The hyperserotonemia of autism spectrum disorders

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The Hyperserotonemia of Autism Spectrum Disorders

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Chapter 1

Introduction

Introduction

The subject of this thesis is the platelet hyperserotonemia of autism. Since Schain & Freedman's (1961) finding of an elevation of whole blood serotonin in individuals with autism and other severe developmental disorders, a body of research has been conducted to clarify this phenomenon. Despite the many studies published, several major issues still need clarification: How can the group differences of platelet serotonin levels be characterized? Is the hyperserotonemia confined to the more severe subjects with so-called Kanner's autism or can it also be found in the other autism spectrum disorders, as Asperger's disorder or pervasive developmental disorder not otherwise specified (PDD-NOS)? How specific is the platelet serotonin elevation for autism as compared to other developmental disorders as mental retardation? Can clinical correlates be identified in the autism spectrum group? How do the genetics of the serotonergic system relate to these probable clinical correlates and hyperserotonemia? What is the exact mechanism causing the elevation of serotonin? Are platelet factors involved or is it merely a consequence of an increased serotonin production in the gastrointestinal tract? Can platelet serotonin levels be used in the prediction of drug effect?

The studies presented in this thesis attempt to contribute to the enlargement of our insight into some of these questions. The current chapter gives background information on autism and autism spectrum disorders, serotonin and its role in autism spectrum disorders. First, the terminology, definitions, assessment, causal factors and treatment of autism and the spectrum will be given. Consecutively, serotonin function and metabolism are considered. Furthermore, the current knowledge available on the serotonergic system in the biological background of autism and autism spectrum disorders will be discussed. Throughout the chapter, specific attention will be paid to the issues examined in this thesis.

Autism Spectrum Disorders

The terminology used to name and describe autism spectrum disorders is divers. 'Pervasive developmental disorders', 'autism and its lesser variants', 'autism and the broader phenotype' are some of the common terms to describe the same group of individuals with severe developmental problems. In the most recent versions of the DSM (DSM-IV-TR; APA, 2000) and the ICD (ICD-10; WHO, 1992) classification systems, developmental disorders at the autism spectrum are classified

in the category pervasive developmental disorders. Both terms autism spectrum disorders (ASD) and pervasive developmental disorders (PDD) will be used interchangeable throughout this thesis.

Diagnosis & classification

The description and criteria of autism and the other pervasive developmental disorders changed considerably since the first monographs of Kanner (1943) and Asperger (1944). According to the DSM-IV-TR, *'pervasive developmental disorders are characterized by severe and pervasive impairment in several areas of development: reciprocal social interaction skills, communication skills, or the presence of stereotyped behavior, interests and activities. The qualitative impairments that define these conditions are distinctly deviant relative to the individual's developmental level or mental age'.* The category includes four specific disorders and one not-otherwise-specified classification: autistic disorder (AD), Asperger's disorder (AS), Rett's disorder, childhood disintegrative disorder and pervasive developmental disorder not otherwise specified (PDD-NOS) (see also table 1.1). Impairments are usually evident from early age. Having a pervasive developmental disorder generally leads to major difficulties in daily living, school and work performance.

Assignment of a diagnosis is complicated, it should involve information from various sources (parents, direct observation of the child, teachers, etc) and time periods (current behavior, developmental milestones, etc) (Volkmar et al., 1999). The use of standardized instruments may be helpful in the diagnostic process, even though an individual diagnosis is never solely based on classification by an interview or observation. Instruments that assist in the diagnosis are the Autism Diagnostic Interview (ADI-R, Rutter et al., 2003) and the Autism Diagnostic Observation Schedule (ADOS, Lord et al., 1998). The ADI-R is a standardized investigator-based interview that aims to provide data on the behavior of a child or young adult to discriminate between AD and non-AD. The ADI-R focuses on the three domains of autism, based on the DSM-IV and ICD-10. The ADI-R is conducted in an interview with parents or caregivers and is applicable for mental ages from about 24 months into adulthood (Rutter et al. 2003). The ADOS is a semi-structured observational instrument, developed for children, adolescents and adults who may have a pervasive developmental disorder, based on the DSM-IV. Scores on the ADOS are

divided into three categories: AD, PDD-NOS and non-PDD. The assessment consists of various standardized situations, in which certain behavior (social, communicative, play or stereotyped) is expected to be elicited. The ADOS consists of four modules, each applicable for children, adolescents or adults of different levels of language and development. Interrater reliability, internal consistency, test-retest reliability and diagnostic validity are reported to be high, on item, domain and classification levels for autism and non-spectrum diagnoses (Lord et al., 2000).

Table 1.1: Diagnostic criteria for the Pervasive Developmental Disorders, DSM-IV-TR (APA, 2000)

299.00 Autistic Disorder

A. A total of six (or more) items from (1), (2), and (3), with at least two from (1), and one each from (2) and (3):

(1) qualitative impairment in social interaction, as manifested by at least two of the following:
  (a) marked impairment in the use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction
  (b) failure to develop peer relationships appropriate to developmental level
  (c) a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest)
  (d) lack of social or emotional reciprocity

(2) qualitative impairments in communication as manifested by at least one of the following:
  (a) delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)
  (b) in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others
  (c) stereotyped and repetitive use of language or idiosyncratic language
  (d) lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level

(3) restricted repetitive and stereotyped patterns of behavior, interests, and activities, as manifested by at least one of the following:
  (a) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus
  (b) apparently inflexible adherence to specific, nonfunctional routines or rituals
  (c) stereotyped and repetitive motor mannerisms (e.g. hand or finger flapping or twisting, or complex whole-body movements)
  (d) persistent preoccupation with parts of objects

B. Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years: (1) social interaction, (2) language as used in social communication, or (3) symbolic or imaginative play.

C. The disturbance is not better accounted for by Rett's Disorder or Childhood Disintegrative Disorder.

299.80 Asperger's Disorder

A. Qualitative impairment in social interaction, as manifested by at least two of the following:
  (1) marked impairment in the use of multiple nonverbal behaviors, such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction
  (2) failure to develop peer relationships appropriate to developmental level
  (3) a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest to other people)
  (4) lack of social or emotional reciprocity

B. Restricted, repetitive, and stereotyped patterns of behavior, interests, and activities, as manifested by at least one of the following:
  (1) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus

continued on next page

299.80 Asperger's Disorder*, continued*

(2) apparently inflexible adherence to specific, nonfunctional routines or rituals
(3) stereotyped and repetitive motor mannerisms (e.g., hand or finger flapping or twisting, or complex whole-body movements)
(4) persistent preoccupation with parts of objects

C. The disturbance causes clinically significant impairment in social, occupational, or other important areas of functioning.

D. There is no clinically significant general delay in language (e.g., single words used by age 2 years, communicative phrases used by age 3 years).

E. There is no clinically significant delay in cognitive development or in the development of age-appropriate self-help skills, adaptive behavior (other than in social interaction), and curiosity about the environment in childhood.

F. Criteria are not met for another specific pervasive developmental disorder or schizophrenia.

299.80 Rett's Disorder

A. All of the following:

(1) apparently normal prenatal and perinatal development
(2) apparently normal psychomotor development through the first 5 months after birth
(3) normal head circumference at birth

B. Onset of all of the following after the period of normal development:

(1) deceleration of head growth between ages 5 and 48 months
(2) loss of previously acquired purposeful hand skills between ages 5 and 30 months with the subsequent development of stereotyped hand movements (i.e., hand-wringing or hand washing)
(3) loss of social engagement early in the course (although often social interaction develops later)
(4) appearance of poorly coordinated gait or trunk movements
(5) severely impaired expressive and receptive language development with severe psychomotor retardation

299.10 Childhood Disintegrative Disorder

A. Apparently normal development for at least the first 2 years after birth as manifested by the presence of age-appropriate verbal and nonverbal communication, social relationships, play, and adaptive behavior.

B. Clinically significant loss of previously acquired skills (before age 10 years) in at least two of the following areas:

(1) expressive or receptive language
(2) social skills or adaptive behavior
(3) bowel or bladder control
(4) play
(5) motor skills

C. Abnormalities of functioning in at least two of the following areas:

(1) qualitative impairement in social interaction (e.g., impairment in nonverbal behaviors, failure to develop peer relationships, lack of social or emotional reciprocity)
(2) qualitative impairments in communication (e.g., delay or lack of spoken language, inability to initiate or sustain a conversation, stereotyped and repetitive use of language, lack of varied make-believe play)
(3) restricted, repetitive, and stereotyped patterns of behavior, interests, and activities, including motor stereotypies and mannerisms.

D. The disturbance is not better accounted for by another specific pervasive developmental disorder or by schizophrenia.

299.80 Pervasive Developmental Disorder-Not Otherwise Specified (Including Atypical Autism)

This category should be used when there is a severe and pervasive impairment in the development of reciprocal social interaction associated with impairment in either verbal or nonverbal communication skills or with the presence of stereotyped behavior, interests, and activities, but the criteria are not met for a specific Pervasive Developmental Disorder, Schizophrenia, Schizotypical Personality Disorder, or Avoidant Personality Disorder. For example, this category includes ‘atypical autism’ - presentations that do not meet the criteria for Autistic Disorder because of late age at onset, atypical symptomatology, or subtreshold symptomatology, or all of these.

Prevalence

Pervasive developmental disorders are relatively rare, but not very uncommon compared to other childhood psychiatric problems like anxiety and ADHD. Pervalences have been reported to vary between 4/10,000 to most recently 6/1,000 (Fombonne, 2005). The more severe disorders like autistic disorder and Asperger's disorder have prevalences of 1.3/1.000 and 0.4/1.000, respectively. PDD-NOS accounts for the remaining 4.3/1.000 (Fombonne, 2005). There is a markedly higher prevalence of pervasive developmental disorders in mental retardation of approximately 26 times the prevalence in the typically developing population (de Bildt et al., 2005). Rett's disorder and childhood desintegrative disorder are even more rare then the other pervasive developmental disorders, prevalences have been estimated at 3.8 and 1.7/100,000 (Fombonne, 2005). The male to female ratio of all PDD's is 4:1.

Causal factors

Although described as a disorder with a possible biological background in the original descriptions of Kanner (1943) and Asperger (1944), autism has long been viewed as caused by 'bad parenting'. Since the 1960's biological factors have become more and more apparent to play a role in the etiology of autism (Rutter, 2005). Today the autism spectrum disorders are regarded as a neurodevelopmental problem, and eludicating the underlying biological causes is the puzzle for current researchers.

In neuroimaging studies several brain structures are implicated in pervasive developmental disorders. Among them are the fusiform gyrus, which has shown to have an important role in face recognition, the amygdala and the cerebellum (Schultz, 2005). Structural MRI studies show larger brain volumes especially in younger indiviuals with pervasive developmental disorders (Palmen et al., 2004a). These findings are corroborated by findings of a larger headcircumference in very young children with autism, followed by normal headcircumferences in older children. This emphasizes the role of disturbed developmental processes in the pathogenesis of autism and related disorders (Courchesne & Pierce, 2005). Neuropathological findings include abberations in the limbic system, the cerebellum and the cerebral cortex (for a review see Palmen et al., 2004b).

Autism is one of the most hereditary disorders in psychiatry. Heredity has been

calculated to be approximately 90%. Concordance rates of 60-91% are reported in monozygotic twins, in contrast to 10% concordance in dizoygotic twins (Bailey et al., 1995). Recurrence rates in siblings have been estimated at 4,5% (Jorde et al., 1991). The search for genes related to pervasive developmental disorders has proven to be very complex, due to the considerable clinical and genetic heterogeneity of the disorder. Supposedly more than 15 genes are involved (Risch et al., 1999). The initial genome screens suggested significant linkage on chromosome 2q and 3q, later studies added regions of interest on chromosomes 7q, 13q, 16p and 17q (see for an excellent review Veenstra-VanderWeele & Cook, 2004). Linkage findings on chromosomes 7q and 17q have been reported several times. The latter is the only one replicated in a independent sample so far (Cantor et al., 2005; Bacchelli & Maestrini, 2006). The linkage findings on chromosome 17q are of particular interest for this thesis, since this region of interest contains the gene for the serotonin transporter molecule, SLC6A4 (Ramamoorthy et al., 1993).

Treatment

Until now no curative therapy is available for autism and the other pervasive developmental disorders. Treatment aims at decreasing unwanted problem behaviors like aggression, stereotyped/rigid/compulsive behaviors and hyperactivity and attention problems (Veenstra-VanderWeele et al., 2000). The neuroleptics, especially risperidone, have been found to be effective in treating aggression (RUPP, 2002; Troost et al., 2005). Serotonin reuptake inhibitors (SSRI's) appear to decrease stereotyped behaviors in some individuals with pervasive developmental disorders (McDougle et al., 2000). Hyperactivity and attention problems tend to be treated by methylphenidate with some succes (Di Martino et al., 2004; Handen et al., 2000). Non-pharmacologal treatments like early intervention programs and cognitive behavioral therapy interventions appear to be of help in stimulating development and reducing specific behavioral problems, but lack systematic randomized controlled research (Howlin, 2005).

Serotonin

Since its first identification (Ersparmer, 1940; Ersparmer & Asero, 1952; Rapport et al., 1948) the monoamine serotonin has been proven to have a variety of functions. It serves as a 'signalling molecule' in neurotransmission in the brain and

the gastro-intestinal tract (Frazer & Hensler, 1999; Gershon, 1999), in the cardiovascular system throughout the whole body (Gershon & Tamir, 1985), and as a neurohormone in neurodevelopment (Whitaker-Azmitia, 2001). More recently, serotonin has also been found to fullfil a pivotal role in the early steps of embryonic development (Levin et al., 2006) as well as in liver regeneration (Lesurtel et al. 2006).

Physiology

In the brain serotonergic cell bodies are located mostly in the raphe nuclei and project throughout the brain (Frazer & Hensler, 1999). Serotonin generally acts as an inhibiting neurotransmitter, which influences a broad range of physiological systems. These systems include the cardiovascular and respiratory systems, thermoregulation, and a variety of behavioral functions, including circadian rhythm entrainment, sleep-wake cycle, appetite, aggression, sexual behavior, sensorimotor reactivity, pain sensivity and learning. The serotonergic system has been shown, mostly through pharmacological regulation, to be a factor in many psychiatric disorders also, such as depression, anxiety disorder, schizophrenia, anorexia nervosa and autism (Lucki, 1998).

Peripheral serotonin is found in the enterochromaffin cells in the intestine, where it serves as an enteric neurotransmitter and an autocrine hormone. Serotonin is active in many more organs and tissues. Some of its functions are stimulation of platelet aggregation, activating vascular, bronchial and intestinal smooth muscle (causing vasoconstriction), and hypersensitivity reactions (Gershon & Tamir, 1985). Apart from its role as a signalling molecule serotonin, is also the precursor of the neurohormone melatonin (Brzezinski, 1997).

Metabolism

Figure 1.1 gives a comprehensive schematic overview of serotonin metabolism in the human body. Serotonin is synthesized from the essential amino acid tryptophan (TRP). The serotonin pathway, and its sidebranch the melatonin pathway, constitute a minor route of the metabolism of tryptophan. About 2% of tryptophan is available for the synthesis of serotonin. The remaining 98% is used for the synthesis of proteins or catabolized via the enzyme tryptophan/indoleamine 2,3 dioxygenase (TDO2) into kynurenin and 3-hydroxyanthranilic acid (Tyce, 1985; Heyes et al., 1992).

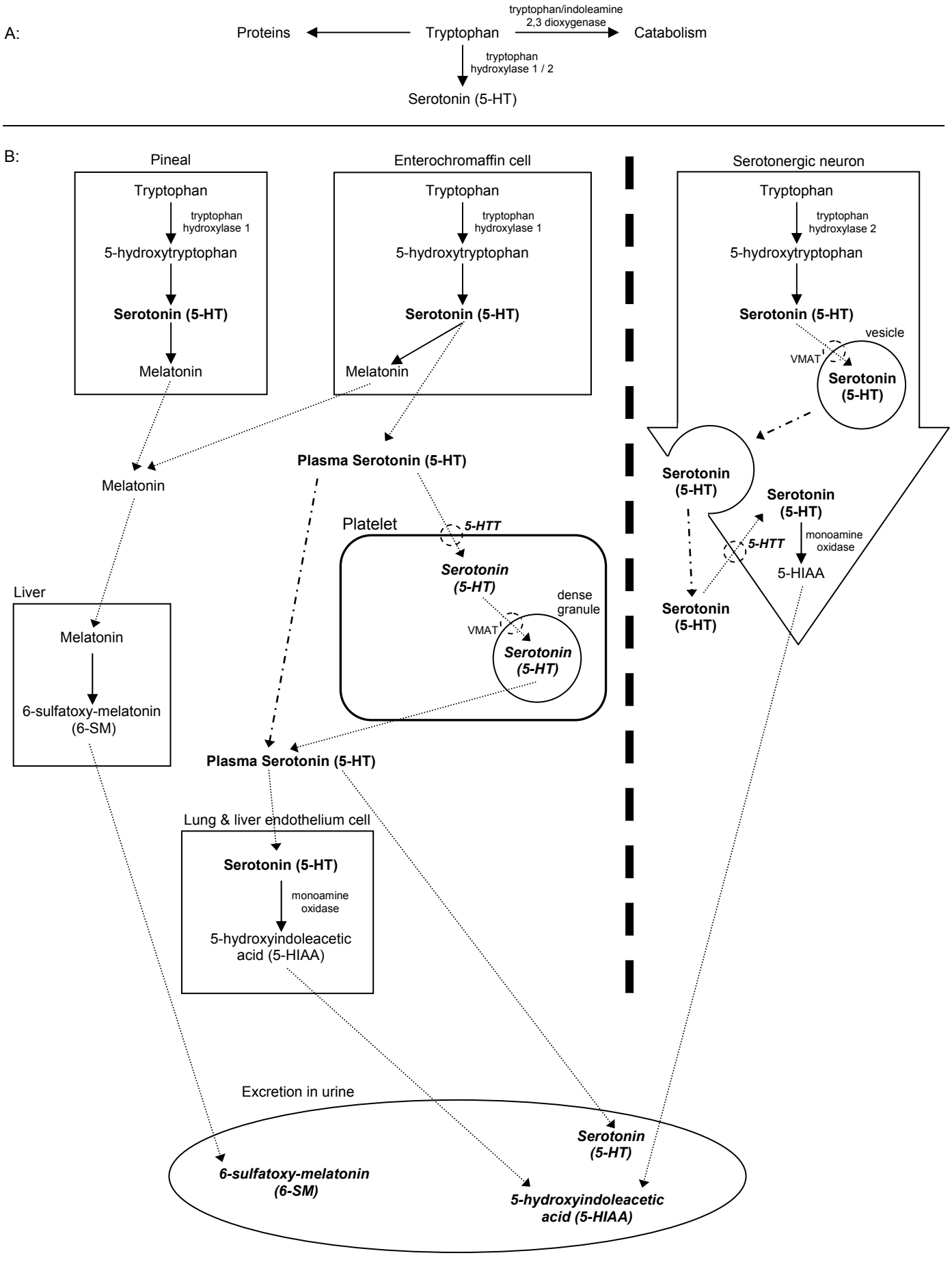

Figure 1.1: Schematic overview of tryptophan and serotonin metabolism. A: three metabolic routes of tryptophan. B: routes and compartments of serotonin metabolism. Normal arrows are used when chemical reactions are taking place, dotted arrows depict transport of a molecule over a cell membrane, dashed arrows mean transport within a compartment. The bold dashed line depicts the blood brain barrier.

Tryptophan hydroxylase (TPH) is the rate-limiting enzyme in the synthesis of serotonin. Recently two isoforms of TPH have been identified, TPH1 and 2 (Walther et al., 2003). TPH1 is found to be present in the gastro-intestinal tract and the pineal gland and TPH2 in the serotonergic neurons in the raphe nuclei (Sakowski et al., 2006). Serotonin synthesis rate is also dependent on tryptophan availability and the activity of the other involved enzym aromatic-L-amino acid decarboxylase (AADC). In normal physiological circumstances the activity of AADC is higher, compared to the availability of tryptophan and lower than the TPH activity (Hamon, 1974; Tyce, 1985). Serotonin does not cross the brain blood barrier, however tryptophan does. Brain and peripheral serotonin metabolism are for the most part separate systems (Tyce, 1985).

In the brain serotonin is synthesized in the serotonergic neurons and consequently taken up by a vesicular monoamine transporter (VMAT) into vesicles. Here it is protected from the catabolic enzym monoamine oxidase (MAO) and stored awaiting its release into the synaptic cleft (Frazer & Hensler, 1999). Two isoforms of VMAT are reported in the literature (Henry et al., 1994). In serotonergic neurons and platelets VMAT2 is expressed (Lesch et al., 1993a). Upon depolarization of the neuron the serotonin containing vesicles release their content into the synaps, where serotonin binds to its postsynaptic (e.g. 5-HT_2) and presynaptic (e.g. 5-$HT_{1a,b}$) receptor thus constituting its neurotransmittorial role. Consequently, serotonin is taken up back into the neuron by the serotonin transporter (5-HTT, SERT). In the neuron serotonin is oxidized by the enzyme monoamine oxidase (MAO) into 5 hydroxy-3-indoleacetic acid (5-HIAA), which is secreted into the blood (Frazer & Hensler, 1999). The serotonin transporter is the site of action of the serotonin reuptake inhibitors, SSRI's. Blockade by an SSRI of the serotonin transporter results in a block of reuptake of serotonin in the neuron and hence a longer availabilty of serotonin for neurotransmission (Frazer & Hensler, 1999).

Most peripheral serotonin is synthesized in the enterochromaffin cells of the gastro-intestinal tract. When released from the enterochromaffin cells it serves as a enteric neurotransmitter and hormone (Gershon, 1999). Eventually, serotonin is released into the portal circulation, and taken up in the liver. The serotonin that escapes the liver is transported to the lung and removed from the blood rapidly. In the endothelium cells of both the liver and the lung serotonin is oxidized into 5-HIAA by MAO. After passing the liver and the lung, about 99% of serotonin is cleared from the circulation (Gershon & Tamir, 1985; Gillis, 1985).

An additional site of minor production of serotonin is the pineal gland. Here serotonin is converted to melatonin, that serves its role in sleepregulation and circadian rhythm processes. Melatonin is released into the circulation, cleared by the liver and metabolized into 6-sulfatoxy-melatonin (6-SM; Brzezinski, 1997).

Both 5-HIAA and 6-SM, together with a small amount of free circulating serotonin, are excreted in urine through the kidney (Frazer and Hensler, 1999).

Platelet storage

A small amount of serotonin is not cleared in the liver and the lung. About 99% of this remaining circulating serotonin is stored in platelets and a small proportion circulates as free serotonin in the circulation (Anderson et al., 1987a). The serotonin transporter is responsible for the uptake of serotonin from the circulation into the platelet. Once inside the platelet, serotonin is stored in the dense granules. The process of transport over the dense granules membrane mimicks the process of storing serotonin in the neuron's vesicles. The same transporter (VMAT2) transports the serotonin actively over the granules membrane (Lesch et al., 1993a). The uptake process takes place during the entire life-time of the platelet, which constitutes about 8-10 days (Stolz, 1985). Blood serotonin half-life is estimated to be about 4 days, the same as that of the platelet. The maximum storage capacity of the human platelet is not reached during its lifetime in the circulation when serotonin concentrations are within the normal range (Kema et al., 1993). Although there is supposed to be some MAO activity in the platelet cytosol, most serotonin from the platelets eventually ends up in the liver and lung clearence system and is metabolized to 5-HIAA (Stolz, 1985).

Serotonin transporter

As described, the serotonin transporter plays a central role in the metabolism of serotonin. It executes the re-uptake of serotonin in the neuron as well as the uptake of serotonin into the platelet. The gene coding for the serotonin transporter molecule is located at chromosome 17q (Ramamoorthy et al., 1993). The molecular structure of the transporter is found to be identical in both the serotonergic neuron membrane and the platelet membrane (Lesch et al., 1993b). Since the discovery of two common, supposedly functional, polymorphisms in the serotonin transporter gene, this gene became subject of intense reseach. One is an insertion/deletion polymorphism in the promotor (5-HTTLPR), which has a long (L) and a short (S)

variant (Heils et al., 1995). The other concerns a variable number of tandem repeats polymorphism in intron 2 (Int2VNTR), with three alleles (with 9, 10 and 12 copies of the tandem repeat; Heils et al., 1996). Most functional studies of the 5-HTTLPR (Heils et al., 1995; Hanna et al., 1998; Greenberg et al., 1999; Nobile et al., 1999; Anderson et al., 2002), but not all (Kaiser et al., 2002), have found allelic effects on 5-HT uptake. In a transfection experiment, the intron 2 VNTR has been reported to affect transporter expression in the mouse hindbrain during embryonic development (MacKenzie & Quinn, 1999).

