Determinants of Neurotransmitters in Cerebrospinal Fluid and Plasma

from Seasonality to Quantitative Genetics

Jurjen Luykx

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Determinants of Neurotransmitters in Cerebrospinal Fluid and Plasma

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van Seizoenen tot Kwantitatieve Genetica

(met een samenvatting in het Nederlands)

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Jurjen Justin Luykx

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Promotoren: Prof. dr. ir. R.A. Ophoff
Prof. dr. R.S. Kahn

Co-promotoren: Dr. S.C. Bakker
Dr. M.P.M. Boks

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General Introduction

What is this Thesis About?

The prime goal of this thesis is to quantify the factors that explain inter-individual variation in neurotransmitter (metabolite) concentrations. More specifically, the thesis deals with seasonal and genetic factors that influence such constituents in cerebrospinal fluid (CSF) and plasma. At first sight, seasonality and genetics seem to have little in common. In the context of CSF, however, their commonality lies in the degree to which they explain phenotypic variation in such levels.

What are the implications of the findings outlined in this thesis? As the genetic dissection of psychiatric disorders has proven highly convoluted [1], here we reasoned that targeting the abovementioned CSF and plasma components would indirectly contribute to the elucidation of biological determinants underlying neuropsychiatric conditions. Consistent with this contention, the genomic regions highlighted in this thesis provide insight into the genetic groundwork of some of these intermediate phenotypes. Such knowledge may in turn result in exploring the functionality of these genetic variants in neuropsychiatric disorders. Furthermore, they will potentially facilitate disease prediction and pharmacotherapeutic treatment tailoring, as demonstrated for genetic variation of serum analytes [2]. Finally, the presented findings on seasonality of serotonin (5-HT) in CSF may open avenues for the clarification of biological processes mediating (seasonal) affective disorders.

Psychiatric Genetics

Human genetics

The "genotype" and "phenotype" concepts were coined in 1905 by the Danish botanist Wilhelm Johannsen [3]. By his definitions, the first referred to the whole of genes an organism possesses and the second to all traits, from the molecular to the behavioral level. It was not until the second half of the twentieth century that the idea of a gene as an indivisible unit of heredity was replaced by more dynamic viewpoints. These outlooks, partly derived from the identification of the double-helix structure of DNA[4], formed the basis for modern genetics that regards genomic complexity as fundamental to our understanding of genotype-phenotype correlations. The intricacies of the human genome likely also apply to behavioral phenotypes, as outlined below.

Psychiatric phenotypes

Emerging from the abovementioned developments in human genetics came the notion that the majority of genetically determined conditions are complex genetic and not inherited as a monogenic trait. Many psychiatric conditions indeed pertain to the first category: while in some patients penetrant deleterious copy number variants (CNVs)

mediate disease susceptibility, the largest proportion of genetic variance is likely to derive from many genetic variants of small effect. That genes play a role in the etiology of psychiatric illness is supported by their consistently reported high heritability (h^2) estimates - between 60 and 80% for schizophrenia, bipolar disorder, ADHD, and autism [1, 5]. Furthermore, although linkage studies have indicated large effects of genes such as *DISC1* [6] and *MAOA* [7] in certain families, for the majority of psychiatric cases with high h^2 the abovementioned complex genetic scenario holds. Nonetheless, for schizophrenia (the psychiatric disorder with largest available sample sizes) only ~3% of the phenotypic variance may currently be explained by genetic factors [1], although this number will likely rise as sample sizes are ramped up.

Most phenotypes do not directly emanate from a genotype but instead are the product of both (epi)genetic and environmental factors. The interplay of such variables particularly applies to the field of psychiatric genetics, as has been demonstrated for the interaction of the 5-HT-transporter-linked polymorphic region (the 5-HTTLPR) with stressful life events [8]. Other phenomena additionally contribute to the complexity of psychiatric genetics. For instance, the brain is the most variegated of all mammalian organs in its neuronal connections and number of mRNA transcripts, giving rise to pathways that impact a range of behavioral characteristics. Moreover, the classification criteria that define disease phenotypes in psychiatric genetic research predominantly stem from Diagnostic Statistical Manual (DSM) consensus meetings. The resulting categorical disease entities are consequently heterogeneous and it is still open to debate to what degree DSM-based classifications are amenable to contemporary human genetic approaches. In summary, the "genotype – categorical phenotype" paradigm is unlikely the sole path to follow in the quest for genotypes influencing behavioral abnormalities.

Bridging the Genotype-Phenotype Gap in Psychiatry: Intermediate Phenotypes

As outlined in the previous section, dissecting the neurobiological mechanisms underlying neuropsychiatric disorders has not proven to be straightforward. Intermediate phenotypes - quantifiable continuous traits pertinent to (disease) phenotypes- may be more directly linked to genetic mechanisms than the abovementioned heterogeneous disorders. The term "endophenotype" is more narrowly defined and incorporates assumptions about h^2 and co-segregation with the disease (the "exophenotype") in families [9]. Although most constituents mentioned in this thesis were shown to be heritable in serum [10], little is known about their h^2 in CSF and their co-segregation patterns. "Intermediate phenotype" is therefore the preferred term in this thesis.

Intermediate phenotypes have opened avenues for the genetic dissection of medical conditions. For instance, genetic linkage studies of the QT-interval [11], serum iron [12] and polyps [13] led to the discovery of genes responsible for the long-QT syndrome, hemochromatosis and familial polyposis coli, respectively. Along similar lines, by quantitatively analyzing genetic mechanisms influencing molecule concentrations in CSF, the genetic studies outlined in this thesis target intermediate phenotypes. In fact, the clinical relevance of genetic variants influencing metabolites in serum was recently demonstrated [2]. For instance, variants associated with serum metabolite levels mediate response to medication and were shown to be involved in a range of metabolic and other diseases [2].

Identifying genetic variants associated with such intermediate phenotypes may uncover neurobiological pathways underlying one or more psychiatric conditions. These conditions may involve both narrowly defined psychiatric disorders and symptoms that cross the diagnostic classification boundaries. For instance, psychotic features may be observed in major depressive and bipolar disorders, while hyperprolinaemia is associated with both schizophrenia and schizoaffective disorder [14,15].

To recapitulate, intermediate phenotypes relevant to psychiatry may be more closely linked to underlying genetic mechanisms than psychiatric diagnoses. Thereby, they may deepen the understanding of biological determinants underlying psychiatric illness.

Neurotransmitters and Coagonists in Psychiatry

Neurotransmitters, coagonists and a neurotrophin constitute the focus of this thesis as evidence converges on their implication in psychiatric disorders. Neurotransmitters are endogenous proteins or polypeptides that transmit signals from a neuron to a target cell. They may be divided into neuropeptides, small molecules and other, recently discovered chemicals. Neuropeptides and small molecules differ in the speed at which they give rise to action potentials: small molecules are typically involved in rapid-response signaling [16]. Binding of coagonists to specific sites at neurotransmitter receptors is a necessary step for signal transduction by especially Glutamate. Neurotrophins mediate a variety of nervous system processes. Below, the actions of neurotransmitters, coagonists and the neurotrophin relevant to this thesis are briefly discussed: the monoamine neurotransmitters, γ-Aminobutyric acid (GABA), the N-methyl-D-aspartate receptor (NMDAR) coagonists (all of which are small molecules), and brain-derived neurotrophic factor (BDNF).

Monoamine neurotransmitters

Monoamine neurotransmitters contain at least one amine-group. Dopamine (DA), norepinephrine (NE) and serotonin (5-HT) are the prime monoamine neurotransmitters of interest for the study of neurobehavioral disorders as they are associated with psychiatric disorders and a range of behavioral and cognitive processes [17].

5-HT regulates several behavioral, cognitive and physiological functions, such as mood, aggression, reward, sexuality, attention, memory, perception, bowel motility, and cardiac rhythm [18]. 5-HT neurons project from the raphe nucleus to a plethora of cortical regions. Although 15 5-HT receptors have been discovered, the exact mechanisms by which they interact and how they influence behavior have remained poorly understood [19]. Strong evidence indicates that 5-HT plays a role in depression. For instance, depletion of tryptophan (a 5-HT precursor) results in depressive symptoms [20] and drugs targeting 5-HT (SRIs, serotonin reuptake inhibitors) are efficacious in severe depression [21].

The functions of DA become manifest when studying the dopamine pathways in the central nervous system (CNS):

- the nigrostriatal pathway (from the substantia nigra to the striatum) is mostly involved with motor control;
- the mesolimbic pathway (from the ventral tegmental area (VTA) to the limbic system) is primarily implicated in reward;
- the mesocortical pathway (from the VTA to the cortex, in particular the frontal lobes) mediates cognitive, motivational and emotional processes;
- the tuberoinfundibular pathway (from the hypothalamus to the pituitary gland) regulates prolactine release.

Dopamine is also implicated in neuropsychiatric conditions, as demonstrated by confluent evidence: antagonizing agents are effective in the treatment of psychosis [22], while psychotic features constitute adverse reactions to dopamine agonists prescribed for Parkinson's disease.

Norepinephrine projections originate in the locus coeruleus and act on the amygdala and cortex where they modulate attention, fear responses and possibly decision-making [23]. Norepinephrine uptake promoting agents are effective antidepressants with generally favorable adverse effect profiles [24].

Inhibitory small molecules: GABA and Glycine

GABA is the main inhibitory neurotransmitter of the CNS: approximately a third of brain synapses use GABA as their inhibitory neurotransmitter [16]. Glutamic acid decarboxylase (GAD) catalyzes the conversion of Glutamate to GABA. GABA acts on three types of postsynaptic receptors ($GABA_A$, $GABA_B$, and $GABA_C$). Most GABA is removed from the synaptic cleft by transporters called GATs after which GABA is finally converted to succinate [16].

Glycine –which is synthesized from Serine- is the other dominant inhibitory neurotransmitter of the CNS and is mainly active in the spinal cord [16]. GABA and Glycine receptors are ligand-gated chloride channels. In addition to being an inhibitory neurotransmitter, Glycine acts as a coagonist at the Glycine binding site of the NMDAR (Figure 1).

Excitatory small molecules: the NMDAR coagonists

Glutamate is the prime excitatory neurotransmitter of the CNS as nearly all excitatory synaptic transmission is Glutamate-mediated and approximately half of all brain synapses release Glutamate [16]. The most ubiquitous Glutamate receptors are the AMPAR (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor) and ionotropic NMDAR, the latter of which contains coagonist binding sites (Figure 1). Binding of Glycine or D-Serine to the Glycine binding site of the NMDAR substantially potentiates Glutamate-dependent neurotransmission and plays an important role in long-term potentiation (LTP) [25-28]. The other NMDAR coagonists include L-Proline and possibly D-Alanine (Chapter 6).

BDNF: a neurotrophic factor

BDNF is one of the four members of the neurotrophin family (in addition to nerve growth factor (NGF), neurotrophin-3 and neurotrophins 4 and 5) [16]. Neurotrophins are key regulators of three cellular nervous system processes: neural process growth/retraction, synapse stabilization/elimination and cell survival/death [16]. In contrast to the first described neurotrophin (NGF), BDNF predominantly operates in the CNS [29]. Although rodent knock-outs of NGF receptor TrKA (tyrosine kinase A)

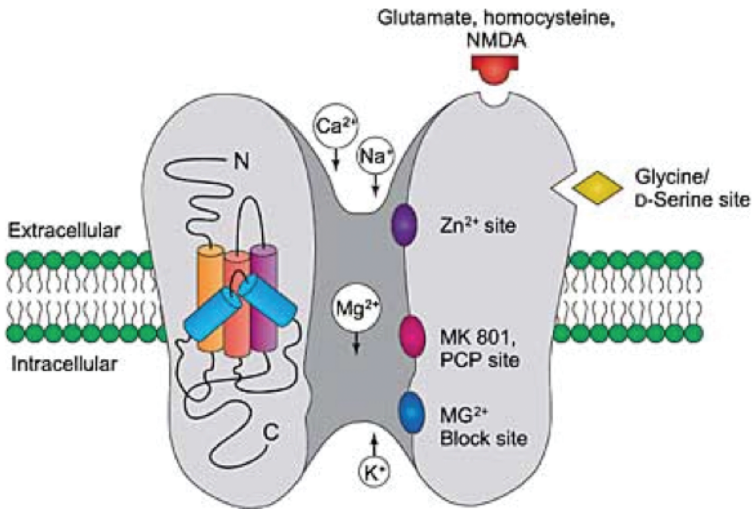

Figure 1. The NMDA receptor displayed with the Glycine/D-Serine binding site.

display clear peripheral nervous system (PNS) deficits, deletion of TrkB (the prime BDNF receptor) genes has minimal impact on the CNS [29]. While a number of other studies have investigated BDNF involvement with CNS processes and neuropsychiatric disorders over the past two decades, its CNS functionality has thus remained partly elusive.

Neurotransmitters, coagonists and neurotrophins as intermediate phenotypes
The links between neurotransmitters, coagonists, BDNF and neuropsychiatric disorders (psychosis in particular) range from pharmacological to pathophysiological in nature. Benzodiazepines –that target the benzodiazepine binding site at the $GABA_A$ receptor- have anxiolytic and sedative properties. Antipsychotics and antidepressants act by inhibiting monoamine reuptake and blockade of receptors. Decreased levels of D-Serine have been reported in CSF of schizophrenia patients [30]. Moreover, D-cycloserine, D-Alanine and Glycine constitute experimental pharmacological options in the treatment of schizophrenia (Chapter 6). Furthermore, a polymorphism in the coding region of *BDNF* (rs6265, Chapter 5) mediates susceptibility to depression [31] and smoking initiation in humans [32]. Finally, a number of studies on genes involved with NMDAR coagonist degradation have provided evidence for their role in schizophrenia (Chapter 6). Polymorphisms associated with the abovementioned neurotransmitters, their metabolites and coagonists may set the stage for follow-up studies targeting psychosis beyond the confines of DSM classifications. Such knowledge in turn will possibly yield insights into neurobiological mechanisms mediating psychosis.

Cerebrospinal Fluid (CSF) and Plasma

CSF is a body fluid that engulfs the brain and spinal canal. It is divided into a ventricular, a spinal and a subarachnoid part. Figure 2 provides an overview of the anatomy relevant to CSF in the human CNS. The human body has approximately 140-150 mL of CSF circulating at any moment of the day, of which approximately a third is contained within the spinal canal. CSF encompasses ~8% of total intracranial brain volume during adolescence, rising to ~25% in the eight decade of life due to gray matter loss [33]. Enhanced CSF volume is a prominent feature in several neuropsychiatric disorders, e.g. schizophrenia [34]. Moreover, in the 1980s and 1990s CSF was a frequently investigated body fluid in biological psychiatry. Possibly due to limited power and discrepant detection techniques, the results of those studies are highly inconsistent.
Blood plasma ("plasma") is the other body fluid targeted for the purpose of this thesis. Plasma is the intravascular part of the extracellular fluid, i.e. whole blood after removal

of red and white blood cells by centrifugal force. In addition to water (93%), it is composed of proteins, glucose, clotting factors, ions, hormones, carbon dioxide, and neuropeptides. The difference with serum is that plasma contains fibrinogen and clotting factors. Preliminary evidence furthermore suggests that reproducibility of metabolite level measurements is superior in plasma compared to serum [35]. An advantage of plasma over CSF is that the former is straightforward to obtain. Aberrations in concentrations of plasma neurotransmitters and genetic associations may therefore be more readily followed up by upcoming projects.

What are the functions of CSF?

CSF has traditionally been thought of as merely serving a protective role against impact on the skull and tension on the nerve roots. In the past decades, however, accumulating evidence hints at a pivotal role of the choroid plexus – CSF system in CNS homeostasis [37, 38]. Besides transporting essential molecules across the blood-CSF barrier (such as neurotransmitters), the choroid plexus (CP) synthesizes a range of neuropeptides, growth factors and cytokines [39]. By diffusion into brain parenchyma, such polypeptides contribute to restoration of neuron integrity following ischemic and neurodegenerative brain injury [37, 39, 40]. On the other hand, CSF has drainage properties since solutes from the interstitial fluid diffuse down a concentration gradient to the CSF [41]. Finally, by generating pressure and trophic factors during embryogenesis, CSF regulates neuroepithelium morphogenesis and thus plays a role in early brain development [39].

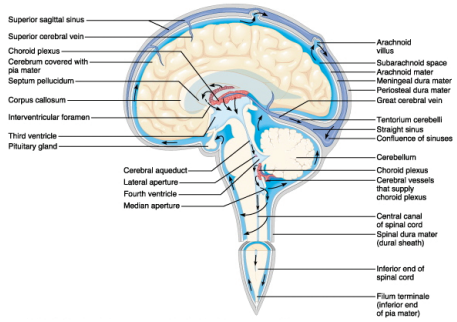

Figure 2. Anatomy and Flow of CSF in the Human CNS (Courtesy of Benjamin Cummings, 2001, an imprint of Addison Wesley Longman, Inc). CSF drains from the lateral ventricles through the foramina of Monro (or interventricular foramina) to the third ventricle; then through the aqueduct of Sylvius (or the cerebral aqueduct) into the fourth ventricle; and finally through the foramina of Luschka (or lateral aperture) and the single foramen of Magendie (median aperture) into the cisterna magna, which is part of the subarachnoid space. The subarachnoid space is a CSF and artery containing area located between the arachnoid mater and pia mater. These latter layers together with the dura mater constitute the meninges. CSF drains into the blood via the arachnoid villi (into the superior sagittal sinus), the spinal nerve roots and the olfactory tract [36].

Synthesis of CSF

At least 90% of CSF is produced by the choroid plexuses (CPs, at a rate of 0.5 L per day); ~10% comes from brain interstitial fluid, partly by ultrafiltration across cerebral microvessels [36]. CPs are highly vascularized (10 times more than the cortex) [42], leafy-like structures (cilia) located in the lateral, the third and the fourth ventricles [36]. Villous processes on the apical (CSF-facing) and extensive folding on the basolateral (blood-facing) side further ensure the surface enlargement that makes CPs such efficient membranes [36]. CP capillaries are fenestrated and -as opposed to the rest of the outer ventricular lining- CP epithelium possesses tight junctions [43], allowing passage of hydrophilic molecules to blood [36]. The hypertonic make-up of CSF indicates this fluid is not simply formed by ultrafiltration from plasma [36, 44]. CP epithelium possesses a unique distribution of ion channels and transporters, ensuring a unidirectional flux of NaCl and $NaHCO_3$ to the apical side, upon which osmosis stimulates water to be transferred into the apical space [45]. Such unidirectional H_2O transport is a characteristic feature of CSF as most other secretory epithelia allow for both trans and paracellular water fluxes.

Composition of CSF

Despite differences between plasma and CSF composition, the fluid compartments share many commonalities regarding the presence of ions (e.g. NaCl), hormones (e.g. leptin), neurotransmitters (e.g. dopamine), (poly)peptides (e.g. substance P), and glucose. Temporal concentration shifts in CSF may be indicative of underlying pathologies, e.g. ferritine increase, glucose decrease, and lactate increase in bacterial meningitis [46]. The composition of CSF can be studied after obtaining CSF by a lumbar puncture (LP; Figure 3). While it is beyond the scope of this introduction to discuss the concentrations of each constituent, amino acids merit some attention as these are a focus of this thesis. Based on animal studies, amino acid ratios (e.g. of Proline, Hydroxyproline and Glycine) between CSF and plasma are small [47, 48] and directionality of passage across the CP likely depends on the amino acid and its concentration [49].

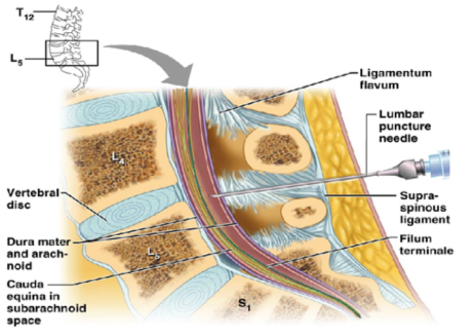

Figure 3. Lumbar Puncture. (Courtesy of Benjamin Cummings, 2006, Pearson Education Inc). A lumbar puncture (LP) is the procedure of inserting a needle between the lumbar vertebrae past the ligamentum flavum and (except for epidural punctures) the dura mater to reach the subarachnoid space. In neurology, LPs are diagnostic and sometimes therapeutic procedures that aim to withdraw CSF from the spinal canal. In anesthesia, LPs are carried out to administer anesthetics to the subarchnoid space (spinal anesthesia) or to the epidural space. The former technique was employed for the purpose of obtaining the CSF studied in this thesis. Given the narrowness of spinal anesthesia needles (25-27G), CSF was suctioned into tubes. This procedure allowed us to reduce tapping time by 10-fold to an average of 40 seconds.

Summary of Methods

CSF being the body fluid most proximate to the brain, it allows for *in vivo* studies of molecules relevant to CNS homeostasis. CSF thereby bypasses the limitations inherent in postmortem brain research, e.g. confounding by the postmortem interval. As opposed to brain imaging techniques -such as magnetic resonance spectroscopy- quantitation of all sorts of metabolites and neurotransmitters is feasible in CSF. As delineated in the previous sections, several lines of research have advanced the understanding of CSF physiology over the past decades. On the other hand, lacunae in the appraisal of mechanisms mediating neurotransmitter concentration variability in CSF and plasma have driven the make-up of the studies described in this thesis.

Study populations

To approximate the general population, neurologically healthy subjects undergoing spinal anesthesia were chosen as the study population for all studies except the one in Chapter 5. Individuals who underwent lumbar punctures for the purpose of spinal anesthesia were asked to donate 6mL of CSF. In addition, blood for DNA and plasma extraction was tapped from an I.V. that also formed part of standard perioperative care. Finally, age restrictions were applied to the inclusion of participants given the diminished CSF turnover rate at advanced age [46]. For the BDNF study, healthy

individuals were selected from a population-based cohort called the Utrecht Health Project (Chapter 5).

Metabolomics

Metabolomics is the research area that aims to identify biological determinants of metabolite levels. Metabolomics consortia have installed specimen collection and reporting standards that may vary according to the biological sample under investigation. As CSF has not been targeted in metabolomics [10], the standards applied here to assure such homogeneity are both derived from these consortia and based on our own experiences with CSF, as outlined below.

DA, 5-HT and NE concentrations in CSF being unstable and diminutive, their main metabolites (homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxy-4-hydroxyphenylglycol (MHPG), respectively) are thought to most reliably reflect monoamine turnover [50]. Monoamine metabolite (MM) peak detection was conducted using High-Performance Liquid Chromatography (HPLC) with electrochemical detection. HPLC is a sensitive technique in which the components to be separated are distributed between liquid stationary and mobile phases. As electrochemical detectors are preferred for catecholamines, this detection method was applied to MM quantitation. For amino acid quantitation, HPLC was coupled to mass spectrometry (MS), the latter of which ionizes a molecule and then identifies it by its mass-to-charge ratio [51]. MS coupled to HPLC is highly sensitive as both chromatographic separation and mass spectra are obtained. Tandem MS –two sequentially arranged mass spectrometers- was chosen as it offers the additional advantage of characterizing a compound by its precursor and product ion masses [51]. For BDNF quantitation, an enzyme-linked immunosorbent assay (ELISA) was used since this is a sensitive technique with low inter-assay variation and little cross-reactivity with other neurotrophic factors (Chapter 5).

Standard Operating Procedures (SOPs) were applied to both CSF and plasma collection to assure between-sample processing homogeneity and reproducibility. Biological samples were immediately worked up since > 24 hours storage duration prior to centrifugation results in plasma metabolite leakage [52].

Quantitative trait locus (QTL) analysis

Single nucleotide polymorphisms (SNPs) are the most commonly used polymorphic markers for association studies, i.e. relating genetic variants to phenotypes (the genetic technique employed for the purpose of this thesis). QTL mapping is the statistical technique that allows mapping of quantitative phenotypes to genomic regions. Examples range from human height and body mass index [53] to analytes in body fluids. Since 2007, genome-wide association studies (GWASs) have revolutionized QTL analysis as high-density arrays have uncovered genetic mechanisms underlying a

plethora of disease and other biological traits. With regard to human metabolomics, large consortia have undertaken elegant GWAS approaches to detect QTLs for serum and urine metabolites [2, 54-56]. However, analytes in plasma or CSF have not been targeted in genome-wide QTL analyses. Here we coin the term neurometabolic QTL to refer to QTLs underlying metabolites that have relevance to the CNS, e.g. genetic variation associated with NDMAR coagonist levels.

Hypothesis-driven vs hypothesis-generating approaches in human genetics
One of the methodological subdivisions in human genetics is between hypothesis-driven and hypothesis-generating strategies. The first hinges on the suspicion that a certain genotype influences a specific trait, the second on a predefined level of statistical evidence. Chapters 3, 4 and 5 describe single-genotype association tests inspired by the literature, thereby increasing power at the potential cost of type-I error. Upon inclusion of > 400 participants, we reasoned that a hypothesis-free genome-wide association approach would allow us to uncover novel quantitative trait loci (Chapters 6 and 7).

Seasonality in Monoaminergic Turnover

As outlined in the first section, the prime goal of this thesis is to quantify the factors that explain inter-individual variation in neurotransmitter concentrations. To that end, we first investigated to what degree covariates influence monoaminergic transmission. Hypothesized variables influencing any MM include time of day of sampling, use of psychotropic or other medication, storage duration, psychiatric history, type of procedure, duration of aspiration, season of sampling, season of birth, and CSF amount (the rostrocaudal gradient resulting from a lumbar puncture negatively influences MM concentrations in lumbar CSF) [57, 58]. Notably, 5-HIAA levels were found to be substantially higher in spring and season of sampling seemed to influence 5-HIAA more than any other variable. In light of the conflicting literature on seasonality of CSF 5-HT turnover (Chapter 2), these observations led us to build parametric and non-parametric statistical models to substantiate the contribution of seasonality to 5-HT turnover in CSF (Chapter 2). After obtaining a large enough study population collected over 3 years, we were able to confirm the spring 5-HIAA peak but falsified suspicions that season of birth influences 5-HIAA (Chapter 3). Having appraised the contribution of seasonal factors to 5-HT turnover, seasonal variation in 5-HIAA was then linked to the one genotype associated with 5-HT seasonality, the 5-HTTLPR (Chapter 3). Moreover, correction for seasonality was applied to the quantitative genetic analyses (Chapter 7).

Outline of the thesis

To substantiate the hitherto tenuous evidence that seasonal and genetic factors contribute to variation in CSF constituents, the following structure of studies was chosen.

In *Chapter 2*, circannual variation in CSF 5-HIAA concentrations is modeled parametrically and non-parametrically. *Chapter 3* extends these findings by corroborating the increased 5-HT turnover in spring using a multi-year analyses design in over twice as many participants. Moreover, the association of 5-HT seasonality with the genotype most strongly implicated in a range of 5-HT seasonal measures –the 5-HTTLPR (the 5-HT transporter-linked polymorphic region)- was tested. The same study additionally highlights how 5-HT seasonality correlates with depressive symptomatology. *Chapter 4* was inspired by a genotype-phenotype correlation described in the literature: the association of a common variant in *ERBB4* with *in vivo* GABA measured using a neuroimaging technique. The hypothesis that the same variant would be associated with overall CNS GABA turnover as reflected by CSF GABA resulted in the first genetic association with CSF GABA. *Chapter 5* demonstrates that hypothesis-based designs may result in negative findings. The *BDNF* Val66Met polymorphism, one of the most thoroughly investigated genetic variants in psychiatry, has long been hypothesized to mediate plasma BDNF. However, no difference in plasma BDNF between Val and Met homozygous groups was detected. *Chapter 6* establishes that neurometabolic quantitative trait loci (QTLs) can be detected in CSF and plasma using genome-wide association analysis. Variants in three biologically tenable transporter and metabolic genes were found to be associated with NMDAR coagonists in CSF and plasma at a genome-wide significant level. *Chapter 7* describes the other genome-wide study of CSF constituents in the current study population. Using expression QTL (eQTL) analyses, the impact of a genome-wide significant variant on expression of *PDE9A* – a gene involved with depression and antidepressant response- is highlighted. The approach outlined in *Chapter 8* was adopted to determine the effects of the NMDAR coagonists on two quantitative behavioral phenotypes, smoking and alcohol consumption. *Chapter 9* concludes this thesis by integrating the highlighted findings, discussing their relevance and offering an outlook for future projects.

In summary, we first investigated the impact of seasonal changes on fluctuations in monoamine metabolite levels. The second stage comprised hypothesis-driven approaches to identify genetic variants associated with 5-HT seasonality, GABA and BDNF. NMDAR coagonists and monoamine metabolites were then subjected to genome-wide studies. Finally, to translate the possible implications of NMDAR coagonist concentration variability to the behavioral level, we tested whether such levels differ according to smoking and alcohol drinking habits.

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[1] *Rudolf Magnus Institute of Neuroscience, Department of Psychiatry, University Medical Center Utrecht, Utrecht, The Netherlands*
[2] *Department of Psychiatry, ZNA hospitals, at Stuivenberg, Antwerp, Belgium*
[3] *Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, The Netherlands*
[4] *Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands*
[5] *Clinical Research Unit, Academic Medical Center, Amsterdam, The Netherlands*
[6] *Department of Biostatistics, Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, the Netherlands*
[7] *Department of Medical Genetics, University Medical Center Utrecht, Utrecht, the Netherlands*
[8] *Department of Human Genetics, David Geffen School of Medicine, University of California, Los Angeles, USA*
[9] *Department of Anesthesiology, Intensive Care & Pain Management, Diakonessenhuis, Utrecht, The Netherlands*
[10] *Central Military Hospital, Utrecht, The Netherlands*
[11] *Intensive Care Unit, Vrije Universiteit Medical Center, Amsterdam, The Netherlands*
[12] *Department of Anesthesiology, Intensive Care & Pain Management, St Antonius Hospital, Nieuwegein, The Netherlands*
[13] *Division of Anesthesiology, Intensive Care and Emergency Medicine, University Medical Center Utrecht, Utrecht, The Netherlands*
[14] *Biostatistics School of Public Health, University of California, Los Angeles, USA*
[15] *Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, University of California Los Angeles, Los Angeles, CA, USA*

2

Season of Sampling and Season of Birth Influence Serotonin Metabolite Levels in Human Cerebrospinal Fluid

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Jurjen J. Luykx[1,2]
Steven C. Bakker[1]
Eef Lentjes[3]
Marco P.M. Boks[1,4]
Nan van Geloven[5]
Marinus J.C. Eijkemans[6]
Esther Janson[7]
Eric Strengman[7,8]
Anne M. de Lepper[1]
Herman Westenberg[1]
Kai E. Klopper[9]
Eric-Jan (H.C.) Hoorn[9]
Harry P.M.M. Gelissen[10,11]
Julian Jordan[10]
Noortje M. Tolenaar[12]
Eric (H.) P.A. van Dongen[12]
Bregt Michel[13]
Lucija Abramovic[1]
Steve Horvath[8,14]
Teus Kappen[13]
Peter Bruins[12]
Peter Keijzers[10]
Paul Borgdorff[9]
Roel A. Ophoff[1,8,15]
René S. Kahn[1]

Abstract

Background

Animal studies have revealed seasonal patterns in cerebrospinal fluid (CSF) monoamine (MA) turnover. In humans, no study had systematically assessed seasonal patterns in CSF MA turnover in a large set of healthy adults.

Methodology / Principal Findings

Standardized amounts of CSF were prospectively collected from 223 healthy individuals undergoing spinal anesthesia for minor surgical procedures. The metabolites of serotonin (5-hydroxyindoleacetic acid, 5-HIAA), dopamine (homovanillic acid, HVA) and norepinephrine (3-methoxy-4-hydroxyphenylglycol, MPHG) were measured using high performance liquid chromatography (HPLC). Concentration measurements by sampling and birth dates were modeled using a non-linear quantile cosine function and locally weighted scatterplot smoothing (LOESS, span=0.75). The cosine model showed a unimodal season of sampling 5-HIAA zenith in April and a nadir in October (p-value of the amplitude of the cosine = 0.00050), with predicted maximum (PC_{max}) and minimum (PC_{min}) concentrations of 173 and 108 nmol/L, respectively, implying a 60% increase from trough to peak. Season of birth showed a unimodal 5-HIAA zenith in May and a nadir in November (p=0.00339; PC_{max}=172 and PC_{min}=126). The non-parametric LOESS showed a similar pattern to the cosine in both season of sampling and season of birth models, validating the cosine model. A final model including both sampling and birth months demonstrated that both sampling and birth seasons were independent predictors of 5-HIAA concentrations.

Conclusion

In subjects without mental illness, 5-HT turnover shows circannual variation by season of sampling as well as season of birth, with peaks in spring and troughs in fall.

Introduction

Seasonal patterns in behavior and psychiatric symptoms are present in both healthy and clinical populations. For example, in healthy humans mood is lowest in fall [1, 2], bipolar patients are at the highest risk of suffering an episode in fall [3] and suicide peaks in spring in both hemispheres [4-8]. Moreover, season of birth has been associated with several behavioral traits, such as smoking, novelty seeking and suicide [9-13]. Finally, meta-analyses have linked season of birth with schizophrenia [14, 15].

From an evolutionary perspective, seasonal adaptation of several kinds of behaviors, e.g. mating and degree of physical activity, may be advantageous. Seasonal variation in psychiatric illness may reflect such season-dependent behavioral variations in that psychiatric symptoms are at the extremes of normal behavior. Clarifying the mechanisms underlying seasonal variation in behavioral processes could thus further our understanding of the etiology of mental disorders.

Animal studies have revealed pronounced correlations between mating behavior and energy consumption (i.e. hibernation) on the one hand and serotonin (5-hydroxytryptamine, 5-HT) metabolism on the other [16-19]. For example, in rhesus macaques, the main metabolite of 5-HT (5-hydroxyindoleacetic acid, 5-HIAA) in cerebrospinal fluid (CSF) is highest during the mating season and correlates positively with several measures of successful mating [17, 19]. In addition, 5-HIAA levels and monoamine oxidase (MAO)-activity increase during arousal from hibernation in ground squirrels [20]. However, knowledge about seasonal patterns in 5-HT, dopamine (DA), and norepinephrine turnover in the human brain is scant. One study describing a sample of 34 volunteers reported that concentrations of 5-HIAA and the main DA metabolite (homovanillic acid, HVA) in CSF were highest in summer [21]. Monoamine (MA) metabolite levels in the CSF of 283 newborn febrile infants showed seasonal variation [22], but it has remained unclear whether season of sampling or season of birth was the main determinant and it is unknown how this relates to MA metabolite levels in adulthood. And finally, preliminary evidence exists that in fall and winter human dopamine synthesis and storage are increased in the putamen [23].

We hypothesized that human MA turnover shows circannual fluctuations but given the inconclusiveness of the available data were unable to form any prior assumptions about their pattern. To elucidate the seasonal variation of physiological human MA turnover, we prospectively collected standardized amounts of CSF in a homogeneous sample of 223 healthy individuals undergoing spinal anesthesia for minor elective procedures and studied the effects of season of sampling and season of birth on MA metabolites.

Results

Subject Characteristics and Data Completeness (Supplemental Table S1)
The characteristics of the procedures and 223 included subjects are listed in Supplemental table S1. One hundred sixty-seven subjects were male (75%) and the mean (S.D.) age was 39 (+-11). For the two main covariates -age and sex- no data were missing. The maximum percentage of missing data for the other covariates was 5%. Seven subjects used psychotropic medication. Knee arthroscopy mostly due to meniscus injuries was by far the most common surgical procedure, comprising 77% of all operations. No correlation between storage time and metabolite concentrations or between birth and sampling dates was found.

Concentrations of Metabolites per Season (Table 1)
All MA metabolite concentrations were normally distributed. Median concentrations per season are shown in table 1. Only for 5-HIAA, a clear per-season pattern of season of both sampling and birth was observed, with highest concentrations in spring and lowest in fall.

Season of Sampling (Models 1 and 2; Table 2 and Figure 1)
The best fitting nlqr-model (1-peak) was significant for 5-HIAA (Figure 1 and table 2): the A (amplitude) was -32.3, p=0.00050; t_{max} = 4.04 (April) and t_{min} 10.0 (October). HVA and MHPG amplitude results were non-significant (table 2).
The predicted concentrations at t_{max} (PC_{max}) and at t_{min}(PC_{min}) for 5-HIAA were: 173 and 108 nmol/L, respectively, implying a 60% increase from October to April. Deviances and β's of the nlqr-models are given in Supplemental table S2.
The LOESS for 5-HIAA followed a similar pattern to the cosine function (Figure 1, Kruskal-Wallis p-value of the three peak months that turned out to be sequential –April, May, June- vs the other months = 3.90×10^{-6}), validating the chosen cosine model. Supplemental figure S1 provides the raw data points with cosine and LOESS lines.

Season of birth (Models 1 and 2; Table 2 and Figure 2)
The best fitting model (1-peak) was significant for 5-HIAA (Figure 2 and table 2): the A was -22.7 (p=0.00339); t_{max} = 5.34 (May) and t_{min}=11.3 (November). Amplitudes for the other MA metabolites were non-significant (table 2).
The predicted concentrations at t_{max} (PC_{max}) and at t_{min} (PC_{min}) for 5-HIAA were: 172 and 126 nmol/L, respectively, implying a 37% higher concentration for those born in May compared to November. Deviances and β's of the nlqr-models are given in Supplemental table S2. The LOESS for 5-HIAA followed a similar pattern to the cosine function (Figure 2, no Kruskal-Wallis computed as the median peak months –May,

June, September- were not sequential), suggesting our cosine modeling approach was also valid for the season of birth 5-HIAA analysis. Supplemental figure S2 provides the raw data points with cosine and LOESS lines.

Table 1. Median concentrations of monoamine metabolites (in nmol/L) in cerebrospinal fluid with standard deviations (S.D.), per season (italics indicate totals).

Season of Sampling	Number	Median	S.D
5-HIAA	*223*	*141*	*65*
Winter	88	139	67
Spring	53	183	67
Summer	25	139	58
Fall	57	115	49
HVA	*223*	*201*	*77*
Winter	88	202	82
Spring	53	200	69
Summer	25	249	71
Fall	57	181	75
MHPG	*223*	*24.2*	*5.4*
Winter	88	23.9	4.4
Spring	53	23.2	5.1
Summer	25	25.6	6.4
Fall	57	26.3	6.0
Season of Birth			
5-HIAA			
Winter	51	126	70
Spring	57	163	56
Summer	71	136	63
Fall	44	126	70
HVA			
Winter	51	174	79
Spring	57	221	85
Summer	71	187	63
Fall	44	217	77
MHPG			
Winter	51	24.8	5.7
Spring	57	24.0	5.0
Summer	71	24.7	5.1
Fall	44	24.2	5.4

Table 2. Amplitude of the best fitting models per metabolite with significance levels (in bold: Bonferroni-corrected significant results).

	Amplitude	p-value	t-statistic	t_{max}	t_{min}	Model
Season of sampling						
- 5-HIAA	**-32.26**	**0.00050**	**-3.53**	**April**	**October**	**1-peak**
- HVA	-14.95	0.09027	-1.70	Feb/August	May/Nov	2-peaks
- MHPG	1.75	0.02252	2.30	March	September	1-peak
Season of birth						
- 5-HIAA	**-22.70**	**0.00339**	**-2.96**	**May**	**November**	**1-peak**
- HVA	17.93	0.07157	1.81	March/Sept	June/Dec	2-peaks
- MHPG	-1.34	0.04730	-1.99	May/Nov	August/Feb	2-peaks

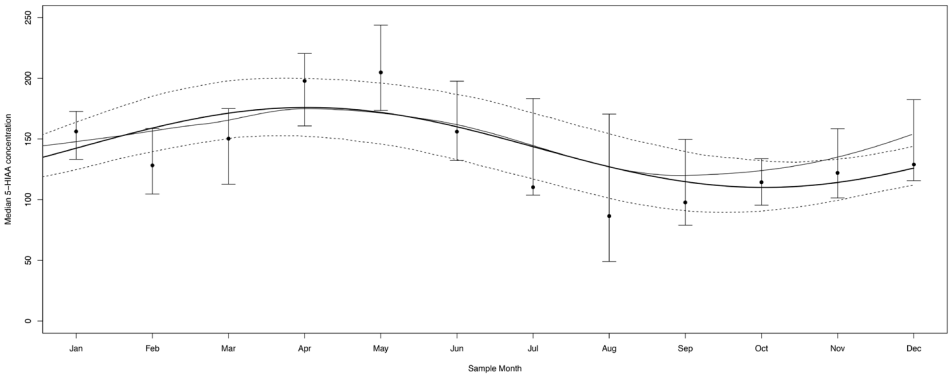

Figure 1. Median CSF 5-HAA Concentrations (in nmol/L) are plotted against Month of CSF Sampling. Bold line represents cosine, thin line represents LOESS and dashed lines represent 95% CIs of the cosine. Whiskers indicate 95 % CIs of 5-HIAA concentrations.

