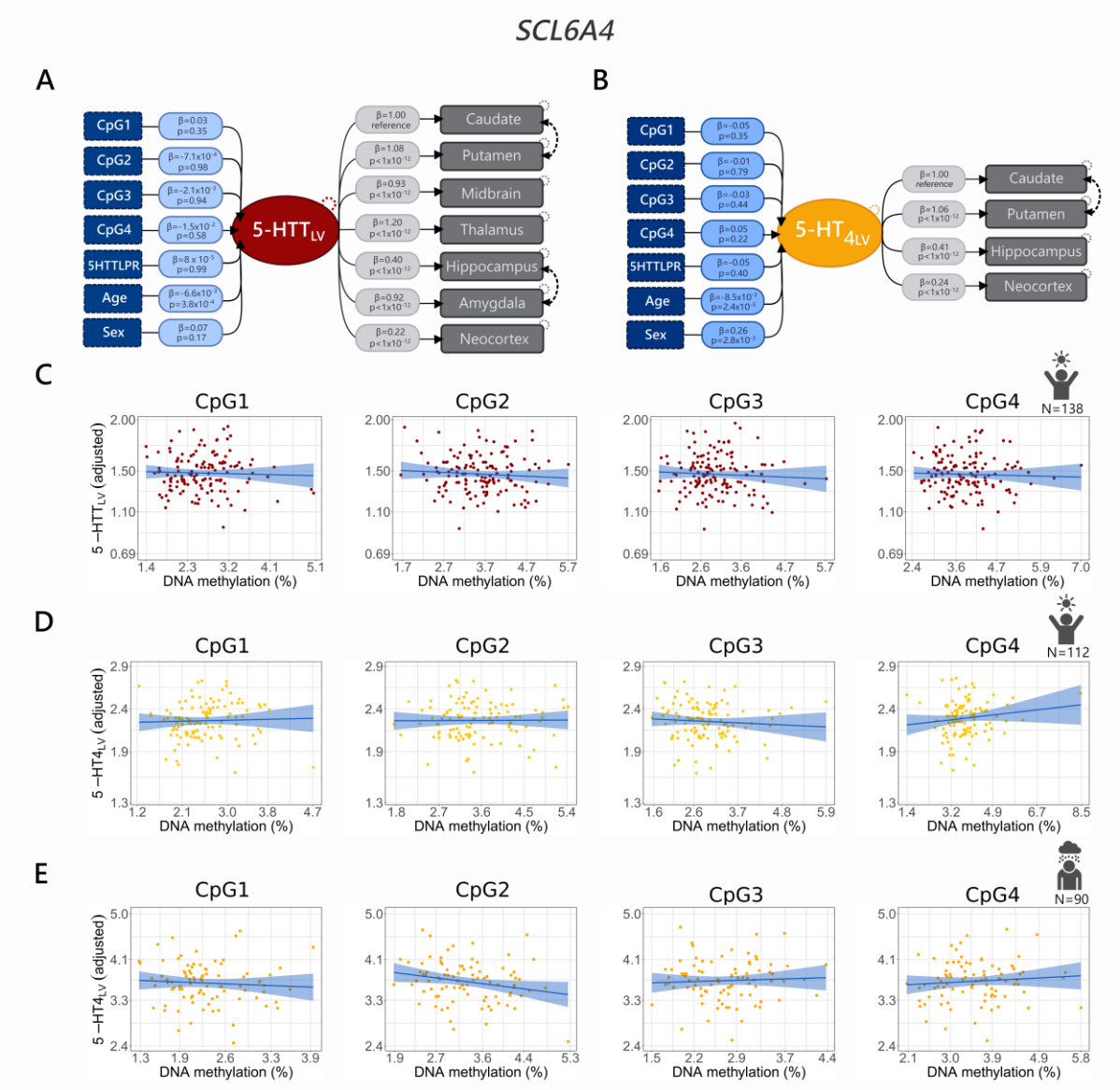

Figure 9. Latent variable models modelling the association between *SLC6A4* methylation and 5-HTT (A) or 5-HT$_4$ (B) binding. Results for associations between each CpG site and 5-HTT binding in healthy participants (C), 5-HT4 in healthy participants (D) and in patients with depression (E). Reprint from Study II by Bruzzone et al., Clinical Epigenetics, 2024. DOI: https://doi.org/10.1186/s13148-024-01678-y. This work is licensed under Creative Commons Attribution 4.0 International License (CC BY 4.0). To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

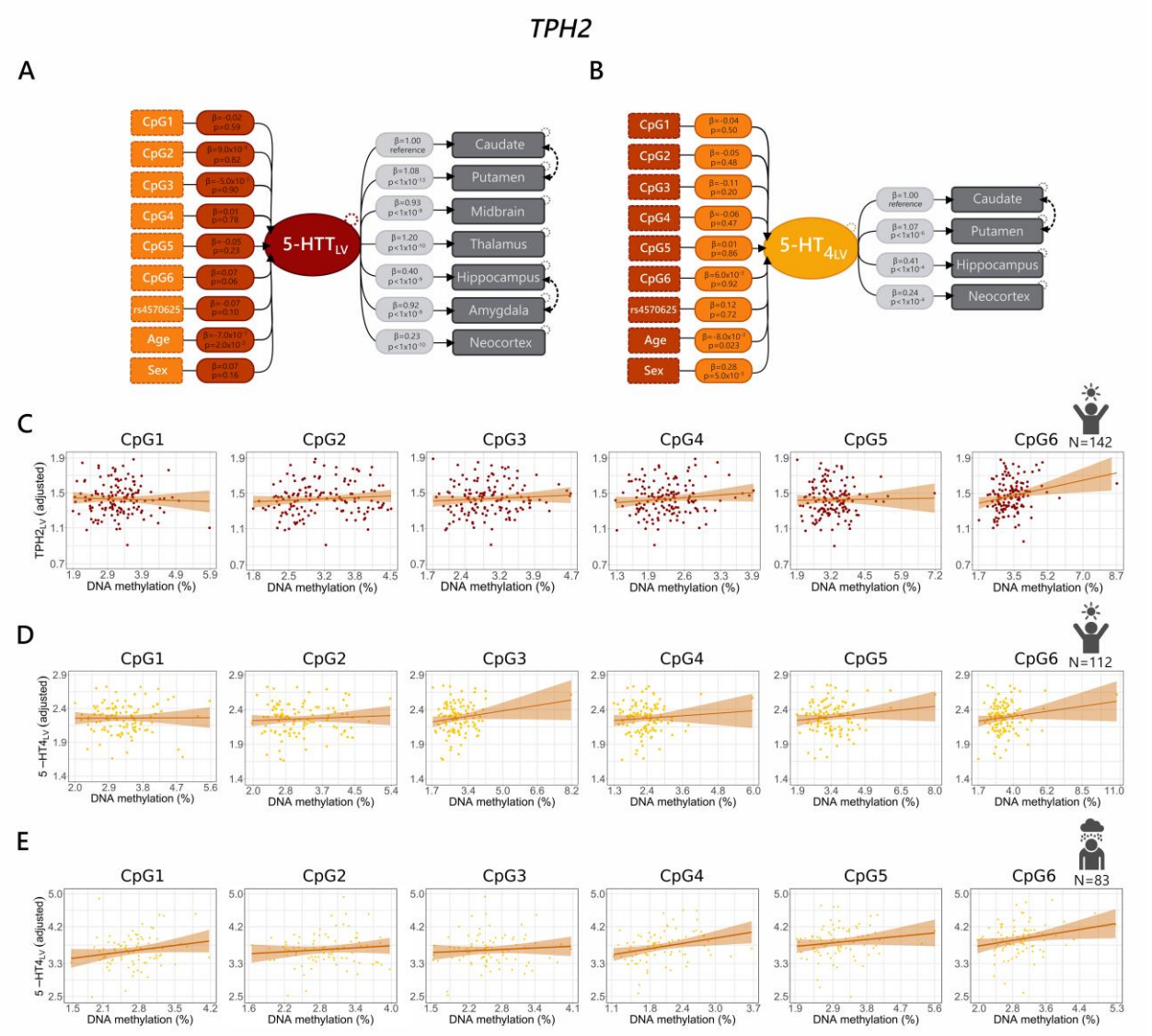

Figure 10. Latent variable models modelling the association between *TPH2* methylation and 5-HTT (A) or 5-HT4 (B) binding. Results for associations between each CpG site and 5-HTT binding in healthy participants (C), 5-HT4 in healthy participants (D) and in patients with depression (E). Reprint from Study II by Bruzzone et al., Clinical Epigenetics, 2024. DOI: https://doi.org/10.1186/s13148-024-01678-y. This work is licensed under Creative Commons Attribution 4.0 International License (CC BY 4.0). To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

Association between measures of environmental stress or psychiatric state traits and peripheral SLC6A4/TPH2 methylation

Among all the statistical models, the association between *SLC6A4* CpG2 and PBI overprotection in the healthy cohort was the only one that remained significant after correcting for multiple comparisons (β: − 0.83; p_{unc}= 0.01; p_{adj}=0.04; 95% CI:[-1.48;-0.19]). **Figure 11** (not shown in Bruzzone et al., (2024)). However, this was not confirmed in the MDD patients (β: 0.53; p_{unc}=0.56; 95% CI [-1.26; 2.32]) and no association was found between *SLC6A4* methylation and PBI overprotection when accounting for blood cell proportions (p>0.42).

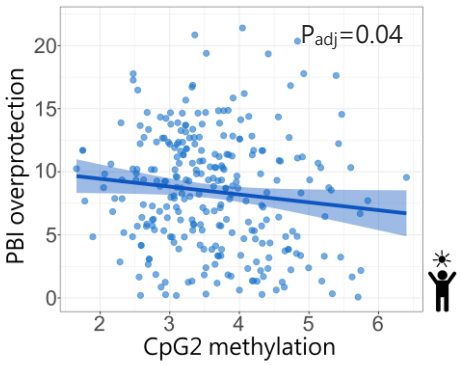

Figure 11. Association between overprotection scores from the parental bonding inventory (PBI) and *SLC6A4* CpG2 methylation in healthy participants (without accounting for blood cell proportions).

Accounting for blood cells proportions

Model fit improved when including blood cell proportions in all models including data from the healthy cohort (all likelihood ratio tests: p<0.01). In the models including data from the MDD patients, model fit improved only for the models relating *TPH2* methylation and BDI, GAD10, HAMD16 and PSS. Nonetheless, accounting for blood cell proportions did not affect the relation observed in the models that did not account for this information in neither cohort.

17. Study III

In this study, we aimed to evaluate: 1) the association between baseline *SLC6A4/TPH2* methylation and clinical outcomes after 8 weeks of SSRI treatment; 2) whether baseline *SLC6A4/TPH2* methylation predicts clinical outcomes after 8 weeks of SSRI treatment; 3) whether *SLC6A4/TPH2* methylation changes over 12 weeks of SSRI treatment; 4) whether we could confirm previous findings linking *SLC6A4* CpG1 and CpG2 to clinical outcomes after SSRI treatment.

Association between baseline SLC6A4/TPH2 methylation and clinical outcomes after 8 weeks of SSRI treatment

Results from LVMs are depicted in **Figure 12**. We found that patients with higher $TPH2_{LV}$ at baseline would be classified as responders at week 8 and would show a greater improvement in depressive symptoms. Specifically, each 0.1 increase of $TPH2_{LV}$ was linked to a 4.5% reduction in depressive symptoms (p=0.01). However, we found no evidence for an association between *SLC6A4* and any clinical outcome at week 8 (categorical outcome: p=0.23; continuous outcome: p=0.98).

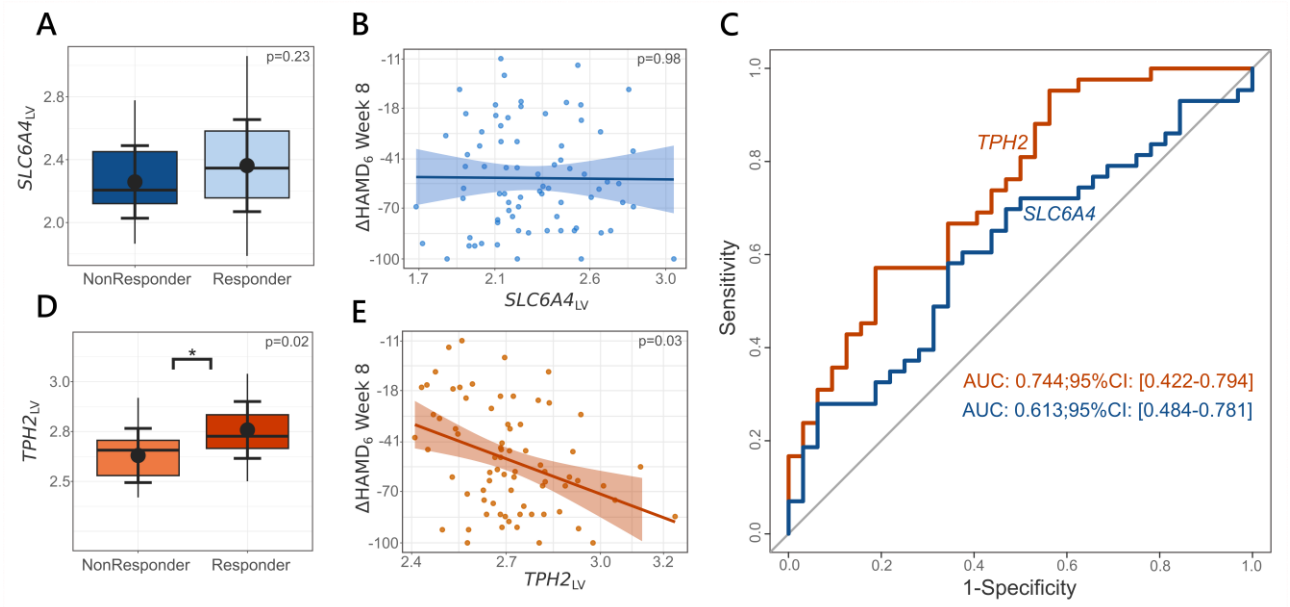

Figure 12. Results from latent variable models linking baseline *SLC6A4/TPH2* methylation and clinical outcomes after 8 weeks of SSRI treatment. A and B depict findings for associations between *SLC6A4* methylation and either categorical (A) or continuous (B) outcomes. D and E show results from associations between *TPH2* methylation and categorical (D) or continuous (E) outcomes. Receiver operating characteristics curves (ROC) depict the predictive potential of *SLC6A4/TPH2* methylation for clinical outcomes following antidepressant treatment.

Predicting baseline clinical outcomes after 8 weeks of SSRI treatment using SLC6A4/TPH2 methylation

AUC suggested small predictive value of *SLC6A4* (AUC: 0.613; 95% CI: [0.484-0.741]) and moderate predictive value of *TPH2* (AUC: 0.744; 95 CI: [0.422-0.794]) for clinical outcomes at week 8 (**Figure 12**). Nonetheless, confidence intervals do not allow us to conclude that *SLC6A4* or *TPH2* can predict clinical outcome after 8 weeks of SSRI treatment.

Changes in TPH2 methylation SLC6A4/TPH2 methylation over 12 weeks of SSRI treatment

Before adjusting for multiple comparisons, we observed a trend (p_{unc}=0.02) where *TPH2* CpG2 methylation decreased from week 0 to week 8 but no difference at week 12. Nonetheless, statistical significance disappeared after Bonferroni correction (p_{adj}=0.2). We observed no evidence for a change in *SLC6A4* methylation across the 12 weeks of treatment (all $p_{adj} \geq 0.33$). In the models accounting for escitalopram plasma concentration, we observed trends for: 1) an increase in *SLC6A4* CpG2 methylation from baseline to week 8 (p_{unc}=0.03; p_{adj}=0.12); 2) a decrease in *TPH2* CpG2 methylation from baseline to week 12 (p_{unc}=0.03; p_{adj}=0.18). However, none of these trends withstood adjustments for multiple comparisons.

Confirming previous findings linking SLC6A4 CpG1 and CpG2 to clinical outcomes after SSRI treatment

We observed no statistically significant association between *SLC6A4* CpG1 or CpG2 methylation and clinical outcomes at week 8, as assessed with multiple linear regression models. This was the case both when attempting to confirm findings on CpG2 from Domschke et al. (2014) (β: 2.33; p-value: 0.65, 95% CI [-7.79; 12.45]), and when trying to confirm findings on CpG1 from Schiele et al. (2021), (clinical response: (β: 0.31; p-value: 0.49, 95% CI [-0.56; 1.21]); remission: (β: -0.40; p-value: 0.41; 95% CI [-1.41; 0.54]); change in $HAMD_{17}$: β: 2.06; p-value: 0.73, 95% CI [-9.97; 14.09])). In addition, when available, 95% CI reported in previous studies did not overlap with the ones observed in our analyses.

Finally, we observed no statistically significant association between *SLC6A4* CpG2 methylation and $\Delta HAMD_{17}$ at week 12, as assessed with Pearson's correlation (r=0.18; p-value=0.11; 95% CI: [-0.044; 0.390]). Nonetheless, we observed that the direction (positive association) and correlation coefficients (Kang et al., (2013): r=0.19) were consistent with what previously reported by Kang et al. (2013). Yet, 95% CI were not reported by Kang et al. (2013), which does not allow us to directly compare the findings between our and the previous study. Results from

associations with change in depressive symptoms are illustrated in **Figure 13** (not shown in the manuscript of Study III).

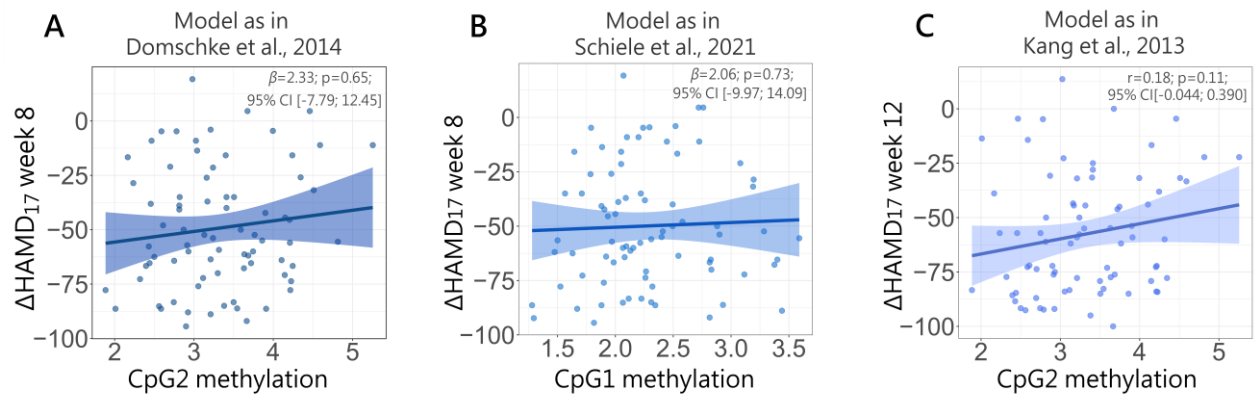

Figure 13. Replication of previous findings linking either CpG1 or CpG2 to clinical outcomes after treatment with antidepressant medications in our study cohort (NP1). A depicts results from replication of findings by Domschke et al. (2014); B depicts results from replication of findings by Schiele et al. (2021); C depicts results from findings by Kang et al (2013).

18. Study IV

In this study, we aimed to: 1) replicate previous findings relating *SLC6A4/TPH2* methylation and depression status or early life stress; 2) test for enrichment of serotonin-related genes in the association between DNA methylation and depression; 3) determine whether 27 centrally relevant genes for serotonin transmission are associated with depressive symptoms, childhood trauma or depression chronicity, with a focus on depression chronicity in patients that were taking serotonergic-acting antidepressant medications.

Replication of findings on SLC6A4/TPH2

Only 7/22 CpG sites described in literature were available in NESDA and GSMS; 2/22 in TD; 4/22 in MDDbrain. We observed no statistically significant association between *SLC6A4/TPH2* methylation and any of the outcomes of interest (all p≥0.06). We only observed a trend for a negative association between

One *SLC6A4* CpG site (chr17:28563424) that was closest to previously reported sites (see Table 2 in Study IV) and depression in the MDDbrain cohort but not in the other cohorts, although the previous finding was based on a CpG site that was .

Enrichment of serotonin-related genes in the association between depression or childhood trauma

We also observed no evidence for enrichment of serotonin-related genes in associations with depression status/childhood trauma, in any of the examined cohorts (p≥0.1).

Association of 27 serotonin-related genes with depressive symptoms, childhood trauma and depression chronicity

We found no evidence for an association between DNA methylation of serotonin-related genes and questions i), ii), iii), iiia) or iiib); all FDR-corrected p-values (q-values)>0.1 (**Figure 14**).

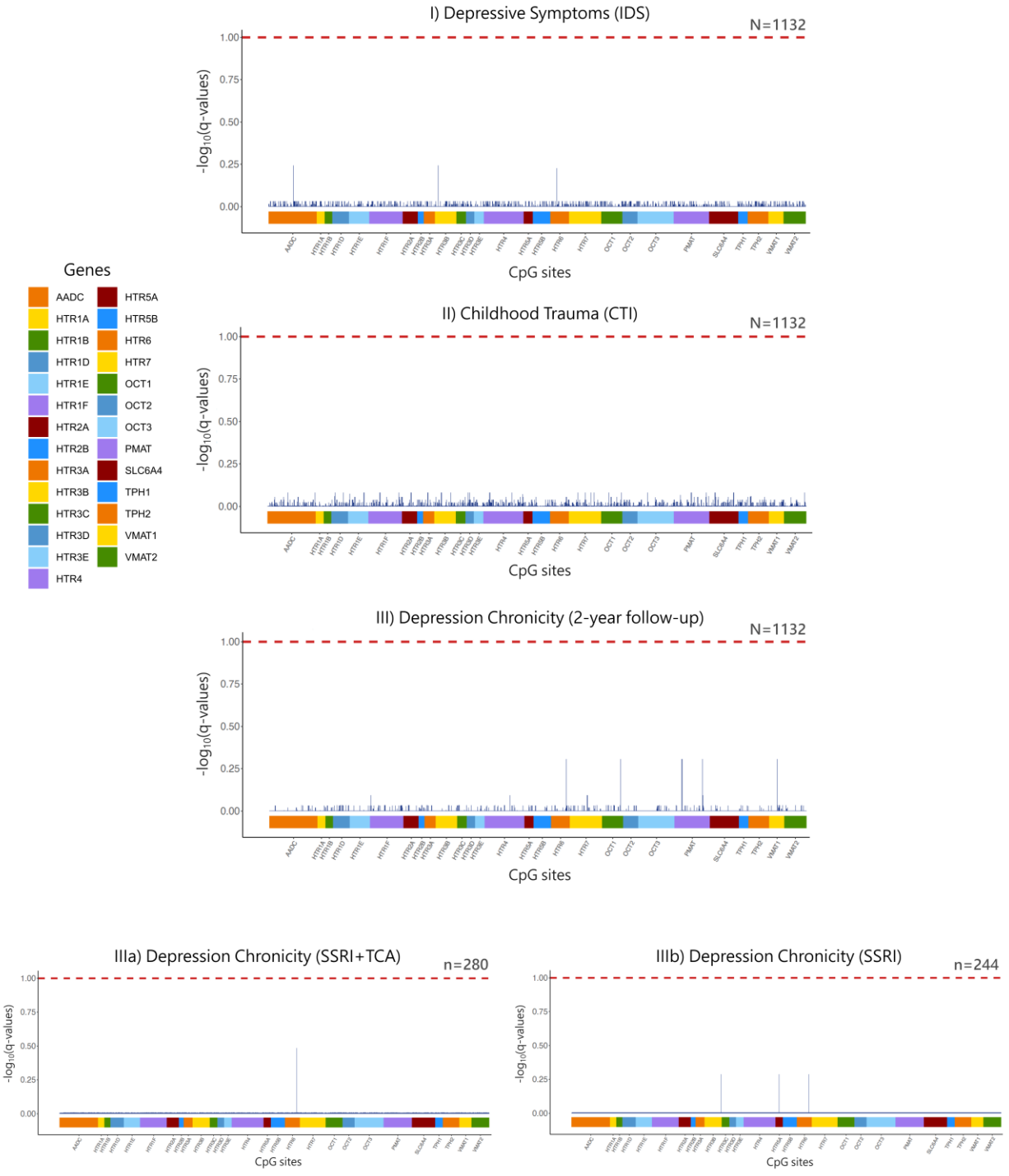

Figure 14. Results from multiple linear regression models evaluating the association between DNA methylation of 27 serotonin-related genes and aims i), ii) iiia) and iiib). Vertical bars represent -log$_{10}$ (q-values). Significance level was set to 0.1, which is represented by the red dashed line. Reprinted from Study IV.

DISCUSSION

Using data from healthy and patient cohorts, we evaluated the genetic and epigenetic contributions to serotonin neurotransmission and the potential of DNA methylation of serotonin-relevant genes as a biomarker for early life stress, depression and clinical outcomes after antidepressant treatment. The main points of the thesis are discussed below. For more in-depth discussion of the individual studies, see the articles attached in the Appendix.

19. Study I

Genetic variation within the serotonin system and serotonergic neurotransmission

In a cohort of healthy participants, we observed that T-carriers of the *MAOA* 1137070 variant had higher 5-HTT binding in both cortical and subcortical regions. The highest binding was observed in caudate (~11%) and the lowest binding was observed in amygdala (~2%). The T allele has been previously associated with low *MAOA* activity (Hotamisligil & Breakefield, 1991; Pinsonneault et al., 2006), suggesting that T-carriers may have higher 5-HT levels compared to C-carriers.

Based on our observations, we speculate that individuals with this genotype might have higher 5-HTT levels to compensate their genetically-determined higher serotonin levels. This would allow them to maintain constant levels of extracellular serotonin by reuptaking the "extra" serotonin. Nonetheless, studies based on animal models support an opposite explanation (e.g. *MAOA* knock-outs present lower 5-HTT levels compared to wild-types) (Evrard et al., 2002; Godar et al., 2014) and studies conducted in humans report conflicting results (Bi et al., 2021; Huang et al., 2009; Zhang et al., 2010), which makes the interpretation of our findings difficult.

In addition, as previously reported in the same cohort (Fisher et al., 2017), we observed that *BDNF* rs6265 (or val66met) met-carriers had lower 5-HTT binding in subcortical regions compared to individuals with val/val genotype.

No other genotype was associated with 5-HTT binding, suggesting that although these variants may affect the expression of the receptors or transporters that they encode for, these effects may not be sufficient to directly affect 5-HTT binding in the adult healthy brain. Interestingly, 5-HTTLPR, which is known to affect 5-HTT expression in-vitro, was not associated with 5-HTT brain binding in this study, which comprises the largest dataset of 5-HTT scans currently available. 5-HTTLPR was previously linked to 5-HTT brain binding in some studies (Praschak-Rieder et al., 2007) but this finding had not been confirmed by all studies (Fisher et al., 2017; Murthy et al., 2010). Notably, this study, along with the study by Fisher et al. (2017), which was based on the

same cohort, is the best-powered study to date that could allow detecting an effect of 5-HTTLPR on 5-HTT availability. Yet, we did not observe an effect, suggesting that small sample size might not be the reason behind this negative finding. Instead, our findings might be explained in light of the complex in-vivo biological interactions, which we will discuss in the "Methodological Considerations" section.

Finally, genotype information did not successfully predict brain 5-HTT binding, except for caudate, in which we observed a marginal effect. This may be due to the low number of examined variants, each one of which may provide only a small contribution to 5-HTT levels, not allowing us to have a strong predictive power.

20. Study II

Epigenetic variation within the serotonin system and serotonergic neurotransmission

We observed no association between *SLC6A4* or *TPH2* methylation and brain 5-HTT or 5-HT_4 binding in healthy participants or with 5-HT_4 binding in patients with depression. Notably, while we evaluated the link between *SLC6A4* methylation levels and 5-HTT brain binding, we did not directly evaluate an association between *TPH2* methylation and brain TPH2 levels, as no radiotracer targeting TPH2 is currently available. Thus, we cannot exclude a possible link between peripheral *TPH2* methylation and brain TPH2 binding.

As serotonin transmission and changes in DNA methylation are crucial for brain development, *SLC6A4* or *TPH2* methylation might have been associated with brain 5-HTT or 5-HT_4 levels earlier in development. Both DNA methylation and 5-HTT and 5-HT_4 levels significantly fluctuate across the lifespan (Fisher et al., 2015; Mulder et al., 2021) and in response to environmental factors (Dam et al., 2024; Jakobsen et al., 2016; Kim et al., 2005; Praschak-Rieder et al., 2008; Vulpius et al., 2023). Nonetheless, DNA methylation of about 50% of the total CpG sites remains unchanged after the first years of life (Dor & Cedar, 2018; Mulder et al., 2021). We observed this was the case for three of our CpG sites (*SLC6A4* CpG and CpG4; *TPH2* CpG2). However, we cannot exclude a more dynamic change in methylation for the other CpG sites.

Importantly, although we identified no association between peripheral *SLC6A4/TPH2* methylation and brain proxies of serotonin neurotransmission, *SLC6A4/TPH2* methylation might still be relevant biomarkers for different psychiatric conditions. As DNA methylation was measured in immune cells and peripheral serotonin signaling mediates inflammatory responses (Chen et al., 2015; Herr et al., 2017), alterations in *SLC6A4* or *TPH2* peripheral methylation could provide valuable insights into immune function alterations, which have been widely reported in patients with depression (Hunt et al., 2020; Milaneschi et al., 2020). In addition, the

relation between peripheral DNA methylation, gene expression and protein levels is complex, and is discussed more in depth in the "Methodological Considerations" section.

SLC6A4/TPH2 methylation and environmental stress and psychiatric state traits

We found no statistically significant association between *SLC6A4* or *TPH2* methylation and depressive or anxiety symptoms or measures of recent or early life stress. We only observed a statistically significant association between *SLC6A4* CpG2 methylation and the PBI overprotection item in healthy participants, but only in models that were not accounting for blood cell proportions. Importantly, our models correcting for blood cell proportions included the shared correlations across all CpG sites (as CpG sites were pooled into a latent variable), so we cannot exclude that the link between CpG2 and PBI Overprotection persisted even after correcting for multiple comparisons and was cancelled out by the other CpG sites. Notably, none of the former studies reporting a link between either of these genes and early life stress or psychopathological symptoms corrected for blood cell proportions (Alasaari et al., 2012; Kang et al., 2013; Leibold et al., 2020; Shen et al., 2020b), which are critical for correct interpretation of DNA methylation data (Koestler et al., 2013; Qi & Teschendorff, 2022). Thus, we cannot exclude that lack of correction for blood cell proportions biased previously reported findings. However, other factors might have affected our results: 1) neither healthy participants nor the patients with depression in our cohorts had a history of severe traumas, which might leave a stronger "mark" on DNA methylation; 2) our measurements for environmental stress might not have been sensitive enough; 3) smaller sample size of our cohort of patients with depression compared to previous studies might have masked extremely small effects.

21. Study III

SLC6A4/TPH2 methylation and clinical outcomes after antidepressant treatment

We found that participants who were classified as responders after 8 weeks of treatment had higher *TPH2* methylation at baseline compared to non-responders. Thus, individuals with supposedly lower baseline serotonin levels might be those benefitting the most by SSRI treatment. Conversely, we observed no association between baseline *SLC6A4* methylation and clinical outcomes at 8 weeks. Both findings are in contrast with former studies. About *TPH2*, only one study (Shen et al., 2020a) reported that *TPH2* hypomethylation was associated with better clinical outcomes after 2 weeks of antidepressant treatment.

About *SLC6A4* methylation, we observed a trend for a positive association between baseline *SLC6A4* CpG2 methylation and clinical outcomes after 12 weeks. Although it did not reach the

threshold for statistical significance, confidence intervals and effect sizes were within the same range and direction as the study by Kang et al. (2013), suggesting a partial replication. This is opposite to the other two studies (Domschke et al., 2014; Schiele et al., 2021) reporting that baseline *SLC6A4* CpG1 or CpG2 hypomethylation was linked to better clinical outcomes after 6 weeks.

In addition, we observed a trend for an increase in *SLC6A4* CpG2 methylation and for a decrease in *TPH2* CpG1, CpG2 and CpG4 over treatment when accounting for escitalopram plasma drug concentration, but only before correction for multiple comparisons. If true, this observation points to an escitalopram-dose-dependent increase in serotonin synthesis and an increase in serotonin reuptake, suggestive of an increase in endogenous serotonin levels over treatment. However, this is also in contrast with former literature, which did not describe any change in *SLC6A4* CpG2 levels over 6 weeks (Moon et al., 2023).

The causes of the discrepancies between our and previous findings may lie in differences in study design: 1) clinical outcomes were defined either after 2 weeks of treatment, which is much shorter than what typically done in other trials (Leon, 2001; Trivedi et al., 2006) (Shen et al., 2020a) or after 6 (Domschke et al., 2014; Moon et al., 2023; Schiele et al., 2021) or 12 weeks (Kang et al., 2013), while we defined them after 8 weeks of treatment; 2) participants in all former studies had a more severe history of early life traumas compared to our participants, which might have affected DNA methylation or clinical outcomes differently; 3) generally larger sample sizes (N>110) compared to that of our cohort; 4) different definitions of clinical outcomes, based on different HAMD versions (6 vs 17 vs 21); 5) Participants were either treated with different antidepressant medications (e.g. TCAs, SSRIs, atypical antidepressants) and/or for comorbidities (Domschke et al., 2014; Schiele et al., 2021), or at least with different classes of SSRIs (Kang et al., 2013; Moon et al., 2023); 6) participants were generally older in other cohorts, which might be linked to more severe forms of (e.g. chronic) depression.

Importantly, none of the former studies accounted for blood cell proportions, which can crucially bias DNA methylation (Farré et al., 2015; Zheng et al., 2017). Thus, although we partially replicated a former finding, we suggest that the results of our LVMs, which include blood cell proportions, should be considered as main findings.

22. Study IV

Replicating findings on SLC6A4/TPH2 methylation and depression or childhood trauma

We failed to confirm findings from previous studies, at least for the CpG sites that were present in our datasets. We only observed a trend for an association between one CpG site of *SLC6A4*

that was 249-370 bp away from previously reported sites (Mendonça et al., 2019) and depression. Notably, the previous study examined DNA methylation in whole blood while we observed this only in the MDDbrain cohort but not in the other, better-powered cohorts. This prevents us from interpreting this observation as a solid replication.

There are several strengths in this study: 1) this is the largest study to date to address these research questions and with a focus on replicating former findings; 2) cross-validating previous findings in MWAS datasets allowed us to account for the high degree of inter-correlation across methylation sites that might inflate the risk of false positives in candidate gene methylation studies (Shabalin et al., 2015); 3) using DNA methylation data from post-mortem brains: methylation levels only partially overlap between blood and brain, and this is likely to be the case for genes that are specific to serotonin neurotransmission (e.g. *TPH2*); 4) we accounted for blood cell proportions, which was not done by any of the previous studies focusing on these two genes, except for our Study II and III (Bruzzone et al., 2024; Bruzzone et al., 2025). Nonetheless, the heterogeneity (demographics, methylation assay methods, determination of depression status) across cohorts, along with the fact that not all previously reported CpG sites could be tested in the four independent datasets that we used in this study does not allow us to formulate definitive conclusions about the lack of a relation between methylation of all CpG sites within *SLC6A4/TPH2* and depression or childhood trauma.

SLC6A4/TPH2 methylation and beyond: the other serotonin-related genes

We observed no enrichment of serotonin genes in the associations between DNA methylation and depression status or childhood trauma. Similarly, we observed no link between DNA methylation of serotonin-related genes and depressive symptoms, childhood trauma and depression chronicity, and the latter association did not change in the subgroups of patients taking antidepressant medications with serotonergic outcomes. Our findings suggest that DNA methylation of serotonin-related genes is unlikely to reflect depression status or childhood trauma or be a marker for depression chronicity in DNA measured in blood samples from adolescents or adults or in postmortem brains. Similarly, these genes might not be insightful markers for long-term (2-years) clinical outcomes following antidepressant treatment. However, we cannot exclude a role of DNA methylation in these genes during early development.

23. General Discussion

In summary, we observed that two out of five genetic variants (*MAOA* 1137070, *BDNF* rs6265), which might differentially affect serotonin levels, were linked to greater 5-HTT binding but these

and the other three variants were not sufficient to predict 5-HTT binding in healthy participants per se. We also observed that greater baseline *TPH2* methylation was linked to better clinical outcomes following 8 weeks of antidepressant treatment and partially replicated previous findings linking greater *SLC6A4* CpG2 methylation to better clinical outcomes after 12 weeks of SSRI treatment in patients with depression. Interestingly, we also observed a link between greater *SLC6A4* CpG2 and increased PBI overprotection (reflecting increased early life stress) in healthy participants and that the same CpG site also showed a trend for a decrease in methylation across 12 weeks of treatment. Taken together, results from Study II and III might point to the fact that lower baseline serotonin levels might indicate greater early life stress and better clinical outcomes. These findings are difficult to interpret, as 1) findings on PBI overprotection were only found in healthy participants, 2) greater early life stress is usually linked to worse clinical outcomes (Kornstein & Schneider, 2001) and 3) we observed a link between *SLC6A4* CpG2 and PBI overprotection or clinical outcomes only in models that did not account for blood cell proportions. Nonetheless, we were not able to test this or the CpG sites we analyzed in Study II or III in four large independent cohorts, since they were not present in the MWASs that we used to replicate previous findings. Thus, we cannot completely exclude an association between these methylation sites and early life stress or clinical outcomes following antidepressant treatment. However, it is important to note that baseline *SLC6A4/TPH2* methylation levels were not sufficient to predict individual outcomes after treatment and that changes of their methylation levels over time (as observed in the NP1 cohort) were only at the trend levels, making them unlikely candidates for predicting antidepressant treatment outcomes or informing on treatment mechanisms.