Serotonin and the autism spectrum disorders

The serotonergic system has been implicated in the pathogenesis of autism spectrum disorders for several reasons. First there is the well replicated finding of elevated platelet serotonin levels in groups of individuals with autism and autism spectrum disorders (Anderson, 2002). Additionally, findings of linkage and association of genes involved in the serotonergic system to autism corroborate the notion that some - although until now unknown - factor in this system contributes to the risk or severity for autism spectrum disorders (Veenstra-Vanderweele & Cook, 2004). Furthermore, tryptophan depletion experiments (McDougle et al., 1996) and a fenfluramine challenge study (McBride et al., 1989) add to evidence of a role of serotonin in autism. Another indication for the involvement of the serotonergic system in autism spectrum disorders consitutes the efficacy of drugs aiming at the serotonin receptors and transporter, like risperidone and the SSRI's (McDougle et al., 2000; RUPP, 2002; Troost et al., 2005). Finally the sparse and sometimes unequivocal brain imaging studies of the serotonergic system in autism indicate deviations in the synthesis of serotonin (Chugani et al., 2002). However, the exact role of the serotonin system in the pathofysiology of autism remains unclear to date. Also the meaning of the platelet hyperserotonemia still has to be eludicated.

Whole blood / platelet serotonin in autism

In 1961, Schain and Freedman first studied whole blood serotonin in autism. In their three groups of subjects the individuals with autism had the highest whole blood serotonin values. A control group with mild mental retardation had normal serotonin levels. The third group, subjects with more profound mental retardation had levels higher than the other controls, but not as high as the autistic individuals.

Table 1.2: Whole blood or platelet serotonin studies in autism spectrum disorders

Study	Group (N)	Age[a]	Sample type[b]	Autism, mean ± SD	NC, mean ± SD	% 5-HT increase	Plt count[c]	Method[d]	Remarks
Schain and Freedman (1961)	Autism (23) NC (4)	6-18	WB	141 ± 78 ng/mL	65 ± 17 ng/mL	117%*	-	B	hyper 5-HT: 26%, Aut > Sev MR(7) > NC
Ritvo et al. (1970)	Autism (24) NC (not rep.)	2-8	WB	263 ± 63 ng/mL	216 ± 61 ng/mL	22%*	375 ± 70.8 vs. 330 ± 77.9**	A	
Yuwiler et al. (1971)	Autism (7) NC (4)	4-9	WB	272 ± 53 ng/mL	183 ± 23 ng/mL	49%**	351 ± 24 vs. 365 ± 23**	A	circadian rhythm: Aut = NC
Yuwiler et al. (1975)	Autism (12) NC (12)	3-6	WB	273 ± 30 ng/mL	205 ± 17 ng/mL	33%*	333 ± 23 vs. 310 ± 21**	A	uptake and efflux: Aut = MC
Takahashi et al. (1976)	Autism (30) NC (30)	4.8	PltP	980 ± 357 ng/mL	807 ± 202 ng/mL	21%*	-	N	younger > older parents: Au = NC
Hanley et al. (1977)	Autism (27) Mildly MR (23)	8-22	WB	134.5 ± 56.9 ng/mL	96.5 ± 38.0 ng/mL	39%*	-	A	Sev MR(25) > Aut > NC(6)
Hoshino et al. (1979)	Autism (42) NC (20)	5.7	S	218 ± 79 ng/mL	175 ± 60 ng/mL	25%*	-	A	Hyperactivity corr. 5-HT
Hoshino et al. (1984)	Autism (37) NC (12)	3-11	WB	173 ± 62 ng/mL	124 ± 44 ng/mL	40%*	-	A	Normal Adults(27) < Aut, TRP: Aut = NC
Anderson et al. (1984)	Autism (11) NC (10)	10-24, 17.3 ± 4.4	WB	176 ± 97.1 ng/mL	123 ± 43.5 ng/mL	43%**	466 ± 170/nL vs. 589 ± 109/nL**	Hf	Imipramine binding: Aut = NC
Anderson et al. (1987)	Autism, (21) NC (87)	21-27, 16.8 ± 6.0	WB	205 ± 15.7 ng/mL 776 ± 87 ng/10^9 plt	136 ± 5.4 ng/mL 522 ± 26 ng/10^9 plt	51%* 49%*	279 ± 16/nL vs. 281 ± 16/nL**	Hf	TRP: Aut = NC
Minderaa et al. (1987)	Autism (16) NC (27)	14-28, 20.6 ± 4.6	WB	163 ± 86.3 ng/mL 630 ± 333 ng/10^9 plt	113 ± 24.6 ng/mL 443 ± 112 ng/10^9 plt	44%* 42%*	262/nL vs. 261/nL**	Hf	Urinary 5-HIAA: Aut = NC
Launay et al. (1988)	Autism (22) NC (22)	5-16	WB	Median 1.20 μM	Median .36 μM	~300%*	-	R	TRP, uptake, efflux, ur5-HIAA: Aut = NC; ur5-HT: Aut > NC
Abramson et al. (1989)	Autism (57) NC (17)	13.9 ± 5	WB	399 ± 210 ng/mL	184 ± 97 ng/mL	117%*	-	A	hyper 5-HT: 40%, male = female, AfrAm > Cauc
Minderaa et al. (1989)	Autism (17) NC (20)	19.4 ± 4.9	WB	163 ± 81.7 ng/mL 620 ± 303 ng/10^9 plt	116 ± 35.0 ng/mL 465 ± 162 ng/10^9 plt	41%* 33%*	267 ± 67/nL vs. 258 ± 47/nL**	Hf	TRP: Aut = NC

Singh et al. (1997)	Autism (23) NC (20)	6.3	WB	~180 pmol/mL	~113 pmol/mL	~59%*	-	E	5-HT rec antibodies: Aut < NC
McBride et al. (1998)	Autism (58) NC (38)	2-37, 6.7 ± 3.1	WB	245 ± 78 ng/mL 78 ± 23 ng/ul plt vol (white)	187 ± 50 ng/mL 68 ± 17 ng/ul plt vol (white)	25%* (tot. group)	389 ± 75/nL vs. 337 ± 59/nL* (white)	Hf	MR(22) = NC, black = latin > white, prepub: Aut > NC, postpub: Aut = NC
Leboyer et al. (1999)	Autism (60) NC (118)	3-23	WB	1.02 ± 0.77 umol/L	0.42 ± .14 umol/L	143%*	-	R	no change with age, Aut = sibs = mo = fa > NC
Croonenberghs et al. (2000)	Autism (13) NC (13)	12-18	WB	223 ± 39 ng/mL	206 ± 68 ng/ml	8%**	-	Hf	TRP : Aut < NC
Mulder et al. (2004, ch. 2)	Autism (33) NC (60)	4-20, 11.7 ± 4.0	PRP	4.51 ± 1.61 nmol/10^9 plt	3.58 ± 1.08 nmol/10^9 plt	29%*	428 ± 130/nL vs. 423 ± 151/nL**	Hf	Aut = PDDNOS(43) = Asp(5) > MR(54) = NC, biomodality, no relation beh. correlates

*NOTE: * significant difference, ** non-significant difference; a. Age in years: range and/or mean ± SD; b. Sample type: WB = whole blood, PltP = platelet pellet, S = serum, PRP = platelet rich plasma; c. Plt count: Autism vs. NC, mean ± SD; d Method: B = bioassay, A = acid fluorescence, N = inhydrine fluoroscence, Hf = HPLC fluorometric, R = radioenzymology, E = enzyme immunoassay*

These results have been largely replicated in the studies that followed. See table 1.2 for an overview of relevant studies.

Summarizing the data, it appears that the magnitude of the elevation varies between 25 and 40%. About 38% of individuals with autism can be regarded 'hyperserotonemic' (Anderson, 2002). A range of demographic, descriptive, and behavioral variables have been evaluated for their relationship with platelet serotonin levels, including age, sex, ethnicity, family loading, medication, and platelet count and size (Ritvo et al., 1970, 1971; Kuperman et al., 1985; Anderson et al., 1987b; Geller et al., 1988; Abramson et al., 1989; Minderaa et al., 1989; Leventhal et al., 1990; Cook et al., 1990; Piven et al., 1991; Cook et al., 1993; Cuccaro et al., 1993; Anderson et al., 1996; Leboyer et al., 1999). A landmark study by McBride et al. (1998) elucidated several issues that had been observed, yet had not been clarified in the earlier studies. Their most important finding constituted of race and puberty-status as significant confounders for platelet serotonin levels in individuals with autism spectrum disorders and in typically developing subjects (McBride et al., 1998). They concluded that the estimation of the true elevation of serotonin in autism should be closer to 25% than to 40%. Although this study provides answers to some longstanding issues concerning the characterization of the hyperserotonemia in autism, several questions still remained open. How are the platelet serotonin values distributed within the autism group? Can a distinct hyperserotonemic group be recognized, or is there a shift of the mean upwards in the total group, without a distinction of a separate group? Is the hyperserotonemia confined to core autism or does it also extend to the other autism spectrum disorders, Asperger's disorder and PDD-NOS?

Another issue concerns the relation of hyperserotonemia to the specific autism related behavioral domains and other area's like intellectual functioning. A small number of studies have examined the relationship of platelet serotonin levels to intelligence and mental retardation, and specific aspects of cognitive functioning (Campbell et al., 1975; Cook et al., 1990; Cuccaro et al., 1993; Kuperman et al., 1987; McBride et al., 1998). Most of the studies only addressed one or two of the variables of interest and studied limited numbers of subjects. These issues have never been investigated together in larger groups of individuals with autism spectrum disorders.

Mechanism

With the metabolism of tryptophan and serotonin in mind (see figure 1.1) there are a number of mechanisms by which platelet hyperserotonemia might occur. These processes break down to two principal categories. The first is the way the platelet handles serotonin, i.e. alteration in the uptake of serotonin in the platelet. The second implies an increased exposure of the platelet to serotonin, caused by a higher synthesis of serotonin in the enterochromaffin cells of the gut or a decreased clearence of serotonin from the circulation.

Platelet handling

Although results of serotonin uptake and serotonin transporter binding studies are inconclusive, uptake of serotonin in platelets of some individuals with hyperserotonemia appears to be increased (Anderson et al., 1984; Cook et al., 1993; Launay et al., 1988; Marazziti et al. 2000).

There has been a body of research into the functional polymorphisms and other single nucleotide polymorhisms (SNP's) within the serotonin transporter gene. Significant linkage and association with autism has been reported a number of times and is replicated (see table 1.3). Although results haven't been in accordance with each other with respect to which polymorphism is related to the disorder, the gene is a strong candidate to be a major susceptibility gene for autism (Sutcliffe et al., 2005). Several rare functional mutations have been found to be related to autism spectrum disorders, Asperger's disorder and obsessive compulsive disorders (Ozaki et al., 2003; Sutcliffe et al., 2005).

Severity of impairment in the social interaction and communication domains (Tordjman et al., 2001) and the stereotyped, rigid, compulsive behaviors domain (McCauley et al., 2004) were found to be associated to polymorphisms of the serotonin transporter gene. These findings need replication and extension in independent populations.

The role of mutations of the serotonin transporter gene in causing the platelet hyperserotonemia of autism has not been assessed extensively, but from the available data it appears to be small. Anderson et al. (2002) found a significant increase of serotonin uptake in subjects homozygous for the L-allele of the promoter polymorphism. However, the relation between the polymorphisms and platelet serotonin levels perse was reported to be minor (Persico et al., 2002; Coutinho et al.,

2004) or non existent (Anderson et al., 2002; Betancur et al., 2002). Other factors - until now unknown - presumably are in play in the elevation of serotonin transporter functioning, serotonin uptake and concentration in platelets.

Table 1.3: Serotonin transporter gene (5-HTT, SLC6A4) studies in autism spectrum disorders

Association studies	***Sample***	***Analysis method and markers[a]***	***Results (p-value)[b]***	***Remarks[c]***
Cook et al. (1997)	86 trios	TDT; 5-HTTLPR and Int2VNTR	5-HTTLPR-s (.03)	
Klauck et al. (1997)	65 trios	TDT; 5-HTTLPR and Int2VNTR	5-HTTLPR-l (.032)	
Maestrini et al. (1999)	90 families	TDT; 5-HTTLPR and Int2VNTR	ns	
Zhong et al. (1999)	72 cases	case-control; 5-HTTLPR	ns	
Persico et al. (2000)	91 families	TDT/HHRR; 5-HTTLPR	ns	
Tordjman et al. (2001)	69 families	TDT; 5-HTTLPR	5-HTTLPR-l (.046)	E-TDT (mild soc./comm.) .007[d]
Yirmiya et al. (2001)	34 trios	TDT; 5-HTTLPR	5-HTTLPR-l (.025)	
Kim et al. (2002)	115 trios	TDT; 20 markers	5-HTTLPR-s (.007); 4 SNP's (<.0002)	Mutation screening (10 cases)
Betancur et al. (2002)	96 families	TDT; 5-HTTLPR and Int2VNTR	ns	ANOVA (5-HT) ns
Persico et al. (2002)	153 trios	TDT; 5-HTTLPR and Int2VNTR	ns	Q-TDT (5-HT) ns
McCauley et al. (2004)	125 families	TDT; 8 markers	5-HTTLPR-s (.01)	
Conroy et al. (2004)	84 trios	TDT; 5 markers	5-HTTLPR-s (.033)	
Devlin et al. (2005)	390 families	TDT; 4 markers	5-HTTLPR-s (.007)	
Mulder et al. (2005, ch. 3)	125 families	TDT; 5-HTTLPR and Int2VNTR	ns	Q-TDT (rig./comp.) .015
Wu et al. (2005)	175 families	TDT; 3 SNP's	ns	
Sutcliffe et al. (2005)	384 families	TDT; 2 markers	rs14700 (.03); 5-HTTLPR ns	Mutation screening (24 cases)
Koishi et al. (2006)	104 trios	TDT; 5-HTTLPR	ns	
Ramoz et al. (2006)	352 families	TDT; 9 markers	ns	
Positive linkage studies		***Method***	***LOD score 17q[e]***	***Remarks***
IMGSAC (2001)	152 sib-pairs	whole genome scan (83) + 119 markers (69)	2.34	Higher LOD scores chr. 2, 7, and 16
Yonan et al. (2003)	354 sib-pairs	whole genome scan	2.83	Sign. LOD scores chr. 5, 11, 4, and 8
McCauley et al. (2004)	137 sib-pairs	5-HTT gene locus fine map	2.74	Increase to 3.62 on Rig./Com. subset
Cantor et al. (2005)	109 sib-pairs	whole genome scan + 5-HTT gene locus fine map	1.90	Increase to 4.1 in male only subset

NOTE: a. TDT = transmission disequilibrium test; HHRR = haplotype-based haplotype relative risk test; 5-HTTLPR = serotonin transporter promotor polymorphism, Int2VNTR = serotonin transporter intron2 variable number of tandem repeats. b. 5-HTTLPR-l = long allele, 5-HTTLPR-s = short allele. c. E-TDT = extended TDT, Q-TDT = quantitative TDT, soc./comm. = combined social and communication domain, rig./comp. = rigid/stereotyped/compulsive behavioral domain. d. method (trait) p-value. e. LOD = logarithmic odds, Maximum LOD scores as reported in the papers are reported here, 17q = the locus of the serotonin transporter gene on chromosome 17.

Synthesis and catabolism

Synthesis of serotonin in the enterochromaffin cells and the brain depends on several mechanisms (see figure 1.1). Evidence available for each separate process will be reviewed here.

One process concerns the availability of tryptophan, which appears not to be changed in autism (Hoshino et al., 1979; Minderaa et al., 1987; Anderson et al., 1987; Launay et al., 1988; Croonenberghs et al., 2000). Subsequently, the activity of enzymes TPH1 or 2 and TDO2, the rate limiting enzymes for the conversion of tryptophan into serotonin and kynurenin respectively, influence the amount of serotonin synthesized. There are no reported functional studies of these enzymes in autism. However, association studies in autism of SNP's of the genes for these enzymes suggest minor or no association of TPH2 gene variants (Coon et al., 2005; Delorme et al., 2006) and a possible association with the TDO2 gene (Nabi et al., 2004). The significance of these findings for the hyperserotonemia is unclear pending data directly assesing the association with peripheral serotonin indices.

Altered excretion of the catabolic products of serotonin, 5-HIAA, also points to changes in gut serotonin production (Kema et al., 2000). Although less wel studied also levels of plasma free serotonin and urine serotonin might give insight into the serotonin. Urinary excretion of 5-HIAA as well as platelet serotonin itself have been shown to be markers of gross overproduction of serotonin in carcinoid tumors of the intestine (Kema et al, 2000). Several studies have measured urinary excretion of 5-HIAA in autism in order to address the issue of serotonin production and platelet serotonin exposure. A few studies have also reported urinary serotonin levels and free plasma serotonin concentrations. Most studies have not found differences in urinary 5-HIAA excretion between autistic individuals and normal or mentally retarded controls (Schain and Freedman, 1961; Partington et al., 1973; Minderaa et al., 1987; Launay et al., 1988; Martineau et al., 1992; Herault et al., 1996; Croonenberghs et al., 2000). However, Hanley et al. (1977) reported elevated levels in urinary 5-HIAA and urinary serotonin in autistic subjects compared to a group of subjects with mental retardation. Minderaa et al. (1987) reported borderline significantly higher levels of 5-HIAA excretion in four hyperserotonemic autistic individuals, while finding a very similar urinary 5-HIAA excretion in the whole group of unmedicated autistic individuals compared to normal controls. Three studies have reported elevated urinary serotonin excretion in groups of autistic subjects, but no differences in 5-HIAA

excretion (Launay et al., 1988; Martineau et al., 1992; Herault et al., 1996), while Anderson et al. (1989) reported that urinary excretion of serotonin was unaltered in autism.

The melatonin pathway has sparsly been assessed in autism. Only a minor proportion of tryptophan is used for the production of melatonin (Brzezinkski, 1997). A recent study by Tordjman et al. (2005) reported a decrease of nocturnal melatonin production in prepubertal autistic subjects. No studies have been published that assess the relation of melatonin and its metabolites with platelet serotonin measures.

Finally alteration in the activity of the monoamine oxidase, MAO, might result in higher platelet serotonin levels. Early studies suggest that MAO activity appears not to be deviant in autism (Cohen et al., 1977). Non-functional polymorphisms of the MAO A gene are suggested to be associated to IQ level in autism (Yirmiya et al., 2002; Jones et al., 2004), however these are not evaluated directly in relation to platelet serotonin.

In summary, these data suggest that serotonin production might not be altered greatly in hyperserotonemic individuals with autism. However, increased exposure of the platelet to serotonin can not be ruled out as playing a role in causing an increased serotonin amount in platelets.

Effects of serotonergic system indices on medication

The value of platelet serotonin levels and serotonin transporter gene polymorphisms in the prediction of medication effect is of great interest, since both are relatively easy accessable and are biological, ergo more reliably measurable than psychological factors (Veenstra-VanderWeele et al., 2000). The possible importance of the serotonin transporter polymorphisms has been illustrated by studies into the role of the promotor alleles in the effect of stressfull life events on the development of major depression in young adults (Caspi et al., 2003), the finding of an association between the S-allele and the risk for anti-depressant-induced mania in bipolar disorder (Mundo et al., 2001); and several studies into the relation of transporter alleles to the effect of SSRI's in depression (Smeraldi et al., 1998; Kim et al., 1999).

The usefulness of platelet serotonin levels is not as apparent given the major influence of the SSRI's on platelet serotonin content. By blocking the serotonin transporter the serotonin levels in platelets decrease to levels close to zero in days (Anderson, 2004). Several studies, especially in obsessive compulsive disorder, have

used a paradigm of change in whole blood or platelet serotonin during the use of medication as a measure of medication effect (Humble et al., 2001; Delorme et al., 2004). Claims have been made concerning the relevance of whole blood serotonin variability for elevaluation of drug efficacy, but several aspects of the role of platelets have not been assessed (Humble et al., 2001).

Recently the clinical efficacy of fluvoxamine in autism was studied in relation to serotonin transporter gene levels and whole blood serotonin levels (Sugie et al, 2005). Although the number of subjects was limited particularly the 5-HTTLPR L-allele was suggested to predict a better fluvoxamine response in subjects with autism.

Specific aims and scope of this thesis

The studies described in this thesis aim to enlarge the insight in several aspects of the role of the serotonergic system in autism. In chapter 2 we present a study into diagnostic group differences, within-group distribution and behavioral correlates of platelet serotonin levels. In this large study we compared groups of subjects with autistic disorder, Asperger's disorder, PDD-NOS, mental retardation and a group of typically developing children. In a consequent study (chapter 3) we looked into the relation of variants of the serotonin transporter gene and severity of impairment of social, communicative and rigid/compulsive behaviors in individuals with autism spectrum disorders. We used a quantitative family based approach in order to parcel out the role of the transmission of serotonin transporter gene alleles. Chapter 4 describes the development of a method for the measurement of 5-hydroxyindole-3-acetic acid (5-HIAA), the metabolite of serotonin, in urine. The method improves analytic precision and automation of sample handling. In chapter 5 the issue of possible increased production of serotonin in the gastro-intestinal tract was studied through comparing 24 hour excretion of metabolites of serotonin between hyperserotonemic and normoserotonemic individuals with autism spectrum disorders. Chapter 6 discusses issues of the use of whole blood serotonin in the prediction of serotonergic drug response. Finally, in chapter 7 the results of the several studies will be summarized and discussed. The thesis will be placed in a broader perspective and suggestions for further research will be given.

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Chapter 2

Platelet Serotonin Levels in Pervasive Developmental Disorders and Mental Retardation: Diagnostic Group Differences, Within-Group Distribution, and Behavioral Correlates

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Abstract

Objective: To investigate group differences, the within-group distributions, and the clinical correlates of platelet serotonin (5-HT) levels in pervasive developmental disorders (PDD). Method: Platelet 5-HT levels were measured in Dutch children and young adults, recruited from 2001 through 2003, with PDD (autism, Asperger's, and PDD-not otherwise specified [PDD-NOS]; n = 81) or with mental retardation (MR; n = 54) but without PDD, and in normal controls (n = 60). The distribution of platelet 5-HT levels was assessed using mixture-modeling analyses. Relationships between platelet 5-HT levels and a full range of demographic, clinical, and behavioral variables were examined. Results: Group mean (± SD) platelet 5-HT levels (nmol/10^9 platelets) were significantly higher in the autistic (4.51 ± 1.61, n = 33) and PDD-NOS (4.90 ± 1.54, n = 43) groups compared to the MR (3.48 ± 1.33, n = 54) or the normal control (3.58 ± 1.08, n = 60) groups ($F_{4,190}$ = 9.35, p < .001). Platelet 5-HT values in the combined PDD group showed a bimodal distribution, and an empirical cutpoint for hyperserotonemia was determined. None of the behavioral variables examined was significantly associated with platelet 5-HT levels. Conclusions: The platelet hyperserotonemia of autism was replicated in Dutch subjects. Platelet 5-HT levels were also increased in PDD-NOS, while no elevation was seen in MR. Platelet 5-HT levels appeared to be bimodally distributed in the PDD group, with an apparent hyperserotonemic subgroup.

Introduction

Platelet hyperserotonemia is a longstanding finding in autism (Schain and Freedman, 1961). Although many studies have been conducted attempting to clarify this phenomenon, no definite conclusions can be drawn regarding the mechanism of the elevated group mean serotonin (5-hydroxytryptamine [5-HT]) levels seen in autism (Anderson et al., 1990; Cook and Leventhal, 1996; Anderson, 2002). A number of investigations have attempted to characterize the group differences in platelet 5-HT levels and sought to identify clinical correlates in the autistic group. A range of demographic, descriptive, and behavioral variables have been evaluated for their relationship with platelet 5-HT levels, including age, sex, ethnicity, family loading, medication, and platelet count and size (Ritvo et al., 1970, 1971; Kuperman et al., 1985; Anderson et al., 1987b; Geller et al., 1988; Abramson et al., 1989; Minderaa et al., 1989; Leventhal et al., 1990; Cook et al., 1990; Piven et al., 1991; Cook et al., 1993; Cuccaro et al., 1993; Anderson et al., 1996; Leboyer et al., 1999). A smaller number of studies have examined the relationship of platelet 5-HT levels to intelligence and mental retardation, and specific aspects of cognitive functioning (Campbell et al., 1975; Cook et al., 1993; Cuccaro et al., 1993; Kuperman et al., 1987; McBride et al., 1998). Most of the studies have addressed only one or two of the variables of interest and have studied limited numbers of subjects.

In the present study we investigated the group differences and the clinical and behavioral correlates of platelet 5-HT levels in relatively large groups of Caucasian subjects with autism, Asperger's syndrome, or pervasive developmental disorder-not otherwise specified (PDD-NOS), and in ethnically matched nonautistic mentally retarded and normal controls. Given the prior reports, we hypothesized that 5-HT levels would be elevated in the PDD groups and unchanged in mental retardation (MR). We specifically aimed to enhance the utility of the platelet 5-HT phenotype by attempting to define biochemical subgroups within the PDD groups and by exploratory analysis of the relation between platelet 5-HT levels and behavioral expression.