Sampling and birth within one model (Model 3; Supplemental table S2)

For all MA metabolites, model 3 improved the goodness-of-fit compared to the sampling and birth models 1 and 2. The 5-HIAA one-peak model 3 showed similarly significant sampling (p=0.00017) and birth (p=0.00756) amplitudes to model 1. In addition, the peaks and troughs of birth and sampling remained similar (sampling PC_{max}=3.75 and PC_{min}=9.75; birth PC_{max}=5.40 and PC_{min}=11.4). The amplitude significances of the HVA two-peak model 3 were similarly non-significant to model 2. For MHPG, the one-peak model 3 showed a similarly non-significant birth amplitude to model 2, but a significant sampling amplitude (p=0.00589, in contrast to p=0.02252 in model 1; PC_{max}=3.61 and PC_{min}=9.61).

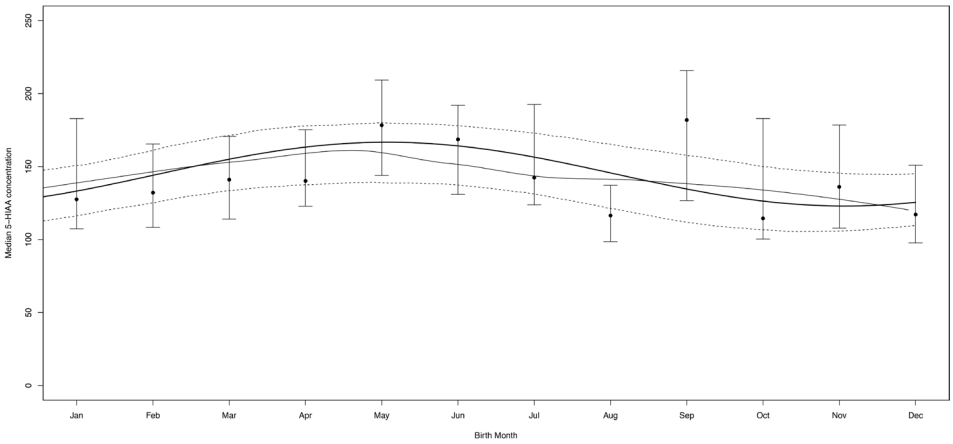

Figure 2. Median CSF 5-HAA Concentrations (in nmol/L) are plotted against Month of Birth. Bold line represents cosine, thin line represents LOESS and dashed lines represent 95% CIs of the cosine. Whiskers indicate 95 % CIs of 5-HIAA concentrations.

Discussion

In the first study on this topic in healthy human participants (n=223), all three main monoamine metabolites in CSF were modeled by sampling and birth dates using a non-linear cosine function and LOESS (locally weighted scatterplot smoothing). Sampling and birth in spring were associated with unimodal peaks in 5-HIAA concentrations.

Hypothesized Functions and Mechanism of High Spring 5-HIAA

Conception is the most seasonally driven behavior across species [24] and peaks in spring in most species, including humans [25]. Evidence supporting a role for 5-HT in mating behavior of animals abounds. CSF 5-HIAA is highest during the mating season and correlates positively with sexual competence in rhesus macaques [17, 19]. In addition, catfish have high levels of monoamine-oxidase (MAO) activity before reproduction whereas the inverse applies to the spawning (reproduction) phase [16]. MAO is the main 5-HT breakdown enzyme and increased MAO levels may consequently correspond with high 5-HT turnover (reflected by the 5-HIAA/5-HT-ratio). Provided in humans 5-HIAA concentrations also positively correlate with successful reproductive behavior, early spring would thus be the most appropriate time of year for 5-HIAA peaks, increasing the likelihood of spring or summer births. Such a hypothesis is in keeping with our findings of highest 5-HIAA concentrations in early spring.

One plausible mechanism underlying our finding of highest 5-HIAA in early spring relates to differences in light exposure throughout the year. Circannual melatonin

(5-methoxy-N-acetyltryptamine) and 5-HT up and down regulation are likely to be interdependent, as 5-HT is the precursor of melatonin (Figure 3). Melatonin is synthesized within pinealocytes from 5-HT by two enzymes – arylalkylamine N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyltransferase (HIOMT) [26]. Its functions range from circadian (most importantly sleep cuing) to circannual (melatonin as a seasonal zeitgeber for reproduction and puberty) across a wide range of species [24, 27]. In humans, only preliminary evidence indicates that pinealectomized subjects are less seasonal than individuals with an intact pineal gland [28], which may be related to blunting of melatonin peaks due to the abundance of artificial light in modern society [27]. In the pineal gland of the European hamster, however, the activity and gene expression of AA-NAT are three to eight times increased in November compared to June [26]. This is in accordance with the higher basal and nighttime melatonin concentrations in fall compared to spring that have been demonstrated for several species [26, 27]. Linking this knowledge about seasonal variation in the melatonin pathway to our findings leads us to posit that more 5-HT is used by the human body to produce melatonin in fall than in spring. This hypothesis would explain two phenomena. First, more 5-HT may be available for other purposes –such as conception-

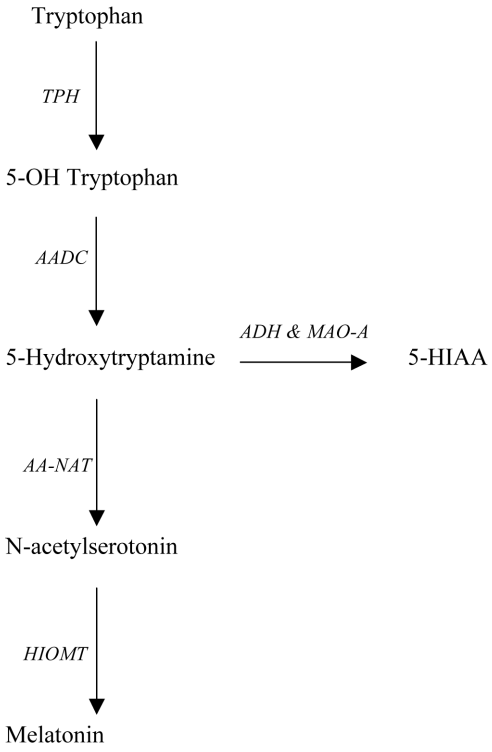

Figure 3. Metabolism of 5-hydroxytryptamine (5-HT, serotonin). Enzymes in italics. TPH = tryptophan hydroxylase; AADC = aromatic amino acid decarboxylase; ADH = alcohol dehydrogenase; MAO = monoamine oxidase; 5-HIAA = 5-hydroxyindoleacetic acid; AA-NAT = arylalkylamine N-acetyltransferase; HIOMT = hydroxyindole-O-methyltransferase.

in spring than in fall. And second, the 5-HIAA/5-HT-ratio would be higher in spring than in fall, which is in keeping with the high concentrations of 5-HIAA we detected in spring.

Season of Birth Effects

Our finding of increased CSF 5-HIAA levels in those born in spring supports available data showing a primary role of 5-HT turnover in fitness and survival. That especially 5-HIAA concentrations in CSF (and to a lesser degree HVA and MHPG) are stable from childhood into adulthood, has been demonstrated in rhesus macaques [29]. Moreover, high 5-HIAA levels are associated with an increased likelihood of survival in these primates [29, 30]. Until recently in human history, early spring was an auspicious period to start the reproduction cycle as the likelihood of a child being born in spring is then also increased. Such reasoning is supported by epidemiological data showing that European women are most likely to become pregnant within six months and only have an approximately 20% chance of becoming pregnant after one month [31]. In fact, a Finnish study demonstrated that births peaked in early spring from 1650 through 1850 [32]. Although no data are currently available to test such a correlation in humans, it is thus conceivable that in the pre-industrialization era high CSF 5-HIAA concentrations in spring-born humans advanced adaptability and survival, which is supported by our data. On the other hand, other studies have found high suicide rates among spring and summer-born individuals [12, 33]. Although speculative as no empirical data are currently available to test such a hypothesis, one may reason that a difference in monoamine turnover seasonality between suicide committers and healthy subjects (our study population) is one of the determinants of suicide predisposition. Those born in summer and spring may thus only be at risk to commit suicide if their 5-HIAA peak is attenuated compared to healthy subjects.

Neurobehavioral Disorders

How may our findings be viewed in light of studies showing highest levels of 5-HIAA in other seasons than spring in neuropsychiatric patients? Differences in seasonal variation between cases and controls may partly explain such apparent disagreement, in that patients may display either an attenuated or an opposite pattern of seasonal variation in MA metabolism. Two studies provide preliminary evidence for the former; one in a sample of children suffering from a variety of psychiatric disorders (n=72) and the other in alcohol dependent patients (n=135). In neither of the two reports seasonal differences in monoamine metabolites were detected [34, 35]. Similarly, given previously detected peaks of MA metabolite levels in winter born psychiatric patients [36], the influence of season of birth on monoamine metabolite levels seems to differ between patients and healthy controls. In this context, a prime disorder of interest is seasonal affective disorder (SAD), in which the prominent characteristic is seasonal

variation in semiology. Interestingly, also its pathophysiology is seasonally determined as melatonin rhythms in SAD patients are delayed compared to controls [37, 38], which in turn may be related to seasonal shifts in the 5-HT-melatonin pathway.
Suicide is another season-associated psychiatric phenotype, for which several studies support an early spring incidence zenith [4-8]. It is conceivable that seasonal variation in the foremost neurotransmitter system associated with suicide -5-HT- plays a role in the pathophysiology of season-associated suicide. As suicide is most frequent among severe psychiatric disorders with a chronic course and we have not included such patients in our study population, possible differences in seasonal variation of 5-HT turnover between such patients and healthy individuals remain hypothetical.

Limitations and Future Directions

To our knowledge, no previous study on seasonal variation in any animal or human metabolite to date incorporated an assumption-free LOESS model into a cosine (that includes both season of sampling and season of birth) after systematically correcting for all possibly relevant covariates. This statistical approach has made our findings robust to type I and II errors, although a methodological concern may be selection bias as only patients undergoing minor elective procedures were included. On the other hand, available epidemiological data obtained from over 400,000 knee arthroscopies (by far the most common procedure in this study) suggest that such patients reflect the general population, for example with regard to history of cancer and comorbidities [39]. In addition, the standardized sampling conditions in our operating rooms are likely to have benefitted the reliability of intersubject MA metabolite concentration comparisons.
To detect possible gender-dependent differences in seasonal patterns, we ran the 1-peak 5-HIAA models for men and women separately. Seasonal variation in both season of birth and season of sampling remained similar with all models yielding Bonferroni-corrected significant amplitudes and similar t_{max} and t_{min}, with the exception of season of birth where the same peaks and troughs were visible but significance decreased to p= 0.042 for women and 0.046 for men. Excluding the 7 subjects on psychotropic medication from our analyses also resulted in similar findings and significance levels (Supplemental table S2).
Limiting the interpretability of our season of birth findings are the moderate significance of the season of birth nlqr model and the unexpectedly high September concentrations. A limitation of our method is that MA metabolites at one point in time per person have been assessed. Future longitudinal studies over several years in the same study population and studies comparing healthy subjects with neuropsychiatric patients may fill the current lacunae in our understanding of seasonal variability in monoamine turnover. In addition, based on our data the potential effects

of MAO-A activity cannot be teased apart, as high 5-HIAA concentrations may either point to increased breakdown or to an altogether upregulated 5-HT system, including more biosynthesis. Although we cannot draw firm conclusions about causal directions based on our findings, animal studies hint at 5-HT up-regulation in spring [40]. In vivo assessments of MAO-A activity (e.g. by PET [41] and gene expression profiling) in subjects with measured CSF 5-HIAA-concentrations may thus provide new insights into the seasonality of human 5-HT physiology.

Materials and Methods

Subjects

This study was approved by the ethics committee at the University Medical Center Utrecht (UMCU) and all local ethics committees. Volunteers were recruited at outpatient pre-operative screening services in four hospitals in and around Utrecht, The Netherlands, from August 2008 until March 2010: UMCU, Central Military Hospital, Sint Antonius Hospital, and Diakonessenhuis. We included patients (i) undergoing spinal anaesthesia for minor elective surgical procedures, (ii) ranging between 18-60 years of age, and (iii) with four grandparents born in The Netherlands or other North-Western European countries (Belgium, Germany, UK, France, and Denmark). Written informed consent was obtained from all participants. Subjects suffering current or past major psychiatric or neurological disorders were excluded during a telephone interview. During this interview J.L. (a psychiatry resident) or a medical student trained by J.L excluded subjects who reported they currently or in the past (had) suffered any psychiatric or neurological illness or had been admitted to a psychiatric or neurological unit (n=5).

Collection of CSF

Subjects had fasted at least 6 hours prior to lumbar puncture (LP). Before administration of medication (either pre-medication or compounds for the purpose of anesthesia), a 25-27 Gauge needle was inserted into the L1/L2, L2/L3, L3/L4, or L4/L5 interspace (estimated by the anaesthesiologist). A single sample of 6 mL of CSF was obtained from each subject. Age, height, weight, time of procedure, duration of aspiration (usually 30-60 seconds), type of procedure, and diagnosis related to the procedure were recorded. In addition, any deviations from the instructed procedure were recorded, such as smaller amounts of CSF drawn or operation complications. CSF was kept at 4°C and transported within 9 hours to the laboratory at UMCU. Each sample was immediately stored in fractions of 0.5mL and 1mL at -80 °C. One fraction of 0.5mL was used for MA metabolite measurements.

Monoamine Metabolite Measurements

Concentrations of CSF MA metabolite levels (3-methoxy-4-hydroxyphenylglycol, MHPG; 5-HIAA; and HVA) were measured using high performance liquid chromatography (HPLC; Dionex-Thermo Fisher, USA) with electrochemical detection (DecadeII, Antec, Leiden, the Netherlands). The CSF samples were thawed, mixed and centrifuged, after which 50 µL was injected on a reverse phase C18 (150x4.6 mm, 3 µm) HPLC column (Supelco, Sigma-Aldrich). The MA metabolites were eluted with a mixture of methanol (8% final concentration) and a phosphate buffer (50 mM, pH3) containing 0.2 g/L octane-sulphonic acid and 0.2 g/L Na-EDTA. Separate stock standards of MHPG, 5-HIAA and HVA (Sigma, St Louis, USA) were prepared in a 0.2 mol/L HCL-154 mmol/L NaCL solution at concentrations of 502, 506 and 502 µmol/L, respectively. A mixture of the working external standards was made by diluting the stock solutions in 154 mmol/L NaCL to a final concentration of 100, 502 and 1012 nmol/L, respectively. In-house quality controls were prepared by mixing separate stock solutions of the MA metabolites to final concentrations of 500 nmol/L for HVA and 5-HIAA and 50 nmol/L for MHPG. All MA metabolites in each sample were measured twice. Peak areas were measured and compared to those of the external standards for quantitation. The electochemical detector settings of the conditioning and analytical cells were +150 mV +400 mV, respectively. Samples showing >10% differences (15% for MHPG) between the first and second measurement were measured again until the difference was < 10% (which applied to a total of <5% of all measurements). For each sample, the mean of the two measurements was used for further analyses. Day-to-day imprecision (for single measurements) ranged from 4 to 10% for 5-HIAA, from 5 to 10% for HVA and 4 to 8% for MHPG.

Statistical Analyses

MA metabolite concentration distributions were verified for normality using the Kolmogorov-Smirnov test (p-values >0.05 were considered indicative of a normal distribution). Median concentrations per season (defined as starting on the 21st and ending on the 20th three months later, e.g. spring starting March 21st and ending June 20th) were computed. Non-linear quantile regression (nlqr) – which models the median values per month - was chosen before inspecting circannual metabolite concentration variability to evaluate the effects of season of sampling and season of birth on MA metabolite concentrations assuming a cosine-shaped relationship. (Median values were used because of uneven distributions of sampling and birth dates). To validate the cosine function and model concentration measurements per day non-parametrically, we used LOESS (locally weighted scatterplot smoothing), which offers the advantage of modeling data points without having to set a function according to which the data are described. The default span in R 2.12.1 (www.r-project.org) of 0.75 was used. In the event three months with highest median MA metabolite concentrations

were sequential within the same season, the non-parametric group comparison Kruskal-Wallis test was used to compare metabolite levels between that season and the other seasons taken together. Kruskal-Wallis test results with p-values < 0.05 were deemed significant. Two covariates, age and sex, were included in all nlqr-models. Other potentially confounding factors (amount of CSF suctioned, type and timing of procedure, comorbidities (psychiatric and other), psychotropic medication and other medication, LP level, and height and weight of participants) were investigated for association with MA metabolites by means of univariate linear regression after multiple (n=5) imputation of missing values in SPSS 18.0 (SPSS for Windows, SPSS Inc). Factors that showed a univariate association ($p<0.05$) and did not show collinearity with age or sex (Pearson's $r<0.6$) were additionally entered into the models (which was the case for LP level and weight in the HVA model). Using Pearson's r, it was checked whether storage time and metabolite concentrations and whether sampling and birth date correlated. Since we hypothesized circannual variation with either one or two zeniths and nadirs per year would apply to all three MA metabolites, nlqr-models were fitted for each of these two scenarios. We measured the fit of each model per MA metabolite and compared the deviances (residual sum of squares). Thus, two nlqr-models were compared for goodness-of-fit for each of the six analyses (season of sampling and season of birth being the predictors for each of the three MA metabolites) and only results for the best fitting model were reported. To determine whether birth and sampling dates independently contributed to MA metabolite concentrations, a third model including both sampling and birth months within one model was created.

Model 1a (5-HIAA and MHPG, one peak):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times t + \beta 3) + (\beta 4 \times Sex) + (\beta 5 \times Age)$$

Model 1b (5-HIAA and MHPG, two peaks):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times 2 \times t + \beta 3) + (\beta 4 \times Sex) + (\beta 5 \times Age)$$

Model 2a (HVA, one peak):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times t + \beta 3) + (\beta 4 \times Sex) + (\beta 5 \times Age) + (\beta 6 \times LPlevel) + (\beta 7 \times Weight)$$

Model 2b (HVA, two peaks):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times 2 \times t + \beta 3) + (\beta 4 \times Sex) + (\beta 5 \times Age) + (\beta 6 \times LPlevel) + (\beta 7 \times Weight)$$

Model 3a (5-HIAA and MHPG, sampling and birth month within one model, one peak):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times t1 + \beta 3) + \beta 4 \times \cos(0.5236 \times t2 + \beta 5) + (\beta 6 \times Sex) + (\beta 7 \times Age)$$

Model 3b (5-HIAA and MHPG, sampling and birth month within one model, two peaks):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times 2 \times t1 + \beta 3) + \beta 4 \times \cos(0.5236 \times 2 \times t2 + \beta 5) + (\beta 6 \times Sex) + (\beta 7 \times Age)$$

Model 3c (HVA, sampling and birth month within one model, one peak):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times t1 + \beta 3) + \beta 4 \times \cos(0.5236 \times t2 + \beta 5) + (\beta 6 \times Sex) + (\beta 7 \times Age) + (\beta 8 \times LPlevel) + (\beta 9 \times Weight)$$

Model 3d (HVA, sampling and birth month within one model, two peaks):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times 2 \times t1 + \beta 3) + \beta 4 \times \cos(0.5236 \times 2 \times t2 + \beta 5) + (\beta 6 \times Sex) + (\beta 7 \times Age) + (\beta 8 \times LPlevel) + (\beta 9 \times Weight)$$

In which:
- metabolite = concentration of MA metabolite
- β1 = baseline level
- β2 = amplitude (A)
- 0.5236 = coefficient of t = 2π/12 (one cosine period in radians divided by the number of months per year; "x 2" added for the two-peaks model)
- t = month of sampling or birth; t1 = month of sampling; t2= birth month
- β3 = phase shift
- β4, β5, β6, β7, β8, and β9 = covariates' coefficients (except for in model 3, where β4 and β5 are amplitude and phase shift, respectively)

For each 1-peak model showing a significant amplitude the t_{max} (month during which a level is at its maximum), the t_{min} (month during which a level is at its minimum), and predicted maximum (PC_{max}) and minimum (PC_{min}) concentrations were computed:
$t_{max} = (\pi - \beta 3) / 0.5236 \ (+12)$
and the predicted concentration at t_{max}: $PC_{max} = \beta 1 + \beta 2 \times \cos(0.5236 \times t_{max} + \beta 3)$
$t_{min} = - \beta 3 / 0.5236 \ (+12)$
and the predicted concentration at t_{min}: $PC_{min} = \beta 1 + \beta 2 \times \cos(0.5236 \times t_{min} + \beta 3)$

In each of the best fitting models, the effect of season of sampling or season of birth was considered significant when the amplitude (A) was significantly different from zero. The nlqr significance level was Bonferroni corrected and set at 0.05/6 = 0.00833 (since three tests for both season of sampling and season of birth were performed). All data analyses were performed with the statistical software package SPSS 18.0 (SPSS for Windows, SPSS Inc) and R 2.12.1 (www.r-project.org).

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Supplemental Information Legends

(available online at www.plosone.org)

Supplemental table S1. Subject characteristics; means are given for all variables except for sex and procedure type (for which absolute numbers and percentages are shown).
Supplemental table S2. Deviances and results of each model per monoamine metabolite.
Supplemental figure S1. Raw values of CSF 5-HAA Concentrations (in nmol/L) are plotted against Month of CSF Sampling. Bold line represents cosine, thin line represents LOESS and dashed lines represent 95% CIs of the cosine.
Supplemental figure S2. Raw values of CSF 5-HAA Concentrations (in nmol/L) are plotted against Month of Birth. Bold line represents cosine, thin line represents LOESS and dashed lines represent 95% CIs of the cosine.

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[1] *Department of Psychiatry, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht (UMCU), Utrecht, The Netherlands*
[2] *Clinical Research Unit, Academic Medical Center, Amsterdam, The Netherlands*
[3] *Department of Biostatistics, Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht, The Netherlands*
[4] *Department of Human Genetics, University of California, Los Angeles, California, USA*
[5] *Biostatistics School of Public Health, University of California Los Angeles, Los Angeles, California, United States of America*
[6] *Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, The Netherlands*
[7] *Department of Medical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands*
[8] *Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, University of California Los Angeles, Los Angeles, CA, USA*
[9] *Department of Anesthesiology, Intensive Care & Pain Management, St Antonius Hospital, Nieuwegein, The Netherlands*
[10] *Department of Anesthesiology, Intensive Care & Pain Management, Diakonessenhuis Hospital, Utrecht, The Netherlands*

3

Seasonal Variation of Serotonin Turnover in Human Cerebrospinal Fluid and the Role of the 5-HTTLPR Polymorphism

Submitted

Jurjen J. Luykx[1]
Steven C. Bakker[1]
Nan van Geloven[2]
Marinus J.C. Eijkemans[3]
S. Horvath[4,5]
Eef Lentjes[6]
Marco P.M. Boks[1]
Eric Strengman[7]
Joseph DeYoung[8]
Jacobine E. Buizer-Voskamp[1]
Rita M. Cantor[5]
Ake Lu[5]
Eric P.A. van Dongen[9]
Paul Borgdorff[11]
Peter Bruins[9]
René S. Kahn[1]
Roel A. Ophoff[1,4,8]

Abstract

Background

Studying monoaminergic seasonality is likely to improve our understanding of neurobiological mechanisms underlying season-associated physiological and pathophysiological behavior. Studies of monoaminergic seasonality and the influence of the serotonin transporter promoter polymorphism (5-HTTLPR) on serotonin seasonality have yielded conflicting results, possibly due to lack of power and absence of multi-year analyses. We aimed to assess the extent of seasonal monoamine turnover and examined the possible involvement of the 5-HTTLPR.

Methods

To unambiguously assess the influence of seasonality on monoamine turnover, 5-hydroxyindoleacetic acid (5-HIAA) and homovanillic acid (HVA) were measured in the cerebrospinal fluid of 479 human subjects from the general population, collected during a 3-year period. Cosine and non-parametric seasonal modeling were applied to both metabolites. We computed serotonin (5-HT) seasonality values and performed an association analysis with the s/l alleles of the 5-HTTLPR. Depressive symptomatology was assessed using the Beck-Depression Inventory-II.

Results

Circannual variation in 5-HIAA fitted a spring-peak cosine model that was significantly associated with sampling month ($P = 0.0074$). Season of sampling explained 5.4% ($P = 1.57 \times 10^{-7}$) of the variance in 5-HIAA concentrations. The 5-HTTLPR s-allele was associated with increased 5-HT seasonality (standardized regression coefficient = 0.12, $P = 0.020$, N=393). 5-HT seasonality correlated with depressive symptoms (Spearman's rho = 0.13, $P = 0.018$, N=345).

Conclusions

We highlight a dose-dependent association of the 5-HTTLPR with 5-HT seasonality and a positive correlation between 5-HT seasonality and depressive symptomatology. The presented data set the stage for follow-up in clinical populations with a role for seasonality, e.g. affective disorders.

Introduction

Seasonal variation in monoaminergic transmission may be one of the mechanisms explaining circannual fluctuations in behavior, which range from suicide in humans [1-4] to mating in animals [5, 6]. The monoamines dopamine (DA) and serotonin (5-HT) play pivotal roles in human behavior. 5-HT regulates a plethora of behavioral, cognitive and physiological functions, such as mood, aggression, reward, sexuality, attention, memory, and perception[7, 8]; DA mediates a similarly diverse list of neuroendocrine, behavioral and neurophysiological actions [9]. While preliminary evidence indicates that monoaminergic transmission in human cerebrospinal fluid (CSF) varies between seasons, the results of such studies are inconsistent [10-12]. Limited sample sizes and absence of multi-year analyses constitute the foremost explanations behind such discrepancies. Insufficient power of these investigations further precludes reliable estimations of the explained variances in monoamine transmission by seasonal factors. As a consequence, it has not been unequivocally determined whether and to what degree seasonal factors explain variation in monoaminergic transmission.

Studying monoaminergic seasonality is likely to improve our understanding of neurobiological mechanisms underlying season-associated physiological and pathophysiological behavior, such as suicide and seasonal affective disorder (SAD). While the heritability (h^2) of seasonality in monoamine turnover is unknown, twin studies indicate that seasonal fluctuations in human behavior (e.g. sleep, mood and weight) are highly heritable, particularly in men ($h^2 = 69\%$) [13]. The genotype most extensively investigated for its role in monoaminergic seasonality is the s/l (short/long) polymorphism of the 5-HT transporter-linked polymorphic region (5-HTTLPR). Notably, seasonality in 5-HT transporter (5-HTT) binding measured with positron emission tomography (PET) in the putamen is associated with the s-allele of the 5-HTTLPR [14]. Other lines of evidence also hint at an impact of this polymorphism on a variety of 5-HT seasonality measures, including 5-HT concentrations in blood and 5-HTT binding measured with PET and SPECT [15-18]. However, these studies investigating the influence of the 5- HTTLPR on measures of 5-HT seasonality are inconsistent with regard to seasonal directionality, i.e. whether the s-allele increases or diminishes 5-HT seasonality. Such incongruities may have originated from the small and diverse study populations they were based on [14-18]. A well-powered genetic study targeting 5-HT seasonality in a homogeneous sample of subjects has the potential to resolve these inconsistencies. In addition, unraveling the seasonality of monoamine turnover and clarifying the impact of the 5-HTTLPR on 5-HT seasonality may open avenues for clinical research. For example, comparisons of seasonal patterns in 5-HT turnover and 5-HTTLPR mediated seasonality between SAD cases and controls may aid in the elucidation of the unknown pathophysiology of this disorder.

Based on a recent study [12] we reasoned that season of sampling and season of birth influence 5-hydroxyindoleacetic acid (5-HIAA) but not homovanillic acid (HVA) concentrations in human CSF. To unambiguously assess the association of such seasonal factors with monoamine turnover, CSF measurements were carried out in 479 individuals from the general population over three consecutive years. Given the influence of 5-HTTLPR s-allele carrier status on seasonality in 5-HTT binding [14], we hypothesized that a relatively large sample size would allow us to detect dose-dependent differences of the 5-HTTLPR in seasonality of 5-HT turnover. We accordingly aimed to clarify whether this polymorphism predicts seasonal variation in 5-HIAA levels in human CSF. To that end, we first computed per-subject 5-HT seasonality values and subsequently analyzed the association of these values with the 5-HTTLPR s/l polymorphism. Finally, to extend the findings to the behavioral level, we correlated 5-HT seasonality with depressive symptomatology.

Methods and Materials

Subjects

Subject collection procedures have been described elsewhere [12]. In brief, 479 volunteers were recruited at outpatient preoperative screening services in four hospitals in and around Utrecht, The Netherlands, from 28 August 2008 until 31 August 2011. The ethics committee at the University Medical Center Utrecht (UMCU) and all local ethics committees approved this study. Written informed consent was obtained from the participants. Seasonal variation in monoaminergic transmission was also analyzed in a subset of the study population (N=223) in a previous study [12]. We included patients (i) undergoing spinal anaesthesia for minor elective surgical procedures, (ii) ranging between 18-60 years of age, and (iii) with four grandparents born in The Netherlands or other North-Western European countries (Belgium, Germany, the UK, France, and Denmark). Each candidate participant received a personal telephone interview to exclude subjects with a history of psychotic or major neurological disorders (stroke, brain tumors, and neurodegenerative diseases) and to record any use of psychotropic medication. A self-reported history of unipolar affective, attention-deficit-hyperactivity and anxiety disorders was allowed and recorded if applicable.

Collection of CSF, DNA and 5-HIAA Measurements

These methods have been described elsewhere [12]. In brief, a single sample of 6 ml of CSF was suctioned from each participant and transported the same day to the laboratory, where fractions of 0.5 and 1 ml were immediately stored at -80 °C. Concentrations of 5-HIAA and HVA in CSF were measured using High-Performance

Liquid Chromatography (HPLC), as described previously [12]. A whole blood sample was used to extract genomic DNA using standard techniques in a subset of the study population (N=414).

Genotyping Procedures

As part of an on-going project, genotype data of 414 subjects were collected using the Illumina HumanOmniExpress Beadchip at the UCLA Neurosciences Genomics Core (UNGC). Quality control (QC) was conducted as described in the Supplemental Methods, leaving 398 individuals. We used a recently developed machine learning method of vertex discriminant analysis validated for Northern European populations to predict the 5-HTTLPR polymorphism [19]. Three of the eight single nucleotide polymorphisms (SNPs) used for this prediction model had been imputed, as described in the Supplemental Methods. Imputation r^2 values for these three SNPs were 1.00 (rs1487971; rs7217677) and 0.94 (rs887469). All eight SNPs passed the QC thresholds outlined in the Supplemental Methods (< 2% genotyping missingness, Hardy-Weinberg equilibrium (HWE) p-value > $1x10^{-6}$, and minor allele frequency (MAF) > 0.05).

Seasonal Modeling

Collinearity between sampling and birth dates was not detected (Spearman's rho = 0.03, P=0.5). To test the association of season of sampling and season of birth with 5-HIAA concentrations, non-linear quantile regression (nlqr) – modeling the median values per month assuming a cosine-shaped relationship – was chosen before inspecting circannual metabolite concentration variability [12]. To validate the cosine function and model concentrations per day non-parametrically, LOESS (locally weighted scatterplot smoothing, span = 0.75) was employed [12]. The same covariates as in a previous study on monoamine metabolite seasonality were incorporated, i.e. age and sex for 5-HIAA and weight and lumbar puncture level in addition to these two for HVA [12]. Nlqr models were fitted for a one and two-peak scenario as outlined in the Supplemental Methods. The best fitting model of these two scenarios (defined as the one showing less deviance) was chosen per metabolite. The nlqr significance level was Bonferroni corrected ($\alpha = 0.05/4 = 0.0125$ as both season of sampling and season of birth effects were tested for 5-HIAA and HVA). Whenever three months with highest median metabolite concentrations were sequential, the Kruskal-Wallis (K-W) test was used to compare metabolite levels between that season and the other seasons taken together ($\alpha = 0.05$). We then computed the explained variance in metabolite concentration by season using analysis of covariance, setting metabolite concentrations as dependent variables; sampling or birth season (dichotomized: three consecutive peak months vs. all other months of the year) as independent variables; and incorporating age and sex as covariates. The sum of squares for seasonal effect

was then divided by the total sum of squares ($\alpha = 0.05$). These analyses were performed with the statistical software packages SPSS (IBM SPSS Statistics for Mac, Version 20.0, Armonk, NY, USA) and RStudio (www.rstudio.com).

Computation of 5-HT Seasonality Values and Association Testing of the 5-HTTLPR

To assess the genetic association with 5-HT seasonality, we first calculated 5-HT seasonality values. We define 5-HT seasonality as the degree to which CSF 5-HIAA levels vary by season of sampling, i.e. the difference between the observed 5-HIAA value and the 5-HIAA value predicted by the best fitting nlqr cosine model. Measured 5-HIAA values above the cosine during the six months surrounding the peak of the cosine (here: January-June) and values below the cosine during the rest of the year were defined as positively seasonal, while all other values were deemed negatively seasonal. In other words, positive 5-HT seasonality values correspond to larger predicted amplitudes than negative values (see Figure 1 for a graph of the measured values and their direction of seasonality). In the Supplemental Methods we give examples of how observed 5-HIAA values translate into seasonality values.

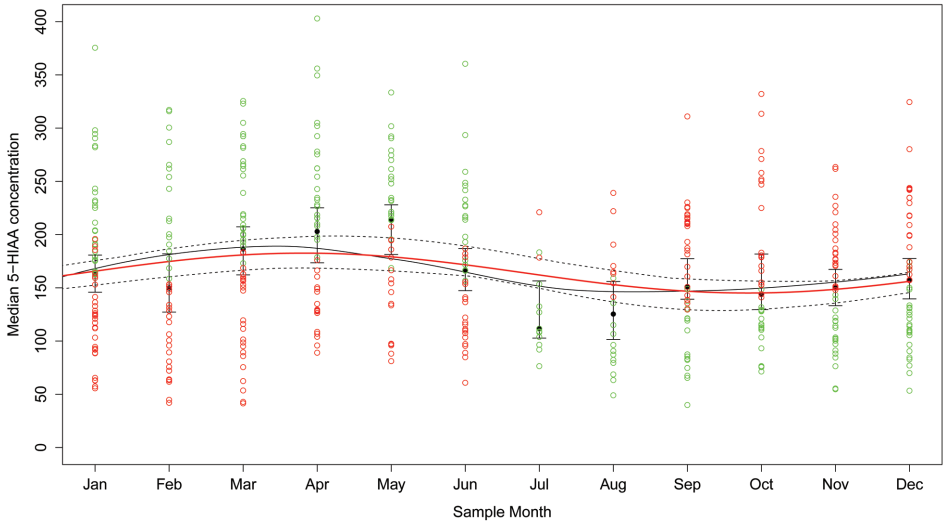

Figure 1. Graph displaying measured 5-HIAA data points per sampling month and 5-HT seasonality values. Measured 5-HIAA concentrations (nmol/L) per subject are plotted against sampling month. Positively seasonal values are green and negatively seasonal values red. The covariates age and sex are included in the model, explaining why some values in January-June are green while under and red while above the cosine curve (and vice versa for July-December). Red line represents the cosine; black line the LOESS; dashed line the 95% confidence intervals of the cosine; and whiskers 95% confidence intervals of 5-HIAA concentrations per sample month.

Genotype summary statistics were generated in Plink V1.07 [20]. To test for the association between the 5-HTTLPR s/l polymorphism and 5-HT seasonality, a linear additive model was run in Plink V1.07 [20]. Since age and sex were included in the model used for computation of 5-HT seasonality values, we provide the following association statistics for linear models with and without these covariates ($\alpha = 0.05$): standardized regression coefficients (β) that correspond to a change in 5-HT seasonality by an increase in the number of s-alleles, i.e. from 0 to 1 to 2; and p-values. As a test of robustness, we repeated the analysis on a subset of patients resulting from excluding subjects currently on psychotropic medication or with a self-reported psychiatric history. To rule out direct associations of the 5-HTTLPR with CSF 5-HIAA, the association of the 5-HTTLPR s/l polymorphism with absolute concentrations of CSF 5-HIAA was tested.

Assessment of Depressive Symptoms and Correlation Analysis with 5-HT Seasonality

Affective disorders (SAD and bipolar II disorder, in particular) are most commonly associated with seasonal behavioral fluctuations [21]. We therefore assessed the association of seasonal variation in 5-HT transmission with depressive symptomatology as a quantitative trait. To that end, all participants were asked to fill out the Dutch version of the Beck Depression Inventory II (BDI-II) [22] online within two weeks after their surgical elective procedure. We chose web-based symptom questionnaires as these have been validated as reliable assessment tools in a range of epidemiological studies [23, 24] and may decrease socially desirable responses compared to face-to-face interviews or questionnaires that are filled out in clinical settings [25, 26]. To test whether depressive symptoms show seasonal variation, we applied the best fitting of models 1a and b to BDI-II scores. We then used the non-parametric Spearman's correlation test to estimate the possible correlation between 5-HT seasonality values and BDI-II total scores ($\alpha = 0.05$) since total BDI-II scores were not normally distributed (K-S two-tailed $P < 0.001$). To rule out direct associations between absolute 5-HIAA values and BDI-II scores, correlations between the CSF 5-HIAA and BDI-II scores were also estimated using Spearman's correlation.

Results

Descriptive Statistics

The study population consisted of 479 subjects, of whom 71% were male (Table 1). The mean (SD) concentrations of 5-HIAA and HVA were 169 (68.6) and 217 (75.1) nmol/L, respectively. Twenty-three subjects (4.8%) had a self-reported history of psychiatric illness and 13 (2.7%) were on psychotropic medication during the lumbar puncture.

Monoamine Metabolite Seasonality (Figures 2 and 3)

For 5-HIAA, the one-peak nlqr-model sampling model showed less deviance than the two-peak model (Supplemental Table 1) and was significant (P of the amplitude of the cosine = 0.0074, Figure 2). The LOESS followed a similar pattern to the cosine, validating the chosen model (Figure 2). Figure 1 provides the raw 5-HIAA measurements per subject and sampling month. Peak and trough concentrations were in late March (t_{max} = 3.8) and September (t_{min} = 9.8), respectively. Concentrations were 44% increased in spring compared to the other months. In Supplemental Table 1 summary statistics per nlqr-model are given. Three consecutive spring months (March, April and May) showed highest mean and median 5-HIAA levels and differed from the other months considered together: the mean 5-HIAA concentration in these three months was 195, while in the other months the mean was 158 nmol/L (K-W test P =

Table 1. Descriptive statistics of the study population (N=479); part of whom were genotyped (N=414).