Overall, our findings suggest that: 1) genetic and epigenetic variation within genes that are relevant for serotonin function is unlikely to have a major impact on the brain serotonergic architecture in adults; 2) *SLC6A4/TPH2* methylation might inform on mechanisms underlying early life stress, depression or antidepressant treatment only to a very limited extent and 3) DNA methylation of serotonin-related genes is unlikely to reflect early life stress or depression status in a clinically relevant context, nor to be used as a predictive biomarker for antidepressant treatment outcome. Our observations might discourage future research from using (epi)genetic variants within the serotonin system as proxies for brain serotonin neurotransmission in adult participants as well as encourage researchers to shift their focus away from methylation of genes of the serotonin system when looking for mechanisms or biomarkers relevant for depression and early life stress.

Importantly, the validity of our findings is supported by some major strengths of our studies, that consist in: using the largest currently available cohorts with 5-HTT/5-HT_4 brain scans (Study I, II); investigating DNA methylation of serotonin-related genes using the largest study cohort to date (Study IV); attempting to directly replicate previous findings (Study III, IV); incorporating blood cell proportions in DNA methylation studies focusing on serotonin-related genes (Study II, III, IV).
However, a number of methodological considerations (listed in the next section) should be taken into account when interpreting our findings.

24. Methodological Considerations

24.1. The candidate gene approach

In this thesis, we used a candidate (epi)gene approach to investigate how genetic and epigenetic variation can affect serotonin neurotransmission and clinical outcomes following antidepressant treatment. There are many limitations in using a candidate gene approach. The main limitation is that focusing on single candidate genes inflates the number of false positive findings, and this risk is even higher in candidate gene methylation studies (Shabalin et al., 2015). This is mostly due to the fact that DNA methylation across different CpG sites (independently on their genomic location) tend to co-vary. This covariance is assumed to be due to a variety of environmental factors such as differences in lifestyle that cannot be captured by e.g. questionnaires. In MWASs, this covariance can be captured using PCA and can be accounted for by regressing relevant principal components out in e.g. multiple linear regression models. Importantly, if the principal components explaining such variance are associated with e.g. the disease status and are not accounted for in the statistical analyses (e.g. as covariates), this can inflate the type I error (Shabalin et al., 2015). In addition, positive findings are also more likely to be published (publication bias) (Keller & Ph, 2011). We suggest that these may be the main reasons behind some of the discrepancies reported by former literature and observed in our studies.
Nonetheless, the main focus of this work was the characterization of the serotonin system in health and disease. Thus, we hypothesized that genes that are directly involved in this signaling pathway would provide the greatest insights. This choice has limitations too, as genetic and epigenetic variation outside serotonin-relevant genes may also affect their expression or their resulting protein levels, e.g. via (epi)genetic variation in distal regulatory regions such as enhancers or silencers, in genes coding for proteins involved in post-translational modifications or protein degradation or in proteins mediating epigenetic modifications such as DNMTs. However, our strategy was also motivated by the fact that we aimed to build our hypotheses on

previous findings and possibly replicate them and that the sample size of the cohorts included in Study I, II and III were not large enough for genome-wide analyses, especially since each variant is likely to provide only a small contribution to in-vivo serotonin neurotransmission. For instance, to observe a link between genetic variants or DNA methylation and 5-HTT levels, we would have needed either a few thousands subjects or a very large difference in 5-HTT BP_{ND} (e.g.≥ 20%) between genotypes or across CpG sites (Cohen's d > 1). This would have been extremely difficult to achieve, given that on average group differences in BP_{ND} do not exceed 10-13% (Fisher, et al., 2015a; Köhler-Forsberg et al., 2023a; Larsen et al., 2020).

24.2. The relation between genotype, peripheral DNA methylation and brain levels of serotonergic markers

In Study I, we found that *MAOA* 1137070 T-carriers, who supposedly have lower MAOA activity (and therefore higher serotonin levels) had increased 5-HTT binding compared to CC individuals. These findings are difficult to interpret, as normally greater serotonin levels would be reflected by lower 5-HTT binding. This might be in part explained by the fact that protein-protein interactions occurring in-vivo might affect available 5-HTT levels, independently from serotonin levels (Anderluh et al., 2014; Gradisch et al., 2024).

In addition, we observed no association between the other transcriptional variants within *SLC6A4*, *HTR1A* and *HTR2A* and brain 5-HTT levels. This might be due to the fact that in-vivo biological interactions are more complex than what observed in vitro: 1) genetic variation is not the only source of variation in gene expression and therefore protein levels; instead, environmental factors, along with epigenetic signatures, can affect gene expression and possibly protein levels (Spies et al., 2015); 2) post-transcriptional and post-translational modifications, as well as protein turnover, can affect protein levels independently of gene expression (Baudry et al., 2019; Bradley & Blakely, 1997; Grohmann et al., 2010; Millan, 2011).

Similarly, in Study II, we found no association between peripheral *SLC6A4/TPH2* methylation and brain 5-HTT or 5-HT_4 levels. While points 2) and 3) can partially explain these findings too, when investigating DNA methylation levels across tissues we should also point that: 1) DNA methylation is tissue-specific and it is not clear to what extent methylation levels at these genes and specific CpG sites correlate between blood and brain; 2) the same methylation signatures may be interpreted differently by different transcription factors expressed in different tissues (Chatterjee & Vinson, 2012). Importantly, serotonin is involved in immune functions in the periphery and T-lymphocytes express several serotonin receptors, along with serotonin

transporters (Wu et al., 2019). Thus, altered peripheral *SLC6A4/TPH2* methylation might most likely reflect altered peripheral immune processes.

24.3. Considerations on study design

In Study I and II, we used a cross-sectional study design. Given that both the composition of the brain serotonin system and DNA methylation can dynamically change throughout life (Beliveau et al., 2020; Madsen et al., 2011; Mc Mahon et al., 2016; Mulder et al., 2021; Yamamoto et al., 2002), longitudinal study designs would have provided more detailed insights into the relation between (epi)genetic variation of serotonin-related genes and serotonin brain architecture.

In Study III, we used data from an open-label, longitudinal trial spanning 12 weeks of antidepressant treatment. Although this study design has some important advantages, such as an easier implementation and results that are closer to what observed in real world (e.g. clinical practice), a main disadvantage is the absence of randomization and placebo administration. Thus, our findings do not allow us to draw causal conclusions, neither about antidepressant treatment outcomes, nor when evaluating changes in DNA methylation over treatment. For this reason, we referred to "clinical outcomes following antidepressant treatment" instead of "antidepressant treatment outcome" throughout Study III.

In Study IV, a meta-analysis approach would have provided more solid evidence for our replication part. Nonetheless, the heterogeneity of the cohorts included in the study (involving different methods for DNA methylation estimates, different age ranges across cohorts and different tissues in which DNA methylation was measured) did not allow us to perform one.
In addition, we could not directly evaluate clinical outcomes following antidepressant treatment, as none of the cohorts we used to cross-validate previous findings was designed with this purpose. However, we assumed that, if patients experiencing depression and taking antidepressant medications at baseline were still experiencing depressive symptoms after 2 years, they likely did not respond to treatment. As biomarkers should be reliable and highly reproducible across different cohorts and contexts, we hypothesized that we would still get some insights into the potential of *SLC6A4* or *TPH2* methylation as a biomarker for clinical outcomes following antidepressant treatment. However, it is also important to note that while previous studies focused on short-term clinical outcomes (e.g. 2-12 weeks) following antidepressant treatment, here we focused on long-term outcomes. As DNA methylation is a dynamic modification, it is likely to change over the course of years. Also, we do not know for how long patients had been taking antidepressant medications, nor if they complied to their prescriptions.

Antidepressant non-compliance can increase the risk of depression relapse (Ho et al., 2016), and might be one explanation for “depression chronicity”.

24.4. Epigenetic modifications

In this thesis, we focused on DNA methylation as a possible mark of gene-by-environment interaction. However, it is important to remark that DNA methylation is only one of many epigenetic modifications that can affect gene expression. Different epigenetic modifications can cross-talk with each other such that, for instance, certain histone modifications or small RNAs can induce DNA methylation, and, vice versa, DNA methylation can direct histone modifications or affect the expression of small RNAs (Cedar & Bergman, 2009; Murr, 2010; O’Donnell & Meaney, 2020). Thus, other epigenetic modifications or a combination thereof might more relevantly affect serotonin genes expression and possibly reflect gene-by-environment interactions in the context of early life stress and depression.

24.5. *MAOA* and *HTR2C* genes and X-linked inactivation

DNA methylation is essential in X-linked inactivation, the process through which one of the X chromosomes in female individuals is silenced, in order to maintain a balanced amount of gene expression between XY and XX individuals (Fang et al., 2019). Notably, two key genes for the serotonin system, namely *MAOA* and *HTR2C*, are located on the X chromosome. A consistent amount of candidate-gene-based studies linked *MAOA* methylation to several psychiatric disorders, including depression (Ziegler & Domschke, 2018). Nonetheless, there are several reasons why X-linked genes are usually not included in MWAS studies and, therefore, why we could not investigate them in Study IV: 1) array-based methods for MWAS measure DNA methylation in alleles from both chromosomes and estimate DNA methylation as an average between alleles. As XY individuals only have one X chromosome, this cannot be done for all study participants; 2) it is unknown what X chromosome is inactivated and how to disentangle information on DNA methylation levels in the inactivated X vs the non-inactivated gene. Thus, there is currently no standardized way to analyze X-linked DNA methylation in MWAS (Inkster et al., 2023).

24.6. Methods for DNA methylation estimation and cross-validating findings

In study II and III, DNA methylation was measured using pyrosequencing, which is a gold-standard for accurate quantitative measurements for DNA methylation in short sequences and is in line with what previously done in most candidate gene studies. However, in Study IV, we used data from MWAS, that were measured either with MBD-seq or Illumina EPIC arrays. Although there is

a good correlation between DNA methylation measured with these different techniques (Aberg, et al., 2020; Li et al., 2010), data provided by these methods can be quite different, which hinders cross-validation across datasets that were created using different methodologies: for instance, MBD-seq does not provide absolute methylation levels, and the single-base methylation estimates provided by this method are not as reliable as those provided by pyrosequencing (Aberg, et al., 2020b). Importantly, the CpG sites that we measured in study II and III are not present in any of the datasets using MBD-seq, and only 3 CpG sites are present in the dataset using EPIC arrays. This is also due to the fact that most of the previously investigated CpG sites are located in promoter regions, that are usually hypomethylated, and most of hypomethylated sites are filtered out from MBD-seq datasets. In general, this also reflects the difficulties to accurately study the methylome and cross-validate findings across studies given currently available methodologies.

In addition, in Study IV, we used DNA methylation data from four large datasets that are based on heterogeneous study populations. For instance, participants' age in the GSMS cohort was lower than that of participants in the NESDA and TD cohorts; study design involved longitudinal data for the NESDA cohort but not for the other cohorts and depression status was defined differently across studies. However, while this heterogeneity can hinder replicability, it also represents a strength: if the same epigenetic signature is detected in all four different datasets, then its link with the trait of interest (e.g. depression status) must be strong enough to be generalized across different demographic characteristics and diagnostic criteria.

24.7. Heterogeneity of depression and sources of early life stress

Importantly, depression is a heterogeneous disorder, and it has been suggested to reflect a variety of brain disorders (Hasler, 2010), possibly originating from a variety of different mechanisms. Thus, if different depression subtypes are linked to different DNA methylation signatures, each one with a small effect size, these might have cancelled each other out. Importantly, participants with MDD included in Study II and III (NP1 cohort) were carefully examined in order to prevent including patients with established depression subtypes e.g. postpartum, bipolar or psychotic depression. In addition, in Study II we used PBI and SLE to estimate early life stress in healthy participants and PBI and CATS in patients with depression, while in Study IV, scores from CTQ were used in NESDA and scores from CAPA/YAPA were used for GSMS. In all cases, we focused on the total scores of these measurements. However, distinct sources of traumas e.g. parental neglect, physical abuse have been linked to trauma-specific methylation signatures in several studies (Smeeth et al., 2024; Vijayendran et al., 2012). Also in

this case, trauma-specific effects might have been cancelled out by a lack of participants stratification. Furthermore, the timing of trauma might be particularly relevant, and given the dynamic nature of DNA methylation, the related DNA methylation signatures might be detectable only for a specific time window, e.g. around the time when the stressful event was experienced (Burns et al., 2018; Dunn et al., 2018; Provençal & Binder, 2015) but no longer visible years after.

24.8. Interplay between genetics and epigenetics

Genetic variation can affect DNA methylation (Villicaña & Bell, 2021). In Study II and III, we included some genetic variants that are known to affect gene expression of our genes of interest, while we did not include information on genetic variation in Study IV. However, other genes that we did not consider might impact DNA methylation levels and possibly their relation to early life stress, depression status or clinical outcomes following antidepressant treatment.

24.9. Considerations on the serotonin system and depression

In this thesis, we found no link between (epi)genetic variation within the serotonin system and depression and early life stress. The link between serotonin and depression is still unclear and the topic has been highly debated, especially in light of a recently published review (Jauhar, et al., 2023; Möller & Falkai, 2023; Moncrieff et al., 2022). As mentioned in the previous sections, there are several limitations in our work, so we cannot draw final conclusions based on our observations. However, it is important to acknowledge that, based on our findings, peripheral blood and post-mortem brain DNA methylation variation within the serotonin system is likely not to play a crucial role in depression or in childhood trauma, at least when measured in adult life or during adolescence. Notably, depression is a highly heterogeneous disease and alterations in DNA methylation of serotonin genes might be present only in a subgroup of patients, which we were not able to identify.

While the causal link between serotonin and depression is missing, it is well established that antidepressant medications acting on the serotonin system are effective for many patients (Cipriani et al., 2018). However, we observed only a marginal role of *SLC6A4/TPH2* methylation in clinical outcomes following antidepressant treatment. Thus, focusing on the serotonin system to find biomarkers for childhood trauma, depression or antidepressant treatment outcomes might not be as informative as we hypothesized. Instead, to this goal, using less hypothesis-driven approaches (e.g. GWAS or MWAS) in well-powered study cohorts might help detect novel, relevant markers. Alternatively, using online resources such as GTExPortal (https://gtexportal.org/home/) might allow to identify genotypes that are associated with gene

expression of specific genes (e.g. *SLC6A4*) in tissues of interest (e.g. the brain). This would result in a smaller number of SNPs compared those explored in GWAS and, consequently, smaller sample sizes needed to observed an effect.

CONCLUSION

The main aims of this thesis were to characterize the role of (epi)genetic variation within serotonin-related genes in the in-vivo brain serotonergic architecture in both healthy participants and patients with depression and to understand to what extent DNA methylation of serotonin-related genes holds potential as a gene-by-environment biomarker for depression and antidepressant treatment outcomes.

Among the five genetic variants that we examined in relation to brain 5-HTT binding, we found that individuals carrying the T-allele of *MAOA* rs1137070 had increased 5-HTT binding in all regions of interest; however, this information was not sufficient to predict brain 5-HTT levels. We found no association between peripheral *SLC6A4/TPH2* methylation and brain 5-HTT or 5-HT_4 in healthy participants and no association with 5-HT_4 in patients with depression. In addition, we found no association between *SLC6A4/TPH2* and depressive or anxiety symptoms or measures of environmental stress, except for a positive link between *SLC6A4* CpG2 and PBI in healthy individuals, but only before accounting for cell proportions. We observed that patients whose clinical outcome improved after 8 weeks of SSRI treatment had baseline *TPH2* hypermethylation but that baseline *TPH2* methylation levels were not sufficient to predict clinical outcomes following SSRI treatment, hinting that biomarkers based on this *TPH2* methylation are unlikely to be used in a clinical setting. *SLC6A4* and *TPH2* methylation levels marginally changed over 12 weeks of treatment in a way that would suggest gradual increase of brain serotonin levels, but effects did not sustain correction for multiple comparisons. Finally, we did not confirm previous findings about *SLC6A4/TPH2* and depression or childhood trauma in a set of independent cohorts and we found no evidence for a link between DNA methylation of serotonin-related genes and childhood trauma, depression status or depression chronicity.

25. Implications and future directions

Taken together, these findings suggest that:

1) (Epi)genetic variation within genes that code for essential constituents of the serotonin system are unlikely to have an impact on the in-vivo serotonergic brain architecture in adults (either healthy controls or patients with depression).
2) DNA methylation of genes that are essential for serotonin signaling are unlikely to be useful gene-by-environment markers reflecting or providing mechanistic insights into childhood trauma, current depression status or depression chronicity. However, more research is needed to confirm our observations.

3) Based on our findings and the discrepancies with previous literature, DNA methylation of *SLC6A4* and/or *TPH2* is unlikely to be implemented in the clinical practice as biomarkers to guide clinicians in the choice of antidepressant treatments.

This involves several implications:

- Other genetic variants or DNA methylation sites might have a stronger effect on in-vivo markers of serotonin neurotransmission. In this case, larger studies including more participants and with a genome-wide focus might help unraveling the association between (epi)genetic variation and brain serotonergic neurotransmission. For instance, data sharing initiatives and consortia would allow to pool different datasets and create study cohorts large enough to reach the needed statistical power. Alternatively, identifying genotypes associated with gene expression of genes of interest based on their expression levels in the brain would help increasing the statistical power by only looking at some, empirically-choosen genes.
- Genetic variation may not be crucial in shaping the in-vivo serotonergic neurotransmission in adult individuals, and other factors e.g. environmental factors or presence of a pathology might have a stronger influence on it. In this case, more accurate deep phenotyping, with measurements capturing more detailed information about the individual exposome (e.g. type and timing of stress/trauma), would help understanding to what extent environmental factors vs genetic factors shape serotonergic neurotransmission. Also, longitudinal studies focusing on early developmental stages e.g. including infants and following them until adulthood might allow us to understand if this is true also in early life. Given the major role of serotonin in neurodevelopment, and the dynamic nature of both DNA methylation and the serotonin system, this relation might be different if examined at early developmental stages and possibly inform on risk for neuropsychiatric conditions (e.g. depression).
- DNA methylation of serotonin-related genes might be relevant in a specific subgroup of patients. Future studies based on large populations and using adequate statistical approaches to stratify patients might address this issue.
- DNA methylation signatures of trauma or depression might only be detectable in study populations with experience of extreme early life stress or with specific types of stress/trauma. Including participants with extreme experiences of stress and stratifying them by e.g. type and timing of stress might help future research to better understand the relation between DNA methylation of serotonin-related genes and early life stress.
- Findings linking peripheral DNA methylation of serotonin genes and brain disease phenotypes should be interpreted with caution, as peripheral modifications are unlikely to

reflect alterations in serotonergic neurotransmission. Instead, they are more likely to reflect DNA methylation signatures of immune cells and be interpreted consequently.

- Study designs e.g. randomized, placebo-controlled trials would allow us to infer causal links to better understand to what extent DNA methylation signatures of *SLC6A4/TPH2* are stable and reproducible and, possibly, be implemented in the clinical setting.
- DNA methylation patterns at genes other than those encoding for essential regulators of serotonin signaling might more accurately capture signatures of early life adversities, depression status and chronicity. MWAS based on longitudinal study designs and large populations might allow us to identify such markers in an unbiased fashion.
- Cross-validation of DNA methylation findings across study cohorts is challenging, especially when different methods are used and some methods are more suitable than others for measuring e.g. hypomethylated sites. More research using complementary methods and appropriate study designs might better elucidate to what extent serotonin-related genes are relevant in the context of depression, childhood trauma or antidepressant treatment outcomes.

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APPENDICES

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STUDY I

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Genetic contributions to brain serotonin transporter levels in healthy adults

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The serotonin transporter (5-HTT) critically shapes serotonin neurotransmission by regulating extracellular brain serotonin levels; it remains unclear to what extent 5-HTT levels in the human brain are genetically determined. Here we applied [^{11}C]DASB positron emission tomography to image brain 5-HTT levels and evaluated associations with five common serotonin-related genetic variants that might indirectly regulate 5-HTT levels (*BDNF* rs6265, *SLC6A4* 5-HTTLPR, *HTR1A* rs6295, *HTR2A* rs7333412, and *MAOA* rs1137070) in 140 healthy volunteers. In addition, we explored whether these variants could predict in vivo 5-HTT levels using a five-fold cross-validation random forest framework. *MAOA* rs1137070 T-carriers showed significantly higher brain 5-HTT levels compared to C-homozygotes (2–11% across caudate, putamen, midbrain, thalamus, hippocampus, amygdala and neocortex). We did not observe significant associations for the *HTR1A* rs6295 and *HTR2A* rs7333412 genotypes. Our previously observed lower subcortical 5-HTT availability for rs6265 met-carriers remained in the presence of these additional variants. Despite this significant association, our prediction models showed that genotype moderately improved prediction of 5-HTT in caudate, but effects were not statistically significant after adjustment for multiple comparisons. Our observations provide additional evidence that serotonin-related genetic variants modulate adult human brain serotonin neurotransmission.

Serotonin neurotransmission mediates a multitude of brain functions, including neurodevelopment, behavior and cognition[1]. As such, identifying sources of individual variation in brain serotonin neurotransmission is relevant to identify mechanisms contributing to variation in behavior and possibly associated risks for disease. Dysregulation in brain serotonin signaling is implicated in a number of neuropsychiatric disorders such as anxiety and depression, which are known to have a prominent genetic component[2–5]. However, the contributions of genetic factors to individual differences in in vivo serotonin neurotransmission are not well understood.

The serotonin transporter (5-HTT) critically shapes serotonin neurotransmission as it facilitates serotonin reuptake, thereby regulating extracellular serotonin levels and associated receptor signaling[1]. It is also the pharmacological target for selective serotonin reuptake inhibitors (SSRIs), the most commonly prescribed class of antidepressants and anxiolytics[6]. 5-HTT availability can be visualized in humans in vivo using [^{11}C]DASB positron emission tomography (PET)[7,8].

Previous research has reported a link between 5-HTT levels and both healthy and pathological behavioral phenotypes. Increased 5-HTT availability (expressed in terms of binding potential, 5-HTT BP) has been linked to depressive symptoms severity in seasonal affective disorder[9], greater negative affective bias[10,11] and reduced amygdala reactivity to threat in healthy individuals[12,13], whereas low 5-HTT availability has been associated with childhood abuse in patients with depression vs patients who did not experience childhood abuse[14]. Notably, some studies have speculated that 5-HTT availability may be used as a marker of serotonin tone and a histochemical marker for serotonergic projections[15,16]. Thus, identifying sources of variation in 5-HTT availability is relevant to advance our understanding of mechanisms driving individual variation in human behavior.

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Evidence from PET studies supports a genetic effect on the brain distribution of receptors involved in serotonin function[17–19]. Similarly, genetic variation is likely to contribute to 5-HTT levels and activity. Multiple genetic variants have been hypothesized to affect brain serotonin signaling and behavior as well as brain function. Whereas human functional neuroimaging genetics with, e.g., BOLD fMRI, is challenging due to the inherently complex processes underlying brain function[20], PET imaging provides a direct estimate of brain protein levels that is highly reproducible, making it a valuable target for relating to genetic variation.

5-HTTLPR, a common insertion/deletion polymorphism occurring in the promoter region of the 5-HTT gene (*SLC6A4*) is the most widely investigated 5-HTT variant. Nonetheless, although 5-HTTLPR was shown to affect 5-HTT gene expression in vitro in early studies[3], PET studies in humans reported conflicting in vivo results[21–24]. In contrast, single nucleotide polymorphisms (SNP) in genes coding for proteins whose activity is related to that of 5-HTT, such as rs6265 (or Val66Met) in the neurotrophic factor (*BDNF*) gene[23] and rs7333412 in the serotonin receptor 2A (5-HT_{2A}) gene (*HT2AR)* were found to be associated with 5-HTT availability in healthy adults[25,26]. Specifically, the *BDNF* rs6265 met-carriers, who presumably have lower BDNF levels, showed increased 5-HTT availability in subcortical regions[23], whereas the rs7333412 A-carriers showed reduced 5-HTT levels. BDNF is a common neurotrophin mediating neurodevelopmental, survival and plasticity functions whose activity levels can affect serotonin release [27] as well as 5-HTT expression in animal models[28], while 5-HT_{2A} is a core regulator of excitatory serotonin neurotransmission. These findings suggest that genetic variants other than those in the *SLC6A4* gene may indirectly modulate 5-HTT availability by affecting serotonergic signaling via other pathways.In this framework, serotonin receptor 1A (5-HT_{1A}, encoded by gene *HTR1A*), which is the principal inhibitory serotonin receptor that can inhibit serotonin release[29], and monoamine oxidase A (MAOA, encoded by gene *MAOA*), the main enzyme involved in serotonin degradation[30] can directly affect serotonin levels, which may in turn affect 5-HTT levels via its downregulation [31]. Previous evidence reports that *HTR1A* rs6295 G-carriers have increased 5-HT_{1A} protein levels in the dorsal raphe, pointing to decreased serotonin tone that may in turn affect 5-HTT levels in humans [34]. *MAOA* rs1137070 has been linked to increased MAOA mRNA levels and higher enzymatic activity, suggesting that this variant may directly affect serotonin levels[35] and it was associated to SSRI treatment outcome[36,37], suggesting a possible interaction with 5-HTT.

For explanatory purposes, an overview of the relationship between the serotonin-related genetic variants mentioned above and clinical or behavioral phenotypes is provided in Supplementary Table 1.

Previous studies have focused on the *association* between genetic variant and 5-HTT availability. However, although observing associations informs on group differences, it does not establish the ability to predict features at the individual level, which could be relevant for, e.g., personalized medicine strategies. To this end, exploring whether genetic variants assist in predicting 5-HTT availability can be of complementary value.

In this study we used the largest currently available dataset (N = 140) of [^{11}C]DASB PET scans from the Cimbi database[38] to explore the role of *BDNF* rs6265 and *SLC6A4* 5-HTTLPR(previously investigated in the same cohort[23]) rs7333412 in *HT2AR*[25,26], rs6295 in *HTR1A*[32,39,40], and rs1137070 in *MAOA*[36,41] in 5-HTT availability in the healthy brain. First, we evaluated whether the genotypes examined were associated with 5-HTT availability. Next, we used random forest to determine whether genetic information predicted regional 5-HTT availability.

Methods

Participants

Cross-sectional data were included that was collected previously and were available from the Cimbi database[38].

We selected healthy participants based on the following inclusion criteria: (1) availability of *BDNF* val66met (rs6265) and *SLC6A4* (5-HTTLPR and SNP rs23351) genotypes, (2) availability of blood samples for additional genotyping, (3) availability of a [^{11}C]DASB PET scan before any intervention, 4) ⩽60 years of age (to avoid age-related biases in brain volumes, as partial volume effects become stronger after 60), 5) self-identification with European ancestry. In addition, we excluded participants who had the following: (1) diagnosis of a severe neurological or systemic disease; (2) diagnosis of a primary psychiatric disease; (3) substance or drug abuse, based on self-reported clinical history and neurological/physical examination.

We identified 140 healthy participants, 84 females and 56 males (mean age: 26.7 ± 7.2; range: 18–51). The sex imbalance is partly because some studies from which data are drawn included only males or females. Demographic data are described in Table 1.

Subsets of the PET and genetic data included in the current study were collected as parts of multiple previous studies and have been included in previous publications. PET scans included in the current analyses were acquired between 2005 and 2015[11,18,42–46]. All research protocols were approved by the Ethics Committee of Copenhagen and Frederiksberg, Denmark ((KF) 01–124/04, (KF) 01–156/04, (KF) 01 2006–20, H-1–2010-085, H-1–2010-91, H-2–2010-108, amendments included). All participants provided written informed consent after receiving a detailed description of the respective study. All experimental procedures were carried out in compliance with the declaration of Helsinki.

Data included in the current study has been utilized in previous studies[9,10,23,45,47], some of which focused on the relation between 5-HTTLPR and/or *BDNF* rs6265 [^{11}C]DASB binding[23,45].

Genotyping

No additional genotyping for the 5-HTTLPR (including rs25531) and *BDNF* rs6265 was performed beyond that which has been described previously[23,23,48,48,49].

The three additional variants were determined from whole-blood derived genomic DNA using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). DNA concentration and purity levels were estimated using an UV–Vis spectrophotometer (Nanodrop 2000, Thermo Scientific).

		BDNF rs6265		*SLC6A4* (5-HTTLPR, rs23351)		*HTR1A* rs6295		*HTR2A* rs7333412		*MAOA* rs1137070	
	Total	Val/val (G)	met- (A)	L_AL_A	S-	CC	G-	AA	G-	CC	T-
N	140	90 (64.3%)	50 (35.7%)	41 (29.3%)	99 (70.7%)	33 (23.6%)	107 (76.4%)	87 (62.1%)	53 (37.9%)	75 (53.6%)	65 (46.4%)
Age (mean ± s.d.)	26.7 ± 7.2	26.4 ± 7.2	27.3 ± 7.1	26.4 ± 6.4	26.9 ± 7.5	25.7 ± 6.5	27.1 ± 7.4	27.3 ± 8.0	25.9 ± 5.5	25.7 ± 5.9	28.0 ± 8.3
Sex (F/M)	84/56	53/37	31/19	24/17	60/39	22/11	62/45	53/34	31/22	35/40	46/16
PET scanner											
(A/H)	42/98	30/60	12/38	13/28	29/70	7/26	35/72	23/64	19/34	26/49	16/49
MRI scanner (T/V)	81/59	52/38	29/21	22/19	59/40	16/17	65/42	52/35	29/24	48/27	33/32
k2' (DASB kinetic modeling parameter)	0.065 ± 0.013	0.065 ± 0.013	0.066 ± 0.012	0.066 ± 0.014	0.065 ± 0.012	0.066 ± 0.014	0.065 ± 0.013	0.065 ± 0.012	0.066 ± 0.014	0.065 ± 0.013	0.066 ± 0.013
[^{11}]DASB-injected mass (μg)	3.2 ± 3.1	3.2 ± 2.9	3.2 ± 3.5	2.9 ± 2.9	3.3 ± 3.2	3.2 ± 3.2	3.2 ± 3.1	2.8 ± 2.7	3.8 ± 3.6	3.6 ± 3.5	2.7 ± 2.6
[^{11}]DASB-injected dose (MBq)	552 ± 78	554 ± 72	548 ± 88	562 ± 73	548 ± 80	570 ± 53	546 ± 84	561 ± 65	537 ± 94	545 ± 82	560 ± 73
cerebellum AUC (Bq ml^{-1})	16,561 ± 4555	16,469 ± 4415	16,726 ± 4839	16,421 ± 4557	16,619 ± 4576	17,468 ± 3712	16,281 ± 4766	17,103 ± 4070	15,671 ± 5175	15,778 ± 4646	17,465 ± 4308
Alcohol units per week	7.8 ± 10.8 (N = 131)	8.2 ± 12.8	7.1 ± 5.9	10.4 ± 16.3	6.8 ± 7.5	6.2 ± 5.5	8.4 ± 12.0	7.5 ± 11.8	8.3 ± 9.1	9.3 ± 13.8	6.1 ± 4.9
Smoking status (smokers/non smokers)	39/96 (N = 135)	24/62	15/34	9/31	30/65	10/21	29/75	24/60	15/36	16/57	23/39
PSQI	3.9 ± 2.0 (N = 97)	3.8 ± 2.2	4.1 ± 1.7	3.8 ± 2.0	4.0 ± 2.0	3.8 ± 2.0	4.0 ± 2.0	4.1 ± 2.0	3.7 ± 2.0	3.5 ± 1.7	4.4 ± 2.1
PSS	9.3 ± 5.1 (N = 126)	9.3 ± 5.1	9.4 ± 5.0	8.4 ± 4.3	9.7 ± 5.3	8.4 ± 4.8	9.6 ± 5.1	9.8 ± 4.9	8.5 ± 5.3	8.8 ± 5.2	10.0 ± 4.9

Table 1. Demographic data of the 140 healthy volunteers included in the study and [^{11}C]DASB scan information. Information regarding lifestyle was available only for subsets of the total participants: alcohol units per week (N = 131); Smoking status (N = 135); PSQI (N = 97); PSS (N = 126). Abbreviations: *BDNF*, brain-derived neurotrophic factor; *SLC6A4*, serotonin transporter gene; *5-HTTLPR*, serotonin-transporter-linked promoter region; *5-HT_{1A}R*, serotonin receptor 1A; *5-HT_{2A}R*, serotonin receptor 2A; *MAOA*, monoamine oxidase A; *F*, female; *M*, male; *PET*, positron emission tomography; *A*, GE-Advance PET scanner; *H*, HRRT PET scanner; *T*, Trio MRI scanner; *V*, Verio MRI scanner; *k2'* [^{11}C]DASB kinetic modeling parameter; *μg*, microgram; *MBq*, megabecquerel; *Bq ml^{-1}*, becquerel per milliliter; *AUC*, area under the curve (i.e., cerebellum reference region time activity curve); PSQI, Pittsburgh Sleep Quality Index; PSS, Cohen Perceived Stress Scale .

The SNPs were determined using TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA) with genotype-specific probes (*BDNF* rs6265: C_11592758_10, *HTR1A* rs6295: C__11904666_10, *HTR2A* rs7333412: C__29235757, *MAOA* rs1137070: C___8878813_20). We performed real-time polymerase chain reaction for allelic discrimination using the LightCycler 480 Real-Time PCR System (Roche Diagnostics, IN).

MRI data acquisition

For each participant, high-resolution T1-weighted structural brain scans were acquired on either a Siemens Magnetom Trio 3 T (N = 81) or a Siemens Verio 3 T (N = 59) (Siemens, Erlangen, Germany magnetic resonance imaging (MRI) scanner. We used structural MRI scans for segmentation and to delineate regions of interest in the PET scans.

[^{11}C]DASB PET data acquisition

We acquired PET scans for each participant on one of two PET scanners: 1) a Siemens ECAT high-resolution research tomography (HRRT) scanner operating in 3D-acquisition mode with an in-plane resolution of approximately 2 mm (N = 98) or 2) an 18-ring GE-Advance scanner (General Electric, Milwaukee, WI, USA) with a three-dimensional (3D) acquisition mode and an in-plane resolution of approximately 6 mm (N = 42). PET images were acquired on the HRRT scanner using a 6-min transmission scan followed by an intravenous bolus injection of [^{11}C]DASB that was given over 20 s while a dynamic 90-min emission scan was acquired over 36 frames (6 × 10 s, 3 × 20 s, 6 × 30 s, 5 × 60 s, 5 × 120 s, 8 × 300 s, 3 × 600 s). Dynamic PET images were reconstructed with an iterative OP-OSEM3D method using resolution modeling (10 iterations, 16 subsets)[50,51]. Images acquired on the GE-Advance scanner involved a 10-min transmission scan followed by a bolus injection given over 12 s while a 90-min dynamic emission scan was acquired over 36 frames (same time frames as HRRT acquisitions). Dynamic PET images from the GE-Advance scanner were reconstructed using filtered back projection and were corrected for attenuation, dead-time and scatter with a 6-mm Hann filter.

For both scanners, we determined single-subject PET scan motion and realignment using an automatic image registration algorithm[52]. Next, we smoothed the PET images using a 10 mm (HRRT) or a 12 mm (GE-Advance) within-frame Gaussian filter and subsequently aligned the volumes. Using the scaled least squares cost function, we estimated the rigid translation/rotation parameters that align each PET frame to a reference frame with sufficient structural information (frame 26: 20-25 min post injection). We resliced non-filtered PET images and co-registered the high-resolution MR with PET using SPM (HRRT) or automatic image registration (GE-Advance). Co-registration was based on the mean of frames 10–26, i.e., a flow-weighted image; the accuracy of this step was confirmed visually. We automatically delineated brain regions on structural MRI scans using Pvelab[53]. We determined the time-activity curves across gray matter voxels within each region except for the midbrain region, where we used the mean time-activity across all voxels. We determined regional 5-HTT non-displaceable binding potential (BP_{ND}) using kinetic modeling of regional time-activity curves in PMOD (Zurich, Switzerland) We applied the multilinear reference tissue model (MRTM/MRTM2) with a fixed k2' estimated for each individual in a high binding region (caudate, putamen, and thalamus) and with cerebellum as reference region[54]. We calculated regional BP_{ND} values bilaterally, computing a volume-weighted mean from the right and left hemisphere.

Data analysis

We carried out the statistical analyses in R v4.1.2 (https://cran.r-project.org/). Consistent with previously related analyses, we considered regional 5-HTT BP_{ND} within caudate, putamen, midbrain, thalamus, hippocampus, amygdala and neocortex as our regions of interest[23]. We selected a single neocortical region (including orbitofrontal-, parietal- and occipital cortex and superior frontal-, pre/post central-, superior temporal- and middle/inferior frontal gyrus) because of previous evidence of high correlation between different cortical areas[55]. For all analyses, we grouped genotypes as follows: *SLC6A4* 5-HTTLPR and rs23351: L_AL_A versus S'- carriers (individuals carrying at least one S- or one L_G allele); *HTR1A* rs6295: G-carriers (carrying at least one G-allele) vs CC homozygotes; *HTR2A* rs7333412: G- carriers vs AA homozygotes; *MAOA* rs1137070: T-carriers versus CC; *BDNF* rs6265: met-carriers versus val/val. In addition, we included age, sex, MRI and PET scanner type as covariates, consistent with previous findings[23]. We mean-centered all continuous variables. Although previous evidence showed a seasonal and body mass index (BMI) effect on 5-HTT availability[45,55], no statistically significant effect of neither daylight minutes nor BMI on 5-HTT BP_{ND} was previously observed on the same cohort[23] so these variables were not included as covariates. We considered additional lifestyle factors, including alcohol consumption (i.e., alcohol units per week), smoking status, total scores of the Pittsburgh Sleep Quality Index[56] and of the Cohen Perceived Stress Scale[57]. None of these measures were significantly associated with regional 5-HTT BP_{ND} ($p > 0.05$) and were not included as covariates in the main analyses. For all the models, we set the statistical significance threshold to $p < 0.05$ (two-sided tests).