Method

Subjects

Children and young adults with pervasive developmental disorders (PDD) or with MR in the absence of a PDD were recruited through an epidemiological survey

being carried out in the north of the Netherlands, and through an autism outpatient clinic affiliated with the Child and Adolescent Psychiatry Center of Groningen. The age range for subjects to be included in the study was 5 to 20 years, corresponding to the school-age range used as inclusion criterion in the epidemiological survey. Subjects were excluded from the study if they had a known genetic condition or severe peri- or prenatal problems. None of the subjects was physically ill or had a history or signs or symptoms of inborn errors of metabolism or chromosomal syndromes, including fragile X syndrome. Thirty of the 81 PDD subjects were receiving medication: 8 were receiving neuroleptics (either the atypical neuroleptic risperidone or pimpamperone, a 'typical' neuroleptic used to treat aggression in MR individuals and in patients with PDD), 7 were receiving methylphenidate, 5 were receiving clonidine, and 10 were receiving antiepileptic drugs. Non-MR normal control subjects were recruited from children seen during routine visits to a pediatric nephrology outpatient clinic of the University Medical Center Groningen. Potential normal control subjects who attended special education schools or who had ever used child psychiatric services were excluded. All subjects in the study were of Dutch descent. Demographic and clinical data for the included subjects are given in Table 2.1. The study was approved by the Medical Ethical Committee of the University Medical Center Groningen, and written informed consent was obtained.

Clinical Assessment

All developmentally impaired subjects were extensively evaluated. PDD subjects were initially evaluated using the Autism Diagnostic Interview-Revised (ADI-R; Lord et al., 1994) and the Autism Diagnostic Observation Schedule-G (ADOS-G; Lord et al., 2000) administered by trained examiners. They were then diagnosed by experienced clinicians using DSM-IV-TR criteria (American Psychiatric Association, 2000). The clinicians were blinded to the ADI-R and ADOS-G outcome. A final diagnosis was established by combining DSM-IV-TR, ADI-R, and ADOS-G information for a best-estimate diagnosis.

To ensure the absence of a PDD in all MR comparison group subjects, the Autism Behavior Checklist (ABC; Krug et al., 1980) was filled out by the parents and the Scale for Pervasive Developmental Disorders in the Mentally Retarded (PDDMRS; Kraijer, 1999) was filled out by teachers and school psychologists. To be

included in the MR comparison group, subjects had to score below the cutoff for PDD on both instruments.

Table 2.1: Demographic and Clinical Characteristics of Subjects

Group	Autism	Asperger	PDD-NOS	MR	Normal Control	Statistic$_{df}$; p
n	33	5	43	54	60	
Age, yr ± SD	11.7 ± 4.0	13.2 ± 3.3	13.1 ± 4.2	13.0 ± 3.3	11.5 ± 3.9	$F_{4,190}$=1.74;.14
Gender (male:female)	29:4	4:1	37:6	43:11	29:31	X^2_4=27.2;<.001
Level of functioning (n)						
Profound/severe MR	10	—	8	2	—	
Moderate MR	10	—	6	15	—	
Mild MR	8	—	17	37	—	
'High-functioning' (>70 IQ)	5	5	12	—	—	X^2_9=56.8;<.001
Vineland scores: total age eq., mo ± SD	44.3 ± 26.8	86.3 ± 3.5	65.1 ± 32.4	70.3 ± 28.2	—	$F_{3,121}$=7.96;<.001
Behavioral ratings						
ADI social	22.1	20.0	15.7	—	—	$F_{2,80}$=12.1;<.001
ADI communication	14.4	15.0	12.1	—	—	$F_{2,80}$=2.92;.06
ADI stereotypies	6.2	5.2	4.8	—	—	$F_{2,80}$=2.50;.09
ADI total	46.8	43.6	36.2	—	—	$F_{2,80}$=8.98;<.001
CBCL internal	59.1	64.6	56.4	53.3	—	$F_{3,132}$=3.42;<.05
CBCL external	52.3	61.2	55.0	52.1	—	$F_{3,132}$=1.30;.28
CBCL total	60.8	65.8	60.4	55.5	—	$F_{3,132}$=3.39;<.05
ABC total	62.8	67.2	42.6	18.5	—	$F_{3,132}$=33.1;<.001
CY-BOCS total	8.6	4.2	5.8	1.8	—	$F_{3,132}$=14.1;<.001
CSBQ total	38.2	42.8	33.6	20.1	—	$F_{3,132}$=11.7;<.001

Note: PDD-NOS = pervasive developmental disorder-not otherwise specified; MR = mental retardation; ADI = Autism Diagnostic Interview; CBCL = Child Behavior Checklist; ABC = Autism Behavior Checklist; CY-BOCS = Children's Yale-Brown Obsessive Compulsive Scale; CSBQ = Children's Social Behavior Questionnaire.

Intellectual functioning was tested in the majority of subjects in the PDD and MR groups. However, because test data were not available for all subjects, subjects were assigned to one of four levels of intellectual functioning: profound/severe MR (total IQ < 36), moderate MR (IQ 36–50), mild MR (IQ 51–70), and 'high' functioning (IQ > 70). In most cases (115/135), this classification was based on information from standardized intelligence tests or developmental tests (Wechsler Intelligence Scale for Children- Revised [WISC-R]; Vander Steene et al., 1986), Wechsler Preschool and Primary Scale for Intelligence [WPPSI]; Vander Steene and Bos, 1997), nonverbal intelligence tests (Snijders-Oomen Niet-verbale intelligentie test; Snijders

et al., 1996) or a Dutch modification of the Bayley scales of Infant Development (Van der Meulen and Smrkovsky, 1983). In 20 of the cases, subjects were assigned to one of these categories by a psychologist (AdB) based on their Vineland Adaptive Behavior Scales Scores (Sparrow et al., 1984), and a clinical review of functioning. No data on intellectual functioning were available for the normal control group.

Information from the parent interview ADI-R was used to assess the severity of social impairment, communication impairment, and stereotypical behaviors. The ADI-R uses a semistructured interview format; for most items, parents are asked about the period between age 4 to 5 years and the present. We chose to use the current scores of the ADI-R to assess relationships between behavior and 5-HT levels ('direct eye gaze' was omitted since this item is only scored before age 6).

For the assessment of speech and language development, three items from the ADI-R were used: 'age at first single words', 'age at first phrases' and 'overall level of language'. The first two items were dichotomized into not delayed and delayed, corresponding to words before or after 24 months and phrases before or after 36 months, respectively.

The Dutch version of the Child Behavior Checklist (CBCL; Achenbach and Edelbrock, 1981; Verhulst et al., 1990) was filled out by parents to explore the presence and extent of other problem behaviors. The Dutch Children's Social Behavior Questionnaire (CSBQ; Luteijn et al., 2000) further evaluates the social behavior of children and has six empirically derived scales: Acting Out, Social Contact Problems, Social Orientation Problems, Social Insight Problems, Stereotypical, and Anxious/Rigid scale. To assess compulsive behavior and stereotypes, the Children's Yale-Brown Obsessive Compulsive Scale (CY-BOCS; Scahill et al., 1997) was administered using parental informants. The ABC (Krug et al., 1980) subscores and total score were used to assess the severity of autistic behaviors in the PDD and MR groups.

Laboratory Measures

Blood samples were collected in 10 mL Vacutainer tubes (Becton-Dickinson, Meylan Cedex, France) containing 0.12 mL (0.34 mol/L) EDTA solution. Platelet-rich plasma was prepared from EDTA blood within 1 hour after sampling by centrifuging for 30 min at 120 × g and 4°C, and a platelet count was obtained. Automated analysis of plasma indoles was performed in batch. Concentrations of 5-HT were

expressed as nmol/10^9 platelets; 5-HT was determined in a quality control sample with within-series and between-series coefficients of variation of 2.8% and 5.4%, respectively (Kema et al., 2001).

Statistical Analyses

The statistical analyses were performed using the SPSS software package (SPSS Inc., 1999) and the Analyse-It software for Microsoft Excel (Analyse-It Software Ltd., 2002). Possible diagnostic group differences in demographic variables and behavioral ratings were analyzed using analysis of variance (ANOVA) or X_2 test where applicable. Relationships between platelet 5-HT levels and demographic variables were evaluated with ANOVAs and Students t-tests. Given some nonnormal subgroup distributions, both ANOVA and the Kruskal-Wallis (KW) test were used to compare platelet 5-HT levels, platelet count, and mean platelet volume between diagnostic groups. Post hoc group comparisons were performed using the Tukey honestly significant difference test and the Mann-Whitney U test. To assess the effect of puberty in the absence of Tanner stage information, prepubertal (subjects 10 and younger) and postpubertal (14 and older) groups were formed on the basis of age.

Normality of the distribution of 5-HT levels in the diagnostic groups and subgroups was tested using the Anderson/Darling goodness-of-fit test (Anderson and Darling, 1952; Lewis, 1961). We used the MIXMOD (MIXture MODeling) program to assess whether the sample consisted of one or a mixture of two or more gaussian distributions (Biernacki et al., 2001). This mixture-model approach to commingling calculates approximate Bayes factors or the posterior odds for one model against the other, assuming neither is favored a priori. The smaller the log Bayes factor, termed the bayesian information criterion, the stronger the evidence for the model (Fraley and Raferty, 1998; Schwartz, 1978).

Relations between the behavioral measures and platelet 5-HT levels were evaluated using Spearman rank correlations. Nonparametric tests were used, since not all behavioral measures showed normal distributions. When significant correlations were found, further analyses were performed using linear and logistic regression analysis, with the platelet 5-HT level as the dependent variable and the behavioral measures as independent variables.

The α-value was set at $p = .05$ for all analyses. To avoid errors of the second type in the exploratory analyses, no correction for multiple comparisons was made.

Effects of age, gender, season, and medication were tested due to prior reports indicating these variables might have some effect on platelet 5-HT levels.

Results

5-HT Level and Age, Gender, Level of Functioning, Use of Medication, and Season

The variables age, gender, level of functioning, use of medication, and season of blood draw were included in an univariate analysis of variance (ANOVA) to test their effect on platelet 5-HT levels in all subjects. The effects of age, gender, level of functioning, use of medication, and season were not significant ($F_{1,194}$ = 0.03, p = .87; $F_{1,194}$ = 2.12, p = .15; $F_{3,194}$ = 0.35, p = .70; $F_{3,194}$ = 1.19, p = .31; and $F_{3,194}$ = 2.24, p = .090, respectively). No significant interaction effects were observed between any of the variables.

Table 2.2: Platelet 5-HT Levels, Platelet Count, and Mean Platelet Volume in PDD, MR, and Normal Control Groups

Subject Group	***n***	***5-HT[a,b] (nmol/10⁹ plts)***	***Platelet Count (10⁹/mL)***	***Mean Platelet Volume (fL)***
Autism	33	4.51 ± 1.61	0.428 ± 0.130	8.17 ± 0.95
Asperger	5	4.00 ± 1.37	0.463 ± 0.158	7.42 ± 0.14
PDD-NOS	43	4.90 ± 1.54	0.408 ± 0.118	8.27 ± 1.13
Combined PDD[c]	81	4.69 ± 1.56	0.429 ± 0.131	8.13 ± 0.97
MR	54	3.48 ± 1.33	0.400 ± 0.119	7.84 ± 1.09
Normal control	60	3.58 ± 1.08	0.423 ± 0.151	8.17 ± 1.01
Kruskal-Wallis		X^2_4 = 30.1	X^2_4 = 2.85	X^2_4 = 7.16
		p < .001	p = .58	p = .13
ANOVA		$F_{4,190}$ = 9.35	$F_{4,190}$ = 1.02	$F_{4,190}$ = 1.76
		p < .001	p = .40	p = .14

Note: 5-HT = serotonin; PDD-NOS = pervasive developmental disorder-not otherwise specified; MR = mental retardation; ANOVA = analysis of variance; HSD = honestly significant difference test; plts = platelets.
a. Post hoc M-W U: autism vs. MR and normal p<.005, p=.01, respectively. PDD-NOS vs. MR and normal, both p<.001.
b. Post hoc Tukey HSD: autism vs. MR and normal p=.005, p=.01, respectively. PDD-NOS vs. MR and normal, both p<.001.
c. ANOVA (5-HT), combined PDD vs. MR and normal, F2,194 = 17.2, p<.001, post hoc Tukey HSD, both p<.001. Combined PDD = autism, Asperger, and PDD-NOS.

5-HT Values in Diagnostic Groups

Group mean platelet 5-HT values are given in Table 2.2. An ANOVA confirmed a robust and statistically significant effect of diagnosis. Post hoc tests showed that platelet 5-HT levels were significantly elevated in the autistic group versus the MR and normal control groups and in the PDD-NOS group versus the MR and normal control groups. The group means seen for platelet 5-HT levels in the autism and PDD-NOS groups were 28.7% and 36.9% greater, respectively, than the mean

observed in the normal control group. When individuals with autism, Asperger's, or PDDNOS were combined in one group, an ANOVA also revealed a significant elevation of 5-HT in the combined PDD group. There were no significant differences in platelet 5-HT between the Asperger's, MR, and normal control groups (all three pairwise p values > .05). Platelet count and mean platelet volume did not differ significantly between any of the groups. Medication status did not influence platelet 5-HT levels in the autism group ($F_{3,32}$ = 0.55, p = .65) or the PDDNOS group ($F_{3,42}$ = 0.45, p = .72) or in the combined PDD group ($F_{3,80}$ = 0.40, p = .76).

5-HT Values and Pubertal Status

Given prior reports of pubertal effects on platelet 5-HT levels, the effect of puberty and the interaction effect of puberty and diagnosis were examined in detail in the combined PDD (autism, Asperger's, and PDDNOS) and combined control (normal and MR) groups. When pubertal status and diagnosis were included as predictor variables in an ANOVA of all subjects (post-, peri- and prepubertal, n = 195), the effect of diagnosis was significant ($F_{4,195}$ = 8.89, p < .001), while the effect of puberty and the interaction effect were nonsignificant ($F_{2,195}$ = 0.13, p = .88 and $F_{4,195}$ = 0.95, p = .50, respectively). Diagnostic group comparison in the prepubertal subgroup revealed a significant difference for platelet 5-HT levels between PDD (n = 32) and control (n = 38) subjects (4.81 ± 1.43 versus 3.58 ± 1.24 nmol/10^9 platelets, t_{69} = 4.0, p = .001). A similar significant increase in the PDD group (n = 39) compared to the control group (n = 40) was also observed in the postpubertal group (4.52 ± 1.54 versus 3.61 ± 1.12 nmol/10^9 platelets, t_{70} = 3.0, p = .004). No significant diagnostic group differences occurred in platelet count or mean platelet volume in either pubertal subgroup.

No significant effects of age on platelet 5-HT levels were observed in either the prepubertal ($F_{6,47}$ = 0.45, p = .84) or postpubertal ($F_{8,63}$ = 1.25, p = .29) groups; the interactions between age and diagnosis were also not significant in either group ($F_{12,47}$ = 0.92, p = .53 and $F_{23,65}$ = 1.49, p = .14, respectively).

Distribution of 5-HT Values

Distribution histograms for 5-HT values in normal control, MR, and combined PDD groups are shown in Figure 2.1. The distribution in the PDD group was clearly nonnormal and apparently bimodal on visual inspection. Anderson-Darling tests of

normality indicated that 5-HT values in the normal control group were normally distributed (A^2_{60} = 0.441, p = .29), but that the distributions in the MR and combined PDD groups were nonnormal (A^2_{54} = 1.55, p < 0.001 and A^2_{81} = 0.78, p = .046, respectively).

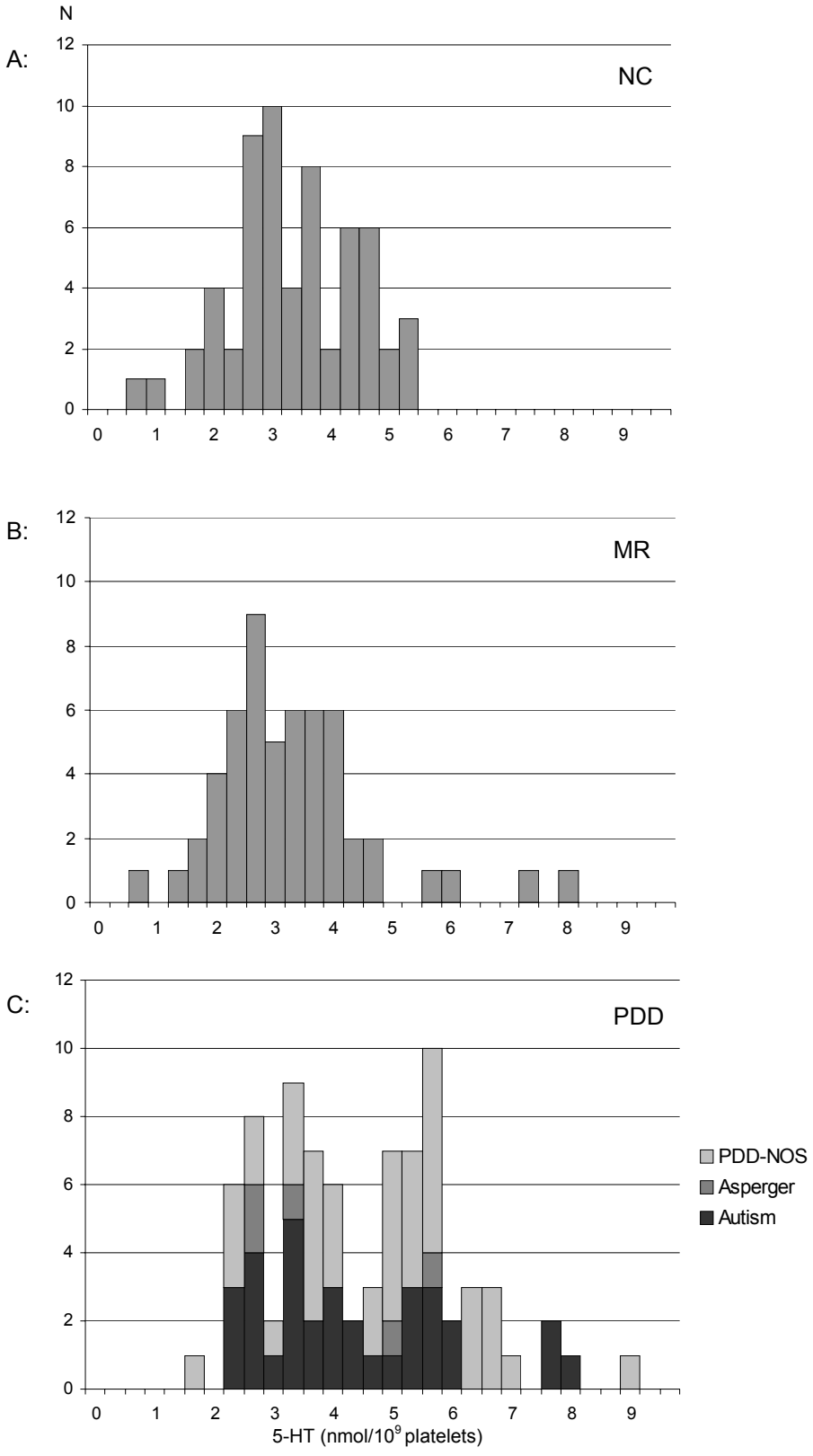

Figure 2.1: Histograms showing the distribution of platelet serotonin (5-HT) levels in the normal control group, n=60 (A), the mentally retarded (MR) group, n=54 (B) and the combined pervasive developmental disorder (PDD) group (autism, Asperger's, and pervasive developmental disorder-not otherwise specified [PDD-NOS]), n=81 (C).

The distributions of platelet 5-HT values in the autism and PDD-NOS subgroups also appeared bimodal by visual inspection, but tests of nonnormality were hindered by reduced sample size (A^2_{33} = 0.73, p = .057 and A^2_{43} = 0.29, p = .60, respectively). The lower mode in the combined PDD group appeared similar in mode, median, and variance to the group distributions observed for the normal control and MR groups. Specifically, if the combined PDD group was dichotomized into normo- and hyperserotonemic subgroups using a valley cutoff value of 4.55 nmol/10^9 platelets, the lower and upper modes had means and SDs of 3.39 ± 0.67 and 6.02 ± 0.98 nmol/10^9 platelets, respectively (t_{79} = 14.1, p < .001). Although a greater proportion of the individuals in the PDD-NOS group were defined as hyperserotonemic using this cutoff value (25/43 [58%] versus 12/33 [36%] in the autistic group), the difference obtained only trend level significance (X^2_1 = 3.54, p = .060). While the mean platelet 5-HT concentration in the small Asperger's group was not elevated, the five observed values (2.7, 2.9, 3.5, 5.2, 5.7 nmol/10^9 platelets) were not inconsistent with the bimodal distribution seen for the combined PDD group.

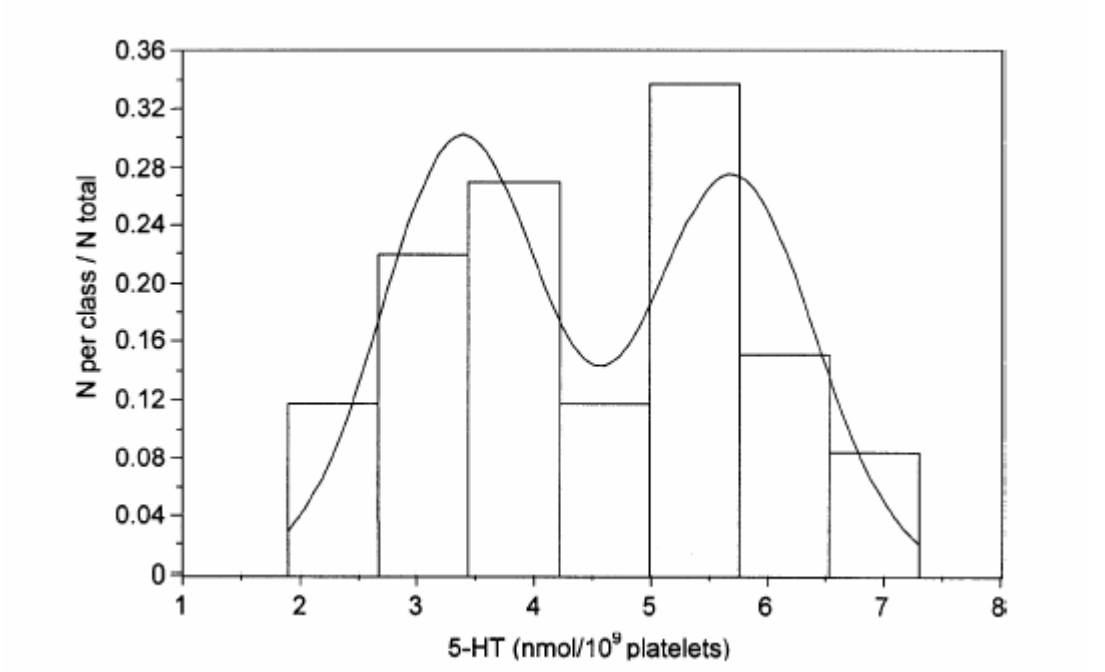

Figure 2.2: The two-component distribution of platelet serotonin (5-HT) (nmol/10^9 platelets) values in the pervasive developmental disorder (PDD) group as determined by commingling analyses with MIXMOD. The calculated MIXMOD means and SDs of components 1 and 2 (3.39 ± 0.67 and 5.80 ± 0.63, respectively) were similar to those determined by dichotomizing at the valley point (3.39 ± 0.67 and 6.02 ± 0.98).

Although platelet 5-HT values were nonnormally distributed in the MR group according to the Anderson- Darling test, this nonnormality was due to four individuals with levels more than 3 SD above the mean. These four apparent outlier individuals were carefully examined but did not differ from the group of MR individuals on any of the demographic, diagnostic, or behavioral measures obtained. When these subjects were excluded, the Anderson-Darling statistic was not significant (A^2_{50} = 0.180, p = .92). In contrast, when the four PDD individuals with 5-HT levels more than 3 SD above the mean were excluded, the Anderson- Darling statistic remained significant (A^2_{77} = 1.03, p = .010). Mixture-modeling analysis in the combined PDD group (n = 77) revealed that a mixture of two normal distributions fit the data better than did a single distribution or a mixture of three distributions. Bayesian information criteria for the one-, two-, and threecomponent distribution models were 131.6, 130.8, and 138.4, respectively. The MIXMOD graphical solution for the preferred two-component model is plotted in Figure 2.2. The MIXMOD calculated means and SDs for the upper and lower components (see figure legend) were similar to those determined above by dichotomization at the valley point.