	Mean / N (%)	Std. Deviation
5-HIAA (nmol/L)	169	68.64
HVA (nmol/L)	217	75.07
N male	340 (71)	N/A
Age (years)	40	11.3
Spring sampling (21 March – 20 June)	134 (28)	N/A
Psychiatric History	23 (4.8)	N/A
Psychotropic Medication	13 (2.7)	N/A
Knee Arthroscopies	354 (74%)	N/A

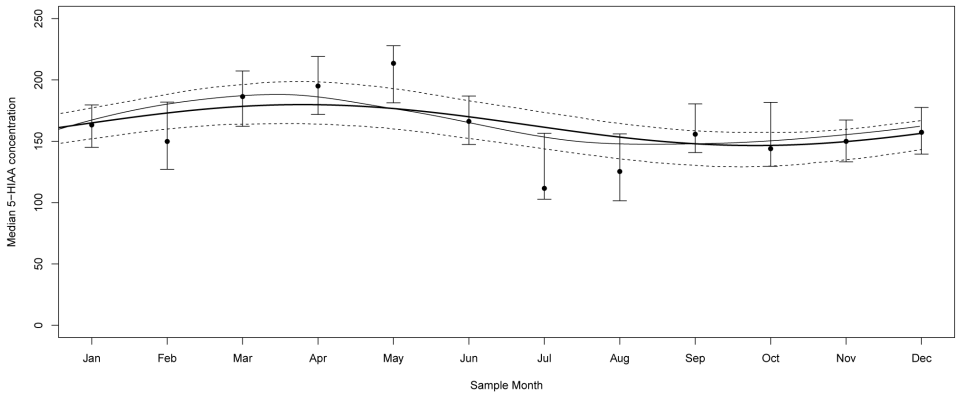

Figure 2. Median CSF 5-HAA concentrations (in nmol/L) are plotted against month of CSF sampling (N=479). Bold line represents cosine, thin line represents LOESS and dashed lines represent 95% CIs of the cosine. Whiskers indicate 95% CIs of 5-HIAA concentrations.

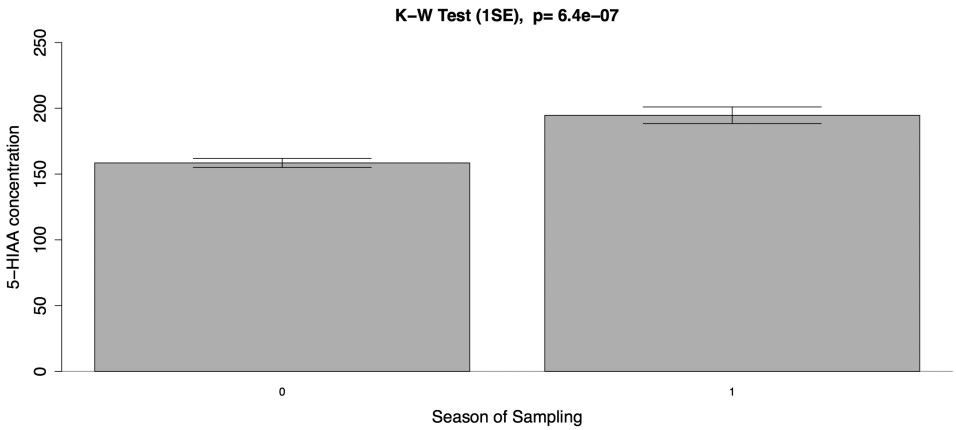

Figure 3. Kruskal-Wallis test results for CSF 5-HAA (+/- SE) in the three consecutive months with highest 5-HIAA concentrations (March-May = 1) vs. the other months of the year (=0).

6.4×10^{-7}, Figure 3). The explained variance in 5-HIAA concentrations by sampling in these three months was 5.4% ($P = 1.57 \times 10^{-7}$). 5-HIAA season of birth results were non-significant. For HVA, no significant associations with sampling month or birth month were found (Supplemental Table 1).

Association of the 5-HTTLPR Genotype with 5-HT Seasonality (Figure 4)

After genotype QC (Supplemental Methods), 398 subjects remained, for 393 from whom 5-HIAA levels were available. The genotypes were in Hardy-Weinberg equilibrium (P>0.5) and the numbers of subjects per genotype were: 73 (19%) S/S, 192 (49%) S/L, and 128 (33%) L/L. The mean (SE) 5-HT seasonality values per genotype were: 11.39 (7.61), 0.13 (4.96), and -10.22 (5.41), respectively (Figure 4). A dose-dependent positive association of the s-allele with 5-HT seasonality was detected in the models with and without covariates age and sex ($\beta = 0.12$, $P = 0.020$; and $\beta = 0.11$, $P = 0.023$, respectively). When excluding subjects on psychotropic mediation or with a self-reported psychiatric history, the results did not change ($\beta = 0.12$, $P = 0.019$). Absolute concentrations of CSF 5-HIAA were not associated with the 5-HTTLPR (P= 0.2).

Correlation between 5-HT Seasonality and Depressive Symptoms

Four hundred and six (85%) of the 479 subjects filled out the BDI-II and the mean total score (SD) was 4.48 (5.32). For 345 subjects, both 5-HT seasonality values and BDI-II scores were available. No seasonal patterns in mood symptoms were detected (Supplemental Table 1). 5-HT seasonality correlated positively with total BDI-II scores (Spearman's rho = 0.13, P = 0.018), while CSF 5-HIAA did not correlate with BDI-II scores (Spearman's rho = 0.04, P = 0.45).

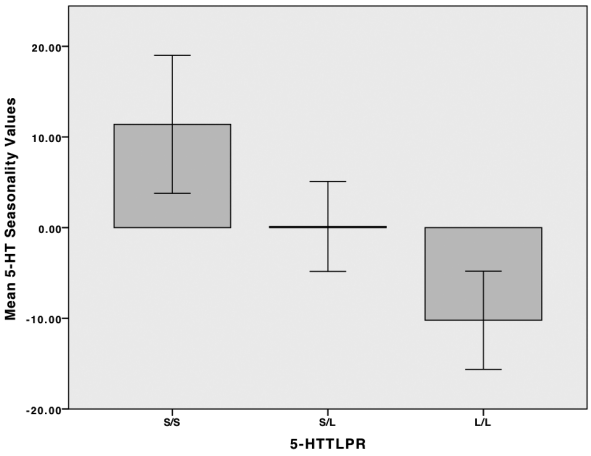

Figure 4. Mean 5-HT seasonality values (+/- SE) are shown per 5-HTTLPR genotype: S/S (N=73): 11.39 ± 7.61; S/L (N=192): 0.13 ± 4.96; L/L (N=128): -10.22 ± 5.41.

Discussion

In this three-year study of CSF monoamine turnover (N=479), we confirm that 5-HIAA concentrations fit a cosine model that displays a peak in spring and a trough in fall. We additionally show that a significant proportion of the variance (5.4%) in serotonergic transmission is explained by seasonality. Furthermore, we demonstrate a dose-dependent association of the 5-HTTLPR s-allele with seasonality of 5-HT turnover, while this polymorphism was not associated with absolute 5-HIAA levels. Finally, 5-HT seasonality correlated positively with depressive symptoms.

The current data are based on the largest number of years and participants in seasonal research of CSF monoaminergic transmission in humans or non-human primates to date. Our design has thus enabled us to clarify some of the issues related to the much debated and inconsistent findings in seasonal research of CSF monoaminergic transmission [5, 6, 10-12, 27, 28]. The CSF 5-HIAA peak in spring that we detected is in agreement with a previous study (for which part of the current study population was used) [12]. 5-HIAA increases from trough to peak and the summary statistics of the non-quantile regression were similar to those previous findings. We furthermore demonstrate that seasonality explains over 5% of the variation in CSF 5-HIAA, which is substantial for a biological trait. Explanations behind explained variance in 5-HIAA by season may be divided into conception-related and melatonin metabolism-dependent, as discussed elsewhere [12]. Although season of birth and 5-HIAA fitted a cosine model in a previous study [12], the exceptionally high September concentrations constituted a limitation of that study. The current sample size, which is more than twice the previous one and collected over almost a two-fold longer period, allows for more reliable conclusions and thus makes it unlikely that 5-HIAA in human CSF is

influenced by season of birth. The lack of HVA seasonality findings is in agreement with the previous study [12].

The genetic findings imply that in our study population 5-HTTLPR s-allele homozygous subjects are positively seasonal (i.e. tend to have relatively high CSF 5-HIAA in spring or low 5-HIAA in fall), while heterozygotes follow the expected cosine circannual pattern (i.e. their mean seasonality value is ~0) and L-homozygotes are negatively seasonal (i.e. show a flattened seasonal curve). We thereby extend the results of a PET study comparing s-allele carriers to l-homozygous subjects [14]. To our knowledge, no genetic study on CSF 5-HIAA seasonality in healthy subjects had been reported. In addition, the approaches adopted by all but one group [14] investigating seasonality of 5-HT measures entailed dividing the year into four seasons, as opposed to per-month modeling of 5-HT seasonality. Moreover, in contrast to the previous studies on 5-HT seasonality [14-18], the current sample size (N=414) yields adequate genotype distributions to analyze 5-HTTLPR dose-dependent associations with 5-HT seasonality.

Our observation of the positive correlation between 5-HT seasonality and depressive symptomatology suggests that not *absolute* 5-HIAA concentrations but *seasonal* patterns in 5-HT turnover variability could influence susceptibility to depressive symptomatology. Such a contention would be in line with epidemiological evidence pointing to a latitude-dependent seasonal variation in suicide rates, i.e. spring peaks in high latitude regions and absence of seasonal variation around the equator [29-31]. Speculatively, seasonal variation in CSF 5-HIAA may be more pronounced at higher latitudes, which in turn would confer risk for season-associated depression, and thereby suicide.

A limitation of this study is that psychiatric illness was not systematically assessed. However, running the same nlqr models after excluding the subjects on psychotropic medication during the lumbar puncture (N=13) and those with a self-reported history of psychiatric illness (N=23) did not change the CSF 5-HIAA seasonality findings (summary statistics not shown). A second potential caveat is that the 5-HTTLPR prediction model that we applied [19] explains 85% of the variation in the 5-HTTLPR, although this figure exceeds the R^2 of a different SNP-based 5-HTTLPR prediction model [32]. Notably, there is no 'gold standard' for 5-HTTLPR genotyping and direct genotyping is notoriously cumbersome [19]. Moreover, the genotype distributions reported here are identical to previously reported distributions in Dutch healthy individuals [19]. Regarding the correlation between 5-HT seasonality and depressive symptomatology, the sample size (N=345) and Spearman's rank correlation coefficient (r=0.13) were relatively small. We also recognize that lack of an independent replication cohort is a constraint given the uniqueness of this CSF cohort from the general population.

Our findings contribute to unmasking the molecular basis of seasonality of 5-HT turnover and the potential role of the 5-HTTLPR. Larger sample sizes are needed to

replicate our findings and further investigate the genetic basis of 5-HT seasonality. Targeted studies, such as PET, could be performed to examine the role of MAO-A, a major enzyme in the 5-HT pathway. MAO-A PET has demonstrated relevance for studying psychiatric phenotypes [33-35]. Our findings suggest a genetic association of the 5-HT transporter polymorphism with seasonal changes of 5-HIAA. Future studies should involve sampling around the equator as well as at different latitudes in the northern and southern hemispheres to gain a better understanding of how 5-HT turnover in CSF varies between seasons and around the globe. Moreover, such a study would provide the means to investigate the latitude-dependent role of the 5-HTTLPR and other genetic factors in 5-HT seasonality and possible relationships with neurobehavioral traits (such as SAD, bipolar disorder and suicide). When complemented with assessments of additional 5-HT-associated phenotypes (e.g. 5-HTT binding measured with PET), such projects may comprehensively dissect the genetic determinants of 5-HT physiology. This in turn may open avenues for applications in clinical populations with an established role for seasonality, such as affective disorders.

In summary, we highlight a dose-dependent association of the 5-HTTLPR s/l polymorphism with 5-HT seasonality and demonstrate that increased 5-HT seasonality is associated with depressive symptomatology. Notably, 5-HIAA seasonal variability and not absolute 5-HIAA concentrations show associations at genetic and behavioral levels, underscoring the biological relevance of 5-HT seasonality.

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Supplemental Information Legends

Supplemental Methods. *(page 158)* Genotyping quality control procedures; non-linear quantile regression models; 5-HT seasonality values.

Supplemental Table 1. *(page 161)* Non-linear quantile regression results.

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[1] *Department of Psychiatry, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht (UMCU), Utrecht, The Netherlands*
[2] *Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neuroscience, Utrecht University, Utrecht, The Netherlands*
[3] *Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht/Wilhelmina Children's Hospital, Utrecht, The Netherlands*
[4] *Department of Medical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands*
[5] *Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, University of California Los Angeles, Los Angeles, CA, USA*
[6] *Department of Anesthesiology, Intensive Care & Pain Management, Diakonessenhuis Hospital, Utrecht, The Netherlands*
[7] *Department of Anesthesiology, Central Military Hospital, Utrecht, the Netherlands*
[8] *Department of Anesthesiology, Intensive Care & Pain Management, UMCU, Utrecht, The Netherlands*
[9] *Department of Anesthesiology, Intensive Care & Pain Management, St Antonius Hospital, Nieuwegein, The Netherlands*
[10] *Department of Genetics, University Medical Center Groningen, Groningen, The Netherlands*

4

A Common Variant in *ERBB4* Regulates GABA Concentrations in Human Cerebrospinal Fluid

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Jurjen J. Luykx[1 #]
Christiaan H. Vinkers[1,2 #]
Steven C. Bakker[1]
Wouter Visser[3]
Loes van Boxmeer[1]
Eric Strengman[4,5]
Kristel R. van Eijk[4]
Judith A. Lens[6]
Paul Borgdorff[6]
Peter Keijzers[7]
Teus H. Kappen[8]
Eric P.A. van Dongen[9]
Peter Bruins[9]
Nanda M. Verhoeven[3]
Tom J. de Koning[3, 10]
René S. Kahn[1]
Roel A. Ophoff[1,5]

[#] *these authors contributed equally to this work*

Abstract

The neuregulin 1 (NRG1) receptor ErbB4 is involved in the development of cortical inhibitory GABAergic circuits and NRG1-ErbB4 signaling has been implicated in schizophrenia. A magnetic resonance spectroscopy (^{1}H-MRS) study has demonstrated that a single nucleotide polymorphism in *ERBB4*, rs7598440, influences human cortical GABA concentrations. Other work has highlighted the significant impact of this genetic variant on expression of *ERBB4* in the hippocampus and dorsolateral prefrontal cortex in human post mortem tissue. Our aim was to examine the association of rs7598440 with cerebrospinal fluid (CSF) GABA levels in healthy volunteers (n=155). We detected a significant dose-dependent association of the rs7598440 genotype with CSF GABA levels (G-allele standardized β=-0.23; 95% CIs: -0.39 - -0.07; p=0.0066). GABA concentrations were highest in A homozygous, intermediate in heterozygous, and lowest in G homozygous subjects. When excluding subjects on psychotropic medication (three subjects using antidepressants), the results did not change (G-allele standardized β=-0.23; 95% CIs: -0.40 - -0.07; p=0.0051). The explained variance in GABA by rs7598440 in our model is 5.2% (p=0.004). The directionality of our findings agrees with the aforementioned ^{1}H-MRS and gene expression studies. Our observation therefore strengthens the evidence that the A-allele of rs7598440 in *ERBB4* is associated with increased GABA concentrations in the human central nervous system (CNS). To our knowledge, our finding constitutes the first confirmation that CSF can be used to study genotype-phenotype correlations of GABA levels in the CNS. Such quantitative genetic analyses may be extrapolated to other CSF constituents relevant to schizophrenia in future studies.

Introduction

Single nucleotide polymorphism (SNP) and haplotype analyses have implicated neuregulin-1 (NRG1) in schizophrenia (SCZ) [1,2]. A genome-wide copy number analysis found evidence of structural ERBB4 (OMIM 600543) abnormalities in SCZ [3]. In addition, enhanced signaling of NRG1-ErbB4 in SCZ patients has been reported [4]. Converging evidence thus pleads for a role of the NRG1-ErbB4 pathway in SCZ, although the underlying mechanisms are currently unknown.

While the physiological ramifications of this pathway in the healthy human brain have yet to be fully understood, preclinical studies indicate that NRG1 is involved in neurodevelopment and brain plasticity, acting via ERBB receptor tyrosine kinases, such as ErbB4 [5]. *ERBB4* expression in the brain is largely restricted to parvalbumin-expressing GABAergic cortical interneurons (basket and chandelier cells) in humans, rodents and non-human primates [6,7]. NRG1-induced GABA release depends on ErbB4[8], resulting in suppression of firing of pyramidal neurons [9] and affecting interneuronal long-term potentiation and contextual fear conditioning [10].

Recently, a study highlighted the effect of a common variant in *ERBB4* on cortical GABA concentrations in healthy volunteers using proton magnetic resonance spectroscopy (^{1}H-MRS) [11]. The authors reported that A allele carriers of single nucleotide polymorphism (SNP) rs7598440 within the *ERBB4* gene possessed higher anterior cingulate cortical GABA concentrations than G-allele homozygous subjects. This suggests that genetic variation in ERBB4 signaling affects *in vivo* cortical GABA levels. As *in vivo* GABA concentrations in the prefrontal cortex measured by ^{1}H-MRS reflect a fraction of whole-brain GABAergic neurons [12], it remains unclear how this finding applies to other brain regions. GABA concentrations in the cerebrospinal fluid (CSF) have been proposed to reflect overall central GABA activity [13], which is supported by a rodent study demonstrating a high correlation (r=0.92) between CSF and brain GABA [14] and the rostrocaudal CSF GABA gradient hinting at a central nervous system (CNS) origin of CSF GABA [15].

In another study, rs7598440 was found to be associated with gene expression levels of *ERBB4* in the dorsolateral prefrontal cortex (DLPFC) and hippocampus [16], suggesting a cis-effect of this variant (or one that is tagged by it) on CNS *ERBB4* expression. We therefore hypothesized that the effect of the *ERBB4* rs7598440 genotype on GABA concentrations would not be limited to the anterior cingulate cortex but that the SNP mediates overall central GABA turnover. Additionally, our aim was to assess the validity of CSF to study genotype-phenotype correlations of central GABA activity. To our knowledge, no genetic linkage or association study on CSF GABA has been published to date. We thus investigated the association of rs7598440 with CSF GABA levels in healthy volunteers.

Materials and methods

Subjects

The ethics committee at the University Medical Center Utrecht (UMCU) and all local ethics committees approved this study. Volunteers were recruited at outpatient pre-operative screening services in four hospitals in and around Utrecht, The Netherlands, from August 2008 until March 2010: UMCU, the Central Military Hospital, Sint Antonius Hospital, and Diakonessenhuis. We included patients (i) undergoing spinal anaesthesia for minor elective surgical procedures, (ii) ranging between 18-60 years of age, and (iii) with four grandparents born in The Netherlands or other North-Western European countries (Belgium, Germany, UK, France, and Denmark). Written informed consent was obtained from the participants. Each candidate participant received a personal telephone interview by J.L. (a psychiatry resident) or a medical student trained by J.L. During this non-standardized interview, subjects with psychotic or neurological disorders were excluded and any use of psychotropic medication was assessed. A history of unipolar affective and anxiety disorders was allowed. To gauge the possible association of anxiety with CSF GABA levels, a Pearson correlation between the State and Trait Anxiety Inventory (STAI) and GABA was computed (α = 0.05) [17].

CSF collection

Subjects had fasted at least 6 hours prior to lumbar puncture (LP). Before administration of medication (either pre-medication or compounds for the purpose of anaesthesia), a 25-27 Gauge needle was inserted into the L1/L2, L2/L3, L3/L4, or L4/L5 interspace (estimated by the anaesthesiologist). A single sample of 6 mL of CSF was obtained from each subject. Any deviations from the instructed procedure were recorded, such as smaller amounts of CSF drawn. CSF was kept at 4°C and transported within 9 hours to the laboratory at UMCU. Each sample was immediately stored in fractions of 0.5mL and 1mL at -80 °C. One fraction of 0.5mL was used for GABA measurements.

CSF GABA measurements

The free GABA quantification method employed here is similar to an ultra-performance liquid chromatography – mass spectrometry (UPLC-MS/MS, multiple stage tandem mass spectrometry) based method for quantification of D-amino acids described elsewhere [18]. Out of a 0.5 ml aliquot, 50 µl was mixed with 25 µl of an internal standard solution containing 80 µM of [$^{13}C_3$]-L-serine (obtained from Cambridge Isotope Laboratories Inc., Andover, MA, USA). Subsequently, the sample was deproteinized by the addition of acetonitrile and derivatized with the chiral reagent (S)-NIFE (purchased from Sigma-Aldrich, Zwijndrecht, The Netherlands). Analyses were carried

out with a Waters Acquity UPLC system equipped with an Acquity 1.7 µm BEH-C18 2.1*100mm column and a VanGuard BEH-C18 2.1x5 mm pre-column. The UPLC system was coupled to a Waters Xevo MS operated in positive electron spray mode. The following mass reaction monitoring (MRM) settings were employed for GABA: parent ion=353.35 Da; daughter ion = 120.1 Da; cone voltage=20V, collision energy=24V; and for [$^{13}C_3$]-L-serine: parent ion=358.3; daughter ion=120.1; cone voltage=18V; collision energy=26V. The retention time of GABA was 10.27 min and that of [$^{13}C_3$]-L-serine 9.14 min. A calibration curve covering the concentration range of interest was included in each measurement session. The peaks were integrated using the computer software TargetLynx 4.1 (Waters, Milford, MA, USA).

Genotyping procedures and quality control

As part of another, larger study whole-genome SNP data were generated at the UCLA Neuroscience Genomic Core (UNCG) facility using the Illumina Human OmniExpress Beadchip and genotype data of rs7598440 were extracted for use in this study. All genetic quality control (QC) checks were performed using Plink v1.07 in 240 genotyped individuals, 155 of whom had available CSF GABA levels (see Supplemental Methods). Given the prior evidence on rs7598440 and our limited study population size resulting in insufficient power to perform whole-genome analyses, we studied the association of this single SNP with CSF GABA.

Quantitative trait locus (QTL) analyses

Normality of the CSF GABA distribution was checked using SPSS version 17 (SPSS Inc., Chicago, IL, USA) and defined by a Kolmogorov-Smirnov (K-S) test asymptotic two-tailed p-value >0.05. A linear model accounting for all six patient- and procedure-specific factors known to influence CSF GABA levels was performed in Plink v1.07. These patient covariates include age and sex [11, 19] while procedure-specific covariates include time elapsed prior to storage (number of hours from lumbar puncture until storage at -80 °C), storage duration, the rostrocaudal concentration gradient (reflected by subjects' height), and amount of CSF drawn [20, 21]. A maximum of 5% missing data per covariate was allowed and subjects with more than two missing covariates were excluded. Missing covariate data were replaced by the mean (in the event of height, means were computed separately for the two sexes). Two tests of robustness were carried out. First, subjects on psychotropic medication were left out and the same linear model was run. Second, only covariates that showed suggestive ($p<0.1$) Spearman's rho correlations with GABA were included in the model. Given the prior evidence for this locus, the significance threshold of the association analysis of rs7598440 with CSF GABA levels was set at $p<0.05$. Outcome measures (standardized regression coefficients, β), the minor allele frequency (MAF), the genotyping success rate, and Hardy-Weinberg equilibrium (HWE) of rs7598440 were computed in Plink.

Boxplots of genotype-phenotype associations were generated in SigmaPlot 11. The explained variance by rs7498440 was computed by subtracting the R^2 in a linear regression model including covariates that showed suggestive Spearman's rho correlations ($p<0.1$) with logGABA from the R^2 of a linear model additionally including the rs7598440 genotype (logGABA being the dependent variable; $\alpha = 0.05$).

Results

Subject characteristics

Quality control based on genetic data resulted in the exclusion of four subjects with available CSF GABA levels, leaving 151 subjects with measured CSF GABA concentrations for further study (Supplemental Methods). No correlation between CSF logGABA and anxiety state and trait measures (which were normally distributed and filled out by 87% of the subjects) was detected ($p=0.9$). While data were complete for three covariates, missing data ranged from 1-5% per covariate for the other three. For one individual multiple covariates were missing, leaving a total of 150 subjects for the linear model. Three of the 151 subjects were on psychotropic medication (SSRIs and an SNRI). An overview of the study population, GABA measurements, covariates, and genotype distributions is shown in Table 1.

Table 1. Subject characteristics; means (S.D.) of subject characteristics and GABA levels. Only subjects passing genetic quality control are shown.

Parameter	Total sample	AA genotype	AG genotype	GG genotype
Subjects (N)	151	50	79	22
Age, mean	40.4	38.6	41.8	39.6
Sex (% male)	70	74	67	73
CSF GABA in µmol/L (S.D.)	0.47 (0.28)	0.54 (0.31)	0.44 (0.21)	0.40 (0.36)
log CSF GABA in µmol/L (S.D.)	-0.40 (0.24)	-0.33 (0.23)	-0.40 (0.21)	-0.51 (0.29)
Time elapsed prior to storage in hours (S.D.)	5.68 (2.16)	6.34 (2.19)	5.48 (2.04)	4.89 (2.23)
Storage time in months (S.D.)	9.01 (3.08)	9.63 (3.46)	8.61 (2.92)	9.69 (2.47)
Subject height in cm (S.D)	180.4 (9.5)	180.4 (8.3)	180.5 (10.0)	180.3 (10.7)
Amount CSF drawn in ml (S.D.)	5.62 (0.60)	5.57 (0.65)	5.61 (0.57)	5.75 (0.61)

CSF GABA

As CSF GABA was not normally distributed (K-S p=0.001), values were logarithm (log) transformed. This resulted in a normal distribution (K-S p=0.84, Figure 1). Mean (S.D.) CSF GABA and logGABA levels of these subjects were 0.47 (0.28) and -0.40 (0.24) µmol/L, respectively (Table 1).

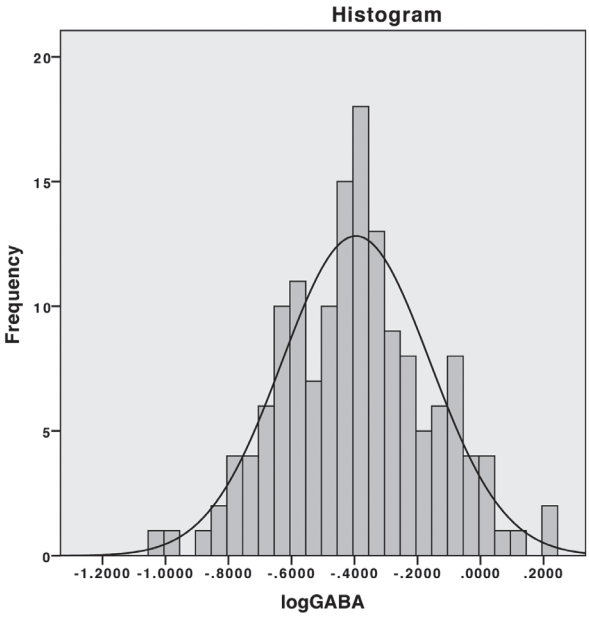

Figure 1. Histogram showing the distribution of log transformed CSF GABA levels (µmol/L).

Association of rs7598440 with CSF GABA levels

Genotyping was successful in all subjects and no departure from HWE was detected (p=0.11); the MAF was 0.42, which is equal to the previously reported frequency based on the 1000 genomes project (CEU population; http://www.ncbi.nlm.nih.gov/snp).

A significant dose-dependent association of the rs7598440 genotype with CSF logGABA levels was detected (β=-0.23; p=0.0066; Figure 2 and Table 2), i.e. logGABA (and therefore GABA) concentrations were highest in A homozygous, intermediate in heterozygous, and lowest in G homozygous subjects. The explained variance in logGABA by the rs7598440 genotype in our data set is 5.2% (p=0.004). The two tests of robustness resulted in similar findings. When excluding the three subjects on psychotropic medication, the results did not change (β=-0.23; p=0.0051, Table 2). The only covariate showing suggestive Spearman's rho correlations with logGABA was time elapsed prior to storage (Spreaman's rho= -0.21, p=0.011). The results for the linear model correcting for only this covariate were: β=-0.25; p=0.0031 (Table 2).

Table 2. Summary Statistics of the Linear Models: on All Subjects for whom GABA, Genotypes, and Covariates Were Measured (N = 150); on Those Who Were Not on Psychotropic Medication N = 147); and for the Analysis Including Only Covariates that Correlated with GABA (N = 150). β= standardized regression coefficient; SE= standard error; 95%CI= 95% confidence interval.

N	β	SE	95% CI	t-statistic	p-value
150	-0.225	0.082	-0.39 - -0.07	-2.76	**0.0066**
147	-0.234	0.082	-0.40 - -0.07	-2.85	**0.0051**
150	-0.245	0.081	-0.40 - -0.09	-3.01	**0.0031**

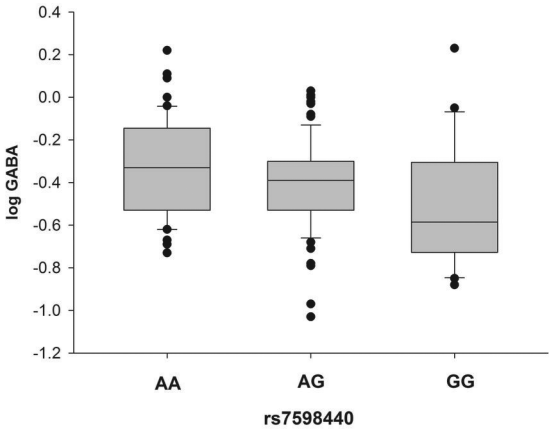

Figure 2. Log transformed GABA levels (μmol/L) by rs7598440 genotype: interquartile ranges (boxes) with medians (lines in boxes), whiskers (10-90 percentiles), and dots (values falling outside the 10-90 percentiles).

Discussion

In the first endeavor to identify a quantitative trait locus associated with cerebrospinal fluid GABA levels, a dose-dependent association of the common variant rs7598440 in *ERBB4* was detected. These results confirm a ^{1}H-MRS finding that the A-allele of rs7598440 increases GABA concentrations in the human CNS [11] and strengthen the evidence that this variant is implicated in *in vivo* GABA metabolism in the CNS.
The role of *ERBB4* in controlling cortical GABA circuitry development was previously demonstrated [6]. At a general genetic level, it is known that synonymous [22] and intronic [23] SNPs may contribute to phenotypic variation by means of genotype-specific differences in gene expression levels, RNA stability, RNA splicing, as well as in protein translation rate and protein folding. The demonstrated cis-effect of intronic rs7598440 on human post-mortem *ERBB4* expression and splicing in the hippocampus (p=0.009) and DLPFC (p=0.03) may be viewed in light of such phenomena [16]. Our results are in keeping with this *ERBB4* expression finding in that the A-allele that increases *ERBB4* expression in these tissues [16] was associated with elevated CSF GABA levels in the current study.

The exact genetic mechanisms underlying rs7598440-induced effects on CSF GABA levels are currently unknown. For example, there is no evidence suggesting marked sequence conservation between species at this intronic region of *ERBB4* or the presence of a regulatory element in the sequence immediately surrounding rs7598440 (http://genome.ucsc.edu/). In addition, the link between the NRG1-ErbB4 pathway and the pathophysiology of SCZ has yet to be elucidated, although a detected relation between NRG1, ErbB4, glutamate, and dopamine implicates the pathway in neurotransmitter systems relevant to SCZ [24]. The agreement in directionality between the ^{1}H-MRS signal [11] and the signal in our study indicates a high validity of both approaches in genetic quantitative analyses of CNS GABA. In this context, our thorough assessment of and correction for all covariates that are known to influence CSF GABA levels are likely to have benefited the reliability of our analyses. The ^{1}H-MRS study corrected for the same patient-related covariates (age and sex) but clearly different procedure-related covariates were accounted for [11].

A limitation of the current study is that no standardized psychiatric interview was employed to screen for psychiatric illness in the study population. Future studies may overcome this caveat by relating psychiatric diagnoses (especially anxiety disorders, alcohol dependence and abuse) to GABA levels and SNP data. On a similar note, the current design does not allow to parse possible age, sex or other genotype specific modulations of preoperative stress influences on CSF GABA levels. Quantifying perioperative stress in upcoming projects may be a way to address such uncertainties.

To our knowledge, our finding constitutes the first confirmation that CSF can be used to study genotype-phenotype correlations of CNS GABA levels. Such quantitative genetic analyses may be extrapolated to other CSF constituents relevant to SCZ in future studies, e.g. amino acids involved in glutamatergic pathways. An interesting question remains whether intra-individual changes in CSF GABA concentrations reflect central GABA activity. This may only be addressed in prospective studies with longitudinal measures of CSF GABA concentrations within the same individuals so that possible changes in these levels can be correlated with genotypes and expression of *ERBB4*.

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Supplemental Information Legend

(available online at www.nature.com/npp)

Supplemental Methods. Description of genotyping quality control measures.

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[1] *Department of Psychiatry, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht (UMCU), Utrecht, The Netherlands*
[2] *Department of Medical Genetics, UMCU, Utrecht, The Netherlands*
[3] *Department of Pharmacy, University of Pisa, Italy*
[4] *Department of Clinical and Experimental Medicine, University of Pisa, Italy*
[5] *Julius Center, UMCU, Utrecht, The Netherlands*
[6] *Department of General Practice & Elderly Care, VU Medical Centre Amsterdam, The Netherlands*
[7] *Department of Human Genetics, University of California, Los Angeles, California, USA*
[8] *Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, University of California Los Angeles, Los Angeles, CA, USA*

5

BDNF Val66Met Homozygosity Does Not Influence Plasma BDNF Levels in Healthy Human Subjects

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Jurjen J. Luykx[1,2]
Marco P.M. Boks[1]
Elemi J. Breetvelt[1]
Maartje F. Aukes[1]
Eric Strengman[2]
Eleonora da Pozzo[3]
Liliana Dell'osso[4]
Donatella Marazziti[4]
Annelies van Leeuwen[1]
Annabel Vreeker[1]
Lucija Abramovic[1]
Claudia Martini[3]
Mattijs E. Numans[5,6]
René S. Kahn[1]
Roel A. Ophoff[1,7,8]

Abstract

A putative pathway by which the BDNF Val66Met polymorphism (rs6265) leads to aberrant phenotypes is its influence on plasma BDNF. Research into the impact of rs6265 on plasma BDNF has given rise to conflicting results. Moreover, most such studies have compared Met-carriers with Val-homozygous subjects. We therefore genotyped subjects from a population-based cohort (the Utrecht Health Project, N=2,743) and assessed whether plasma BDNF differs between rs6265 homozygous groups. We maximized the number of Met-homozygous subjects in whom we measured plasma BDNF, resulting in plasma BDNF being available for 19 Met-homozygous and 42 matched Val-homozygous subjects. Mean concentrations (S.D.) were 1963.1 (750.1) and 2133.2 pg/ml (1164.3) for the Val/Val and Met/Met groups, respectively. Using ANOVA, no differences in plasma BDNF between the two groups were detected. In conclusion, these results add to a growing body of evidence indicating that allelic variation at rs6265 does not have medium to large effects on plasma BDNF concentrations.

Introduction

The involvement of the *BDNF* Val66Met polymorphism (rs6265) in psychiatric disorders has been extensively studied, in particular for major depressive disorder [1] and smoking [2]. The polymorphism furthermore mediates BDNF secretion by neurons [3] and is implicated in human memory [4], hippocampal volume [5], and anxiety in mice [6].

A hypothesized pathway by which the *BDNF* Val66Met polymorphism leads to aberrant phenotypes is its influence on plasma BDNF levels. In the body fluids of healthy volunteers, associations of this single nucleotide polymorphism (SNP) with serum BDNF levels have been reported [7, 8]. Moreover, as indicated by amniotic fluid measurements [9], the influence of rs6265 on BDNF protein levels may start as early as during fetal development. Notably, most of the associations between the *BDNF* Val66Met polymorphism and BDNF in blood are weak and non-replication papers have been published for serum [10, 11] and plasma [12] BDNF. An open question remains how this lack of replication is best explained. A confounding factor may be the relatively low minor allele frequency (MAF) of the Met-variant in populations with European ancestry [13], which has given rise to designs that combine homozygotes and heterozygotes of the minor allele in one group. However, the effect of a homozygous genotype may have greater impact on a phenotype than heterozygosity. For the *Bdnf* Val66Met polymorphism, this was illustrated in mice: increased anxiety behavior was observed for the $Bdnf^{Met/Met}$ genotype compared to heterozygotes and a dose-dependent effect of the Met allele on memory was demonstrated [6]. We accordingly hypothesized that the effect size of rs6265 homozygosity-dependent impact on plasma BDNF is medium to large.

To study possible differences in plasma BDNF levels between the two rs6265 homozygous groups, 2,743 subjects from a population-based cohort were genotyped. We maximized the number of Met-homozygous subjects in whom plasma BDNF was measured and matched these to a twice as large Val-homozygous group.

Methods

Study subjects

Participants were part of the Building Blocks study, which was approved by the Medical Ethics committee of the University Medical Center Utrecht. The advantage of our "forward genetics" approach whereby we selectively sampled participants with the most informative genotypes was described previously [14]. Subject recruitment started in January 2009 and was outlined previously [15]. In brief, subjects aged between 18 and 65 years of age were recruited from the Utrecht Health Project [16], an ongoing population-based study in a Dutch residential area (the Leidsche Rijn, Utrecht).

The first 2,743 Caucasian participants with four Dutch grandparents who consented to participate were genotyped. Relatives of these participants were excluded from participation. To maximize the number of rs6265 Met-homozygous participants, all 97 subjects with this genotype and a more than twice as large Val-homozygous group matched for age, sex, and residential neighborhood were invited to participate.

Genotyping

DNA was extracted from peripheral blood lymphocytes using established procedures. Genotyping of SNP rs6265 in the BDNF gene was determined using a validated Taqman® SNP Genotyping assay (Applied Biosystems, Foster City). PCR was executed using an Applied Biosystems GeneAmp 9700 thermal cycler. Allelic discrimination was conducted on an Applied Biosystems 7900HT Real-Time PCR System using the Sequence Detection Software (SDS v2.3, Applied Biosystems, Foster City) according to the manufacturer's protocol. Genotyping was performed twice for each subject to detect possible sample mix-ups. Only subjects with corroborated genotypes were included.

Blood sample handling and BDNF assay

Blood was drawn into 6cc EDTA-tubes, an hour after which plasma was extracted. At least 4 cc of blood from each study subject were centrifuged at 2000g during 15 minutes and subsequently at 10,000g during 5 minutes, both at 2-8°C. We transferred plasma into 2 polypropylene tubes and stored these at -20 °C until plasma BDNF levels were measured.

To measure BDNF concentrations, poor-platelet-plasma was aliquoted and plasma levels of BDNF were determined with an enzyme-linked immunosorbent assay (ELISA) method (BDNF Emax Immunoassay System, Promega, Wallisellen, Switzerland), with a specific detection of BDNF (less than 3% cross-reactivity with other related neurotrophic factors at 100ng/ml), a sensitivity of 15.6pg/ml and an inter-assay variation measured at 8.8% (low concentration), 2.9% (medium concentration) and 2.2% (high concentration). To measure the amount of total BDNF, acidification and subsequent neutralization of the samples were followed before proceeding with the ELISA protocol, as previously reported [17].

Statistical analyses

We used SPSS version 20 (SPSS Inc., Chicago, Il) for the statistical analyses. Normality of total plasma BDNF was checked by Kolmogorov-Smirnov (K-S) testing (two-tailed p-value ≥ 0.05). BDNF plasma outliers - defined as exceeding more than 3 standard deviations (SD) from the mean - were excluded. We used GPower 3.1 [18] to calculate a critical F and effect size, setting power at 80% and the type I error rate at 0.05.

Age and weight [19], but not time of day of sampling [20] are considered relevant covariates for plasma BDNF. Age, weight and sex were therefore investigated for a possible impact on BDNF plasma levels in a general linear model (entering sex, age and weight at the day of plasma withdrawal as independent variables; p-value cut-off of 0.05 for inclusion of these possible covariates). ANOVA (or ANCOVA in the event associated covariates were detected) was used to assess possible differences in BDNF plasma levels per genotype (Met/Met vs Val/Val). Variances were considered equal if the Levene's test p-value was ≥ 0.05.