Association analyses. We fit a linear latent variable model (LVM) to evaluate associations between genotypes and 5-HTT BP_{ND} within our set of pre-defined brain regions (i.e., caudate, amygdala, hippocampus, putamen, thalamus, midbrain and neocortex) as described previously[23]. LVMs are a type of multivariate linear regression that effectively models associations in the presence of inter-correlated variables. In this case, 5-HTT BP_{ND} is highly intercorrelated between the brain regions that we considered[23]. Thus, using the *lava* package v 1.6.10[58] in R, we modeled this shared correlation of regional 5-HTT BP_{ND} values with a latent variable (5-HTT_{LV}). Next, we modeled all the genotype and covariate effects on 5-HTT_{LV}. We included additional covariance links (caudate-putamen, amygdala-hippocampus and thalamus-midbrain) based on the model framework previously reported[23]. In addition, we used Wald tests of improvement in model fit to find additional paths. To control the

false-discovery rate across all possible paths, we included the paths with a false-discovery rate of $p_{FDR} < 0.05$ calculated using the Benjamini–Hochberg test across all paths.

We used caudate as a reference region, so that the covariate effects reported here can be interpreted as effects on caudate BP_{ND} (corresponding to the reference scale).

In addition, we estimated multiple linear regression models including all genotypes and covariates (age, sex, PET and MR scanner type) for each brain region and we used them to report percent differences in 5-HTT BP_{ND} between genotype groups. We reported all results with parameter estimates and 95% confidence intervals within brackets, including the associated units (Fig. 1).

Finally, we tested for a main effect of each variant using a likelihood ratio test comparing a model including all covariates and genotypes with a model including all covariates but not genotypes.

Prediction analyses. To determine whether genotype information predicts regional 5-HTT BP_{ND}, we trained and tested a random forest model[59]. We used the *randomForest* package v.4.7–1.1 in R with default data sampling and model fitting parameters; this included p/3 sampled features per tree (where p represents the total number of features) and 500 trees per forest. As we were specifically interested in how well genetic information predicted 5-HTT BP_{ND}, above and beyond other covariates, we constrained our feature set to include only the five genotypes and we evaluated the prediction of regional 5-HTT BP_{ND}, adjusted for relevant covariates. First, we fitted a multiple linear regression model regressing each region, e.g., caudate 5-HTT BP_{ND}, against age, sex, PET-scanner, and MR-scanner. The residuals from this multiple linear regression model were used as the outcome in our random forest machine learning models with genotype status for rs6265, 5-HTTLPR and rs23351, rs6295, rs7333412, and rs1137070 as model features. Prediction models were estimated using five-fold cross-validation; prediction of 5-HTT BP_{ND} on held out datasets (test data) within each fold was used to determine unbiased, predictive model performance. Model performance was calculated as the root mean-squared error (RMSE) of prediction on test data, across all folds. To account for fold-assignment related variance, model performance was assessed by repeating the five-fold cross-validation 10 times; overall performance was the mean of these 10 resamples. Statistical significance of performance was calculated from an empirical null distribution derived from 10,000 permutations of the resampled residuals. Notably, we performed five-fold cross-validation with 10 resamples within each of the 10,000 permutations so that each permutation more closely reflects our observed model structure. We fitted models for 5-HTT BP_{ND} in each of our seven regions of interest separately, i.e., seven different prediction models. In addition to expressing model performance in terms of RMSE, we express the percent change in RMSE of the prediction models accounting for genotype information ($RMSE_{genotype}$) compared to the RMSE computed on residual 5-HTT BP_{ND} values ($RMSE_{residual}$, i.e., $\Delta RMSE = 100*(RMSE_{residual} - RMSE_{genotype})/RMSE_{residual}$). Statistical significance (p-value) of model performance is expressed both uncorrected (p_{unc}) and corrected (p_{FWE}) for the seven models estimated, using Bonferroni-Holm, which controls the family-wise type-I error rate[60].

Results

Genotyping

Genotype distribution and allelic frequencies are depicted in Table 1. rs6265, rs1137070, rs7333412, rs6295 and rs1137070 were in Hardy–Weinberg equilibrium (all $p > 0.16$). *MAOA* is x-linked so the allele frequency for rs1137070 in men was compared with the frequency in women using a chi-squared test to determine whether there were significant differences between allele frequencies in males and females. We did not find a statistically significant difference in allele frequencies between males and females ($P = 0.67$). Assessment of Hardy–Weinberg for the 5-HTTLPR is not valid because this polymorphism was an inclusion criterion for some studies from which these data are derived, i.e., participants were not sampled independent of 5-HTTLPR genotype[9,43].

Association analyses

The likelihood ratio showed that the LVM including genotype information was statistically significantly different from the LVM not including this information ($p = 0.002$), suggesting that genotype significantly contributed to the model.

The results from the LVM are depicted in Fig. 1. Consistent with previous observations, we observed that all regional 5-HTT BP_{ND} values loaded strongly on to 5-HTT_{LV} (all $p < 10^{-12}$). After adding genetic variants and covariates (sex, age, PET and MRI scanner) to the model a Wald test did not support the inclusion of additional paths to the model ($p_{FDR} > 0.05$).

Within our final model, we found a statistically significant association between *MAOA* rs1137070 T-carriers (vs CC homozygotes) and 5-HTT_{LV} (estimate: 0.07, 95% CI: [8.08×10^{-6}, 0.14], $p = 0.039$). Across regions, rs1137070 T-carriers showed ~ 2–11% higher 5-HTT BP_{ND} compared to CC individuals, the largest differences were observed in caudate (~ 11%) and putamen (~ 9%) and the lowest in amygdala (~ 2%).

As expected based on our previous evaluation of this model, we observed: (1) higher caudate 5-HTT BP_{ND} in males vs females (estimate: 0.14, 95% CI: [0.06, 0.21], $p = 3.88 \times 10^{-4}$); (2) a negative association between age and 5-HTT_{LV} (estimate: − 0.009, 95% CI: [− 0.015, − 0.0038], $p < 0.001$); (3) increased subcortical 5-HTT BP_{ND} in *BDNF* rs6265 met-carriers vs val/val individuals (estimate: − 0.06, 95% CI: [-0.04, -0.01], p = 0.07), corresponding to a 2–6% increase in 5-HTT BP_{ND} across subcortical areas. Conversely, an additional direct path from *BDNF* rs6265 to neocortex 5-HTT BP_{ND} effectively nullified the genetic effect on this brain region, which corresponds to the sum of indirect (*BDNF* rs6265—> 5-HTT_{LV}—> neocortex BP_{ND}) and direct (*BDNF* rs6265—> neocortex BP_{ND}) effects. Although the estimate for statistical significance for rs6265 decreased in the presence of the three other genotypes, the effect size remained very similar, suggestive of independent main effects of *BDNF* rs6265 and *MAOA* rs1137070.

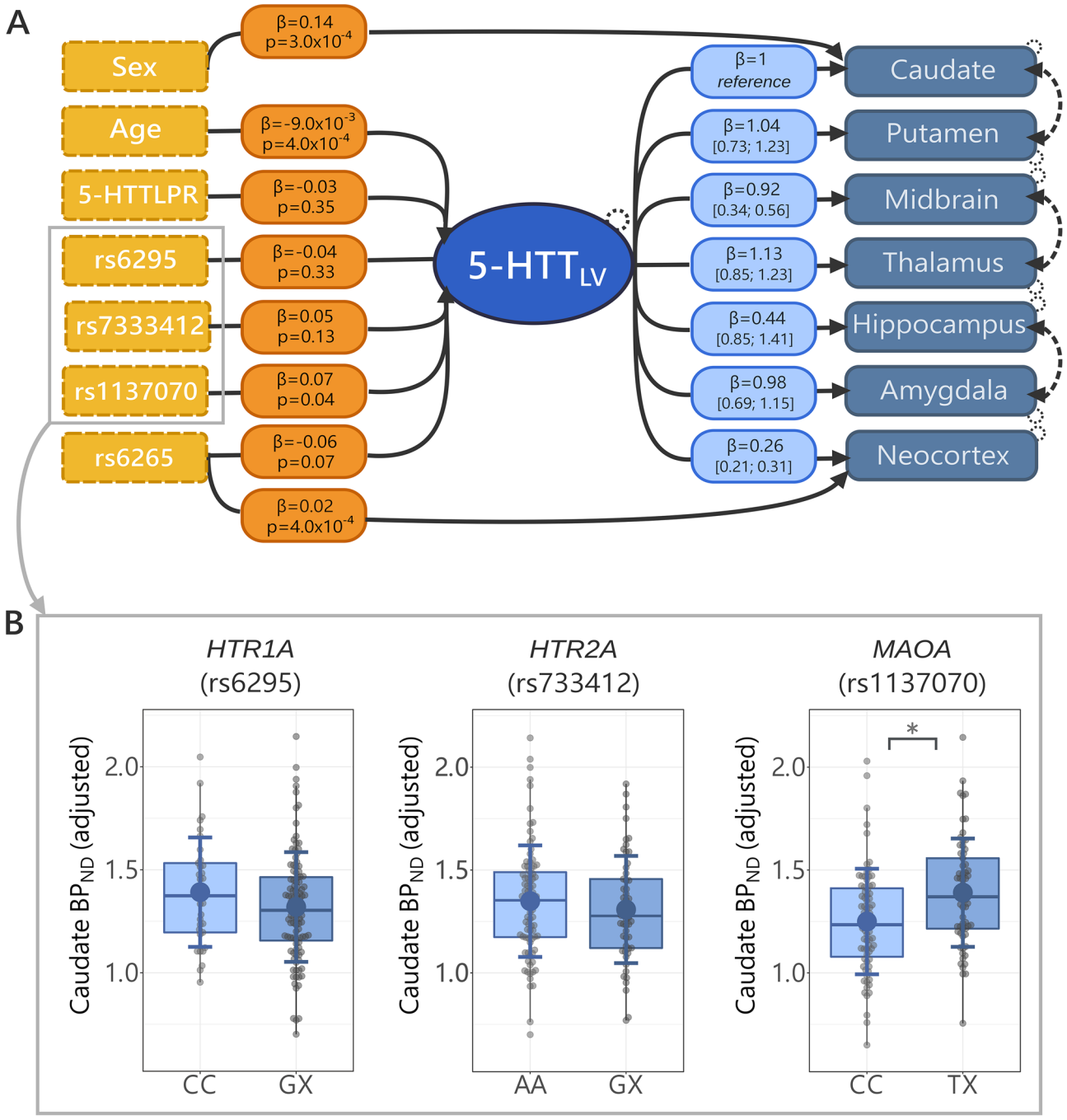

Figure 1. (**A**) Latent variable model (LVM) used to compute the association between each genotype independently from the other genotypes and 5-HTT BP_{ND} in caudate, putamen, midbrain, thalamus, hippocampus, amygdala and neocortex. PET and MRI scanner are not shown but were included as covariates. Yellow hatched boxes to the left represent the genetic variants and the covariates. The genetic variants depicted correspond to the following genes respectively: 5-HTTLPR- > *SLC6A4*, rs6295- > *HTR1A*, rs7333412- > *HTR2A*, rs1137070- > *MAOA*, rs6265- > *BDNF*. The orange boxes represent the covariate effects on the latent variable ($5\text{-}HTT_{LV}$), which is represented by the central blue ellipse. Light blue boxes show the loadings on the latent variable of observed regional 5-HTT BP_{ND} (in the blue solid boxes to the right). β values in light blue and orange boxes indicate the parameter estimates for each model parameter with either its respective p-value (orange boxes) or 95% confidence interval (light blue). Hatched lines between regions indicate interregional shared correlations. Hatched circles on the brain regions represent the included error estimates. Arrows from the yellow to the blue boxes (sex- > caudate, rs6265- > neocortex) represent direct covariate effects on binding. All brain regions significantly loaded on to $5\text{-}HTT_{LV}$ ($p < 1 \times 10^{-12}$). (**B**) Boxplots showing representative effects of *HTR1A* rs6295, *HTR2A* rs7333412 and *MAOA* rs1137070 on caudate 5-HTT BP_{ND}. The y axis represents 5-HTT BP_{ND}, adjusted for age, sex, MRI scanner, and PET scanner. Gray dots represent 5-HTT BP_{ND} from each participant adjusted for age, sex, MRI and PET scanner. The larger solid dots and lines represent respective group means and ± 1 SD. The boxes represent datapoints from the 25% to the 75% quantile.5-HTT: serotonin transporter; *SLC6A4*: 5-HTT gene; *HTR1A*: serotonin receptor 1A gene; *HTR2A*: serotonin receptor 2 gene; *MAOA*: monoamine oxidase A gene; *BDNF*: brain-derived neurotrophic factor gene; MRI: magnetic resonance imaging (MRI); PET: positron emission tomography; BP_{ND}: non-displaceable binding potential.

We did not observe evidence for an association between 5-HTTLPR ($p = 0.35$), *HTR1A* rs6295 ($p = 0.33$), nor *HTR2A* rs7333412 ($p = 0.13$) and 5-HTT$_{LV}$.

Prediction analyses

Across the seven regions evaluated, we observed that the set of genetic variant features slightly improved prediction of caudate 5-HTT BP$_{ND}$ compared to the model not including genetic information (caudate: RMSE$_{residual}$ = 0.262, RMSE$_{genotype}$ = 0.266, ΔRMSE = 1.6%, p$_{unc}$ = 0.036) (Table 2). However, this effect, and the effect in all other regions was not statistically significant after correction for seven models (Table 2).

Discussion

We observed that *MAOA* rs1137070 T-carriers had higher 5-HTT availability compared to CC individuals. T-carriers showed higher 5-HTT availability in all the seven brain regions examined, with the highest binding in caudate (~ 11%) and the lowest in amygdala (~ 2%). Conversely, variants in the *HTR1A* and *HTR2A* genes were not associated with 5-HTT availability. Despite observing evidence for a statistically significant association, the genetic variants were not significantly informative for predicting brain 5-HTT available above chance. Taken together, our findings support that genetic variation in the *MAOA* contributes to variation in brain 5-HTT availability in the healthy adult human brain.

MAOA degrades monoamines in the brain, including serotonin, for which it has a preferential affinity compared to its other substrates[61]. *MAOA* knock-out rodents have increased extracellular serotonin levels and abnormal affective behavior[30,62–64] as well as reduced 5-HTT expression[63,65], suggesting that genetically altered MAOA signaling can affect regulation of 5-HT levels, which may in turn modulate 5-HTT levels e.g. via downregulation[31]. Similarly, inhibition of MAO activity by monoamine oxidase inhibitors increases extracellular serotonin levels[62] and is associated with reduced 5-HTT BP$_{ND}$ in rhesus monkey and rats[66]. Notably, reductions in 5-HTT BP$_{ND}$ following an acute pharmacologically-induced serotonin increase may not only reflect a down-regulation of 5-HTT but also increased serotonin levels competing for the radioligand rather than a change in 5-HTT gene expression[67].

The rs1137070 T-allele has been previously associated with lower MAOA enzymatic activity compared to the C-allele both in human fibroblasts in vitro and in post-mortem brains[35,68]. Studies on clinical populations have reported mixed findings[41,69,70]. Although some studies suggest a link between the T-allele and increased MAOA mRNA expression in peripheral blood of patients with depression compared to healthy controls[41], as well as increased vulnerability to depression[41,69], other studies reported an association between the C-allele and impaired antidepressant treatment outcome in women[70].

Conversely, we found that healthy human rs1137070 T-carriers, previously associated with low MAOA activity compared to C-carriers, had greater 5-HTT availability. In this case, putatively lower MAOA activity would correspond to greater amounts of intra- and extracellular serotonin. We can speculate that increased 5-HTT availability reflects increased 5-HTT levels, which might be a compensatory mechanism put in place to reuptake the excess serotonin and maintain extracellular serotonin levels constant. Nonetheless, findings from preclinical research point towards an effect opposite to what we observed, whereas the studies in humans provided mixed findings. Thus, the ambiguity provided by previous evidence in humans does not allow us to draw conclusions about the relationship that we detected between *MAOA* rs1137070 and 5-HTT BP$_{ND}$.

We did not observe evidence for an effect of *HTR2A* rs7333412 on 5-HTT availability. A previous study in patients with major depressive disorder, bipolar disorder, and healthy participants reported an effect on thalamus 5-HTT levels[25]. We did not replicate this effect in our study, as indicated by a comparison of Fig. 2 of their manuscript and our observed group differences. This is possibly because our study is based on a larger and more homogeneous cohort of healthy participants, whereas the previous study included patients with major depressive disorder and bipolar disorder as well as individuals with varying ethnic backgrounds. Taken together, our findings do not support that this *HTR2A* variant is associated with changes in 5-HTT availability in healthy adults.

Similarly, we did not observe evidence that *HTR1A* rs6295 is associated with 5-HTT availability, suggesting that whatever effects this polymorphism may have directly on the serotonin 1A receptor, those effects do not significantly modulate 5-HTT availability in healthy adults as measured with [^{11}C]DASB PET.

Notably, we previously reported an association between *BDNF* rs6265 and 5-HTT BP$_{ND}$, such that met-carriers showed reduced binding in subcortical areas compared to val-homozygotes[23]. Although this effect was marginally

Region	RMSE$_{residual}$	RMSE$_{genotype}$	ΔRMSE	p$_{unc}$	p$_{FWE}$
Caudate	0.266	0.263	1.61%	0.036*	0.252
Putamen	0.301	0.300	0.43%	0.112	0.560
Midbrain	0.251	0.253	− 1.01%	0.078	0.468
Thalamus	0.305	0.302	0.88%	0.205	0.652
Hippocampus	0.121	0.121	− 0.07%	0.468	0 .936
Amygdala	0.272	0.268	1.37%	0.163	0.652
Neocortex	0.056	0.057	− 1.75%	0.752	0.936

Table 2. Uncorrected (p$_{unc}$) and corrected (p$_{FWE}$) p-values for each brain region; root mean squared error computed using residual 5-HTT BP$_{ND}$ values (RMSE$_{residual}$), using genotype information (RMSE$_{genotype}$) and percent change in RMSE between RMSE$_{genotype}$ and RMSE$_{residual}$ (ΔRMSE). * indicates p$_{unc}$ < 0.05.

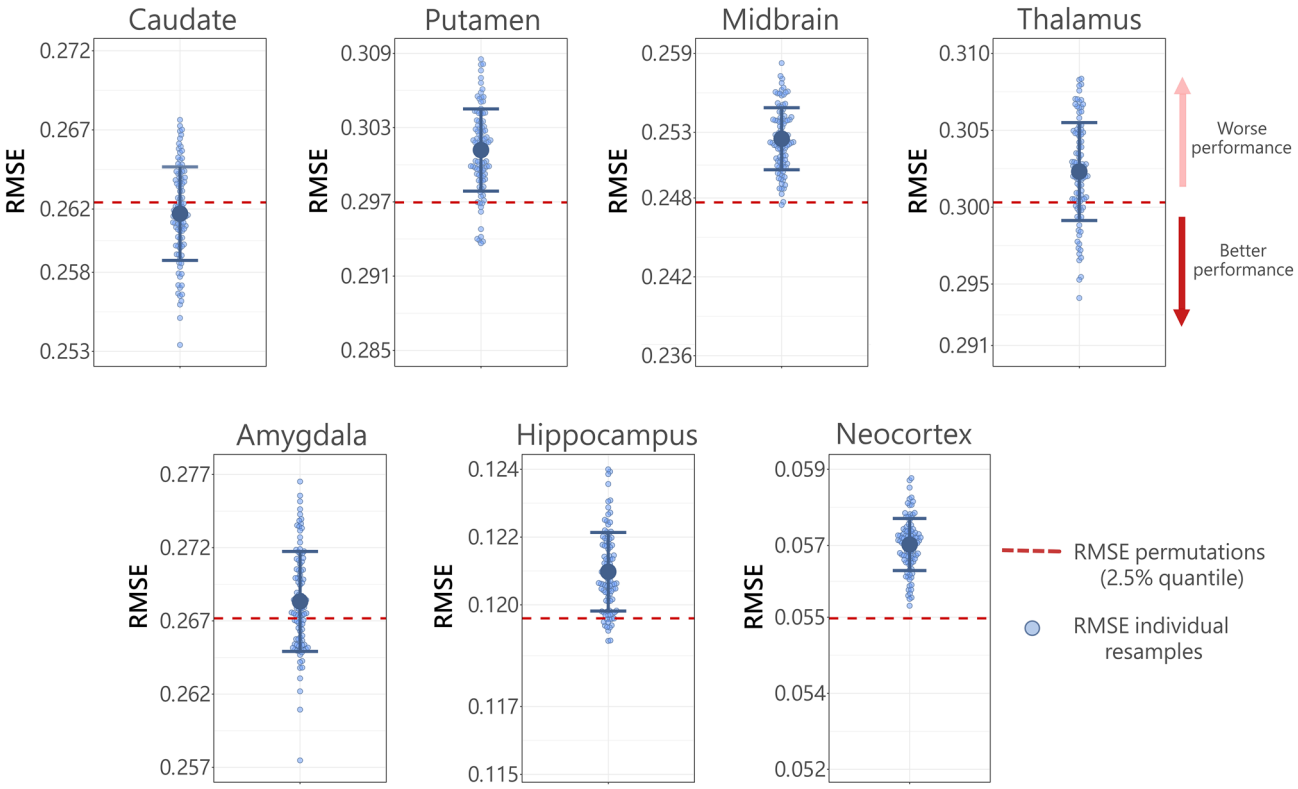

Figure 2. Random forest model performance. The light blue dots represent the individual RMSE values obtained from resampling (for display purposes, the distribution of the RMSE values from resampling is derived from a model run with 100 instead of 10 resamples) of the model including genotype information ($RMSE_{genotype}$). The dark blue error plot displays the mean ± standard deviation of the distribution. The red hatched line indicates the 2.5% quantile of the average RMSE value derived from 10,000 permutations in the model that did not include genotype information ($RMSE_{residual}$). Dark blue dots below the red hatched line indicate that the model performed significantly better than chance upon adding genotype information.

above our threshold for statistical significance in the current model, the effect size was very similar, suggesting independent contributions of *MAOA* rs1137070 and *BDNF* rs6265 to 5-HTT availability in healthy humans. As we reported previously, 5-HTTLPR was not significantly associated with 5-HTT BP_{ND} in this cohort[23,23].

Regarding our prediction model, we observed that using genotype information led to a marginal improvement in predicting caudate 5-HTT BP_{ND} vs not using genotype information, but this effect was not significant after correcting for multiple comparisons. In addition, we could not predict 5-HTT BP_{ND} in any other brain regions. This limited performance may be because we evaluated only five variants, whereas genetically induced variation in 5-HTT levels likely stems from many variants.

Previous studies underscore the limited extent to which candidate variants exert main effects on complex behavioral traits or related features of brain activity[20,71]. Direct measures of discrete neurobiological features, e.g., serotonin transporter protein levels, may however be more susceptible to genetic variants that modulate the relevant neurotransmission pathways[72,73]. Nevertheless, alternative genetic analysis strategies such as GWAS would undoubtedly provide a more comprehensive evaluation of genetic contributions to 5-HTT levels in the human brain. However, an exploratory GWAS requires either thousands of datasets or very large effect sizes (i.e., > 20% difference in 5-HTT BP_{ND}, Cohen's d > 1) to establish statistical significance. GWAS-based polygenic risk scores, e.g., for psychiatric disorders or independent-dataset, hypothesis-generation via, e.g., expression quantitative trait loci (eQTL) databases may instead provide informative and statistically viable strategies for resolving genetic contributions to variation in brain serotonin neurotransmission measured with PET. Our cohort of 140 healthy participants stands as the largest single database of 5-HTT PET brain scans in the world. Future studies probing genetic contributions to brain serotonin-related PET scans would likely benefit from pooling data via, e.g., OpenNeuro (https://openneuropet.github.io/).

Although genetic variation is plausible and partially supported by our findings, environmental factors are also likely to contribute to 5-HTT levels[67]. A previous study based on the same cohort used for the present study reported no association between 5-HTT levels and daylight minutes or body mass index, contrarily to what reported by earlier studies based on smaller cohorts, part of which are included in our analyses[42,55]. In addition, we could not find any effect of variables reflecting lifestyle measures such as smoking, alcohol consumption, sleep and perceived stress on 5-HTT levels, suggesting that such environmental contributions in this cohort did not confound the observed genetic effects.

All participants self-identified with European ancestry. Self-reports of ancestry can be inaccurate[74] and the lack of ethnic diversity in our sample limits the generalizability of our findings.

Notably, we exclude that the association between 5-HTT availability and the rs6265 and rs1137070 genotypes is due to a direct effect of genotype on BP_{ND}. BP_{ND} is proportional to the amount of target proteins available for binding (B_{avail}), i.e. 5-HTT, the affinity constant of the radioligand for its target (K_D) and the free fraction of ligand in the non-displaceable tissue compartment (f_{ND})[75]. We infer the observed genetic effects to be primarily related to change in B_{avail}, although we cannot rule out effects on K_D or f_{ND}. Nonetheless, this seems unlikely because rs6265 and rs1137070 are proximal to *SLC6A4*, but a two scan study structure could more directly disentangle effects of B_{avail} and K_D[76].In conclusion, we report evidence for the association between *MAOA* rs1137070 genotype and brain 5-HTT availability. We did not observe evidence for an effect of *HTR1A* and *HTR2A* variants previously associated with brain serotonin markers, suggesting that their contribution may not be relevant to 5-HTT availability in the healthy adult human brain. Future studies considering additional genetic variants as well as environmental factors in larger datasets are critical for improving our understanding of the factors shaping serotonergic neurotransmission in health and disease.

Data availability

The R code employed for statistical analyses can be made available upon request to the corresponding author (patrick.fisher@nru.dk). Data can be made available upon reasonable request via this form (https://cimbi.dk/index.php/documents/category/3-cimbi-database) and with an appropriate data sharing agreement.

Code availability

The R codes used for the latent variable model and the random forest model described in this manuscript can be made available upon request.

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Author contributions

S.E.P.B.: Data analysis, writing of the original draft, figures design and execution, manuscript editing and reviewing. A.N.: Data analysis, manuscript editing and reviewing. S.S.A.: Data analysis, manuscript editing and reviewing. M.S.: Conceptualization, funding, manuscript editing and reviewing. B.O.: Data analysis, manuscript editing and reviewing. P.S.J.: Data management, manuscript editing and reviewing. G.M.K.: Funding, manuscript editing and reviewing, supervision. V.G.F.: Data acquisition, manuscript editing and reviewing, supervision. P.M.F.: Conceptualization, funding, data analysis, data acquisition, manuscript editing and reviewing, supervision.

Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Materials I

Genetic variant	Alleles	Functional effects	Role in serotonergic neurotransmission	References
***BDNF* (rs6265)**	**G/A** (more commonly **Val/Met**)	Lower BDNF circulating levels in met- (or G-) carriers vs val/val (or A/A) homozygotes.	Increased subcortical 5-HTT BP_{ND} in healthy human met-carriers.	(Fisher et al., 2017)
			Decreased 5-HT_{1A} BP_{ND} in healthy human met-carriers but not in patients with depression.	(Lan et al., 2014)
			Lower 5-HTT BP_{ND} in healthy human male met-carriers but no association with $5HT_{1A}$ BP_{ND}.	(Henningsson et al., 2009)
			Increased 5-HT_4 BP_{ND} in healthy human met-carriers.	(Fisher et al., 2015)
			No association between SNP and 5-HTT nor 5-HT_{1A} BP_{ND} in healthy human volunteers.	(Kraus et al., 2014)
			No association with neither 5-HTT nor $5HT_{2A}$ BP_{ND} nor BDNF blood levels in healthy human volunteers.	(Klein et al., 2010)
***SLC6A4* (5-HTTLPR, rs23351)**	**L_AL_A** vs **S-** (L_GL_A, L_AS, L_GS, SS)	Higher 5-HTT expression in L_AL_A homozygotes compared to S- carriers.	Increased 5-HTT BP_{ND} in L_AL_A homozygotes.	(Kalbitzer et al., 2009; Praschak-Rieder et al., 2007; Matthias Reimold et al., 2011)
			No association with 5-HTT BP_{ND}.	(Parsey et al., 2006, Fisher et al., 2017)
			Lower 5-HT_4 neocortical BP_{ND} in S- carriers.	(Fisher et al., 2012)
***HTR1A* (rs6295)**	**C/G**	Higher 5-HT_{1A}R expression in G- carriers vs CC homozygotes.	Higher 5-HT_{1A} BP in in G- carriers.	(Parsey et al., 2005)
			No association between SNP and 5-HT_{1A} BP in either healthy volunteers or patients with depression.	(Kaufman et al., 2015)

			G- allele linked to increased *5HT1AR* expression.	(Lemonde et al., 2003; Czesak et al., 2006; Pernhornst et al., 2013)
***HTR2A* (rs7333412)**	**G/A**	Unknown	Lower 5-HTT BP_{ND} in A- carriers.	(Laje et al., 2010)
			Impaired response to antidepressant treatment in GG patients with depression vs A-carrier patients.	(Qesseveur et al., 2016)
MAOA (rs1137070)	**T/C**	C allele associated with higher MAOA activity.	C- allele associated with poorer antidepressant treatment outcome when the C- allele of *MAOA* rs6323 is present.	(Xu et al., 2011)
			C- allele associated with greater MAOA activity levels in cultured human fibroblasts.	(Hotamisligil and Breakefield, 1991)
			C- allele associated with greater *MAOA* expression in human post-mortem brains.	(Pinsonneault et al., 2006)
			Increased risk for depression and greater MAOA mRNA levels in T-carriers.	(Zhang et al., 2010)
			Impaired antidepressant (venlafaxine) treatment outcome in female C- carriers.	(Bi et al., 2021)

Table S1.

Overview of the alleles, the respective functional effects of the serotonin-related genetic variants examined, and previous findings about their role in serotonergic neurotransmission. 5-HTT: serotonin transporter; *SLC6A4*: 5-HTT gene; $5\text{-}HT_{1A}$: serotonin 1A receptor; *HTR1A*: serotonin receptor 1A gene; $5\text{-}HT_{2A}$: serotonin 2A receptor; *HTR2A*: serotonin receptor 2 gene; $5HT_4$: serotonin 4 receptor; *MAOA*: monoamine oxidase A gene; *BDNF*: brain-derived neurotrophic factor gene; BP: binding potential; BP_{ND}: non-displaceable binding potential.

STUDY II

Clinical Epigenetics

RESEARCH

Open Access

No association between peripheral serotonin-gene-related DNA methylation and brain serotonin neurotransmission in the healthy and depressed state

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Abstract

Background Methylation of serotonin-related genes has been proposed as a plausible gene-by-environment link which may mediate environmental stress, depressive and anxiety symptoms. DNA methylation is often measured in blood cells, but little is known about the association between this peripheral epigenetic modification and brain serotonergic architecture. Here, we evaluated the association between whole-blood-derived methylation of four CpG sites in the serotonin transporter (*SLC6A4*) and six CpG sites of the tryptophan hydroxylase 2 (*TPH2*) gene and in-vivo brain levels of serotonin transporter (5-HTT) and serotonin 4 receptor (5-HT_4) in a cohort of healthy individuals ($N=254$) and, for 5-HT_4, in a cohort of unmedicated patients with depression ($N=90$). To do so, we quantified *SLC6A4/TPH2* methylation using bisulfite pyrosequencing and estimated brain 5-HT_4 and 5-HTT levels using positron emission tomography. In addition, we explored the association between *SLC6A4* and *TPH2* methylation and measures of early life and recent stress, depressive and anxiety symptoms on 297 healthy individuals.

Results We found no statistically significant association between peripheral DNA methylation and brain markers of serotonergic neurotransmission in patients with depression or in healthy individuals. In addition, although *SLC6A4* CpG2 (chr17:30,236,083) methylation was marginally associated with the parental bonding inventory overprotection score in the healthy cohort, statistical significance did not remain after accounting for blood cell heterogeneity.

Conclusions We suggest that findings on peripheral DNA methylation in the context of brain serotonin-related features should be interpreted with caution. More studies are needed to rule out a role of *SLC6A4* and *TPH2* methylation as biomarkers for environmental stress, depressive or anxiety symptoms.

Keywords Serotonin transporter, 5-HT, Tryptophan hydroxylase 2, TPH2, Serotonin 4 receptor, Depression, Human brain imaging, PET, Mood disorders, Epigenetics, Early life stress

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Background

Most psychiatric disorders, including major depressive disorder (MDD), arise from a complex etiology, with contributions from genetic and environmental factors. The serotonin system mediates a variety of different functions from the very early stages of development and throughout life, including cognition, mood and sleep as well as adaptation to environmental challenges [1–4]. For instance, serotonin-mediated neuroplasticity has been suggested to allow us to adapt to the ever-changing environment [5]. In this case, alterations in serotonin function might translate into resilience or vulnerability to MDD [2, 6].

DNA methylation of genes coding for key regulators of the serotonin system, such as the serotonin transporter (*SLC6A4*) and the tryptophan hydroxylase 2 gene (*TPH2*), has been proposed as a possible gene-by-environment mechanism involved in several psychiatric disorders, including MDD [7, 8]. However, DNA methylation is often measured in peripheral samples (e.g. blood or saliva) and little is known of the effect of this modification on in-vivo brain serotonin transmission. In this study, we investigated the association between methylation of peripheral *SLC6A4* and *TPH2* and brain proxies of serotonin transmission measured with in-vivo positron emission tomography (PET) imaging.

The serotonin transporter (5-HTT) and tryptophan hydroxylase 2 (TPH2) critically shape serotonin signalling by regulating serotonin levels. Specifically, 5-HTT regulates synaptic levels of serotonin available for neurotransmission and is the main target of selective serotonin reuptake inhibitors (SSRI), the most widely used class of antidepressant medications. TPH2 is the rate-limiting enzyme for serotonin synthesis in the brain [9], thereby directly affecting presynaptic serotonin levels.

A role of *SLC6A4* in gene-by-environment interaction was initially described by Caspi and colleagues [10], reporting that 5-HTTLPR s-carriers, who had lower *SLC6A4* expression, were more vulnerable to stress in terms of developing depressive episodes when experiencing stressful life events. Nonetheless, these findings have not been replicated by all larger studies [11, 12], suggesting that 5-HTTLPR per se may not be as relevant to MDD or anxiety-related traits as previously thought. Instead, a combination of genetic and epigenetic factors may affect *SLC6A4* gene expression levels [13] in a way that may be relevant to the development of psychopathology [14].

Over the last two decades, several studies have pointed to a possible role of DNA methylation levels in the transcriptional control region of *SLC6A4* as a marker of gene-by-environment interaction [7, 15–17]. Specifically, alterations in *SLC6A4* methylation have been associated with recent [15] and early life stress [7, 18], depressive symptoms [19], panic disorder [16] and likelihood to respond positively to antidepressant treatment [20, 21], although the relation with depressive symptoms and antidepressant treatment outcome was not confirmed by all studies [20, 22].

More recently, methylation of *TPH2* gene has also been suggested as a biomarker for vulnerability to depression and antidepressant treatment outcome [23, 24].

DNA methylation at cytosine-guanine dinucleotides (CpG) is a common epigenetic modification which can affect gene expression in response to environmental cues [25]. Early studies reported an association between *SLC6A4* methylation and 5-HTT mRNA levels measured in lymphocytes [13] and peripheral whole blood [26, 27]. However, it is not known whether such altered peripheral 5-HTT mRNA levels also correlate with brain 5-HTT protein levels. Given the fundamental role of 5-HTT and TPH2 in serotonin neurotransmission, understanding whether peripheral *SLC6A4* or *TPH2* methylation mirrors serotonin brain architecture is essential to interpret previous findings and to shed light on the role of peripheral methylation in the context of health and disease, e.g. psychiatric disorders.

Indeed, it is important to note that DNA methylation is cell-type specific [28]. However, as brain tissue of living human participants is mostly unavailable for biomarker assessment, blood and saliva are the most used tissue types for the investigation of DNA methylation. Peripheral blood and postmortem brain DNA methylation partially correlate at multiple CpG sites, but there is not a perfect correspondence between the two tissues [29–31]. Evaluating DNA methylation associations with in vivo brain serotonin markers allows to estimate its relevance as a peripheral marker of serotonin neurotransmission. However, to our knowledge, no study has investigated whether peripheral methylation of *SLC6A4* or *TPH2* is associated with brain levels of 5-HTT or with other markers of serotonin neurotransmission, such as serotonin 4 receptor (5-HT_4), a post-synaptic serotonin receptor that has been proposed as a biomarker for brain serotonin tonus [32]. Only one study reported an association between *SLC6A4* promoter methylation and brain serotonin synthesis measured in terms of brain tryptophan levels [27].

PET imaging allows quantification of serotonin system protein levels in the living brain [33, 34]. In this study, we used PET scans of 254 healthy participants and 90 patients with MDD to determine the relation between peripheral *SLC6A4* and *TPH2* methylation and two key features of the serotonergic brain signalling system, i.e. 5-HTT and 5-HT_4, imaged with combined with [^{11}C]DASB and [^{11}C]SB207145 PET radiotracers, respectively.