Relation Between ADI-R-Rated Autistic Behavioral Expression and 5-HT Level

As the first step in an extensive exploratory analysis of possible behavioral associations with platelet 5-HT levels, we examined possible correlations between the 5-HT level, and the ADI-R total score, the social interaction, communication, and stereotyped behavior ADI-R domain scores, and ADI-R subdomain scores in the combined PDD group (autism, Asperger's, and PDD-NOS, n = 81). No significant Spearman correlations were found between any of the ADI-R scores examined and platelet 5-HT levels: r values observed for the total, social interaction, communication, and stereotyped behavior scores and platelet 5-HT level were -0.008, 0.083, -0.064, and 0.044, respectively. A linear regression analysis of the same four behavioral scores also showed no significant association with platelet 5-HT level, with standardized β values ranging from .39 to -.035.

Language Development Items and 5-HT Level

The relationship between several speech and language items from the ADI and platelet 5-HT levels was assessed by comparing 5-HT levels in dichotomized subgroups. There were no significant differences in mean platelet 5-HT levels

between verbal (mean = 4.55 ± 1.50 nmol/10^9 platelets, n = 66) and nonverbal (mean = 5.29 ± 1.72 nmol/10^9 platelets, n = 15; t_{79} = 1.69, p = .096) PDD subjects, and between subjects who spoke their first single word after (4.69 ± 1.47 nmol/10^9 platelets, n = 45) and before (mean = 4.69 ± 1.69 nmol/10^9 platelets, n = 36; t_{79} = 0.01, p = 1.0) the age of 24 months. Mean levels also did not differ in subjects speaking their first sentence after 33 months of age (mean = 4.71 ± 1.61 nmol/10^9 platelets, n = 60) versus those with their first sentence before 33 months of age (mean = 4.61 ± 1.43 nmol/10^9 platelets, n = 21; t_{79} = 0.25, p = .80).

Relations Between Other Behavioral Expression Measures and 5-HT Level

The potential relationships between 5-HT and the total scores and subscores on the CBCL, CY-BOCS, ABC, and CSBQ were examined in the combined PDD group (autism, Asperger's, and PDD-NOS, n = 81) and in the combined developmentally delayed group (PDD and MR, n = 135). No significant correlations were seen in the PDD group; however, in the combined (PDD + MR) group, the ABC total score (r = 0.238, p = 0.006), three of the ABC subscores (Sensory, Body/Object Use, and Sensory, r values 0.253, 0.186, and 0.243, p values .003–.030), two of the CBCL subscales (Thought and Attention Problems, r values 0.172 and 0.173, p = .046), and the CSBQ item Stereotypical/Sensory Problems (r = 0.184, p = 0.033) did tend to correlate with 5-HT level. When these seven scores showing some trend to correlate with 5-HT level were entered into a forward linear regression analysis, only the ABC Sensory score was significantly associated (platelet 5-HT; standardized β = .216, t_{135} = 2.53, p = 0.013). To control for the influence of diagnosis, group status (PDD or MR control) was entered into a linear regression model with ABC Sensory score. This analysis revealed that when controlling for diagnostic group, there was clearly no significant separate effect of severity of PDD-related behaviors as measured with the ABC (group and ABC β weights, .357 and .016, p values <.0001 and .87, respectively).

Behavioral and Demographic Variables in Biochemically Defined Subgroups

To explore any influence of the major behavioral and demographic variables on group membership for hyper- versus normoserotonemia in the PDD group, a logistic regression analysis was performed. In the PDD group (n = 81), none of the variables significantly predicted membership in the hyper- and normoserotonemic groups.

Discussion

Several findings have emerged from this study of large, ethnically homogenous, groups of subjects. As expected, the finding of hyperserotonemia in autism was replicated. The 29% group mean elevation seen in the autistic subjects compared to normal controls was consistent with the study of McBride et al. (1998) and was in the lower end of the range of reported elevations (Anderson et al., 1990; Cook and Leventhal, 1996). Importantly, a significant and substantial elevation of 37% was also seen in the PDD-NOS group. This first report of group mean platelet 5-HT levels in PDD-NOS strongly indicates that platelet hyperserotonemia also occurs in this other category of patients on the autism spectrum. No mean elevation was seen for the Asperger's group, but only five patients were studied. A previous report of platelet 5-HT levels in Asperger's also found no elevation in a small group (n = 5) of subjects (Anderson et al., 1996).

The finding of similar group means and variances for the MR group and the normal control group confirms data reported by McBride et al. (1998) and more firmly establishes the absence of hyperserotonemia in nonautistic MR. The absence of any relationship between intelligence or level of functioning and platelet 5-HT level in either the PDD or MR groups supports the group mean data. Although there are several reports of hyperserotonemia in groups of MR subjects (Schain and Freedman, 1961; Partington et al., 1973; Hanley et al., 1977), the groups studied were small and the subjects were not as well characterized as in the present study. We did note that there were four MR subjects with 5-HT values more than 3 SD above the control mean (> 7.5 nmol/10^9 platelets); however, no distinguishing characteristics could be discerned for these four subjects, and they constituted only 7% of the MR group.

A major finding of the present study was the apparent bimodal distribution of platelet 5-HT levels in the PDD group. While previous studies have consistently found group mean elevations of 5-HT in autism, it has not been possible to draw any conclusions regarding the modality of the measure. Previously, it was not at all clear whether the entire group distribution had shifted upward or whether a hyperserotonemic subgroup existed. The large, homogenous groups that we studied were probably crucial to our being able to detect bimodality. It now appears that approximately half of the PDD subjects closely overlap the normal distribution in

platelet 5-HT level, while a second mode of hyperserotonemic subjects exists. Thus, a biochemically defined subgroup of patients can now be identified and an initial empirically derived cutoff (4.55 nmol/10^9 platelets) now determined for the hyperserotonemia of PDD. Surprisingly, the prevalence of hyperserotonemia was not higher in autism (36%) compared to PDD-NOS (58%).

A range of demographic and clinical variables, including age, pubertal status, gender, use of medication, and season of blood draw, did not appear to influence platelet 5-HT levels. The lack of an age effect is consistent with most previous studies (Ritvo et al., 1971; Anderson et al., 1987; Abramson et al., 1989). The absence of an effect of puberty is at some variance with the report of McBride et al. (1998) that indicated a lowering of platelet 5-HT levels after puberty. Most prior studies had not observed a puberty effect (Ritvo et al., 1971; Anderson et al., 1987; Abramson et al., 1989), and the effect reported by McBride et al. was much less marked in their Caucasian subjects. Two previous studies also suggested that the use of medication (other than reuptake inhibitors) did not substantially influence platelet 5-HT levels (Kuperman et al., 1987; Minderaa et al., 1989). Previously reported seasonal differences have been variable and generally of relatively minor effect (Badcock et al., 1987; McBride et al., 1998).

The absence of any significant behavioral correlates with platelet 5-HT levels or hyperserotonemic status was remarkable given the extensiveness of the behavioral assessments. The language and communication domain was examined in particular depth in this context, given prior reports of possible associations with platelet 5-HT levels (Cook et al., 1990; Cuccaro et al., 1993) and our own limited data indicating that the group mean value of 5-HT was not increased in Asperger's subjects.

In summary, we have replicated the platelet hyperserotonemia of autism in Dutch subjects, found that platelet 5-HT levels are also increased in PDD-NOS, identified bimodality of the measure in autistic and PDD-NOS subjects, and established an absence of a group mean elevation in MR. The absence of detectable behavioral correlates was somewhat surprising, but it will serve to focus our interest on understanding the genetic and biochemical mechanisms underlying the altered platelet 5-HT levels seen in a subgroup of individuals with PDD. The recent report of the extremely high heritability of platelet 5-HT is encouraging in this regard (Ober et al., 2001).

Limitations

Although relatively large, ethnically homogeneous subject groups were studied, sample size still limited the commingling or admixture analysis even in the combined PDD group. Indications of bimodality in the separate autism and PDD-NOS groups are especially tentative. More generally, it is not certain that the findings regarding the distribution of platelet 5-HT levels and the absence of behavioral correlates can be generalized to other populations.

Clinical Implications

If replicated, the defining of normo- and hyperserotonemic subgroups in PDD may provide a route to illuminating the neurobiology and genetics of autism. The biochemically defined groups may also differ in their response to pharmacological and behavioral intervention.

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Chapter 3

Serotonin Transporter Intron 2 Polymorphism Associated With Rigid-Compulsive Behaviors in Dutch Individuals With Pervasive Developmental Disorder

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Abstract

Two putatively functional polymorphisms of the serotonin transporter gene (HTT, SLC6A4) were examined for associations with risk for pervasive developmental disorders (PDDs) and specific autism phenotypes. Dutch patients diagnosed with PDD (n = 125, age range 5–20 years, DSM-IV-TR based criteria, ADI-R and ADOS behavioral assessments) and their parents (n = 230) were genotyped for promoter ins/del (5-HTTLPR) and intron 2 variable number of tandem repeats (VNTR) alleles. Using the transmission disequilibrium test (TDT), no disorder-specific preferential transmission of promoter (long and short) or intron 2 (10- and 12-repeat) alleles was observed. However, multivariate analysis of continuous autism-related behavioral measures revealed that subjects with intron 2 12/12 genotype were significantly more impaired in the rigid-compulsive domain (p = 0.008). Quantitative TDT (QTDT) analysis also showed significant association of the intron 2 VNTR 12-repeat allele with rigidcompulsive behavior (p = 0.015). These results suggest that intron 2 VNTR alleles or nearby polymorphisms in linkage disequilibrium may play a role in specific aspects of the behavioral phenotype of autism.

Introduction

The serotonin transporter gene (HTT, SLC6A4) is of special interest in autism given the platelet hyperserotonemia of autism (Mulder et al., 2004), the treatment effects of serotonergic agents (McDougle et al., 2000), the role of serotonin (5-hydroxytryptamine, 5-HT) in neurodevelopment (Whitaker-Azmitia, 2001) and prior reports of genetic associations with disorder risk and specific autism phenotypes (Lauritsen and Ewald, 2001; Anderson, 2002; Kim et al., 2002). Over 20 HTT polymorphisms have been identified and two are of particular interest in neuropsychiatry given their apparent effects on serotonin transporter expression and functioning. One of the two functional variants is a 44-bp insertion/deletion polymorphism in the promoter region of the gene (5-HTTLPR) with long and short alleles, L and S; the other a variable number of tandem repeats (VNTR) polymorphism in the second intron with 9, 10, or 12 copies of the 17-bp repeat sequence. Most (Lesch et al., 1996; Hanna et al., 1998; Greenberg et al., 1999; Nobile et al., 1999; Anderson et al., 2002), but not all (Kaiser et al., 2002), functional studies of the 5-HTTLPR have found allelic effects on 5-HT uptake. In a transfection experiment, the intron 2 VNTR has been reported to affect transporter expression in the mouse hindbrain during embryonic development (MacKenzie and Quinn, 1999).

Possible linkage and association of HTT and its variants with autism have been examined by genome scanning, familybased, and case-control studies. Although initial genome scans in autism did not find significant linkage at 17q11-12, a chromosomal region that includes the SLC6A4 locus (IMGSAC, 1998; Barrett et al., 1999; Risch et al., 1999), the three most recent reports have all reported significant linkage at 17q11-12 (IMGSAC, 2001; McCauley et al., 2004; Yonan et al., 2003). Studies examining transmission of the promoter alleles in autism have produced inconsistent findings, with preferential L allele (Klauck et al., 1997; Yirmiya et al., 2001), preferential S allele (Cook et al., 1997; Kim et al., 2002; McCauley et al., 2004; Conroy et al., 2004) or no preferential transmission (Maestrini et al., 1999; Persico et al., 2000; Tordjman et al., 2001; Persico et al., 2002; Betancur et al., 2002) having been reported. Several of the studies also explored the transmission of intron 2 VNTR alleles, with most reporting an absence of preferential transmission (Cook et al., 1997; Klauck et al., 1997; Maestrini et al., 1999; Betancur et al., 2002). However, Kim et al. (2002) did observe preferential transmission of the 12-repeat allele. In studies examining combinatorial effects, two groups have found preferential

transmission of an S-12 haplotype consisting of the 5-HTTLPR short allele and the intron 2 VNTR 12- repeat allele (Cook et al., 1997; Kim et al., 2002) and another has reported preferential transmission of a three variant haplotype that included the S-allele and the intron 2 VNTR 12- repeat allele (Conroy et al., 2004). Preferential transmission of a L-12 haplotype has also been reported (Klauck et al., 1997).

Much less attention has been paid to possible linkage or association with specific components or domains of autism related behavior. Tordjman et al. (2001) used Autism Diagnostic Interview-Revised (ADI-R; Lord et al., 1994) domain scores to construct severely or mild/moderately handicapped subgroups and found preferential transmission of the L-allele in the mild/moderately social and communication handicapped group. In a more recent study of multiplex families, McCauley et al. (2004) reported their highest genome-wide LOD score (3.62 at 17q11.2) in a subset of families with high ADI-Rderived rigid-compulsive factor scores.

In this study, we aimed to re-examine possible associations between 5-HTTLPR and intron 2 VNTR alleles and disorder risk, and between the alleles and domain severity. The associations were examined in a large, relatively homogeneous group of well-characterized Northern Dutch subjects.

Method

Subjects and Assessment

Children and young adults with pervasive developmental disorders (PDD) and their parents were recruited through an epidemiological survey carried out in the north of the Netherlands, and through an Autism Outpatient Clinic affiliated with the Child and Adolescent Psychiatry Center of Groningen. The age range for subjects included in the study was 5-20 years, corresponding to the school-age range used as inclusion criterion in the epidemiological survey. Subjects were excluded from the study if they had a known genetic condition or severe peri/prenatal problems. All subjects were of Northern Dutch descent.

Subjects were assessed using the Autism Diagnostic Interview-Revised (ADI-R; Lord et al., 1994) and the Autism Diagnostic Observation Schedule-G (ADOS-G; Lord et al., 2000) administered by trained examiners. Subjects were diagnosed and their intellectual functioning evaluated as previously described (Mulder et al., 2004). Behavioral expression in the social-communication and stereotyped domains was

assessed using factor scores based on items from the ADI-R as per Tadevosyan-Leyfer et al. (2003). As described, 6 factors were obtained of which the factors 'social intent' and 'rigid-compulsive' most closely represented these two domains (Tadevosyan-Leyfer et al., 2003). The 'spoken language' factor was also included in our analyses, since this factor contains items measuring communicative aspects of autism.

DNA was collected by means of mouth-swaps from 125 patients and their parents and/or siblings. In five cases where attributed parentage was inconsistent with child and parent genotypes, typings were repeated and the families subsequently excluded. From the remaining 120 families, 110 were trios of 2 parents and one patient, 7 trios consisted of a mother, patient and an unaffected sibling and 3 were duo's of a mother and a patient. Group characteristics for the 120 patients are presented in Table 3.1.

Table 3.1. Clinical Characteristics of the Sample (mean ± SD)

	Autism	***Asperger***	***PDD-NOS***	***total group***	***Statistic$_{df}$; p***
N	51	4	65	120	
Age, yrs	11.0 ± 4.0	11.6 ± 1.7	11.7 ± 3.7	11.4 ± 3.8	$F_{2,117}$=0.52; .60
IQ	44 ± 25	111 ± 13	52 ± 29	52 ± 29	$F_{2,117}$=11.8; <.001[a]
Gender, n, M/F	44/7	3/1	48/17	95/25	
Gender, % male	86.3%	75%	73.8%	79.2%	X^2_2=2.72; .26
ADI-R Social Intent	28.8 ± 9.0	19.5 ± 6.8	21.1 ± 9.5	24.3 ± 9.9	$F_{2,117}$=10.4 ; <.001[b]
ADI-R Spoken Language	18.7 ± 12.0	6.5 ± 2.7	10.8 ± 9.9	14.0 ± 11.4	$F_{2,117}$ =8.89; <.001[c]
ADI-R Compulsions	6.4 ± 4.2	5.3 ± 4.0	5.1 ± 4.1	5.7 ± 4.1	$F_{2,117}$=1.42; .25

NOTE: a. Posthoc Tukey's HSD: Autism & PDD-NOS vs Asperger, p<.001. b. Posthoc Tukey's HSD: Autism vs PDD-NOS, p<.001. c. Posthoc Tukey's HSD: Autism vs PDD-NOS, p<.001.

DNA Analyses

DNA was extracted from cheekcells obtained by mouth-swap using the Epicentre MasterAmp™ DNA Extraction Solution Kit (BiozymTC, Landgraaf, the Netherlands). The intron 2 VNTR and the promoter variant were analyzed using the method of Cook et al. (1997) with slight modification. Primers were synthesized on an Applied Biosystems 380B DNA synthesizer at the General Clinical Laboratory of the University Medical Center Groningen.

Statistical Analyses

Possible diagnostic group differences in demographic variables and behavioral ratings were analyzed using analysis of variance (ANOVA) or X^2 test where applicable. The X^2 test was performed to test for deviations from the Hardy–Weinberg equilibrium. The transmission disequilibrium test (TDT) for family-based association analysis (Spielman et al., 1993) was used to test association of any allele with pervasive developmental disorder (PDD), autism or PDD-NOS. Possible association of 5-HTTLPR–intron 2 VNTR haplotypes with risk was examined by TDT analysis, cell size considerations precluded such analyses in behaviorally-defined subgroups. Haplotypes were constructed by hand. The ADI-R-derived domain scores, 'social intent,' 'spoken language,' and 'rigid-compulsive' behaviors (Tadevosyan-Leyfer et al., 2003), observed across genotypes were normally distributed and were compared using one-way ANOVA. When an ANOVA was significant, post-hoc Tukey HSD tests were performed to test the differences between the genotype groups. When the genotype comparisons revealed significant or trends to significant differences, association patterns of the ADI-R-derived domains with the HTT alleles were further evaluated with a family-based quantitative transmission disequilibrium test (QTDT, Abecasis et al., 2000a and 2000b).

In order to compare directly our results with prior reports, we also performed subgroup TDT analyses in the social/communicative and the rigid/compulsive domains using the same analytic approaches reported by Tordjman et al. (2001) and McCauley et al. (2003).

The α-value was set at $p = 0.05$ for all analyses and the p values presented are uncorrected. Bonferroni correction factors of 2 (number of polymorphisms tested) and 3 (number of domains) could be applied to P values obtained for the risk analyses and the behavioral domain analyses, respectively.

Results

Risk Association Analyses

The observed genotype distribution did not deviate significantly from that expected according to the Hardy-Weinberg equilibrium in the parents and siblings for both the 5-HTTLPR and intron 2 VNTR polymorphisms. However, there tended to be a greater number of S/L probands than predicted by H-W considerations (S/S, S/L, and L/L: 17, 72, and 30 observed versus 24, 59, and 37 predicted; $X^2_2 = 6.013$, p =

0.05). The family-based TDT did not indicate preferential transmission of 5-HTTLPR or intron 2 VNTR alleles in the combined PDD group. In total, 54 S-alleles were transmitted versus 51 L-alleles (TDT $X^2_1 = 0.086$, $p = 0.77$). For the intron 2 VNTR, 2 of 7 9-repeat alleles (TDT $X^2_1 = 1.286$, $p = 0.26$), 67 of 125 10-repeat alleles (TDT $X^2_1 = 0.648$, $p = .42$), and 58 of 122 12-repeat alleles (TDT $X^2_1 = 0.295$, $p = 0.59$) were transmitted. Preferential transmission was also not observed when the autism and PDD-NOS groups were examined separately (data not given).

The transmission of 5-HTTLPR-intron 2 VNTR haplotypes, S/10, S/12, L/10, and L/12 was examined. TDT analysis indicated that S/10 (16 out of 32) and S/12 (29 out of 58) haplotypes were not preferentially transmitted; however, trends to increased transmission of the L/10 (36 out of 57, TDT $X^2_1 = 3.947$, $p = .047$) and reduced transmission of L/12 were observed (20 out of 52, TDT $X^2_1 = 2.769$, $p = 0.096$).

Behavioral Domain Association Analyses

Genotype comparisons. Promoter alleles: groups defined by HTT promoter genotype (S/S, L/S, and L/L: n=19, 72, 30, respectively) did not differ significantly in terms of mean ADI-R factor scores for 'social intent' ($F_{2,118} = 1.01$, $p = 0.37$) and 'spoken language' ($F_{2,118} = 0.129$, $p = 0.88$). The 'rigid-compulsive' factor scores showed a trend towards a genotype effect ($F_{2,118} = 2.13$, $p = 0.12$), with the highest mean (± SD) scores being observed in the S/S subgroup (S/S, L/S, and L/L scores 7.35 ± 3.53, 5.13 ± 3.82, 5.73 ± 4.71, respectively).

Intron 2 VNTR alleles: neither the ADI-R factor 'social intent' ($F_{2,117} = 0.637$, $p = 0.53$) nor 'spoken language' ($F_{2,117} = 1.481$, $p = 0.23$) factor scores differed across intron 2 VNTR genotypes. However, as seen in Figure 3.1, the 'rigid-compulsive' factor scores were significantly dependent upon genotype, with the highest severity scores observed for the 12/12 genotype, intermediate severity scores seen for the 10/12 genotype, and lowest severity scores seen in the 10/10 group ($F_{2,117} = 5.068$, $p = 0.008$, post-hoc Tukey's HSD: 10/10 versus 12/12, $p = 0.022$, 10/12 versus 12/12, $p = 0.003$). Genotypes containing the 9-repeat allele were not included in these analyses, since only two subjects carried this allele (one 9/10 genotype and one 9/12 genotype).

Family-based QTDT analyses. Possible association of the 'rigid-compulsive' factor with 5-HTTLPR or intron 2 VNTR alleles was further evaluated by performing

QTDT with each polymorphism. Results of these two QTDT's revealed no significant association of either of the promoter alleles (F_{118} = 0.00, p = 1.0), but a significant association of the 12-repeat allele of the intron 2 polymorphism with the 'rigid-compulsive' factor was found (F_{119} = 6.16, p = 0.015).

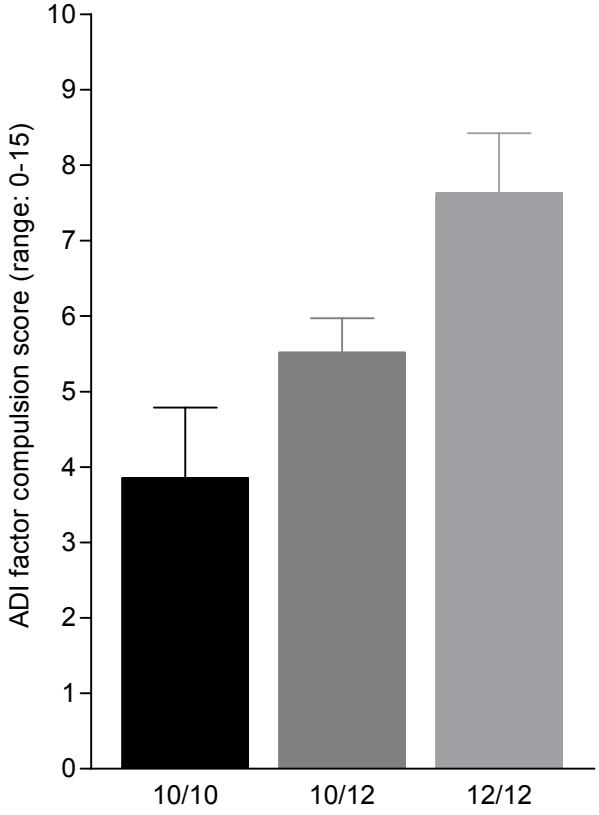

Figure 3.1: Autism Diagnostic Interview-Revised (ADI-R) rigid-compulsive factor score (mean and SE) per HTT intron 2 variable number of tandem repeats (VNTR) genotype (10/10 (n=20), 10/12 (n=74), 12/12 (n=24)). The rigid-compulsive score ranges from 0 to 15. Differences between genotype groups were significant ($F_{2,117}$=5.068, P=0.008, post-hoc Tukey's HSD: 10.10 vs. 12/12, P=0.022, 10/12 vs. 12/12, P=0.003).

Subset TDT Analyses. Following the method of Tordjman et al. (2001), the transmission of alleles was examined in subsets defined by subjects' combined social/communication severity score. TDT in the mild/moderately impaired group showed that neither 5-HTTLPR allele was preferentially transmitted (L-allele transmitted: 35, not-transmitted: 40, TDT X^2_1 = 0.333, p = .56). TDT analysis was not performed in the severely impaired group, as the group only contained 10 subjects. Also when domain scores were compared across genotype groups using the same method as Tordjman et al (2001) no significant differences were found (average soc-comm domain scores were 1.59, 1.59, 1.47 in the S/S, L/S and L/L genotype groups, respectively; Kruskal-Wallis X^2 = 0.515, p = .77).