Results

Genotype Distributions and Subject Characteristics

Genotype corroboration resulted in the exclusion of 111 out of 2,743 subjects (4.0%). rs6265 was in Hardy-Weinberg equilibrium (P = 0.64), the MAF was 0.19 (in line with Hapmap CEU frequencies [13]) and the genotype distributions were: 97 TT (Met-homozygous, 3.7%); 836 CT (30.5%) and 1,731 CC (61.9%).

Nineteen Met-homozygous subjects consented to come to our institution for blood withdrawal that served for plasma extraction. These were included in the current study and matched to 42 Val-homozygous participants. For the Val-Val and Met-Met groups respectively, the mean ages (SD) were 48.6 (11.5) and 41.5 (9.80) years; the mean body weights 82.5 (23.5) and 75.9 (8.8) kgs; the mean IQs 106.0 (15.8) and 107.7 (19.9) points; and the percentage of male participants 56 and 63. Using t and chi-square tests, none of these subject characteristics was found to differ between the two selected groups.

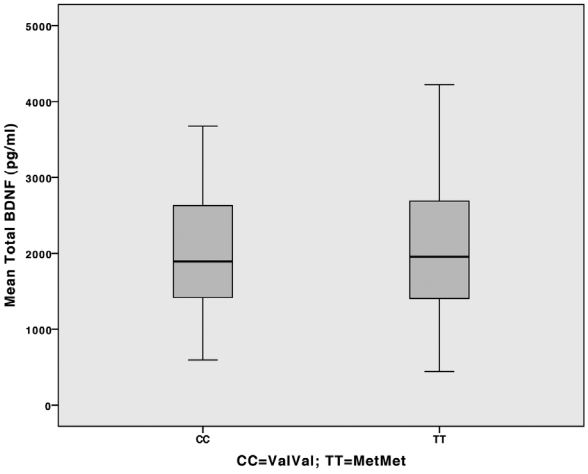

Figure. Mean total BDNF did not differ between the two homozygous genotype groups: the mean concentrations (S.D.) per genotype group were 1963.1 (750.1) in the Val/Val group (N=41) and 2133.2 pg/ml (1164.3) in the Met/Met group (N=18). The boxes represent the interquartile range; the lines in the boxes the median; and the whiskers indicate the range of measured values.

Plasma BDNF levels

Two outliers were excluded: one in the Met-Met and one in the Val-Val group. The total BDNF plasma concentrations were normally distributed (K-S test P = 0.39). Age, sex and weight did not influence BDNF plasma levels.

We had 80% power to detect an effect size of 0.37 (critical F = 4.0). Mean concentrations (S.D.) per genotype group were: 1963.1 pg/ml (750.1) in the Val/Val group (N=41) and 2133.2 pg/ml (1164.3) in the Met/Met group (N=18). Considering that the variances were unequal between groups (Levene's test P = 0.041) with the larger variance in the smaller Met/Met group, the ANOVA may be biased towards rejecting the null hypothesis. In other words, the chance to find between-group differences was inflated. However, plasma levels did not differ by homozygous genotype group (P = 0.6, Figure).

Discussion

Despite enrichment of our sample with subjects homozygous for the rare *BDNF* rs6265 Met allele, we found no effect of *BDNF* Val66Met homozygosity on plasma BDNF.

A plausible account for these negative findings is that the polymorphism does not substantially control plasma or serum BDNF, as indicated by a growing body of evidence [21-23]. The authors of those studies were unable to detect an impact of rs6265 on plasma, serum or whole blood BDNF. Moreover, meta-analytical evidence also points to a lack of association between serum BDNF and the Val66Met polymorphism[23]. Lack of power may additionally explain the negative findings, particularly considering that actual effect sizes may be smaller than the one hypothesized and detectable according to our power calculation. On the other hand, this study has employed the largest rs6265 Met-homozygous group to study effects on plasma BDNF in healthy human subjects to date. Furthermore, the plasma BDNF measurements varied substantially across subjects in the current study, which is reflected by the high standard deviations and may have resulted from low test-retest measurement correlations [21]. Alternatively, this substantial between and within-subject variability may arise from barely quantifiable factors such as mental activity, stress and diet. Finally, given the weak correlation between serum and plasma BDNF [19, 24, 25] meta-analyses including both serum and plasma BDNF studies may parse body fluid-dependent effects on genotype-phenotype relationships.

Conclusion

We conclude that allelic variation at rs6265 does not have medium to large effects on plasma BDNF in the Dutch population. To investigate the impact of rs6265 on plasma

BDNF further, large sample sizes allowing for stratification by sex may be useful given sex-dependent differences in the impact of the polymorphism on serum BDNF [26] and depression [1]. Substantial disparities in rs6265 Met allele frequencies and *BDNF* linkage disequilibrium patterns exist across populations [13]. Therefore, we cannot rule out rs6265 homozygosity-dependent effects on plasma BDNF in other populations.

Acknowledgements

We regret the loss of Professor Herman Westenberg, who unexpectedly passed away during this project. His contributions to the set-up of the study have proven invaluable. We thank the Utrecht Health Project investigators for the inclusion of the study cohort participants.

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[1] *Department of Psychiatry, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht (UMCU), Utrecht, The Netherlands*
[2] *Department of Medical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands*
[3] *Department of Metabolic Diseases, University Medical Center Utrecht/Wilhelmina Children's Hospital, Utrecht, The Netherlands*
[4] *Computer Science Department, University of California, Los Angeles, California, USA*
[5] *Department of Human Genetics, University of California, Los Angeles, California, USA*
[6] *Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, University of California Los Angeles, Los Angeles, CA, USA*
[7] *Department of Anesthesiology, Intensive Care & Pain Management, UMCU, Utrecht, The Netherlands*
[8] *Department of Anesthesiology, Intensive Care & Pain Management, Diakonessenhuis Hospital, Utrecht, The Netherlands*
[9] *Department of Anesthesiology, Intensive Care & Pain Management, St Antonius Hospital, Nieuwegein, The Netherlands*
[10] *University of Groningen, University Medical Center Groningen, Department of Genetics, Groningen, the Netherlands*

6

Genome-Wide Study of NMDA Receptor Coagonists in Cerebrospinal Fluid and Plasma Identifies Metabolic and Transporter Pathways

Submitted

Jurjen J. Luykx[1, 2]
Steven C. Bakker[1]
Wouter F. Visser[3]
Nanda Verhoeven-Duif[3]
Jacobine E. Buizer-Voskamp[1]
Jonas M. den Heijer[1]
Marco P.M. Boks[1]
Jae Hoon Sul[4]
Eleazar Eskin[4, 5]
Rita M. Cantor[5]
Jacob Vorstman[1]
Eric Strengman[2]
Joseph DeYoung[6]
Teus H. Kappen[7]
Esther Pariama[8]
Eric P.A. van Dongen[9]
Paul Borgdorff[8]
Peter Bruins[9]
Tom J. de Koning[3, 10]
René S. Kahn[1]
Roel A. Ophoff[1, 5, 6]

Abstract

The N-methyl-D-aspartate receptor (NMDAR) coagonists Glycine, D-Serine, L-Proline and possibly D-Alanine play crucial roles in NMDAR-dependent neurotransmission and are associated with a range of neuropsychiatric disorders. We conducted the first genome-wide association study (GWAS) of concentrations of these coagonists and their enantiomers in plasma and cerebrospinal fluid (CSF) of human subjects from the general population (N=414). Genetic variants at chromosome 22q11.2, located in and near *PRODH*, were associated with L-Proline in plasma ($\beta = 0.29$; $P = 6.38 \times 10^{-10}$). The missense variant rs17279437 in the Proline transporter SLC6A20 was associated with L-Proline in CSF ($\beta = 0.28$; $P = 9.68 \times 10^{-9}$) and the L-Proline plasma-CSF ratio ($\beta = -0.30$; $P = 2.88 \times 10^{-9}$). Suggestive evidence of association was found for the D-serine plasma-CSF ratio at the D-amino-acid oxidase (*DAO*) gene ($\beta = -0.28$; $P = 9.08 \times 10^{-8}$), whereas a variant in *SRR* (that encodes serine racemase) constituted the most strongly associated locus for the L-Serine to D-Serine ratio in CSF. All these genes are highly expressed in rodent meninges and choroid plexus. The enzymes and transporters they encode may be targeted to further construe the nature of NMDAR coagonist involvement in NMDAR gating. Moreover, the highlighted genetic variants may be followed up in clinical populations, e.g. schizophrenia and 22q11 deletion syndrome. We conclude that CSF is a meaningful and informative body fluid for quantitative trait locus analyses.

Introduction

The N-methyl-D-aspartate receptor (NMDAR) coagonists Glycine, D-Serine, L-Proline and possibly D-Alanine play crucial roles in NMDAR-mediated neurotransmission. While D-Serine primarily mediates synaptic NMDAR gating [1, 2], Glycine acts on extraynaptic NMDARs [2, 3]. D-Alanine and L-Proline, although understudied in preclinical settings, also act on NMDAR binding sites [4-8].

NMDAR coagonists are involved in neuropsychiatric disorders, as demonstrated by pharmacological and genetic findings. For example, hyperprolinemia is associated with schizophrenia (SCZ) [9] and schizoaffective disorder [10]. Decreased levels of D-Serine have been reported in cerebrospinal fluid (CSF) of SCZ patients [11, 12] and low Glycine levels were observed in CSF of patients suffering from affective disorders [13]. Moreover, D-cycloserine, D-Alanine and Glycine constitute (experimental) adjuvant pharmacological options in the treatment of SCZ [14-19] and anxiety disorders [20-24]. Finally, a number of studies on genes hypothesized to be involved with NMDAR coagonist metabolism (e.g. *PRODH* and *DAO*) have provided evidence for a role of such genes in SCZ [25-29].

Heritability estimates for amino acids in serum range from 0.23 to 0.55 [30]. Genome-wide association studies (GWASs) of amino acid levels in urine and serum have yielded quantitative trait loci (QTLs) in genes encoding enzymes (e.g. *NAT2* and *AGXT2* [31]) and several members of the solute carrier family (e.g. *SLC2A4*, *SLC25A1* [30] and *SLC6A20* [31]). Such associations were detected for (ratios of) several amino acids, whereas L and D-enantiomers have not yet been subjected to GWASs. More importantly, it is unknown how urine and serum findings should be interpreted for the central nervous system (CNS) given the presence of the blood-brain-barrier. When studying the genetic control of NDMAR coagonists in the CNS, CSF may be the most informative body fluid as it is most proximate to the CNS. In addition, the critical role of CSF in CNS signaling was recently demonstrated [32]. Furthermore, while knowledge regarding the differential effects of amino acid L and D-enantiomers on the CNS has been accumulating over the past decade, genetic mechanisms underlying stereoisomer concentration variations remain elusive. For instance, it has not been established whether conversion from L-Serine constitutes the prime source of endogenous D-Serine [29, 33]. The discovery of the D-amino acid synthesizing enzyme Serine Racemase (SRR) in the mammalian brain and its implication in neurotransmission have illustrated the relevance of D-amino acid signaling molecules for the CNS [34-36]. As genes that moderate D-amino acid concentration variation have not been established, quantitatively analyzing the genetic mechanisms underlying such traits may elucidate D-amino acid metabolism and transporter pathways. Moreover, clarifying the genetic underpinnings of NMDAR coagonist concentration variations may deepen the understanding of NMDAR physiology. Such insight in turn may open avenues for

preclinical studies aimed at dissecting the localization of NMDAR coagonist-dependent gating [2] and clinical applications, e.g. improvements in add-on pharmacotherapy in SCZ and disease prediction, as was shown for diabetes [37].

We thus set out to study the genetic underpinnings of variation in NMDAR coagonists in both plasma and CSF by analyzing genome-wide data of 414 healthy human subjects. We hypothesized that our design of measuring both L and D-amino acids in two body fluid compartments using a tandem mass spectrometer in addition to liquid chromatography would augment power to detect single nucleotide polymorphisms (SNPs) relevant to biological processes underlying amino acid turnover in the CNS. Several genome-wide significant neurometabolic quantitative trait loci (QTLs) in genes encoding transporter proteins and enzymes were detected. We confirmed the robustness of the QTLs by permutation testing and, for a plasma QTL, replication in human serum. Finally, the highlighted genes have rodent orthologues that are all highly expressed in the choroid plexus and meninges, regions of prime interest to CSF physiology.

Results

Descriptive Statistics (Table 1) and Quality Control

Study population and NMDAR coagonist characteristics are tabulated in Table 1. On average, 4.2 outliers (range: 0-11) were removed per measured metabolite, leaving measured values for an average of 378 subjects (range: 362-393). None of the QQ-plots was suggestive of population stratification as the genomic inflation correction factors (λ_{GC}) ranged between 0.98 and 1.05. Supplemental Figure S1 shows the QQ-plots of the associations mentioned in the text, all of which had λ_{GC} of 1.00 and 1.01.

Genome-Wide Significant Loci Detected for L-Proline in Plasma and CSF

Genome-wide association signals in plasma were strongest for L-Proline (Figure 1A). Five variants on 22q11 in complete linkage disequilibrium (LD) -intronic in *DiGeorge Syndrome Critical Region Gene 5 (DGCR5)* and 35kb from *Proline Dehydrogenase (PRODH)*- showed association at a genome-wide significant level (rs2518810, rs2540640, rs2540641, rs2518814, and rs2540644; chr 22q11.2; $\beta = 0.29$; $P = 6.38 \times 10^{-10}$; Figure 2A). Loci within *PRODH* showed similarly strong associations with L-Proline in plasma. Two variants in pairwise LD ($r^2 = 0.87$) were strongly associated with L-Proline in plasma: rs3970551 ($\beta = 0.28$; $P = 2.62 \times 10^{-9}$) and rs2904552 ($\beta = 0.27$; $P = 1.65 \times 10^{-8}$). Both these SNPs are in LD with the abovementioned SNPs in DGCR5 (LD r^2 of 0.60 and 0.54, respectively). The latter is a missense variant resulting in an Arg to His change in the *PRODH* coding region. We observed no evidence of genome-wide significant associations with the other metabolic traits in plasma.

Table 1. Subject characteristics of the 414 genotyped subjects and of measured L and D-enantiomers after removal of values exceeding the mean ± 3 S.D. (in µmol/L).

	N	Mean	S.D.	Minimum	Maximum
Age (years)	414	40	11	18	60
Sex (male)	299				
Storage time (months)	414	7.8	5.0	1	33
L-Alanine in CSF	392	31.6	7.92	16.1	72.0
D-Alanine in CSF	383	0.16	0.09	0.04	0.56
L-Alanine in plasma	367	334	74.0	185	561
D-Alanine in plasma	365	0.85	0.47	0.09	2.71
Glycine in CSF	390	6.61	3.03	3.03	35.5
Glycine in plasma	365	165	49.4	5.66	330
L-Serine in CSF	388	23.7	3.91	14.7	47.0
D-Serine in CSF	393	1.26	0.23	0.78	2.21
L-Serine in plasma	367	84.6	16.9	22.0	141
D-Serine in plasma	369	1.09	0.26	0.42	1.78
L-Proline in CSF	392	0.79	0.60	0.07	6.39
D-Proline in CSF	389	0.01	0.01	0.00	0.08
L-Proline in plasma	363	176	43.9	86.8	324
D-Proline in plasma	362	0.32	0.18	0.01	1.18

The strongest signal in CSF was also found for L-Proline. SNP rs17279437 in the Proline transporter *SLC6A20* showed significant association with L-Proline in CSF (chr 3p21.31; $\beta = 0.28$; $P = 9.68 \times 10^{-9}$). Two other SNPs in strong LD with rs17279437 ($r^2 = 0.9$) showed genome-wide significance for L-Proline in CSF (rs73058498 and rs73060324; chr 3p21.31; $\beta = 0.28$; $P = 6.57 \times 10^{-9}$; Figures 1B and 2B).

We next computed ratios between plasma and CSF concentrations of the same amino acid and ratios between L and D-stereoisomers within the same compartment (resulting in 1 ratio for Glycine and 4 for the other amino acids). In GWASs of serum and urine metabolites, using ratios yielded a number of QTLs additional to the ones detected for measured values [31, 37]. Furthermore, whenever a shared enzymatic pathway metabolizes a pair of metabolites, association tests of ratios increase power [38]. Again, genome-wide significance was reached: the same variant as mentioned above for L-Proline in CSF -rs17279437 in the Proline transporter *SLC6A20*- was associated with the L-Proline plasma-CSF ratio ($\beta = -0.30$; $P = 2.88 \times 10^{-9}$; Figures 1C and 2C). That genome-wide significant association with this nonsynonymous variant was detected for both L-Proline in CSF and the plasma-CSF ratio internally validated the strength of the association at the *SLC6A20* locus.

When testing candidate polymorphisms previously associated with NMDAR coagonists in GWASs, we observed no evidence of additional loci involved in these traits.

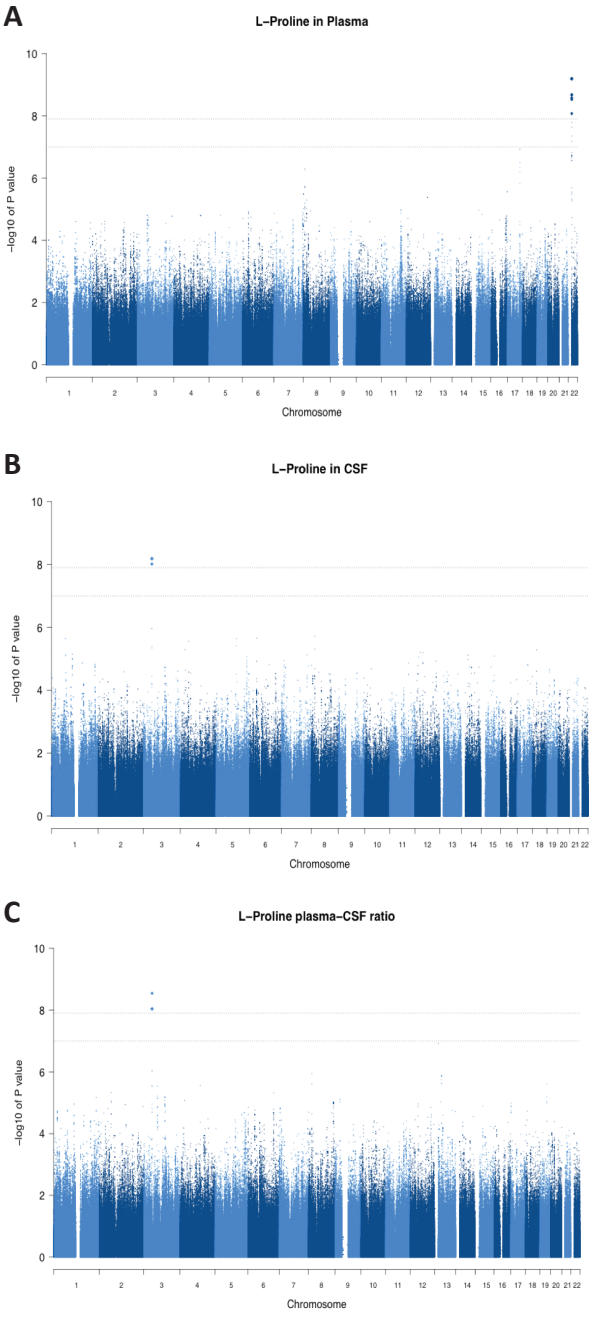

Figure 1. Manhattan Plots of Genome-Wide Significant Neurometabolic Quantitative Trait Loci:
A. L-Proline in Plasma
B. L-Proline in CSF
C. The L-Proline Plasma-CSF ratio

Replication in Human Serum

We used an online tool [39] to try and replicate the abovementioned association for L-Proline in plasma. Strong evidence of replication was found for the most strongly associated locus (rs2540641 on 22q11): rs2023634 (LD r^2=0.71) is associated with the Proline/Valine ratio in one of the two study populations of that study (N= 1,768) at P = 1.21 x 10^{-25}, while two other SNPs in strong LD with rs2540641 (r^2 > 0.8) are associated with other Proline ratios in the other study population (N= 1,052) at P = 1.03 x 10^{-5} and P = 1.18 x 10^{-6}. Although genotyping coverage was incomplete for the *PRODH* region in that study, the authors consider the rs2023634 signal the lead locus for Proline within/near *PRODH* [39].

Permutation Testing Confirms Neurometabolic QTLs

As a test of robustness we next carried out 1 / 1.25 x 10^{-8} = 8 x 10^{7} permutations in Plink v1.07 [40] to establish whether the null hypothesis (H_0) -lack of association- could be rejected. This label swapping procedure annuls all genotype-phenotype relationships, thereby providing a virtual dataset sampled under the H_0 [40]. Permutation testing indeed confirmed the results for L-Proline in Plasma (rs2540641 and all variants in compete LD), the L-Proline plasma-CSF ratio (rs17279437) and the CSF L-Proline (rs73058498 and rs73060324) associations, while an empirical P-value of 2.5 x 10^{-8} was computed for the QTL at rs17279437 with L-Proline in CSF.

SNP Conditioning Determines Independency of L-Proline Association Signals

To determine whether the highlighted loci independently contribute to the abovementioned L-Proline association signals, we conditioned on SNPs. When conditioning on the SNPs associated with L-Proline in plasma, the association results for rs17279437 with L-Proline in CSF did not change (β = 0.28; P = 7.36 x 10^{-9}). Vice versa, when conditioning on rs17279437, the association results for the L-Proline in plasma locus did not change (β = 0.29; P = 7.58 x 10^{-10}). We thus confirmed the independency of the L-Proline QTLs.

CSF D-Serine Findings Suggest Involvement of D-amino acid oxidase (DAO)

Suggestive evidence of association (P < 10^{-7}) was found between two common intronic variants in complete LD located in D-amino acid oxidase (*DAO*) and the D-serine plasma-CSF ratio (rs2070587 and rs4964766; chr 12q24.11; β = -0.28; P = 9.08 x 10^{-8}; Figure 2D). Other suggestive signals (Table 2) –all of which are outside genes with known functions- were found for the L-Alanine plasma-CSF ratio (rs4377332, rs13414424, and rs4674607; chr 2q36.1; β = -0.28; P = 2.91 x 10^{-8}), the D-Alanine plasma-CSF ratio (rs352052 and rs422140; chr 4q13.3; β = -0.30; P = 3.38 x 10^{-8}) and Glycine in CSF (rs115153038; chr 14q32.12; β = 0.27; P = 3.56 x 10^{-8}).

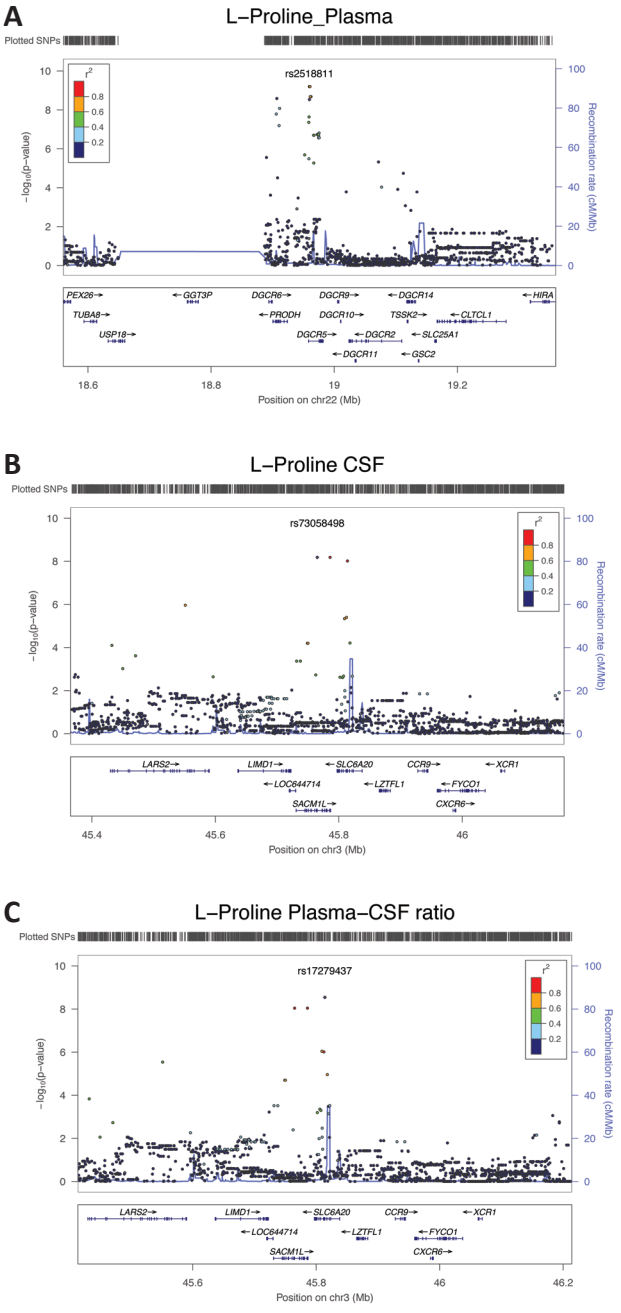

Figure 2. Regional Association Plots of Neurometabolic Quantitative Trait Loci:
A. Genome-wide significant L-Proline (plasma) association in *DGCR5*/ 35 kb from *PRODH*
B. Genome-wide significant L-Proline (CSF) association in *SLC6A20*
C. Genome-wide significant L-Proline (plasma-CSF ratio) association in *SLC6A20*

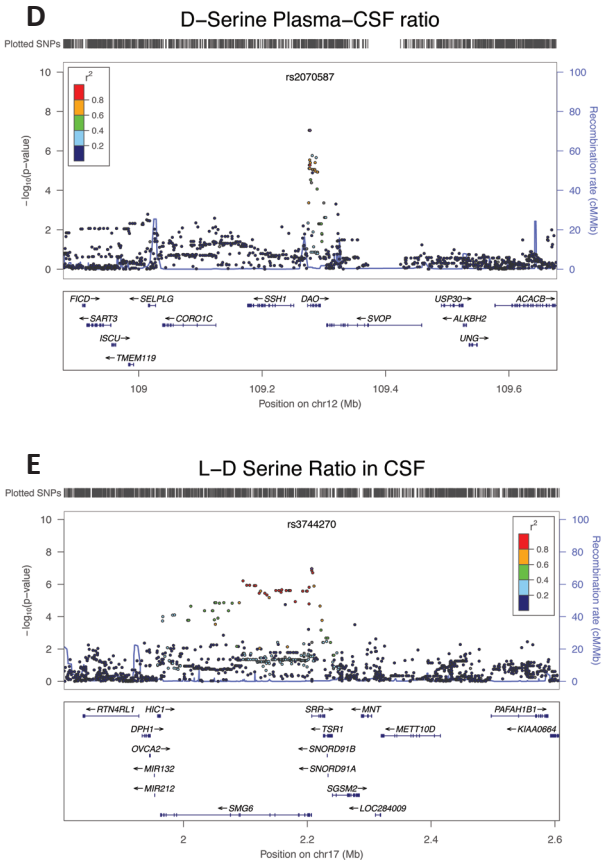

Figure 2 (ctd). Regional Association Plots of Neurometabolic Quantitative Trait Loci:
D. Suggestive D-Serine (plasma-CSF ratio) association in *DAO*
E. Non-significant L-D-Serine ratio (CSF) association in *SRR*

Computation of Explained Variances

The variances explained by the genome-wide and suggestively significant SNPs ranged between 3.8 and 8.2 (Table 2). This range is consistent with a previous metabolic trait GWAS [31] and our power calculation. Supplemental Figure S2 provides boxplots of the concentrations per genotype.

Rodent Synteny, Gene Expression and Functionality of Neurometabolic QTLs

Online tools were used to assess rodent synteny, gene expression patterns, and the functions of the genes located in or in close proximity to the detected QTLs. All highlighted transporter and metabolic genes (*SLC6A20*, *PRODH* and *DAO*) are primarily expressed in the brain and expression patterns are stable across the lifespan. Proline transporter *SLC6A20* is syntenic with two highly homologous genes in mouse and rat, *Slc6a20a* and *Slc6a20b*, the latter of which is predominantly expressed in the choroid plexus and meninges [41]. These are the areas where most CSF transport from and to brain parenchyma occurs, in addition to the choroid plexus being the region

where CSF is produced. *PRODH* encodes proline dehydrogenase 1 (PRODH) that catalyzes the transformation of L-Proline to D1-pyrroline-5-carboxylate [42]; the mouse orthologue is *Prodh*. *DAO* (or *DAAO, D-Amino Acid Oxidase*) is syntenic to *Dao* that encodes DAO, an enzyme that degrades D-Serine. In humans the function of *DAO* had remained largely elusive. In addition to *Slc6a20b*, both *Dao* and *Prodh* are highly expressed in the choroid plexus and meninges [43, 44].

Table 2. The top genome-wide significant ($P < 1.25 \times 10^{-8}$, in bold) and suggestively significant ($P < 10^{-7}$) associations are shown per quantitative trait, ordered by decreasing P-values. Only one SNP per locus is shown.

Phenotype	SNP	Function	Position	Locus	MAF	β	P-value	R^2
L-Proline (P)	rs2540641	Unknown*	22q11.2	DGCR5 / 35kb from PRODH	0.11	**0.30**	6.38×10^{-10}**	8.2
L-Proline (P/CSF)	rs17279437	Missense	3p21.31	SLC6A20	0.09	**–0.30**	2.88×10^{-9}	3.8
L-Proline (CSF)	rs73058498	Unknown***	3p21.31	SACM1L / 22 kb from SLC6A20	0.09	**0.28**	6.57×10^{-9}	7.8
L-Alanine (P/CSF)	rs4377332	Unknown	2q36.1	198kb from EPHA4	0.36	**–0.28**	2.91×10^{-8}	7.5
D-Alanine (P/CSF)	rs352052	Unknown	4q13.3	Inter-genic between CXCL3 and CXCL5	0.48	**–0.30**	3.38×10^{-8}	6.5
Glycine (CSF)	rs115153038	Unknown	14q32.12	4.2kb from TC2N	0.05	**0.27**	3.56×10^{-8}	8.1
D-serine (P/CSF)	rs2070587	Unknown	12q24.11	DAO	0.19	**–0.28**	9.08×10^{-8}	7.7

P = Plasma; CSF = cerebrospinal fluid; MAF = Minor Allele Frequency; β = standardized beta; R^2 = explained variance (%). * This SNP is in LD (r^2=0.54) with missense variant rs2904552 in *PRODH* that was also associated with L-Proline in plasma ($P = 1.65 \times 10^{-8}$). ** This SNP is in LD with rs2023634 (r^2=0.71) that is associated with the Proline/Valine ratio at $P = 1.21 \times 10^{-25}$ in an independent dataset of human serum (N= 1,768). *** This SNP is in strong LD (r^2=0.9) with rs17279437 in *SLC6A20*.

Discussion

In the first genome-wide study of N-methyl-D-aspartate receptor (NMDAR) coagonists and enantiomers in CSF and plasma, we observed strong genetic evidence for loci influencing L-Proline levels. A missense variant in Proline transporter *SLC6A20* and variants in *DGCR5* and *PRODH* were strongly associated with L-Proline levels in CSF and plasma, respectively. In *DAO*, suggestive evidence of association was found with D-Serine levels.

The association between rs17279437 in *SLC6A20* and both L-Proline in CSF and the L-Proline Plasma-CSF ratio suggests that L-Proline levels in CSF are primarily

controlled by a transporter mechanism and not by endogenous synthesis within the CNS. The discrepancy between the loci detected for CSF and plasma L-Proline may imply that this coagonist is mainly produced peripherally where degradation occurs by PRODH, while a proportion is transferred across the blood-brain-barrier to the CNS by SLC6A20. This hypothesis is further supported by the high L-Proline plasma-CSF ratio (223:1 in the current study) and demonstrated highest expression levels of *Slc6a20b* in the choroid plexus and meninges [45]. L-Proline concentrations in plasma on the other hand, were strongly associated with several SNPs within DiGeorge Syndrome Critical Region (*DGCR5*) and *PRODH* at chromosome 22q11.2. Whereas *DGCR2* is implicated in SCZ [46], little is known about the function of *DGCR5*. However, this locus is in close proximity (~35kb upstream) of *PRODH*. The finding that these variants in *DGCR5* tag a missense variant in *PRODH* (LD $r^2 = 0.54$) that itself was strongly associated with L-Proline in Plasma (rs2904552, $P = 1.65 \times 10^{-8}$) may imply that allelic variation at this missense variant mediates L-Proline concentrations in plasma. A GWAS of Proline in human serum detected a locus in close vicinity to the one we detected [39], which may be due to the different SNP array platforms used and imputation being carried out on the most recent 1000 Genomes release in the current study, which in turn has increased coverage of the 22q11 region. Furthermore, here we show that strong associations with L-Proline itself instead of ratios to other amino acids can be achieved.

By demonstrating that the missense variant rs17279437 in *SLC6A20* mediates L-Proline concentration variability in the CSF of healthy subjects we extend previous work demonstrating a role for this variant in iminoglycinuria (IG), an autosomal recessive abnormality in renal Glycine and Proline transport [47]. Furthermore, rs17279437 was recently ranked as the fifth most strongly associated SNP with amino acid metabolism in human urine [31]. Our findings thus portend an extensive influence of this variant on amino acid metabolism.

To our knowledge, the present GWAS constitutes the first on D-amino acids in any body fluid, precluding direct comparisons with previous findings. Since their discovery in human biological fluids [48], there has been considerable debate regarding the origin and biological functions of D-amino acids [33]. Although not genome-wide significant, the SNP most strongly associated with Serine (the D-Serine plasma-CSF ratio, intronic rs2070587, $P = 9.08 \times 10^{-8}$) is located in *DAO*. The findings of the current study are highly suggestive of a major role for this variant or one that is tagged by it in determining D-Serine concentration gradients between CSF and plasma in humans. How rs2070587 contributes to DAO functionality remains elusive because this SNP does not result in frame-shifts, splice-site variation or altered *DAO* mRNA in postmortem cerebellar tissue [49]. We found similarly strong associations - although not suggestively significant by our predefined criteria - for *SRR* (*Serine Racemase*) at rs3744270 on 17p13.3 with the L-Serine/D-Serine ratio in CSF ($\beta = 0.27$; $P = 1.10 \times$

10^{-7}; Figure 2E). This being the strongest associated locus for this ratio, our data lend support to the contention that in humans SRR variants control L to D-Serine conversion [50]. Notably, *Srr* is also highly expressed in mouse choroid plexus and meninges [44]. No associations at $P < 5 \times 10^{-7}$ were found for the L-Serine/D-Serine ratio in plasma, suggesting that either CSF is a more reliable fluid compartment for Serine quantitative genetics or that Serine Racemase mediates L to D-Serine conversion mainly in the CNS. We found no evidence for involvement of the D-amino acid oxidase activator (*DAOA*) gene (also known as G72) in D-Serine levels (no association results at $P < 10^{-4}$ for this gene ± 20kb with any of the Serine traits). Finally, the associations in the genes encoding D-Serine degrading (DAO) and producing (SRR) enzymes imply that a bacterial or dietary origin of this D-amino acid is unlikely.

Several limitations must be borne in mind, however. The size of our study population was modest compared to metabolic trait GWASs in other bodily fluids [30, 31, 39]. In addition, owing to the uniqueness of our sample and the enterprising nature of CSF collection from the general population, a similarly sized replication cohort with CSF measurements was unavailable. Furthermore, due to high correlations between ratios and enantiomers we corrected for the number of NMDAR coagonists instead of all measured values and ratios in the two body fluids. Although the plasma L-Proline association finding would survive Bonferroni correction for all these traits ($\alpha = 5 \times 10^{-8} / 27 = 1.85 \times 10^{-9}$), future projects that include a wider range of neurometabolic traits will require larger sample sizes and replication cohorts.

The presented data constitute a proof-of-principle that CSF is a meaningful and informative body fluid for QTL analyses. Our findings may have particular implications for 22q11 deletion syndrome and hyperprolinemia, both of which may entail cognitive deficits and psychosis [10, 51, 52]. Based on the current data, one may speculate that pharmacologically blocking of SLC6A20 in patients suffering from these disorders may reduce the detrimental effects of excess CNS L-Proline. In addition, chromosome 22q11 deletion syndrome is the strongest genetic risk factor for psychosis [53] and L-Proline was recently found to be the prime metabolite altered in SCZ [54]. Thus, genetic studies of the highlighted 22q11.2 variants in SCZ patients may elucidate trajectories pertinent to L-Proline metabolism in psychosis. Finally, recent research has indicated how the localization of NMDAR coagonist gating may be deduced from modulation of enzymes and transporters relevant to NMDAR coagonists [2]. The genetic variants, enzymes and transporters highlighted here may accordingly be targeted to further construe the nature of NMDAR coagonist involvement in synaptic and extrasynaptic glutamate signaling.

In summary, loci in genes encoding both a transporter protein (*SLC6A20*) and an enzyme (*PRODH*) were identified. Furthermore, we provide preliminary evidence that in humans a variant in *DAO* mainly determines D-Serine levels in the CNS, whereas variants in *SRR* affect L to D-Serine conversion in CSF. As biologically tenable

associations in both plasma and CSF were detected, we conclude that NMDAR coagonists in both body fluids constitute informative targets for preclinical and human follow-up studies.

Materials and Methods

Subjects and Collection of Samples

Subject description and collection of samples were described in detail previously [55]. In brief, 414 volunteers were recruited at outpatient pre-operative screening services in four hospitals in and around Utrecht, The Netherlands, from August 2008 until June 2011. We included subjects (i) undergoing spinal anaesthesia for minor elective surgical procedures, (ii) aged between 18 and 60 years, and (iii) with four grandparents born in The Netherlands or other North-Western European countries (Belgium, Germany, UK, France, and Denmark). Each candidate participant received a personal telephone interview to exclude subjects with psychotic or major neurological disorders (stroke, brain tumors, and neurodegenerative diseases) and to record any use of (psychotropic) medication. Whole blood was collected in EDTA tubes for DNA and plasma extraction. Plasma was extracted by centrifuging whole blood at ambient temperature for 10 minutes at 2,500 g, after which plasma was stored at -80 °C. Per subject, 6 mL of CSF were suctioned and stored at -80 °C. Informed consent was obtained from the subjects. The ethics committee of the University Medical Center Utrecht and all local ethics committees approved the study. All experiments were conducted according to the principles expressed in the Declaration of Helsinki.

NMDAR Coagonist Measurements

We quantified Glycine (that is not chiral) and L and D-enantiomers of Serine, Proline, and Alanine in CSF and plasma of the same subjects using ultra-performance liquid chromatography – mass spectrometry (UPLC-MS/MS), as described previously [56].

Genotyping, Imputation and Quality Control Procedures

Genotype data of 414 subjects were collected using the Illumina HumanOmniExpress Beadchip (730,525 single nucleotide polymorphisms (SNPs)), at the UCLA Neurosciences Genomics Core (UNGC). All genetic analyses were performed using Plink v1.07 [40]. Quality control (QC) was performed as described in the Supplemental Methods, leaving 398 individuals. We used the BEAGLE [57] software to phase the genotype data and the minimac [58] software (a computationally efficient implementation of MACH [59]) for genotype imputation on these 398 subjects. The reference panel used for imputing markers in autosomes is the EUR population of 1000 Genomes Phase I version 3 that includes 379 individuals and approx. 17 million markers. After applying

the quality control (QC) standards outlined in the Supplemental Methods (< 2% genotyping missingness, Hardy Weinberg Equilibrium (HWE) p-value > $1x10^{-6}$, and minor allele frequency (MAF) ≥ 0.05), a total of 5,749,079 autosomal SNPs (567,521 genotyped) remained for the association analyses. Imputation quality scores (r^2) of imputed SNPs that showed significant or suggestive associations were high: rs2904552 and rs3970551 had respective scores of 0.64 and 0.60, while the other quality scores ranged between 0.84 and 1.0. QQ plots were generated and the genomic inflation factor was computed using the R package (www.r-project.org).