Both the 5-HTT and 5-HT_4 are known to play a role in healthy brain function and in MDD pathology and can be considered as key markers for serotonin neurotransmission [35–38].

Furthermore, as primary sensitivity analyses, we evaluated the association between DNA methylation and self-reported early life stress and stressful life events, as well as state measures of perceived stress and anxiety and depressive symptoms in 297 healthy participants.

Finally, blood is a heterogeneous tissue containing different cell types. Interindividual differences in blood cell proportions can be a source of bias on DNA methylation measurements carried out on whole blood [39], hindering comparability between individuals. Nonetheless, while epigenome-wide studies routinely correct for blood cell proportions [40], most of previous studies linking *SLC6A4* and *TPH2* methylation to environmental stress [15, 41] or psychiatric conditions [7, 16, 21, 23] did not account for blood cells proportions. Thus, we used blood cell counts to estimate blood cell proportions in a subgroup of participants for whom this information was available. Then, we included cell proportions in all our statistical models as secondary sensitivity analyses.

Methods

Participants

All participants included in this study were recruited as part of neuroimaging projects conducted at Neurobiology Research Unit, Copenhagen University Hospital Rigshospitalet, in compliance with the Declaration of Helsinki and Good Clinical Practice guidelines. An overview of the methods is depicted in Fig. 1.

Healthy Cohort

We included data of healthy volunteers from the Cimbi database and biobank [42]. The data was included based on the following criteria: (1) availability of [^{11}C]DASB PET or [^{11}C]SB207145 PET; (2) availability of whole blood or buffy coat samples matching scan date (blood samples drawn maximum one week before or after the PET scan were also included for $N=3$) and (3) self-identification with European ancestry. Before inclusion in any of the original studies, all participants were screened for psychiatric disorders and underwent a physical and neurological examination. Participants with a history of psychiatric illness or current use of psychotropic drugs or drugs potentially affecting PET measurements were excluded.

We identified a cohort of 142 participants with [^{11}C]DASB PET scans and a cohort of 112 participants with [^{11}C]SB207145 PET scans. Demographic data relative to the cohorts included in the study are depicted in Table 1.

To evaluate the potential association between DNA methylation and early life stress history or state measures of perceived stress, depressive or anxiety symptoms, data from an additional 43 healthy participants without PET scans was available, resulting in a total of 297 healthy participants. Demographics for the participants included in all analyses are depicted in Table S1.

MDD patient cohort

We included baseline data from 90 unmedicated patients with moderate to severe unipolar MDD that were originally part of the NeuroPharm-1 study [43], an open-label, non-randomized longitudinal clinical trial. Patients were included based on the availability of both blood samples and [^{11}C]SB207145 PET scans that were collected no more than one week apart.

The primary outcome of the trial involved measures of molecular neuroimaging and cognitive functions and is described in previous publications [36, 43, 44]. Shortly, previously unmedicated patients with MDD were recruited for an open-label clinical trial aiming to uncover biomarkers predicting clinical outcome after 12 weeks of antidepressant treatment. In this study, we included blood samples, PET scans and psychometric data collected at baseline. Analyses carried out in this study involving *SLC6A4* are planned secondary analyses. Analysis including *TPH2* are unplanned exploratory analyses. Participants were evaluated in face-to-face interviews and diagnosed by a certified psychiatrist. Individuals between 18–65 years of age, scoring > 17 in the Hamilton Depression Rating Scale 17 items ($HAMD_{17}$) [45] and who were unmedicated for at least two months

(See figure on next page.)

Fig. 1 Overview of the data and methods used in this study. **a**, **b**, **c**, **d** depict the primary analyses, in which a latent variable model was used to determine the association between peripheral *TPH2* and *SLC6A4* methylation and brain levels of 5-HTT and 5-HT_4. **e**, **f**, **g** describe the sensitivity analyses evaluating the association between *SLC6A4*/*TPH2* methylation and measures of environmental stress, depressive and anxiety symptoms. **h** and **i** show sensitivity analyses used to evaluate potential influence of blood cell proportions in the A-G analyses. Abbreviations: 5-HT_4: serotonin 4 receptor; 5-HTT: serotonin transporter; CpG: CpG site; TSS: transcription start site; SLE: stressful life events; PBI: parental bonding inventory; BDI: Beck's depressive index; GAD10: generalized anxiety disorder 10-item; CATS: childhood abuse trauma scale; HAMD6: Hamilton depressive rating scale 6; PSS: perceived stress scale; CpG_{LV}: latent variable including all CpG methylation values

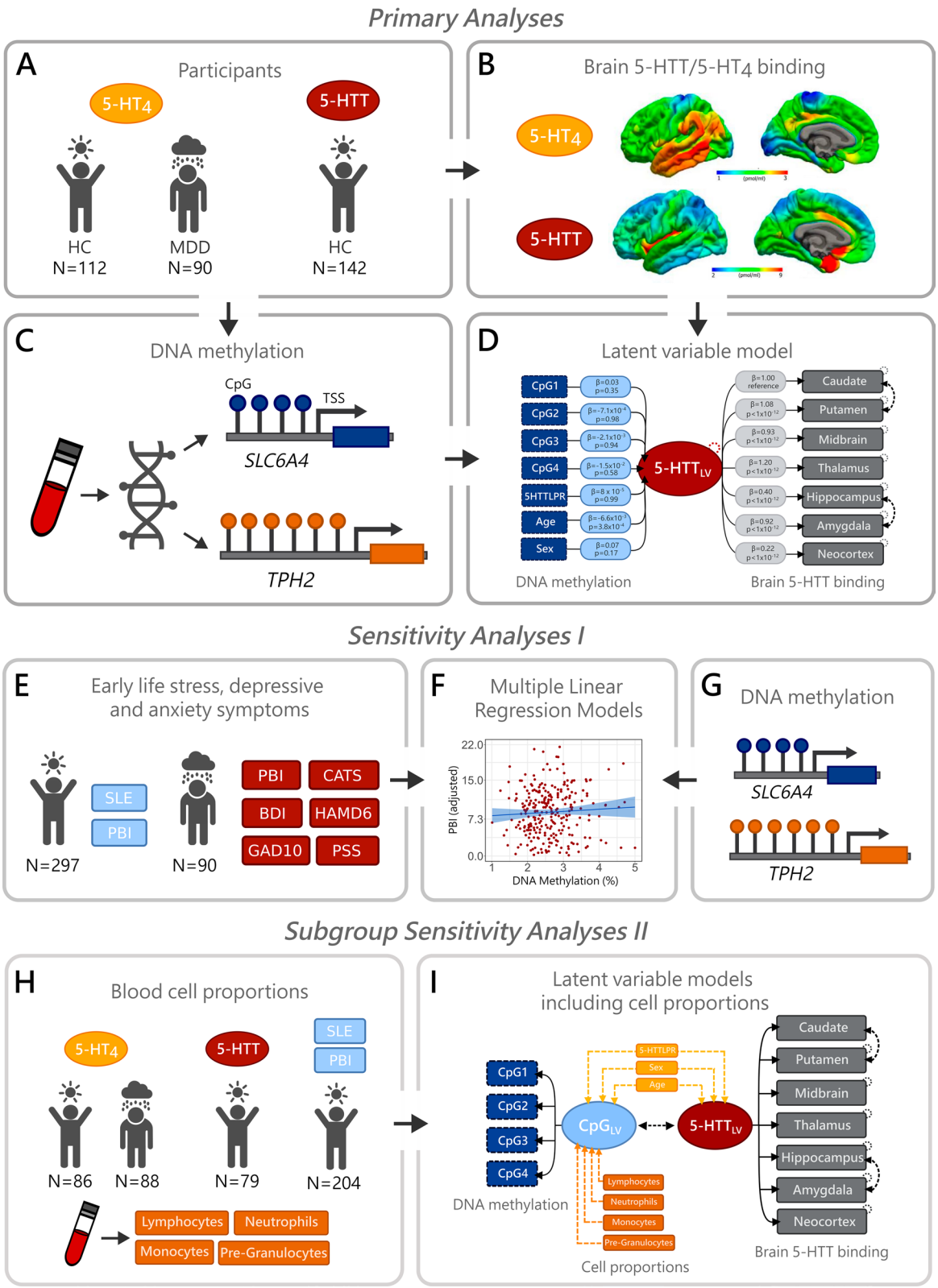

Fig. 1 (See legend on previous page.)

Table 1 Demographics of the participants included in the primary analyses

		5-HTT	5-HT_4	
		HC	HC	MDD
N		**142**	**112**	**90**
Age (mean ± s.d. [min–max])		29.0 ± 11.8 [18.4–80.3]	29.3 ± 12.9 [19.2–86.2]	26.7 ± 7.6 [18.2–56.4]
Sex (F/M)		81/61	65/47	64/26
PET scanner (A/H)		50/92	20/92	90/-
MRI scanner		*T* = 92/*V* = 50	*T* = 59/*P* = 50/*V* = 3	*P* = 90
[^{11}C]DASB/SB207145-injected mass (µg)		0.045 ± 0.041	0.023 ± 0.024	0.013 ± 0.015
[^{11}C]DASB/SB207145-injected dose (MBq)		546 ± 83.7	557 ± 91.3	578 ± 56.3
Cerebellum AUC (Bq ml^{-1})		18,200 ± 3450	10,200 ± 2450	10,200 ± 2520
SLC6A4 methylation (%)	CpG1	2.65 ± 0.67 [1.4–5.05]	2.54 ± 0.58 [1.24–4.71]	2.3 ± 0.55 [1.28–3.94]
	CpG2	3.61 ± 0.80 [1.66–5.7]	3.53 ± 0.77 [1.75–5.42]	3.29 ± 0.71 [1.88–5.26]
	CpG3	2.97 ± 0.73 [1.56–5.72]	2.92 ± 0.74 [1.56–5.9]	2.73 ± 0.61 [1.5–4.38]
	CpG4	3.94 ± 1.07 [2.44–11.8]	3.78 ± 0.85 [1.4–8.48]	3.54 ± 0.76 [2.06–5.8]
TPH2 methylation (%)	CpG1	3.22 ± 0.66 [1.89–5.88]	3.26 ± 0.65 [2–5.58]	2.7 ± 0.49 [1.47–4.18]
	CpG2	3.14 ± 0.67 [1.8–4.51]	3.14 ± 0.69 [1.96–5.38]	2.88 ± 0.53 [1.65–3.98]
	CpG3	2.91 ± 0.62 [1.7–4.68]	2.93 ± 0.75 [1.74–8.18]	2.6 ± 0.54 [1.54–4]
	CpG4	2.27 ± 0.51 [1.27–3.93]	2.33 ± 0.61 [1.28–5.96]	2.02 ± 0.49 [1.14–3.66]
	CpG5	3.25 ± 0.73 [1.92–7.18]	3.43 ± 0.88 [1.87–8.02]	3.06 ± 0.66 [1.94–5.26]
	CpG6	3.34 ± 0.80 [1.72–8.72]	3.42 ± 1.05 [1.7–10.8]	2.96 ± 0.52 [1.96–4.78]
SLC6A4 5-HTTLPR/rs25531 (L_AL_A / S-)		41/101	36/76	24/62
TPH2 rs4570625 (GG/TX)		90/52	71/40	54/34
BDNF Val/Met (rs6265) (Val/Val / Met-carriers)		87/50	60/52	46/20
MAOA rs1137070 (CC/T-)		75/58	–	–
Blood cells counts available (yes/no)		79/63	86/26	88/2

HC:Healthy Control; *MDD:* patients with major depressive disorder; *5-HTT*: serotonin transporter; *5-HT$_4$*: serotonin 4 receptor; *F*: female; *M*: male; *PET*: positron emission tomography; *A*: GE-Advance PET scanner; *H*: HRRT PET scanner; MRI: magnetic resonance imaging; *T*: Trio MRI scanner; *V*: Verio MRI scanner; *P*: Prisma MR scanner; *µg*: microgram; *MBq*: megabecquerel; *Bq ml^{-1}*: becquerel per milliliter; *AUC*: area under the curve (i.e., cerebellum reference region time activity curve); *SLC6A4*: serotonin transporter gene; *TPH2*: tryptophan hydroxylase 2 gene; 5-HTTLPR, serotonin-transporter-linked promoter region; *BDNF*: brain-derived neurotrophic factor; *MAOA*: monoamine oxidase A gene

before the start of the trial were included in the study. Additional details about the trial as well as inclusion and exclusion criteria are specified in [43].

DNA methylation analysis

SLC6A4 methylation percentages were estimated at four CpG sites (Table S2) that were previously linked to clinical phenotypes, including depressive symptoms [7, 16, 26, 46], early-life adversities [7], recent environmental stress [15], antidepressant treatment outcome [20, 21] and panic disorder [16]. *TPH2* methylation was estimated at 6 CpG sites (Table S2) based on previous studies showing an association with gene expression (13, 26, 28), early life stress, depressive symptoms, antidepressant treatment outcome [23, 24, 47] and attention deficit hyperactivity disorder [48].

Genomic DNA was isolated from peripheral blood cells from whole blood or buffy coat samples that were stored at − 20 °C (MDD patient cohort) or − 80 °C (healthy cohort) in EDTA tubes. DNA was purified using the FlexiGene Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. 500 ng DNA of each sample were bisulfite-converted using the EpiTect 96 Bisulfite Kit (Qiagen). The sequence of interest was amplified via polymerase chain reaction (PCR) using the PyroMark PCR Kit (Qiagen) and a forward (F) and a reverse (R) biotinylated primer (**Table S3**). The quality of PCR amplification was visually evaluated using gel electrophoresis. The target DNA sequence was isolated and then sequenced using the PyroMark Q96 ID (Qiagen) pyrosequencing system, with target-specific primers (**Table S3**). CpG methylation rates (in %), pyrograms and quality reports were obtained using the PyroMark software (Qiagen). Analyses were run in duplicates and pairs of duplicates differing more than 3% from each other were excluded from the analyses. Average DNA methylation value between each pair of duplicates was used for statistical analyses. Pyrograms and quality reports provided by PyroMark were used to quality check the data. Commercially available (Epitect PCR Control DNA Set, Qiagen)

fully methylated, fully non-methylated and 50%-methylated DNA samples as well as DNase free H_2O were included in all experiments as controls. Methylation data of two samples for *SLC6A4* and three samples for *TPH2* were excluded due to failed bisulfite conversion, as indicated by the PyroMark software.

Genotyping

All samples were genotyped for *SLC6A4* 5-HTTLPR and rs25531, *TPH2* rs4570625, *BDNF* rs6265 and *MAOA* rs1137070 polymorphisms. Genotyping for *SLC6A4* 5-HTTLPR and rs25531, *BDNF* rs6265 and *MAOA* rs1137070 was performed as previously described [35, 49–51]. Hardy–Weinberg equilibrium was tested using Chi-squared test in R. Table 1 and **S1** show allele frequencies within all cohorts.

PET and MR data acquisition and processing

The acquisition, preprocessing and quantification of [^{11}C]SB207145 and [^{11}C]DASB PET and MR images has been previously reported [37, 50, 52]. For each participant, both PET and concomitant MR scans were acquired. MR scans coregistered to PET were used to delineate brain regions and quantify regional PET signal.

Shortly, all participants were scanned for a 120-min ([^{11}C]SB207145) or a 90-min ([^{11}C]DASB) dynamic scan after bolus injection of the respective radioligand. Two different PET scanners were used for data collection: a High-resolution Research Tomography (HRRT) PET scanner (CTI/Siemens) with an approximate in-plane resolution of 2 mm, or an 18-ring GE-Advance PET scanner (General Electric, Milwaukee, USA) with an approximate in-plane resolution of 6 mm. T1-weighted MPRage images were acquired using three different Siemens 3-Tesla magnetic resonance (MR) scanners: Prisma, Trio or Verio. Regions of interest (ROI) were automatically delineated using PVElab and the individual T1-weigthed images [53]. Mean time-activity curves for average grey matter voxels in each hemisphere was determined using the Simplified Reference Tissue Model for [^{11}C]SB207145 scans and multilinear reference tissue model (MRTM/MRTM2) for [^{11}C]DASB scans. Cerebellum (except for vermis) was used as a reference region for all scans. The non-displaceable binding potential (binding or BP_{ND}) was used as an outcome measure of tracer binding (and therefore as an estimate of 5-HTT and 5-HT_4 levels) for both tracers.

Statistical analyses

All statistical analyses were conducted in R v4.1.2 [54].

Primary analyses

The association between *SLC6A4*/*TPH2* methylation and 5-HTT or 5-HT_4 was evaluated using three different linear latent variable models (LVM): one for healthy controls with DASB scans (5-HTT binding), one for healthy controls with SB scans (5-HT_4 binding) and one for MDD patients with SB scans. LVM is a type of multivariate linear regression that allows to model associations between a variable of interest and the shared variance of a set of inter-correlated variables (e.g. 5-HTT or 5-HT_4 binding in different brain regions).

Regions of interest (ROI) for the 5-HTT LVM were chosen based on 5-HTT distribution in the human brain [55] and comprised caudate, amygdala, hippocampus, putamen, thalamus, midbrain and neocortex. Similarly, ROIs for the 5-HT_4 LVMs include caudate, putamen, hippocampus and neocortex, reflecting brain regions across low, moderate to high density of 5-HT_4 receptor in these areas [55] and aligning with previous findings investigating the $5HT_4$ receptor system and MDD [32, 56].

Analyses were carried out in R and the LVMs were modelled using the *lava* v 1.6.10 [57] package. First, the shared correlations of regional 5-HT_4 or 5-HTT binding were modelled into a latent variable for each model (referred to as 5-HT_{4LV} or 5-HTT_{LV} respectively). Next, *SLC6A4* CpG1-CpG4 or *TPH2* CpG1-6 methylation and the covariate effects were modelled on the 5-HTT_{LV}. In all models, covariates included age, sex, PET scanner type (Advance vs HRRT) and MR scanner type (Prisma vs Trio vs Verio). 5-HTTLPR/rs25531 genotype was included in the statistical models including *SLC6A4* methylation based on previous studies suggesting a combined effect of genotype and DNA methylation on 5-HTT transcription [13, 17]. Similarly, *TPH2* rs4570625 was included in the models evaluating *TPH2* methylation [48]. Models evaluating associations between DNA methylation and 5-HT_4 binding also included information of 5-HTTLPR/rs25531 and *BDNF* rs6265, based on previous findings [58]. In addition, *BDNF* rs6265 and *MAOA* rs1137070 genotype information, which have been previously shown to affect 5-HTT [49, 50, 58], were included in a separate model as sensitivity analyses, as information for the latter genotype was not available for all subjects. Region-specific effects of each CpG site were evaluated as the product of the CpG effect on the latent variable multiplied by the loading of each region on the latent variable and were used as a measure of effect sizes.

PET and MR scanner type were modelled as region-specific effects, based on previous findings [58]. Additional covariance links were identified using an iterative procedure where score tests are used to detect model misspecification. *P*-values for these score test were adjusted using Benjamini–Hochberg [57].

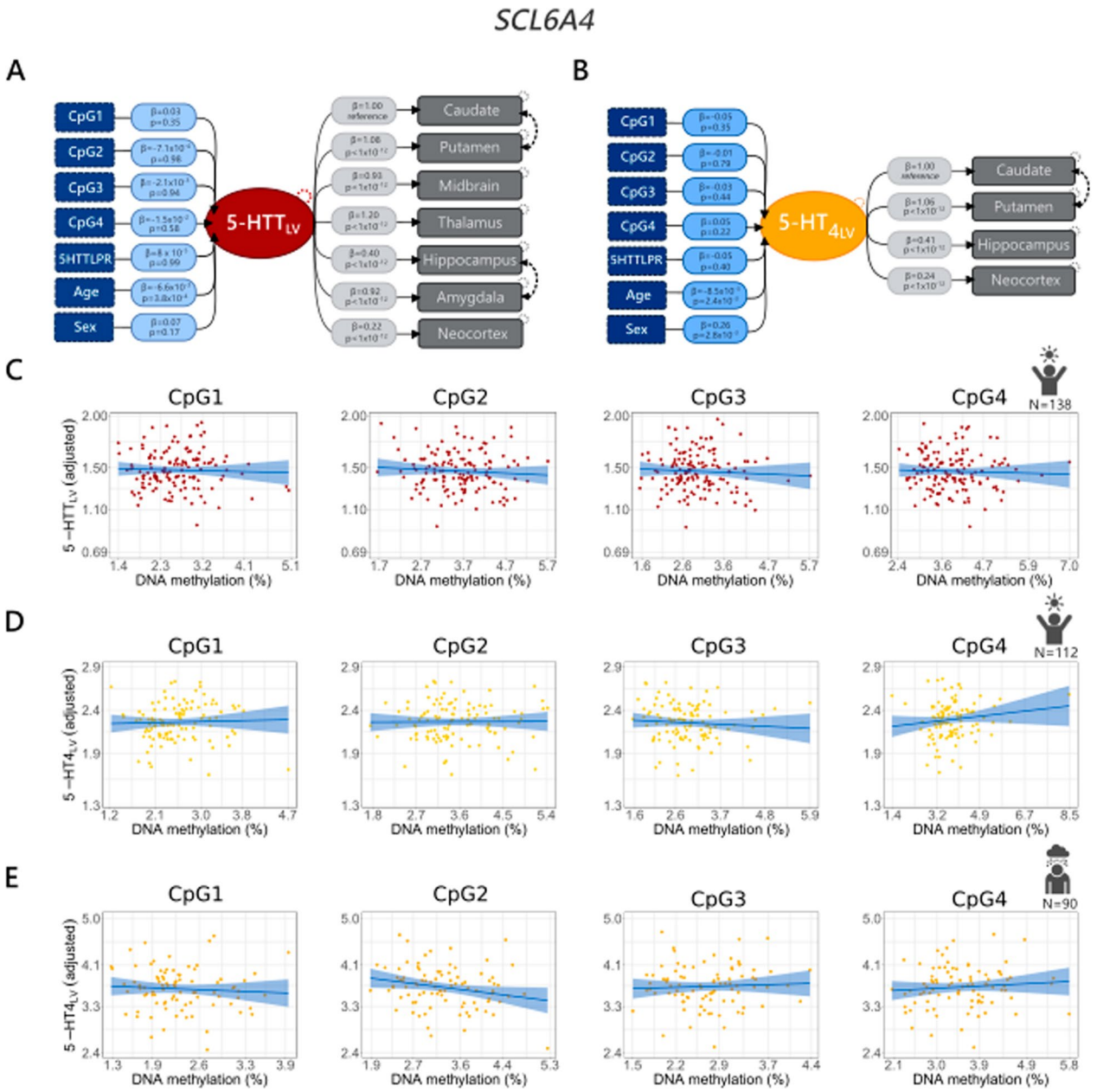

Fig. 2 Associations between peripheral *SLC6A4* methylation and brain 5-HTT binding (**a**) or 5-HT_4 binding (**b**). Blue dashed boxes depict the *SLC6A4* CpG sites and the covariates included in the model. The light blue boxes indicate the CpG and covariate effects on the latent variable (5-HTT_{LV} or 5-HT_{4LV}). Dark grey boxes to the right represent the observed 5-HTT or 5-HT_4 binding in the brain regions of interest. β values refer to the parameter estimates; they are reported either with their respective *p*-values or with their 95% confidence intervals. Dashed arrows connecting brain regions show interregional correlations, while dashed circles on the brain regions show error estimates. For representation purposes, PET and MR scanner covariates are not reported in the **a** and **b** models. Similarly, although included in the 5-HT_4 latent variable model, 5-HTTLPR/rs25531 and *BDNF* rs6265 genotypes are not reported in (**b**). Scatter plots in **c** and **d** depict the relation between *SLC6A4* methylation and 5-HTT_{LV} or 5-HT_{4LV} in healthy controls (**c**, **d**), while the relation between *SLC6A4* methylation and 5-HT_4 binding in patients with MDD is shown in (**e**)

The LVMs used for primary analyses are graphically represented in Figs. 2 and 3.

Caudate was used as a reference region in all LVMs. Thus, covariate effects can be interpreted as effects on caudate binding. Statistical significance was set at $p < 0.05$ for all the statistical models.

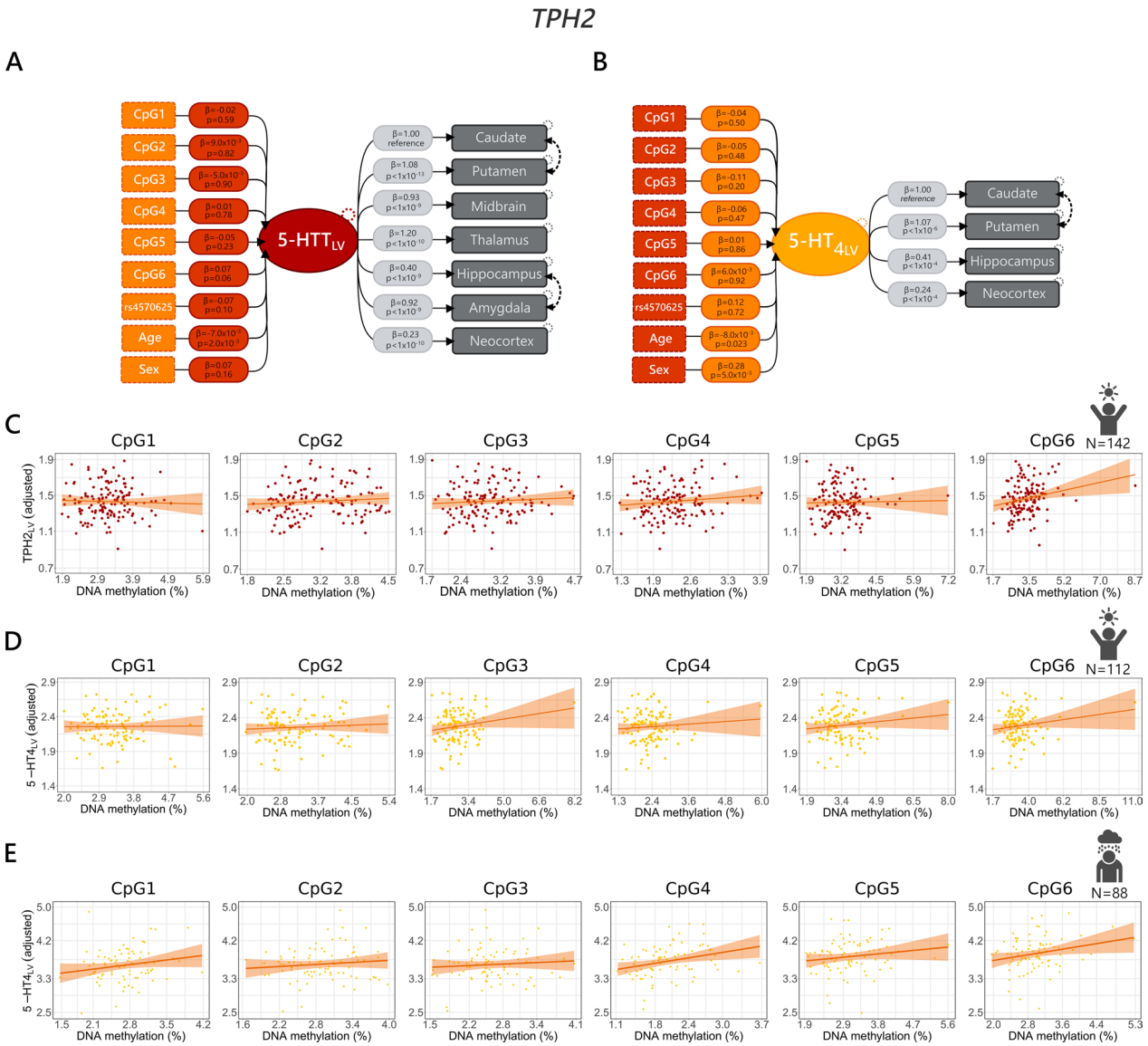

Fig. 3 Associations between peripheral *TPH2* DNA methylation and brain 5-HTT binding **a** or 5-HT$_4$ binding **b** in the healthy cohort. Orange dashed boxes to the left depict the *TPH2* CpG sites and the covariates included in the model. Rs45706210 stands for *TPH2* rs45706210 G/T SNP. For representation purposes, PET and MR scanner covariates are not reported in the **a** and **b** models. Similarly, although included in the 5-HT$_4$ latent variable model, 5-HTTLPR/rs25531 and *BDNF* rs6265 genotypes are not reported in (**b**). Scatter plots in **c** and **d** depict the relation between *TPH2* methylation and 5-HTT$_{LV}$ or 5-HT$_{4LV}$ in healthy controls (**c**, **d**), while the relation between *TPH2* methylation and 5-HT$_4$ binding in patients with MDD is showed in (**e**)

Sensitivity analyses I: DNA methylation vs measures of environmental stress, depressive and anxiety symptoms

Multiple linear regression models were used to explore the relation between methylation of each CpG site and measures of environmental stress in both healthy participants and MDD patients. Associations with depressive and anxiety symptoms were also evaluated in the MDD patients. Data of all the healthy controls were pooled together with data of 58 additional healthy participants, for a total of $N=297$ (**Table S1**). Data from the MDD patients were the same as those used for the LVM analyses. The stressful life events (SLE) questionnaire was used as an estimate of both lifetime (total SLE score) and recent stress (recent SLE score) in the healthy cohort. The parental bonding inventory (PBI) was used as a proxy estimate of early life stress in both the healthy and the MDD cohorts. Scores from both parents were combined into a measure for the "care" (PBI care score) and

one for the "overprotection" (PBI overprotection score) subscales. In addition, models exploring the association between *SLC6A4* or *TPH2* DNA methylation and the following measurements were carried out in the MDD cohort: (1) Beck's Depression Inventory (BDI) indexing recent depressive symptoms; (2) childhood abuse trauma scale (CATS) as a measure of early life stress; (3) generalized anxiety disorder 10-item (GAD10); (4) Hamilton depression rating scale 6 item ($HAMD_6$) indexing current depressive symptoms; (5) perceived stress scale (PSS) indexing recent stress symptoms.

All statistical models included age, sex and genotype (5-HTTLPR in the case of *SLC6A4* or rs4570625 for *TPH2*) as covariates. Bonferroni correction for four and six tests was applied for analyses including *SLC6A4* and *TPH2* data, respectively (*SLC6A4*: $p=0.01$; *TPH2*: $p=0.008$).

Sensitivity analyses II: analyses accounting for cell type proportions

Sensitivity analyses were conducted to evaluate whether different blood cell type proportions affected the associations evaluated in the primary analyses and in the primary sensitivity analyses. Blood cell counts information was available only for a subset of the total participants used in the analyses (Table 1; panel H of Fig. 1). Corrections were done for lymphocytes, monocytes, granulocyte precursors and neutrophils proportions. The term granulocyte precursors used here refers to the sum of granulocyte precursors metamyelocytes, myelocytes and promyelocytes. Cell proportions were calculated by dividing the cell counts of each cell type by the number of leukocytes, multiplied by 100.

For the models evaluating the association with brain 5-HTT or $5\text{-}HT_4$ levels, the correction for cell type involved first modelling two latent variables, one including the shared correlations among DNA methylation across the four CpG sites (CpG_{LV}) and one including the shared correlations of 5-HTT ($5\text{-}HTT_{LV}$) or $5\text{-}HT_4$ binding ($5\text{-}HT_{4LV}$) across model-specific ROIs. CpG_{LV} was adjusted for age, sex, genotype and cell proportions by regressing out all cell proportions but neutrophils proportions ($CpG_{LV+cells}$). Finally, the covariance between $CpG_{LV+cells}$ and $5\text{-}HTT_{LV}$ or $5\text{-}HT_{4LV}$ was estimated (panel I of Fig. 1).

To account for cell type proportions when evaluating the association between peripheral *SLC6A4* or *TPH2* DNA methylation and measures of environmental stress, depressive or anxiety symptoms, LVMs were used instead of linear regressions. For a given gene, a latent variable reflecting DNA methylation at all CpG sites was modelled (CpG_{LV}) and adjusted for cell types ($CpG_{LV+cells}$). Next, for every psychometric measurement, LVMs containing $CpG_{LV+cells}$ were regressed out on the psychometric score.

In all models including $CpG_{LV+cells}$, the effect of cell types was tested using a likelihood ratio test between the LVM including CpG_{LV} (without adjustment for cell proportions) and the corresponding LVM including $CpG_{LV+cells}$ (adjusted for cell proportions). Whenever significant, cell type specific effects estimated by the LVM were reported without adjustment for multiple comparisons.

Results

Genotyping

Alleles were in Hardy–Weinberg equilibrium ($p>0.1$) in all cohorts used for statistical analyses (5-HTT, MDD and healthy participants used in sensitivity analyses I) except for rs4570625 in the $5\text{-}HT_4$ cohort (χ^2: 6.12; $p=0.01$). However, rs4570625 did not deviate from Hardy–Weinberg equilibrium for the whole population used in this study ($N=389$; χ^2: 0.34; $p=0.56$), suggesting that the lack of equilibrium might be due to chance and not to biases in genotyping.

Association between peripheral DNA methylation and brain serotonergic markers

Loadings, i.e. parameters evaluating the association between the latent variable and the 5-HTT or $5\text{-}HT_4$ binding values, were all significantly different from 0 (all $p<10^{-4}$), indicating evidence for shared variance among the 5-HTT and $5\text{-}HT_4$ in the respective ROIs.

The LVMs did not reveal a statistically significant association between *SLC6A4* or *TPH2* methylation and $5\text{-}HTT_{LV}$ or $5\text{-}HT_{4LV}$ in the healthy cohort nor the cohort of MDD patients (unadjusted *p*-values ranged between 0.06 and 0.97; Table 2). A graphical representation of the LVMs including the results is reported in Figs. 2 and 3.

In line with previous studies [49, 50, 58, 59] based on the same cohort, we observed: (1) a negative association between age and $5\text{-}HTT_{LV}$ and $5\text{-}HT_{4LV}$ in the healthy cohort ($p<0.01$) but no association between age and $5\text{-}HT_4$ binding in the MDD cohort; (2) a non-significant effect of 5-HTTLPR on $5\text{-}HTT_{LV}$; (3) an association between *MAOA* rs1137070 and $5\text{-}HTT_{LV}$ (*MAOA* T- carriers vs CC, β: 0.1, 95% CI: [0.02; 0.18], $p=0.01$) and between neocortex binding and *BDNF* rs6265 (with lower subcortical binding for met-carriers, estimate (β): − 0.02, 95% CI: [− 0.04; 0.01], $p=0.005$) in the subset of the sample with this information available ($N=130$), 4) higher $5\text{-}HT_4$ binding in male compared to female participants. Contrary to previous observations based on a subgroup ($N=68/112$) of the participants included in this study (Fisher et al. 2015: β: 0.070, 95% CI: [0.018; 0.122], $p=0.008$), we did not observe a statistically significant

Table 2 Results of latent variable models (LVM) evaluating the association between *SLC6A4/TPH2* methylation and 5-HTT and 5-HT_4 brain binding

SLC6A4	**5-HTT**			**5-HT_4**					
	HC (*N* = 138)			**HC (*N* = 112)**			**MDD (*N* = 90)**		
Variable	***β***	***P*-value**	**95% CI**	***β***	***P*-value**	**95% CI**	***β***	***P*-value**	**95% CI**
CpG1	0.02	0.56	[− 0.051; 0.092]	− 0.01	0.83	[− 0.126; 0.102	− 0.03	0.80	[− 0.229; 0.178]
CpG2	− 0.01	0.78	[− 0.069; 0.052]	− 0.01	0.78	[− 0.106; 0.080]	− 0.13	0.09	[− 0.293; 0.023]
CpG3	− 0.01	0.67	[− 0.084; 0.054]	− 0.07	0.18	[− 0.177; 0.035]	0.11	0.27	[− 0.084; 0.300]
CpG4	− 0.01	0.73	[− 0.073; 0.051]	0.09	0.06	[− 0.003; 0.189]	0.08	0.32	[− 0.076; 0.227]
Age	− 0.01	1.4×10^{-3}	[− 0.011; − 0.003]	− 0.01	0.01	[− 0.015; − 0.003]	− 0.01	0.18	[− 0.023; − 0.004]
Sex (Male)	0.05	0.32	[− 0.055; 0.164]	0.28	4.2×10^{-3}	[0.092; 0.460]	0.01	0.95	[− 0.221; 0.235]
5-HTTLPR	-4.5×10^{-3}	0.93	[− 0.095; 0.087]	− 0.04	0.57	[− 0.172; 0.097]	0.02	0.87	[− 0.215; 0.252]
BDNF rs6265	–	–	–	− 0.08	0.19	[− 0.214; 0.044]	-1.5×10^{-3}	0.99	[− 0.211; 0.208]
TPH2	**5-HTT**			**5-HT_4**					
	HC (N = 140)			**HC (N = 112)**			**MDD (*N* = 88)**		
Variable	***β***	***P*-value**	**95% CI**	***β***	***P*-value**	**95% CI**	***β***	***P*-value**	**95% CI**
CpG1	− 0.02	0.59	[− 0.079; 0.045]	− 0.04	0.50	[− 0.150; 0.075]	0.04	0.76	[− 0.225; 0.305]
CpG2	0.01	0.82	[− 0.066; 0.083]	− 0.05	0.48	[− 0.182; 0.088]	0.02	0.88	[− 0.254; 0.294]
CpG3	− 0.01	0.90	[− 0.095; 0.084]	0.11	0.20	[− 0.059; 0.270]	− 0.25	0.09	[− 0.549; 0.039]
CpG4	0.01	0.78	[− 0.082; 0.109]	− 0.06	0.47	[− 0.243; 0.115]	0.25	0.13	[− 0.076; 0.575]
CpG5	− 0.05	0.23	[− 0.131; 0.032]	0.01	0.86	[− 0.135; 0.162]	0.00	0.97	[− 0.271; 0.262]
CpG6	0.07	0.06	[− 0.004; 0.140]	0.01	0.92	[− 0.120; 0.132]	0.17	0.22	[− 0.110; 0.457]
Age	− 0.01	1.7×10^{-3}	[− 0.011; − 0.003]	− 0.01	0.02	[− 0.014; − 0.001]	− 0.01	0.10	[− 0.026; − 0.002]
Sex (Male)	0.07	0.16	[− 0.030; 0.176]	0.28	5.0×10^{-3}	[0.090; 0.472]	0.02	0.86	[− 0.210; 0.250]
TPH2 rs4570625	− 0.07	0.09	[− 0.150; 0.012]	0.12	0.72	[− 0.558; 0.799]	0.02	0.86	[− 0.204; 0.242]
5-HTTLPR	–	–	–	− 0.03	0.70	[− 0.167; 0.113]	0.09	0.45	[− 0.154; 0.343]
BDNF rs6265	–	–	-	− 0.08	0.21	[− 0.212; 0.049]	0.03	0.78	[− 0.193; 0.255]

HC healthy controls; *MDD* patients with major depressive disorder; *β* LVM estimated parameters; *95% CI* 95% confidence intervals

association between 5-HTTLPR or *BDNF* rs6265 genotypes and 5-$HT_{4LV.}$ In addition, we did not find any association between *TPH2* rs4570625 and 5-HTT_{LV} or 5-HT_{4LV} (Table 2). Estimated effect sizes and respective 95% CI for effects of each CpG site on each brain region are reported in Tables S4 and S5 for models including *SLC6A4* and *TPH2* respectively. Compared to age, which is known to affect 5-HTT and 5-HT_4 binding by about 9% and 1% per decade, respectively [59, 60], the effect sizes of our study were minimal. The largest effect sizes in our dataset indicated that 5-HT_4 binding decreases by 0.14% for each one-unit increase in *SLC6A4* methylation (Table S4) and by 0.24% for each one-unit increase of *TPH2* methylation (Table S5).