Analyses of possible preferential transmission of 5-HTTLPR and intron 2 VNTR alleles was also performed in subsets defined by a median-split of probands based on 'rigid-compulsive' factor scores. The 'high' and 'low rigid-compulsive' subsets so

formed had mean (± SD) factor scores of 2.2 ± 1.7 and 9.0 ± 2.8, respectively. No preferential transmission was observed for 5-HTTLPR alleles; and also the 12-repeat intron 2 allele was found not to be preferentially transmitted in the 'high compulsions' subset (L-alleles–transmitted: 25, not transmitted: 24, TDT X^2_1 = 0.020, p = .89 / 12 repeat alleles–transmitted: 29, not transmitted: 27, TDT X^2_1 = 0.071, p = .79).

Discussion

This study revealed no significant associations between PDD disorder risk and either 5-HTTLPR or intron 2 VNTR alleles, and this was true for the autism and PDD-NOS subgroups as well. These findings are not inconsistent with the accumulated prior studies. Of the 11 family-based studies of the 5-HTTLPR, 5 showed no association, 2 showed an association to the L-allele, and 4 to the S-allele of the HTT promoter. Five of the six studies examining the intron 2 polymorphism reported no association with autism.

There was also no strong indication that 5-HTTLPR-intron 2 VNTR haplotypes were preferentially transmitted. While two prior studies (Kim et al., 2002; Conroy et al., 2004) have found nominal or trend level significant preferential transmission of the short/12 repeat haplotype, we observed no tendency (29 transmissions observed; 29 expected) for this haplotype to be transmitted to a greater extend that expected by chance.

The intron 2 VNTR 12-repeat allele was associated with severity of rigid-compulsive behaviors, both when using a genotype comparison approach and a QTDT analysis. On one hand, the results are consistent with McCauley et al.'s (2004) recent report of increased 17q11.2 linkage scores in the high rigid-compulsive subset. However, these investigators failed to find any preferential transmission of SLC6A4 markers in the rigid-compulsive subset. Their more recent examination of intron 2 VNTR allele transmission in the rigid-compulsive subset indicated that these alleles were also not preferentially transmitted (personal communication, J. S. Sutcliffe). In addition, in their sample, intron 2 VNTR genotypes were not significantly associated with rigid-compulsive factor scores and a QTDT analysis did not find significant association with rigid-compulsive factor scores.

Further examination of possible preferential transmission of intron 2 alleles and related haplotypes appears warranted. It is possible that the association we observed is due to specific patterns of linkage that may be present in the Northern Dutch

sample that was studied. Relative isolates might offer special advantages when attempting to identify the underlying genetic basis for the inconsistent, but intriguing, findings that have been reported for the HTT gene and autism. Although the genetic determinants of 'rigid-compulsive' behavior in autism are far from clear, the area appears to be critical and may provide general insight into the genetic control of obsessive-compulsive behaviors. Our and others' interest in the role of the HTT in compulsive and social behavior has recently increased due the reported association of a gain-of-function mutation in the HTT with obsessive-compulsive behavior and social problems (Ozaki et al., 2003). It can be noted that the apparent functional effect of the 12-repeat allele is to increase transporter expression in the developing mammalian brainstem (MacKenzie and Quinn, 1999), an effect that is at least superficially consistent with the physiology of the gain-of-function mutation. It can also be pointed out that both the gain-of-function mutation and the platelet hyperserotonemia of autism involve a presumed or apparent increase of the intracellular pool of serotonin relative to the extracellular compartment. This speculation concerning disposition or compartmentalization of serotonin is quite tenuous as there is no direct evidence of altered extracellular serotonin associated with the gain-of-function mutation or with autism, and the genetic and biochemical basis of the platelet hyperserotonemia of autism (and its relationship to central serotonin) is unclear.

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Chapter 4

Automated On-Line Solid-Phase Extraction Coupled with HPLC for Measurement of 5-Hydroxyindole-3-acetic Acid in Urine

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Abstract

Background: Quantification of 5-hydroxyindole-3-acetic acid (5-HIAA) in urine is useful in diagnosing and monitoring of patients with carcinoid tumors and in the study of serotonin (5-hydroxytryptamine) metabolism in various disorders. We describe an automated method that incorporates on-line solid-phase extraction (SPE) and HPLC to measure urinary 5-HIAA. Methods: Automated prepurification of urine was accomplished with HySphere-resin GP SPE cartridges containing strong hydrophobic polystyrene resin. The analyte (5-HIAA) and internal standard [5-hydroxyindole-3-carboxylic acid (5-HICA)] were eluted from the SPE cartridge, separated by reversed-phase HPLC, and detected fluorometrically with a total cycle time of 20 min. Urinary excretion of 5-HIAA was measured in a group of patients with known and suspected carcinoid tumors (n = 63) and in 20 patients with autism. Results: The internal standard (5-HICA) and 5-HIAA were recovered in high yields (87.2%–114%). Within- and between-series CVs for the measurement of 5-HIAA in urine ranged from 1.2% to 3.9% and 3.2% to 7.6%, respectively. For urine samples from patients with known or suspected carcinoid tumors, results obtained by the automated method were highly correlated (r = 0.988) with those from an established manual extraction method. For samples from autistic patients, urinary excretion of 5-HIAA was similar to that reported for healthy individuals. Conclusion: This SPE-HPLC method demonstrated lower imprecision and time per analysis than the manual solvent extraction method.

Introduction

The neurotransmitter/neurohormone serotonin [5-hydroxytryptamine, (5-HT)] is synthesized from the essential amino acid tryptophan in the enterochromaffin cells of the gut and in serotonergic neurons in the central nervous system (Tyce, 1985; Young and Teff, 1989). Peripheral serotonin is metabolized mainly in the lung and the liver through the action of monoamine oxidase-A. 5-Hydroxyindole-3-acetic acid (5-HIAA) is the predominant end-product of serotonin metabolism and is subsequently excreted in urine (Grahame-Smith, 1988).

Serotonin is clearly involved in carcinoid syndrome and is hypothesized to play a role in schizophrenia, depression, migraine, and autism (Kema et al, 2000; Naughton et al., 2000; Anderson, 2002). The most pronounced deviations in serotonin metabolism are found in patients with carcinoid tumors, which can secrete large amounts of serotonin (Kema et al., 1999). Quantification of urinary 5-HIAA is important in the diagnosis and follow-up of such patients. Diagnosis can be established by measuring urinary 5-HIAA or platelet serotonin. When serotonin production of the tumor exceeds platelet storage capacity, however, changes in production will not be reflected in platelet serotonin concentrations, whereas changes in the amount of 5-HIAA excreted in urine will still occur (Kema et al., 1992). Increased platelet serotonin has also been observed in autistic patients, with typical reported group mean increases of 25%–50% (Anderson, 2002). For the study of peripheral serotonin metabolism in autism, measurement of 5-HIAA in urine is useful to evaluate a possible role of altered production or catabolism in observed changes in platelet serotonin (Anderson, 2002).

Since the early 1950s, many analytical methods have been described to measure 5-HIAA in urine (Deacon, 1994). The first methods used colorimetric methodology, paper and thinlayer chromatography, or gas chromatography. Later, various HPLC analyses were reported that used colorimetric, fluorometric, or electrochemical detection (Deacon, 1994). Recently, liquid chromatographic–tandem mass spectrometric methods have been reported for urine and whole blood (Kroll et al., 2002; Danaceau et al. 2003).

We developed an automated method with on-line solid-phase extraction (SPE) and HPLC with fluorometric detection to measure urinary 5-HIAA and applied this method to the determination of urinary 5-HIAA excretion rates in individuals with known or suspected carcinoid tumors and in individuals with autism.

Materials and Methods

Reagents and stock solutions

HPLC-grade acetonitrile and methanol were obtained from Rathburn; 5-HIAA and disodium EDTA were from Sigma; tetrabutylammonium bromide was from Romil; dipotassium EDTA was from Brunschwig; 5-hydroxyindole-3-carboxylic acid (5-HICA) was from Janssen Chimica; and acetic acid, sodium metabisulfite ($Na_2S_2O_5$), ascorbic acid, phosphoric acid, potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were from Merck. All reagents were of at least analytical reagent grade. Reagent-grade water, obtained from a Barnstead system, was used throughout.

Stock solutions containing 0.34 mmol/L 5-HICA or 2.1 mmol/L 5-HIAA were prepared in water containing 20 mL/L glacial acetic acid. The 5-HICA stock solution was stored protected from light at -20 °C for a maximum of 1 year. The 5-HIAA stock solution was prepared on the day of analysis. Saturated stock solutions containing ascorbic acid (300 g/L), dipotassium EDTA (750 g/L), and $Na_2S_2O_5$ (750 g/L) were prepared in water and stored at room temperature for 3 weeks.

Samples and participants

Patient urine samples were collected into brown polypropylene bottles (Sarstedt) containing ~250 mg each of $Na_2S_2O_5$ and disodium EDTA. Urine samples were acidified to pH 4 with acetic acid and stored at -20 °C. Before analysis, aliquots of thawed urine samples (50 µL) were mixed with 300 µL of antioxidant solution (100 µL of ascorbic acid stock solution, 100 µL of dipotassium EDTA stock solution, and 100 µL of $Na_2S_2O_5$ stock solution) and 100 µL of internal standard stock solution (0.34 mmol/L 5-HICA). The mixture was then diluted with 1.2 mL of water, and 20 µL of each sample was injected into the SPE-HPLC system as described below. This injection volume was equivalent to 0.61 µL of urine.

For method-comparison studies, we used spot urine samples from 63 patients with suspected or known carcinoid tumors and 24-hr urine specimens from 20 individuals with autism (Mulder et al., 2004).

Analysis and quantification

Instrumentation. The Prospekt-2 (Spark Holland) on-line SPE unit consisted of 3 modules: an SPE controller unit (automated cartridge exchanger), a solvent delivery

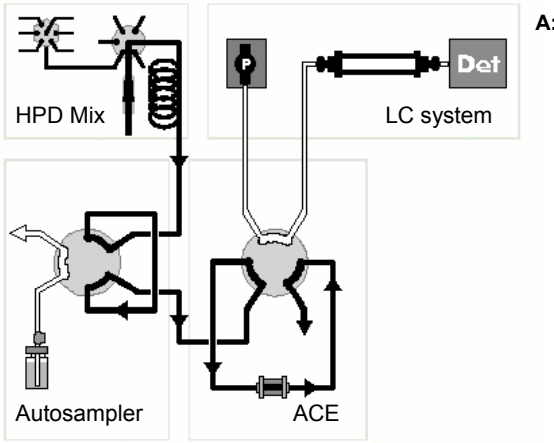
unit (high-pressure dispenser), and an autosampler (Triathlon). A SparkLink software package (Ver. 2.10) was required for control of the Prospekt-2 modules. The main task of the automated cartridge exchangers was exchange of disposable SPE cartridges. The high-pressure dispenser had a single-syringe configuration and delivered solvents for conditioning, sample application, and clean up of SPE cartridges. HySphere-resin GP cartridges (10 x 2 mm; Spark Holland) were used for cleaning up samples and concentrating analytes. Samples were injected with a Triathlon autosampler. The HPLC pump used was a Gynkotek Series P580A binary high-pressure gradient pump (Softron); detection was with a Waters 474 spectrofluorometer (excitation wavelength, 280 nm; emission wavelength, 360 nm). The temperature of the column was regulated with a Mistral column oven (Spark Holland).

For analytical HPLC, a 100 x 4.6 (i.d.) mm Brownlee Spheri-5 RP-18 column filled with 5-µm spherical particles (Inacom) was used. The analytical column was preceded by a 15 x 3.2 (i.d.) mm Aquapore RP18 ODS guard column filled with 7-µm spherical particles (Inacom). Detector output was integrated by ChromPerfect (Ver. 3.51) integration software (Justice Innovations).

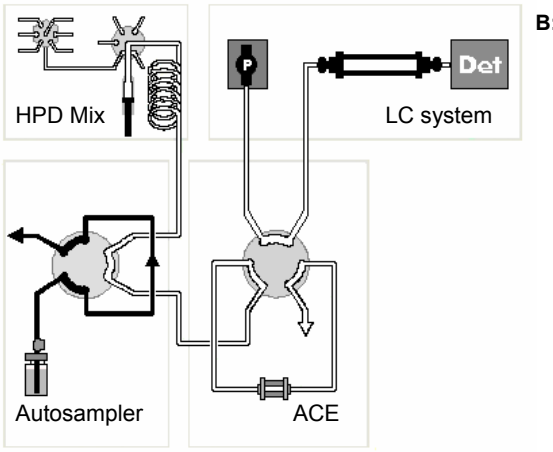
Eluents. The isocratic system consisted of eluent B (11 mmol/L dipotassium hydrogen phosphate and 3 mmol/L tetrabutylammonium bromide in water adjusted to pH 6.0 with phosphoric acid, added to 80 mL of acetonitrile to a total eluent volume of 1 L). Eluent A (22 mmol/L potassium dihydrogen phosphate in water adjusted to pH 2.0 with phosphoric acid, added to 10 mL of acetonitrile to a total eluent volume of 1 L) was delivered to the high-pressure dispenser.

Before use, eluent B was filtered through a 0.45 µm membrane filter (Schleicher and Schuell) and was degassed before entering the HPLC pump. The flow rate of eluent B was 1.0 mL/min, and chromatography was performed at 20 °C.

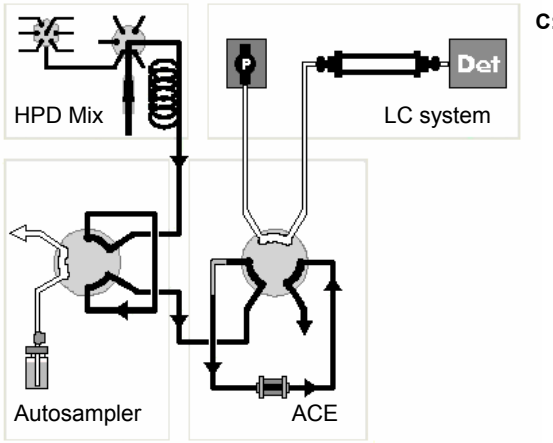
On-line SPE. On-line SPE was performed according to the schedule specified in Table 4.1 and as depicted schematically in Figure 4.1. The system was designed to proceed automatically through a series of programmable routines during which the SPE cartridge was loaded, purged for clean up, and eluted to the analytical column. During the loading routine, the SPE cartridge was conditioned sequentially with methanol, eluent A, 5 g/L dipotassium EDTA in water, and eluent A, all provided by the highpressure dispenser at flow rates of 2000 µL/min. The mixing coil between the



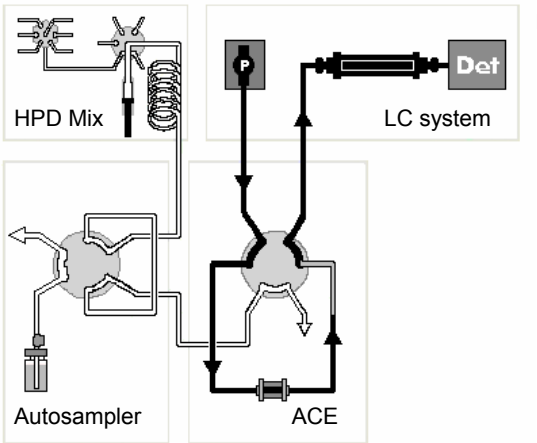
A: Solvation and equilibration



B: Loopfill Autosampler



C: Sample to cartridge



D: Elution sample to column

Figure 4.1: Schematic representation of the on-line SPE system coupled to HPLC with fluorescence detection. (A), solvation and equilibration step, showing the system during conditioning of the cartridge. (B), filling of the autosampler loop, showing the sample as it is processed by the autosampler. (C), flow of sample to cartridge, showing the system as the sample is on its way to the cartridge, where it will be washed. (D), elution of sample from the cartridge to the column, showing the system during the elution of the cartridge. After this, the system can be programmed to change the cartridge before the next sample is introduced. For additional details, see Table 1 and the Materials and Methods. HPD, high-pressure dispenser; LC, liquid chromatography; P, pump; Det, detector; ACE, automated cartridge exchanger.

Table 4.1: Summary of the sequence of events used for on-line SPE, as described in Materials and Methods.

Step	Action	Details		Comment
01	New cartridge	Left clamp		Put cartridge in clamp
02	Solvation	Methanol (2000 µL at 2000 µL/min)	SSM 1A	Activate cartridge with methanol
03	Equilibration	Eluent A (2000 µL at 2000 µL/min)	SSM 1B	Condition cartridge with eluent A
04	Equilibration	5 g/L dipotassium EDTA (2000 µL at 2000 µL/min)	SSM 1D	Condition cartridge with dipotassium EDTA
05	Equilibration	Eluent A (2000 µL at 2000 µL/min)	SSM 1B	Condition cartridge with eluent A
06	Start autosampler	Start loopfill		Fill loop autosampler with sample
07	Sample application	Eluent A (2000 µL at 2000 µL/min)	SSM 1B	Inject sample (load sample on cartridge using eluent A)
08	Wash cartridge	Eluent B (560 µL at 1000 µL/min)	SSM 1C	Wash cartridge with eluent B (removal ofinterferents)
09	Inputs	Input 1 high	Pump ready?	
10	Outputs	Auxiliaries 1 and 2	Start pump and start data	
11	Elution	1.5 min		Elution of sample from cartridge to analytical column
12	Move cartridge	Left to tray		Cartridge back to tray

high-pressure dispenser and the autosampler facilitated mixing of the eluents before they reached the cartridge.

After the mixed eluents reached the cartridge, the autosampler injected the sample, thereby loading the cartridge with the sample, using eluent A. The highpressure dispenser then continued to wash the loaded cartridge with eluent B, thus cleaning the cartridge; the analytes were then eluted from the cartridge to the analytical column with eluent B in 1.5 min. After elution from the cartridge, chromatographic separation on the analytical column occurred. During this separation, the next sample was loaded on the cartridge and washed. When the pump was ready, this sample was eluted to the analytical column. If necessary, cartridges could be changed automatically.

5-HIAA was quantified by calculation of the peak-area ratios in samples relative to those of the internal standard (5-HICA). Sample peak-area ratios were compared with the peak-area ratios obtained for the calibration solutions at 6 different concentrations, which were prepared by addition of known amounts of 0.21 mmol/L 5-HIAA calibrator solution (prepared fresh daily by diluting the stock solution 10-fold with water).

Urine creatinine was measured by a picric acid method on a Merck Mega Analyzer.

Analytical characteristics

In on-line SPE, detection limits depend on the extent of sample preconcentration on the SPE cartridge. For urine, we determined detection limits by injecting samples with decreasing concentrations of 5-HIAA. The detection limit was defined as the injected amount that produced a signal-to-noise ratio of 3. We estimated the percentage of carryover between sequential analyses performed on a single SPE cartridge by alternating injections of blanks and urine samples with high concentrations of 5-HIAA.

Quality control and validation of the automated urinary 5-hiaa method

Recoveries were estimated by the addition of 5-HIAA in 3 different concentrations (160, 350, and 520 µmol/L) to 1 urine specimen. Recoveries were measured in 6 replicates of these samples and calculated relative to the recovery of the internal standard; mean recoveries and ranges are reported. Within- and between-series precision was determined by use of 3 samples with 5-HIAA concentrations in the low, medium, and high ranges. Within-series precision was assessed in 6 replicates analyzed in a single series; between-series precision was assessed on 6 different days over a 1-month period. Experiments were performed using 1 SPE cartridge per series.

The identities of sample 5-HIAA peaks were verified by addition of calibrator (standard addition) and by observation of retention time changes of the sample peak under different chromatographic conditions. The automated urinary 5-HIAA method was also validated by comparison of its results with those obtained by our routinely used manual ether extraction method (Rosano et al., 1982).

Statistics

We compared methods by Deming regression analysis using the slope and intercept; we also calculated the correlation coefficient and the standard error of prediction ($S_{y|x}$). The Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) EP-6P protocol (Passey et al., 1986) was applied to test the linearity of the method.

Results

Chromatography

The chromatographic separation of 2 calibrators by automated on-line SPE and HPLC with fluorometric detection is shown in Figure 4.2A. Total cycle time was ~20 min (Table 4.1). Chromatograms of urine samples obtained from a healthy adult and from a patient with a carcinoid tumor are shown in panels B and C, respectively, of Figure 4.2 (note the different sensitivity scales). Complete separation of the analytes from interferents was achieved within 16 min. Comparison of the two chromatograms shows markedly increased 5-HIAA in the sample from the patient.

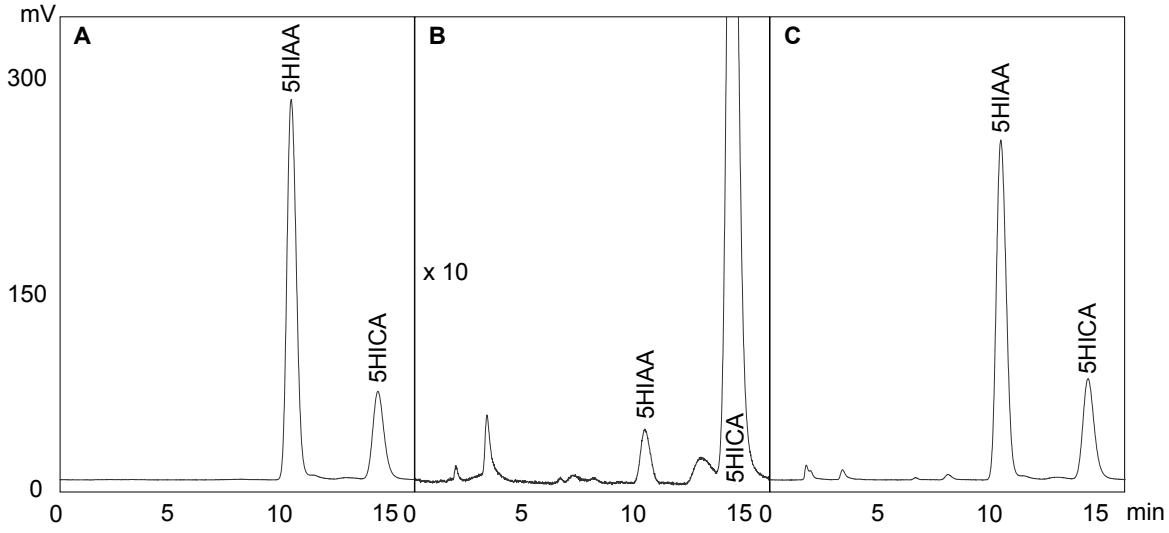


Figure 4.2: Chromatograms of urine from a healthy adult and a patient with carcinoid tumor, as obtained by on-line SPE coupled to HPLC with fluorescence detection. Excitation wavelength, 280 nm; emission wavelength, 360 nm. (A), separation of 5-HIAA and the internal standard 5-HICA (injected amounts, 0.34 nmol for both). (B), 5-HIAA in urine from a healthy adult (note the 10-fold increase in sensitivity). (C), 5-HIAA in urine obtained from a patient with carcinoid tumor. Chromatograms in B and C were obtained with 20 µL injections of diluted urine (equivalent to 0.61 µL of urine). Calculated 5-HIAA concentrations in the urine samples were 8.75 and 506 µmol/L for B and C, respectively.

Analytical characteristics

A signal-to-noise ratio of 3 was achieved at a 5-HIAA concentration of 0.8 µmol/L. Analysis of a split urine specimen with increasing amounts of added 5-HIAA calibrator demonstrated that the assay was linear to 5-HIAA concentrations of at least 2000 µmol/L. The percentage carryover between sequential analyses performed on a single SPE cartridge was <1%.

Quality control and validation of the automated urinary 5-hiaa method

The results of the recovery and precision experiments are presented in Table 4.2. The recovery data represent recoveries for 6 replicates of each of 3 samples

analyzed in 1 analysis time series with 1 SPE cartridge. Recoveries ranged from 87.2% to 114.0%. Within-series imprecision (as CVs) based on 6 measurements of 3 different urine samples were 3.9%, 1.6%, and 1.2% in samples with mean (SD) 5-HIAA concentrations of 2.3 (0.1), 25.0 (0.4), and 147.9 (1.7) µmol/L, respectively. The between-series imprecision (CVs) for analyses performed across 6 series were 7.6%, 3.7%, and 3.2% in samples with 5-HIAA concentrations of 2.5 (0.2), 26.3 (1.0), and 154.8 (3.2) µmol/L, respectively.

Table 4.2: Recovery and precision of the on-line SPE urinary 5-HIAA excretion method

Recovery[a]	
Added, µmol/L[b]	*% (range)*
160	94.0 (87.2-98.9)
350	99.9 (95.0-109.1)
540	102.1 (95.8-114.0)

Precision[c]			
Within-series		*Between-series*	
5-HIAA, µmol/L, (mean ± SD)	*CV, %*	*5-HIAA, µmol/L, (mean ± SD)*	*CV, %*
2.3 ± 0.1	3.9	2.5 ± 0.2	7.6
25.0 ± 0.4	1.6	26.3 ± 1.0	3.8
147.9 ± 1.7	1.2	154.8 ± 3.2	3.2

NOTE: a. Recovery data represent data from 6 measurements of each supplemented sample determined in single series using one SPE cartridge. b. The endogenous concentration of 5-HIAA in the urine sample used in the recovery experiments was (mean ± SD): 29.2 ± 1.2 µmol/L. c. Within-series precision data are derived from 6 measurements of three samples (low, mid and high concentration) made in a single series. Between-series data are from measurements of the same three samples made in 6 different series using one SPE cartridge per series.