Neurometabolic Quantitative Trait Locus Analyses

The statistical analyses described here are based on a previous metabolic trait GWAS [31]. Outliers (defined as data points > 3 standard deviations (S.D.) away from the mean) were excluded. Distributions deviating from normality (defined by a Kolmogorov-Smirnov (K-S) two sided p-value < 0.05) were log-scaled (Supplemental Methods). We applied an age- and sex-adjusted additive linear model in Plink v1.07 [40]. Other covariates tested using Spearman's rho correlations with neurometabolic traits were: time of day of sampling, CSF amount (since in 8% of the samplings the amount of CSF suctioned differed > 1 mL from the planned 6 mL), use of psychotropic medication (binary), use of other medication (binary), storage duration at -80 °C, current or past self-reported psychiatric diagnosis (binary), and type of elective surgical procedure (arthroscopy vs. other). These variables were assessed by telephone interviews, online questionnaires and perioperative recordings. Storage time was added as a third covariate to our model since this was the only impacting variable on ≥ 2 NMDAR coagonists at P < 0.05 (Supplemental Methods). We used a genome-wide significance threshold of $\alpha = (5 \times 10^{-8})/4 = 1.25 \times 10^{-8}$ reflecting a correction for the number of NMDAR coagonists (N=4). Suggestive evidence for association was set at $P < 10^{-7}$. Because of the strong correlations between the chiral forms and measured values in CSF and plasma, we used a less stringent correction factor than Bonferroni correction. Moreover, considering this is the first GWAS of CSF neurometabolites, we thus increased power at the potential cost of type-I error. Furthermore, we performed permutation testing, which offers the advantages of providing a framework for multiple-testing correction and dealing with relatively small sample sizes. To find out whether the null hypothesis (H_0) -lack of association- could be rejected in the event of genome-wide significant associations, we therefore carried out a maximum number of $1 / 1.25 \times 10^{-8} = 8 \times 10^7$ permutations in Plink v1.07 [40]. The other settings were set at adaptive permutation default [40]. In the event several genome-wide significant associations were detected for the same neurometabolic trait, conditioning on SNPs was performed using the '-condition' command in Plink v1.07 [40] to test the independency of the associated SNPs.

Regional association plots were generated in Locuszoom (http://csg.sph.umich.edu/

locuszoom/) and Manhattanplots in R (www.r-project.org). The explained variances were computed by subtracting the R-square of a linear model including the SNP and all covariates as independent variables from the R-square of a model including only the covariates. We estimated the statistical power of our sample with the Genetic Power Calculator [60] using the option for QTL association of singletons and a genome-wide significance threshold of $P < 5 \times 10^{-8}$. Given the sample size (N=400) this study has 80% power to detect a QTL that explains approximately 9.5% of the trait variance.
The Helmholtz Zentrum Metabolomics GWAS server (based on a meta-analysis of human serum QTLs) was interrogated for replication of any genome-wide significant plasma findings using an LD r^2 cut-off of 0.6 [39]. Online tools employed to assess mouse synteny, gene expression patterns and the functional implications of the highlighted SNPs and genes encompass the UCSC genome browser (http://genome.ucsc.edu/), the Brain Gene Expression Map [43], the Eurexpress mouse embryo transcriptome atlas [41], GenePaint [44], and Brain Cloud [61].
Candidate polymorphisms were retrieved by selecting the SNPs reported to be associated at $P < 5 \times 10^{-8}$ with any amino acid in GWASs of metabolic traits in human body fluids published to date (serum, plasma and urine) [30, 31, 38, 39, 62]. In total, 14,670 SNPs were thus selected, of which 2,221 passed the abovementioned QC steps and were available in our dataset. We applied Bonferroni correction for association testing of these candidate polymorphisms on all metabolic traits ($\alpha = 0.05 / (4 \times 2{,}221) = 5.63 \times 10^{-6}$).

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Supplemental Information Chapter 6

Supplemental Methods. *(page 163)* Quality control criteria for subjects and SNP data; Distributions of measured values and ratios (CSF and plasma); Spearman's rho correlations between metabolic traits and storage time.
Supplemental Figure S1. *(page 176)* QQplots of the genome-wide significant associations.
Supplemental Figure S2. *(page 178)* Boxplots show concentrations per genotype of the associations that reached genome-wide and suggestive significance.

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[1] *Department of Psychiatry, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht, Utrecht, The Netherlands*
[2] *Department of Medical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands*
[3] *Department of Clinical Chemistry and Hematology, University Medical Center Utrecht, Utrecht, The Netherlands*
[4] *Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, University of California Los Angeles, Los Angeles, CA, USA*
[5] *Computer Science Department, University of California, Los Angeles, California, USA*
[6] *Department of Human Genetics, University of California, Los Angeles, California, USA*
[7] *Department of Anesthesiology, Ter Gooi Hospitals, Hilversum, The Netherlands*
[8] *Department of Anesthesiology, Intensive Care & Pain Management, St Antonius Hospital, Nieuwegein, The Netherlands*
[9] *Department of Anesthesiology, Central Military Hospital, Utrecht, the Netherlands*
[10] *Department of Anesthesiology, Intensive Care & Pain Management, University Medical Center Utrecht, Utrecht, Utrecht, The Netherlands*
[11] *Department of Anesthesiology, Intensive Care & Pain Management, Diakonessenhuis Hospital, Utrecht, The Netherlands*
[12] *Academic Medical Center, Department of Psychiatry, Amsterdam, The Netherlands*

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Genome-Wide Association Study of Monoamine Metabolite Levels in Human Cerebrospinal Fluid

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Jurjen J. Luykx[1,2]
Steven C. Bakker[1]
Eef Lentjes[3]
Marjolein Neeleman[1]
Eric Strengman[2]
Laura Mentink[1,2]
Joseph DeYoung[4]
Simone de Jong[4]
Jae Hoon Sul[5]
Eleazar Eskin[5,6]
Kristel van Eijk[2]
Jessica van Setten[2]
Jacobine E. Buizer-Voskamp[1]
Rita M. Cantor[6]
Ake Lu[6]
Marjolein van Amerongen[7]
Eric P.A. van Dongen[8]
Peter Keijzers[9]
Teus Kappen[10]
Paul Borgdorff[11]
Peter Bruins[8]
Eske M. Derks[12]
René S. Kahn[1]
Roel A. Ophoff[1,4,6]

Abstract

Studying genetic determinants of intermediate phenotypes is a powerful tool to increase our understanding of genotype - phenotype correlations. Metabolic traits pertinent to the central nervous system (CNS) constitute a potentially informative target for genetic studies of intermediate phenotypes as their genetic underpinnings may elucidate etiological mechanisms. We therefore conducted a genome-wide association study (GWAS) of monoamine metabolite (MM) levels in cerebrospinal fluid (CSF) of 414 human subjects from the general population. In a linear model correcting for covariates, we identified one locus associated with MMs at a genome-wide significant level (standardized $\beta = 0.32$, $P = 4.92 \times 10^{-8}$), located 20kb from *SSTR1*, a gene involved with brain signal transduction and glutamate receptor signaling. By subsequent whole-genome expression quantitative trait locus (eQTL) analysis, we provide evidence that this variant controls expression of *PDE9A* ($\beta = 0.21$; $P_{unadjusted} = 5.6 \times 10^{-7}$; $P_{corrected} = 0.014$), a gene previously implicated in monoaminergic transmission, major depressive disorder and antidepressant response. A post-hoc analysis of loci significantly associated with psychiatric disorders suggested that genetic variation at *CSMD1*, a schizophrenia susceptibility locus, plays a role in the ratio between dopamine and serotonin metabolites in CSF. The presented DNA and mRNA analyses yielded genome-wide and suggestive associations in biologically plausible genes, two of which encode proteins involved with glutamate receptor functionality. These findings will hopefully contribute to an exploration of the functional impact of the highlighted genes on monoaminergic transmission and neuropsychiatric phenotypes.

Introduction

Studying genetic determinants of intermediate phenotypes is a powerful approach to deepen the understanding of genotype - phenotype correlations [1, 2]. This consideration may particularly apply to the field of behavioral disorders where genome-wide association studies (GWASs) have had limited success in identifying susceptibility loci for major depressive disorder (MDD), attention-deficit hyperactivity disorder (ADHD) and panic disorder [3-5]. Metabolic traits pertinent to the central nervous system (CNS) constitute potentially informative targets for genetic studies as their molecular underpinnings may shed light on etiological mechanisms [6]. Such quantitative measurements may be more useful for genetic mapping as they are less subject to genetic heterogeneity than categorical diagnoses. Moreover, elucidation of the genetic determinants underlying these quantitative traits may aid in the delineation of clinical subtypes, the prediction of treatment response and the identification of novel pharmacotherapeutic modalities [6-8].

Of the human CNS metabolic traits, monoamine metabolites (MMs) are particularly relevant to the study of neuropsychiatric disorders as most psychotropic medicines act on monoamine systems. Cerebrospinal fluid (CSF) constitutes the body fluid compartment most proximate to the CNS and therefore metabolite levels in this compartment are thought to reflect CNS processes more accurately than blood constituents. In addition, the blood-brain-barrier hampers the generalizability of serum and plasma quantitative genetic studies to the CNS. The monoamines -serotonin (5-HT), dopamine (DA) and norepinephrine - regulate a variety of processes that are perturbed in psychiatric disorders, e.g. memory, appetite, psychomotor function, mood, sexual behavior, and hormone release [9, 10]. Moreover, neurological selective 5-HT deficiency and secondary neurotransmitter disorders (for which low levels of the main 5-HT metabolite, 5-hydroxyindoleacetic acid -5-HIAA- in CSF were described) have unresolved etiologies, despite candidate gene mutation analyses [11-13]. Given the heritability estimates of MM levels in CSF (ranging from 0.30 for 5-HIAA to 0.52 for the main DA metabolite, homovanillic acid -HVA) [14-16], genetic factors are involved in inter-individual variation of MM concentrations and therefore a well-powered GWAS may elucidate monoamine metabolism pathways.

Thus far, genetic studies of CSF MM concentrations have focused on candidate genes encoding known enzymes pertaining to monoamine metabolism pathways. For example, a repeat length polymorphism in the monoamine oxidase A gene (*MAOA*) was reported to be associated with HVA levels in alcohol dependent patients [17] and with HVA and 5-HIAA levels in a small sample of healthy volunteers [18]. Other MM candidate loci include variants at the dopamine β hydroxylase (*DBH*) and serotonin 2C receptor (*HTR2C*) [19] genes as well as the repeat polymorphism in the promoter of the serotonin transporter (*SLC6A4*), also known as the 5-HTTLPR short/long

polymorphism [20]. Most of these studies suffered from limited power due to small sample sizes and included a mixed sample of patients with psychiatric diagnoses and unaffected controls, increasing the likelihood of sample heterogeneity. To our knowledge, no genome-wide study of MM concentrations in CSF has been performed except for a linkage study in a non-human primate, the vervet monkey, in which a locus was identified for HVA [14].

Here, we report on the first GWAS of MMs measured in CSF of humans, in a relatively large sample (N=414). Genome-wide significant associations were followed up by whole-genome gene expression quantitative trait locus (eQTL) analyses in a subset of the study population (N=240).

Methods

Subjects and Collection of Samples

Sample collection has been described elsewhere [21]. In brief, 414 volunteers were recruited at outpatient pre-operative screening services in four hospitals in and around Utrecht, The Netherlands, from August 2008 until June 2011. We included patients (i) undergoing spinal anaesthesia for minor elective surgical procedures, (ii) ranging between 18-60 years of age, and (iii) with four grandparents born in The Netherlands or other North-Western European countries (Belgium, Germany, the UK, France, and Denmark). Each candidate participant received a personal telephone interview to exclude subjects with psychotic or major neurological disorders (stroke, brain tumors, neurodegenerative diseases) and to record any use of psychotropic medication. A self-reported history of unipolar affective, attention-deficit-hyperactivity and anxiety disorders was allowed. A sample of 6 mL of CSF was obtained from each subject and immediately stored in fractions of 0.5mL and 1mL at -80 °C. A whole blood sample was used to extract genomic DNA using standard techniques and RNA was collected in PAXgene tubes. The study was approved by all applicable ethics committees and participants provided written informed consent.

Monoamine metabolite measurements

Concentrations of three CSF MM levels (5-HIAA; HVA; and 3-methoxy-4-hydroxyphenylglycol, MHPG) were measured using high performance liquid chromatography (HPLC) with electrochemical detection as described before [22]. Ratios between MMs (N=3) were computed given the potential increase in power to detect underlying enzymatic processes [23] and the previously detected biologically plausible signals in a GWAS of metabolite ratios in urine [24]. The following Pearson correlations between MMs were computed using SPSS 20 (SPSS Inc., Chicago, Il): r = 0.594 ($P = 2.77 \times 10^{-40}$) for HVA and 5-HIAA; r = 0.188 (P = 0.004), for HVA and MHPG; r = 0.043

(P = 0.5) for 5-HIAA and MHPG. Given the high correlation between 5-HIAA and HVA, a principal components analysis (PCA) for these two MMs was conducted and factor scores were calculated. Factor scores with Eigenvalues > 1 were entered as a dependent variable in the genetic association analyses.

Genotyping, Imputation and Quality Control Procedures

Genotype data of 414 subjects were collected using the Illumina HumanOmniExpress Beadchip (730 525 single nucleotide polymorphisms (SNPs)), at the UCLA Neurosciences Genomics Core (UNGC). All genetic analyses were performed using Plink v1.07 [25]. Quality control (QC) was conducted as described in the Supplemental Methods, leaving 398 individuals. We used the BEAGLE [26] software to phase the genotype data and the minimac [27] software (a computationally efficient implementation of MACH [28]) for genotype imputation on these 398 subjects. The reference panel used for imputing markers in autosomes is the EUR population of 1000 Genomes Phase I version 3 that includes 379 individuals and approx. 17 million markers. After applying the QC standards outlined in the Supplemental Methods (< 2% genotyping missingness, Hardy Weinberg Equilibrium (HWE) p-value > $1x10^{-6}$, and minor allele frequency (MAF) > 0.05), a total of 5 767 231 SNPs (585 655 genotyped) remained for the association analyses. QQ plots were generated and the genomic inflation factor was computed using the R package (http://www.r-project.org).

Genome-wide Quantitative trait locus (QTL) analyses

A linear, additive model accounting for age, sex and covariates was performed in Plink v1.07 [25] for each of the three MMs, the three ratios and the HVA 5-HIAA factor score. To detect relevant covariates, we computed a Pearson's correlation (r) of these seven traits with the following variables: time of day of sampling, CSF amount (since in 8% of the samplings the amount of CSF suctioned differed > 1 mL from the planned 6 mL), use of psychotropic medication (binary), use of other medication (binary), storage duration at -80 °C, current or past self-reported psychiatric diagnosis (binary), type of procedure (binary: arthroscopy vs other), duration of aspiration (the log was used to reach a normal distribution), and season of sampling (as a dummy variable: spring vs all other months)[11, 22, 29, 30]. The maximum percentage of missing data for covariates was < 10%. Missing covariate data were replaced by the mean (for weight and height separately in men and women; missing categorical covariates were replaced by the value with the highest frequency). Covariates that correlated with MMs, ratios or the factor score at P < 0.05 were included in the linear model separately for each trait. Covariates that proved collinear -defined as a Pearson r > 0.5- with age or sex were excluded. If covariates proved collinear with one or more of the other covariates, the latter of the covariates in the randomly chosen order described above was excluded. MM concentrations exceeding the mean +/- 3 S.D. were considered outliers and

removed. Outcome measures were standardized regression coefficients (β). Genome-wide significance was set at $P < 5 \times 10^{-8}$, a threshold chosen to reduce the likelihood of type-II errors and because of the high intercorrelations of the traits [31]. The explained variance was computed by subtracting the R^2 of a linear model including the SNP and all covariates as independent variables from the R^2 of the model including only the covariates. We estimated the statistical power of our sample with the Genetic Power Calculator [32] using the option for QTL association of singletons and a genome-wide significance threshold of $P < 5 \times 10^{-8}$. Given the sample size (N=400) this study has 80% power to detect a QTL that explains approximately 9.5% of the trait variance.

eQTL analyses

mRNA quantification and genome-wide expression profiling procedures were performed in a subset of the study population (N=240) as described in the Supplemental Methods. Raw data extraction and background correction were performed using GenomeStudio (Illumina, Inc., San Diego, CA). The Lumi package in R was used for robust spline normalization and variance stabilizing transformation of the gene expression data [33, 34]. QC procedures are outlined in the Supplemental Methods. SNPs showing associations with MMs or ratios at a genome-wide significance level ($P < 5 \times 10^{-8}$) were tested for association with mRNA of individual genes at a genome-wide level by means of linear regression using the Limma package in R [35, 36]. Expression values were taken as dependent variables, allele dosage as independent, while outcome measures were β (increase or decrease per minor allele dosage). Significance of eQTL regression results was set at a False-Discovery-Rate (FDR) corrected $P < 0.05$.

Association Testing of Candidate Polymorphisms

The selection process of candidate genetic variants is described in the Supplemental Methods. In brief, given its demonstrated involvement in CSF monoamine metabolism and interactions with depression [20], we used a recently developed machine learning method to predict the 5-HTTLPR polymorphism [37]. Next, we identified SNPs associated with psychiatric traits in GWASs. Candidate polymorphisms in monoamine-related pathway genes were then selected based upon a literature search and a recent enumeration of the known polymorphisms and mutations in these genes [38]. A linear, additive model including covariates as described above was fitted for this total of 86 candidate polymorphisms in Plink v1.07 [25] in the study population after QC (N=398, see Supplemental Methods for SNP lists). For this analysis we applied a Bonferroni correction for multiple testing ($\alpha = 0.05/86 = 5.81 \times 10^{-4}$). Finally, association of SNPs in the 15MB vervet locus syntenic to human chromosome 10:5-20Mb [14] with HVA and its ratios was tested, applying Bonferroni-correction here as well ($\alpha = 0.05 / 40\,770 = 1.23 \times 10^{-6}$).

Table 1. Subject characteristics and mean monoamine metabolite concentrations (nmol/L) with SDs of the 414 genotyped subjects (N/A = not applicable).

	N	Mean (SD) / N (%)	Range
MHPG (nmol/L)	239	24.7 (4.95)	13.3 – 40.4
5-HIAA (nmol/L)	412	164 (67.0)	40.0- 360
HVA (nmol/L)	412	214 (72.8)	32.8 - 422
Age (years)	414	40 (11)	18-60
Sex (male)	299	299 (72%)	N/A
Procedure type (arthroscopies)	414	314 (76%)	N/A
Amount of CSF drawn (mL)	414	5.5 (0.8)	2.5 – 7.0
Psychiatric history	414	17 (4.1%)	N/A

MHPG = 3-methoxy-4-hydroxyphenylglycol; 5-HIAA = 5–hydroxyindoleacetic acid; HVA = homovanillic acid.

RESULTS

Descriptive Information

HVA and 5-HIAA measurements were available for 412 genotyped subjects; MHPG measures were obtained for a subset (N=239). Per MM, two outliers were removed. MM concentrations were distributed normally (Supplemental Figure 1). Mean MM concentrations and subject characteristics are given in Table 1.

Genome-wide Results

QQ and Manhattan plots per metabolite and ratio are shown in Supplemental Figure 2. The genomic inflation correction factors (λ_{GC}) ranged between 0.99 and 1.02, suggesting no inflation of the test statistics. The only collinear covariate was height (with sex). The one genome-wide significant association was found for the 5-HIAA/MHPG-ratio analysis (covariates: age, sex, sampling in spring; N=235) with two genotyped (rs11628551 and rs10498339) and 16 imputed SNPs ($\beta = 0.32$, $P = 4.92 \times 10^{-8}$; Figure 1 and Supplemental Figure 3). These SNPs were in perfect linkage disequilibrium (LD $r^2 = 1.0$) and explained 9.0% of the variance in 5-HIAA/MHPG-ratios. Imputation r^2 values for these SNPs ranged from 0.87 to 1.00. Although their MAF was low (5%), no inflation of the test statistics was detected when parsing QQ results by MAF (Supplemental Figure 4). The association analyses of the other metabolites (N=396 for 5-HIAA and HVA; N=235 for MHPG) and ratios (N=392 for the 5-HIAA/HVA-ratio and N=235 for the HVA/MHPG-ratio) did not result in genome-wide significant findings (Table 2). A PCA of 5-HIAA and HVA revealed one

factor with an Eigenvalue > 1. This factor (Eigenvalue = 1.60) explained 80% of HVA and 5-HIAA variance. The most strongly associated SNP was rs2830487 ($\beta = 0.25$, $P = 4.88 \times 10^{-7}$), the same most strongly associated SNP as for the HVA analysis. Seventeen participants had a self-reported history of past or current minor psychiatric illness (unipolar affective disorders, anxiety disorders and ADHD). When excluding these 17 subjects, the results did not change (data not shown).

eQTL Results

After gene expression QC, 233 of the 240 samples remained (75% male; mean age 39 +/- 11 years). One of the probes, representing the gene *PDE9A*, showed a significant association with abovementioned rs11628551 and the SNPs in complete LD with this SNP ($\beta = 0.21$, $P_{unadjusted} = 5.6 \times 10^{-7}$, FDR corrected P = 0.014; Figure 2).

The mean expression level (SD) of *PDE9A* (ILMN_2306540) in homozygous subjects for the major allele (N=207) was 8.22 (0.18), while expression in heterozygous subjects (N=22) was 8.44 (0.22). Expression levels of *PDE9A* correlated positively with the 5-HIAA/MHPG-ratio (Pearson r=0.189, P = 0.0062), the 5-HIAA/HVA-ratio (r=0.186, P = 0.0046) and 5-HIAA (r=0.168, p=0.0102).

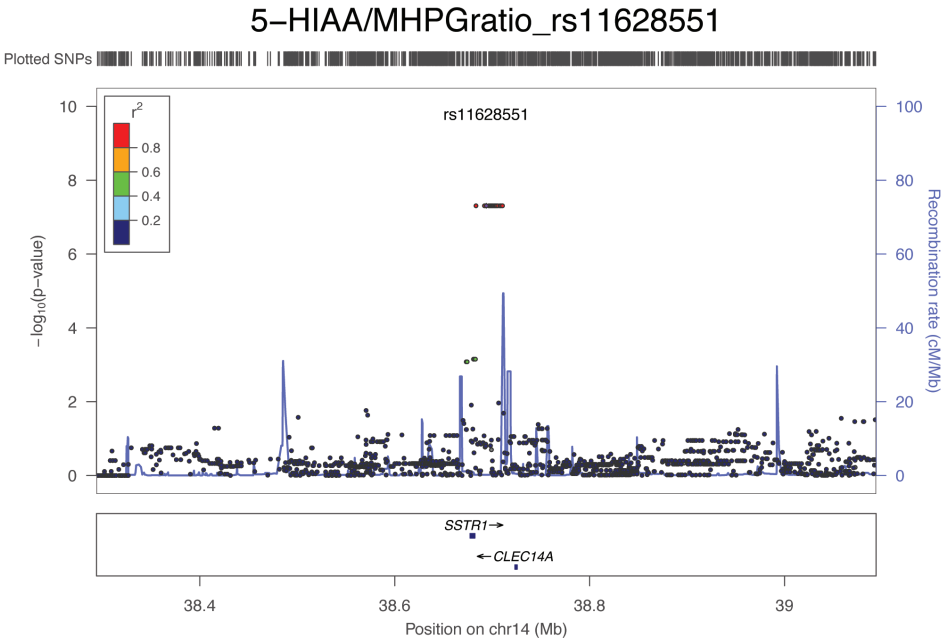

Figure 1. Regional Association Plot of the association with the 5-HIAA/MHPG ratio that reached genome-wide significance ($\beta = 0.32$, $P = 4.92 \times 10^{-8}$). Genotyped and imputed SNPs remaining after quality control procedures and surrounding rs11628551 (±400kb) are displayed.

Candidate Polymorphisms

When examining the top SNPs from psychiatric GWASs and candidate polymorphisms in monoamine metabolic pathways, one locus (genotyped rs10503253) survived correction for multiple testing and showed evidence of involvement with the HVA/5-HIAA-ratio ($\beta = 0.18$, $P_{unadjusted} = 1.82 \times 10^{-4}$; $P_{corrected} = 0.016$; Supplemental Figure 3). The SNP is located in *CSMD1* on chromosome 8p23.2 and was associated with schizophrenia in a large GWAS [39]. Based on our data, allelic variation at rs10503253 explains 2.4% of the variance in the HVA/5-HIAA-ratio ($P = 7.32 \times 10^{-4}$).

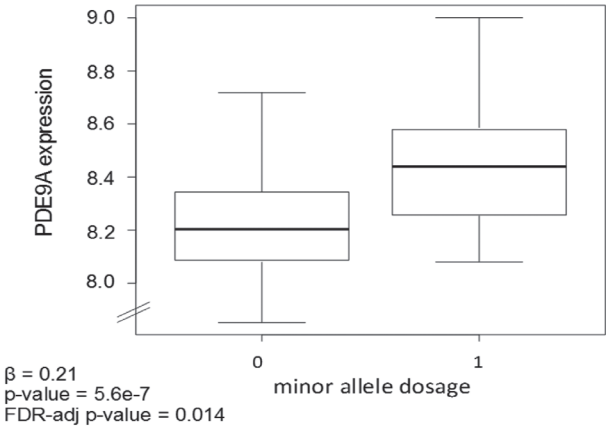

Figure 2. PDE9A gene expression significantly differs between subjects without the minor allele (0) and those heterozygous for the minor allele (1) at rs11628551. β = regression coefficient; FDR-adj = False Discovery Rate adjusted.

Table 2. For all tested traits, the most significantly associated SNP is tabulated. Results are ranked by decreasing p-value (the genome-wide significant SNP is displayed in bold).

Phenotype	SNP	Chr.	Posit.*	MAF	P-value	β	Gene	Distance (kb)	Reg. Mot.
5-HIAA / MHPG	**rs11628551**	14	38.7	0.05	4.92×10^{-8}	0.32	*SSTR1*	11.6	+
HVA	rs2830487	21	28.1	0.46	2.23×10^{-7}	0.37	*ADAMTS1*	62.4	+
MHPG	rs4766646	12	110	0.33	4.05×10^{-7}	0.32	*GIT2*	Intronic	-
5-HIAA	rs11221522	11	129	0.22	4.60×10^{-7}	-0.24	*TP53AIP1/ ARHGAP32*	2.92	+
Factor Score#	rs2830487	21	28.1	0.46	4.82×10^{-7}	0.25	*ADAMTS1*	62.4	+
HVA/ MHPG	rs7483296	11	115	0.15	5.74×10^{-7}	0.34	*CADM1*	23.5	+
HVA / 5-HIAA	rs1881744	12	47.8	0.10	7.76×10^{-6}	0.23	*SNORA64.2*	74.9	+

Chr=chromosome; MAF=Minor Allele Frequency; β = standardized regression coefficient; Reg. Mot.: altered regulatory motifs of the SNP or SNPs in LD ($r^2>0.8$) according to HaploReg (http://www.broadinstitute.org/mammals/haploreg); * hg19 position in Mb; # Factor Score= principal components factor (HVA and 5-HIAA).

We observed no significant association between any of the MM levels or ratios and the 5-HTTLPR, for which genotype summary statistics are described in the Supplemental Methods. Similarly, we observed no evidence for association between HVA or its ratios and the region syntenic to the vervet HVA locus [14] after correction for multiple testing. Borderline significance levels of association with the HVA/MHPG-ratio were observed at rs2489199 ($P = 3.37 \times 10^{-6}$), an intragenic SNP within the *CACNB2* gene (see Supplemental Figure 5 for a regional association plot).

Discussion

We performed the first GWAS of monoamine metabolite levels in human CSF and identified a single genome-wide significant locus. Further analyses in our study sample showed this locus to be important in *trans* regulation of *PDE9A* expression levels, a gene previously implicated in monoaminergic transmission [40]. An analysis of candidate polymorphisms suggested involvement of *CSMD1*, a well-established schizophrenia susceptibility locus, with monoaminergic transmission.

The genome-wide significantly associated SNPs are located in an intergenic region on chromosome 14q21.1, almost 20kb upstream from *SSTR1*, encoding somatostatin receptor 1 and highly expressed in the brain [41]. Somatostatin functions as a neuropeptide that affects the electrical activity of neurons by binding to SSTR1. SSTR1 has several links to CNS signal transduction, e.g. by cell-cell signaling, G-protein coupled receptor signaling, and glutamate receptor signaling. Upon visual inspection of the Manhattan plots (Supplemental Figure 2) the only association peak encompassing several SNPs at a range of p-values was found for 5-HIAA. This peak covers *TP53AIP1* and *ARHGAP32* (Figure 3), the latter of which encodes a GTPase-activating protein involved with NMDA receptor-dependent actin reorganization in dendritic spines and located in cell junctions and synapses throughout the human cortex. None of the other genes (besides *SSTR1* and *ARHGAP32*) listed in Table 2 has established CNS functionality. For the candidate gene analyses no genome-wide significant loci were detected and a separate Bonferroni correction was chosen as a conservative hypothesis-driven approach to weigh the involvement of SNPs relevant to psychiatric phenotypes and monoamine metabolic pathways. The SNP in *CSMD1* that exceeded this threshold for the HVA/5-HIAA-ratio QTL analysis is located in an intron of *CSMD1*, a target of *MIR-137*, another schizophrenia susceptibility locus identified in a recent GWAS [39]. The risk (minor) allele A in the schizophrenia GWAS meta-analysis [39] is associated with an increased HVA/5-HIAA-ratio in our study, implying a relatively high DA turnover in A-allele carriers, which is compatible with the hypothesized hyperdopaminergic state in schizophrenia (reviewed in [42]). We did not find Bonferroni-corrected

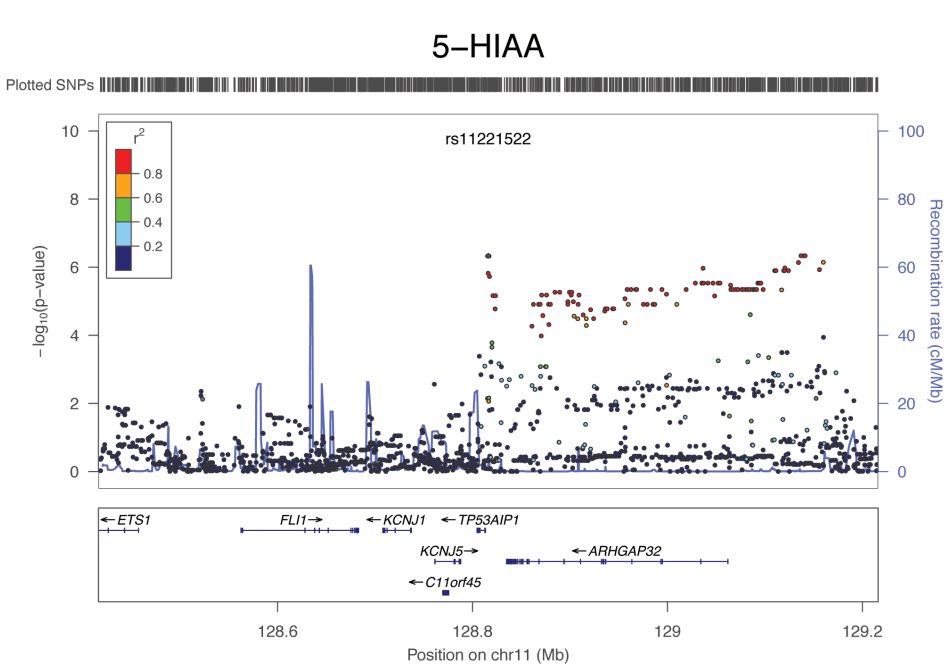

Figure 3. The suggestively significant association peak of 5-HIAA at a locus on chromosome 11 (rs11221522 ± 400kb; β = -0.24, P= 4.60 x 10^{-7}).

significant evidence of association with monoamine levels in the region syntenic to a linkage peak described in a non-human primate pedigree [14]. Suggestive evidence was observed at *CACNB2* (Supplemental Figure 5), a gene encoding the beta-2 unit of a voltage-gated calcium channel with neuronal function that was recently implicated in a GWAS of bipolar disorder [43]. Non-replication here may be due to differences inherent in the nature of linkage and association genetic approaches, incomplete synteny between the human and vervet genomes, or lack of statistical power. At the genome-wide level, we observed a number of suggestively associated ($P < 10^{-5}$) SNPs in other genes reported to be involved with neuropsychiatric phenotypes or neurotransmitter functionality, including *PARK2* (HVA/5-HIAA-ratio) [44, 45], *CSMD2* (HVA/MHPG-ratio) [46, 47], and *NRXN1* (HVA/MHPG-ratio) [48].

Our data show that the genome-wide significant locus is associated with expression levels of *PDE9A*, a gene located on chromosome 21q22.3. Although mRNA was obtained from whole blood, the genetic link between MM levels and *PDE9A* confirms previous findings pointing at PDE9A involvement in monoaminergic transmission [40]. The expression and distribution patterns of phosphodiesterases (PDEs) influence neuronal activity by downstream effects of cAMP and cGMP on ion-gated channels [49] and protein kinases [50]. In rats, administration of antidepressants influences *Pde* cortical and hippocampal expression [51, 52]; in humans, PDE genes have been implicated

in MDD [53] and antidepressant response [54, 55]. *PDE9A*, a key regulator of cGMP, is widely expressed in the CNS and CNS targets of PDE9A inhibitors include cognition and neurodegeneration [50, 56-58]. PDE9A inhibition increases levels of cGMP in the striatum [59] and CSF [40, 60] of rodents and reverses aberrations in rodent behavior that have been linked to monoaminergic transmission, such as auditory gating and working memory deficits [40]. Moreover, out of 21 PDE genes studied, polymorphisms in *PDE9A* and *PDE11A* comprised the strongest association signals in a study of Mexican Americans suffering MDD [54]. Preclinical data thus hint at the importance of PDE9A-dependent CNS pathways, while preliminary clinical evidence suggests a role for PDE9A in MDD.
The effect of the genome-wide significant locus on *PDE9A* expression levels constitutes a *trans*-effect as the SNPs reside on chromosome 14, whereas *PDE9A* localizes to chromosome 21. Because the sequences of the *PDE9A* probes are not known to contain common (MAF > 0.05) nucleotide polymorphisms affecting hybridization and probe sequences map uniquely to this gene, a cryptic *cis*-effect is unlikely. In addition, *trans*-eQTLs have been increasingly identified as key regulators of both quantitative traits and disease phenotypes over the past years [61, 62]. Moreover, the intergenic location of the genome-wide significant SNPs does not preclude regulatory effects –*cis* or *trans*-on gene expression, as has been demonstrated in a meta-analysis of GWAS data from over 712 000 samples [63], in which 39 percent of the associated SNPs were intergenic and several *trans*-regulatory effects were shown relevant to clinical parameters [63].
Although this is the first GWAS of MMs, the size of our study population is modest compared to GWASs of serum and urine metabolites [24, 64]. According to our calculation, statistical power was sufficient to detect the actual effect size for the genome-wide significant locus (9%), but we had reduced power to detect smaller effect sizes or effects resulting from genetic complexities such as interactions. On the other hand, aspects of our methods that have likely benefited the reliability of the results comprise: choosing HPLC as the analytical tool (as opposed to nuclear magnetic resonance, a less sensitive technique [65]); the comprehensive collection of and correction for covariates; the stringent inclusion criteria in terms of age and genetic homogeneity; and the selection of participants subjected to lumbar punctures under standardized conditions after > 6 hours of fasting. In addition, subjects with knee arthroscopy indications -by far the most common procedure in this study- reflect the general population, e.g. with regard to comorbidities [66]. The absence of another set of homogeneously collected CSF samples in which MMs were measured precluded us from retrieving a replication cohort elsewhere. Because the nature of standardized CSF collection from human subjects is highly enterprising, maximizing sample sizes of CSF metabolite studies remains challenging.
In summary, the presented DNA and mRNA QTL analyses yielded genome-wide and suggestive associations in biologically plausible genes, two of which (*SSTR1* and *ARHGAP32*) encode proteins involved with glutamate receptor functionality.

Alterations in CSF MM concentrations and glutamate have been associated with both neurological and neuropsychiatric traits (such as epilepsy and MDD) [9, 11, 67]. Our findings will hopefully contribute to an exploration of the functional impact of *PDE9A*, *SSTR1*, *CACNB2*, *ARHGAP32*, and *CSMD1* on monoaminergic transmission and neuropsychiatric phenotypes. Furthermore, sequencing of such genes in patients suffering selective serotonin deficiency and secondary neurotransmitter disorders may contribute to the elucidation of the unresolved etiologies of these conditions [11-13]. To our knowledge, no *Pde9a* or *Csmd1* knock-out mouse has been described and thus future rodent studies may aid in clarifying the pathways that connect these genes to monoaminergic transmission.

URLs used for statistical analyses, to generate plots and as information resources
http://csg.sph.umich.edu/locuszoom/
http://genome.ucsc.edu/
http://www.ncbi.nlm.nih.gov/pubmed/
http://www.r-project.org
http://www.broadinstitute.org/mammals/haploreg

Supplemental Information Legends

(available online at www.nature.com/mp)

Supplemental Methods. Further details on materials and methods.
Supplemental Figure 1. Distributions of monoamine metabolite concentrations, ratios and the HVA 5-HIAA factor score.
Supplemental Figure 2. QQ and Manhattan plots of imputed and genotyped SNPs per association test.
Supplemental Figure 3. Boxplots showing concentrations and concentration ratios of the SNPs that reached genome-wide significance (rs11628551) and Bonferroni-corrected significance of candidate variant testing (rs10503253).
Supplemental Figure 4. QQ plot of the 5-HIAA-MHPG ratio association results parsed by minor allele frequency (MAF), suggesting no inflation of the test statistic for lower frequency variants ($0.05 < MAF < 0.1$).
Supplemental Figure 5. Regional Association Plot surrounding the most significantly associated SNP (with the HVA-MHPG ratio, rs2489199, $P = 3.37 \times 10^{-6}$, that did not reach Bonferroni-corrected significance) within the 15Mb locus syntenic to the previously detected HVA vervet locus on chromosome 10. The SNP is located in *CACNB2*, which is outside the non-human primate linkage peak.

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[1] *Department of Psychiatry, Rudolf Magnus Institute of Neuroscience, University Medical Center Utrecht (UMCU), Utrecht, The Netherlands*
[2] *Department of Medical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands*
[3] *Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences and Rudolf Magnus Institute of Neuroscience, Utrecht University, Utrecht, The Netherlands*
[4] *Department of Anesthesiology, Intensive Care & Pain Management, St Antonius Hospital, Nieuwegein, The Netherlands*
[5] *Department of Anesthesiology, Intensive Care & Pain Management, Diakonessenhuis Hospital, Utrecht, The Netherlands*
[6] *University of Groningen, University Medical Center Groningen, Department of Genetics, Groningen, The Netherlands*
[7] *Center for Neurobehavioral Genetics, Semel Institute for Neuroscience and Human Behavior, University of California Los Angeles, Los Angeles, CA, USA*

8

D-Amino acid Aberrations in Cerebrospinal Fluid and Plasma of Smokers

Submitted

Jurjen J. Luykx[1,2]
Steven C. Bakker[1]
Loes van Boxmeer[1]
Christiaan H. Vinkers[1, 3]
Eva Smeenk[1]
Wouter F. Visser[2]
Nanda M. Verhoeven-Duif[2]
Eric Strengman[2]
Jacobine E. Buizer-Voskamp[1]
Lizzy de Groene[4]
Eric P.A. van Dongen[4]
Paul Borgdorff[5]
Peter Bruins[4]
Tom J de Koning[2, 6]
René S. Kahn[1]
Roel A. Ophoff[1, 7]

Abstract

The glutamatergic neurotransmission system and the N-methyl-D-aspartate receptor (NMDAR) have been implicated in smoking and alcohol consumption behavior. Preclinical studies have demonstrated that nicotine and ethanol influence NMDAR functionality, which may play a role in tendencies to consume these substances. Nonetheless, little is known about concentrations of NMDAR coagonists in the cerebrospinal fluid (CSF) and plasma of individuals who smoke or consume alcohol. Glycine and L and D-stereoisomers of Alanine, Serine and Proline were therefore measured using ultra-high performance liquid chromatography-tandem mass spectrometry in 403 healthy subjects. Nicotine and alcohol consumption were quantified using questionnaires. Subjects were divided into two groups per substance: smokers vs. non-smokers; and mild (<1 alcohol unit/day) vs. moderate (≥1 alcohol units/day) alcohol consumers. Possible differences in NMDAR coagonist concentrations in plasma and CSF were investigated using ANCOVA with age and storage duration as covariates. The significance threshold was Bonferroni-corrected ($\alpha = 0.00625$). Compared to non-smokers, smokers displayed lower levels of D-Proline in plasma ($p = 0.0030$, Cohen's $d = -0.40$) and D-Proline in CSF ($p = 0.0027$, Cohen's $d = -0.33$). D-Serine in CSF was higher in smokers than in non-smokers ($p = 0.0056$, Cohen's $d = 0.36$). After subdividing participants based on smoking quantity (< 1 cigarette; 1-10 cigarettes; and > 10 per day), dose-dependent decreases were demonstrated for smokers in D-Proline in CSF ($F= 8.15$, $p= 3.56 \times 10^{-4}$) and plasma ($F= 6.29$, $p= 0.0021$). No differences in NMDAR coagonist levels between the two alcohol consumption groups were detected. To our knowledge, this is the first report to implicate D-amino acids in smoking behavior of humans. Whether such concentration differences lie at the root of or result from smoking habits may be addressed in prospective studies.