DNA methylation and measures of environmental stress

Results from multiple linear regressions on all cohorts are reported in Table S6 and S7. Among all statistical tests, only three associations reached the threshold for statistical significance before correction for multiple comparisons, and only the association between *SLC6A4* CpG2 and PBI overprotection item remain statistically significant at the 5% level after Bonferroni correction (β: − 0.83; p_{UNC} = 0.01; 95% CI: − 1.48; − 0.19).

Corrections for cell type

Loadings of DNA methylation at single CpGs onto CpG_{LV} were all significantly different from 0 (all $p < 0.01$). Likewise, loadings of regional 5-HTT or 5-HT_4 binding significantly loaded onto their corresponding latent variables 5-HTT_{LV} or 5-HT_{4LV} (all $p < 10^{-11}$).

Likelihood ratio tests showed an improved model fit when including cell proportions in all the healthy cohorts (all $p < 0.01$). However, in the MDD cohort only the models evaluating the association between *TPH2* methylation and BDI, $HAMD_6$, PSS and GAD10 showed improved model fit after adding cell proportions.

Lymphocytes proportion was significantly associated with CpG_{LV} in the *TPH2* models including the healthy cohort (DASB: β: 0.017, $p = 0.017$, 95% CI: [0.004; 0.04];

Table 3 Association between a latent variable including blood cell proportions ($CpG_{LV+cells}$) and a latent variable including serotonin transporter (5-HTT) or serotonin 4 receptor ($5\text{-}HT_4$) binding

Brain 5-HT proxy	Cohort	Gene	Estimate	*P*-value	95% CI
5-HTT	HC	*SLC6A4*	− 0.01	0.95	[− 0.28; 0.26]
		TPH2	− 0.01	0.95	[− 0.28; 0.26]
$5\text{-}HT_4$	HC	*SLC6A4*	− 0.22	0.08	[− 0.48; 0.03]
		TPH2	− 0.20	0.11	[− 0.45; 0.04]
	MDD	*SLC6A4*	0.08	0.56	[− 0.20; 0.37]
		TPH2	0.20	0.10	[− 0.04; 0.44]

5-HT: serotonin; *5-HTT*: serotonin transporter; *5-HT*$_4$: serotonin 4 receptor; *HC*: healthy controls; *MDD*: patients with depression; *SLC6A4*: serotonin transporter gene; *TPH2*: tryptophan hydroxylase 2 gene

SB: β: 0.016, $p=0.019$, 95%CI: [0.003; 0.03]) and both in the *SLC6A4* and *TPH2* models based on the MDD patients cohort (*SLC6A4*: β: 0.014, $p=0.03$, 95% CI: [0.002; 0.03]; *TPH2*: β: 0.01; $p=0.01$; 95% CI: [0.002; 0.02]). No statistically significant association was found between CpG_{LV} and any cell type, age or sex in the *SLC6A4* model.

Accounting for blood cells proportion did not affect the conclusions about the associations between *SLC6A4* or *TPH2* methylation and $5\text{-}HTT_{LV}$ or $5\text{-}HT_{4LV}$ in the healthy cohort nor the cohort of MDD patients (Table 2), as all *p*-values were greater or equal to 0.08 (Table 3).

All models evaluating the association between *SLC6A4*/*TPH2* methylation and measures of early stress, anxiety or depressive symptoms and including cells proportions showed a significant association between lymphocytes proportions and CpG_{LV} (Table S8). Contrarily, granulocyte precursors were marginally statistically significantly associated only with *SLC6A4* CpG_{LV} in the model including healthy participants (Table S8). The other cell types considered showed no association. Age was statistically significantly associated with *TPH2* CpG_{LV} but not with *SLC6A4* CpG_{LV}. Before adjusting for multiple comparisons, *TPH2* CpG_{LV} was associated with sex in the healthy participants but not in the MDD participants, with higher *TPH2* CpG_{LV} values in males compared to females (Table S8).

Associations between $CpG_{LV+cells}$ and measures of environmental stress or mood or anxiety symptoms are depicted in Table S9 and showed no statistically significant association.

Discussion

In this study we found no statistically significant associations between peripheral DNA methylation of two key regulatory genes of serotonin neurotransmission (*SLC6A4* and *TPH2)* and brain levels of 5-HTT and $5\text{-}HT_4$ in a cohort of healthy participants or $5\text{-}HT_4$ in a cohort of unmedicated patients with MDD.

Previous evidence supports an association between the CpG sites observed in our study and psychopathological features [7, 16, 48]. However, little is known about how peripheral DNA methylation of serotonin genes maps onto the brain serotonergic architecture. Only one study reported that increased *SLC6A4* methylation was associated with reduced in-vivo TPH2 brain levels in a cohort of adult males that experienced childhood aggression [27]. Nonetheless, the study was based on a relatively small cohort ($N=25$) that experienced high childhood aggression while our study, although based on a notably larger cohort, includes participants that did not experience extreme childhood traumas.

The lack of an association observed in our study should be considered also in light of the intricate nature of gene regulation. First, DNA methylation levels can differ across tissues. Previous epigenome-wide association studies reported that methylation of some CpG sites correlate between peripheral blood cells and entorhinal cortex, cerebellum, superior temporal gyrus and prefrontal cortex in postmortem brains of elderly adults [29, 61]. To evaluate a correspondence of DNA methylation between the two tissues, we consulted the online database created by Hannon et al. (2015) and found that only *SLC6A4* CpG4 methylation levels correlate with methylation in entorhinal cortex ($p_{UNC}=0.02$) and superior temporal gyrus ($p_{UNC}=0.04$). *SLC6A4* CpG1 and *TPH2* CpG2 did not show any correlation, while information on the other CpG sites included in our study or other brain regions was not available in their database, so we cannot exclude a correspondence between the two tissues at other sites.

Second, even in the case DNA methylation was consistent across tissues, different transcription factors might interact differently with similar DNA methylation patterns in different tissues [62]. Thus, assuming similar DNA methylation levels between the two tissues, it is not known whether *SLC6A4* or *TPH2* expression would be affected in the brain in the same way that it is known to be affected in peripheral blood [13, 26, 27, 63]. Third, gene expression does not always directly correspond to protein levels as post-transcriptional and post-translational modifications can affect protein levels and function, and this notion seems to be true for both genes [55, 64–67]. This might also help explain why we did not observe any association between 5-HTTLPR or *TPH2* rs4570625, which are polymorphisms known to affect *SLC6A4* and *TPH2* expression, and 5-HTT or $5\text{-}HT_4$ levels, which is in line with former studies [50, 68].

Nonetheless, most of this evidence is based on studies in adult individuals and we cannot rule out an effect of genetic variation or DNA methylation within

serotonergic genes on early brain development, which is critically driven by serotonin transmission [69, 70]. Indeed, while brain 5-HTT and 5-HT_4 levels may vary substantially throughout the lifespan [35] and in response to environmental changes [71–74], DNA methylation remains stable at about half of the total CpG sites after the first years of life [75, 76]. Using the online database provided by Mulder et al. [76], we observed no change in DNA methylation at *SLC6A4* CpG1 or CpG4 or *TPH2* CpG2 over the first 17 years of life of healthy individuals, although information on the other CpG sites relevant to our study was not provided. Longitudinal study designs with methylation sampling and PET imaging would allow to better understand if this was the case.

Notably, the lack of association between peripheral DNA methylation and adult brain levels of serotonergic markers does not necessarily imply that *SLC6A4* or *TPH2* methylation cannot be used as an informative biomarker for mental health. Instead, it might reflect peripheral alterations, e.g. of the immune system which can be critical for mental health. Altered immune function has been described in individuals who have experienced early life stress [77] and stress-related disorders, including depression [78]. Likewise, previous findings relating *SLC6A4* and *TPH2* methylation to measures of early life stress [7, 79] or depressive symptoms [7] might reflect alterations in peripheral immune function rather than in the brain serotonergic transmission.

In this regard, our sensitivity analyses revealed a borderline significant association between *SLC6A4* CpG2 methylation at these genes and the parental bonding inventory (PBI) overprotection subscale, i.e. a proxy for suboptimal early social environment. However, this association was no longer statistically significant after including cell proportions in the model. Nonetheless, it is relevant to note that information of blood cells counts was available only for a subset of the total participants used for the sensitivity analyses, so such changes may be due to lower statistical power instead of the removal of unwanted variance. Thus, we suggest that this finding should be interpreted only if replicated in other cohorts.

Our study is the largest ($N=297$) to date investigating the association between *SLC6A4*/*TPH2* DNA methylation and early life stress in a healthy cohort. In line with our study, the second largest study based on a cohort of healthy participants ($N=133$) [41] reported no association between *SLC6A4* methylation and measures of early life stress. Our measurements for early life stress were PBI and SLE, which are based on retrospective self-reports and may not be as sensitive as other measurements in capturing early life stress. However, Wankerl et al. [41] did not find an association with early life stress, although both information of early-life stress reported by the participants' mothers and self-reported were used. Thus, we can speculate that alterations in DNA methylation levels might only become detectable in case of more extreme (early) environmental stressors or in pathological conditions. Indeed, most previous studies linking peripheral *SLC6A4* or *TPH2* methylation to stress-related phenotypes were based on patients with mood disorders [7, 16] or individuals who were exposed to intense environmental stress [15, 27]. Our MDD cohort is smaller ($N=90$) than some of those previously investigated ($N>100$) [7, 16], so the results of our sensitivity analyses might be ascribable to a lack of power. Alternatively, the psychometric measurements used in our study might not be as sensitive at capturing early life stress as those used in other studies.

Importantly, the relation between *SLC6A4* and *TPH2* methylation and early life stress or brain levels of 5-HT_4 or 5-HTT might also be affected by other environmental factors that were not considered in our study. For example, smoking [80], alcohol consumption [77, 81] or exposure to air pollutants [82] are known to affect gene expression through epigenetic modifications such as DNA methylation. Thus, we cannot exclude that future study designs including extreme exposure groups may inform on the potential effects of such environmental factors on a link between DNA methylation and serotonergic brain architecture.

In line with previous studies, we found that 5-HT_4 binding was higher in men compared to women in healthy participants cohort [59] but not in the MDD cohort [83]. Previous studies investigating *SLC6A4* and *TPH2* methylation suggests that DNA methylation levels might be affected by sex [79, 84]. However, in our dataset we did not observe any conclusive effects of sex on neither gene, except for a trend in *TPH2* CpG_{LV} in the healthy participants. Notably, we observed it only before correcting for multiple comparisons, which we therefore interpret with caution. We did not observe the same effect on the MDD cohort.

It is important to mention some strengths of the present study compared with previous literature. First, it is based on the currently largest dataset in the world for brain molecular imaging for 5-HTT and 5-HT_4. This also allows us to validate previous findings based on the same cohort such as in the case of *BDNF* rs6265 and 5-HT_4 [58], which was initially found in a subset ($N=68$) of the participants included in this cohort but could not be replicated in the more recent and larger current cohort ($N=112$). Second, it includes both healthy participants and MDD patients, allowing us to investigate potential associations unique to healthy or pathological states; third, in our analyses we included blood cells proportions, which has rarely been done in former studies

evaluating methylation at *SLC6A4* or *TPH2* and can be the main driver of methylation variability across individuals [39, 85].

However, this study also comes with several limitations. First, we examined four and six CpG sites for *SLC6A4* and *TPH2* respectively, which is only a small fraction of the total CpG sites in these genes and even smaller of those across the genome. An epigenome-wide exploration would be more informative, although a much larger sample size would be needed to capture potential peripheral epigenetic signatures associated with brain serotonergic transmission. For this reason, we chose a candidate epigenetic marker strategy for this study. Second, we considered only 5-HTT and 5-HT_4 as proxies for serotonergic neurotransmission. Although this was based on previous evidence, there are many more contributors to serotonergic neurotransmission. In addition, in the MDD cohort we were only able to explore the association between DNA methylation and 5-HT_4 levels since we did not have data on 5-HTT brain binding from this group. Alterations in brain 5-HTT levels have been reported in MDD patients [86] and future studies are needed to explore the relation between peripheral *SLC6A4* methylation and brain 5-HTT levels in MDD. Nonetheless, preclinical and clinical studies show that 5-HT_4 levels vary in response to serotonin levels [32, 87, 88] and in-vivo 5-HT_4 levels deviate between healthy participants and MDD patients [36], pointing to the relevance of this target as a proxy for serotonin transmission in the context of MDD.

Third, although we included cell proportions in our sensitivity analyses, we could not take into account all blood cells subtypes (e.g. lymphocyte subtypes such as CD4+ or CD8+) but only the broader classes or cell types (monocytes, lymphocytes, neutrophils and granulocytes precursors) that are commonly evaluated in clinical routine. Thus, although we have accounted for some of the variance deriving from blood cell composition, we cannot assume that our analyses have accounted for all the variance.

Fourth, we could not account for the timing of possible traumas experienced by our participants. Cumulative evidence shows that the timing at which environmental stress was experienced can differentially affect DNA methylation [89, 90] as well as the vulnerability to developing psychiatric disorders [91, 92]. Future studies carried out on naturalistic cohorts should consider collecting data on the timing but also on the type of stress experienced in early life, to better capture the individual exposome. Finally, DNA methylation is tightly associated with genetic variation [93]. Several studies reporting associations with early life stress or mood disorder symptoms are based on populations of Asian ancestry, whereas our study only included participants of European ancestry. Hence, although we took into account two genetic variants that might influence the expression of *SLC6A4* or *TPH2*, our findings may only be generalizable to populations of European ancestry. In addition, we used the online mQTL database browser (http://www.mqtldb.org/) to investigate potential genetic influences on the methylation loci considered in our study. We did not find any variant associated with any of our CpG sites of interest. However, as only three out of the ten loci were available on their dataset, we cannot exclude that other genetic variants might affect *SLC6A4* or *TPH2* methylation. Future studies considering more CpG sites and genetic variants as well as including participants from different ancestries would help clarify this limitation.

Conclusions

To conclude, our findings do not support an association between *SLC6A4* or *TPH2* methylation and 5-HTT or 5-HT_4 brain levels or measures of early life stress, anxiety or depressive symptoms. We suggest that caution should be used when interpreting findings on peripheral DNA methylation in relation to the adult serotonergic brain architecture and to measures of early life stress or mood disorders symptoms. However, our findings do not rule out a role of peripheral DNA methylation in serotonergic neurotransmission and (mal)adaptation to environmental stress, which should be further elucidated by future studies considering more CpG sites and related genetic variants, larger sample sizes, more sensitive measures of early environmental stress, blood cell composition and longitudinal cohorts.

Abbreviations

SLC6A4	Serotonin transporter gene
TPH2	Tryptophan hydroxylase 2 gene
5-HTT	Serotonin transporter
5-HT_4	Serotonin 4 receptor
MDD	Major depressive disorder
5-HTTLPR	Serotonin-transporter-linked promoter region polymorphism
CpG	Cytosine–Guanine dinucleotide
HAMD17	Hamilton depression rating scale 17-item
HAMD6	Hamilton depression rating scale 6-item
MRTM/MRTM2	Multilinear reference tissue model
LVM	Latent variable model
5-HTT_{LV}	Latent variable based on 5-HTT binding values
5-HT_{4LV}	Latent variable based on 5-HT_4 binding values
ROI	Region of interest
SLE	Stressful life events
PBI	Parental bonding inventory
BDI	Beck's depression index
CATS	Childhood abuse trauma scale
GAD10	Generalized anxiety disorder 10-item
PSS	Perceived stress scale
CpG_{LV}	Latent variable based on CpG values
$CpG_{LV+cells}$	CpG_{LV} adjusted for cell proportions
HC	Healthy control

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-024-01678-y.

Table S1. Demographics of the participants included in the secondary analyses (XLSX 12 kb)

Table S2. Genomic locations of the *SLC6A4* and *TPH2* CpG sites included in this study (XLSX 10 kb)

Table S3. Primers used for pyrosequencing *SLC6A4* and *TPH2* (XLSX 9 kb)

Table S4. Effect sizes and respective 95% confidence intervals of models including *SLC6A4* data (XLSX 11 kb)

Table S5. Effect sizes and respective 95% confidence intervals of models including *TPH2* data (XLSX 12 kb)

Table S6. Associations between *SLC6A4* methylation and environmental stress in healthy controls (HC) and mood and anxiety disorder symptoms in patients with depression (MDD) (XLSX 11 kb)

Table S7. Associations between *TPH2* methylation and environmental stress in healthy controls (HC) and mood and anxiety disorder symptoms in patients with depression (MDD) (XLSX 12 kb)

Table S8. Results of associations between $CpG_{LV+cells}$ and cell proportions, sex, age and genotype (XLSX 10 kb)

Table S9. Results of associations between $CpG_{LV+cells}$ and measures of environmental stress or mood or anxiety symptoms (XLSX 10 kb)

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Author contributions
SEPB took care of conceptualization, DNA methylation measurements, genotyping, data analysis, writing of the original draft, design and execution of figures and tables, manuscript editing and reviewing and funding. BO supported conceptualization, statistical analyses, manuscript editing and reviewing. PMF helped in conceptualization, data analysis, manuscript editing and reviewing and supervision. GO contributed to the DNA methylation measurements and genotyping. PSJ helped with data management, manuscript editing and reviewing. VHD was involved in data collection, data management, manuscript editing and reviewing. CS contributed to PET data analysis, manuscript editing and reviewing. GMK provided funding, manuscript editing and reviewing and supervision. KPL supplied supervision, funding, manuscript editing and reviewing. VGF contributed to conceptualization, funding, clinical care of MDD patients, manuscript editing and reviewing and supervision. All co-authors approved the final draft of the manuscript.

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Availability of data and materials
The R codes used for statistical analyses can be made available upon request to the corresponding author (vibe.frokjaer@nru.dk). Data can be made available upon reasonable request via this form (https://cimbi.dk/index.php/documents/category/3-cimbi-database) and with an appropriate data sharing agreement.

Declarations

Ethics approval and consent to participate
All studies protocols comply with the Declaration of Helsinki. All participants provided written and informed consent before being included in the research studies. Data of healthy participants included in this study are from the Cimbi database (https://doi.org/10.1016/j.neuroimage.2015.04.025). All research projects generating the data included in the Cimbi database have been approved by the Danish Data Protection Agency and the local scientific ethics committee. Data of patients with major depressive disorder included in this study are from the NeuroPharm 1 study, a clinical trial registered at clinicaltrials.gov (NCT02869035) on 16 August 2016. The study protocol was approved by the Health Research Ethics Committees of the Capital Region of Denmark (reference number: H-15017713), the Danish Medicines Agency (protocol number: NeuroPharm-NP1, EudraCT-number 2016–001626-34) and the Danish Data Protection Agency (04711/RH-2016–163).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Supplementary Materials II

	HC	MDD
N	297	90
Age (mean ± s.d.)	30.5 ± 13.5 [18.4-86.2]	26.6 ± 7.2 [18.3-56.4]
Sex (F/M)	173/124	64/26
BDI	-	33.4 ± 7.2 [17-50]
CATS	-	30 ± 18.5 [0-81]
GAD10	-	23.6 ± 9.0 [7-47]
HAMD6	-	12.3 ± 1.6 [7-17]
PSS	-	26.6 ± 4.6 [16-39]
PBI care	26.3 ± 6.3 [5-36]	23.8 ± 7.4 [4.5-36]
PBI Overprotection	9.5 ± 4.9 [0.5-22]	10.3 ± 5.2 [1-23.5]
Recent SLE	3.6 ± 3.3 [0-35]	-
Total SLE	1.5 ± 1.4 [0-7]	-
***SLC6A4* CpG 1**	2.61 ± 0.65 [1.01-5]	2.23 ± 0.67 [0-3.59]
***SLC6A4* CpG 2**	3.62 ± 0.87 [1.66-6.4]	3.33 ± 0.87 [1.88-8.14]
***SLC6A4* CpG 3**	2.97 ± 0.77 [0-5.92]	2.72 ± 0.75 [0-4.38]
***SLC6A4* CpG 4**	3.88 ± 0.85 [1.4-8.48]	3.53 ± 0.88 [1.83-7.67]
***TPH2* CpG1**	3.24 ± 0.68 [1.66-5.88]	2.71 ± 0.51 [1.47-4.18]
***TPH2* CpG2**	3.15 ± 0.68 [1.8-5.38]	2.94 ± 0.80 [1.65-8.4]
***TPH2* CpG3**	2.95 ± 0.68 [1.7-8.18]	2.66 ± 0.76 [1.54-7.52]
***TPH2* CpG4**	2.36 ± 0.88 [1.2-14.4]	2.06 ± 0.63 [1.14-5.58]
***TPH2* CpG5**	3.38 ± 1.07 [1.86-15.8]	3.13 ± 0.90 [1.94-8.77]
***TPH2* CpG5**	3.43 ± 1.03 [1.32-11.7]	2.98 ± 0.549 [1.96-4.78]
***SLC6A4* 5-HTTLPR/rs25531 (L_AL_A / S-)**	94/203	23/67
***TPH2* rs4570625 (GG/TX)**	181/115	51/34

Abbreviations: *BDI*, Beck's Depression Index; *CATS*: Child and Adolescence Trauma Screen; *GAD10*: Generalized Anxiety Disorder 10; *HAMD-6*: Hamilton Depression Rating Scale 6; *PBI*: parental bonding inventory; *SLE*: Stressful Life Events; *SLC6A4*, serotonin transporter gene; *5-HTTLPR*, serotonin-transporter-linked promoter region; *TPH2,* tryptophan hydroxylase 2

Table S1. Demographics of the participants included in the secondary analyses.

Gene	CpG site	Genomic location (GRCh38/hg38)
SLC6A4	**CpG1**	chr17:30 236 071
	CpG2	chr17:30 236 083
	CpG3	chr17:30 236 088
	CpG4	chr17:30 236 090
TPH2	**CpG1**	chr12:71 938 979
	CpG2	chr12:71 938 928
	CpG3	chr12:71 938 922
	CpG4	chr12:71 938 902
	CpG5	chr12:71 938 898
	CpG6	chr12:71 938 877

Table S2. Genomic locations of the *SLC6A4* and *TPH2* CpG sites included in this study.

		SLC6A4	*TPH2*	
Reaction	**Direction**	Sequence(5'-3')		Sequence (5'-3')
PCR	**Forward**	TGGGGAGGTGTTAGAGGTTAAGAGAAA	**Forward**	GGAAAATATTATTATTGTTGGTTGTATGGA
	Reverse	[Btn]ATCCTAACTTTCCTACTCTTTAACT	**Reverse**	[Btn]CATTACTCTTCAACACCAAAATTCTA
Pyrosequencing	**CpG site**		**CpG site**	
	CpG1-4	GTGTGTAGTTTTGTGGG	**CpG1**	ATTGTTGGTTGTATGGAT
			CpG2-3	GGTATTAGAGGGGTAG
			CpG4-5	GAGATTGAGAGGAAGG
			CpG6	ATTAGTTGTTTGTTTGGG

Table S3. Primers used for pyrosequencing *SLC6A4* and *TPH2*.

		CpG1		CpG2		CpG3		CpG4	
		Effect size	**95% CI**	**Effect size**	**95% CI**	**Effect size**	**95% CI**	**Effect size**	**95% CI**
5HTT	Caudate	0.003	-0.06;0.066	0.007	-0.045;0.06	-0.014	-0.08;0.052	0.009	-0.033;0.051
	Amygdala	0.003	-0.054;0.06	0.007	-0.041;0.055	-0.013	-0.073;0.047	0.008	-0.03;0.047
	Hippocampus	0.001	-0.024;0.026	0.003	-0.018;0.024	-0.006	-0.031;0.02	0.004	-0.013;0.02
	Putamen	0.004	-0.065;0.072	0.008	-0.049;0.065	-0.015	-0.086;0.056	0.010	-0.036;0.055
	Thalamus	0.004	-0.071;0.079	0.009	-0.054;0.072	-0.017	-0.095;0.062	0.011	-0.04;0.061
	Midbrain	0.003	-0.055;0.061	0.007	-0.042;0.055	-0.013	-0.074;0.048	0.008	-0.031;0.047
	Neocortex	0.001	-0.014;0.015	0.002	-0.01;0.014	-0.003	-0.018;0.012	0.002	-0.008;0.012
5HT4 (Healthy Cohort)	Caudate	-0.012	-0.116;0.092	-0.013	-0.097;0.072	-0.071	-0.167;0.025	0.093	0.005;0.181
	Hippocampus	-0.005	-0.048;0.038	-0.005	-0.04;0.03	-0.029	-0.069;0.01	0.039	0.003;0.074
	Putamen	-0.013	-0.124;0.099	-0.014	-0.105;0.077	-0.076	-0.179;0.027	0.100	0.007;0.194
	Neocortex	-0.003	-0.028;0.022	-0.003	-0.024;0.018	-0.017	-0.041;0.006	0.023	0.002;0.044
5HT4 (MDD Cohort)	Caudate	-0.026	-0.214;0.163	-0.135	-0.281;0.012	0.108	-0.07;0.286	0.075	-0.065;0.216
	Hippocampus	-0.008	-0.071;0.054	-0.045	-0.093;0.004	0.036	-0.023;0.094	0.025	-0.022;0.071
	Putamen	-0.024	-0.205;0.156	-0.129	-0.269;0.011	0.103	-0.067;0.273	0.072	-0.062;0.206
	Neocortex	-0.004	-0.037;0.028	-0.023	-0.049;0.002	0.019	-0.012;0.049	0.013	-0.011;0.037

Table S4. Effect sizes and respective 95% confidence intervals of models including *SLC6A4* data.

		CpG1		CpG2		CpG3		CpG4		CpG5		CpG6	
		Effect size	95% CI	Effect size	95% CI	Effect size	95% CI	Effect size	95% CI	Effect size	95% CI	Effect size	95% CI
5HTT	Caudate	-0.017	-0.076;0.042	0.010	-0.061;0.08	-0.008	-0.094;0.077	0.010	-0.082;0.101	-0.048	-0.126;0.03	0.075	0.001;0.149
	Amygdala	-0.016	-0.071;0.04	0.009	-0.057;0.075	-0.008	-0.088;0.072	0.009	-0.077;0.095	-0.045	-0.118;0.029	0.070	0.0002;0.14
	Hippocampus	-0.007	-0.03;0.017	0.004	-0.024;0.032	-0.003	-0.037;0.031	0.004	-0.033;0.04	-0.019	-0.05;0.012	0.030	0.001;0.059
	Putamen	-0.019	-0.084;0.047	0.011	-0.067;0.088	-0.009	-0.103;0.085	0.011	-0.09;0.111	-0.052	-0.139;0.034	0.082	0.001;0.164
	Thalamus	-0.020	-0.091;0.051	0.011	-0.072;0.095	-0.010	-0.112;0.092	0.011	-0.098;0.121	-0.057	-0.15;0.036	0.090	0.001;0.178
	Midbrain	-0.016	-0.072;0.04	0.009	-0.057;0.075	-0.008	-0.088;0.072	0.009	-0.077;0.095	-0.045	-0.118;0.029	0.070	0.001;0.14
	Neocortex	-0.004	-0.017;0.01	0.002	-0.014;0.018	-0.002	-0.021;0.018	0.002	-0.019;0.023	-0.011	-0.029;0.007	0.017	0.0002;0.034
5HT4 (Healthy Cohort)	Caudate	-0.011	-0.094;0.072	-0.060	-0.169;0.048	0.011	-0.121;0.143	0.061	-0.079;0.202	0.039	-0.084;0.163	-0.012	-0.111;0.087
	Hippocampus	-0.004	-0.039;0.03	-0.025	-0.07;0.02	0.005	-0.05;0.06	0.026	-0.033;0.084	0.016	-0.035;0.068	-0.005	-0.046;0.036
	Putamen	-0.012	-0.1;0.077	-0.064	-0.18;0.051	0.012	-0.129;0.153	0.066	-0.084;0.216	0.042	-0.089;0.173	-0.013	-0.118;0.093
	Neocortex	-0.003	-0.024;0.018	-0.015	-0.043;0.012	0.003	-0.031;0.036	0.016	-0.02;0.051	0.010	-0.021;0.041	-0.003	-0.028;0.022
5HT4 (MDD Cohort)	Caudate	0.053	-0.18;0.286	0.025	-0.214;0.264	-0.244	-0.49;0.003	0.208	-0.056;0.473	0.017	-0.203;0.237	0.189	-0.06;0.438
	Hippocampus	0.018	-0.06;0.095	0.008	-0.071;0.088	-0.081	-0.163;0.001	0.069	-0.019;0.157	0.006	-0.067;0.079	0.063	-0.02;0.146
	Putamen	0.052	-0.179;0.284	0.025	-0.213;0.262	-0.242	-0.486;0.001	0.207	-0.055;0.468	0.017	-0.201;0.235	0.188	-0.059;0.434
	Neocortex	0.009	-0.032;0.051	0.004	-0.038;0.047	-0.043	-0.087;0.001	0.037	-0.01;0.084	0.003	-0.036;0.042	0.034	-0.011;0.078

Table S5. Effect sizes and respective 95% confidence intervals of models including *TPH2* data.

	SLC6A4															
	HC															
	CpG1			**CpG2**			**CpG3**			**CpG4**						
Psychometric measure	**β**	**P_{UNC}**	**95% CI**	**β**	**P_{UNC}**	**95% CI**	**β**	**P_{UNC}**	**95% CI**	**β**	**P_{UNC}**	**95% CI**				
PBI Care (N=290)	-0.09	0.88	[-1.20; 1.03]	0.73	0.08	[-0.08; 1.54]	-0.01	0.99	[-0.94; 0.92]	0.54	0.17	[-0.23; 1.31]				
PBI Overprotection (N=290)	0.46	0.32	[-0.44; 1.35]	-0.83	**0.01**	[-1.48; -0.19]	0.10	0.79	[-0.85; 0.64]	0.17	0.59	[-0.45; 0.79]				
SLE Recent (N=295)	0.46	0.13	[-0.13; 1.06]	0.20	0.37	[-0.23; 0.64]	0.47	0.06	[-0.03; 0.97]	0.08	0.70	[-0.33; 0.50]				
SLE Total (N=295)	0.004	0.98	[-0.23; 0.24]	-0.07	0.41	[-0.24; 0.10]	-0.12	0.23	[-0.32; 0.08]	-0.06	0.46	-0.22; 0.10				
	MDD															
BDI (N=89)	-1.474	0.319	[-4.40; 1.45]	-0.16	0.89	[-2.41; 2.09]	-0.36	0.79	[-3.05; 2.34]	0.32	0.76	[-1.80; 2.44]				
CATS (N=76)	-2.58	0.56	[-11.39; 6.23]	-0.68	0.84	[-7.36; 6.01]	3.05	0.44	[-4.83; 10.93]	1.42	0.66	[-4.97; 7.81]				
GAD10 (N=89)	0.89	0.63	[-2.72; 4.50]	-0.08	0.96	[-2.84; 2.69]	-3.37	**0.04**	[-6.60; -0.14]	-0.35	0.79	[-2.95; 2.26]				
HAMD-6 (N=90)	-0.15	0.65	[-0.79; 0.50]	0.02	0.95	[-0.48; 0.51]	0.21	0.49	[-0.38; 0.79]	0.13	0.57	[-0.33; 0.60]				
PBI Care (N=76)	1.26	0.46	[-2.08; 4.59]	0.10	0.94	[-2.44; 2.63]	-1.76	0.24	[-4.73; 1.21]	-1.14	0.35	[-3.55; 1.27]				
PBI Overprotection (N=76)	-0.90	0.45	[-3.26; 1.46]	0.53	0.56	[-1.26; 2.32]	-0.44	0.68	[-2.57; 1.68]	-0.01	0.99	[-1.73; 1.70]				
PSS (N=89)	0.41	0.65	[-1.41; 2.23]	-0.39	0.58	[-1.78; 1.01]	0.18	0.83	[-1.49; 1.85]	0.33	0.62	[-0.98; 1.64]				

β: linear model estimate; **P_{UNC}**: uncorrected p-value; **HC**: healthy controls; **MDD**: patients with depression. **PBI**: Parental Bonding Inventory; **SLE**: Stressful life events; **BDI**: Beck's Depressive Index; **CATS**: Childhood Abuse Trauma Scale; **GAD10**: Generalized Anxiety Disorder 10-item; **HAMD-6**: Hamilton's Depressive Rating Scale; **PSS**: Perceived Stress Scale

Table S6. Associations between *SLC6A4* methylation and environmental stress in healthy controls (HC) and mood and anxiety disorder symptoms in patients with depression (MDD).

	HC																			
	CpG1			CpG2			CpG3			CpG4			CpG5			CpG6				
Measure	**β**	**P_{UNC}**	**95% CI**	**β**	**P_{UNC}**	**95% CI**	**β**	**P_{UNC}**	**95% CI**	**β**	**P_{UNC}**	**95% CI**	**β**	**P_{UNC}**	**95% CI**	**β**	**P_{UNC}**	**95% CI**
PBI Care (N=292)	0.34	0.55	[-0.76; 1.44]	-0.06	0.92	[-1.14; 1.02]	-0.15	0.79	[-1.27; 0.96]	-0.82	0.049	[-1.64; -0.005]	-0.49	0.17	[-1.18; 0.21]	-0.53	0.17	[-1.29; 0.22]
PBI Overprotection (N=292)	0.10	0.83	[-0.79; 0.99]	-0.02	0.96	[-0.9; 0.85]	-0.45	0.32	[-1.36; 0.45]	0.48	0.16	[-0.18; 1.14]	0.29	0.31	[-0.27; 0.85]	0.20	0.52	[-0.41; 0.81]
SLE Recent (N=297)	0.28	0.36	[-0.31; 0.87]	-0.14	0.65	[-0.72; 0.45]	-0.02	0.95	[-0.62; 0.58]	0.17	0.46	[-0.28; 0.61]	-0.12	0.51	[-0.50; 0.25]	0.07	0.73	[-0.33; 0.47]
SLE Total (N=297)	-0.005	0.96	[-0.23 0.22]	0.05	0.67	[-0.18; 0.28]	-0.06	0.61	[-0.30; 0.17]	-0.003	0.98	[-0.18; 0.17]	-0.014	0.85	[-0.16; 0.13]	0.008	0.92	[-0.15; 0.17]
	MDD																	
BDI (N=88)	1.53	0.13	[-0.74; 5.70]	1.99	**0.05**	[-0.004; 4.08]	1.46	0.15	[-0.56; 3.67]	0.95	0.35	[-1.40; 3.96]	1.20	0.23	[-0.73; 2.94]	0.91	0.37	[-1.64; 4.38]
CATS (N=76)	1.97	**0.05**	[-0.09; 7.60]	1.73	0.09	[-0.75; 10.42]	1.64	0.11	[-1.02; 10.40]	1.49	0.14	[-1.80; 12.36]	1.30	0.20	[-1.70; 8.08]	0.84	0.41	[-4.80; 11.74]
GAD10 (N=88)	-0.07	0.95	[-4.10; 3.83]	0.24	0.81	[-2.23; 2.84]	-0.32	0.75	[-3.02; 2.18]	-0.31	0.76	[-3.78; 2.75]	-0.42	0.67	[-2.72; 1.76]	-0.92	0.36	[-5.35; 1.96]
HAMD6 (N=89)	-0.21	0.84	[-0.79; 0.64]	0.08	0.93	[-0.44; 0.48]	-0.46	0.65	[-0.58; 0.36]	-1.00	0.32	[-0.89; 0.29]	-0.82	0.41	[-0.57; 0.24]	0.05	0.96	[-0.65; 0.68]
PBI Care (N=76)	-1.95	0.06	[-6.67; 0.08]	-1.90	0.06	[-4.14; 0.11]	-1.86	0.07	[-4.19; 0.14]	-1.45	0.15	[-4.67; 0.73]	-1.48	0.14	[-3.24; 0.48]	-1.69	0.10	[-5.74; 0.48]
PBI Overprotection (N=76)	0.72	0.47	[-1.56; 3.32]	-0.31	0.76	[-1.77; 1.30]	-0.05	0.96	[-1.61; 1.53]	-0.03	0.97	[-1.97; 1.90]	-0.44	0.66	[-1.63; 1.04]	0.27	0.79	[-1.93; 2.55]
PSS (N=88)	0.10	0.92	[-1.88; 2.09]	1.05	0.30	[-0.59; 1.93]	0.72	0.47	[-0.83; 1.77]	0.69	0.49	[-1.07; 2.20]	0.78	0.44	[-0.68; 1.56]	0.77	0.45	[-1.13; 2.54]

β: linear model estimate; **P_{UNC}**: uncorrected p-value; **HC**: healthy controls; **MDD**: patients with depression. **PBI**: Parental Bonding Inventory; **SLE**: Stressful life events; **BDI**: Beck's Depressive Index; **CATS**: Childhood Abuse Trauma Scale; **GAD10**: Generalized Anxiety Disorder 10-item; **HAMD-6**: Hamilton's Depressive Rating Scale; **PSS**: Perceived Stress Scale

Table S7. Associations between *TPH2* methylation and environmental stress in healthy controls (HC) and mood and anxiety disorder symptoms in patients with depression (MDD).