As seen in Figure 4.3, 5-HIAA values determined in 63 clinical samples by the automated SPE method were highly correlated with results obtained by the manual ether extraction method (Rosano et al., 1982). Deming regression gave a slope of 0.9996 (range, 0.9811–1.0181) and a y-intercept of -3.50 µmol/L (95% confidence interval, -9.92 to 2.92 µmol/L). The Spearman correlation coefficient was 0.988 ($p < 0.0001$), and the SE of prediction ($S_{y|x}$) was 3.21 µmol/L. The lack of linear fit test according to the CLSI EP-6P protocol indicated no significant deviance from linearity ($F = 0.22$; $p = 0.80$). In the autism group, mean (SD) 24-h urinary 5-HIAA excretion [0.0165 (0.0086) mmol/24 h or 1.74 (0.79) mmol/mol creatinine] was similar to that

reported previously for control groups and autistic patients (Launay et al., 1987; Minderaa et al., 1987; Anderson et al., 1989; Deacon, 1994).

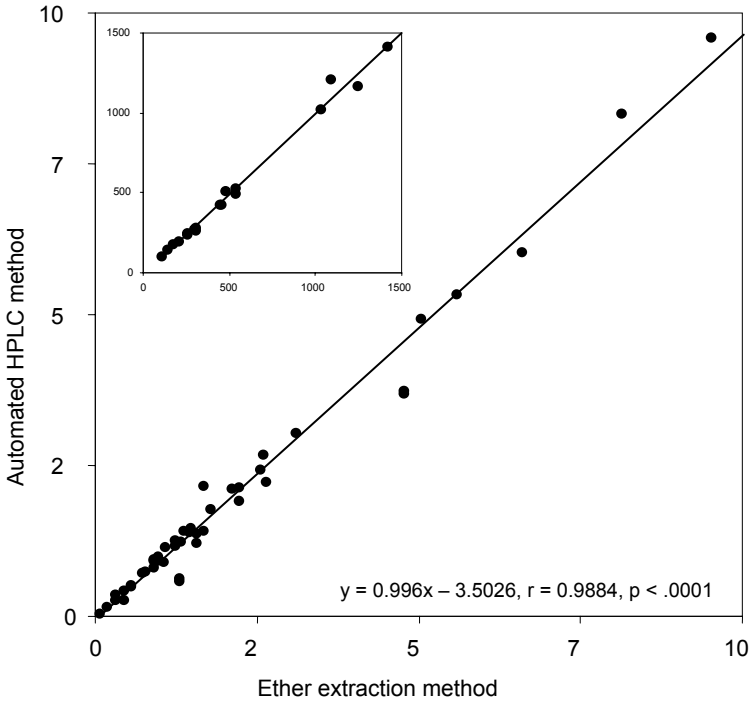


Figure 4.3: Comparison of 5-HIAA results for 63 urine samples as derived from the automated SPE-HPLC method (y axis) and the ether extraction method (x axis). The main panel shows values from 0 to 100 µmol/L. The inset shows values from 100 to 1500 µmol/L.

Discussion

A variety of methods for urine 5-HIAA analysis have been described, including several HPLC methods (Deacon et al. 1994). The automated SPE-HPLC method we developed reduced the per sample analysis time considerably, with a concomitant increase in sample throughput. The CVs for this method were ~50% lower than those for the manual method. We found that use of the on-line SPE system allows regeneration of SPE cartridges up to 40 times when small amounts of urine or plasma are extracted (Kema et al., 2001). This can be done with negligible loss of accuracy. During the 2-year period that this method has been in operation, interferences from drugs and other substances have been extremely rare (and discernable). The group mean urine concentrations and excretion rates reported here for 5-HIAA were within the reference intervals established at the University Medical Center Groningen and were in excellent agreement with previous reports (Launay et al., 1987; Minderaa et al., 1987; Anderson et al., 1989; Deacon, 1994).

In summary, our automated SPE-HPLC method for the determination of urinary 5-HIAA appears to offer advantages in time and precision compared with manual chromatographic methods.

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Chapter 5

Urinary Excretion of 5-Hydroxyindoleacetic Acid, Serotonin and 6-Sulphatoxymelatonin in Normoserotonemic and Hyperserotonemic Autistic Individuals

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Abstract

The presence of elevated levels of platelet serotonin (5-hydroxytryptamine, 5-HT) in a substantial proportion of individuals with autism is one of the more well replicated findings in neuropsychiatry. The issue of gut 5-HT production and platelet hyperserotonemia was studied by comparing urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA) and 5-HT in 10 normoserotonemic and 10 hyperserotonemic age-matched autistic individuals. The relationship of urinary 6-sulfatoxymelatonin (6-SM) excretion to platelet 5-HT, and to urinary 5-HT and 5-HIAA excretion, was also examined. In the hyperserotonemic group, a trend-level significant increase for urinary excretion of 5-HIAA or 5-HT and a significant decrease for 6-SM were found, while the urinary 5-HIAA / 5-HT ratio was similar in the two groups. The results suggest that the catabolism of 5-HT does not differ in the groups, but that greater exposure of the platelet to 5-HT can not be ruled out as a cause of the platelet hyperserotonemia of autism. Larger studies are needed to examine the relationship between gut 5-HT production and the platelet 5-HT elevation of autism, and to characterize the relationship between melatonin production and platelet hyperserotonemia more thoroughly.

Introduction

Elevated levels of the neurotransmitter/neurohormone serotonin (5-hydroxytryptamine, 5-HT) in blood platelets of individuals with autism is one of the most well replicated findings in neuropsychiatry (Anderson, 2002). Typically, the group mean has been reported to be elevated 25-50% in groups of individuals with autism compared to normally developing individuals. Recently, we have reported data that suggest a bimodal distribution of platelet serotonin in Dutch individuals diagnosed with autism and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS) (Mulder et al., 2004). It appeared that about half of the individuals with autism or PDD-NOS had platelet 5-HT values distributed in an upper mode, with a mean upper mode value nearly twice that seen for the lower mode. The lower mode in the autistic group appeared to largely overlap the distribution seen in normal control and mental retardation contrast groups.

While this further characterization of the platelet hyperserotonemia of autism does not immediately shed light on the mechanism responsible for the elevation, it may lead to improved strategies for elucidating underlying alterations and mechanisms. The mechanism has been hypothesized to be due either to increased exposure of the platelet to 5-HT or to an alteration in the platelet's handling of 5-HT (Anderson, et al., 1990; Anderson, 2002).

More than 90% of peripheral 5-HT is synthesized from the essential amino acid tryptophan in the enterochromaffin cells of the gut wall, stored within the enterochromaffin cells and released into the mucosal and myenteric layers of the gut, where 5-HT plays a critical role in stimulating peristalsis and other enteric processes (Gershon, 2003). Released 5-HT is metabolized locally by uptake into enterocytes and after entering the general circulation by efficient clearance by the endothelium, lung and liver. In both cases, 5-HT is predominantly metabolized by monoamine oxidase-A (MAO-A) to 5-hydroxyindoleacetic acid (5-HIAA) (Grahame-Smith, 1988).

Plasma free 5-HT can also be taken up by circulating platelets and nearly all 5-HT in the blood (>99%) is found stored within the platelet, with the typical blood concentration being approximately 150 ng/ml (~600 ng/109 platelets, ~3.5 nmol/109 platelets). Levels of free plasma 5-HT in the general circulation appear to be quite low (<300 pg/ml), and it is not clear whether most loading of platelets with 5-HT occurs in gut capillaries and the portal circulation or in the general circulation. It is clear that increased gut production of 5-HT, such as seen in carcinoid syndrome, a

cancerous proliferation of the enterochromaffin cells, leads to increased levels of platelet 5-HT and increased urinary excretion of 5-HIAA (Anderson et al., 1987; Kema et al., 2000). Urinary 5-HT is less well established to be proportional to gut serotonin synthesis; however, much of urine 5-HT can also be assumed to be derived from the enterochromaffin cell.

Several studies have measured urinary excretion of 5-HIAA in autism in order to address the issue of 5-HT production and platelet 5-HT exposure. A few studies have also reported urinary 5-HT levels and free plasma 5-HT concentrations. Most studies have not found differences in urinary 5-HIAA excretion between autistic individuals and normal or mentally retarded controls (Schain and Freedman, 1961; Partington et al., 1973; Minderaa et al., 1987; Launay et al., 1988; Martineau et al., 1992; Herault et al., 1996; Croonenberghs et al., 2000). However Hanley et al. (1977) reported elevated levels in urinary 5-HIAA and urinary serotonin in autistic subjects compared to a group of subjects with mental retardation. Minderaa et al. (1987) reported borderline significantly higher levels of 5-HIAA excretion in four hyperserotonemic autistic individuals, while finding a very similar urinary 5-HIAA excretion in the whole group of unmedicated autistic individuals compared to normal controls. Three studies have reported elevated urinary 5-HT excretion in groups of autistic subjects, but no differences in 5-HIAA excretion (Launay et al., 1988; Martineau et al., 1992; Herault et al., 1996), while Anderson et al. (1989) reported that urinary excretion of serotonin was unaltered in autism. Taken together, the prior studies tend to suggest that gut 5-HT synthesis is not altered in autism. However, in general, the group sizes have been small and only a limited number of subjects with elevated platelet 5-HT have been studied.

The neurohormone melatonin is synthesized from 5-HT, predominantly in the pineal gland, and has a major role in the regulation of circadian rhythms, sleep and mood. To date, melatonin production has not been assessed in relation to the issue of hyperserotonemia in autism. Production of pineal melatonin is regulated by photic information from the retina and during the day circulating melatonin levels are quite low. Pineal melatonin production increases swiftly after the onset of darkness and stays high until dawn (see for a review Brzezinksi, 1997). Another source of melatonin is the enterochromaffin cells of the gut wall. Several studies show that the melatonin concentration in the gastro-intestinal tract surpasses the blood concentration 10 to 100 times. Studies in animals indicate that melatonin derived

from the gastro-intestinal tract accounts for most of the meager day-time blood concentration of melatonin, whereas night time circulating levels of melatonin are determined by pineal gland production (as reviewed by Bubenik, 2002). Melatonin is metabolized in the liver and kidney to 6-sulphatoxymelatonin (6-SM) and excreted in urine. Urinary excretion of 6-SM closely follows the melatonin blood concentration (Lynch et al., 1975). The limited available data regarding melatonin levels in autism suggest that group mean melatonin production is reduced in autism (Nir et al., 1995; Hayashi et al., 2000; Kulman et al. 2000; Tordjman et al., 2005). These prior results, and the fact that 5-HT is the immediate precursor for melatonin in the pineal and the gut, suggested that it would be of interest to examine 6-SM excretion along with that of 5-HT and 5-HIAA.

We examined the issue of gut 5-HT production and platelet hyperserotonemia by comparing urinary excretion of 5-HIAA and 5-HT in groups of normoserotonemic and hyperserotonemic autistic individuals. Possible group differences in melatonin excretion and the interrelationships between the urinary measures were also examined.

Method

Subjects

Subjects from our prior study (Mulder et al., 2004), examining the distribution of platelet 5-HT values in autism spectrum disorders, were invited to participate in a follow-up study of indole metabolism. Diagnoses were established through comprehensive assessment, using the Autism Interview Schedule-Revised (ADI-R, Lord et al., 1994), Autism Diagnostic Observation Schedule-Generic (ADOS-G, Lord et al., 2000) and DSM-IV-TR diagnosis of autism, Asperger disorder or PDDNOS (APA, 2000) as described previously. All subjects (N = 20) were male, unmedicated and had an ADI-R classification of autism corroborated by an ADOS-G and DSM-IV-TR diagnosis of PDD. Autistic subjects were assigned to hyperserotonemic and normoserotonemic subgroups using a cut-off of 4.55 nmol/10^9 platelets derived from the aforementioned study (Mulder et al., 2004). Hyper- and normoserotonemic subjects were matched on age: group mean ages [mean ± SD, (median)] 15.3 ± 4.4, (15.5) and 15.3 ± 4.0, (15.5) yrs., respectively (Mann-Whitney U; Z_2 = 0.038, p = .971). Group mean IQs were 80.5 ± 36.4, (82.0) and 46.3 ± 20.5, (55.0) respectively; Mann-Whitney U; Z_2 = 1.965, p = .052. Approval for the study was given by the

Central Committee for Research in Humans in The Hague, NL. Informed consent/assent was obtained from all subjects and their parents.

Laboratory Measures

Blood samples were collected in 10-mL Vacutainer tubes (Becton-Dickinson, Meylan Cedex, France) containing 0.12 mL (0.34 mol/L) dipotassium EDTA solution. Platelet-rich plasma was prepared within 1 hour after sampling by centrifuging for 30 min at 120 × g and 4°C, and a platelet count was obtained. Platelet rich plasma 5-HT and plasma tryptophan were determined using a previously described HPLC method with fluorometric detection (Kema et al., 2001).

Twenty four-hour urine samples were collected from the subjects in brown propylene bottles (Sarstedt). Instructions concerning dietary intake (Kema et al., 2000) were issued to the parents and no consumption of prohibited foods was reported. One subject was excluded because of incompleteness of the 24 hour urine collection. Urinary 5-HIAA and 5-HT were measured using previously described methods (Kwarts et al., 1984; Mulder et al., 2005). 6-Sulphatoxymelatonin (6-SM) analysis was performed by enzyme-linked immunoabsorbent assay (ELISA) using a 6-SM ELISA Kit from Bühlmann AG (Schönenbuch, Switzerland). The urine samples were diluted prior to assay (1/200). The intra–assay and inter–assay coefficients of variation were 13.6% and 7.8% (n = 35) respectively for a 23.9 ng/mL (~100 nmol/mL) control sample value (based on approximately 1 liter per day urine volume this control sample would be equivalent to 0.10 nmol/24 hr).

Statistical Analysis

Age, IQ, platelet 5-HT, urinary 5-HIAA, urinary 5-HT, urinary 6-SM, plasma tryptophan levels, and urinary 5-HIAA / 5-HT ratios between hyper- and normoserotonemic groups were compared using the non-parametric Mann-Whitney U test. Spearman correlations were used to examine relationships between biochemical variables. For urinary 5-HIAA and urinary 5-HT the analyses are one-tailed, since there was an a priori expected direction of the difference. Despite the number of planned comparisons, α was set to .05 to reduce the potential for errors of the second type.

Results

Group means, SDs and medians observed for platelet 5-HT levels and urinary 5-HIAA, 5-HT and 6-SM excretion rates are given in Table 5.1 and the individual data plotted in Figure 5.1. Comparison of urinary 5-HIAA and of urinary 5-HT excretion in hyper- and normoserotonemic groups revealed trend-level significant differences between groups, with one-tailed p-values just above the .05 level (p = .061 and p = .071 for urinary 5-HIAA and 5-HT respectively). Excretion of urinary 6-SM appeared to be decreased in the hyperserotonemic group at a trend-level when expressed per 24 hours and was significantly lower when expressed per mol of creatinine (see Table 5.1). Plasma tryptophan concentrations were not significantly different in the two groups [mean ± SD, (median): 52.3 ± 15.2, (50.1) vs. 55.3 ± 9.89, (53.6) μM; Mann-Whitney U; Z_2 = 0.643, p = .523].

Table 5.1: Twenty four-hour urinary excretion (mean ± SD, median) of 5-HIAA, 5-HT and 6-SM in normoserotonemic (N=10) and hyperserotonemic (N=9) autistic subjects.

	Normoserotonemic *(< 4.5 nmol/10^9 plts)*	***Hyperserotonemic*** *(≥ 4.5 nmol/10^9 plts)*
Platelet Rich Plasma (PRP) 5-HT		
nmol/10^9 platelets [a]	3.14 ± 0.70, 2.95	6.02 ± 1.33, 5.60
Urinary 5-HIAA Excretion		
μmol/24 hours [b]	14.0 ± 6.27, 14.2	19.2 ± 10.3, 16.8
mmol/mol creat [c]	1.69 ± 0.86, 1.70	1.79 ± 0.75, 1.60
Urinary 5-HT Excretion		
μmol/24 hours [d]	0.52 ± 0.20, 0.55	0.68 ± 0.26, 0.65
μmol/mol creat [e]	60.1 ± 21.4, 59.0	63.2 ± 19.2, 56.0
Urinary 6-SM Excretion		
nmol/24hours [f]	77.1 ± 35.1, 84.2	53.0 ± 32.7, 57.2
μmol/mol creat [g]	9.03 ± 4.55, 9.07	4.90 ± 2.84, 4.81

NOTE: a. Mann-Whitney U; $Z_{(2)}$=3.787, p<.0001, b. Mann-Whitney U; $Z_{(2)}$=1.55, p=.061 (one tailed) c. Mann-Whitney U, $Z_{(2)}$=0.328, p=.390 (one-tailed) d. Mann-Whitney U; $Z_{(2)}$=1.47, p=.071 (one-tailed) e. Mann-Whitney U, $Z_{(2)}$=0.368, p=.360 (one-tailed) f. Mann-Whitney U, $Z_{(2)}$=1.470, p=.142 g. Mann-Whitney U; $Z_{(2)}$=2.205, p=.027

Across all subjects, urinary 5-HIAA and 5-HT excretion rates (per 24 hours) were not significantly correlated with platelet serotonin (r = 0.128, p = .60 and r = 0.149, p = .54, respectively); however, urinary 5-HIAA and urinary serotonin were highly correlated (r = 0.840, p < .0001). Urinary 6-SM was trend-level significant

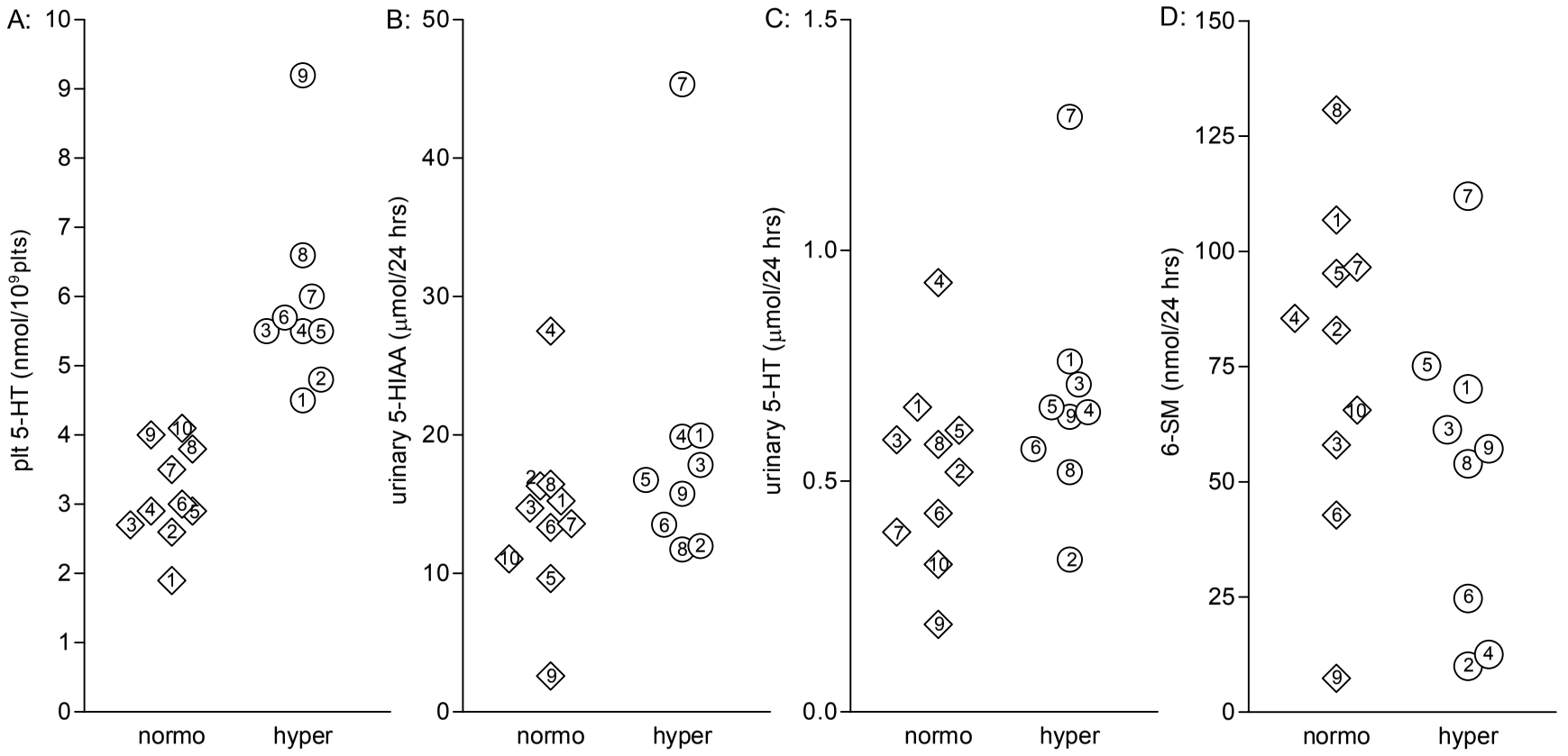

Figure 5.1. Individual platelet 5-HT concentrations (nmol/10^9 plts; A) and twenty four-hour urinary excretion of 5-HIAA (µmol/24 hrs; B), 5-HT (µmol/24 hrs; C) and 6-SM (nmol/24hrs) of the normoserotonemic (N=10) and hyperserotonemic (N=9) autistic subjects. The enclosed numbers are the subjects' ID numbers; diamonds depict the normoserotonemic and circles the hyperserotonemic subjects.

negatively correlated with platelet serotonin and positively correlated with urinary 5-HIAA and 5-HT when expressed on a 24 hour basis ($r = -0.365$, $p = .125$, $r = 0.458$, $p = .049$, $r = 0.433$, $p = .064$, respectively). No significant correlations were observed between plasma tryptophan and any of the other three biochemical measures, apart from urinary 5-HT where a positive correlation of $r = 0.633$, $p = .004$ was found. Similar correlative relationships were observed between the biochemical measures when excretion was expressed per mol of creatinine (platelet 5-HT vs. urinary 5-HIAA and 5-HT; $r = -0.034$, $p = .89$ and $r = -0.069$, $p = .78$, respectively; urinary 5-HIAA vs. 5-HT; $r = 0.927$, $p < .0001$). However when 6-SM excretion was expressed per mol of creatinine all correlations were significant ($r = -0.491$, $p = .033$, $r = 0.553$, $p = .014$, $r = 0.515$, $p = .024$, respectively). Correlations within the normoserotonemic and hyperserotonemic subgroups were similar as compared to the whole group (data not given).

When urinary product / substrate (5-HIAA / 5-HT) ratios were compared between hyper- and normoserotonemic groups, no significant difference was observed between groups (mean ± SD, median; 270 ± 70, 280 and 280 ± 50, 260, respectively; Mann-Whitney U: $Z_2 = 0.82$, $p = .935$).

The group means (± SD) for moles of creatinine/24 hr excreted in the hyper- and normoserotonemic groups were similar (11.3 ± 3.83, 12.40 versus 9.07 ± 3.48, 7.91 mmol/24hrs; Mann-Whitney U, $Z_2 = 1.470$, $p = .156$); the amount of urine collected per 24 hours was also similar in the two groups (1,327 ± 552, 1,330 versus 1,075 ± 555, 975 ml/24hrs; Mann-Whitney U, $Z_2 = 1.184$, $p = .236$, respectively).

The excretion of 5-HIAA, 5-HT and 6-SM expressed on a 24 hour basis was uncorrelated with age ($r = -0.194$, $p = .43$, $r = 0.091$, $p = .71$ and $r = -0.042$, $p = .89$, respectively). The excretion rates of 5-HIAA, 5-HT and 6-SM expressed per mole of creatinine were negatively correlated with age ($r = -0.82$, $p < .0001$. $r = -0.80$, $p < .0001$ and $r = -0.402$, $p = .087$), apparently mainly due to a positive correlation of creatinine with age ($r = 0.62$, $p = .005$).

Discussion

Urinary excretion rates of 5-HIAA, 5-HT and 6-SM were examined in subgroups of autistic patients as part of an investigation of the underlying causes of the platelet hyperserotonemia of autism. The present study revealed trend-level significant differences in urinary excretion of 5-HIAA and 5-HT between hyperserotonemic and

normoserotonemic individuals with autism. Group mean excretion of 6-SM appeared to be decreased in the hyperserotonemic individuals. In normoserotonemic as well as in hyperserotonemic individuals with autism, excretion rates of 6-SM were negatively correlated to platelet 5-HT and positively correlated to urinary excretion rates of 5-HIAA and 5-HT. Additionaly, plasma tryptophan concentration was correlated to urinary 5-HT excretion. The group mean excretion values reported here for 5-HIAA, 5-HT and 6-SM were within normal ranges established at the University Medical Center Groningen and were in excellent agreement with the prior reports (Minderaa et al., 1987; Launay et al., 1988; Anderson et al., 1989; Deacon, 1994).