Introduction

In Europe, the prevalence of smoking was ~ 28.6% in 2005 [1, 2]. Smoking decreases life expectancy by 12-20 years and is one of the major mortality risk factors in the world: more than 5 million people worldwide die of the consequences of tobacco consumption yearly [1].

Although genetic variants in genes encoding neuronal nicotinic acetylcholine receptor (nAChR) subunits (e.g. CHRNA3 and CHRNA5) are associated with smoking quantity, the explained variances by such single nucleotide polymorphisms (SNPs) are low [3, 4]. This suggests that other pathways, such as the glutamatergic neurotransmission system, play a role in smoking behavior. Glutamate is the prime excitatory neurotransmitter in the central nervous system (CNS) and binds to several receptors, including the ionotropic N-methyl-D-aspartate receptor (NMDAR). Animal studies have reported that nicotine increases glutamate concentrations in the ventral tegmental area (VTA) [5, 6] and exerts an excitatory effect on the NMDAR in dopaminergic neurons in the VTA [6-8], the nucleus accumbens [9] and the central nucleus of the amygdala [10]. In addition, nicotine self-administration in rats up-regulates the expression of NMDAR subunits in the VTA and amygdala [10]. In vivo blockade of the NMDAR by NMDAR antagonists diminishes nicotine-induced dopamine release [9] and nicotine self-administration [10], but promotes tolerance to nicotine administration in rats [11, 12]. NMDAR antagonism could therefore constitute a target in the treatment of nicotine dependence, although to date this has not been successful in humans [13].

The other substance frequently associated with burden of disease in Western society is alcohol. The World Health Organization estimated that alcohol is currently the world's third largest risk factor for burden of disease [14]. In contrast to nicotine, ethanol has been reported to block the NMDAR [15-18]. Chronic alcohol administration increases the number of NMDARs, thus playing a role in alcohol dependence and withdrawal seizures [15, 19, 20]. Moreover, NMDAR antagonists (e.g. MK-801 and ketamine) cause ethanol-like effects in animals and humans [21-23].

Glycine, D-Serine, D-Alanine, and L-Proline are coagonists at the NMDAR [24-34]. To date, neither in plasma nor cerebrospinal fluid (CSF) have these constituents been investigated for their role in smoking or alcohol consumption tendencies. Elucidating possible substance use-associated abnormalities in NMDAR coagonist levels may deepen the understanding of NMDAR involvement in substance use and susceptibility to nicotine and alcohol dependence. Moreover, the success rates of pharmacotherapies in the treatment of nicotine [35] and alcohol [36] dependence are limited and therefore novel agents may contribute to treatment optimization. Elucidating NMDAR coagonist functionality in subjects who consume these substances will potentially set the stage for further research into pharmacological modulations of NMDAR gating in preclinical settings and human consumers.

As the NMDAR is involved in smoking and alcohol use, we hypothesized that consumers of nicotine and alcohol display altered levels of NMDAR coagonists in CSF and plasma. Given the demonstrated increase in glutamate concentrations in the rat brain and up-regulation of the NDMAR after nicotine administration [5, 7, 37], we postulated that coagonist levels are elevated in smokers. As ethanol blocks the NMDAR [15-18], we expected NMDAR coagonists in alcohol consumers to be decreased. Enantiomers of the abovementioned coagonists were included in the current study as synthesis and degradation of stereoisomers are likely to be interdependent. We thus measured concentrations of Glycine (that is not chiral) and the enantiomers of Alanine, Serine, and Proline in a unique study population with CSF and plasma available for 403 subjects. We then compared these levels across categories of nicotine and alcohol consumption to detect possible concentration differences associated with the number of cigarettes or alcoholic beverages consumed.

Materials and methods

Subjects

Subject recruitment was described in detail previously [38]. In brief, from August 2008 until November 2011, 403 subjects were recruited at outpatient preoperative screening services in and around Utrecht, the Netherlands. At these services, subjects are advised by the anesthesiologist to start fasting at least 6 hours preoperatively and refrain from smoking and alcoholic beverages at least 24 hours before the procedure. This enabled us to study sustained effects -i.e. effects persisting for at least 24 hours- of smoking and alcohol on amino acid concentrations. Subjects undergoing spinal anesthesia for minor elective surgical procedures and aged between 18-60 years were included. Each candidate participant received a personal telephone interview to exclude subjects with psychotic or major neurological disorders (stroke, brain tumors, neurodegenerative diseases) and to record any self-reported history of other psychiatric illness or any use of psychotropic medication. Informed consent was obtained from the participants and the ethics committee of the UMCU and all local ethics committees approved the study.

CSF and Plasma Collection and Chemical Analyses

Whole blood was collected in EDTA tubes for plasma extraction. Plasma was extracted by centrifuging whole blood at ambient temperature for 10 minutes at 2,500 g, after which plasma was stored at -80 °C. The standardized operating procedures adopted to collect 6mL of CSF from each subject were described previously [38]. Chemical analyses of the L- and D-isomers of Alanine, Serine, Proline,

and Glycine (no D-isomer) were conducted using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) according to a validated method [39].

Questionnaires

During a two-week period after the elective procedure, subjects filled out online questionnaires about their health. The following questions regarding current smoking and alcohol consumption habits were asked:

- Do you smoke? If so, how many cigarettes do you smoke per day?
- Do you drink alcohol? If so, how many units of alcohol do you drink per day?

Smokers were asked to choose between the following divisions of number of cigarettes smoked per day: < 1; 1-10; 11-20; 21-30; and > 30. Alcohol consumption was quantified using the following cut-offs: < 1; 1-3; 4-6; and > 6 alcoholic beverages.

We chose web-based symptom questionnaires as these have been validated as reliable assessment tools in a range of epidemiological studies [40, 41] and may decrease socially desirable responses compared to face-to-face interviews or questionnaires that are filled out in clinical settings [42, 43].

Statistical Analyses

Regarding smoking habits, the participants were divided into two groups: non-smokers (no current smoking) and smokers (any number of cigarettes smoked daily). Alcohol users were divided into mild alcohol consumers (<1 alcohol unit/day) and moderate alcohol consumers (≥1 alcohol units/day) as only 11.2% of the study population proved abstinent.

Outliers were defined as subjects having at least one measurement 3 or more standard deviations (SDs) from the mean and were excluded from further analyses. As no generally accepted covariates for amino acid measurements are available, we comprehensively assessed possible covariates during the study period. The following variables were tested: age, sex, the rostrocaudal concentration gradient (reflected by the participants' height), lumbar puncture level (binary, i.e. lumbar vertebrate levels ≤ 3 vs. > 3 as estimated by the anesthesiologist), time elapsed prior to storage (continuous, in hours), time of the day of lumbar puncture (continuous, rounded to the half hour), storage duration until chemical analyses (continuous, in months), and amount of CSF drawn (continuous, as in 8% of the cases > 7 or < 5 mL of CSF were drawn). Relevant covariates were defined as variables that correlated with more than one amino acid at a Spearman's ρ p-value < 0.05. If covariates were collinear (r > 0.5), the covariate with most missing data was excluded from the model.

Normality of the distributions was verified with a Kolmogorov-Smirnov (K-S) test and defined as a two-tailed asymptotic p-value ≥ 0.05. Non-normally distributed amino acids were logarithm (log) transformed. Homogeneity of variances (defined as

a Levene's test p-value > 0.05) and homogeneity of regression slopes (by visual inspection of the scatterplots between the amino acids and the covariates) between groups were verified. A one-way ANCOVA was conducted correcting for all relevant covariates. Significance was Bonferroni corrected (= 0.05 / 8 = 0.00625 as 4 NMDAR coagonists were tested for both nicotine and alcohol consumption, while stereoisomers in CSF and plasma were highly correlated). Stratification by sex was not performed due to the relatively small number of female participants (N=99). In the event Bonferroni-corrected significance was attained, we tested whether concentration differences were substance dose-dependent. To that end, covariates that correlated at p< 0.05 with that amino acid were determined and ANCOVA correcting for these covariates (or ANOVA in the event no covariate correlated with that amino acid) was conducted for the following substance consumption categories: < 1 consumption a day (category 1); 1-10 cigarettes or 1-3 alcoholic beverages a day (category 2); and > 10 cigarettes or > 3 alcoholic beverages a day (category 3). Given the small numbers of subjects smoking > 20 cigarettes a day or consuming > 4 alcoholic beverages daily this subdivision resulted in the most equal numbers of subjects per category. All statistical analyses were conducted using SPSS version 20 (SPSS, Chicago, IL).

Results

Subject and NMDAR coagonist characteristics

Information about smoking and alcohol consumption habits in addition to NMDAR coagonist levels in either CSF or plasma were available for 403 subjects. Exclusion of outliers (55 subjects) brought the study population to 348 subjects (249 men and 99 women). Characteristics of these 348 subjects (N=325 for whom CSF was available; N=307 for whom plasma was available) are summarized in Table 1. The only significant differences in subject characteristics across substance use categories were found for psychiatric comorbidity and psychotropic medication (both increased in smokers vs. non-smokers; χ-square p-values of 0.030 and 0.018, respectively). Six NMDAR coagonists were normally distributed (L-Serine and D-Serine in CSF; L-Alanine, Glycine, D-Serine, and L-Proline in plasma). The other NMDAR coagonists were log transformed, resulting in a normal distribution by K-S testing for all concentrations except L-Proline in CSF (that approximated normality upon visual inspection).

NMDAR coagonist concentrations in smokers and non-smokers

The covariates age and storage duration were the only covariates that correlated with > 1 amino acid. These showed correlations (p<0.05) with most of the amino acids. For these covariates no data were missing.

Table 1. Subject characteristics; means (SD) are given unless stated otherwise. The only significant differences between substance use categories were found for smoking: psychiatric comorbidity and psychotropic medication (non-smokers vs. smokers respective χ-square p-values of 0.030 and 0.018).

Parameter	Total	Non-smokers	Smokers	Mild alcohol users	Moderate alcohol users
Subjects (N)	348	251	97	228	120
Age (y)	39.5 (10.9)	40.5 (10.5)	36.7 (11.5)	40.0 (10.5)	38.6 (11.7)
Sex (% males)	71.6	71.3	72.2	65.8	82.5
Height (cm)	179.7 (8.9)	179.8 (9.2)	179.7 (8.3)	179.1 (9.4)	181.1 (7.9)
Storage duration (months)	7.8 (4.8)	7.6 (4.5)	8.1 (5.5)	7.9 (4.8)	7.4 (4.8)
CSF quantity (ml)	5.4 (1.1)	5.4 (1.0)	5.3 (1.1)	5.4 (1.1)	5.5 (1.0)
Arthroscopy (%)	66.1	66.9	63.9	63.6	70.8
Psychiatric comorbidity (%)	4.3	2.8	8.2	3.9	5.0
Psychotropic Medication (%)	4.6	2.4	10.3	5.3	3.3
Other Medication (%)	14.7	16.7	9.28	13.6	16.7

After correction for these two covariates, all D-enantiomers in plasma were lower in smokers than in non-smokers, but for plasma only D-Proline reached Bonferroni-corrected significance: the D-Proline concentration in plasma was lower in smokers than in non-smokers ($F_{1,303}$= 8.93, p= 0.0030, Cohen's d= -0.40; Table 2 and Figure 1A). The plasma D-Alanine difference between smokers and non-smokers was nominally significant (p = 0.022) and plasma D-Serine was only slightly decreased in smokers (p = 0.19).

D-Proline in CSF was also lower in smokers than in non-smokers ($F_{1,321}$= 9.13, p= 0.0027, Cohen's d= -0.33; Figure 1B), while D-Serine in CSF was higher in smokers than in non-smokers ($F_{1,321}$= 7.78, p= 0.0056, Cohen's d= 0.36; Figure 1C).

We then assessed whether the concentration differences were substance dose-dependent for the Bonferroni-corrected significant results. While for D-Serine in CSF no smoking dose-dependent effect was detected, we found significant differences between the three categories for D-Proline (Table 3):

- D-Proline in plasma (no significantly correlated covariates with the outcome variable): $F_{2,304}$= 6.29, p= 0.0021 (Figure 2A).
- D-Proline in CSF (covariate: storage duration): $F_{2,321}$= 8.15, p= 3.56 x 10^{-4} (Figure 2B).

Finally, as psychiatric comorbidity and psychotropic medication significantly differed between smokers and non-smokers, we tested whether these variables were correlated with any of the abovementioned amino acids. D-Serine in plasma correlated with psychiatric comorbidity (Spearman's rho = 0.16, p = 0.005), but correcting for this variable did not change the results (data not shown). Variances and regression slopes were homogeneous between groups for all reported differences.

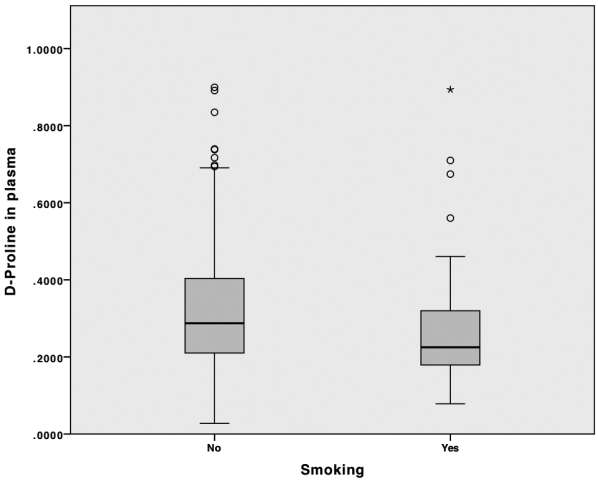

Figure 1 A. D-Proline in Plasma in Smokers vs Non-Smokers.

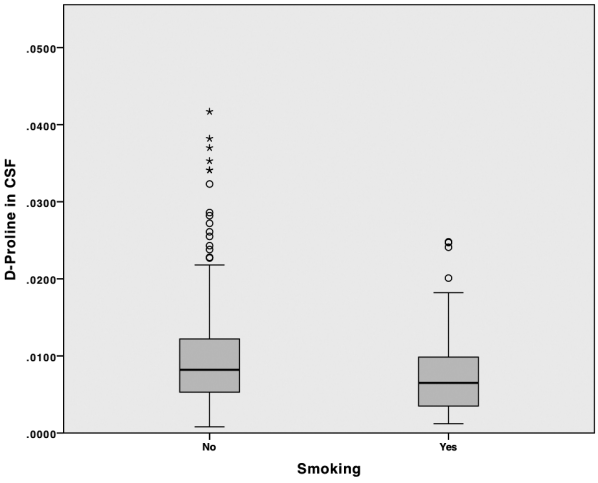

Figure 1 B. D-Proline in CSF in Smokers vs Non-Smokers.

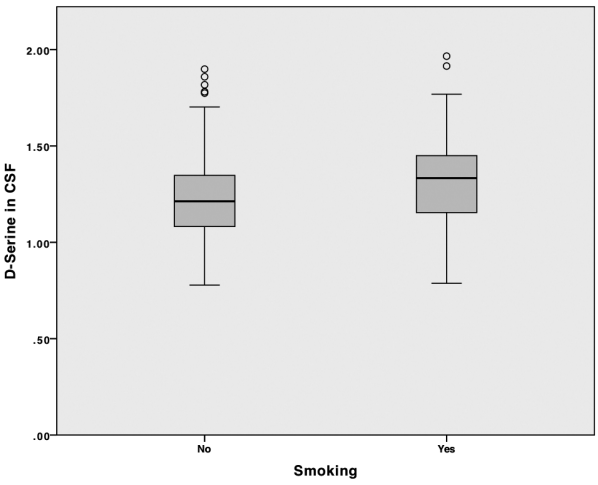

Figure 1 C. D-Serine in CSF in Smokers vs Non-Smokers.

NMDAR coagonist concentrations in mild and moderate alcohol consumers

No differences in NMDAR coagonist levels between mild (<1 unit/day) and moderate alcohol consumers (≥1 units/day) were detected.

Amino acid Reference Values

As reference values for most D- and L-amino acids in CSF and plasma are currently lacking or based on limited study populations, we provide these values before removal of any outliers to give an impression of their naturally occurring variation (Table 4).

Table 2. Concentrations (in µmol/L, ±SD) of all measured amino acid enantiomers in non-smokers vs. smokers. Significant findings are in bold ($p < 0.00625$). F-ratios and effect sizes are reported when the p-value is significant; nominally significant results ($p < 0.05$) are in italics.

	Mean ± SD Non-smokers	Mean ± SD Smokers	P-value	F-ratio	Cohen's d
L-Alanine CSF	31.5 ± 7.20	30.4 ± 6.63	0.75		
D-Alanine CSF	0.16 ± 0.08	0.15 ± 0.09	0.46		
Glycine CSF	6.21 ± 2.04	6.37 ± 1.69	0.08		
L-Serine CSF	23.4 ± 3.55	24.06 ± 3.74	0.28		
D-Serine CSF	1.23 ± 0.21	1.31 ± 0.23	**0.0056**	7.78	0.36
D-Proline CSF	0.01 ± 0.007	0.008 ± 0.005	**0.0027**	9.13	-0.33
L-Proline CSF	0.69 ± 0.35	0.74 ± 0.34	0.20		
L-Alanine plasma	337 ± 71.2	327 ± 77.5	0.22		
D-Alanine plasma	0.86 ± 0.47	0.76 ± 0.41	*0.022*		
Glycine plasma	164 ± 45.3	172.4 ± 46.7	0.072		
L-Serine plasma	84.2 ± 15.4	86.8 ± 16.1	0.44		
D-Serine plasma	1.10 ± 0.26	1.08 ± 0.22	0.19		
D-Proline plasma	0.32 ± 0.16	0.26 ± 0.14	**0.0030**	8.93	-0.40

Table 3. Per smoking category, concentrations (µmol/L, ± SD) of the amino acids that differed significantly between smokers and non-smokers are given.

	< 1 cigarette / day (N=243)	1-10 cigarettes / day (N=31)	>10 cigarettes / day (N=51)	F-Statistic	P-Value
D-Proline in CSF	0.010 ± 0.007	0.008 ± 0.006	0.007 ± 0.005	8.15	3.56×10^{-4}
D-Proline in Plasma	0.32 ± 0.16	0.29 ± 0.16	0.24 ± 0.10	6.29	0.0021

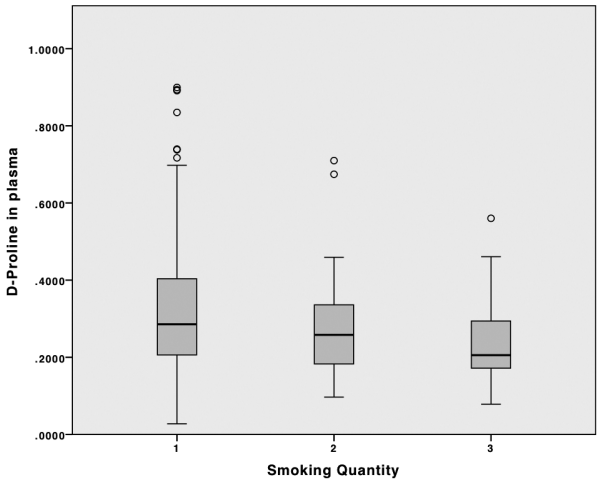

Figure 2 A. D-Proline in Plasma per Smoking Quantity Category (1= < 1 cigarette; 2= 1-10 cigarettes; 3= > 10 cigarettes per day).

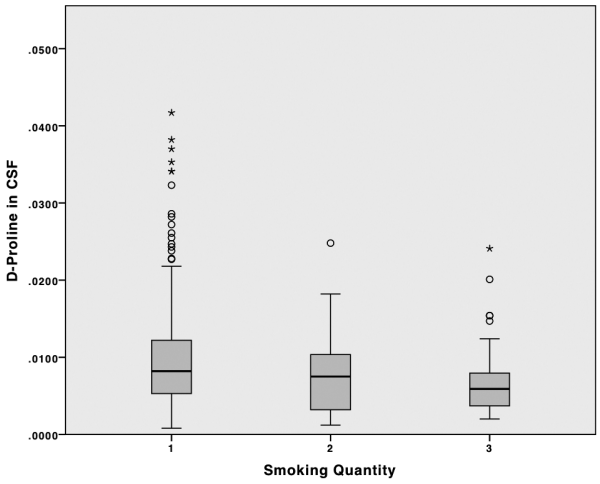

Figure 2 B. D-Proline in CSF per Smoking Quantity Category (1= < 1 cigarette; 2= 1-10 cigarettes; 3= > 10 cigarettes per day).

Table 4. L and D-enantiomer reference values (N=451) in plasma and CSF before removal of outliers.

	Cerebrospinal fluid (µmol/L)						
	L-Alanine	**D-Alanine**	**Glycine**	**L-Serine**	**D-Serine**	**L-Proline**	**D-Proline**
Total (N=406)	31.5 ± 7.69	0.17 ± 0.13	6.57 ± 2.91	23.9 ± 4.33	1.26 ± 0.23	0.78 ± 0.56	0.011 ± 0.011
Men (N=286)							
18-30 yrs (N=90)	29.1 ± 6.47	0.14 ± 0.08	5.94 ± 2.45	24.7 ± 3.53	1.31 ± 0.22	0.67 ± 0.33	0.008 ± 0.008
30-45 yrs (N=90)	31.3 ± 7.56	0.20 ± 0.16	6.68 ± 2.57	23.2 ± 4.11	1.23 ± 0.24	0.80 ± 0.51	0.010 ± 0.008
45-60 yrs (N=106)	35.4 ± 8.17	0.19 ± 0.131	6.62 ± 2.25	22.4 ± 3.68	1.20 ± 0.22	0.87 ± 0.53	0.015 ± 0.015
Women (N=120)							
18-30 yrs (N=24)	27.3 ± 6.52	0.20 ± 0.21	5.86 ± 2.98	24.5 ± 3.29	1.39 ± 0.22	0.59 ± 0.37	0.006 ± 0.005
30-45 yrs (N=43)	29.0 ± 6.49	0.13 ± 0.053	7.23 ± 5.03	25.9 ± 7.00	1.29 ± 0.25	0.76 ± 0.72	0.007 ± 0.005
45-60 yrs (N=53)	32.4 ± 7.31	0.17 ± 0.18	7.20 ± 3.11	25.1 ± 3.89	1.32 ± 0.23	0.85 ± 0.90	0.012 ± 0.013

	Plasma (µmol/L)						
	L-Alanine	**D-Alanine**	**Glycine**	**L-Serine**	**D-Serine**	**L-Proline**	**D-Proline**
Total (N=389)	335 ± 74.1	0.87 ± 0.52	169 ± 49.9	85.5 ± 19.1	1.10 ± 0.25	178 ± 48.4	0.34 ± 0.25
Men (N=278)							
18-30 yrs (N=83)	346 ± 76.6	0.88 ± 0.46	177 ± 48.4	92.7 ± 27.3	1.17 ± 0.27	202 ± 45.8	0.32 ± 0.20
30-45 yrs (N=91)	340 ± 73.9	1.01 ± 0.65	164 ± 46.6	82.3 ± 14.9	1.12 ± 0.29	189 ± 53.2	0.38 ± 0.20
45-60 yrs (N=104)	351 ± 70.9	0.88 ± 0.50	158 ± 35.9	80.8 ± 15.3	1.07 ± 0.22	178 ± 39.7	0.43 ± 0.32
Women (N=111)							
18-30 yrs (N=21)	289 ± 70.0	0.88 ± 0.60	133 ± 44.8	85.7 ± 16.4	1.00 ± 0.24	153 ± 62.9	0.26 ± 0.16
30-45 yrs (N=41)	297 ± 73.9	0.66 ± 0.34	186 ± 54.9	89.7 ± 17.7	1.04 ± 0.25	150 ± 33.5	0.25 ± 0.14
45-60 yrs (N=49)	321 ± 65.7	0.83 + 0.51	190 ± 64.8	86.9 ± 15.5	1.01 ± 0.31	151 ± 37.3	0.32 ± 0.21

Discussion

Here, we demonstrate an increase in CSF D-Serine and a decrease in CSF and plasma D-Proline in smokers compared to non-smokers (N=348). The D-Proline concentration differences proved substance dose-dependent. No differences in NMDAR coagonists between mild and moderate alcohol consumers were found.

The current study being the first to comprehensively compare NMDAR-coagonist levels in the CSF and plasma of smoking and non-smoking subjects, our results cannot be directly compared to previous findings. To our knowledge, only in smoking pregnant women and smoking schizophrenia patients and controls have amino acid levels been compared to non-smokers. None of these two studies detected smoking behavior-dependent differences in plasma amino acid levels [44 45]. However, instead of parsing the results by enantiomer only total amino acid concentrations were investigated in those studies. As the primary contribution to total amino acid concentrations comes from L-enantiomers such previous findings combined with the data presented here suggest that smoking may be associated only with D-enantiomers of amino acids.

A possible mechanism whereby smoking leads to D-amino acid aberrations relates to the direct effect of nicotine on the NMDAR. Nicotine-dependent activation of nAChRs enhances glutamatergic transmission [8]. Glutamate can then activate the NMDAR, leading to long-term potentiation (LTP) [8]. Conceivably, LTP may thereafter result in D-Serine upregulation in the CNS as D-Serine is the most important ligand at the synaptic NMDAR Glycine binding site [46]. Such increased D-Serine consequential to nicotine effects in the CNS is in line with the high CSF D-Serine concentrations we observed in smokers. Relatively high CSF D-Serine in smokers is further consistent with a burgeoning body of evidence demonstrating lack of efficacy of D-cycloserine in reducing tobacco consumption [47-50]. Further increasing already high CNS D-Serine levels by D-cycloserine administration in smokers would likely be unsuccessful for this indication.

To our knowledge, no significant differences in NMDAR coagonist levels between mild and moderate alcohol consumers have been reported, although increased Glutamate and Proline levels in plasma of alcohol dependent patients with positive alcohol breath tests have been reported [51]. This suggests that alcohol consumption shortly prior to sampling affects Glutamate and Proline levels. The subjects included in the current study had fasted prior to lumbar puncture and were instructed to be abstinent for at least 24 hours prior to lumbar puncture. Possibly, such previously reported effects of alcohol on Proline concentrations are short-lived. Higher Proline levels in plasma in subjects with alcohol abuse or dependence compared to healthy controls have been described [45]. Our study population differed in that here analyses were performed on subjects from the general population – not a patient-control cohort.

A limitation of this study is that no formal assessments of alcohol and nicotine dependence and abuse were conducted. Therefore, the effects of such diagnoses on D-amino acid levels cannot be construed from our data. In addition, the data we provide are cross sectional and therefore inferences about causality cannot be made. Furthermore, the limited number of study participants precluded us from running linear regression as few smokers fell in high consumption categories. While designs quantitatively assessing smoking habits and measuring NMDAR coagonists in the plasma of a substantial number of participants are feasible, CSF would be a more cumbersome target. On a similar note, this study was underpowered to stratify analyses by sex given the small number of female subjects. Finally, whether nicotine-dependent influences on NMDAR coagonist levels are sustained over time cannot be deduced from our data as measurements were not performed at different time points. However, as participants were advised to refrain from smoking at least 24 hours preoperatively, it is possible that D-amino acid aberrations in smokers are not short-lived.

In conclusion, the differences in D-amino acids in CSF and plasma that we detected support their involvement in smoking behavior. Animal studies may determine whether nicotine-dependent activation of the NMDAR is mediated by D-Serine and D-Proline. Future longitudinal designs in humans that incorporate measurements of D-amino acid levels before and after substance consumption may clarify whether differences in such levels are state or trait-dependent. Furthermore, formally diagnosing nicotine dependence in such projects may tease apart associations of substance use phenotypes with D-amino acid aberrations.

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9

Summary and General Discussion

Summary

The aim of the studies described in this thesis was to quantify the contributions of genetic and seasonal factors to cerebrospinal fluid (CSF) constituents presumed to play a role in brain function and/or neuropsychiatric disorders. The conclusions that follow from the data presented in each chapter are:

- Concentrations of the prime 5-HT (serotonin) metabolite in CSF, 5-hydroxy-indoleacetic acid (5-HIAA), follow a seasonal pattern with a peak in spring and a trough in fall (Chapter 2).
- Seasonality explains over 5% of the variance in CSF 5-HIAA. The s-allele of the 5-HT transporter polymorphic region (5-HTTLPR) polymorphism dose-dependently determines the degree to which 5-HT turnover varies across seasons. Increased 5-HT seasonality in turn is correlated with depressive symptomatology (Chapter 3).
- An intronic variant in *ERBB4* is associated with CSF GABA levels in a dose-dependent manner (Chapter 4).
- *BDNF* Val66Met homozygosity does not exert substantial effects on plasma BDNF levels (Chapter 5).
- By genome-wide analyses of N-methyl-D-aspartate receptor (NDMAR) coagonists, quantitative trait loci at genes encoding a transporter protein (*SLC6A20*) and enzymes (*PRODH* and *DAO*) were identified. Biologically tenable associations in both plasma and CSF were thus detected (Chapter 6).
- A locus at *SSTR* – encoding a neuropeptide relevant to CNS signal transduction- was associated with CSF monoamine metabolite (MM) levels. This locus in turn was found to control mRNA expression of *PDE9A*, a gene previously implicated in depression and antidepressant response (Chapter 7).
- Although causality and directionality are unknown, concentrations of D-amino acids in plasma and CSF differ between smoking and non-smoking individuals (Chapter 8).

Lessons Learned

Equally relevant as the main findings outlined above are the lessons learned from the set-up and methods applied to obtain such results.

Statistical analyses

Standard Operating Procedures (SOPs) outlined in a comprehensive review [1] were extended and adapted to the constituents and biofluids under investigation here to assure methodological homogeneity. Although age and sex are the covariates usually

accounted for in metabolomics [1], storage duration was additionally corrected for (Chapter 6). While approximately 50% of the investigated amino acids showed concentration gradients with increasing storage duration, Glutamate concentrations most rapidly increased during storage (approximately 10-fold over the course of less than a year). This increase, which was most likely due to the conversion from Glutamine [2], precluded any further analyses on this neurotransmitter. For the NMDAR coagonists D-Serine and L-Proline, however, storage-dependent concentration gradients were small (yet significant) and after correction for this variable biologically plausible association signals were obtained (Chapter 6).

A statistical concern in genome-wide association studies (GWASs) is how to deal with multiple testing. In the current targeted metabolomics GWASs we corrected the generally accepted genome-wide significance threshold for the number of independent traits, although ratios were also tested (Chapters 6 and 7). Future non-targeted CSF metabolomics projects that measure hundreds of metabolites will be methodologically sound only if sample sizes are ramped up (probably to the order of thousands), a replication cohort is available and Bonferroni-correction is applied.

Behavioral assessments

Recent evidence suggests large sample sizes are required to detect genetic association with clinical disease entities and even larger samples for (behavioral) quantitative traits. For instance, 50,000 schizophrenia patients are estimated to deliver sufficient power to explain 10% of the phenotypic variation [3]. In comparison, genome-wide studies of 180,000 subjects were needed to identify genomic loci that explain the same proportion of phenotypic variance in human height [4]. To illustrate the complexity of behavioral genetics, the genetic architecture of personality traits has remained elusive, despite inclusion of >17,000 participants and 2.4M markers in meta-analyses [5]. Reasons for such limited power of behavioral genetic analyses include genetic heterogeneity, genetic complexities such as gene-environment interactions, limited validity and generalizability of behavioral questionnaires, and behavioral state influences on trait measurements.

In light of the abovementioned evidence, we were underpowered to run genetic association analyses of behavioral traits. The presented behavioral findings relate to concentrations of investigated constituents and are restricted to two categories, depressive symptoms (Chapter 3) and smoking behavior (Chapter 8). No correlations between CSF 5-HIAA and depressive symptoms (Chapter 3) or CSF GABA and anxiety (Chapter 4) were detected. Here, the limited number of behavioral measurements precluded us from running further behavioral analyses. For instance, early life adversity has increasingly been identified as a moderator for schizophrenia [6], suicide [7] and other psychiatric conditions over the past decade. Not having included a scale measuring early life stress, we were unable to assess the influence of such adversity on CSF

measurements or detect interactions with genomic variation. Upcoming projects investigating relationships between human behavior and CSF constituents may thus include a wider range of behavioral assessments (e.g. a childhood trauma questionnaire). Moreover, such designs should incorporate diagnostic tools to detect possible differences in CSF constituents between psychiatric cases and controls.

Hypothesis-driven vs hypothesis-generating approaches

This thesis demonstrates that for quantitative trait locus (QTL) analysis of CSF constituents both hypothesis-driven and hypothesis-generating human genetic approaches can be informative. Which of the two to adopt is primarily dependent upon the available sample size and the presence or absence of *a priori* hypotheses. A relatively small study population (Chapter 4) yielded sufficient power for a hypothesis-based association test, after which inclusion of > 400 subjects resulted in genome-wide significant association signals (Chapters 6 and 7). On the other hand, despite applying similarly stringent SOPs to the monoamine metabolite GWAS, no direct genetic associations in genes involved with monoamine metabolism were detected. In line with the modest heritability estimates of CSF monoamine metabolites (0.30 – 0.52)[8-10], environmental factors (e.g. seasonality and dietary intake) or gene-environment interactions may explain a proportion of their variance.

The ultimate goal of hypothesis-driven and hypothesis-generating strategies is to detect causal variants that control a quantitative phenotype. Given linkage disequilibrium (LD, the non-random co-occurrence of alleles), associated SNPs may not be responsible for alterations of biological quantitative phenotypes, but merely tag such causal variants. Formally, proof that allelic substitutions are causal may only be obtained by introducing the allele in a mouse strain against equal genetic and environmental backgrounds[11]. However, the influence of associated variants on traits may be corroborated by alternative approaches, e.g. replication; demonstrating genotype-dependent differences in gene expression (eQTLs); showing that mRNA is expressed in a tissue relevant to the phenotype under study; and verifying that the associated variant is located in or near a biologically plausible gene[12]. All four strategies have proven key in validating the QTLs highlighted in Chapters 6 and 7. The strength of the associations outlined in Chapter 6 lies in their location near or within genes involved with amino acid metabolism and transporter mechanisms. For example, L-Proline in CSF was found to be associated with a gene encoding a Proline transporter that is highly expressed in rodent meninges and choroid plexus, *SLC6A20*. Moreover, the same chapter describes how replication in a publicly available dataset confirmed the strongest QTL presented in this thesis. In Chapter 7, associations of MMs with polymorphisms in a range of genes expressed in the brain and involved with neurotransmitter signaling are described. Furthermore, an eQTL was found for the genome-wide significant SNP. Nonetheless, more variants than the ones highlighted

here -among which those with lower minor allele frequencies and of smaller effect- possibly together explain a substantial proportion of the variance in analyte levels. Only by increasing sample sizes and the availability of a replication cohort may firm conclusions regarding the number of loci per neurotransmitter (metabolite) be drawn. Moreover, while the SNP array employed here offers good yet incomplete coverage of the human genome, exome SNP chips containing over 200,000 nonsynonymous SNPs may yield a more nuanced picture of functional variants implicated in CSF QTLs.

Methodological Strengths

Sample collection

Besides the clear advantages of not having to perform invasive procedures to collect CSF or blood, the standardized conditions under which CSF was drawn have probably resulted in accurate and generalizable measurements. Moreover, the SOPs alluded to in the introduction have likely benefitted the reliability and reproducibility of the measurements.

Study populations

Stringent criteria were applied to the inclusion of participants, thereby increasing genetic homogeneity and rendering age-dependent concentration gradients unlikely. Moreover, subjects undergoing spinal anesthesia reflect the general population, for example with regard to history of cancer and comorbidities [13]. The protocol that we developed furthermore allowed us to collect CSF at a relatively fast pace, i.e. from an average of ~160 subjects per year. This resulted in the largest study population to date for CSF studies in human subjects representative of the general population. Nonetheless, ramping up the scale of study cohorts will likely yield more CSF quantitative trait loci. Our design may fill gaps in the current knowledge of human metabolomics as metabolomics data were lacking for CSF before the current studies [1].

Testing of ratios

The highly sensitive HPLC methods employed here guarantee more sensitive quantitation of concentrations than high-throughput nuclear magnetic resonance techniques generally used in non-targeted metabolomics. Ratio testing methodology was extended from previous designs [1] to include ratios between both L and D-enantiomers and the two biofluids. As highlighted in Chapter 6, the genetic association analyses of ratios flagged up genes encoding enzymes and transporters. For instance, by computing L to D-Serine ratios in CSF, evidence of association in the gene encoding serine racemase -the enzyme hypothesized to mediate L to D Serine

conversion in the CNS- was detected. Resulting in an even stronger genetic association was the analysis of the L-Proline plasma to CSF ratio that suggested a transporter mechanism is mostly implicated in L-Proline transport from peripheral tissues to the CNS.

Genotyping techniques

Genotyping was performed on a single platform for the purpose of the studies in this thesis except Chapter 5: the recently developed Illumina HumanOmniExpress Beadchip (730,525 single nucleotide polymorphisms (SNPs)). Data from all three phases of the HapMap project have optimized the coverage of this array that captures 73% of common (MAF > 5%) SNP variation in the CEU population (www.illumina.com). Moreover, imputation based on the latest release of 1000Genomes has likely improved coverage and imputation accuracy compared to HapMap-based imputation techniques (Chapters 6 and 7). Improved imputation coverage and quality may particularly apply to the 22q11 region where low copy number repeats and pseudogenes restrict genetic analyses [14-16]. Strikingly, the strongest association signal was found in the 22q11 region for L-Proline and was in close proximity to a locus associated with the ratio of Proline to Valine in a meta-analysis [17].

Outlook

The presented data may have implications for several fields of research. First, in the 1980s and 1990s CSF was one of the most popular targets in biological psychiatry research. At the time, investigators aimed to uncover case-control differences in MM concentrations. Although meta-analytical evidence points to 5-HIAA alterations in suicide [18], CSF findings with regard to affective and psychotic disorders are highly inconsistent. The largest CSF study on psychiatric patients was performed on ~200 subjects [19] and likely still lacked power to pick up CSF constituent abnormalities in psychiatric cases. Although quantitative behavioral measurements in healthy subjects differ from dichotomized psychiatric traits, Chapters 3 and 8 hint that the effect sizes of CSF aberrations associated with behavioral phenomena are indeed likely to be small. Alternatively, many CSF components may not directly correlate with behavioral features. Indeed, not absolute 5-HIAA concentrations but seasonal variation in 5-HIAA correlated with depressive symptomatology (Chapter 3). This finding and the signaled D-amino acid abnormalities in subjects who smoke (Chapter 8) should be replicated in independent cohorts before firm conclusions may be drawn. Longitudinal designs and repetitive CSF sampling will further disentangle the directionality of such correlations.