		HC (N=204)			MDD (N=86)		
Gene	**Variable**	**β**	**P-value**	**95% CI**	**β**	**P-value**	**95% CI**
SLC6A4 **$CpG_{LV+cells}$**	Lymphocytes	0.01	$4.0x10^{-3}$	[0.004 ; 0.020]	0.01	0.04	[0.0003; 0.021]
	Monocytes	-0.02	0.09	[-0.054; 0.004]	$-2.0x10^{-3}$	0.84	[-0.026; 0.021]
	Granulocytes	-0.40	0.04	[-0.77; -0.020]	0.16	0.28	[-0.126;0.442]
	5-HTTLPR	-0.02	0.75	[-0.137; 0.100]	-0.07	0.23	[-0.198; 0.048]
	Age	$5.0x10^{-3}$	0.08	[-0.001; 0.010]	$2.42x10^{-5}$	0.99	[-0.006; 0.006]
	Sex (Male)	0.05	0.35	[-0.060; 0.168]	-0.06	0.29	[-0.182; 0.054]
TPH2 **$CpG_{LV+cells}$**	Lymphocytes	0.02	$1.8x10^{-6}$	[0.011; 0.026]	0.01	$4.0x10^{-3}$	[0.004;0.023]
	Monocytes	$-4.0x10^{-3}$	0.67	[-0.021; 0.014]	$3.0x10^{-3}$	0.85	[-0.025;0.030]
	Granulocytes	-0.13	0.25	[-0.364; 0.095]	-0.21	0.18	[-0.502; 0.092]
	TPH2_rs4570625 (T-)	0.25	0.32	[-0.237;0.733]	-0.02	0.76	[-0.135; 0.098]
	Age	0.01	$2.3x10^{-5}$	[0.005; 0.014]	0.01	0.04	[0.0003; 0.016]
	Sex (Male)	0.08	0.03	[0.006; 0.154]	0.10	0.13	[-0.030; 0.226]

Table S8. Results of associations between $CpG_{LV+cells}$ and cell proportions, sex, age and genotype.

HC						
	SLC6A4			*TPH2*		
	Estimate	**P-value**	**95% CI**	**Estimate**	**P-value**	**CI 2.5%**
PBI Care (N=200)	0.01	0.20	[-0.003; 0.016]	$1.0x10^{-3}$	0.84	[-0.006; 0.007]
PBI Overprotection (N=197)	$-4.0x10^{-3}$	0.42	[-0.015; 0.006]	$1.0x10^{-3}$	0.76	[-0.006; 0.008]
SLE Recent (N=204)	0.01	0.13	[-0.004; 0.029]	$-2.0x10^{-3}$	0.65	[-0.012;0.008]
SLE Total (N=204)	-0.01	0.73	[-0.054; 0.037]	$3.0x10^{-3}$	0.84	[-0.025; 0.031]
MDD						
BDI (N=86)	$-2.0x10^{-3}$	0.53	[-0.009; 0.005]	0.01	0.05	[0.00001; 0.015]
CATS (N=75)	$-3.0x10^{-4}$	0.61	[-0.002; 0.001]	$2.0x10^{-3}$	0.11	[-0.001; 0.005]
GAD10 (N=86)	$-2.0x10^{-3}$	0.5	[-0.006; 0.003]	$3.0x10^{-4}$	0.90	[-0.005; 0.006]
HAMD6 (N=86)	-0.02	0.24	[-0.049; 0.012]	-0.01	0.47	[-0.041; 0.019]
PBI Care (N=75)	$-2.0x10^{-4}$	0.9	[-0.003; 0.004]	-0.01	0.13	[-0.013; 0.002]
PBI Overprotection (N=75)	$-2.0x10^{-3}$	0.6	[-0.007; 0.004]	$5.9x10^{-5}$	0.99	[-0.010; 0.010]
PSS (N=86)	$3.0x10^{-3}$	0.51	[-0.007; 0.014]	$2.0x10^{-3}$	0.73	[-0.009; 0.013]

Table S9. Results of associations between $CpG_{LV+cells}$ and measures of environmental stress or mood or anxiety symptoms.

STUDY III

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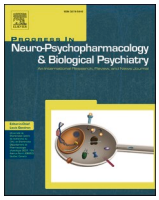

DNA methylation of serotonin genes as predictive biomarkers of antidepressant treatment response

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ABSTRACT

Selective serotonin reuptake inhibitors (SSRI) are frequently ineffective in treating depressive episodes and biomarkers are needed to optimize antidepressant treatment outcomes. DNA methylation levels of serotonin transporter (*SLC6A4*) and tryptophan hydroxylase 2 genes (*TPH2*) have been suggested to predict antidepressant clinical outcomes but their applicability remains uncertain. In this study, we: 1) evaluated *SLC6A4/TPH2* methylation biomarker potential for predicting clinical outcomes after escitalopram treatment; 2) evaluated whether changes in *SLC6A4/TPH2* methylation are informative of treatment mechanisms. We used a cohort of 90 unmedicated patients with major depressive disorder that were part of a 12-week open-label longitudinal trial and compared our observations with previous findings. Depressive symptoms were measured at baseline and after 8 and 12 weeks of treatment using the Hamilton Depression Rating Scale ($HAMD_{6/17}$). We found an association between baseline *TPH2* methylation and both clinical response (β:3.43; $p = 0.01$; 95 % CI:[0.80; 6.06]) and change in depressive symptoms after 8 weeks (β:−45.44; p = 0.01; 95 %CI:[− −78.58; −12.30]). However, we found no evidence for predictive value of any gene (*TPH2* AUC: 0.74 95 % CI:[0.42;0.79]; *SLC6A4*: AUC: 0.61; 95 % CI: [0.48–0.78]). Methylation levels changed at the trend level for CpG sites of *SLC6A4* and *TPH2* over the course of 12 weeks of treatment. In addition, similar to previous observations, we found a trend for an association between methylation of *SLC6A4* CpG2 (chr17:30,236,083) and $HAMD_{17}$ change after 12 weeks. Our findings suggest that although *TPH2* and *SLC6A4* methylation may be informative of antidepressant treatment outcome, they are unlikely to prove useful as clinical predictor tools.

1. Introduction

Most antidepressant medications affect extracellular serotonin levels (Artigas et al., 2002). Selective serotonin reuptake inhibitors (SSRI) are the most commonly prescribed class of antidepressants (Abbing-Karahagopian et al., 2014) and target the serotonin transporter (5-HTT) (Artigas et al., 2002). However, only about 50 % patients with major depressive disorder (MDD) respond to SSRI treatment (Berton and Nestler, 2006; Trivedi et al., 2006).

Genetic variation affecting gene expression key to serotoninergic neurotransmission might play a role in interindividual variability of antidepressant treatment outcomes, though not confirmed by all studies (Culverhouse et al., 2018; Porcelli et al., 2011). Among these, variants within the 5-HTT-encoding gene (*SLC6A4*) and tryptophan hydroxylase 2 (*TPH2*), which is the rate-limiting enzyme for serotonin production in the brain, have been largely investigated (Lesch and Gutknecht, 2005;

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Porcelli et al., 2011; Walther and Bader, 2003). To date, however, neither candidate genotype approaches nor polygenic risk scores have succeeded in identifying reliable predictive biomarkers of antidepressant treatment outcomes (Nøhr et al., 2022).

Genetic and environmental factors (e.g. early life stress) are likely to affect recovery after using antidepressants. In this framework, DNA methylation is an epigenetic modification that can occur in response to environmental changes and can affect gene expression (Villicaña and Bell, 2021). Specifically, DNA methylation of *SLC6A4* and *TPH2* has been associated to early life stress and childhood trauma in patients with depression (Beach et al., 2010; Kang et al., 2013; Shen et al., 2020; Van Ijzendoorn et al., 2010) and has been suggested as a predictor of clinical response to antidepressant medications (Hack et al., 2019).

Four studies have linked pre-treatment methylation of the *SLC6A4* promoter region to antidepressant treatment outcome (Domschke et al., 2014; Kang et al., 2013; Okada et al., 2014; Schiele et al., 2021). Two of these, including partially overlapping populations (Domschke et al., 2014; Schiele et al., 2021), reported that lower pre-treatment CpG1 (chr17:28563090, GRCh37/hg19) and CpG2 (chr17:28563102) methylation was linked to impaired recovery after 6 weeks of SSRI treatment. Conversely, another study (Kang et al., 2013) reported that higher baseline CpG2 methylation correlated with poorer treatment outcome after 12-weeks antidepressant treatment.

Finally, one study described that low baseline *TPH2* methylation was associated with poor treatment outcome after 2 weeks of antidepressant treatment (Shen et al., 2020). Notably, previous studies evaluated associations between methylation levels and clinical outcomes but did not perform prediction analyses.

DNA methylation can also be altered by antidepressant treatment (Dong et al., 2008; Perisic et al., 2010; Zimmermann et al., 2012) and this may inform mechanisms underlying treatment. One study did not detect a difference between pre- and post-treatment *SLC6A4* methylation levels (Okada et al., 2014), while another reported both increased and decreased methylation at different CpG sites (Moon et al., 2023). To our knowledge, changes in *TPH2* methylation levels following treatment have not yet been examined.

While some of the discrepancies reported by previous studies might be due to differences in e.g., ethnic background, study designs, or inclusion criteria, another source of variation could arise from the lack of correction for blood cell types (Moore and Kobor, 2017). Indeed, accounting for cell heterogeneity is critically important to remove confounding effects on epigenetic variation (Farré et al., 2015; Zheng et al., 2017).

In this study, we aimed to evaluate whether: a) pre-treatment *SLC6A4/TPH2* methylation is associated with and can predict recovery after antidepressant treatment; b) *SLC6A4/TPH2* methylation changes over the course of treatment and c) we could confirm previous findings. We included measured blood cell proportions in our analyses to account for cell heterogeneity. *SLC6A4/TPH2* methylation was measured in peripheral DNA in a cohort of 90 unmedicated patients with MDD who were started on the SSRI escitalopram and followed for 12 weeks.

2. Materials and methods

2.1. Participants and study design

Participants were part of the open-label, non-randomized, longitudinal clinical trial NeuroPharm, involving a 12-week treatment with the SSRI escitalopram. The detailed research protocol has been previously described (Köhler-Forsberg et al., 2020). Seven participants switched to duloxetine after 4 weeks because of escitalopram inefficacy (Köhler-Forsberg et al., 2020). Drug plasma concentrations were taken after eight weeks to monitor treatment compliance. Patients were evaluated by a certified psychiatrist in face-to-face interviews, scored >17 in the 17-item Hamilton Depression Rating Scale ($HAMD_{17}$) (Hamilton, 1967) and had not taken antidepressant medications for at least two months before the start of the trial. Data from 90 patients with moderate to severe depression were included in the current study based on the availability of blood samples and blood cell counts. Follow-up was available for $N = 76$ patients at week 8 and $N = 72$ at week 12 (Table 1). This study was carried out in compliance with the Declaration of Helsinki. The study was approved by the Danish Data Protection Agency, the Health Research Ethics Committee of the Capital Region of Denmark, and Danish Medicines Agency (H-15017713 and H-KF-2006-20).

2.2. DNA methylation analysis

DNA methylation was estimated at four CpG sites within the CpG island in the promoter region of *SLC6A4* and six CpG sites at the 5' UTR of *TPH2*, as previously reported (Bruzzone et al., 2024). Genomic DNA was purified from whole blood using the FlexiGene Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. First, 500 ng DNA were bisulfite-converted using the EpiTecht 96 Bisulfite Kit (QIAGEN). Next, the sequences of interest were amplified with polymerase chain reaction (PCR) (Bruzzone et al., 2024). The resulting amplicon was sequenced using target-specific primers (Bruzzone et al., 2024) and the pyrosequencing system PyroMark Q96 ID (QIAGEN). Quality check was based on the PyroMark software (QIAGEN) reports and on visual inspection of the pyrograms. Measurements were run in duplicates. Each

Table 1
Demographic data of the participants with major depressive disorder (MDD) included in the study.

	Week 1	Week 8	Week 12
N	89	76	72
Age	26.7 ± 7.7 [18.2–56.4]	26.9 ± 7.6 [18.3–56.4]	26.9 ± 7.9 [18.3–57.3]
Sex (F/M)	63/26	55/21	51/21
$HAMD_6$	12.3 ± 1.7 [7–17]	5.89 ± 3.8 [0–16]	4.73 ± 3.7 [0–14]
$HAMD_{17}$	22.8 ± 3.4 [18–31]	11.4 ± 6.4 [1−31]	9.4 ± 6 [0–25]
SLC6A4 CpG1 (%)	2.29 ± 0.55 [1.28–3.94]	2.46 ± 0.68 [1.4–4.15]	2.41 ± 0.56 [1.32–4.19]
SLC6A4 CpG2 (%)	3.28 ± 0.71 [1.88–5.26]	3.49 ± 0.85 [1.91–5.62]	3.53 ± 0.89 [1.9–5.58]
SLC6A4 CpG3 (%)	2.73 ± 0.61 [1.5–4.38]	2.7 ± 0.63 [1.6–4.07]	2.75 ± 0.7 [1.46–4.74]
SLC6A4 CpG4 (%)	3.54 ± 0.75 [2.06–5.8]	3.53 ± 0.83 [1.96–6.8]	3.53 ± 0.75 [2.22–5.52]
TPH2 CpG1 (%)	2.7 ± 0.5 [1.47–4.18]	2.74 ± 0.49 [1.32–4.14]	2.88 ± 0.74 [1.54–5.88]
TPH2 CpG2 (%)	2.95 ± 0.79 [1.65–8.4]	2.87 ± 0.66 [1.66–5.6]	2.86 ± 0.8 [1.45–6.8]
TPH2 CpG3 (%)	2.68 ± 0.77 [1.54–7.52]	2.62 ± 0.6 [1.46–5.63]	2.69 ± 0.69 [1.5–5.46]
TPH2 CpG4 (%)	2.07 ± 0.62 [1.14–5.58]	2.11 ± 0.61 [0.735–5.69]	2.11 ± 0.63 [0.9–4.38]
TPH2 CpG5 (%)	3.16 ± 0.92 [1.94–8.77]	3.12 ± 0.76 [1.83–6.96]	3.19 ± 0.91 [1.76–6.12]
TPH2 CpG6 (%)	3.02 ± 0.6 [1.96–5.26]	3.06 ± 0.66 [1.78–5.44]	2.92 ± 0.66 [1.4–5.68]
SLC6A4 5-HTTLPR/rs25531 (L_AL_A / S-)	24 / 65	21 / 55	21 / 49
TPH2 rs4570625 (GG/T-)	53 / 35	45 / 31	44 / 28
Available blood cells counts (yes/no)	85/4	74/2	67/5
Plasma escitalopram concentration (nM)		79.4 ± 44.9 [1.12–263]	

HAMD: Hamilton Depressive Rating Scale (6- or 17-items); *SLC6A4*: serotonin transporter gene; *TPH2*: tryptophan hydroxylase 2 gene; L_AL_A: high *SLC6A4* expression haplotype; s-: low *SLC6A4* expression haplotype; GG: normal *TPH2* expression genotype; T-: reduced *TPH2* expression allele. DNA methylation values are expressed in percentages (methylated vs non methylated DNA template).

batch of analyses included non-methylated, 50 %-methylated, fully methylated samples and DNase-free H_2O as controls.

2.3. Genotyping

All participants were genotyped for the functional polymorphisms *SLC6A4* 5-HTTLPR/rs25531 and *TPH2* rs4570625, which have been reported to affect gene expression of these genes in combination with DNA methylation (Akhrif et al., 2023; Philibert et al., 2007; Van Ijzendoorn et al., 2010). Genotyping procedures involved using PCR and gel electrophoresis, as previously described (Fisher et al., 2015; Gutknecht et al., 2007). Based on chi-squared tests, alleles of both *SLC6A4* 5-HTTLPR/rs25531 and *TPH2* rs4570625 did not significantly deviate from Hardy-Weinberg equilibrium ($p > 0.1$).

2.4. Clinical outcomes

Depressive symptoms were measured using $HAMD_6$ and $HAMD_{17}$ (Hamilton, 1967). For statistical analyses, were used: a) a categorical outcome, which classified the participants as responders if they showed a ≥ 50 % reduction in $HAMD_{17}$ after 8 weeks of treatment, otherwise as non-responders, as previously done (Fisher et al., 2022); b) a continuous outcome, consisting in the percent change of $HAMD_6$ scores at week 8 ($\Delta HAMD_6$) compared to baseline, as mentioned in the original research protocol (Köhler-Forsberg et al., 2020). This was calculated as: ($HAMD_6$ at week 8 - $HAMD_6$ at baseline / $HAMD_6$ at baseline)*100).

2.5. Statistical analyses

The analyses including *SLC6A4* were indicated as secondary analyses in the research protocol, whereas analyses including *TPH2* are

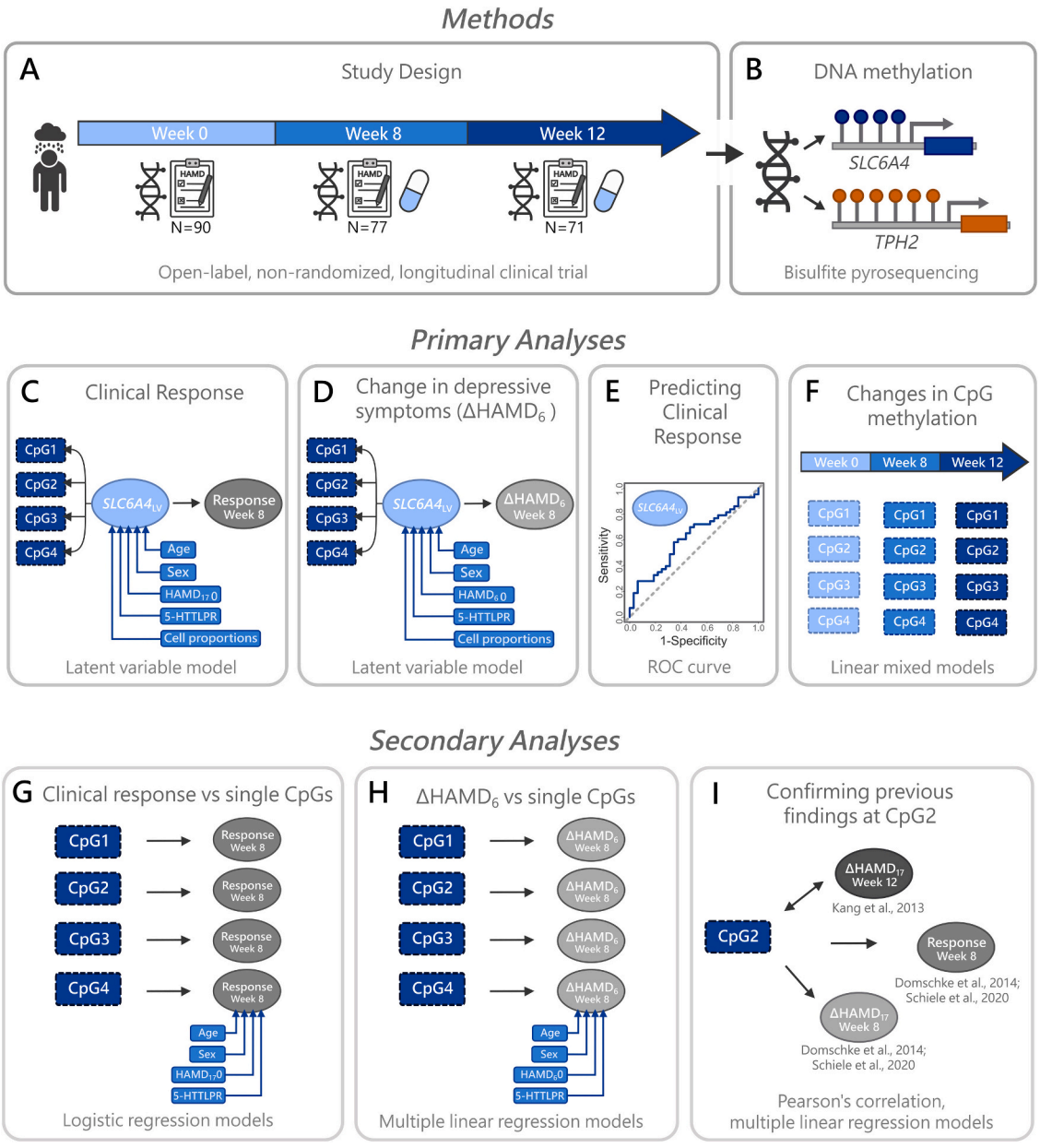

Fig. 1. Overview of the statistical analyses carried out in the study. A and B depict the study design and experimental methods. C-f depict the primary analyses, involving latent variable models with categorical (C) or continuous (D) outcomes, the ROC curve (E) and linear mixed models (E). G-I illustrate the secondary analyses, involving separate general linear models (F), multiple linear regression models (G, I) or Pearson's correlation models for separate CpG sites. For illustrative purposes, only models for SLC6A4 are shown. Analogous models were used for TPH2.

exploratory analyses as to what was initially described (Köhler-Forsberg et al., 2020). All statistical analyses were carried out in R (R Core Team, 2021). A graphical representation of statistical analyses is reported in Fig. 1.

2.6. Primary analyses

2.6.1. Association between pre-treatment SLC6A4/TPH2 methylation and clinical outcome or $\Delta HAMD_6$

Using the *lava* package v 1.6.10 (Holst and Budtz-Jørgensen, 2013), we modelled two latent variable models (LVM), one including *SLC6A4* CpG1–4 and one including *TPH2* CpG1–6 methylation. LVMs are multivariate linear regressions which allow to evaluate the association between the shared variance of a set of inter-correlated variables (e.g. methylation of multiple CpG sites) and a variable of interest (e.g. clinical outcome).

LVMs included a latent variable ($SLC6A4_{LV}$ or $TPH2_{LV}$) capturing the shared correlations across CpG sites of either *SLC6A4* or *TPH2* regressed against age, sex, genotype (5HTTLPR/rs25531 for *SLC6A4* and rs4570625 for *TPH2*) and cell proportions. Cell proportions included lymphocytes, monocytes, granulocytes, basophils and eosinophils. Baseline $HAMD_{17}$ or $HAMD_6$ scores were included as covariates in models including the categorical and continuous outcomes respectively. $SLC6A4_{LV}$ and $TPH2_{LV}$ were regressed against clinical response or $\Delta HAMD_6$. 95 % confidence intervals (CI) and *p*-values were derived from the model-based standard errors, assuming normally distributed estimates and test statistics. As a sensitivity analysis, we compared these CI to the studentized CI based on 1000 resamples from a non-parametric bootstrap.

2.6.2. Prediction analyses

To evaluate whether *SLC6A4* or *TPH2* methylation predicted clinical outcome after 8 weeks of SSRI treatment, we constructed a receiving operating characteristics (ROC) curve for each gene. ROC curves were constructed using clinical outcome information and the latent variable values that were estimated for the LVMs used in the primary analyses. For each curve, the area under the curve (AUC) was estimated to quantify the predictive value of each gene with respect to clinical response. Confidence intervals for AUCs were obtained from a non-parametric bootstrap with 10,000 resamples, by taking 2.5 % and 97.5 % percentiles of the AUC bootstrap estimates. As this (in-sample) estimated AUC may be subject to optimism bias, it was compared to the average AUC estimated by repeated 8-fold cross-validation framework (15 repetitions leading to a total of 120 resamples). Two of the randomly-generated folds were excluded from analyses due to an imbalanced responder vs non-responder ratio (e.g. one responder out of nine data points), resulting in a total of 118.

2.6.3. Changes in DNA methylation over time

Linear mixed models (LMM) were used to evaluate whether the mean *SLC6A4* or *TPH2* methylation change from baseline differed between groups (responders vs non-responders) after 8 or 12 weeks of treatment. We added an interaction between week (8 or 12) and tested the corresponding parameters against zero using Wald test. Week, age, sex, genotype and cell proportions were included as covariates. An unstructured pattern was used to model the within-subject residual variance.

Additionally, we used the plasma drug concentrations that were taken at week 8 to evaluate possible dose-dependent changes in DNA methylation in the subgroup of patients that were treated exclusively with escitalopram throughout the trial ($N = 69$). We used LMM_S similar to those described above but including plasma drug concentration as a covariate. We corrected the *p*-values to account for multiple CpG sites examined in each gene.

2.7. Secondary analyses

2.7.1. Single-CpG analyses

Although methylation levels of adjacent CpG sites in promoter regions tend to be highly correlated (Jajoo et al., 2023), isolated CpG sites have been associated with various clinical phenotypes (Bock, 2012; Houtepen et al., 2016; Schiele et al., 2021). Thus, to investigate whether potential associations at the single CpG level were overlooked by modelling all CpG sites into latent variables, we modelled associations between methylation levels at each CpG site and clinical outcome or $\Delta HAMD_6$. We used logistic regressions to evaluate associations with the categorical clinical outcome and multiple linear regression models to evaluate the associations with $\Delta HAMD_6$. We used the same covariates included in the LVMs for these models, except for cell proportions.

2.7.2. Comparison with previous findings

To evaluate whether we could confirm previous findings on *SLC6A4* methylation and clinical outcome (Domschke et al., 2014; Kang et al., 2013; Schiele et al., 2021) in our dataset, we focused our analyses on *SLC6A4* CpG2 for findings by Domschke et al. (2014) and by Kang et al. (2013) and CpG1 for findings based on the SSRI/SNRI patients subgroup by Schiele et al. (2021). We did not consider findings from Okada et al. (2014) as DNA methylation estimated with mass spectrometry is not directly comparable to that estimated by pyrosequencing.

We used the same statistical models that were used in the original studies. To reproduce findings by Domschke et al. (2014), we used a multiple linear regression model evaluating the relation between CpG2 and $\Delta HAMD_{17}$, using age, sex, smoking status and depressive symptoms at baseline as covariates. Lifetime duration of depression and lifetime number of depressive episodes were not included in our analyses as this information was unavailable in our dataset. Co-medication with antipsychotics or mood stabilizers and lifetime number of hospitalizations were not included as covariates as no patient in this cohort was on such medication or hospitalized. Both studies used $HAMD_{21}$ to evaluate depressive symptoms after 6 weeks of treatment, while outcomes in our dataset were measured using the $HAMD_6$ or $-$ 17 after 8 weeks from treatment start. Thus, we defined clinical response as a reduction of 50 % or more in $HAMD_{17}$ from baseline to week 8; clinical remission was defined by a $HAMD_{17} \leq 7$ at week 8. To reproduce findings reported for the SSRI/SNRI subgroup examined by Schiele et al. (2021), we used multiple linear regressions to model the association between CpG1 and $\Delta HAMD_{17}$; we used logistic regressions to model the association between CpG1 and remitter or responder status.

To cross-validate findings by Kang et al. (2013), we used Pearson's correlation to evaluate the association between CpG2 and depressive symptoms change at week 12. We used the percent change at week 12 in $\Delta HAMD_{17}$ scores to evaluate symptoms change.

3. Results

As previously reported (Fisher et al., 2022), 44 (59 %) patients were classified as responders and 32 as non-responders (41 %).

3.1. Primary analyses

3.1.1. Association between baseline SLC6A4/TPH2 methylation and clinical outcome or $\Delta HAMD_6$

All loadings (parameters reflecting the association between CpG methylation values and the latent variable) were significantly different from 0 (all $p < 0.01$).

Results of LVMs are reported in Table 2 and illustrated in Fig. 2. We found no statistically significant association between $SLC6A4_{LV}$ and clinical outcome or $\Delta HAMD_6$ after eight weeks of treatment ($p = 0.98$). Instead, we found that each increase of 0.1 in $TPH2_{LV}$ corresponded to a 4.5 % depressive symptoms reduction ($p = 0.01$, Table 2). Likewise, LVMs including the categorical clinical outcome suggested that higher

Table 2
Primary Analyses: Association between baseline SLC6A4/TPH2 methylation and clinical outcome or $\Delta HAMD_6$. Results of LVMs evaluating an association between $SLC6A4_{LV}$ or $TPH2_{LV}$ and either clinical outcome or change in $HAMD_6$ scores.

	Treatment Outcome			Change in $HAMD_6$ score ($\Delta HAMD_6$)		
Gene	β	p	95 % CI	β	p	95 % CI
$SLC6A4_{LV}$	0.68	0.22	[−0.40; 1.77]	0.28	0.98	[−23.45; 24.00]
$TPH2_{LV}$	3.43	0.01	[0.80; 6.06]	-45.44	0.01	[−78.58; −12.30]

$TPH2_{LV}$ values were associated to higher probability of response (+3.43 per $TPH2_{LV}$ unit on the probit scale – see Fig. S1 for interpretation in term of probability of response).

Visual inspection of the data pointed to a single data point possibly driving the observed associations (Fig. S2). However, statistical significance remained in a sensitivity analysis without this extreme value (clinical outcome: Estimate (β): 3.59; p-value: 0.023; $\Delta HAMD_6$: β: −58.75; p-value: 0.03). The plots in Fig. 2 depict results without the extreme value. No technical issue was observed for the measurement of that data point, so we had no reason to exclude it from our main analyses. Covariate effects on $SLC6A4_{LV}/TPH2_{LV}$ are reported in Table S1. 95 % CIs obtained with non-parametric bootstrapping procedures were comparable with the model-based CIs, e.g. [−0.11; 0.41] vs [−0.40; 1.77] (Table S2).

3.1.2. Prediction analyses

The AUC for *SLC6A4* was 0.613 (95 % CI: 0.484–0.741), while that for *TPH2* was 0.744 (95 % CI: 0.422–0.794). In either case, the CI included 0.5 (AUC value under the null hypothesis of no predictive value) and AUC values corresponding to good predictors (e.g. 0.74). The AUC value derived from cross-validating *TPH2* ROC curve (AUC: 0.726) was very similar to the AUC obtained from the main model. Our bootstrap estimates showed skewed distributions, due to lack of convergence of some of the models, possibly due to limited sample size.

3.1.3. Changes in DNA methylation over time

Before Bonferroni correction, we observed a trend ($p = 0.02$; $p_{adj} = 0.1$) for a decrease in *TPH2* CpG2 methylation from week 0 to week 8 but not at week 12 in non-responders. We found no statistically significant change in *SLC6A4* methylation from baseline to 8 or 12 weeks of treatment (all p_{adj}-values>0.33) (Table 3). Covariate effects from these models are reported in Table S3.

When accounting for escitalopram plasma concentration at week 8, we found a trend ($p = 0.03$) for a positive *SLC6A4* CpG2 methylation change and negative *TPH2* CpG2 change from baseline to week 12 in non-responders. We also observed that *TPH2* CpG1 (p = 0.02), CpG2 ($p = 0.05$) and CpG4 (p = 0.03) methylation were positively associated with escitalopram plasma concentration. However, none of these observations remained statistically significant after Bonferroni correction (all p_{adj}-values>0.11, Table S4).

3.2. Secondary analyses

3.2.1. Single-CpG analyses

Results from multiple linear regressions and logistic regressions are reported in Table S5. We found no association between methylation of single *SLC6A4* CpG sites and $\Delta HAMD_6$ (all p_{adj} values>0.94) nor clinical outcome (all p_{adj} values>0.8). Conversely, before correction for multiple comparisons, *TPH2* CpG3-CpG6 were statistically significantly associated with categorical clinical outcome, whereas CpG2-CpG6 were statistically significantly (p = 0.01–0.03) associated with $\Delta HAMD_6$ (Table S5). None of these associations remained statistically significant after Bonferroni correction, although CpG5 and CpG6 remained at a trend level ($p = 0.06$–0.08).

3.2.2. Comparison with previous findings

The multiple linear regression model based on Domschke et al. (2014) revealed no statistically significant association between *SLC6A4* CpG2 and $\Delta HAMD_{17}$ after 8 weeks (β: 2.33; p-value: 0.65, 95 % CI [−7.79; 12.45]). Similarly, we found no statistically significant association between CpG1 and clinical response (β: 0.31; p-value: 0.49, 95 % CI [−0.56; 1.21]), nor remission (β: −0.40; p-value: 0.41; 95 % CI [−1.41; 0.54]), nor $\Delta HAMD_{17}$ at week 8 (β: 2.06; p-value: 0.73, 95 % CI

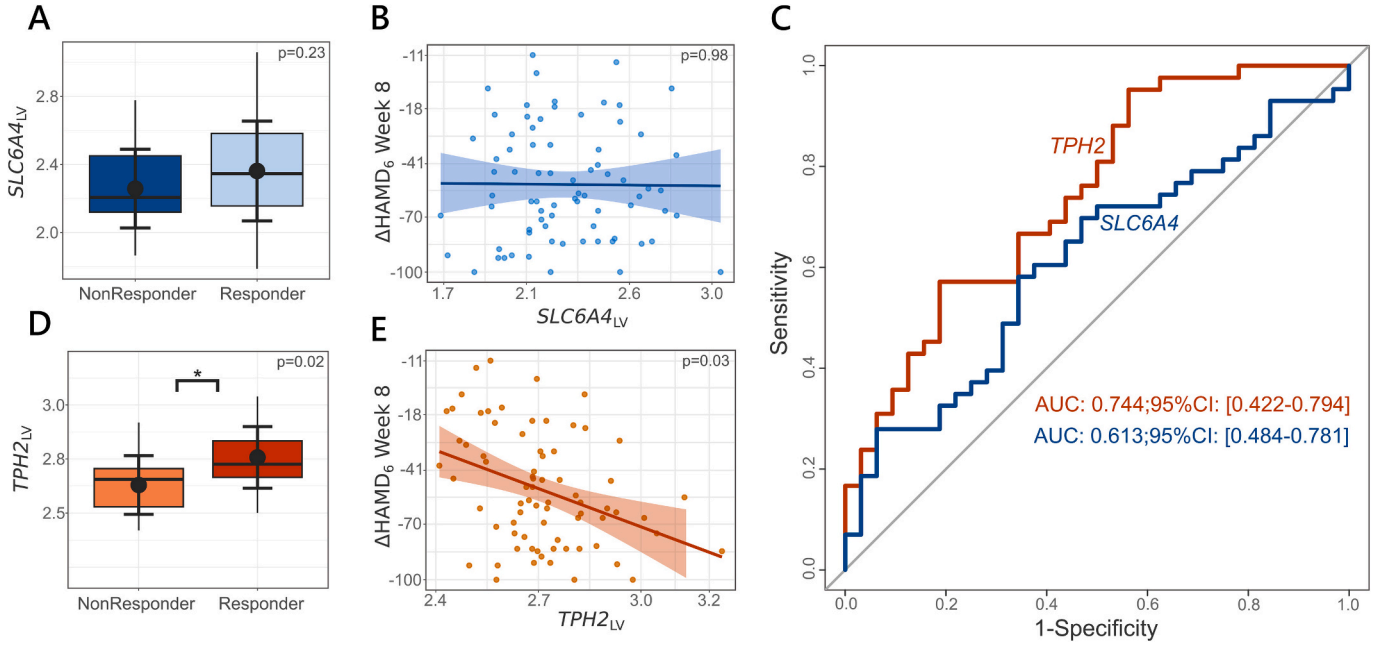

Fig. 2. Main findings. Results from LVM evaluating group differences between responders and non-responders are reported in A for serotonin transporter gene (*SLC6A4*) and D for tryptophan hydroxylase gene (*TPH2*). B and E depict associations between DNA methylation and change in depressive symptoms for *SLC6A4* and *TPH2* respectively. C shows the ROC curves evaluating whether *SLC6A4/TPH2* methylation predicts clinical outcome.

Table 3
Results from linear mixed models evaluating changes in *SLC6A4/TPH2* methylation over 12 weeks of treatment by stratifying responders vs non-responders. Adjustment for multiple comparisons is performed over CpG sites, i.e., 4 tests for *SLC6A4* and 6 tests for *TPH2*.