This test of the relative excretion rates of 5-HIAA and 5-HT excretion in well defined hyperserotonemic and normoserotonemic subgroups suggests that greater exposure of the platelet to 5-HT due to greater gut production of 5-HT can not be ruled out as a cause of the platelet hyperserotonemia of autism. However, the similar urinary 5-HIAA / 5-HT ratios observed across normo- and hyperserotonemic groups suggests that the catabolism of 5-HT does not differ in the groups. The decreased excretion of 6-SM in hyperserotonemic individuals and the negative correlation of urinary excretion of 6-SM with platelet serotonin provide internally consistent data suggesting that there may be some link between altered (elevated) platelet 5-HT and abnormal (lower) melatonin production in autism.

In summary, larger studies in well characterized autistic and contrast groups and subgroups are needed to sort out the underlying cause(s) of, and possible relationship between, the platelet hyperserotonemia and the reduced melatonin production observed in autism.

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Chapter 6

Reactivity of Serotonin in Whole Blood

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This chapter is a comment on
Humble M, Bejerot S, Bergqvist PB, Bengtsson F (2001)
Reactivity of serotonin in whole blood: Relationship with drug response in obsessive-compulsive disorder. Biological Psychiatry 49:360–368, it has been published as a letter to the editor in
Biological Psychiatry, 51, 266-267, 2002

To the Editor:

We read with interest the recent paper of Humble and colleagues (Humble et al 2001) on the relationship between changes in whole blood serotonin (5-HT) and drug response in obsessive-compulsive disorder (OCD). The authors concluded that a rapid decrease of whole blood (i.e., platelet) 5-HT during treatment with the serotonin reuptake inhibitors paroxetine and clomipramine was associated with poor clinical response. We would like to make several comments regarding the study's data and interpretation.

1. It appears that circulating platelets use their membrane 5-HT transporter to take up 5-HT from the plasma throughout their 8- to 12-day life span; once taken up, little platelet 5-HT is released until the platelet is cleared from the circulation (Aranda et al., 1994; Heyssel, 1961). Changes in platelet 5-HT usually occur slowly because of the slow turnover of this pool. Conversely, the rate of 5-HT decrease occurring after transporter blockade will be more rapid in subjects with shorter platelet life spans. The authors' suggestion that changes in metabolic processes might account for the observed differences in rates of 5-HT decrease seems unlikely given the sequestered nature of platelet 5-HT.

2. Future studies on the relationship between changes in platelet 5-HT levels and drug response should attempt to assess the role of platelet lifespan in possible subgroup differences in 5-HT decreases or 'reactivity.' The possible influence of platelet life span could be approached through measurement of platelet count and estimation of platelet half-life.

3. Inspection of Figure 2 of the report reveals that all patients with less than a 60% decline in platelet 5-HT in the first week of treatment were receiving paroxetine. These patients contributed greatly to the negative correlation ($r = 0.61$) observed between percent decrease in 5-HT at 1 week and the OCD response at 12 weeks. Thus, it appears that differences in the immediate biochemical effects of paroxetine versus clomipramine, along with differences in long-term clinical response, might have contributed to the apparent association between 5-HT decline and medication response.

4. One patient with the poorest clinical response (~30% increase in OCD symptom score) and the greatest decrease in 5-HT (-98% at 1 week) also contributed substantially to the negative association between response and 5-HT decrease. This subject appears to be an outlier in the context of the other study subjects and when compared with the previously reported decreases seen after several weeks of 5-HT reuptake inhibitors, however. Rarely do platelet 5-HT levels decline by more than 95%, even after months of treatment. Such an extensive decline is even less likely after only 1 week of treatment given the slow turnover of platelet 5-HT.

5. The reported mean baseline level of whole blood 5-HT (654 nmol/L, 115 ng/mL) is lower than most previously published values (e.g., Flachaire et al., 1990; Kema et al., 1992). It is not clear that the methods used for sample preparation would have led to complete or corrected recoveries of 5-HT. Serotonin is particularly vulnerable to oxidative degradation during thawing of whole blood; losses at that stage would not have been accounted for if the internal standard was added afterward.

6. The authors cited two previous reports of increased platelet 5-HT efflux in autism; however, it should be noted that the cited findings were not replicated in subsequent studies and that the present consensus is that 5-HT efflux is not altered in autism (Boullin et al., 1982).

Although of potential importance, several factors lead us to suggest that the reported association be viewed as quite tentative and in need of replication. If the negative correlation between 5-HT decrease and response is substantiated, the relevant platelet physiology prompts consideration of the role of platelet life span in the association.

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Chapter 7

Summary and General Discussion

Introduction

This chapter summarizes the studies in this thesis and is focused on the discussion of the hyperserotonemia in autism spectrum disorders. The findings described in this thesis as well as relevant findings from other studies are considered in combination. Potential implications for the pathogenesis and treatment of autism spectrum disorders and perspectives for further research are discussed.

This thesis addresses the following themes: I) the further characterization of the hyperserotonemia of autism with respect to differences between autism spectrum disorders, mental retardation and typically developing children; within-group distribution; and demographic, diagnostic and behavioral correlates (*chapter 2*). II) Questions concerning the role of variants of the serotonin transporter gene (*chapter 3*). III) The possible increased exposure in autism of the platelet to serotonin (*chapters 4 & 5*), and IV) Issues concerning the value of peripheral serotonin measures in the management of psychotropic medication (*chapter 6*).

Characterization of the platelet hyperserotonemia of autism

Chapter 2 describes a study into the further characterization of platelet hyperserotonemia in autism. Through studying rather large groups of individuals with autism spectrum disorders, mental retardation and typical development we attempted to elucidate several questions that were still open. Our first aim was to replicate the finding of elevated platelet serotonin levels in a homogeneous group of well characterized subjects. Subsequently we assessed whether the hyperserotonemia is confined to 'core' autism or whether it is also present in the other autism spectrum disorders, i.e. Asperger's disorder and PDD-NOS. Additionally our large (n = 81) group of subjects enabled us to investigate the distribution of the platelet serotonin values in the autism spectrum disorder group. Finally, we evaluated the relation between platelet serotonin values and possible specific demographic, clinical and behavioral factors.

Several findings have emerged from this study. The elevation of serotonin was replicated, with a magnitude of the elevation in individuals with autism that was in the same range as most recent studies (McBride et al, 1998; Anderson, 2002). Following this expected outcome, the current study is the first to report elevated serotonin levels in individuals with PDD-NOS. Individuals with Asperger's disorder did not have

significantly elevated platelet serotonin levels; although our group was (n = 5), this was in accordance with another small study (Anderson et al., 1996). Group mean serotonin in individuals with mental retardation was similar as the mean in the typically developing group. This corroborates earlier observations (McBride et al., 1998) of normal platelet serotonin values in mentally retarded groups. Taking these results together we concluded that increased serotonin levels are associated not only with Kanner's 'core' autism, but also with the milder forms of autism spectrum disorders. Moreover, elevated platelet serotonin levels appear to be specifically linked to developmental problems on the autism spectrum and not to cognitive impairment.

What exactly this association looks like, was illustrated by probably the most notable finding from this study. The large number of participating subjects allowed us to demonstrate a bimodal distribution in the autism spectrum group. Apparently, a 'hyperserotonemic' subgroup of approximately half of the autism spectrum group can be distinguished from a subgroup with mean serotonin values in the same range as the mentally retarded and typically developing groups. Previously, it was not at all clear whether the entire group distribution had shifted upward or whether a hyperserotonemic subgroup existed. It is noteworthy that approximately 40% of individuals with autism and approximately 60% of subjects with PDD-NOS fell in the hyperserotonemic group suggesting that serotonin elevation is not directly related to severity of autistic impairment.

Although the finding of a hyperserotonemic subgroup seems promising for further parcelling out specific demographic, clinical and/or behavioral patterns, thorough analyses of a range of variables showed no relationship with serotonin levels or serotonemia status. This is remarkable given the extensive assessment of development and behavior by several state-of-the-art diagnostic instruments, like the ADI-R (Rutter et al., 2003) and the ADOS (Lord et al., 2000). However, despite the large group, the number of subjects may have been too limited to have sufficient power for detecting more subtle effects.

The apparent bimodality in the distribution of platelet serotonin values designates this biochemical measure as a valuable candidate to serve as an endophenotypic marker for further research into the neurobiology and genetics of autism spectrum disorders. The biochemically defined groups may also differ in their response to pharmacological and behavioral interventions.

Serotonin transporter gene variants in autism spectrum disorders

The serotonin transporter gene is a critical aspect of the serotonergic system. It is of interest for its potential role in autism and the associated domains of impairment and for its functional role in the elevation of platelet serotonin levels. The study reported in *chapter 3* concerns the assessment of the relation between variants of the serotonin transporter gene and the severity of impairment of social, communicative and rigid/compulsive behaviors in individuals with autism spectrum disorders. Additionally the association with the risk for having an autism spectrum disorders was investigated. We applied a family based approach in a sample of trios consisting of an individual with an autism spectrum disorder and both parents or a parent and an unaffected sibling. Two variants with supposed functional consequences for expression of the serotonin transporter were evaluated: the promoter insertion-deletion polymorphism (5-HTTLPR) and the variable number of tandem repeats (VNTR) polymorphism in intron 2.

No significant associations between autism spectrum disorder risk and either 5-HTTLPR or intron 2 VNTR alleles were found. These findings are not inconsistent with the accumulated prior studies. Of the 17 family-based studies of the 5-HTTLPR to date, 9 showed no association, 3 showed an association to the L-allele, and 5 to the S-allele of the HTT promoter. Five of the six studies examining the intron 2 polymorphism reported no association with autism. Additionally, in the current study, no strong indication emerged that 5-HTTLPR-intron 2 VNTR haplotypes were preferentially transmitted, this was in contrast with other studies that evaluated the transmission of several haplotypes (Kim et al., 2002; Conroy et al., 2004). More recent studies reported associations with other markers and thus other haplotypes within the transporter gene, suggesting allelic heterogeneity with respect to autism spectrum disorders (Sutcliffe et al., 2005; Devlin et al., 2005).

Severity of rigid-compulsive behaviors appeared to be associated with the intron 2 VNTR 12-repeat allele, suggesting a modulating influence of serotonin transporter gene functionality on this domain of autism spectrum disorders. This result was in part consistent with existing data of increased 17q11.2 linkage scores in a subset of families with high rigid-compulsive scores (McCauley et al., 2004). However, in that sample no preferential transmission of serotonin transporter gene polymorphisms was found in the rigid-compulsive subset. Also, in their sample, intron 2 VNTR genotypes were not significantly associated with rigid-compulsive factor scores.

The outcome of our study implies that further examination of possible preferential transmission of intron 2 alleles and related haplotypes appears warranted. It is possible that the association we observed is due to specific patterns of linkage that may be present in the Northern Dutch sample that was studied. However these findings add to the notion that the serotonin transporter gene can modify the expression of autism spectrum disorders. As pointed out by Devlin et al. (2005) the number of negative studies is quite high, but still the amount of studies reporting an association of autism with the serotonin transporter gene is much higher than expected by chance.

Mechanism of platelet hyperserotonemia: Exposure of the platelet to serotonin

The amount of serotonin present in the platelet depends on two, probably interplaying, processes: the way the platelet handles serotonin and the amount of serotonin the platelet is exposed to. Increased serotonin synthesis in the enterochromaffin cells of the gastro-intestinal tract can lead to higher exposure of the platelet to serotonin (Anderson et al., 1987). Although research so far tends to favor platelet handling as being the most important mechanism for causing the elevated platelet serotonin in autism spectrum disorders, there is no conclusive evidence that rules out higher exposure of the platelet to serotonin (Anderson, 2002).

In order to efficiently and specifically measure urinary excretion of the principal metabolite of serotonin, 5 hydroxy-3-indoleacetic acid (5-HIAA), we developed a new analytic method, which is described in *chapter 4.* The method consists of automated prepurification of urine by SPE cartridges, followed by elution and separation of the measure compounds by reversed-phase HPLC and fluorometric detection. The use of this automated on-line SPE system reduced the per sample analysis time to approximately 20 minutes, consequently creating an important increase in sample throughput. The method proved to be reliable and accurate in normal controls, in patients with carcinoid tumors of the gastro-intestinal tract and in individuals with autism. The automation of the determination of urinary excretion of this serotonin metabolite offers advantages in time and precision compared to known manual method, thus allowing the analyses of larger groups of subjects with the preservation of accuracy in future studies.

The new method was implemented in a consequent study (*chapter 5*) into the 24 hour urinary excretion of serotonin-related compounds – 5-HIAA, serotonin and 6-sulfatoxy-melatonin (6-SM), the metabolite of melatonin – in individuals with autism spectrum disorders. The excretion of these substances was assessed in a normoserotonemic and a hyperserotonemic group of unmedicated, age and IQ matched, male individuals with autism spectrum disorders. We aimed to evaluate the contribution of a possible increase of serotonin synthesis in the enterochromaffin cell of gastro-intestinal tract to the hyperserotonemia of autism spectrum disorders.

The results of this study still leave the issue of an alteration of gastro-intestinal tract serotonin synthesis open. Although in the hyperserotonemic group twenty four-hour urinary excretion of 5-HIAA and serotonin were found to be increased, the differences were only in the trend-level significant range. Twenty four-hour urinary excretion of 6-SM appeared to be significantly decreased in the hyperserotonemic group. The exact meaning of a decrease of 6-SM excretion for platelet serotonin levels is unclear. This result and the negative correlation of urinary excretion of 6-SM with platelet serotonin provide internally consistent data suggesting that there may be a link between altered (elevated) platelet 5-HT and abnormal (lower) melatonin production in autism. However, the similar urinary 5-HIAA / 5-HT ratios observed across normo- and hyperserotonemic groups suggests that the catabolism of 5-HT does not differ in the groups.

The results of this study were partly in accordance with the results from two earlier studies. Both reported normal urinary 5-HIAA excretion in autism itself, but showed an increased excretion of 5-HIAA in urine in a small subset of autistic individuals with elevated whole blood serotonin levels (Hanley et al., 1977; Minderaa et al., 1987).

Our study indicates that elevated serotonin synthesis can not be ruled out as a contributing factor to the elevation of platelet serotonin in autism spectrum disorders. Thus, altered production of serotonin by the gastro-intestinal system is still a candidate as a source of the excess of platelet serotonin in autism spectrum disorders.

Serotonergic system indices and psychotropic medication

Chapter 6 comments on the methodology and interpretation of an article reporting the results of a study into the relation between decline of whole blood

serotonin and the effect of a serotonin reuptake inhibitor (paroxetine) and a tricyclic antidepressant (clomipramine) in subjects with obsessive-compulsive disorders. The authors concluded that a rapid decrease of whole blood serotonin predicted poor clinical response to either of these substances.

However, when studying whole blood (i.e. platelet) serotonin as a marker for response to especially pharmacological interventions not only serotonin and its metabolism should be accounted for, but also the platelet, its handling of serotonin and its life-span are to be taken into consideration. Consequently, the interpretation of the authors, that decreasing levels of whole blood serotonin during the use of antidepressant medication mean that alterations of serotonin metabolism are present in obsessive-compulsive disorders, was questioned.

General discussion and future perspectives: The hyperserotonemia of autism spectrum disorders – important and clinically relevant endophenotype or interesting but secondary epiphenomenon?

The results of the studies described in this thesis perfectly illustrate the way science works. Although some issues concerning the hyperserotonemia of autism spectrum disorders appear to be elucidated, the results provoke a number of fascinating new questions that open possibilities for generating altered or new hypotheses and initiating further research.

The apparent bimodality of platelet serotonin values enables us to use this biological marker in a variety of studies. Most obviously intervention studies, especially pharmacological studies, can probably benefit from the availability of an objective biological measure, which is relatively easily available through a single blood draw. Also neuroimaging and genetic studies can take advantage of the possibility of including a more reliably measurable variable in their designs as opposed to behavioral measures. Although the clinical meaning of this marker has yet to be established and measuring platelet serotonin levels does not have any consequences for the individual diagnosis and treatment of individuals with autism spectrum disorders, the results warrant further study into the usefulness of platelet serotonin as a biological marker in autism. In this light the comments described in chapter 6 emphasize the importance of careful utilization of the available knowledge

in the fields of biochemistry and molecular biology in designing future studies into this subject.

The functional variants of the serotonin transporter gene and other polymorphisms within the gene constitute a factor that appears to be important in autism related behaviors, as illustrated by the results presented in chapter 3 and the accumulated studies so far. Additionally, the variability in the serotonin transporter gene affects, however not in a large way, platelet serotonin levels itself (Anderson et al., 2002; Persico et al. 2002; Couthino et al. 2004). As mentioned before, the serotonin transporter gene has to be taken into account at least as a co-variate in studies into autism spectrum disorders themselves and into hyperserotonemia, although the association between autism spectrum disorders, platelet serotonin and the serotonin transporter gene appears to be complex.

The mechanism that causes the hyperserotonemia of autism spectrum disorders still remains unclear. The results of our study implicate that a possible higher exposure of the platelet to serotonin merits more explicit attention. After the initial studies of Hanley et al. (1977) and Minderaa et al. (1987) suggesting a minor role of altered serotonin synthesis in gastro-intestinal tract, research focussed mainly on platelet handling of serotonin (Anderson et al., 1990, Cook, 1996). Our study, combined with the observations on serotonin producing carcinoids, where platelet serotonin in itself appears to be a measure of serotonin production in the tumors (Kema et al., 1992), warrants renewed attention for possible aberrations in serotonin synthesis. However, when investigating these processes, it seems of utmost importance to incorporate serotonin transporter gene polymorphism status into the design of these studies.

What does this research teach us in order to better understand the hyperserotonemia of autism, its role in the pathogenesis of autism and subsequently autism itself? It is impossible to state anything conclusive about the role of platelet hyperserotonemia and the serotonergic system in autism. Obviously a number of possible associated factors have not been covered in this thesis. For instance several rare functional mutations of the serotonin transporter gene have been found to be related to autism spectrum disorders, Asperger's disorder and obsessive compulsive disorders (Ozaki et al., 2003; Sutcliffe et al., 2005). Other important results have been published on the involvement of several other genes from the tryptophan and serotonergic pathways in autism. Polymorphisms in the tryptophan 2,3 dioxygenase 2

(Nabi et al., 2004) and monoamine oxidase A (Jones et al., 2004) genes have been found to be associated to autism. Additionally, there is a body of research available on the functionality of the serotonin 2a receptor and its gene, suggesting a role of this receptor in autism (for a review see Cook et al., 1996, as well as Goldberg et al., in preparation). Also, the integrin beta3 (ITGB3) gene, which is located next to the serotonin transporter gene on chromosome 17, appears to be associated to whole blood serotonin levels in the general population (Weiss et al., 2004). Very recently, assocations between ITGB3 and whole blood serotonin in autism have been found, as well as interactions between this gene and the serotonin transporter gene (Weiss et al., 2006a, 2006b). All these observations are in need of replication and should be followed up to further evaluate their contributions to autism and its hyperserotonemia.

An additional area of research that merits further exploration with respect to hyperserotonemia and autism in general is the newly emerging field of epigenetics. Epigenetics concentrates on processes that regulate the expression of genes in certain cells or tissues (Pray, 2004). These processes include genomic imprinting (expression of genes inherited from one particular parent), pleiotropy (expression of genes in different stages of development) and methylation (regulation of the expression of genes through methylation of promoter regions) (Tchurikov, 2005). Epigenetic mechanisms are believed to play a role in the inheritance of traits over several generations, in contrast to genetic information in the DNA that is more or less static over long times (Levenson and Sweatt, 2005). Also, environmental factors like exposure to folate and stress are proposed to affect methylation status (Jiang et al., 2004a; Oommen et al., 2005). The great difference between concordance rates of monozygotic and dizygotic twins in autism spectrum disorders together with the known familiality of serotonin levels in the general population and in autism might point to one of these mechanisms being at work in the pathogenesis of autism (Jiang et al., 2004b).

The research presented in this thesis once again emphasizes the by now scientific fact that alterations in the serotonergic system are unequivocally present in autism spectrum disorders. We showed that apart from the ‘core’ autism group also lesser variants have elevated serotonin levels. Consequently bimodality of the measure was demonstrated. The role of the serotonin transporter in autism and the possible role of gut serotonin synthesis in platelet serotonin content was corroborated. Unfortunately our studies do not solve the question of what role

alterations in the serotonergic system have in autism, yet. An important reason for this is that all measures were done in peripheral compartments of the human body. The meaning of peripheral findings in the human body for brain functioning is not at all clear. Although the human platelet is considered to be a representative model for serotonergic neurons by some authors (Stahl, 1977) and the platelet and the neuron have several structures in common: the serotonin transporter (Lesch et al., 1993a), the vesicle monoamine transporter (Lesch et al., 1993b) and the 5-HT2 receptor (Cook et al, 1994). There are also important differences from the serotonergic system in the brain as well. The different isoforms of the rate-limiting enzyme tryptophan dehydrogenase in the brain and the rest of the body are only one illustration of these differences (Walther et al., 2003). Also the magnitude of different functions of serotonin, as described in chapter 1, warrants careful reasoning when trying to translate findings from the periphery to the brain. Several provoking hypotheses regarding serotonergic abnormalities in the 'autistic brain' have been put forward mainly by scientists with a background in animal research (see Janusonis, 2005a, 2005b and Whitaker-Azmitia, 2005 for two examples). Janusonis (2005a and 2005b) argues that a dysfunction of serotonin receptors in individuals with autism leads to a hampered feedback mechanism in the gastro-intestinal tract and in the brain, thus causing platelet hyperserotonemia as well developmental problems of the serotonergic system in the brain. Whitaker-Azmitia (2005) on the other hand suggests that the excess of serotonin itself activates a negative feedback loop through the serotonin receptors (mainly 5-HT1a), leading to the loss of serotonergic neurons in the brain during neurodevelopment. However these theoretical constructs need confirmation in humans and until then their validity for the pathogenesis of autism and possible consequences for treatment remains unclear.

In conclusion it appears that the hyperserotonemia of autism spectrum disorders remains an intriguing phenomenon. Although 45 years of research has better characterized the basic finding, still the main questions regarding the underlying mechanism and whether the hyperserotonemia of autism disorders is an important and clinically relevant endophenotype or an interesting but secondary epiphenomenon is open for further investigation in the future.

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Samenvatting

Samenvatting

Dit proefschrift gaat over de plaatjes hyperserotonemie van autisme. In *hoofdstuk 1* worden de achtergronden van het onderzoek beschreven. Het begint met een overzicht van autisme en autisme spectrum stoornissen (ASS): de terminologie, definities, diagnostiek, oorzakelijke factoren en behandeling van. Vervolgens worden de functie en het metabolisme van serotonine besproken. Het hoofdstuk sluit af met een uitgebreide beschouwing over de huidige stand van zaken met betrekking tot de beschikbare kennis over de rol van het serotonerge systeem in autisme spectrum stoornissen.

Autisme is een ernstige neuropsychiatrische ontwikkingsstoornis, die zich uit op verschillende ontwikkelingsgebieden. Er is sprake van een combinatie van beperkingen op het gebied van sociale interactie, taal en communicatie en rigide en stereotiepe gedragingen. De ontwikkelingsproblemen zijn vaak al vanaf jonge leeftijd aanwezig. De uitingsvormen van autisme zijn divers, daarom spreekt men van een spectrum van autistische problematiek, of autisme spectrum stoornissen. In het DSM-IV-TR classificatiesysteem wordt de categorie pervasieve ontwikkelings-stoornissen gebruikt om autisme spectrum stoornissen te classificeren. Binnen deze categorie vallen de autistische stoornis, de stoornis van Asperger, de stoornis van Rett, de desintegratieve stoornis van de kinderleeftijd en de Pervasieve Ontwikkelingsstoornis, Niet Anderszins Omschreven (in het Engels: Pervasive Developmental Disorder, Not Otherwise Specified, afgekort: PDD-NOS). Het stellen van een diagnose binnen het autisme spectrum is moeilijk, omdat het beeld zo van patiënt tot patiënt verschilt. In de meest recente literatuur wordt geschat dat 6 van de 1000 kinderen en adolescenten een autisme spectrum stoornis hebben. De verhouding tussen jongens en meisjes is 4:1. De oorzaak van autisme spectrum stoornissen is onbekend. Uit onderzoek blijkt dat erfelijkheid een belangrijke rol speelt in het ontstaan van autisme spectrum stoornissen, daarnaast zijn er in groepen mensen met deze problematiek afwijkingen in het emotionele systeem van de hersenen gevonden bij beeldvormend onderzoek, zoals MRI en PET scans. Er is geen behandeling beschikbaar, die tot genezing van autisme spectrum stoornissen leidt.