Second, metabolomics is a rapidly evolving area of research that recently showed

relevance to pharmacogenomics and a range of medical conditions [17]. Psychopharmacogenomics consortia (e.g. the International Consortium on Lithium Genetics [20]) will hopefully shed light on the genetic variants implicated in response and adverse reactions to psychotropic medication. Extrapolating the pharmacogenomic value of metabolic QTLs from serum [17] to CSF suggests that neurometabolic QTLs will prioritize genetic research into psychotropic medication response. For instance, the highlighted missense variant in Proline transporter SLC6A20 (Chapter 6) may play a role in transport to the CNS of Proline and also of other small molecules targeted by psychotropics. To unambiguously unmask additional loci influencing CSF metabolites and their explained variances, sample sizes of several thousands are probably warranted. Absence of a replication cohort -the foremost limitation of the presented GWASs- may be overcome by ramping up the scale of future CSF GWASs. Such designs may be complemented by sequencing the highlighted genes, exome or whole-genome next-generation sequencing, analyses of epigenetic mechanisms, and rodent knockdown projects.

Third, CSF studies may be complemented by brain imaging techniques to provide a more nuanced understanding of possible temporal changes in CSF constituents. For example, GABA may be measured with spectroscopy before and after subjecting individuals to stress, thereby providing insight into state-dependent shifts in GABA concentrations per chosen brain region. CSF, on the other hand, provides a "snap shot" of overall CNS GABA turnover and thus yields complementary information regarding CNS GABA functionality.

Fourth, the presented seasonality results fit the rise of interest in 5-HT seasonality research. Here, CSF 5-HIAA seasonal variation and its genetic and behavioral correlates in a relatively high latitude area (Northern Europe) were demonstrated, while it is unknown whether similar phenomena apply to lower latitudes. Follow-up work around the equator may thus unravel 1) how CSF 5-HIAA concentrations differ between seasons in low latitude regions and 2) whether the 5-HTTLPR interacts with month of the year to influence 5-HT seasonality in such areas. Psychiatric-behavioral analyses may be incorporated into such projects to find out whether a 5-HTTLR-by-season interaction mediates susceptibility to season-associated psychiatric conditions, e.g. affective disorders.

Finally, human genetics and preclinical neuroscience research may benefit from the QTLs identified in this thesis. For instance, as the SNP in *ERBB4* found to be associated with GABA in CSF is intronic (Chapter 4), sequencing this gene may clarify functional elements. Furthermore, the genetic variants in *SLC6A20*, *DAO* and *PRODH* (Chapter 6) and gene expression of *PDE9A* (Chapter 7) may be followed up in genetic studies of psychiatric patients to unravel their contributions to psychiatric phenotypes. For instance, allelic variation at the highlighted polymorphisms may be compared between psychiatric cases and controls. Moreover, their functional implications in both patients

and controls may be clarified by neuropsychological tests and brain imaging techniques, such as magnetic resonance imaging and spectroscopy. As mentioned above, the possible role these genetic variants play in response and adverse reactions to psychotropic medication may be defined by pharmacogenomic approaches. Speculatively, efficacy of D-cycloserine -prescribed to speed up response to psychotherapy for anxiety disorders- may depend on genetic variation influencing D-Serine availability in the CNS. Along similar lines, the variants in *PRODH* and *SLC6A20* may determine outcome and frequency of psychotic symptoms in patients suffering hyperprolinaemia and 22q11 deletion syndrome. The signaled metabolic and transporter pathways may further be targeted in preclinical studies to construe the much-debated nature of NMDAR coagonist involvement in glutamatergic signaling. A recent study provides examples of how knowledge about NMDAR coagonist transporter systems may elucidate the localization of NMDAR coagonist involvement in synaptic and extrasynaptic NMDA gating [21].

In conclusion, the studies presented in this doctoral thesis demonstrate that CSF is a powerful and informative target for genetic and seasonality research. Similarly to most psychiatric conditions, CSF constituents are influenced by genetic (polymorphisms) and environmental (seasonal) factors. Ultimately, the highlighted metabolic, cell signaling and transporter pathways may provide novel targets for psychopharmacological treatment modalities.

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10

Samenvatting in het Nederlands

Samenvatting in het Nederlands

Dit proefschrift gaat over genetische en seizoensinvloeden op neurotransmitters (*hoofdstuk 1*). Neurotransmitters zijn 'boodschappermoleculen' die signalen van de ene hersencel op de andere overbrengen. In dit proefschrift worden niet alleen de neurotransmitters zelf, maar ook hun metabolieten (afbraakproducten) en co-agonisten (helpers) onderzocht.

Op het eerste oog lijken genetica en de seizoenen weinig met elkaar te maken te hebben. Waarom gaat het proefschrift dan over beide aspecten? Deze hebben gemeen dat ze van invloed zijn op concentraties neurotransmitters in hersenvocht (liquor). Hersenvocht is een vloeistof die het brein en het ruggenmerg omhult, waarvan de mens ongeveer 150 mL bezit (zie figuur 2 van hoofdstuk 1).

De studies beschreven in dit proefschrift komen voort uit de onderzoeken die de laatste jaren naar de genetica van psychiatrische aandoeningen zijn verricht. Hierdoor is duidelijk geworden dat veel psychiatrische aandoeningen deels erfelijk bepaald zijn (vooral schizofrenie, bipolaire stoornis, autisme en ADHD). Men is echter nog niet in staat geweest een groot deel van de verantwoordelijke polymorfismen (genetische factoren) te achterhalen. Waarschijnlijk is dit te verklaren doordat psychiatrische aandoeningen heterogeen zijn, omdat ze uit symptoomclusters bestaan en omdat het brein een zeer complex orgaan is (ongeveer twee derde van de menselijke genen komt in het brein tot expressie). Wellicht is het daarom efficiënter om de genetische grondslagen te onderzoeken van biologische fenomenen (in dit geval neurotransmitters) waarvan een oorzakelijke rol voor psychiatrische aandoeningen bewezen is of vermoed wordt. De genetica van deze zogenaamde intermediaire fenotypen (meetbare biologische kenmerken die geassocieerd zijn met een bepaalde ziekte) onderzoeken is één van de uitgangspunten van dit proefschrift. Een voorbeeld: hyperprolinemie (een verhoogde concentratie Proline in het bloed) verhoogt de kans op psychose. Door de genetica van Proline zowel in bloed als in liquor te onderzoeken kunnen wellicht mechanismen worden ontrafeld die bijdragen aan hyperprolinemie en daarmee iets kunnen vertellen over hoe psychose ontstaat. Uiteindelijk zou de kennis van dergelijke genetische mechanismen kunnen leiden tot verbeterde farmacotherapie van psychiatrische aandoeningen (waarin nog veel terrein te winnen is).

Hoewel de meeste hoofdstukken voornamelijk op genetica gericht zijn, beschrijven hoofdstukken 2 en 3 hoe de jaargetijden bijdragen aan variaties van neurotransmitters in hersenvocht.

Hoe zijn de studies ontworpen om bovenstaande te onderzoeken? Bij het opzetten van de studies was onze intentie op weinig belastende wijze (non-invasief) liquor en bloed te verzamelen. Daarom kozen we ervoor liquor af te nemen bij patiënten die voor een kleine ingreep (zoals een operatie aan de knie) regionale verdoving krijgen.

Middels een lumbaalpunctie (ruggenprik) wordt bij hen een anestheticum toegediend. Bij de mensen die instemmen mee te doen wordt eerst gedurende een halve minuut 6mL liquor opgezogen voordat het verdovingsmiddel in dit liquorkanaal wordt gespoten. Verder krijgen zij ten behoeve van de operatie een infuus waaruit wij bloed afnemen voor metingen en DNA-extractie. Hoewel een deel van de beschreven studies op kleinere groepen is gebaseerd, konden tussen 2008 en 2011 na het toepassen van strikte inclusiecriteria zo bijna 500 deelnemers geïncludeerd worden (in het Universitair Medisch Centrum Utrecht, het Antoniusziekenhuis in Utrecht en Nieuwegein, het Centraal Militair Hospitaal en het Diakonessenhuis in Utrecht en Zeist). Alleen hoofdstuk 5 is op een andere onderzoekspopulatie gebaseerd, het Utrechtse Gezondheidsproject in Leidsche Rijn.

De eerste studie (*hoofdstuk 2*) die we verrichtten toonde aan dat seizoen van invloed is op de voornaamste metaboliet van serotonine (een belangrijke neurotransmitter die onder andere stemming en concentratie reguleert), genaamd 5-hydroxyindolaatzuur (5-HIAA). We stelden vast dat 5-HIAA in liquor een piek laat zien in de lente en een dal in de herfst. Bijna anderhalf keer zo hoog bleek de concentratie in de lente. Bepaalde psychiatrische aandoeningen en verschijnselen, zoals seizoensdepressies, bipolaire II stoornis en suïcide, zijn met de jaargetijden geassocieerd. Aangezien deze studie voor het eerst in een representatieve groep gezonde mensen seizoensinvloeden op de serotoninehuishouding aantoonde, bieden de uitkomsten aanknopingspunten voor vergelijkende onderzoeken van gezonde mensen en patiënten met bovenbeschreven aandoeningen.

Hoofdstuk 3, ook over seizoensinvloeden op serotonine, gaat verder dan de vorige studie. De onderzoeksgroep werd verdubbeld en het aantal onderzoeksjaren bijna verdubbeld tot drie. Daardoor kon worden berekend dat 5% van de variantie in serotoninemetabolietconcentraties in hersenvocht door seizoensinvloeden wordt verklaard. Verder laten we in deze studie zien dat een polymorfisme in de promotorregio van het gen dat de serotonineтransporter codeert, de mate van seizoensvariatie in 5-HIAA beïnvloedt. Dit polymorfisme, genaamde korte/lange arm van de 5-HTTLPR, is één van de sterkste kandidaatgenen betrokken bij depressie. Tenslotte tonen we aan dat niet *absolute* concentraties 5-HIAA maar *seizoensvariaties* in 5-HIAA zwak gecorreleerd zijn met depressieve klachten. Mogelijk zijn de mensen wier serotoninehuishouding met pieken en dalen op de jaargetijden reageren gevoeliger voor depressie dan mensen wier serotonine weinig seizoensvariatie laat zien. Verschillende vragen kunnen in toekomstig onderzoek worden beantwoord: gelden deze seizoenpatronen ook op het zuidelijk halfrond of rond de evenaar?; is daar de 5-HTTLPR ook betrokken bij deze seizoensinvloeden?; speelt seizoenvariatie in 5-HIAA ook een rol bij het ontstaan van ernstige depressies?

Kortom, kwesties die in grote studies die patiënten met gezonden vergelijken kunnen worden ontrafeld.

In *hoofdstuk 4* wordt een hypothese gestuurde genetische studie beschreven. Deze terminologie geeft aan dat op basis van bestaande literatuur de associatie tussen een bepaalde genetische variant en een fenotype wordt onderzocht. In dit proefschrift worden drie hypothese gestuurde genetische onderzoeken uitgelegd (hoofdstukken 3, 4 en 5). Hoofdstukken 6 en 7 zijn hypothesevrij, wat in deze context wil zeggen dat varianten over het hele genoom (het menselijke DNA dat bestaat uit 3 miljard basenparen) worden bepaald. In hoofdstuk 4 wordt de associatie beschreven tussen een variant in een gen genaamd *ERBB4* en concentraties van GABA in hersenvocht. De bevinding bevestigde dat dit gen bij GABA betrokken is, want dat was met beeldvorming bij mensen en in dieronderzoeken eerder al duidelijk geworden. Een genetische variant die GABA in liquor beïnvloedt was echter niet eerder gevonden. GABA is de voornaamste dempende neurotransmitter in het centrale zenuwstelsel en is onder andere betrokken bij epilepsie. Bovendien werken angstdempers en slaapmiddelen op GABA. Mogelijk biedt de bevestiging dat dit gen bij GABA betrokken is aangrijpingspunten om de behandeling van dergelijke aandoeningen en klachten te verbeteren.

Hoofdstuk 5 laat zien dat hypothese gestuurde onderzoeken ook een negatief resultaat kunnen opleveren. Dit onderzoek gaat over BDNF. BDNF is een neurotrofine, een stof die de groei en sterfte van verbindingen in het zenuwstelsel regelt. In het bijzonder gaat het over een genetische variant die al vaak onderzocht is (vooral voor depressie), het Val66Met polymorfisme. Het nieuwe aan de opzet was dat alleen homozygoten (personen met twee kopieën van hetzelfde allel) met elkaar werden vergeleken, waardoor de kracht om concentratieverschillen te vinden groter werd geacht. Deze bekende genetische variant in het gen *BDNF* bleek echter niet van invloed op concentraties BDNF in bloed. De twee voornaamste verklaringen voor deze bevinding zijn dat deze variant niet betrokken is bij de regulering van concentraties BDNF in bloed of dat de onderzoekspopulatie te klein was om een effect aan te tonen.

Hoofdstuk 6 beschrijft een genoom-wijde associatiestudie. Genoomwijd houdt in dat hypothesevrij een groot aantal varianten (in dit geval bijna 6 miljoen) als een soort vertegenwoordiging van het genoom worden onderzocht. Onderwerp waren de NMDAR-co-agonisten. Dit zijn aminozuren die de effecten van Glutamaat -de voornaamste exciterende neurotransmitter van het centraal zenuwstelsel- op de NMDAR (een receptor van Glutamaat) faciliteren. Zowel de links- als rechtsdraaiende vormen van deze aminozuren werden onderzocht. Enerzijds werden genen gevonden die enzymen coderen die al eerder in verband waren gebracht met aminozuren, zoals

DAO (het enzym waarvan bekend was het D-Serine afbreekt in dieren). D-Serine is de belangrijkste synaptische co-agonist op de NMDAR. Anderzijds werd een transportmechanisme gevonden: L-Proline in liquor bleek vooral geassocieerd met een variant in *SLC6A20*, een transporteiwit. Interessant bleek dat de ratio's tussen L- en D-aminozuren en tussen liquor en plasma verschillende genen aan het licht brachten. De sterkste associatie werd gevonden tussen L-Proline in bloed en *PRODH*, dat op 22q11 ligt. Dit is een gebied waar een relatief vaak voorkomende deletie kan voorkomen die tot allerlei ziektebeelden kan leiden, waaronder schizofrenie. Het is zelfs de grootste genetische risicofactor voor schizofrenie, omdat een persoon met deze deletie 30 keer meer kans op schizofrenie heeft dan een persoon zonder de deletie. Het locus op 22q11 dat in hoofdstuk 6 wordt beschreven kan daarom het inzicht in de implicaties van 22q11 deleties vergroten. Bovendien zou dit stukje genoom voor schizofrenie met fijnere technieken, zoals sequencing (een gebied basenpaar voor basenpaar genetisch onderzoeken), verder kunnen worden onderzocht.

Het onderwerp van *hoofdstuk 7* is een genoom-wijde analyse van neurotransmittermetabolieten in liquor. De associatie tussen genetische varianten en metabolieten van de monoamines serotonine, dopamine en noradrenaline -alle belangrijke neurotransmitters voor cognitief en affectief functioneren- werd getest. Een variant die het genoom-wijde significantieniveau haalde, werd vervolgens op expressie (in bloed) van alle menselijke genen onderzocht. De expressie van *PDE9A*, een gen dat eerder bij depressie en respons op antidepressiva was betrokken, bleek samen te hangen met deze genoom-wijd significante genetische variant. De kennis opgedaan met dit onderzoek zou kunnen worden gebruikt om de betekenis van de samenhang tussen *PDE9A* en *SSTR1*, het gen waarin deze variant ligt, voor depressie en respons op antidepressiva verder te onderzoeken.

In *hoofdstuk 8* ligt de nadruk op een gedragsfenotype, roken. Gezien aanwijzingen dat Glutamaat betrokken is bij roken, vergeleken we concentraties van de NMDAR co-agonisten in bloed en hersenvocht van rokers ten opzichte van niet-rokers. Inderdaad bleken enkele concentraties duidelijk te verschillen tussen rokers en niet-rokers, vooral van D-aminozuren. Een beperking van de methode is dat slechts één keer in de tijd gemeten is en niets over causaliteit kan worden gezegd op basis van de gegevens.

Hoofdstuk 9 vormt de algemene discussie van het proefschrift. Conclusies, sterke en zwakke kanten aan de methoden en suggesties voor toekomstig onderzoek worden gegeven. Samengevat zijn enkele sterke punten: de unieke en homogene studiepopulatie, de moderne genetische analyses en het corrigeren voor covariaten (factoren die associaties kunnen beïnvloeden, zoals opslagtijd van liquor) in de statistische analyses. De voornaamste beperking aan de studies is de betrekkelijk

kleine studiepopulatie ten opzichte van andere genoom-wijde studies van metabolieten in urine of bloed. Dat biologisch plausibele genetische signalen in liquor werden gevonden bewijst echter hoe informatief deze vloeistof kan zijn voor het detecteren van loci die concentraties neurotransmitters, metabolieten en co-agonisten bepalen. Zoals geldt voor de meeste psychiatrische aandoeningen, worden ook deze stoffen beïnvloed door omgevingsfactoren (de seizoenen) en genen.

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Supplemental Information per Chapter

Supplemental Information Chapter 3

Supplemental Methods

Genotyping Quality Control Procedures

To check for gender errors, excess homozygosity, cryptic relatedness, and ethnic outliers within our study population (N=414), and to exclude subjects of non-European descent, we made a selection of best performing SNPs. To that end, only SNPs with a minor allele frequency (MAF) >0.1, genotyping missingness < 2% and in Hardy-Weinberg equilibrium (HWE, $p>1x10^{-5}$) were kept and then pruned for redundancy due to linkage disequilibrium (LD) using a cut-off of $r^2=0.2$ (i.e. SNPs showing pairwise LD >0.2 were filtered out), leaving 80 979 SNPs. With these best performing SNPs the following were ascertained in Plink v1.07 [1]: gender errors (none); cryptic relatedness (pi_hat > 0.2; none); and excess homozygosity using a threshold of >3 standard deviations (SDs) from the F-statistic (three subjects, whom we excluded from further analyses). To identify subjects of non-European origin, we compared the genotyped data in the discovery phase with HapMap 3 using principal components analysis and removed six outliers of non-European descent, leaving only individuals clustering within CEU. After additionally removing samples with > 5% missing genotype data (seven), 398 individuals remained for further analyses. To that end, we used the BEAGLE software to phase the genotype data and the minimac software (a computationally efficient implementation of MACH) for genotype imputation on these 398 subjects, using 10 332 781 SNPs of 1000G Phase I version 3 as the reference dataset. The reference panel used for imputing markers in autosomes is the EUR population of 1000 Genomes Phase I version 3 that includes 379 individuals and approx. 17 million markers. Among those SNPs, 1 103 560 SNPs failed the r^2 threshold of 0.3, leaving 9 229 221 SNPs for the final QC standards: < 2% genotyping missingness, HWE p-value > $1x10^{-6}$, and MAF > 0.05. This in turn resulted in a total of 5 767 231 (585 655 genotyped) SNPs.

Non-Linear Quantile Regression Models

Model 1a (5-HIAA, one peak):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times t + \beta 3) + (\beta 4 \times Sex) + (\beta 5 \times Age)$$

Model 1b (5-HIAA, two peaks):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times 2 \times t + \beta 3) + (\beta 4 \times Sex) + (\beta 5 \times Age)$$

Model 2a (HVA, one peak):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times t + \beta 3) + (\beta 4 \times Sex) + (\beta 5 \times Age) + (\beta 6 \times LPlevel) + (\beta 7 \times Weight)$$

Model 2b (HVA, two peaks):

$$metabolite = \beta 1 + \beta 2 \times \cos(0.5236 \times 2 \times t + \beta 3) + (\beta 4 \times Sex) + (\beta 5 \times Age) + (\beta 6 \times LPlevel) + (\beta 7 \times Weight)$$

In which:
- Metabolite = concentration of MA metabolite
- β1 = baseline level
- β2 = amplitude (A)
- 0.5236 = coefficient of t = 2π/12 (one cosine period in radians divided by the number of months per year; "x 2" added for the two-peaks model)
- t = month of sampling or birth; t1 = month of sampling; t2= birth month
- β3 = phase shift
- β4, β5, β6, and β7 = covariates' coefficients

For each 1-peak model showing a significant amplitude the t_{max} (month during which a level is at its maximum), the t_{min} (month during which a level is at its minimum), and the predicted concentration increase from t_{max} to t_{min} (PC_i) were computed:
$t_{max} = (\pi - \beta 3) / 0.5236 \ (+12)$
$t_{min} = - \beta 3 / 0.5236 \ (+12)$
$PC_i = PC_{max} - PC_{min} / PC_{min} \times 100\%$, in which:
$PC_{max} = \beta 1 + \beta 2 \times \cos(0.5236 \times t_{max} + \beta 3)$; and $PC_{min} = \beta 1 + \beta 2 \times \cos(0.5236 \times t_{min} + \beta 3)$.

5-HT Seasonality Values

In the table below we show how raw 5-HIAA values translate into 5-HT seasonality values based on the model outlined in the methods section. Per sample month, five 5-HIAA measurements were randomly selected. 5-HT seasonality values not only depend on raw 5-HIAA and sample month, but also on age and sex as these covariates are included in the prediction models.

Sample Month	5-HIAA	5-HT Seasonality Values	Age	Sex
January	175.5	20.88	38	M
January	294.6	98.74	50	F
January	195.9	0.00	51	F
January	103.3	-54.12	46	M
January	65.5	-124.30	33	F
February	212.7	19.01	19	F
February	153.3	-5.11	24	M
February	89.4	-79.40	52	M
February	158.6	0.38	23	M
February	315.9	122.18	19	F
March	255.5	84.39	42	M
March	292.3	118.89	48	M
March	183.5	-18.59	25	F
March	206.2	36.76	37	M
March	75.5	-88.27	21	M
April	275.4	100.09	49	M
April	232.8	20.02	51	F
April	228.1	20.27	37	F
April	126.5	-49.29	50	M
April	197.9	25.14	42	M
May	252.0	43.98	47	F
May	168.6	6.06	23	M
May	88.2	-76.85	30	M
May	301.8	130.04	49	M
May	194.6	-6.12	26	F
June	360.4	162.51	39	F
June	232.8	76.24	27	M
June	122.1	-34.79	28	M
June	97.2	-60.08	29	M
June	136.5	-30.04	55	M

Sample Month	5-HIAA	5-HT Seasonality Values	Age	Sex
July	178.4	-33.14	22	M
July	109.0	36.78	24	M
July	76.4	76.15	43	M
July	183.3	6.37	43	F
July	108.2	83.92	50	F
August	68.7	71.58	34	M
August	63.4	77.21	34	M
August	164.9	-26.24	29	M
August	141.4	-5.21	22	M
August	190.6	-54.11	23	M
September	65.4	74.41	50	M
September	110.0	57.09	23	F
September	129.7	0.00	21	M
September	219.1	-90.14	19	M
September	211.2	-82.53	18	M
October	152.9	-25.48	19	M
October	112.9	23.90	46	M
October	178.7	-38.39	55	M
October	93.2	36.34	25	M
October	332.1	-199.46	34	M
November	119.0	15.25	29	M
November	139.5	1.81	49	M
November	227.0	-92.95	29	M
November	118.5	49.72	21	F
November	235.5	-97.06	41	M
December	70.0	72.54	31	M
December	280.2	-136.16	36	M
December	82.8	67.92	54	M
December	130.7	56.18	52	F
December	243.8	-96.64	44	M

1. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. (2007): PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 81:559-575.

Supplemental Table 1

Non-linear quantile regression results

For 5-HIAA, HVA and the Beck-Depression Inventory-II (BDI-II) the best fitting model statistics are given. Significant results are displayed in bold.

5-HIAA, Sample Month
> deviance (1-peak nlqr.model) 12637.97
> deviance (2-peak nlqr.model) 12858.79
>> 1-peak model has better fit:

Coefficients:

	Value	Std. Error	t value	Pr(>|t|)
beta1	**98.98593**	**18.24941**	**5.42406**	**0.00000**
beta2	**-16.68534**	**6.20475**	**-2.68912**	**0.00742**
beta3	**13.72030**	**0.34786**	**39.44189**	**0.00000**
beta4	**37.16651**	**8.43169**	**4.40796**	**0.00001**
beta5	**0.41194**	**0.31901**	**1.29127**	**0.19724**

5-HIAA, Birth Month
> deviance (1-peak nlqr.model) 12828.66
> deviance (2-peak nlqr.model) 12816.94
>> 2-peak model has better fit:

Coefficients:

	Value	Std. Error	t value	Pr(>|t|)
beta1	110.24725	15.78051	6.98629	0.00000
beta2	5.84812	5.00161	1.16925	0.24289
beta3	13.39450	1.01814	13.15590	0.00000
beta4	35.96062	8.47165	4.24482	0.00003
beta5	0.12161	0.30135	0.40353	0.68674

HVA, Sample Month
> deviance (1 peak nlqr.model) 14269.64
> deviance (2-peak nlqr.model) 14277.47
>> 1-peak model has better fit:

Coefficients:

	Value	Std. Error	t value	Pr(>|t|)
beta1	186.91709	22.07680	8.46668	0.00000
beta2	-7.46097	6.93287	-1.07617	0.28240
beta3	12.56199	0.90629	13.86093	0.00000
beta4	32.63685	9.97457	3.27200	0.00115
beta5	-0.55910	0.42994	-1.30041	0.19409

HVA, Birth Month
> deviance (1 peak nlqr.model) 14277.99
> deviance (2 peak nlqr.model) 14131.08
>> 2-peak model has better fit:

Coefficients:

	Value	Std. Error	t value	Pr(>|t|)
beta1	177.15386	20.20673	8.76707	0.00000
beta2	15.07240	6.27811	2.40079	0.01674
beta3	13.61259	0.44590	30.52844	0.00000
beta4	34.63163	8.42684	4.10968	0.00005
beta5	-0.35729	0.44318	-0.80621	0.42053

BDI-II, Sample Month
> deviance (1-peak nlrq.model) 763.775
> deviance (2-peak nlrq.model) 764.0001
>> 1-peak model has better fit:

Coefficients:

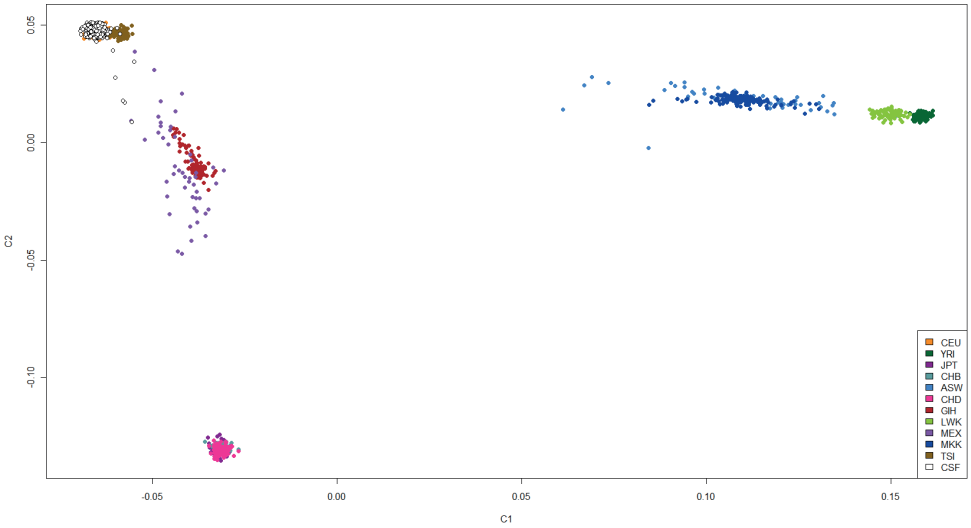
	Value	Std. Error	t value	Pr(>|t|)
beta1	0.66025	1.65928	0.39791	0.69091
beta2	-0.53589	0.52652	-1.01780	0.30939
beta3	13.08994	0.98745	13.25634	0.00000
beta4	1.80385	0.96352	1.87214	0.06191
beta5	0.00000	0.03043	0.00000	1.00000

Supplemental Information Chapter 6

Supplemental Methods

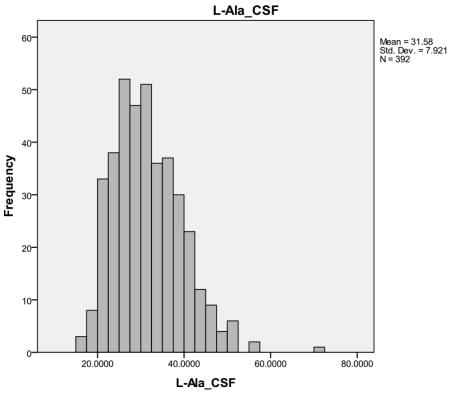
Quality Control of Genotyped Data

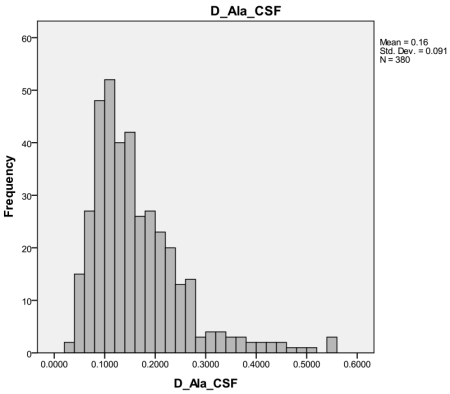
To check for gender errors, excess homozygosity, cryptic relatedness, and ethnic outliers within our study population (N=414), and to exclude subjects of non-European descent, we made a selection of best performing genome-wide SNPs. To that end, only SNPs with a minor allele frequency (MAF) >0.1, genotyping missingness < 2% and in Hardy-Weinberg equilibrium (HWE, $p>1x10^{-5}$) were kept and then pruned for redundancy due to linkage disequilibrium (LD) using a cut-off of r^2=0.2 (i.e. SNPs showing pairwise LD >0.2 were filtered out), leaving 80,979 SNPs. With these best performing SNPs the following were ascertained in Plink v1.07 [1]: gender errors (none); cryptic relatedness (pi_hat > 0.2; none); and excess homozygosity using a threshold of >3 standard deviations (SDs) from the F-statistic (three subjects, whom we excluded from further analyses). To identify subjects of non-European origin, we compared the genotype data in the discovery phase with HapMap 3 using principal components analysis and removed six outliers of non-European descent, leaving only individuals clustering within CEU (see graph below, in which white dots represent the current sample –"CSF"- and the six outliers clearly deviate towards the MEX and GIH populations). After additionally removing samples with > 5% missing genotype data (seven), 398 individuals remained for further analyses. We then conducted imputation on this cleaned set of 398 participants as described in the main document (MACH output was converted to Plink format) using 10,332,781 SNPs of 1000G Phase I version 3 as the reference dataset. Among those SNPs, 1,103,560 SNPs failed the r^2 threshold of 0.3, leaving 9,229,221 SNPs for the final QC standards: < 2% genotyping missingness, HWE p-value > 1x10-6, and MAF > 0.05.

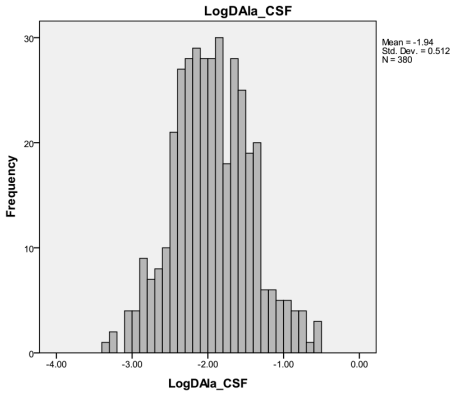


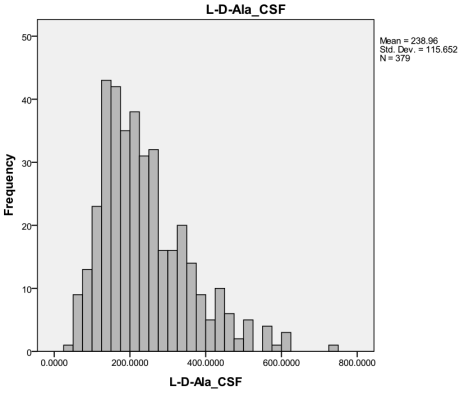
Distributions of measured metabolic traits and ratios in plasma and CSF

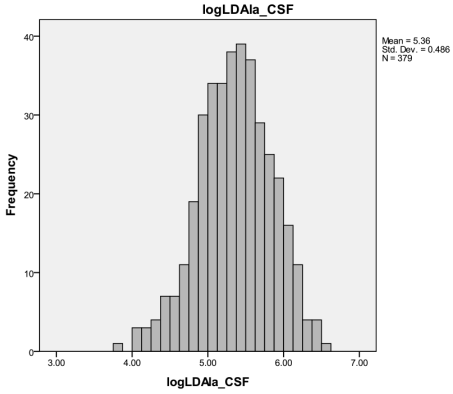
In the event the Kolmogorov-Smirnov (K-S) two-tailed $p < 0.05$, the log transformed distribution was taken for the association analyses (displayed to the right of the original distribution for those with such p-values). "Ala" = Alanine; "Gly" = Glycine; "Ser" = Serine; "Pro" = Proline; "L" and "D" indicate enantiomers; "Pla" = Plasma; "CSF" = cerebrospinal fluid; ratios between enantiomers are indicated as "L-D"; ratios between plasma and CSF are indicated as "Pla-CSF".

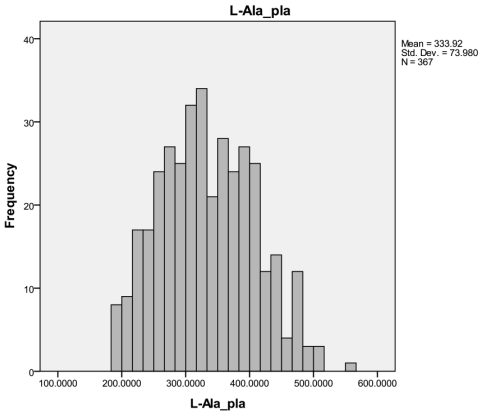

L-Ala_CSF
Mean = 31.58
Std. Dev. = 7.921
N = 392
Frequency
L-Ala_CSF

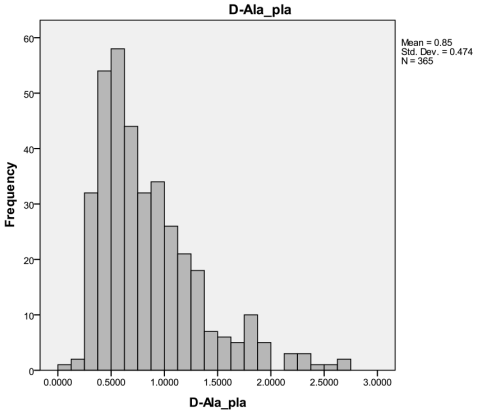

D_Ala_CSF
Mean = 0.16
Std. Dev. = 0.091
N = 380
Frequency
D_Ala_CSF

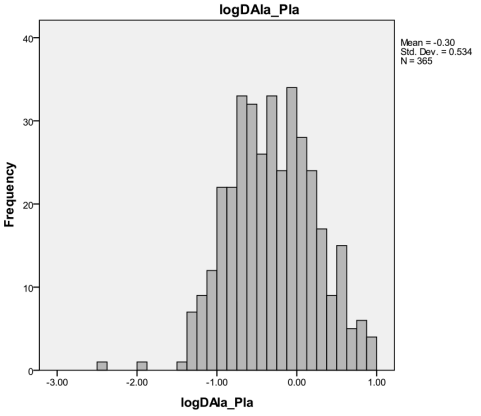

LogDAla_CSF
Mean = -1.94
Std. Dev. = 0.512
N = 380
Frequency
LogDAla_CSF

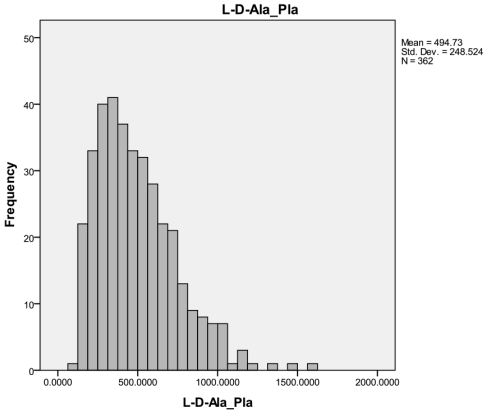

L-D-Ala_CSF
Mean = 238.96
Std. Dev. = 115.652
N = 379
Frequency
L-D-Ala_CSF


logLDAla_CSF
Mean = 5.36
Std. Dev. = 0.486
N = 379
Frequency
logLDAla_CSF


L-Ala_pla
Mean = 333.92
Std. Dev. = 73.980
N = 367
Frequency
L-Ala_pla


D-Ala_pla
Mean = 0.85
Std. Dev. = 0.474
N = 365
Frequency
D-Ala_pla


logDAla_Pla
Mean = -0.30
Std. Dev. = 0.534
N = 365
Frequency
logDAla_Pla


L-D-Ala_Pla
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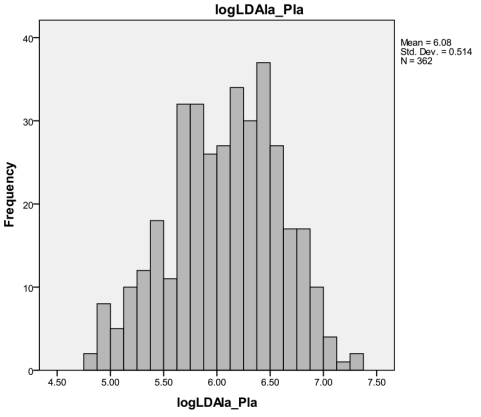
Std. Dev. = 248.524
N = 362
Frequency
L-D-Ala_Pla


logLDAla_Pla
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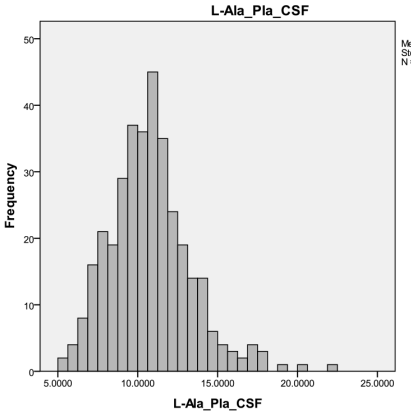
Std. Dev. = 0.514
N = 362
Frequency
logLDAla_Pla