		SLC6A4				***TPH2***			
CpG site	Covariates	*β*	95 % CI	p	p_{adj}	*β*	95 % CI	p	p_{adj}
	Week 8 change (non-resp.)	0.23	[−0.03;0.50]	0.09	0.35	−0.02	[−0.20;0.16]	0.85	1.00
	Change at week 12 (non-resp.)	0.09	[−0.15;0.33]	0.45	1.00	0.26	[−0.06;0.58]	0.11	0.66
CpG1	Difference in change at week 8 (resp. vs non-resp.)	−0.04	[−0.35;0.28]	0.83	1.00	0.05	[−0.17;0.27]	0.64	1.00
	Difference in change at week 12 (resp. vs non-resp.)	0.11	[−0.17;0.39]	0.42	1.00	−0.20	[−0.60;0.20]	0.32	1.00
	Change at week 8 (non-resp.)	0.04	[−0.30;0.38]	0.82	1.00	**−0.26**	**[−0.47;-0.05]**	**0.02**	**0.10**
	Change at week 12 (non-resp.)	0.18	[−0.20;0.57]	0.34	1.00	−0.10	[−0.33;0.12]	0.36	1.00
CpG2	Difference in change at week 8 (resp. vs non-resp.)	0.24	[−0.16;0.65]	0.23	0.92	0.23	[−0.02;0.48]	0.08	0.46
	Difference in change at week 12 (resp. vs non-resp.)	0.17	[−0.28;0.63]	0.45	1.00	0.01	[−0.27;0.29]	0.94	1.00
	Change at week 8 (non-resp.)	−0.16	[−0.42;0.10]	0.24	0.94	−0.14	[−0.34;0.06]	0.18	1.00
	Change at week 12 (non-resp.)	0.00	[−0.29;0.29]	0.98	1.00	0.05	[−0.17;0.27]	0.63	1.00
CpG3	Difference in change at week 8 (resp. vs non-resp.)	0.25	[−0.04;0.55]	0.09	0.37	0.07	[−0.16;0.30]	0.55	1.00
	Difference in change at week 12 (resp. vs non-resp.)	0.03	[−0.32;0.39]	0.85	1.00	−0.08	[−0.35;0.19]	0.54	1.00
	Change at week 8 (non-resp.)	−0.07	[−0.37;0.24]	0.66	1.00	−0.11	[−0.29;0.07]	0.22	1.00
	Change at week 12 (non-resp.)	−0.08	[−0.39;0.22]	0.60	1.00	−0.01	[−0.20;0.19]	0.93	1.00
CpG4	Difference in change at week 8 (resp. vs non-resp.)	0.10	[−0.28;0.47]	0.60	1.00	0.22	[0.00;0.44]	0.05	0.31
	Difference in change at week 12 (resp. vs non-resp.)	0.13	[−0.24;0.49]	0.50	1.00	−0.01	[−0.26;0.23]	0.92	1.00
	Change at week 8 (non-resp.)	–	–	–	–	−0.03	[−0.27;0.21]	0.80	1.00
	Change at week 12 (non-resp.)	–	–	–	–	0.10	[−0.24;0.43]	0.57	1.00
CpG5	Difference in change at week 8 (resp. vs non-resp.)	–	–	–	–	−0.06	[−0.27;0.14]	0.53	1.00
	Difference in change at week 12 (resp. vs non-resp.)	–	–	–	–	−0.10	[−0.37;0.18]	0.48	1.00
	Change at week 8 (non-resp.)	–	–	–	–	−0.05	[−0.28;0.17]	0.65	1.00
	Change at week 12 (non-resp.)	–	–	–	–	−0.09	[−0.30;0.12]	0.38	1.00
CpG6	Difference in change at week 8 (resp. vs non-resp.)	–	–	–	–	0.13	[−0.15;0.41]	0.36	1.00
	Difference in change at week 12 (resp. vs non-resp.)	–	–	–	–	−0.03	[−0.30;0.23]	0.80	1.00

[−9.97; 14.09]). We compared our 95 % CI with those reported by Schiele et al. (2021) and we observed no overlap with clinical response (Schiele et al., 2021): [1.009–1.191]) nor with clinical remission (Schiele et al., 2021): [1.022–1.205]). Confidence intervals were not available for associations with change in depressive symptoms and were not reported in Domschke et al. (2014).

Pearsons's correlations based on the one used by Kang and colleagues (Kang et al., 2013), showed no statistically significant association with $\Delta HAMD_{17}$ after 12 weeks ($r = 0.18$; p-value = 0.11; 95 % CI: [−0.044; 0.390]). However, both direction and correlation coefficients were in line with those found by Kang et al. (2013): $r = 0.19$, p-value<0.05, although the 95 % CI was not reported, so we could not directly compare them with ours.

4. Discussion

We found that higher pre-treatment *TPH2* methylation was associated with better clinical outcomes after 8 weeks of antidepressant treatment in MDD patients. We found no association between *SLC6A4* methylation and clinical outcomes after 8 week of treatment and partially replicated the previously reported (Kang et al., 2013) association between baseline *SLC6A4* CpG2 hypermethylation and lower depressive symptoms after 12 weeks of treatment. However, we observed no evidence of predictive value of neither *TPH2* nor *SLC6A4* methylation. We also observed a trend for an increase in *SLC6A4* CpG2 methylation at week 12 and for a change in *TPH2* CpG1, CpG2 and CpG4 methylation over the study.

Our findings on *TPH2* methylation differ from earlier studies. Shen et al. (2020) reported that hypermethylation at two *TPH2* CpG sites was linked to poor clinical outcomes after 2 weeks of antidepressant treatment, contrary to our results. Notably, their larger cohort ($N = 291$) (Shen et al., 2020) measured clinical response after just two weeks, which is shorter than typical assessments (Leon, 2001; Trivedi et al., 2006). Additionally, Shen et al. observed that *TPH2* hypomethylation was associated with early-life stress, and a sex effect on *TPH2* methylation, which we did not observe (Bruzzone et al., 2024). These differences might be due to inherent population characteristics, such as stress history or sample size.

Greater *TPH2* methylation is linked with lower *TPH2* expression (Zhang et al., 2015), which might correspond to low serotonin brain levels at baseline. Thus, patients with low baseline brain serotonin levels might benefit the most by antidepressant treatment, which increase serotonin neurotransmission. Nonetheless, peripheral *TPH2* methylation might not reflect brain *TPH2* expression or protein levels. Previously, we found no evidence that blood-derived *TPH2* methylation is associated with brain binding of key markers of serotonergic neurotransmission (Bruzzone et al., 2024). Similarly, there may not be a direct relation between *TPH2* peripheral methylation and TPH2 levels in the brain. Nevertheless, peripheral *TPH2* methylation levels might reflect adaptive modifications established early in life, which might have been relevant for brain serotonin tone during development, contributing to changes in e.g. brain wiring or brain structure which might affect clinical outcomes in adult life. Importantly, although we found that depressive symptoms decreased by 4.5 % for each 0.1 increase of $TPH2_{LV}$, our ROC curves suggest that *TPH2* methylation per se cannot predict clinical response to antidepressant treatment. Indeed, while the AUC values suggested that methylation of both *SLC6A4* and *TPH2* might be moderately predictive of clinical outcome, with values comparable to those reported by other large-scale studies for biomarkers of antidepressant treatment outcome (Poirot et al., 2024), the confidence intervals did not support it. Thus, we cannot conclude that *SLC6A4/TPH2* methylation predicts clinical outcome.

In contrast to *TPH2*, we found no association between *SLC6A4* methylation and clinical outcomes. Likewise, when attempting to reproduce earlier findings related to CpG1 and CpG2, we found no association with treatment outcomes after 8 weeks of treatment (Domschke et al., 2014; Schiele et al., 2021), hinting that previous findings on *SLC6A4* should be considered carefully.

Nonetheless, we found a trend for an association between *SLC6A4* CpG2 and change in depressive symptoms after 12 weeks similarly to what reported by Kang et al. (Kang et al., 2013), which hints that *SLC6A4* methylation might be most informative of long-term relative to immediate treatment outcomes. Although our result did not reach statistical significance, we found a very similar effect size and direction, suggesting that our findings are compatible with those previously reported. Notably, if *SLC6A4* methylation corresponds to greater *SLC6A4* expression, it would reflect lower baseline serotonin levels, in line with our *TPH2* findings. Interestingly, when accounting for plasma drug concentration at week 8, we found a trend for an increase of *SLC6A4* CpG2 methylation after 12 weeks of treatment, as well as a trend for changes in *TPH2* CpG1, CpG2 and CpG4 methylation but only before correcting for multiple comparisons. Though the effects were small, *SLC6A4/TPH2* methylation might be informative of mechanisms underlying antidepressant treatment. Clinical studies previously reported changes in *SLC6A4* methylation over antidepressant treatment although not at CpG2 (Moon et al., 2023). In addition, preclinical findings suggest that long-term antidepressant treatment increases *TPH2* expression (Shishkina et al., 2007), pointing to a potential role for changes in *TPH2* expression in treatment mechanisms and supporting our findings.

Our study design shares several characteristics with the previous studies examining *SLC6A4* methylation and antidepressant treatment (Domschke et al., 2014; Kang et al., 2013; Moon et al., 2023; Schiele et al., 2021): first, it is based on an open-label, longitudinal study lasting several weeks; second, previously unmedicated patients were treated with antidepressants and clinical outcomes evaluated; third, *SLC6A4* methylation levels were evaluated at overlapping CpG sites and with the same method (bisulfite conversion and pyrosequencing) and genetic variation within *SLC6A4* was included in the analyses.

However, there are also several differences, which might at least partly explain the discrepancies with previous findings relating *SLC6A4* methylation and antidepressant clinical outcomes. First, our dataset included depressive symptom measurements at weeks 8 and 12, while three previous studies evaluated symptoms after six weeks (Domschke et al., 2014; Moon et al., 2023; Schiele et al., 2021), when the full effect of treatment might not yet have been successful for all patients; only the study by Kang et al. (Kang et al., 2013) evaluated symptoms after 12 weeks. Second, we measured depressive symptoms with the $HAMD_6$ or $HAMD_{17}$, whereas previous studies used $HAMD_{21}$ and only one (Moon et al., 2023) used $HAMD_{17}$. Since $HAMD_{21}$ can capture symptoms from more severe depressed state than $HAMD_{17}$ (i.e. psychotic-like features and diurnal variation), the profiles of depressed states across studies may not be directly comparable. In addition, the population included in the previous studies presented an older age ((Domschke et al., 2014): 47.4 ± 1.7; (Kang et al., 2013): 54.9 ± 14.9; (Moon et al., 2023): 64; (Schiele et al., 2021): 48.26 ± 15.90) compared to ours (Mean age: 26.7 ± 7.7), higher depression chronicity, and, except for one study (Kang et al., 2013), had multiple psychiatric comorbidities and was taking multiple medications. In contrast, participants in our study had mostly first depressive episodes without comorbidities or co-medications. In addition, all previous studies included participants that were treated with a variety of different antidepressant medications (SSRI, SNRI, tricyclic antidepressants…) (Domschke et al., 2014) or at least a variety of different SSRIs (Kang et al., 2013; Moon et al., 2023; Schiele et al., 2021), while in our study 83/90 patients were treated only with escitalopram. Different antidepressant medications and even different SSRI types can have different pharmacological actions (Nemeroff and Owens, 2004; Slattery et al., 2004), with each medication possibly requiring different markers to predict treatment efficacy or having varying effects on DNA methylation. Our study is also based on a smaller sample size compared to that of all previous studies considered ($N = 89$ vs $N = 108–221$), so limited power might partly explain the discrepancies with findings from Schiele et al. (2021) asnd Domschke et al. (2014). Finally, ancestry could affect both genetic and epigenetic variation (Carja et al., 2017). Our study and half of the previous studies (Domschke et al., 2014; Schiele et al., 2021) was based on a population of European ancestry, while the other half (Kang et al., 2013; Moon et al., 2023) was based on a population of Asian ancestry. Nonetheless, our findings are more in line with those by Kang et al. (2013), suggesting that ancestry-related variation might not be relevant in this specific context.

Main limitations of this study include: 1) limited sample size; cross-validating our findings in a larger cohort might provide stronger predictive power; 2) a small number of CpG sites, while many more genes and CpG sites are likely to be involved in treatment resistance or efficacy and 3) lack of a placebo control, which would allow to infer causation on escitalopram treatment response.

In conclusion, although they might be informative of SSRI treatment efficacy and possible mechanisms, *SLC6A4* and *TPH2* are unlikely to be implemented in the clinical setting. Future studies in larger samples and longitudinal study designs and epigenome-wide analyses are needed to confirm our findings and clarify to what extent, if at all, *TPH2* methylation profiles are relevant for treatment mechanisms and clinical care in patients with MDD. In addition, future research should also focus on identifying biomarkers that could inform treatment choices also for other antidepressant medications. Finally, we suggest that combining biomarkers from other realms e.g. genetic, proteomic, psychometric data and combine them with epigenetic data is key to develop more comprehensive and possibly more powerful predictive models.

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Author contributions

SEPB conceptualized the study, collected DNA methylation and genotyping data, analyzed the data, wrote the original draft, designed and executed figures and tables, edited and reviewed the manuscript. BO supported conceptualization, statistical analyses, manuscript editing and reviewing. PMF, GMK and KPL helped with conceptualization, data analysis, manuscript editing and reviewing. GO supported DNA methylation and genotyping data collection. MBJ and VGF recruited and provided clinical care for MDD patients, edited and reviewed the manuscript. VGF also conceptualized and supervised the study.

Ethical statement

This study was carried out in compliance with the Declaration of Helsinki. The study was approved by the Danish Data Protection Agency, the Health Research Ethics Committee of the Capital Region of Denmark, and Danish Medicines Agency (H-15017713 and H-KF-2006-20).

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CRediT authorship contribution statement

Silvia Elisabetta Portis Bruzzone: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Brice Ozenne:** Writing – review & editing, Software, Methodology, Formal analysis, Conceptualization. **Patrick MacDonald Fisher:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Gabriela Ortega:** Writing – review & editing, Methodology. **Martin Balslev Jørgensen:** Data curation, Investigation,

Resources. **Gitte Moos Knudsen:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition. **Klaus-Peter Lesch:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Vibe Gedsoe Frokjaer:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

VGF discloses that she has received honorarium for lectures from SAGE Therapeutics, Lundbeck Pharma A/S, Janssen Cilag A/S, Gedeon-Richter A/S, and Ferring A/S. The other authors have nothing to disclose.

Data availability

Data can be made available upon reasonable request via the form available at: https://cimbi.dk/index.php/documents/category/3-cimbi-database and with an appropriate data sharing agreement. R codes used for statistical analyses can be made available upon request to the corresponding author (vibe.frokjaer@nru.dk).

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Supplementary Materials III

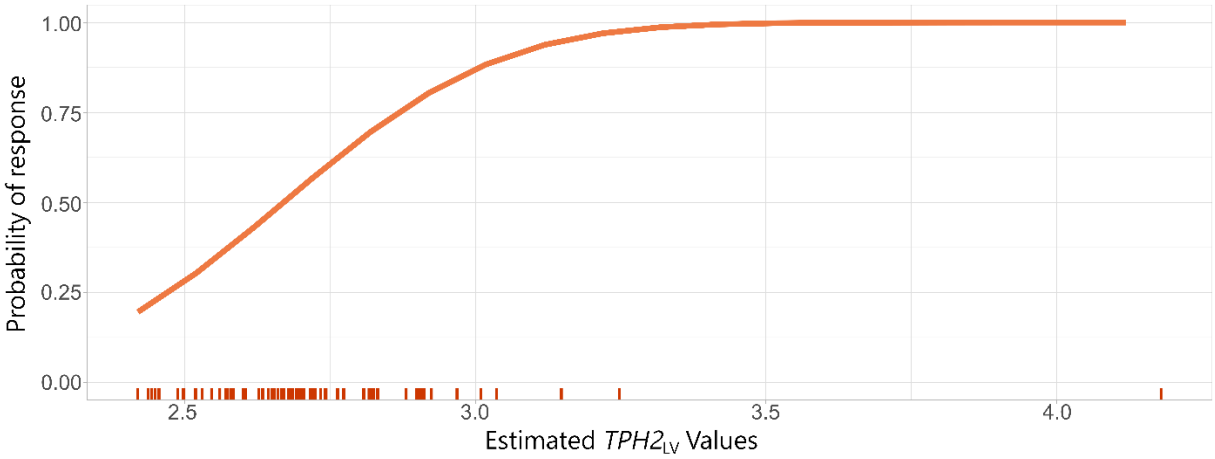

Figure S1. Probit distribution of the latent variable including *TPH2* methylation ($TPH2_{LV}$).This corresponds to e.g. a probability of response of 25% for $TPH2_{LV}$ values of about 2.5 or a probability of response of 50% for $TPH2_{LV}$ values of about 2.7. The rug plot on the axis represents the estimated $TPH2_{LV}$ values e.g. each bar corresponds to $TPH2_{LV}$ of a subject.

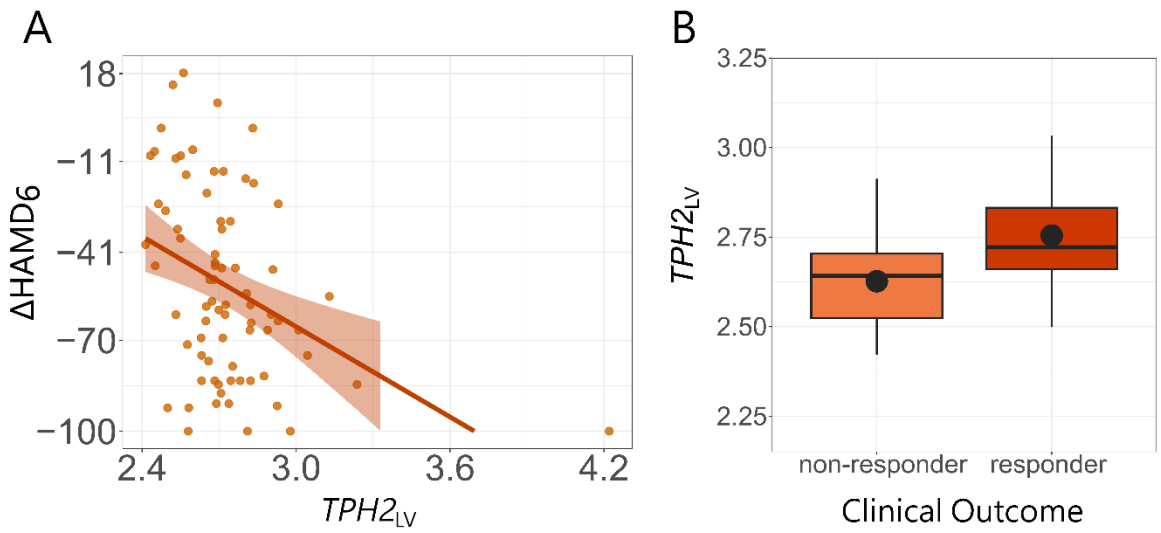

Figure S2. Association between the estimated latent variables of *TPH2* methylation and change in depressive symptoms (A) or clinical outcome after 8 weeks (B).

	SLC6A4			*TPH2*		
Covariates	β	**p**	**95%CI**	β	**p**	**95%CI**
Lymphocytes	0.016	0.010	[0.004;0.03]	0.015	0.001	[0.01;0.02]
Monocytes	0.006	0.784	[-0.04;0.05]	-0.004	0.726	[-0.03;0.02]
Granulocytes	-0.005	0.982	[-0.47;0.46]	-0.180	0.177	[-0.44;0.08]
Eosinophils	-0.008	0.740	[-0.06;0.04]	-0.014	0.344	[-0.04;0.01]
Basophils	-0.064	0.539	[-0.27;0.14]	0.001	0.988	[-0.10;0.10]
Age	0.008	0.214	[0.004;0.02]	0.009	0.022	[0.001;0.02]
$HAMD_{17}$ (Baseline)	-0.0001	0.993	[-0.03;0.03]	-0.007	0.369	[-0.02;0.01]
Sex (male)	0.113	0.263	[-0.08;0.31]	0.083	0.141	[-0.03;0.19]
SLC6A4 5HTTLPR/rs25531 (L_AL_A/SX)	-0.076	0.460	[-0.28;0.13]	-	-	
TPH2 rs4570625 (TX)	-	-	-	-0.014	0.793	-0.12;0.09

Table S1. Primary Analyses: association between pre-treatment *SLC6A4/TPH2* methylation and clinical outcome or ΔHAMD6. Covariate effects on $SLC6A4_{LV}$/$TPH2_{LV}$.

	***SLC6A4*$_{LV}$**		***TPH2*$_{LV}$**	
	Bootstrapped 95% CI	**Model-based 95% CI**	**Bootstrapped 95% CI**	**Model-based 95% CI**
Treatment Outcome	[-0.11; 0.41]	[-0.40; 1.77]	[1.46; 8.08]	[0.80; 6.06]
ΔHAMD$_6$	[-0.18; 0.39]	[-23.45; 24.00]	[-125.03; -31.59]	[-78.58; -12.30]

Table S2. Primary Analyses: association between pre-treatment *SLC6A4/TPH2* methylation and clinical outcome or ΔHAMD6. 95% CI from non-parametric bootstrapping procedures and model-based 95% CI from each LVM.

		SLC6A4				*TPH2*			
CpG site	**Covariates**	β	**95% CI**	**p**	p_{adj}	β	**95% CI**	**p**	p_{adj}
CpG1	**Age**	0.005	[-0.01;0.02]	0.40	1.00	0.003	[-0.01;0.02]	0.68	1.00
	Sex (male)	-0.013	[-0.21;0.18]	0.90	1.00	0.186	[-0.03;0.40]	0.09	0.54
	Lymphocytes	0.005	[-0.01;0.02]	0.34	1.00	0.003	[-0.01;0.01]	0.56	1.00
	Monocytes	-0.019	[-0.06;0.02]	0.37	1.00	-0.016	[-0.06;0.02]	0.43	1.00
	Granulocytes	-0.204	[-0.57;0.17]	0.28	1.00	-0.092	[-0.45;0.27]	0.61	1.00
	Eosinophils	-0.058	[-0.10;-0.01]	0.01	0.04	0.016	[-0.03;0.06]	0.48	1.00
	Basophils	0.015	[-0.14;0.17]	0.85	1.00	0.110	[-0.04;0.26]	0.14	0.86
	5-HTTLPR/rs25531 (sx)	-0.113	[-0.31;0.08]	0.25	1.00	-	-	-	-
	***TPH2* rs4570625 (Tx)**	-	-	-	-	-0.077	[-0.28;0.12]	0.44	1.00
CpG2	**Age**	-0.008	[-0.03;0.01]	0.37	1.00	0.006	[-0.01;0.03]	0.57	1.00
	Sex (male)	0.054	[-0.23;0.34]	0.71	1.00	0.118	[-0.20;0.44]	0.46	1.00
	Lymphocytes	-0.006	[-0.02;0.01]	0.45	1.00	0.014	[0.00;0.03]	0.03	0.20
	Monocytes	-0.014	[-0.07;0.05]	0.64	1.00	0.023	[-0.02;0.07]	0.34	1.00
	Granulocytes	0.130	[-0.40;0.66]	0.63	1.00	0.369	[-0.02;0.75]	0.06	0.36
	Eosinophils	-0.009	[-0.07;0.05]	0.78	1.00	0.022	[-0.03;0.07]	0.38	1.00
	Basophils	0.061	[-0.16;0.29]	0.59	1.00	-0.087	[-0.26;0.08]	0.31	1.00
	5-HTTLPR/rs25531 (sx)	-0.014	[-0.30;0.28]	0.93	1.00	-	-	-	-
	***TPH2* rs4570625 (Tx)**	-	-	-	-	-0.133	[-0.43;0.16]	0.37	1.00
CpG3	**Age**	0.000	[-0.01;0.01]	0.97	1.00	0.013	[0.00;0.03]	0.14	0.84
	Sex (male)	0.198	[-0.03;0.42]	0.09	0.34	0.247	[-0.02;0.52]	0.07	0.44
	Lymphocytes	0.013	[0.00;0.03]	0.03	0.14	0.018	[0.01;0.03]	1.72×10^{-3}	0.01
	Monocytes	-0.0002	[-0.05;0.05]	0.99	1.00	-0.003	[-0.05;0.04]	0.88	1.00
	Granulocytes	-0.102	[-0.50;0.30]	0.62	1.00	0.239	[-0.11;0.59]	0.18	1.00
	Eosinophils	-0.020	[-0.07;0.03]	0.41	1.00	0.013	[-0.03;0.06]	0.57	1.00
	Basophils	0.091	[-0.08;0.27]	0.31	1.00	0.087	[-0.07;0.24]	0.27	1.00
	5-HTTLPR/rs25531 (sx)	-0.168	[-0.40;0.06]	0.15	0.60	-	-	-	-
	***TPH2* rs4570625 (Tx)**	-	-	-	-	-0.145	[-0.39;0.10]	0.24	1.00
CpG4	**Age**	0.004	[-0.01;0.02]	0.65	1.00	0.016	[0.00;0.03]	0.05	0.30
	Sex (male)	0.091	[-0.21;0.40]	0.55	1.00	0.135	[-0.13;0.40]	0.31	1.00
	Lymphocytes	0.023	[0.01;0.04]	0.003	0.01	0.021	[0.01;0.03]	1.39×10^{-4}	8.33×10^{-3}
	Monocytes	-0.043	[-0.10;0.02]	0.15	0.59	-0.015	[-0.06;0.02]	0.46	1.00
	Granulocytes	0.199	[-0.30;0.70]	0.43	1.00	0.085	[-0.24;0.41]	0.60	1.00
	Eosinophils	-0.012	[-0.07;0.05]	0.69	1.00	-0.001	[-0.04;0.04]	0.95	1.00

	Basophils	0.018	[-0.20;0.24]	0.87	1.00	-0.024	[-0.17;0.12]	0.75	1.00
	5-HTTLPR/rs25531 (sx)	-0.023	[-0.33;0.29]	0.88	1.00	-	-	-	-
	***TPH2* rs4570625 (Tx)**	-	-	-	-	-0.148	[-0.39;0.09]	0.22	1.00
CpG5	**Age**	-	-	-	-	0.033	[0.01;0.05]	$1.17x10^{-3}$	0.01
	Sex (male)	-	-	-	-	0.066	[-0.27;0.40]	0.69	1.00
	Lymphocytes	-	-	-	-	0.040	[0.03;0.05]	$5.71x10^{-9}$	$3.43x10^{-8}$
	Monocytes	-	-	-	-	-0.005	[-0.05;0.05]	0.86	1.00
	Granulocytes	-	-	-	-	0.038	[-0.35;0.42]	0.84	1.00
	Eosinophils	-	-	-	-	-0.031	[-0.08;0.02]	0.24	1.00
	Basophils	-	-	-	-	-0.012	[-0.19;0.17]	0.90	1.00
	5-HTTLPR/rs25531 (sx)	-	-	-	-	-	-	-	-
	***TPH2* rs4570625 (Tx)**	-	-	-	-	-0.142	[-0.44;0.15]	0.34	1.00
CpG6	**Age**	-	-	-	-	0.025	[0.01;0.04]	$4.54x10^{-4}$	$2.72x10^{-3}$
	Sex (male)	-	-	-	-	0.246	[0.03;0.46]	0.03	0.17
	Lymphocytes	-	-	-	-	0.018	[0.01;0.03]	$1.10x10^{-3}$	0.01
	Monocytes	-	-	-	-	0.013	[-0.03;0.05]	0.51	1.00
	Granulocytes	-	-	-	-	-0.179	[-0.54;0.19]	0.33	1.00
	Eosinophils	-	-	-	-	0.008	[-0.03;0.05]	0.72	1.00
	Basophils	-	-	-	-	0.031	[-0.12;0.18]	0.69	1.00
	5-HTTLPR/rs25531 (sx)	-	-	-	-	-	-	-	-
	***TPH2* rs4570625 (Tx)**	-	-	-	-	-0.148	[-0.35;0.06]	0.16	0.95

Table S3. Primary Analyses: changes in DNA methylation over time (responder vs non-responder interaction). Covariate effects from linear mixed models evaluating changes in *SLC6A4/TPH2* methylation status over 12 weeks of treatment by accounting for an interaction with responder status (Responder status*Week 8/Week 12).

		SLC6A4				*TPH2*			
CpG site	**Covariates**	***β***	**95% CI**	**p**	**p_{adj}**	***β***	**95% CI**	**p**	**p_{adj}**
CpG1	Change at week 8	0.197	[-0.02;0.41]	0.07	0.28	0.056	[-0.08;0.19]	0.41	1.00
	Change at week 12	0.115	[-0.06;0.29]	0.20	0.79	0.126	[-0.08;0.33]	0.23	1.00
	Escitalopram plasma conc.	-0.018	[-0.04;0.01]	0.13	0.53	**0.027**	**[0.00;0.05]**	**0.02**	**0.11**
	Age	0.005	[-0.01;0.02]	0.40	1.00	0.004	[-0.01;0.02]	0.51	1.00
	Sex (Male)	-0.023	[-0.23;0.18]	0.83	1.00	0.240	[0.03;0.45]	0.03	0.17
	Lymphocytes	0.009	[0.00;0.02]	0.17	0.66	-0.002	[-0.01;0.01]	0.76	1.00
	Monocytes	-0.016	[-0.06;0.03]	0.47	1.00	-0.015	[-0.06;0.03]	0.47	1.00
	Granulocytes	-0.161	[-0.54;0.22]	0.41	1.00	-0.140	[-0.49;0.21]	0.43	1.00
	Eosinophils	-0.049	[-0.11;0.01]	0.09	0.37	0.010	[-0.04;0.06]	0.71	1.00
	Basophils	0.000	[-0.17;0.17]	1.00	1.00	0.131	[-0.02;0.29]	0.10	0.58
	5-HTTLPR/rs25531 (sx)	-0.097	[-0.30;0.10]	0.33	1.00	-	-	-	-
	TPH2 rs4570625 (Tx)	-	-	-	-	-0.118	[-0.32;0.08]	0.24	1.00
CpG2	Change at week 8	0.204	[-0.06;0.46]	0.12	0.49	-0.126	[-0.29;0.04]	0.13	0.80
	Change at week 12	**0.294**	**[0.02;0.56]**	**0.03**	**0.13**	**-0.139**	**[-0.28;0.00]**	**0.05**	**0.32**
	Escitalopram plasma conc.	-0.015	[-0.05;0.02]	0.41	1.00	0.010	[-0.01;0.03]	0.41	1.00
	Age	-0.009	[-0.03;0.01]	0.34	1.00	0.011	[-0.01;0.03]	0.28	1.00
	Sex (Male)	0.091	[-0.21;0.39]	0.55	1.00	0.208	[-0.11;0.53]	0.20	1.00
	Lymphocytes	-0.007	[-0.02;0.01]	0.38	1.00	0.013	[0.00;0.03]	0.06	0.34
	Monocytes	-0.016	[-0.08;0.05]	0.62	1.00	0.026	[-0.02;0.07]	0.27	1.00
	Granulocytes	0.138	[-0.40;0.68]	0.61	1.00	0.348	[-0.03;0.73]	0.07	0.43
	Eosinophils	0.008	[-0.07;0.09]	0.85	1.00	0.033	[-0.03;0.09]	0.28	1.00
	Basophils	0.013	[-0.23;0.26]	0.92	1.00	-0.097	[-0.27;0.07]	0.26	1.00
	5-HTTLPR/rs25531 (sx)	0.012	[-0.29;0.31]	0.94	1.00	-	-	-	-
	TPH2 rs4570625 (Tx)	-	-	-	-	-0.188	[-0.49;0.11]	0.21	1.00
CpG3	Change at week 8	-0.013	[-0.23;0.20]	0.90	1.00	-0.083	[-0.24;0.08]	0.30	1.00
	Change at week 12	0.036	[-0.17;0.24]	0.72	1.00	-0.003	[-0.15;0.15]	0.97	1.00
	Escitalopram plasma conc.	-0.005	[-0.04;0.03]	0.76	1.00	0.004	[-0.02;0.03]	0.70	1.00
	Age	0.001	[-0.01;0.02]	0.85	1.00	0.013	[0.00;0.03]	0.14	0.86
	Sex (Male)	0.220	[-0.02;0.47]	0.08	0.31	0.296	[0.01;0.58]	0.04	0.24
	Lymphocytes	0.012	[0.00;0.02]	0.08	0.34	0.018	[0.01;0.03]	0.01	0.04

	Monocytes	0.000	[-0.05;0.05]	1.00	1.00	-0.003	[-0.05;0.04]	0.91	1.00
	Granulocytes	-0.129	[-0.55;0.29]	0.55	1.00	0.262	[-0.10;0.62]	0.15	0.90
	Eosinophils	-0.006	[-0.07;0.06]	0.85	1.00	0.002	[-0.06;0.06]	0.95	1.00
	Basophils	0.058	[-0.14;0.26]	0.56	1.00	0.081	[-0.09;0.25]	0.34	1.00
	5-HTTLPR/rs25531 (sx)	-0.158	[-0.40;0.09]	0.21	0.82	-	-	-	-
	TPH2 rs4570625 (Tx)	-	-	-	-	-0.153	[-0.41;0.11]	0.25	1.00
CpG4	Change at week 8	-0.013	[-0.23;0.20]	0.90	1.00	0.028	[-0.11;0.16]	0.68	1.00
	Change at week 12	0.036	[-0.17;0.24]	0.72	1.00	-0.019	[-0.16;0.12]	0.78	1.00
	Escitalopram plasma conc.	-0.005	[-0.04;0.03]	0.76	1.00	**0.024**	**[0.00;0.05]**	**0.03**	**0.17**
	Age	0.001	[-0.01;0.02]	0.85	1.00	0.019	[0.00;0.03]	0.03	0.16
	Sex (Male)	0.220	[-0.02;0.47]	0.08	0.31	0.164	[-0.11;0.44]	0.24	1.00
	Lymphocytes	0.012	[0.00;0.02]	0.08	0.34	0.018	[0.01;0.03]	0.002	0.01
	Monocytes	0.000	[-0.05;0.05]	1.00	1.00	-0.017	[-0.06;0.02]	0.41	1.00
	Granulocytes	-0.129	[-0.55;0.29]	0.55	1.00	0.049	[-0.28;0.37]	0.77	1.00
	Eosinophils	-0.006	[-0.07;0.06]	0.85	1.00	-0.019	[-0.07;0.04]	0.49	1.00
	Basophils	0.058	[-0.14;0.26]	0.56	1.00	-0.007	[-0.16;0.15]	0.93	1.00
	5-HTTLPR/rs25531 (sx)	-0.158	[-0.40;0.09]	0.21	0.82	-	-	-	-
	TPH2 rs4570625 (Tx)	-	-	-	-	-0.165	[-0.41;0.08]	0.18	1.00
CpG5	Change at week 8	-	-	-	-	-0.063	[-0.22;0.09]	0.43	1.00
	Change at week 12	-	-	-	-	-0.052	[-0.23;0.12]	0.56	1.00
	Escitalopram plasma conc.	-	-	-	-	0.008	[-0.01;0.03]	0.47	1.00
	Age	-	-	-	-	0.034	[0.01;0.05]	0.001	0.01
	Sex (Male)	-	-	-	-	0.065	[-0.28;0.41]	0.71	1.00
	Lymphocytes	-	-	-	-	0.041	[0.03;0.05]	0.00	0.00
	Monocytes	-	-	-	-	-0.003	[-0.05;0.05]	0.91	1.00
	Granulocytes	-	-	-	-	0.069	[-0.32;0.46]	0.73	1.00
	Eosinophils	-	-	-	-	-0.049	[-0.11;0.01]	0.13	0.77
	Basophils	-	-	-	-	0.002	[-0.18;0.18]	0.98	1.00
	5-HTTLPR/rs25531 (sx)	-	-	-	-	-	-	-	-
	TPH2 rs4570625 (Tx)	-	-	-	-	-0.137	[-0.44;0.17]	0.37	1.00
CpG6	Change at week 8	-	-	-	-	0.041	[-0.13;0.21]	0.64	1.00
	Change at week 12	-	-	-	-	-0.068	[-0.19;0.06]	0.28	1.00

	Escitalopram plasma conc.	-	-	-	-	0.010	[-0.01;0.03]	0.40	1.00
	Age	-	-	-	-	0.025	[0.01;0.04]	0.001	0.004
	Sex (Male)	-	-	-	-	0.257	[0.02;0.49]	0.03	0.19
	Lymphocytes	-	-	-	-	0.015	[0.00;0.03]	0.01	0.05
	Monocytes	-	-	-	-	0.018	[-0.02;0.06]	0.35	1.00
	Granulocytes	-	-	-	-	-0.194	[-0.56;0.17]	0.29	1.00
	Eosinophils	-	-	-	-	-0.014	[-0.07;0.04]	0.60	1.00
	Basophils	-	-	-	-	0.030	[-0.12;0.18]	0.70	1.00
	5-HTTLPR/rs25531 (sx)	-	-	-	-	-	-	-	-
	TPH2 rs4570625 (Tx)	-	-	-	-	-0.140	[-0.36;0.08]	0.20	1.00

Table S4. Primary Analyses: changes in DNA methylation over time (plasma escitalopram concentration). Results from linear mixed models evaluating changes in *SLC6A4/TPH2* methylation status over 12 weeks of treatment by accounting for Escitalopram plasma concentration measured at week 8.