In een grote groep mensen met autisme zijn verhoogde serotonine waarden in bloedplaatjes gevonden. Serotonine is een neurotransmitter/neurohormoon met veel functies in het menselijk lichaam. Het is één van de stoffen die de prikkeloverdracht

in het brein en de darm verzorgt en het speelt het een rol in het hart- en vaat stelsel. Ook heeft serotonine een belangrijke functie als neurohormoon in de ontwikkeling van het zenuwstelsel en in vroege ontwikkelingsstappen van het embryo. Serotonine komt overal in het lichaam voor en wordt op vele verschillende plaatsen gemaakt, zowel in de darm, als in de hersenen. De voorloper van serotonine is tryptofaan. Tryptofaan wordt via de darm opgenomen in het lichaam, het grootste deel van deze stof wordt gebruikt voor de productie van eiwitten en stoffen die te maken hebben met de afweer. Een klein deel wordt in de darmen en de hersenen omgezet in serotonine.

In de hersenen wordt serotonine opgeslagen in het neuron (zenuwcel) en na prikkeling van het neuron in de synapsspleet uitgescheiden. Hier doet het zijn werk als neurotransmitter door het activeren van verschillende serotonine receptoren. Hierna wordt serotonine weer opgenomen in het neuron, via het serotonine transporter molecuul, dat zich in de membraan van het neuron bevindt. Daarna wordt serotonine afgebroken tot de stof 5 hydroxy-3-indoleacetic acid (5-HIAA). De in de darm geproduceerde serotonine komt vrij in de bloedbaan. In de bloedbaan wordt het grootste deel van de serotonine snel opgenomen in de lever en de darm, waar het vervolgens wordt afgebroken tot 5-HIAA. 5-HIAA uit de hersenen en de darm wordt via de nieren in de urine uitgescheiden. Een zijpad van het serotonine metabolisme wordt gevormd door de productie van melatonine, een stof die een belangrijke rol speelt in het dag-nacht-ritme. De productie van melatonine vindt plaats in de pijnappelklier, een klier die zich juist onder de hersenen bevindt. Het afbraakproduct van melatonine (6-sulfatoxy-melatonine, 6-SM) wordt ook via de urine uitgescheiden.

Niet alle in de darm geproduceerde serotonine wordt opgenomen in de lever en de long, een klein deel van de serotonine blijft achter in het bloed. Ongeveer 99% van de achtergebleven serotonine wordt opgeslagen in de bloedplaatjes. Het bloedplaatje heeft een belangrijke rol in de bloedstolling. Dit is een kernloze cel die ongeveer 8 tot 12 dagen in de bloedbaan aanwezig is. Gedurende zijn aanwezigheid in de bloedbaan neemt het bloedplaatje serotonine op via een serotonine transporter molecuul in zijn membraan. De serotonine transporter van het neuron en het bloedplaatje hebben exact dezelfde structuur en worden gecodeerd door hetzelfde gen: het serotonine transporter gen.

Het serotonine transporter gen bevindt zich op chromosoom 17. Er zijn twee varianten van het serotonine transporter gen gevonden die worden verondersteld

invloed te hebben op de expressie van dit gen: een promoter insertie-deletie polymorfisme (5-HTTLPR) en een ‘variable number of tandem repeats’ (VNTR) polymorfisme in intron 2.

In 1961 werd voor de eerste keer een verhoogd serotonine in het bloed van mensen met autisme gevonden. *Hoofdstuk 1* bevat een overzicht van het onderzoek tot nu toe. Er is veel onderzoek gedaan om dit verschijnsel te verklaren. Een verhoogd serotonine in de bloedplaatjes, of plaatjes hyperserotonemie, van patiënten met autisme blijkt de meest gerepliceerde bevinding binnen de psychiatrie. Echter de oorzaak van de hyperserotonemie en de rol hiervan in het ontstaan van autisme is vooralsnog niet gevonden. Ondanks alle onderzoek zijn verschillende belangrijke vragen nog niet beantwoord: Komt een verhoogd serotonine in plaatjes serotonine alleen voor bij ‘kern-autisme’ of is dit ook terug te vinden bij andere autisme spectrum stoornissen, zoals de stoornis van Asperger en PDD-NOS? Is hyperserotonemie in bloedplaatjes specifiek voor autisme of komt het ook voor bij mensen met een verstandelijke handicap? Is er een relatie tussen het klinisch beeld, of een bepaald patroon van gedrag, en plaatjes serotonine waarden in autisme? Wat is de associatie tussen erfelijkheidsaspecten van het serotonerge systeem, hyperserotonemie en het klinisch beeld? Welk mechanisme veroorzaakt eigenlijk de verhoging van de serotonine in de bloedplaatjes? Is de verhoging een gevolg van hoe het bloedplaatje omgaat met serotonine? Of ligt de oorzaak bij een verhoogde productie van serotonine in de darm, waardoor het aanbod van serotonine aan het bloedplaatje eenvoudig groter is? En tot slot: Zijn plaatjes serotonine waarden van nut voor het voorspellen van het effect van medicatie? Met het onderzoek dat wordt beschreven in dit proefschrift is geprobeerd antwoord te geven op een aantal van deze vragen.

Karakterisering van de plaatjes hyperserotonemie van autisme

Hoofdstuk 2 doet verslag van een onderzoek naar verschillende beschrijvende aspecten van de plaatjes hyperserotonemie van autisme in een grote groep patiënten met autisme spectrum stoornissen, een groep met een verstandelijke handicap zonder autisme spectrum stoornis en een zich normaal ontwikkelende groep. Het eerste doel was het repliceren van de bevinding van verhoogde plaatjes serotonine waarden bij kinderen en adolescenten met autisme. In deze groep bleek inderdaad een verhoging van het groepsgemiddelde plaatjes serotonine. Bovendien

toonden we in dit onderzoek voor het eerst aan, dat er in de groep kinderen en adolescenten met PDD-NOS ook sprake was van een significante verhoging van serotonine in het bloedplaatje. Bij de kleine groep patiënten met de stoornis van Asperger werd geen significante verhoging gevonden. In de groep van kinderen en adolescenten met een verstandelijke beperking zonder autisme spectrum stoornissen bleken de serotonine waarden gelijk aan die van de groep die zich normaal ontwikkelde. Samengenomen geven de resultaten van dit deel van de studie aan dat verhoogde plaatjes serotonine waarden niet alleen voorkomen bij 'kern-autisme', maar ook bij de mildere autisme spectrum stoornissen. Voorts blijkt dat verhoogde plaatjes serotonine waarden geassocieerd zijn met ontwikkelingproblemen op het autisme spectrum en niet aan de verstandelijke ontwikkeling.

De belangrijkste bevinding van dit onderzoek echter betrof het aantonen van een tweedeling, of bimodale verdeling, van de serotonine waarden in de autisme spectrum groep. Een zogenaamde 'hyperserotonerge' subgroep, bestaande uit ongeveer de helft van de autisme spectrum groep blijkt te kunnen worden onderscheiden van een subgroep met normale serotonine waarden. Tot op heden was het absoluut niet duidelijk of er sprake was van een verschuiving naar rechts van de hele curve van serotonine waarden bij alle mensen met autisme spectrum stoornissen, of dat er sprake was van een hyperserotonerge subgroep binnen de groep met autisme spectrum stoornissen. Opvallend was dat ongeveer 40% van de proefpersonen met autisme en ongeveer 60% van die met PDD-NOS in de hyperserotonerge groep bleken te vallen. Dit illustreert dat hyperserotonemie kennelijk niet direct samenhangt met de ernst van een autisme spectrum stoornis. Echter, een uitgebreide analyse van verschillende demografische, klinische en gedragsvariabelen leverde geen resultaten op, waardoor aan hyperserotonemie evenmin een bepaald specifiek klinisch beeld of patroon van gedragingen kon worden gekoppeld. Wel biedt de tweedeling in de verdeling van plaatjes serotonine waarden in autisme de mogelijkheid deze biochemische maat te gebruiken, als biologisch endofenotype in verdere neurobiologische, genetische en farmacologische studies in autisme spectrum stoornissen.

Serotonine transporter gen varianten en autisme spectrum stoornissen

Het serotonine transporter gen is een ander onderdeel van het serotonine systeem, dat verder onderzoek verdient vanwege zijn mogelijke rol in autisme, in de gedragsdomeinen van autisme en in verhoogde plaatjes serotonine waarden. Het serotonine transporter gen codeert informatie voor het serotonine transporter molecuul. Deze serotonine transporter zorgt voor de opname van serotonine in het bloedplaatje en in de zenuwcel. In *hoofdstuk 3* werd de associatie onderzocht tussen functionele varianten van het serotonine transporter gen enerzijds, en het risico op het hebben van autisme en de ernst van aan autisme gerelateerde gedragsdomeinen anderzijds. Er werd een benadering gekozen, waarbij gebruik werd gemaakt van trio's, die bestaan uit een proefpersoon met een autisme spectrum stoornis, zijn/haar biologische ouders of één biologische ouder en een biologische broer of zus zonder een autisme spectrum stoornis. De twee varianten van het serotonine transporter gen, die worden verondersteld invloed te hebben op de expressie van dit gen werden onderzocht: het promoter insertie-deletie polymorfisme (5-HTTLPR) en het 'variable number of tandem repeats' (VNTR) polymorfisme in intron 2.

Er werd geen significante associatie gevonden tussen het risico op het hebben van een autisme spectrum stoornis en de 5-HTTLPR of de intron 2 VNTR allelen. De ernst van rigide, stereotiep en dwangmatig gedrag bleek samen te hangen met het '12-repeat' allel van het intron 2 VNTR polymorfisme. Hoewel deze bevinding replicatie behoeft, wijst deze uitkomst erop dat verder onderzoek naar de rol van de VNTR polymorfisme van het intron 2 en andere gerelateerde polymorfismen belangrijk is. Samen met reeds bekende gegevens uit eerdere onderzoeken draagt dit onderzoek bij aan de vaststelling dat het serotonine transporter gen een kwetsbaarheidsgen is voor autisme spectrum stoornissen.

Het mechanisme van de plaatjes hyperserotonemie: Het aanbod van serotonine aan het bloedplaatje

De hoeveelheid serotonine in bloedplaatjes wordt globaal gezien bepaald door twee processen: de manier waarop het bloedplaatje omgaat met serotonine en de hoeveelheid serotonine die aan het plaatje wordt aangeboden. Een verhoogd aanbod van serotonine aan het bloedplaatje zou worden veroorzaakt door een verhoogde productie van serotonine in de darm. Een eventuele verhoging van de productie van

serotonine in de darm is onder andere te onderzoeken door afbraakproducten van serotonine in de urine te meten. Hoewel op basis van de beschikbare literatuur tot op heden de manier waarop het bloedplaatje omgaat met serotonine het belangrijkste mechanisme lijkt, is er geen sluitend bewijs dat een verhoogd aanbod van serotonine helemaal geen rol speelt.

Hoofdstuk 4 beschrijft een nieuwe laboratoriumbepaling die wij hebben ontwikkeld, voor het meten in urine van 5 hydroxy-3-indoleacetic acid (5-HIAA), het belangrijkste afbraakproduct van serotonine. Delen van het analytisch proces zijn in deze bepaling geautomatiseerd. De tijd die nodig is om een urinemonster te analyseren is door dit zogenaamde 'automated on-line SPE' systeem verminderd tot 20 minuten. Hierdoor werd een belangrijke toename bereikt van het aantal monsters dat verwerkt kan worden in korte tijd. De methode bleek zowel betrouwbaar als nauwkeurig, in een normale controle groep, in patiënten met bepaalde maagdarm tumoren, die serotonine produceren en in kinderen en adolescenten met autisme.

Deze nieuwe bepaling hebben wij vervolgens gebruikt in een uitgebreide studie bij kinderen en adolescenten met autisme beschreven in *hoofdstuk 5*. In deze studie werd de 24 uurs uitscheiding in urine bepaald van verschillende stoffen uit het serotonine metabolisme: 5-HIAA, serotonine en 6-sulfatoxy-melatonine (6-SM, het afbraakproduct van melatonine). De uitscheiding van deze stoffen in de urine is onderzocht in twee groepen proefpersonen met autisme spectrum stoornissen, één groep met verhoogde plaatjes serotonine waarden en een andere groep met normale plaatjes serotonine waarden. Het doel was een eventuele toename van de serotonine productie in de darm in relatie tot de hyperserotonemie van autisme te onderzoeken.

De resultaten van de studie geven geen uitsluitsel over de rol van serotonine productie in de darm in de verhoging van serotonine in bloedplaatjes bij mensen met autisme. Hoewel de 24 uurs uitscheiding in urine van 5-HIAA en serotonine was verhoogd in de groep met een verhoogd serotonine, waren de verschillen net niet significant. Wel werd een significant lagere 24 uurs uitscheiding in de urine van het afbraakproduct van melatonine, 6-SM, gevonden. De exacte betekenis hiervan is vooralsnog onbekend.

Hoe dan ook geeft dit onderzoek aan dat, in tegenstelling tot een voorzichtige consensus tot dit moment, een verhoogde serotonine productie in de darm als mogelijke oorzaak van hyperserotonemie in stoornissen op het autisme spectrum

niet uitgesloten kan worden. Verder onderzoek in grotere groepen is noodzakelijk om deze bevinding verder te onderbouwen.

Plaatjes serotonine en psychotrope medicatie

Hoofdstuk 6 bevat een commentaar op een gepubliceerd onderzoek naar de relatie tussen een daling van het serotonine gehalte in het bloed enerzijds, en het effect van anti-depressiva anderzijds in patiënten met een obsessief-compulsieve stoornis. In dit soort studies blijken niet alleen de eigenschappen van de psychiatrische stoornis, het onderzochte medicament en het serotonine systeem van belang, maar vooral ook de eigenschappen van het bloedplaatje zelf. In het commentaar wordt aan de orde gesteld dat dit laatste door de auteurs van dit artikel onvoldoende werd onderkend. Voorts worden vraagtekens gezet bij de bewering van de auteurs, dat een daling van het serotonine gehalte in het bloed tijdens het gebruik van antidepressiva iets zegt over mogelijke afwijkingen in het serotonine systeem zelf bij een obsessief-compulsieve stoornis.

Discussie en toekomstperspectief: De hyperserotonemie van autisme spectrum stoornissen – belangrijk en klinisch relevant endofenotype of interessant maar secundair bijverschijnsel?

Hoofdstuk 7 besluit met een beschouwing over de bevindingen van de onderzoeken in dit proefschrift en de mogelijke implicaties voor verder onderzoek. De bevindingen in dit proefschrift illustreren bij uitstek de manier waarop wetenschap werkt. Hoewel een aantal vragen met betrekking tot de hyperserotonemie van autisme spectrum stoornissen lijkt te zijn beantwoord, lokken de bevindingen vele nieuwe fascinerende vragen uit. Deze scheppen mogelijkheden voor het ontwikkelen van nieuwe hypothesen en verder onderzoek.

De klaarblijkelijke bimodale verdeling van plaatjes serotonine waarden onderbouwt de mogelijke waarde van deze biologische marker als endofenotype in een verscheidenheid aan onderzoeken. In de eerste plaats zal interventie onderzoek, in het bijzonder medicatie onderzoek, kunnen profiteren van de beschikbaarheid van een objectieve, relatief eenvoudig toegankelijke, biologische maat. Verder kunnen beeldvormend en genetisch onderzoek baat hebben hij het gebruik van een betrouwbaar meetbare variabele naast, of in plaats van, gedragsmaten. De klinische

betekenis en de bruikbaarheid van plaatjes serotonine voor autisme spectrum stoornissen moet nog worden vastgesteld en gebruik van plaatjes serotonine metingen heeft vooralsnog geen enkele plaats in de individuele zorg voor patiënten. Desondanks geven de resultaten van dit onderzoek voldoende aanleiding tot het verder onderzoeken van de bruikbaarheid van deze marker in het onderzoek naar en de behandeling van autisme spectrum stoornissen

De functionele varianten van het serotonine transporter gen, evenals andere polymorfismen in dit gen lijken belangrijk voor gedrag gerelateerd aan autisme. Daarnaast is er een, weliswaar klein, effect van varianten van het serotonine transporter gen op de hoogte van plaatjes serotonine waarden. Hoewel de relatie tussen autisme spectrum stoornissen en het serotonine transporter gen zeer complex is, zou het serotonine transporter gen, op zijn minst als co-variaat, moeten worden meegenomen in verder onderzoek naar het serotonine systeem bij autisme spectrum stoornissen.

Het mechanisme dat ten grondslag ligt aan de verhoging van de waarden van plaatjes serotonine in autisme spectrum stoornissen is onbekend. Volgend uit de bevindingen van ons onderzoek zou een mogelijk verhoogd aanbod van serotonine aan het bloedplaatje meer aandacht moeten krijgen. In verder onderzoek naar dit mechanisme zou echter ook, zoals eerder genoemd, de invloed van serotonine transporter gen varianten moeten worden meegenomen.

Wat leert dit onderzoek ons nu over de hyperserotonemie van autisme spectrum stoornissen, de rol van hyperserotonemie in het ontstaan van autisme spectrum stoornissen en uiteindelijk over autisme stoornissen zelf? Het is onmogelijk om een sluitende conclusie te formuleren over de rol van hyperserotonemie en het serotonerge systeem in autisme. Hoewel verschillende factoren in dit proefschrift aan de orde kwamen, zijn ook een aantal belangrijke factoren niet onderzocht. Zo zijn er bijvoorbeeld in het serotonine transporter gen verschillende zeldzame mutaties, die van invloed zijn op het functioneren van de serotonine transporter. Ook zijn er vele andere processen en genen betrokken bij het serotonerge systeem en autisme spectrum stoornissen, zoals verschillende enzymen en de serotonine receptoren. Ten slotte worden er ook genen onderzocht die weliswaar niet rechtstreeks met het serotonine systeem te maken lijken te hebben, maar die op een chromosoom zeer dichtbij het serotonine transporter gen liggen en daardoor mogelijk van invloed zijn.

Een geheel ander onderzoeksgebied, dat aandacht behoeft als het gaat om autisme spectrum stoornissen en het serotonine systeem is het opkomende gebied van de epigenetica. De epigenetica bestudeert werkingsmechanismen, die te maken hebben met het reguleren van de expressie van genen. Epigenetische mechanismen lijken een rol te spelen bij de overerving van eigenschappen van generatie op generatie, terwijl informatie opgeslagen in het DNA zorgt draagt voor de overdracht van erfelijkheidsinformatie op de langere termijn. De manier van overerving van autisme en autisme spectrum stoornissen suggereert een rol van epigenetische mechanismen in het ontstaan van deze problematiek.

Samenvattend kan gesteld worden dat het onderzoek gepresenteerd in dit proefschrift opnieuw aantoont dat afwijkingen in het serotonine systeem zonder enige twijfel aanwezig zijn in autisme spectrum stoornissen. We hebben laten zien dat verhoogde plaatjes serotonine waarden voorkomen in zowel ‘kern-autisme’ als in de mildere autisme spectrum stoornissen. Daarnaast hebben we een bimodale verdeling van deze marker aangetoond. We bevestigden de rol van het serotonine transporter gen in aan autisme gerelateerd gedrag. Daarnaast kan ons onderzoek de eventuele rol van een verhoogde serotonine productie in de darm in de plaatjes hyperserotonemie van autisme spectrum stoornissen niet uitsluiten.

De betekenis van deze bevindingen voor eventuele afwijkingen van het serotonine systeem in het brein van mensen met autisme is niet duidelijk. Immers de betekenis van metingen in het bloed en de urine voor processen in het brein is niet of nauwelijks bekend. Op basis van de literatuur is er echter reden aan te nemen, dat er grote overeenkomsten zijn tussen de serotonine systemen in het brein en de rest van het lichaam, in het bijzonder het bloedplaatje. Het is dus zinvol om onderzoek naar perifere maten als deze voort te zetten, zeker gezien het feit dat direct onderzoek naar het levende brein van mensen met autisme onmogelijk is.

Concluderend blijkt de hyperserotonemie van autisme spectrum stoornissen nog steeds een intrigerend fenomeen. Hoewel in 45 jaar van intensief en nauwkeurig onderzoek verschillende vragen zijn beantwoord, blijft het de vraag of we te maken hebben met een belangrijk en klinisch relevant endofenotype van autisme spectrum stoornissen of met een interessant maar secundair verschijnsel.

Dankwoord

Dankwoord

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Curriculum Vitae

Curriculum Vitae

Erik Joan Mulder werd geboren op 1 september 1971 te Emmen. In 1989 behaalde hij zijn VWO diploma aan de Christelijke Scholengemeenschap aldaar. Na één jaar fysiotherapie te hebben gestudeerd, startte hij in 1990 met de studie Geneeskunde aan de Rijksuniversiteit te Groningen. Hij liep zijn co-assistentschappen in de Deventer Ziekenhuizen te Deventer. Aan het einde van zijn studie bracht hij een onderzoeksstage van vier maanden door aan het Yale Child Study Center van Yale University in New Haven, Connecticut, in de Verenigde Staten.

Na zijn afstuderen als basisarts in januari 1997 werkte hij als assistent geneeskundige niet in opleiding (AGNIO) en vervolgens als assistent in opleiding tot klinisch onderzoeker (AGIKO) bij het Universitair Centrum voor Kinder- en Jeugdpsychiatrie te Groningen (UCKJP). Dit centrum is onderdeel van de noordelijke kinder- en jeugdpsychiatrie instelling Accare. Hij werkte hier op de polikliniek en was gedetacheerd bij het Autisme Team Noord-Nederland, de Polikliniek Psychiatrie Verstandelijk Gehandicapten (beiden onderdeel van de GGz Groningen) en de Jeugdkliniek Groenendaal (onderdeel van Jeugdzorg Groningen). Vanaf maart 2000 werkte hij aan het in dit proefschrift beschreven onderzoek.

Met als opleider prof. dr. R.B. Minderaa deed hij van 1 september 2002 tot 1 september 2003 het keuzejaar Kinder- en Jeugdpsychiatrie binnen het UCKJP. Vanaf 1 september 2003 tot 10 augustus 2006 volgde Erik de basisopleiding tot psychiater aan het Universitair Centrum Psychiatrie, voorheen de afdeling Psychiatrie, voorheen de Psychiatrische Universiteits Kliniek, van het Universitair Medisch Centrum Groningen, zijn opleider hier was prof. dr. R.J. van den Bosch. Op het moment van de verdediging van dit proefschrift is hij in dienst van de GGz Zuid-Oost Friesland te Drachten, hier vervult hij zijn stage Sociale Psychiatrie. Na het voltooien van de opleiding tot psychiater in maart 2007 treedt hij opnieuw in dienst van Accare voor het volbrengen van het aantekeningsjaar kinder- en jeugdpsychiatrie en het voortzetten van de onderzoekslijn waarvan de start beschreven is in dit proefschrift.

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Abbreviations

5-HIAA – 5-Hydroxyindole-3-Acetic Acid
5-HICA – 5-Hydroxyindole-3-Carboxylic Acid
5-HT – 5-Hydroxytryptamine - Serotonin
5-HTTLPR – Serotonin Transporter gene promoter polymorphism
6-SM – 6-Sulfatoxymelatonin
ASD – Autism Spectrum Disorder
ADOS – Autism Diagnostic Observation Schedule
ADI-R – Autism Diagnostic Interview - Revised
AADC – Aromatic-L-amino Acid Decarboxylase
ANOVA – Analysis of Variance
DSM-IV-TR – Diagnostic and Statistical Manual of Mental Disorders, 4^{th} edition, text revision
ELISA – Enzyme-linked Immunoabsorbent Assay
ETDT – Extended Transmission Disequilibrium Test
HPLC – High Performance Liquid Chromatography
HHRR – Haplotype-based Haplotype Relative Risk test
ICD-10 - International Classification of Diseases and related health problems, 10^{th} revision
ITGB3 - Integrin Beta 3
LOD – Logarithmic Odds
MAO-A – Monoamine Oxidase A
MR – Mental Retardation
PDD – Pervasive Developmental Disorder
PDD-NOS – Pervasive Developmental Disorder, not otherwise specified
PRP – Platelet Rich Plasma
QTDT – Quantitave Transmission Disequilibrium Test
SERT/HTT – Serotonin Transporter
SLC6A4 – Serotonin Transporter gene
SNP – Single Nucleotide Polymorphism
SPE – Solid-Phase Extraction
SSRI – Selective Serotonin Reuptake Inhibitor
TDO – Tryptophan/indoleamine 2,3 Dioxygenase
TDT – Transmission Disequilibrium Test
TPH – Tryptophan Hydroxylase
TRP – Tryptophan
VMAT – Vesicular Monoamine Transporter
VNTR – Variable Number of Tandem Repeats