L-Ala_Pla_CSF
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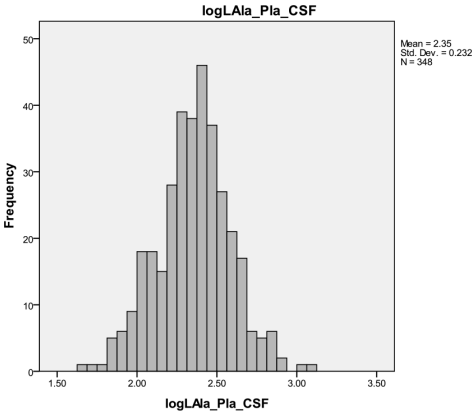
Std. Dev. = 2.532
N = 348
Frequency
L-Ala_Pla_CSF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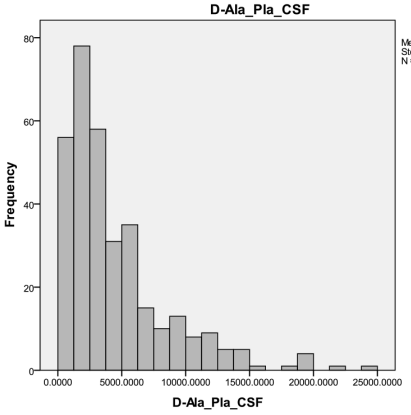
Std. Dev. = 0.232
N = 348
Frequency
logLAla_Pla_CSF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Std. Dev. = 4066.467
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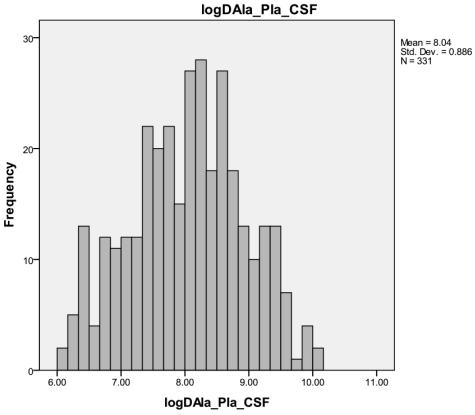
Frequency
D-Ala_Pla_CSF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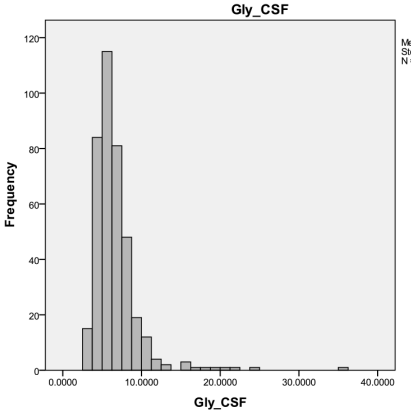
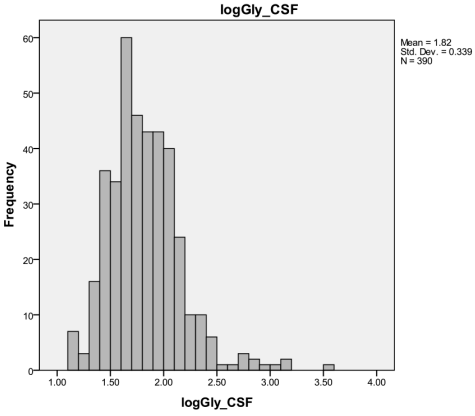
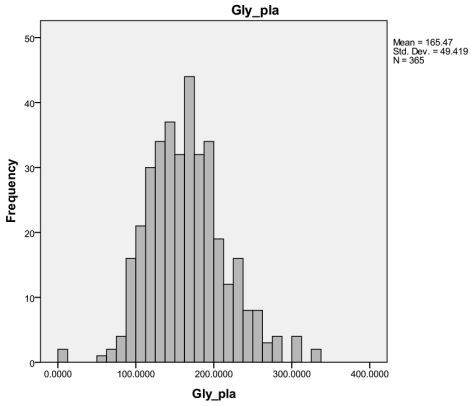
Std. Dev. = 0.886
N = 331
Frequency
logDAla_Pla_CSF


Gly_CSF
Mean = 6.61
Std. Dev. = 3.026
N = 390
Frequency
Gly_CSF


logGly_CSF
Mean = 1.82
Std. Dev. = 0.339
N = 390
Frequency
logGly_CSF


Gly_pla
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Std. Dev. = 49.419
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Frequency
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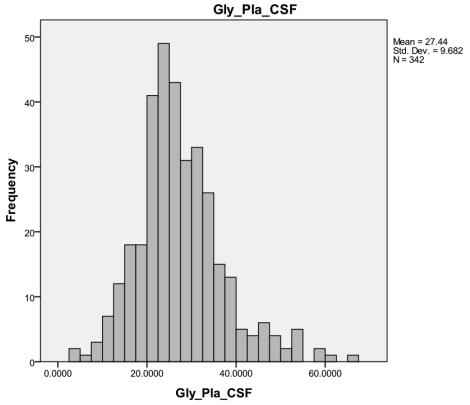
100.0000
200.0000
300.0000
400.0000
Gly_pla


Gly_Pla_CSF
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N = 342
Frequency
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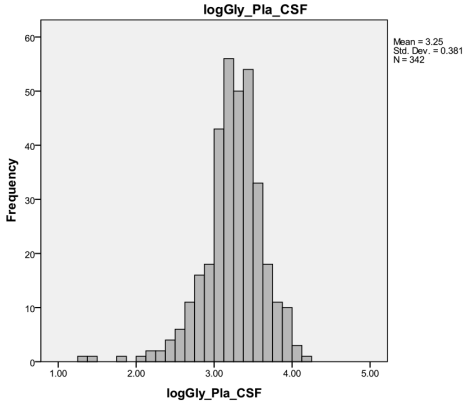
20.0000
40.0000
60.0000
Gly_Pla_CSF


logGly_Pla_CSF
Mean = 3.25
Std. Dev. = 0.381
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Frequency
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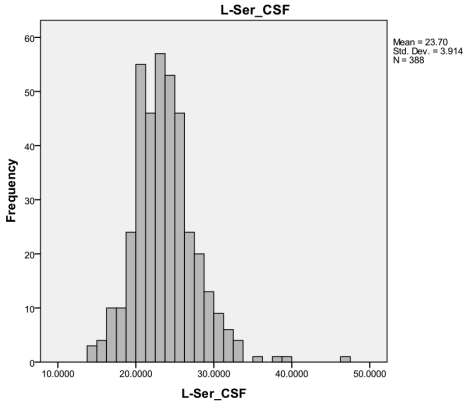
2.00
3.00
4.00
5.00
logGly_Pla_CSF


L-Ser_CSF
Mean = 23.70
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Frequency
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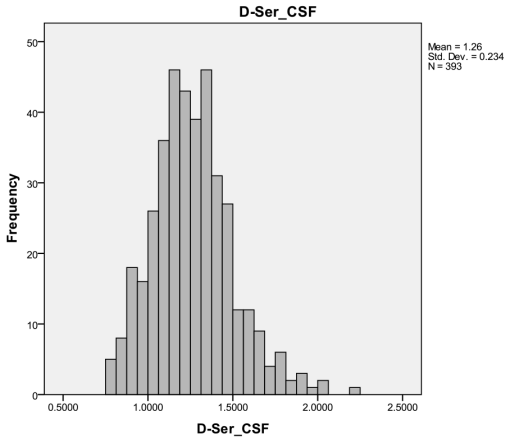
20.0000
30.0000
40.0000
50.0000
L-Ser_CSF


D-Ser_CSF
Mean = 1.26
Std. Dev. = 0.234
N = 393
Frequency
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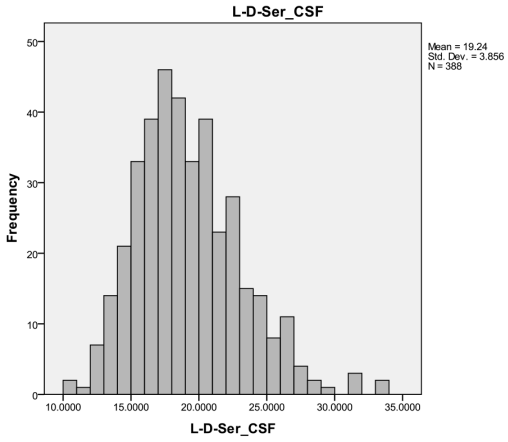
10
20
30
40
50
0.5000
1.0000
1.5000
2.0000
2.5000
D-Ser_CSF


L-D-Ser_CSF
Mean = 19.24
Std. Dev. = 3.856
N = 388
Frequency
0
10
20
30
40
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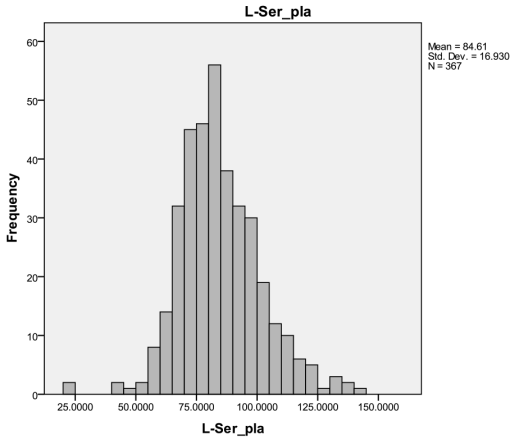
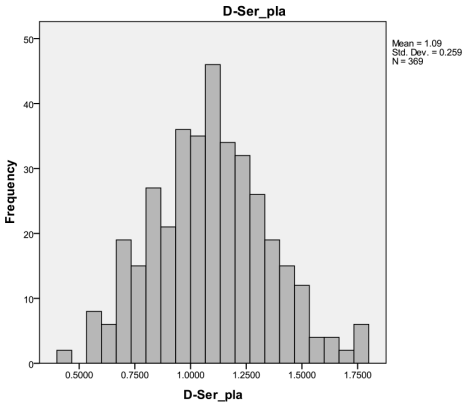
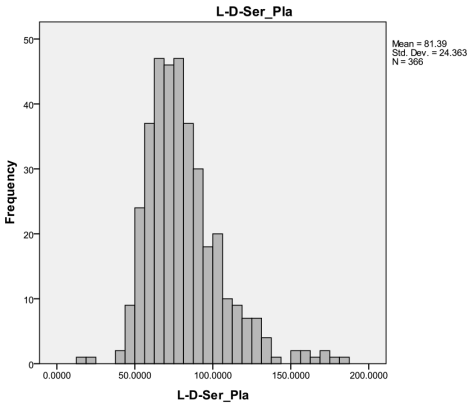
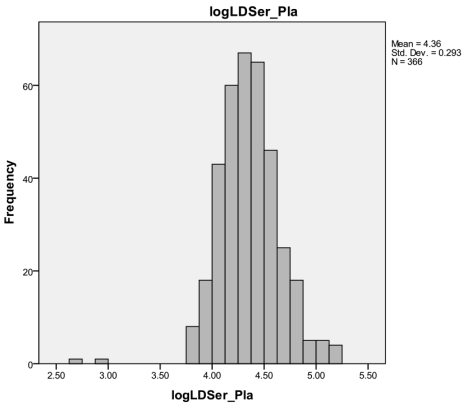
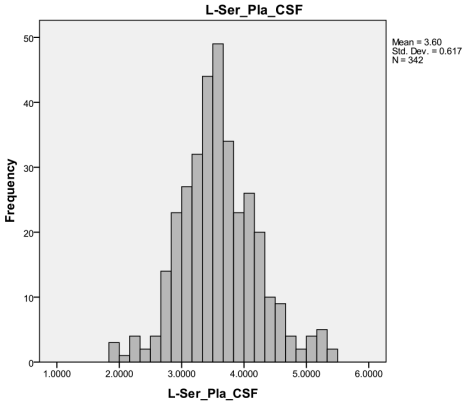
10.0000
15.0000
20.0000
25.0000
30.0000
35.0000
L-D-Ser_CSF


L-Ser_pla
Mean = 84.61
Std. Dev. = 16.930
N = 367
Frequency
0
10
20
30
40
50
60
25.0000
50.0000
75.0000
100.0000
125.0000
150.0000
L-Ser_pla


D-Ser_pla
Mean = 1.09
Std. Dev. = 0.259
N = 369
Frequency
D-Ser_pla


L-D-Ser_Pla
Mean = 81.39
Std. Dev. = 24.363
N = 366
Frequency
L-D-Ser_Pla


logLDSer_Pla
Mean = 4.36
Std. Dev. = 0.293
N = 366
Frequency
logLDSer_Pla


L-Ser_Pla_CSF
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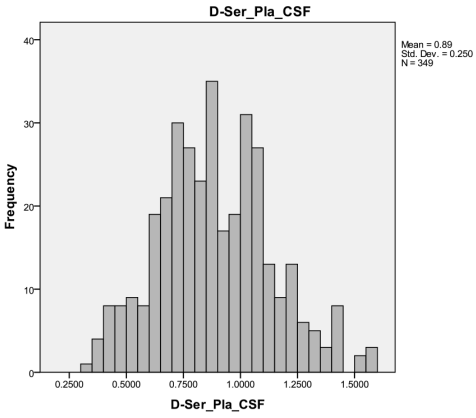
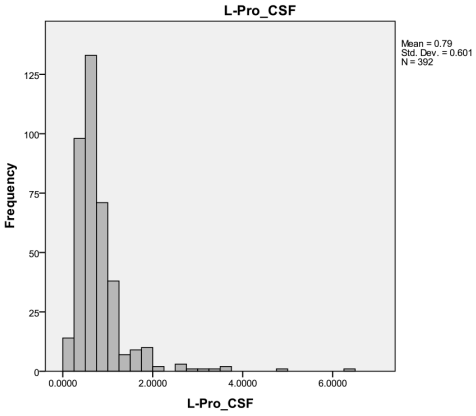
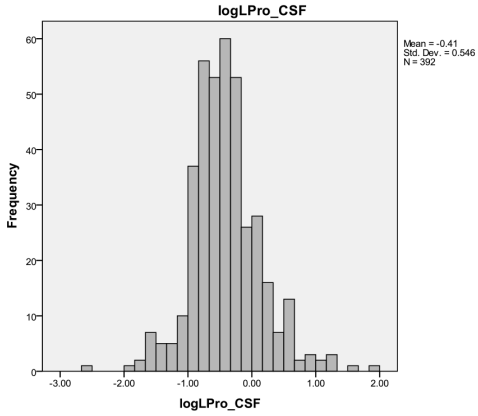
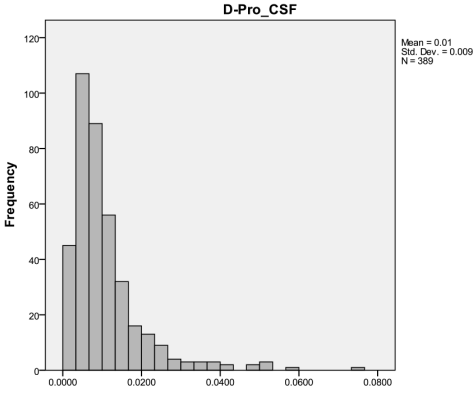
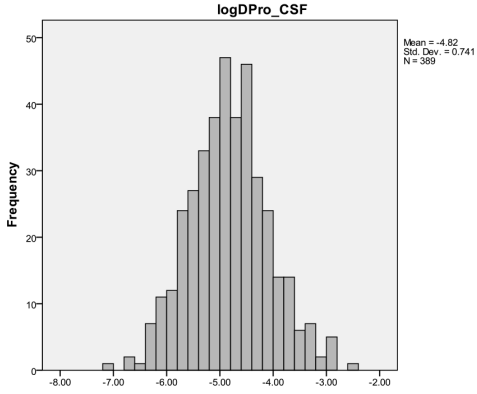
Std. Dev. = 0.617
N = 342
Frequency
L-Ser_Pla_CSF

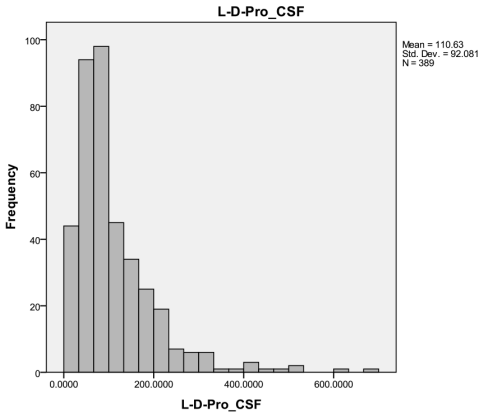

D-Ser_Pla_CSF
Mean = 0.89
Std. Dev. = 0.250
N = 349
Frequency
D-Ser_Pla_CSF

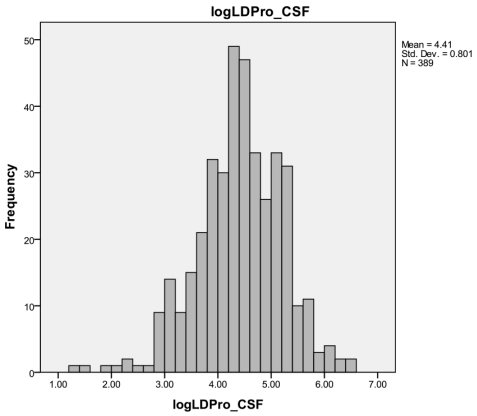

L-Pro_CSF
Mean = 0.79
Std. Dev. = 0.601
N = 392
Frequency
L-Pro_CSF


logLPro_CSF
Mean = -0.41
Std. Dev. = 0.546
N = 392
Frequency
logLPro_CSF


D-Pro_CSF
Mean = 0.01
Std. Dev. = 0.009
N = 389
Frequency


logDPro_CSF
Mean = -4.82
Std. Dev. = 0.741
N = 389
Frequency


L-D-Pro_CSF
Mean = 110.63
Std. Dev. = 92.081
N = 389
Frequency
L-D-Pro_CSF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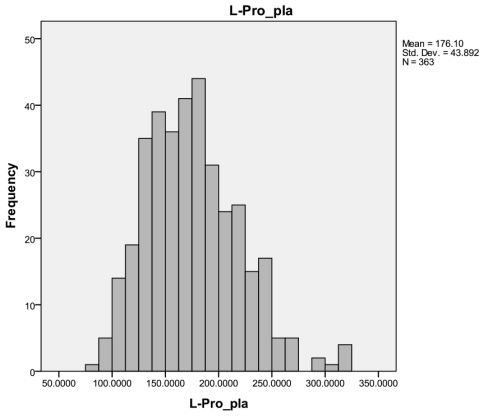
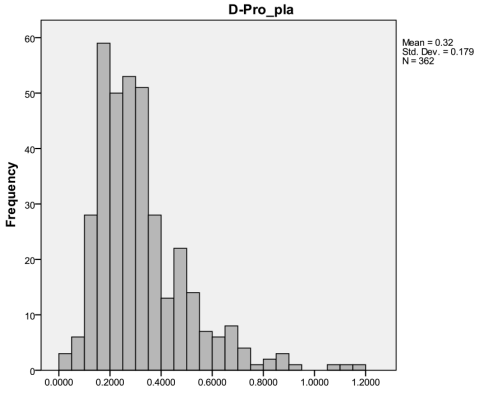
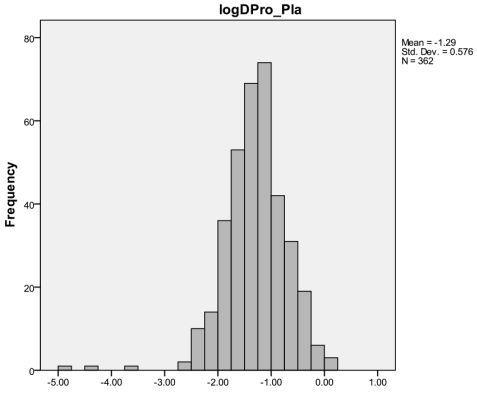
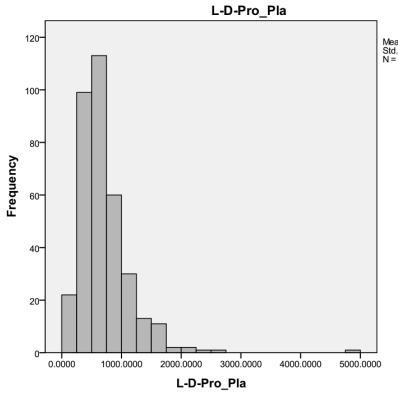
Mean = 4.41
Std. Dev. = 0.801
N = 389
Frequency
logLDPro_CSF


L-Pro_pla
Mean = 176.10
Std. Dev. = 43.892
N = 363
Frequency
L-Pro_pla


D-Pro_pla
Mean = 0.32
Std. Dev. = 0.179
N = 362
Frequency


logDPro_Pla
Mean = -1.29
Std. Dev. = 0.576
N = 362
Frequency


L-D-Pro_Pla
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Std. Dev. = 440.242
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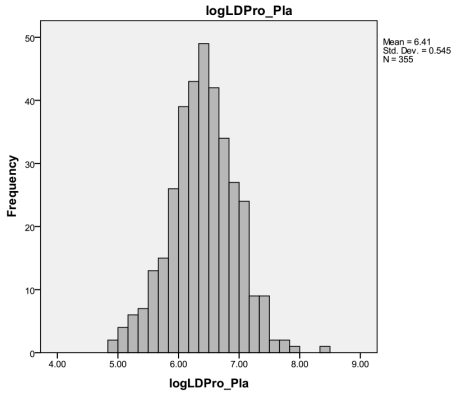
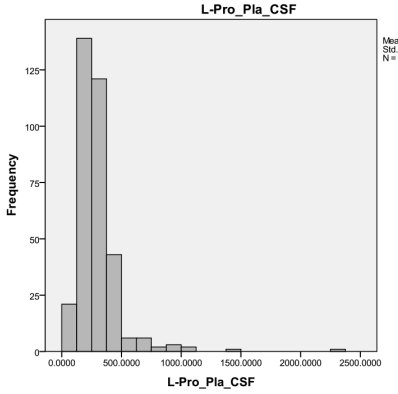
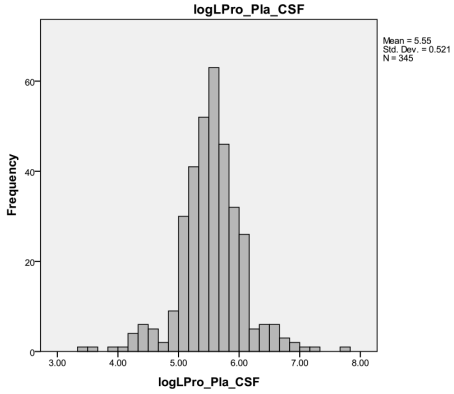
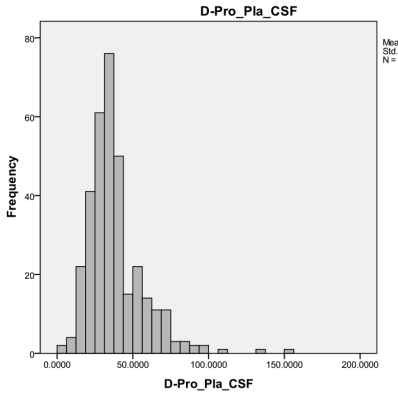
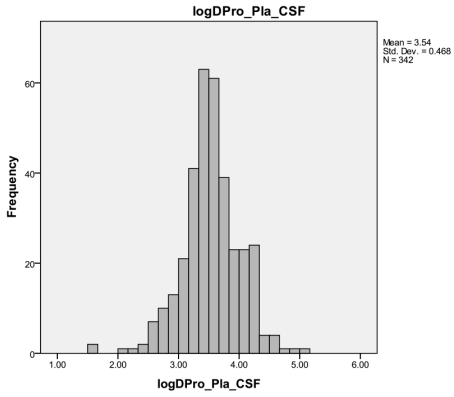
Frequency
L-D-Pro_Pla


logLDPro_Pla
Mean = 6.41
Std. Dev. = 0.545
N = 355
Frequency
logLDPro_Pla


L-Pro_Pla_CSF
Mean = 295.10
Std. Dev. = 196.764
N = 345
Frequency
L-Pro_Pla_CSF


logLPro_Pla_CSF
Mean = 5.55
Std. Dev. = 0.521
N = 345
Frequency
logLPro_Pla_CSF


D-Pro_Pla_CSF
Mean = 38.40
Std. Dev. = 18.895
N = 342
Frequency
D-Pro_Pla_CSF


logDPro_Pla_CSF
Mean = 3.54
Std. Dev. = 0.468
N = 342
Frequency
logDPro_Pla_CSF

Spearman's rho Correlations between Metabolic Traits and Storage Time
Metabolic traits showing correlations at $p < 0.05$ with storage time (in months) are in bold (N = 15 out of 27).

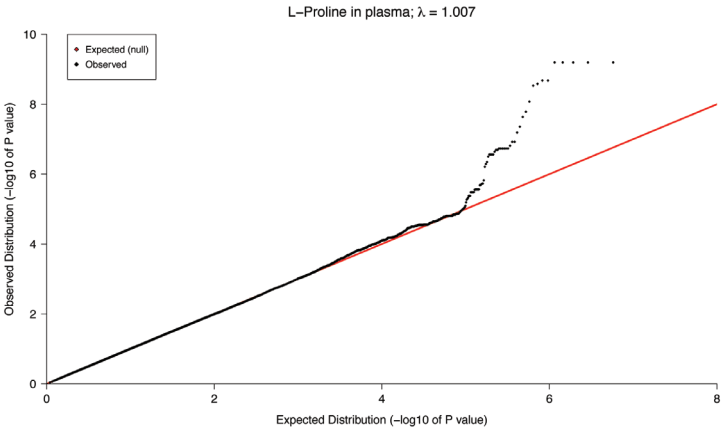
L-Ala_CSF	Correlation Coefficient	.024	L_D_Ala_CSF	Correlation Coefficient	-.093
	Sig. (2-tailed)	.643		Sig. (2-tailed)	.068
	N	391		N	387
D-Ala_CSF	Correlation Coefficient	.114*	**L_D_Ala_Pla**	Correlation Coefficient	-.127*
	Sig. (2-tailed)	.024		Sig. (2-tailed)	.015
	N	389		N	366
L-Ala_pla	Correlation Coefficient	.118*	L_Ala_Pla_CSF	Correlation Coefficient	.081
	Sig. (2-tailed)	.023		Sig. (2-tailed)	.132
	N	368		N	348
D-Ala_pla	Correlation Coefficient	.171**	D_Ala_Pla_CSF	Correlation Coefficient	.058
	Sig. (2-tailed)	.001		Sig. (2-tailed)	.283
	N	368		N	344
Gly_CSF	Correlation Coefficient	-.056	Gly_Pla_CSF	Correlation Coefficient	-.102
	Sig. (2-tailed)	.274		Sig. (2-tailed)	.057
	N	389		N	346
Gly_pla	Correlation Coefficient	-.226**	L_D_Ser_CSF	Correlation Coefficient	.054
	Sig. (2-tailed)	.000		Sig. (2-tailed)	.291
	N	368		N	390
L-Ser_CSF	Correlation Coefficient	.122*	**L_D_Ser_Pla**	Correlation Coefficient	-.145**
	Sig. (2-tailed)	.016		Sig. (2-tailed)	.006
	N	390		N	365
D-Ser_CSF	Correlation Coefficient	.049	L_Ser_Pla_CSF	Correlation Coefficient	-.059
	Sig. (2-tailed)	.335		Sig. (2-tailed)	.279
	N	393		N	344
L-Ser_pla	Correlation Coefficient	.049	**D_Ser_Pla_CSF**	Correlation Coefficient	.112*
	Sig. (2-tailed)	.347		Sig. (2-tailed)	.036
	N	365		N	350
D-Ser_pla	Correlation Coefficient	.193**	**L_D_Pro_CSF**	Correlation Coefficient	-.206**
	Sig. (2-tailed)	.000		Sig. (2-tailed)	.000
	N	370		N	385
L-Pro_CSF	Correlation Coefficient	-.038	**L_D_Pro_Pla**	Correlation Coefficient	.105*
	Sig. (2-tailed)	.453		Sig. (2-tailed)	.045
	N	389		N	365
D-Pro_CSF	Correlation Coefficient	.181**	**L_Pro_Pla_CSF**	Correlation Coefficient	.197**
	Sig. (2-tailed)	.000		Sig. (2-tailed)	.000
	N	388		N	346
L-Pro_pla	Correlation Coefficient	.208**	**D_Pro_Pla_CSF**	Correlation Coefficient	-.258**
	Sig. (2-tailed)	.000		Sig. (2-tailed)	.000
	N	368		N	344
D-Pro_pla	Correlation Coefficient	.004			
	Sig. (2-tailed)	.946			
	N	367			

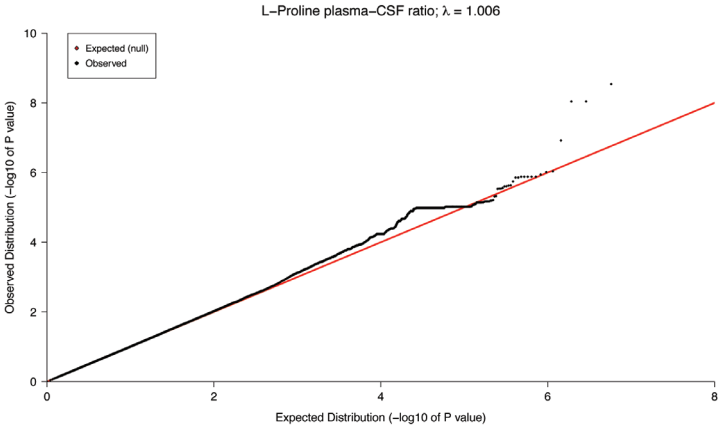
* Correlation is significant at the 0.05 level (2-tailed). ** Correlation is significant at the 0.01 level (2-tailed).

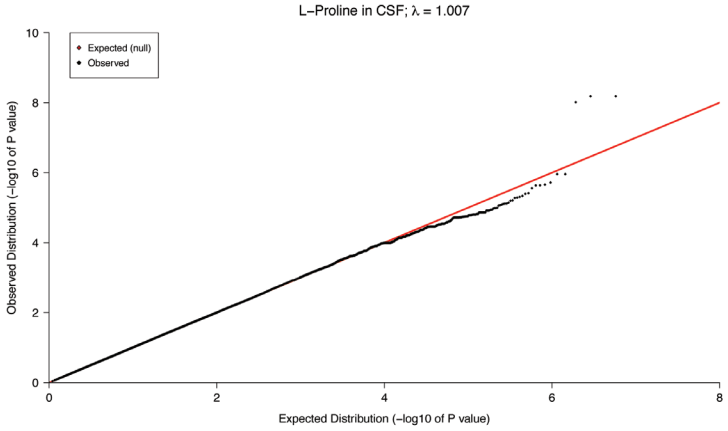
1. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. American journal of human genetics 2007; 81(3): 559-575.

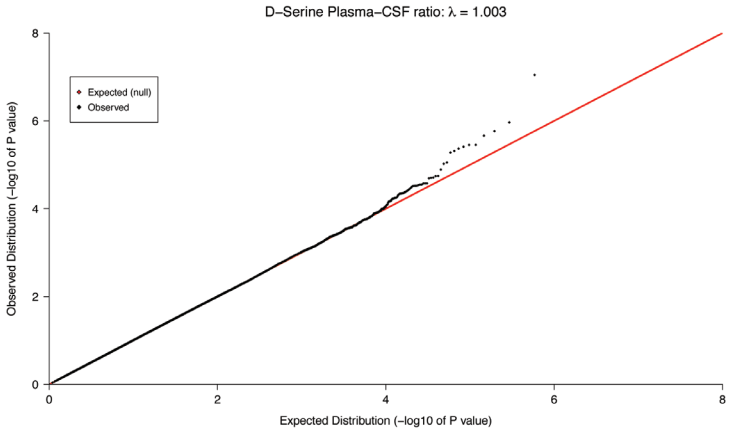
Supplemental Figure S1

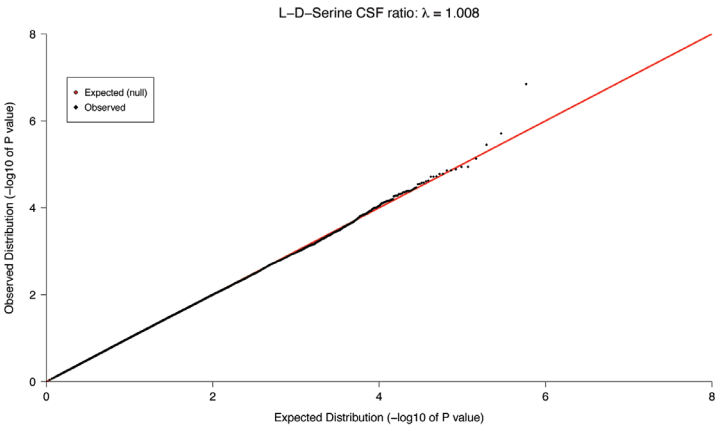
QQplots of genome-wide significant and other associations mentioned in the text: L-Proline in Plasma; The L-Proline Plasma-CSF ratio; L-Proline in CSF; D-Serine Plasma-CSF ratio; L-Serine to D-Serine ratio in CSF.





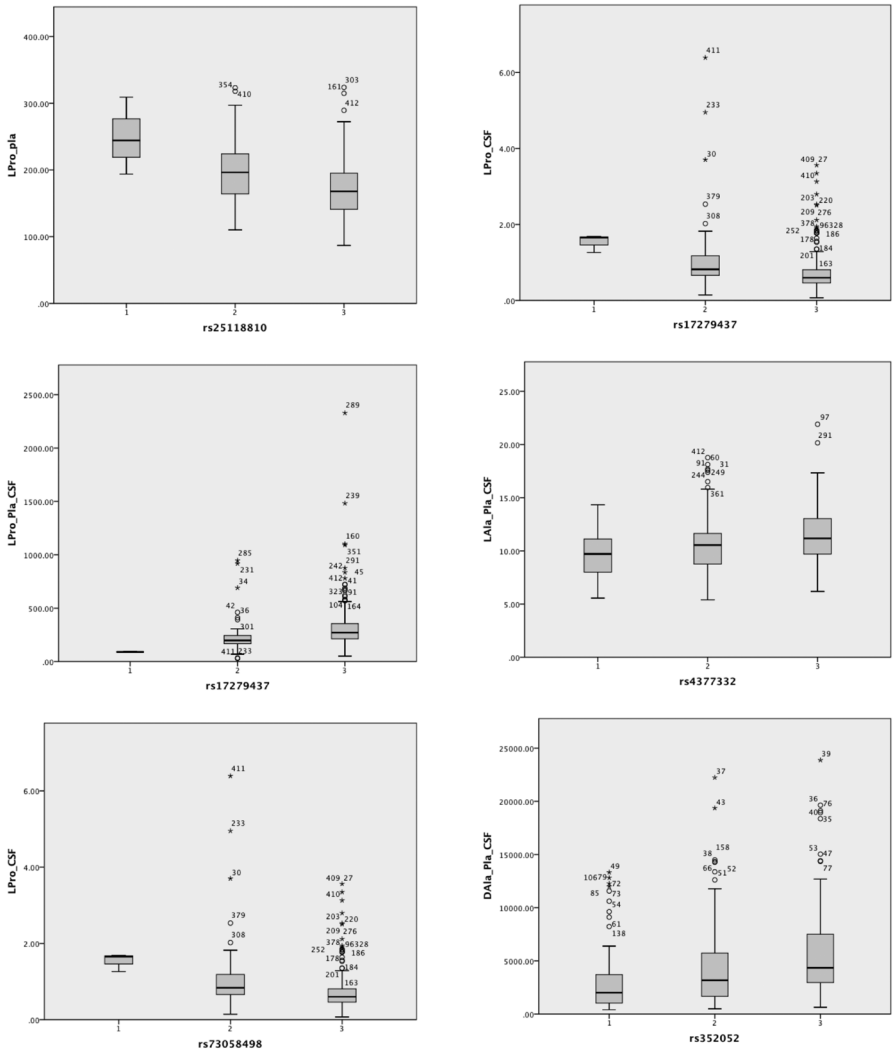

L–Proline in CSF; λ = 1.007
Expected (null)
Observed
Observed Distribution (–log10 of P value)
Expected Distribution (–log10 of P value)
0
2
4
6
8
10

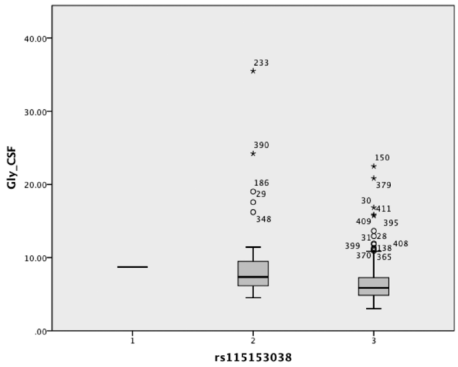

D–Serine Plasma–CSF ratio: λ = 1.003
Expected (null)
Observed
Observed Distribution (–log10 of P value)
Expected Distribution (–log10 of P value)
0
2
4
6
8


L–D–Serine CSF ratio: λ = 1.008
Expected (null)
Observed
Observed Distribution (–log10 of P value)
Expected Distribution (–log10 of P value)
0
2
4
6
8

Supplemental Figure S2

Boxplots show concentrations per genotype of the associations that reached genome-wide and suggestive significance. The boxes represent the interquartile range; the lines in the boxes the median; circles values falling between 1.5 and 3.0 box lengths from the upper or lower edge of the box; asterisks values > 3 box lengths from the upper or lower edge of the box; and the whiskers indicate the range of measured values excluding such outliers. 1=minor allele homozygous group; 2=heterozygous group; 3=homozygous group for the wild type allele.




Gly_CSF
40.00
30.00
20.00
10.00
.00
233
390
186
29
348
150
379
30
411
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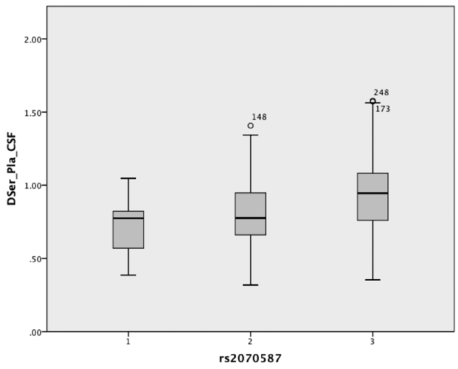
399
370
365
408
1
2
3
rs115153038


DSer_Pla_CSF
2.00
1.50
1.00
.50
.00
148
248
173
1
2
3
rs2070587

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Publications

J.J. Luykx, S.C. Bakker, E. Lentjes, M. Neeleman, E. Strengman, L. Mentink, J. DeYoung, S. de Jong, J. Sul, E. Eskin, K. van Eijk, J. van Setten, J. Buizer-Voskamp, R. Cantor, A. Lu, M. van Amerongen, E. van Dongen, P. Keijzers, T. Kappen, P. Borgdorff, P. Bruins, E. Derks, R.S. Kahn, and R.A. Ophoff.
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Abstracts

Genome-wide association studies of NMDA receptor coagonists in plasma and cerebrospinal fluid identify transporter and metabolic pathways (oral presentation at the *Society of Biological Psychiatry* annual meeting, 2013).

Seizoensinvloeden op serotoninemetabolisme in liquor: samenhang met depressieve klachten en het serotoninetransportergen (oral presentation at the *Dutch Society of Psychiatry* annual meeting, 2013).

GABA and NMDAR coagonists in human cerebrospinal fluid: From hypothesis-driven to genome-wide association studies (oral presentation at the *World Congress of Psychiatric Genetics,* 2012).

Season of sampling and season of birth influence serotonin metabolite levels in human cerebrospinal fluid (poster presentation at the *ECNP young scientists* meeting, 2012).

Curriculum Vitae

Jurjen Justin Luykx werd geboren op 6 april 1980 in Naarden. Aan het Gemeentelijk Gymnasium in Hilversum rondde hij in 1998 zijn middelbare school af. Daarna studeerde hij een half jaar aan de University of Florida in Gainesville (V.S.); aansluitend deed hij een half jaar vrijwilligerswerk in Guatemala en reisde door Argentinië. In 1999 begon hij met de studie geneeskunde aan de Universiteit Utrecht. Coschappen en wetenschappelijke stages deed hij in León (Nicaragua), Puebla (Mexico), Napels (Italië), Madrid (Spanje) en verschillende ziekenhuizen in de regio Utrecht. Zijn propedeuse Italiaanse taal en cultuur behaalde hij in 2003 en zijn doctoraal geneeskunde in 2005. Aansluitend werkte hij een jaar als ANIOS neurologie in Tergooi Ziekenhuizen. In 2006/2007 was hij een jaar werkzaam in het Center for Neurobehavioral Genetics aan de University of California in Los Angeles (UCLA), waar hij doctoraatvakken humane neurogenetica volgde en betrokken was bij multicenterstudies in samenwerking met instituten in Costa Rica en Colombia.
In 2007 begon hij aan een AGIKO-functie (assistent-geneeskundige in opleiding tot klinisch onderzoeker) op de afdeling psychiatrie van het Universitair Medisch Centrum Utrecht (UMCU) onder begeleiding van professoren Kahn en Ophoff, hetgeen heeft geresulteerd in dit proefschrift. In mei 2012 rondde hij zijn opleiding tot psychiater af. Hij is thans werkzaam als psychiater in ZNA (Ziekenhuis Netwerk Antwerpen) en wetenschappelijk verbonden aan het UMCU.