Gene	Categorical Treatment Outcome				$\Delta HAMD_6$			
SLC6A4	β	**p**	p_{adj}	**95% CI**	β	**p**		**95% CI**
CpG1	0.06	0.90	1.00	[-0.86; 0.98]	7.95	0.23	1.00	[-5.21; 21.11]
CpG2	-0.17	0.62	1.00	[-0.85; 0.51]	3.51	0.48	1.00	[-6.29; 13.30]
CpG3	0.51	0.23	0.95	[-0.29; 1.36]	-4.86	0.40	1.00	[-16.20; 6.49]
CpG4	0.29	0.38	1.00	[-0.35; 0.95]	1.35	0.77	1.00	[-7.75; 10.46]
TPH2	β	**p**		**95% CI**	β	**p**		**95% CI**
CpG1	0.67	0.20	1.00	[-0.31; 1.76]	-12.93	0.07	0.47	[-26.77; 0.92]
CpG2	0.81	0.10	0.50	[-0.05; 1.81]	-9.48	0.03	0.28	[-18.02; -0.93]
CpG3	0.98	0.04	0.26	[0.11; 2.00]	-11.45	0.01	0.08	[-20.21; -2.65]
CpG4	1.17	0.04	0.19	[0.15: 2.34]	-12.84	0.02	0.12	[-23.77 -1.91]
CpG5	1.22	0.01	0.07	[0.36; 2.22]	-9.85	0.01	0.08	[-17.28; -2.41]
CpG6	1.61	0.01	0.08	[0.44; 2.98]	-14.10	0.03	0.06	[-26.85; -1.35]

Table S5. Secondary Analyses: single-CpG analyses. Results from multiple linear regressions and logistic regressions evaluating the association between *SLC6A4* and *TPH2* methylation and categorical or continuous outcomes.

STUDY IV

DNA methylation of serotonin-related genes in depression and depression chronicity: a large-scale replication study

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Abstract

DNA methylation of serotonin genes, especially the serotonin transporter (*SLC6A4*) and the tryptophan hydroxylase 2 (*TPH2*) gene has been repeatedly linked to early life stress and depression as well as antidepressant treatment outcomes. However, findings are mixed and previous studies present various technical limitations, such as no correction for blood cell proportions and small sample sizes. In this study, we aimed to replicate previous findings linking *SLC6A4* and *TPH2* to childhood trauma and to depression status in four large datasets (N_{tot}=16,341) from methylome-wide analysis studies (MWAS), including three datasets based on blood-derived DNA methylation and one dataset on post-mortem brains, using either the untargeted MBD-seq platform or the EPIC Illumina array. Then, we evaluated whether any of the genes relevant for serotonin neurotransmission (e.g. all serotonin receptors, transporters and enzymes involved in serotonin synthesis) were enriched in the associations with depression or childhood trauma. Finally, in one large cohort (N=1132), we investigated whether DNA methylation of 27 centrally relevant serotonin-related genes was linked to depressive symptoms, childhood trauma and depression chronicity in both the full sample and subgroups of patients taking antidepressant medications with serotonergic action.
We did not replicate previous findings linking *SLC6A4*/*TPH2* methylation to childhood trauma or depression. In addition, we found no enrichment of serotonin-related genes in the associations with depression or childhood trauma, depressive symptoms or depression chronicity. Our findings did not confirm that DNA methylation of serotonin-related genes reflect childhood trauma or depression status, suggesting that DNA methylation of serotonin-related genes is unlikely to be used as a marker of these associations of inform on their underlying mechanisms.

Introduction

Major depressive disorder is a highly debilitating and difficult-to-treat disorder, and its incidence is rapidly increasing (World Health Organization, 2017).
The serotonin system is the target of most antidepressant medications (Artigas et al., 2002) and alterations in serotonin function have been linked to major depressive disorders (Dam et al., 2024; Erritzoe et al., 2023; Jauhar et al., 2023; Köhler-Forsberg et al., 2023), although with mixed findings (Kendrick & Collinson, 2022).

Individual vulnerability to depressive symptoms likely arises from a combination of genetic and environmental factors. In this framework, epigenetic mechanisms (e.g. DNA methylation) can regulate gene expression in response to environmental challenges (e.g. early life stress) and have been proposed to play a role in the pathogenesis of depression (Penner-Goeke & Binder, 2019) as well as in antidepressant efficacy (Menke & Binder, 2014).

A large body of literature describes associations between alterations in DNA methylation of serotonin-relevant genes (e.g. serotonin transporter gene [*SLC6A4*], tryptophan hydroxylase 2 gene [*TPH2*], monoamine oxidase a [*MAOA*]) and depression and/or environmental stress (Leibold et al., 2020; Palma-Gudiel & Fañanás, 2017; Provenzi et al., 2016; Shen et al., 2020; Ziegler & Domschke, 2018). DNA methylation levels of some of these genes have also been suggested as predictive biomarkers informing on clinical outcomes after antidepressant treatment (Domschke et al., 2014; Kang et al., 2013; Schiele et al., 2021; Shen et al., 2020a), although not confirmed by all studies (Bruzzone et al., 2025; Olsson et al., 2010; Wannemüller et al., 2024; Xu et al., 2013).

However, former studies present several issues. First, they all used a candidate gene approach, which is more likely to provide false positive findings (Shabalin et al., 2015). Indeed, CpG sites across the genome tend to co-vary, possibly reflecting variation in lifestyle or demographic variables. In some cases, this covariance can be associated with the disease status, inflating the number of false positives. Conducting methylome-wide studies (MWAS), which cover the entire genome or a large portion thereof, allows to capture and account for this covariance, reducing the risk for false positives (Shabalin et al., 2015). Second, most studies have focused on *SLC6A4* DNA methylation, whereas methylation of other genes that are also essential to serotonin neurotransmission (e.g. serotonin receptors, other transporters or enzymes involved in serotonin synthesis and degradation) have been rarely considered, or not at all. Third, almost no study accounted for blood cell proportions, which can critically

influence DNA methylation measurements. Fourth, former studies mostly involved small study cohorts (N<200), which limits statistical power to detect small effects. Fifth, findings relating DNA methylation of serotonin-relevant genes to early life stress or depression have not been confirmed by larger epigenome-wide studies (Chan et al., 2020; Clark et al., 2020a; Starnawska et al., 2021). Sixth, studies evaluating the biomarker potential for antidepressant treatment outcomes have been based on short-term trials (e.g. 6-12 weeks), which do not inform on longer-term outcomes. Seventh, most studies were cross-sectional. Finally, previous findings were with mixed.

Furthermore, *MAOA* is located on the X chromosome. As there is currently no standardized pipeline to analyze DNA methylation on sex chromosomes, interpretation of findings in X-linked genes is difficult (Inkster et al., 2023). Consequently, observations on *MAOA* methylation are ambiguous and difficult to cross-validate.

In this study, we aimed to: 1) replicate previously observed associations between *SLC6A4* and *TPH2* methylation and depression and childhood trauma in four large cohorts; 2) evaluate whether genes that are centrally relevant for serotonin transmission (including all serotonin receptors and their isoforms, all transporters with affinity for serotonin, all enzymes essential for serotonin synthesis and degradation) are enriched in the associations with depression; 3) broaden our focus to all genes that are centrally relevant for serotonin neurotransmission and evaluate their association between DNA methylation and: I) Depressive symptoms; II) Childhood trauma; III) Depression chronicity; IV) Depression chronicity specifically in patients taking antidepressants with a serotonergic action (selective serotonin reuptake inhibitors [SSRIs]; tricyclic antidepressants [TCA]), as an indirect proxy of antidepressant non-response.

To do so, we leveraged data from four methylome-wide association studies (MWAS): two large longitudinal studies in whole blood samples (Costello et al., 2016; Penninx et al., 2021) and two cross-sectional studies, one in whole blood and one in post-mortem brains (Aberg, et al., 2020a).

Methods

Participants

Participants were part of four different cohorts that have been previously described (Aberg, Dean, et al., 2020a; Costello et al., 2016; Penninx et al., 2021). General characteristics are reported in **Table 1**. Briefly, we used data from: the Netherlands Study of Depression and Anxiety (NESDA) (Penninx et al., 2021), consisting of N=1132 participants; the Great Smoky Mountains Study (GSMS) (Costello et al., 2016), including 1034 samples from multiple measurements of N=560 participants from; TD, which includes N=14443 participants who performed the commercial test TruAge test (TruDiagnostic); MDDbrain, which consists of data of post-mortem brains (specifically, Broadmann areas 25 and 10) from N=206 donors (Aberg, Dean, et al., 2020a).

	NESDA	GSMS	TD	MDDbrain
N	1132	560 (1034 blood samples)	14443	206
Age	41.6 (±13.0)	18.1 (±6.1)	53.2 (±12.9)	
Sex (F/M)	731/401	(521/513)	5979/8464	121/85
MDD at baseline (%)	71%	-	9%	55%
Follow-up status (MDD/remitted/HC)	319/500	-	-	-

Table 1. General demographics of the cohorts included in this study.

Outcome measures

Depression status was defined as follows: NESDA: by trained clinicians based on the Composite International Diagnostic Interview (CIDI) (Andrews & Peters, 1998); GSMS: based on the Depression Symptoms counts from the Child and Adolescent Psychiatric Assessment (CAPA/YAPA) (Angold & Jane Costello, 2000); TD: based on a "yes" or "no" answer to the question: "were you ever diagnosed or treated for MDD?"; MDDbrain: based on family reports. Childhood trauma scores were obtained from the Childhood Trauma Inventory (CTI) (De Graaf et al., 2004) for NESDA and from the "Early life events and Posttraumatic stress" sections of the CAPA/YAPA (Angold & Jane Costello, 2000) for cohort 2.

In NESDA, depressive symptoms severity was evaluated with the Inventory of Depressive Symptomatology (IDS) (Rush et al., 1996), while depression chronicity was defined as presence of depression both at baseline and at 2-year follow-up. For subgroup analyses on depression chronicity in patients taking antidepressants with serotonergic action, participants were grouped based on presence of depression at baseline and their use of: a) tricyclic antidepressants (TCA) or selective serotonin reuptake inhibitors (SSRI) at baseline; b) based on SSRI use at baseline.

DNA methylation assay

DNA methylation assays have been previously described in details (Aberg et al., 2012; Clark et al., 2020b). DNA methylation was evaluated using an optimized protocol for Methyl-CG Binding Domain sequencing (MBD-seq) in cohorts 1, 2 and 4 (Aberg et al., 2012; Clark et al., 2020b) and using Illumina Infinium EPIC BeadChip arrays in cohort 3. Shortly, reads obtained from MBD-seq were aligned using Bowtie2 (Langmead & Salzberg, 2012). Then, data were quality-controlled and faulty reads (or probes) and samples were excluded using the RaMWAS package (Guintivano et al., 2020; Shabalin et al., 2018).

Genes selection

We selected genes encoding for all serotonin receptors, serotonin/monoamine transporters and for the enzymes that are essential for serotonin synthesis and degradation. Genes were chosen based on the genes at the serotonergic synapse from the KEGG Atlas (Okuda et al., 2008) and on literature (Daws, 2021; Sharp & Barnes, 2020). We identified 29 genes. However, the genes encoding for the serotonin receptor 2C (*HTR2C*) and monoamine oxidase A (*MAOA*) were excluded from analyses as they are located on the X-chromosome and a validated method to analyze DNA methylation on X or Y chromosomes is currently missing (Inkster et al., 2023). Genomic sequences were extracted from the UCSC Genome Browser (https://genome.ucsc.edu/, assembly GRCh37/hg19). We included an additional 10,000bp sequence upstream of gene start as a putative promoter region. Finally, 13967 CpG sites from 27 genes and their putative promoter regions were included in the statistical analyses (**Table S1** for coordinates of the genomic regions included in the analyses).

Statistical Analyses

MWASs were conducted for all cohorts separately in RaMWAS (Shabalin et al., 2018) and involved multiple linear regression models. Four sets of covariates were used: assay-related variables (e.g. batch, assay type, total number of reads), demographic variables (e.g. age, sex, ethnicity, smoking, alcohol use and BMI), estimated blood cell proportions. Blood cell proportions were estimated for T-cells, B-cells, granulocytes and macrophages in the NESDA and GSMS cohorts. For the TD cohort, blood cell proportions were estimated for neutrophils, basophils, eosinophils, B-memory cells, naïve B cells, memory and naïve T-helper and cytotoxic T-cells, T regulatory cells, natural killer cells and monocytes. Finally, cell types in the MDDbrain involved neurons and glial cells.

Finally, the first principal component obtained from principal component analysis (PCA), which was selected based on a scree test (Aberg, Dean, et al., 2020b), was regressed out to account for the residual variance.

Replication of findings linking SLC6A4 and TPH2 methylation to depression and/or early life stress

We considered a broad set of studies that previously described an association between *SLC6A4* or *TPH2* methylation and depression or different measures of early life stress, which are reported in **Table S3** and **S3**.

Replication approach

We selected MWAS results for the CpG sites within *SLC6A4* and *TPH2* that were previously linked to depression and/or childhood trauma or childhood adversities.

Most of previous literature focused on CpG sites within promoter regions, that are hypomethylated. As hypomethylated probes are filtered out from MBD-seq datasets, and EPIC arrays only cover a fraction (~850,000 CpG sites) of the total CpG sites (~28 million CpG sites), we focused on the CpG sites available in our datasets that were closest to the CpG sites of interest. The CpG sites that were used to replicate previous findings are reported in **Table S2 and S3**. Significance threshold was set to p-value = 0.05, based on previous studies.

Gene-set enrichment of serotonin-related genes

We performed enrichment tests with the R package shiftR (https://cran.r-project.org/web/packages/shiftR/index.html) in each cohort. First, the MWAS CpGs were mapped to genomic locations of the serotonin-related genes, allowing for an additional 10kb region upstream of transcription start site (putative promoter region). Next, the top MWAS p-values (defined as the 0.1%, 0.5%, 1% lowest p-values) were cross-classified against gene status, which was defined as a gene being or not (yes/no) a serotonin-related gene. Cramer's V was used to test the null hypothesis that the enrichment odds ratio equals one. These tests were performed using circular permutations (Cabrera et al., 2012). As multiple thresholds were used to define the top MWAS findings, the same thresholds were used for the permutations. The resulting empirical test statistic distributions under the null hypothesis were generated based on the most significant (combination of) thresholds. More details about this approach have been previously described (Aberg, Dean, et al., 2020b; Chan et al., 2020).

Associations with depressive symptoms, childhood trauma and depression chronicity

As NESDA included more detailed demographics data and a longitudinal design, we also evaluated whether DNA methylation of any of the serotonin-related genes was associated with: I) depressive symptoms (IDS score); II) childhood trauma (CTI score); III) depression chronicity at 2-year follow-up (yes/no depression still present after 2 years). Research question III) was addressed: a) in the full cohort of patients (n=812); b) in patients taking TCA or SSRI at baseline (n=280); c) in patients taking SSRI at baseline (n=244). a) and b) were motivated by the fact that several studies previously linked *SLC6A4*/*TPH2* methylation to clinical outcomes following antidepressant treatment (Bruzzone et al., 2025; Domschke et al., 2014; Iga et al., 2016; Kang et al., 2013; Schiele et al., 2021; Shen et al., 2020b). In this cohort we could not directly address this question. Thus, we evaluated whether participants with depression that were taking antidepressants at baseline were still classified as patients with depression after 2-years, with the assumption that if they still had depression despite pharmacological treatment, it meant that they did not respond to treatment.

For these analyses, instead of using MWAS data, we used raw DNA methylation data (following quality control) and multiple linear regression models for each of the outcomes of interest. Specifically, we first regressed 19 technical covariates out of the data. Then, PCA was performed on the residuals from this model and the top 10 principal components were selected based on a scree plot (**Figure S1**). Main multiple linear regression models included the residualized DNA methylation data as outcome and, as covariates, the 10 top principal components, age, sex, BMI, smoking and cell proportions. False discovery rate (FDR) was used to correct for multiple comparisons. Threshold for statistical significance was set to FDR-corrected p-values (q-values) <0.1.

Results

Replication of findings linking SLC6A4 and TPH2 methylation to depression and/or early life stress

We included 22 studies investigating the association between *SLC6A4/TPH2* methylation and depression or early life stress. Among these, twelve studies reported statistically significant associations between *SLC6A4* (ten studies) or *TPH2* (two studies) and depression (**Table S2**) and ten studies reported positive findings regarding the association between *SLC6A4* (nine studies) or *TPH2* (one study) and childhood trauma (**Table S3**).

Replication of previous findings

We identified twenty-two CpG sites (twelve in *SLC6A4*; ten in *TPH2*) that were previously linked to depression (**Table S2**) and fifteen CpG sites (thirteen in *SLC6A4*; two in *TPH2*) that were linked to childhood trauma or early life stress (**Table S3**). Among the CpG sites associated with depression, only 7/22 CpG sites were present in NESDA and GSMS, 2/22 in TD and 4/22 in MDDbrain. Among CpG sites associated with childhood adversities, 7/15 were present in NESDA and GSMS.

We found no statistically significant association between *SLC6A4* or *TPH2* methylation and depression status or childhood trauma in any of the four cohorts ($p>0.06$), except for a trend in statistical significance for the association between depression and decreased methylation of one CpG site in *SLC6A4* (chr17: 28563424) ($p=0.06$), with the same direction of what previously reported (Mendonça et al., 2019). However, the CpG site was located 249-370 bp away from CpG sites reported by literature (**Table 2, 3**).

Gene	CpG site	NESDA				GSMS				TD				MDDbrain			
		Closest CpG	Min. dist. (bp)	Tstat	p-value	Closest CpG	Min. dist.	Tstat	p-value	Closest CpG	Min. dist.	Tstat	p-value	Closest CpG	Min. dist.	Tstat	p-value
***SLC6A4* (Chr17)**	28562474	28562474	0	0.80	0.42	28562474	0	-0.7	0.48	28562474	0	1	0.32	28562436	38	-1.2	0.24
	28562685	28562685	0	-0.59	0.55	28562685	0	1.41	0.16	28562685	0	-0.9	0.39	28562436	249	-1.2	0.24
	28562813	28562813	0	0.20	0.84	28562813	0	1.09	0.27	28562686	127	-0.9	0.39	28562436	377	-1.2	0.24
	28563054	28562987	67	0.79	0.43	28562915	139	0.54	0.59		368	-0.9	0.39	28563424	370	-1.9	0.06
	28563090		103	0.79	0.43		175	0.54	0.59		404	-0.9	0.39		334	-1.9	0.06
	28563102		115	0.79	0.43		187	0.54	0.59		416	-0.9	0.39		322	-1.9	0.06
	28563107		120	0.79	0.43		192	0.54	0.59		421	-0.9	0.39		317	-1.9	0.06
	28563109		122	0.79	0.43	28563300	191	0.69	0.49		423	-0.9	0.39		315	-1.9	0.06
	28563120		133	0.79	0.43		180	0.69	0.49		434	-0.9	0.39		304	-1.9	0.06
	28563144		157	0.79	0.43		156	0.69	0.49		458	-0.9	0.39		280	-1.9	0.06
	28563160		173	0.79	0.43		140	0.69	0.49		474	-0.9	0.39		264	-1.9	0.06
	28563175		188	0.79	0.43		125	0.69	0.49		489	-0.9	0.39		249	-1.9	0.06
***TPH2* (chr12)**	72335393	72335393	0	-0.50	0.62	72335393	0	0.45	0.66	72335306	87	1.19	0.23	72335393	0	1.1	0.27
	72335435	72335435	0	0.58	0.56	72335435	0	0.45	0.65	72335306	129	1.19	0.23	72335435	0	1.49	0.14
	72348000	72348000	0	1.27	0.20	72348000	0	0.82	0.41	72347053	947	-1.8	0.07	72348000	0	-0.5	0.60
	72372868	72372868	0	-0.58	0.56	72372868	0	-1.2	0.25	72376553	3685	0.41	0.68	72372868	0	-0.2	0.80

Table 2. Results from our replication of the association between SLC6A4/TPH2 methylation and childhood trauma. CpG site: CpG site previously reported to be associated with childhood trauma; Closest CpG: closest CpG site to the previously reported one that was present in the dataset; Min. dist (bp): minimum distance between the CpG sites present in our datasets and the previously reported CpG site in base-pairs (bp); Tstat: test statistics. 0 values within Min. dist. Indicate that the CpG site was present in the dataset.

		NESDA				GSMS			
		Closest CpG	**Min. dist. (bp)**	**Tstat**	**p-value**	**Closest CpG**	**Min. dist.**	**Tstat**	**p-value**
***SLC6A4* (Chr17)**	28562142	28562142	0	-1	0.34	28562142	0	0.14	0.89
	28562220	28562220	0	-0.4	0.71	28562220	0	1.29	0.20
	28562388	28562388	0	0.46	0.65	28562388	0	1.63	0.10
	28562401	28562401	0	0.45	0.66	28562401	0	1.07	0.29
	28562436	28562436	0	0.69	0.49	28562436	0	0.84	0.40
	28562673	28562673	0	-0.3	0.73	28562673	0	1.06	0.29
	28562685	28562685	0	-0.2	0.85	28562685	0	0.85	0.39
	28563090	28562987	103	0.94	0.35	28562915	175	1.20	0.23
	28563102	28562987	115	0.94	0.35	28562915	187	1.20	0.23
	28563109	28562987	122	0.94	0.35	28563300	191	-0.03	0.98
	28563120	28562987	133	0.94	0.35	28563300	180	-0.03	0.98
	28563144	28562987	157	0.94	0.35	28563300	156	-0.03	0.98
	28563160	28562987	173	0.94	0.35	28563300	140	-0.03	0.98
	28563300	28563388	88	-0.9	0.36	28563300	0	-0.03	0.98
***TPH2* (chr12)**	72340051	72339839	212	0.28	0.78	72339841	210	-0.59	0.56
	72400979	72401604	625	0.02	0.98	72401600	621	1.33	0.18

Table 3. Results from our replication of the association between SLC6A4/TPH2 methylation and childhood trauma. CpG site: CpG site previously reported to be associated with childhood trauma; Closest CpG: closest CpG site to the previously reported one that was present in the dataset; Min. dist (bp): minimum distance between the CpG sites present in our datasets and the previously reported CpG site in base-pairs (bp); Tstat: test statistics. 0 values within Min. dist. Indicate that the CpG site was present in the dataset.

Gene-set enrichment of serotonin-related genes

Using gene-set enrichment analysis, we found no evidence for enrichment of serotonin-related genes in the associations with depression or childhood trauma (all p-values >0.12) (**Table S4**).

Associations with depressive symptoms, childhood trauma and depression chronicity

A total of 13967 CpG sites were tested against the outcomes of interest in the NESDA cohort (N=1132). We observed no statistically significant association between DNA methylation of any serotonin-related genes and depressive symptoms, childhood trauma or depression chronicity (neither in the full sample nor in the subgroups of patients that were taking antidepressant medications with serotonergic action) (all q-values>0.1) (**Figure 1**).

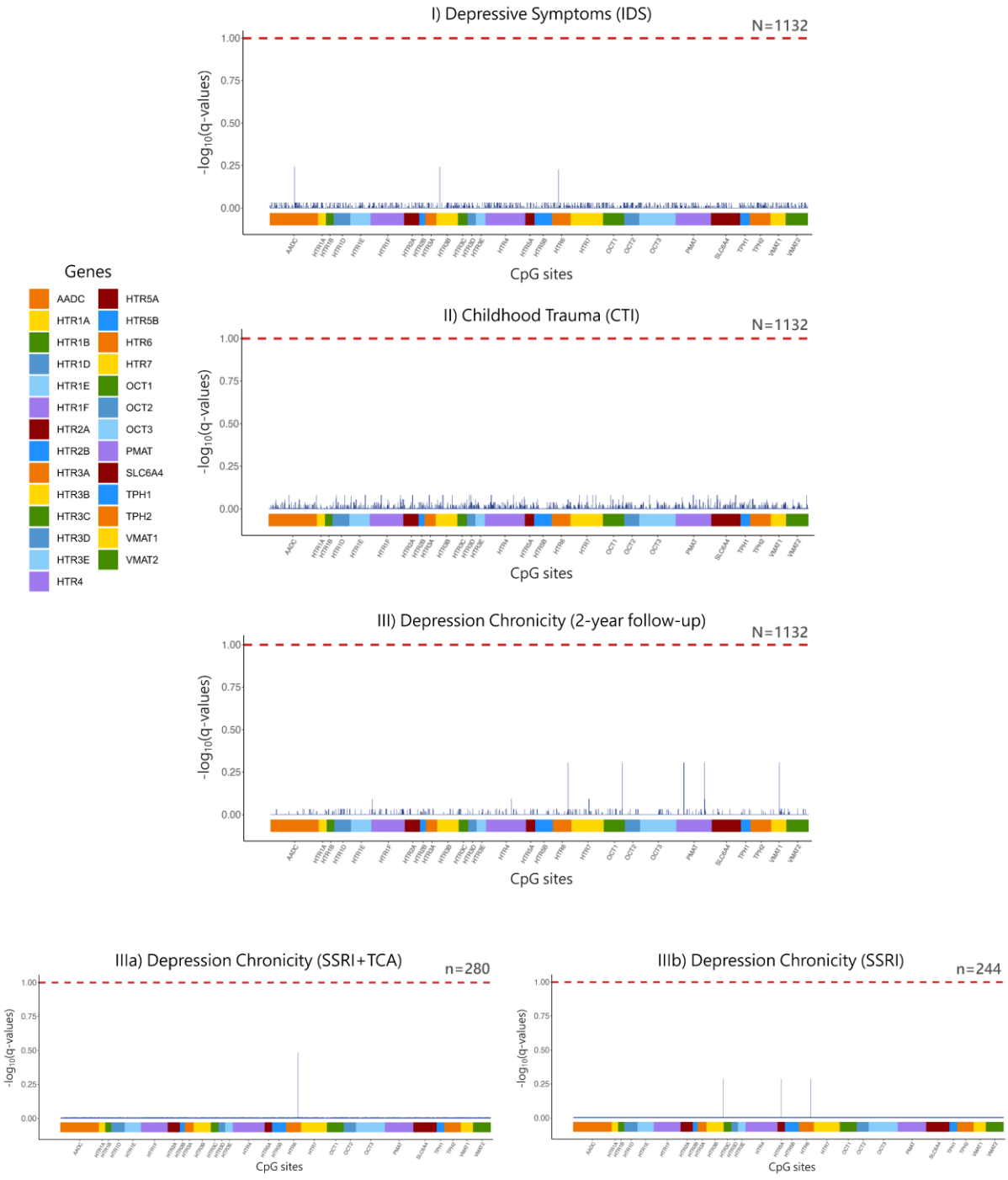

Figure 1. Results from multiple linear regression models evaluating the association between DNA methylation of 27 serotonin-related genes and I) Depressive symptoms, measured with the Inventory of Depressive Symptoms (IDS); II) Childhood trauma, evaluated using the Childhood Trauma Inventory (CTI); III) Depression chronicity after two years in the full sample and in a) patients who were taking SSRIs or TCAs at baseline or b) that were taking SSRIs at baseline. The bars correspond to the -log$_{10}$ of the FDR corrected p-values (q-values). The dashed red line represents statistical significance for q-values, which was set at 0.1.

Discussion

In this study, we aimed to replicate previously reported findings relating *SLC6A4* and *TPH2* DNA methylation to depression or childhood adversities. Next, we expanded our focus to other serotonin-relevant genes and evaluated whether these genes were enriched in the associations with depression and childhood trauma in all cohorts. Finally, we investigated the association between DNA methylation of such genes and depression, childhood trauma and depression chronicity, with a focus on the subgroups of patients that were taking antidepressant medications with serotonergic action in NESDA.

We were unable to replicate previous findings linking DNA methylation of CpG sites within *SLC6A4* or *TPH2* and depression status and childhood trauma for the CpG sites that were present in our data (~31% of the previously described sites for depression and 47% for childhood trauma). Notably, our study represents the largest study to date to address these research questions.

Furthermore, we found no enrichment of any of the serotonin-related genes in depression or childhood trauma in any cohort. Similarly, we found no association between DNA methylation of these genes and depressive symptoms or depression chronicity in NESDA. Also, the link between DNA methylation of serotonin-related genes and depression chronicity did not change in patients that were taking antidepressant medications acting on serotonergic signaling, suggesting that clinical outcomes upon using medications acting on the serotonin system might not be impacted by pre-existing DNA methylation levels of serotonin-related genes, opposite to what was previously suggested (Bruzzone et al., 2025; Domschke et al., 2014; Kang et al., 2013; Schiele et al., 2021).

Strengths and limitations

DNA methylation data are subject to a vast amount of variation due to the high inter-correlation among CpG sites across the genome (Shabalin et al., 2015). This inter-correlation, if related to the outcome of interest (e.g. depression, early life stress), can confound findings, resulting in an increased risk of detecting false positive findings. A major strength of this study is that we used data from MWAS in which the greatest amount of variation was removed using PCA, reducing the risk of false positive findings. In addition, previous studies rarely accounted for blood cell proportions, which can crucially bias DNA methylation levels by, e.g. masking cell-type-specific effects (Chan et al., 2020; Moore & Kobor, 2017; Qi & Teschendorff, 2022; Reinius et al., 2012). Furthermore, in this study we also complemented our findings in blood-

based DNA methylation with DNA methylation estimates in post-mortem brains. Although several studies suggested an overlap between DNA methylation levels measured in peripheral blood and in the brain, this correspondence is not perfect (Aberg, et al., 2020a; Hannon et al., 2015; Riese et al., 2014). This might be the case especially for genes that are mostly expressed in the brain (e.g. *TPH2*) (Bruzzone et al., 2024). Finally, we tested our research questions in cohorts with diverse demographics and depression measurements, allowing us to evaluate the generalizability of previous findings.

However, there are some important limitations that should be considered when interpreting our findings: first, we could only directly test findings for 7/22 CpG sites linked to depression and 7/15 CpG sites linked to childhood adversities, as they were available in our datasets. Instead, we focused on DNA methylation at the neighboring CpG sites, which in some cases were located a few hundreds of base pairs away. Thus, we cannot completely rule out that DNA methylation of the other CpG sites that were described by literature is associated with our outcomes of interest. Also, although DNA methylation of neighboring CpG sites tends to correlate (Aberg, et al., 2020), we do not know if DNA methylation of the CpG sites missing from our datasets correlates with the closest available CpG sites. Second, different datasets contained different numbers of CpG sites, which did not completely overlap across all datasets. This was in part due to the fact that DNA methylation was estimated in different cohorts using different types of DNA methylation assays (MBD-seq, Illumina EPIC arrays), which have different coverage and sensitivity to e.g. hypomethylated sites. Notably, the most consistently investigated CpG sites (Devlin et al., 2010; Iga et al., 2016; Kang et al., 2021; Kim et al., 2023; Schiele et al., 2019) were located in hypomethylated regions, which were not available in our datasets. Third, depression status was evaluated differently across different cohorts: in cohort 3, participants only reported if they had or not a history of depression. As DNA methylation is a dynamic modification (Bergman & Cedar, 2013; Mulder et al., 2021), its levels might vary between individuals with a current depressive episode vs remitted individuals. Fifth, we cannot exclude that DNA methylation of these genes played a role in mediating alterations in serotonin neurotransmission during early stages of neurodevelopment and that this affected individual risk of developing depression later in life. Finally, other co-morbid disorders might have masked some relevant effects. We did not account for co-morbid disorders in the NESDA cohort, although many participants suffered from comorbid anxiety, and we had no information on co-morbid psychiatric disorders for the other cohorts.

Taken together, we did not confirm that DNA methylation of serotonin-related genes play a crucial role in depression pathogenesis, reflect depressive symptoms or childhood trauma. We suggest that our observations should be taken into account when designing future studies and that large-scale, collaborative MWAS approaches meta-analyzing epigenetic data across cohorts, should be preferred over candidate genes approaches in single (small) studies.

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Supplementary Materials IV

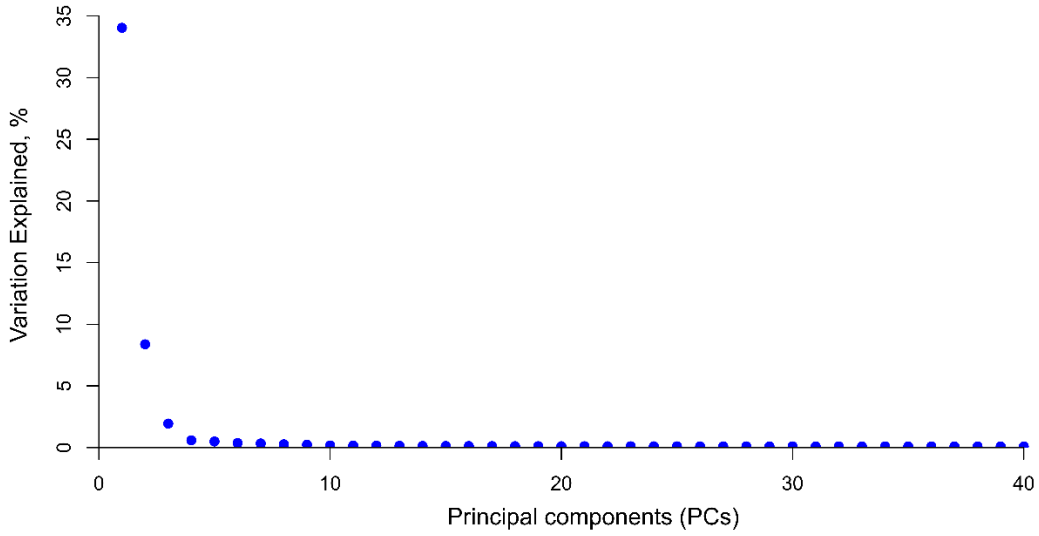

Figure S1. Scree plot depicting principal components (PC), in the x axis, versus the percentage of variation they explain, in the y axis. PCs were obtained from applying principal component analysis (PCA) on Cohort 1 DNA methylation data. PC1 explains more than 30% variance within the dataset.

Supplementary Tables

Gene function	Gene	Genomic location
Serotonin receptors	*HTR1A*	chr5:63253701-63268272
	HTR1B	chr6:78170641-78183208
	HTR1D	chr1:23518388-23553995
	HTR1E	chr6:87637246-87726397
	HTR1F	chr3:87831856-88042989
	HTR2A	chr13:47405681-47482217
	HTR2B	chr2:231972944-231999756
	HTR3A	chr11:113835830-113861035
	HTR3B	chr11:113765518-113817283
	HTR3C	chr3:183760835-183778461
	HTR3D	chr3:183739332-183757157
	HTR3E	chr3:183804852-183824783
	HTR4	chr5:147830595-148044090
	HTR5A	chr7:154852034-154879102
	HTR5B	chr2:118607003-118661256
	HTR6	chr1:19981368-20007459
	HTR7	chr10:92500580-92627796
Essential enzymes for serotonin synthesis	*TPH1*	chr11:18039102-18077816
	TPH2	chr12:72322625-72426220
	AADC	chr7:50526140-50643102
Serotonin transporters	*SLC6A4*	chr17:28521337-28572715
	OCT1	chr6:160532847-160579750
	OCT2	chr6:160637787-160689853
	OCT3	chr6:160759410-160873609
	VMAT1	chr8:20002366-20050647
	VMAT2	chr10:118990625-119038941
	PMAT	chr7:5312574-5346543

Table S1. Gene names, main function and genomic locations that were included in the statistical analyses.

Gene	Chr	CpG	Study
TPH2	12	72335393	Shen et al., 2020
	12	72335435	
	12	72348000	
	12	72372868	
SLC6A4	17	28562474	Lei et al., 2015
SLC6A4	17	28562685	Lei et al., 2015
SLC6A4	17	28562813	Lei et al., 2015
SLC6A4	17	28563054	Lei et al., 2015
SLC6A4	17	28563090	Devlin et al., 2010; Kim et al., 2013; Lei et al., 2015; Iga et al., 2016; Schiele et al., 2019; Handschuh et al., 2024
SLC6A4	17	28563102	Kim et al., 2013
SLC6A4	17	28563107	Devlin et al., 2010; Iga et al., 2016; Schiele et al., 2019; Handschuh et al., 2024
SLC6A4	17	28563109	Devlin et al., 2010; Kim et al., 2013; Iga et al., 2016; Schiele et al., 2019; Handschuh et al., 2024
SLC6A4	17	28563120	Devlin et al., 2010; Kim et al., 2013; Iga et al., 2016
SLC6A4	17	28563144	Devlin et al., 2010; Iga et al., 2016; Schiele et al., 2019
SLC6A4	17	28563160	Philibert, 2008; Devlin et al., 2010; Iga et al., 2016; Schiele et al., 2019
SLC6A4	17	28563175	Devlin et al., 2010; Iga et al., 2016
SLC6A4	17	28563388-28563878	Mendonca et a., 2019

Table S2. Genomic locations of the CpG sites that were previously linked to depression and references to the study. SLC6A4: serotonin transporter gene; TPH2: tryptophan hydroxylase 2 gene (TPH2). Chr: chromosome; CpG: CpG site genomic location (GRCh37/hg19).

Gene	Chr	CpG	Study
SLC6A4	17	28562142	Vijayendran et al., 2011
	17	28562220	
	17	28562388	Beach et al., 2011
	17	28562401	
	17	28562436	Wankerl et a., 2014
	17	28562673	
	17	28562685	Koenen et al., 2011
	17	28563090	Kang et al., 2013
	17	28563102	
	17	28563109	
	17	28563120	
	17	28563144	
	17	28563160	
	17	28563300	Vijayendran et al., 2011
TPH2	12	72340051	Shen et al., 2020
	12	72400979	

Table S3. Genomic locations of the CpG sites that were previously linked to childhood trauma and references to the study. SLC6A4: serotonin transporter gene; TPH2: tryptophan hydroxylase 2 gene (TPH2). Chr: chromosome; CpG: CpG site genomic location (GRCh37/hg19).

Cohort	Outcome	P-value
NESDA	Depression	1
GSMS	Depression	1
	Trauma	0.13
TD	Depression	1
MDDbrain	Depression	1

Table S4. Results from gene-set enrichment analysis, performed by testing serotonin-related genes against all the other genes in the four datasets.