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

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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).

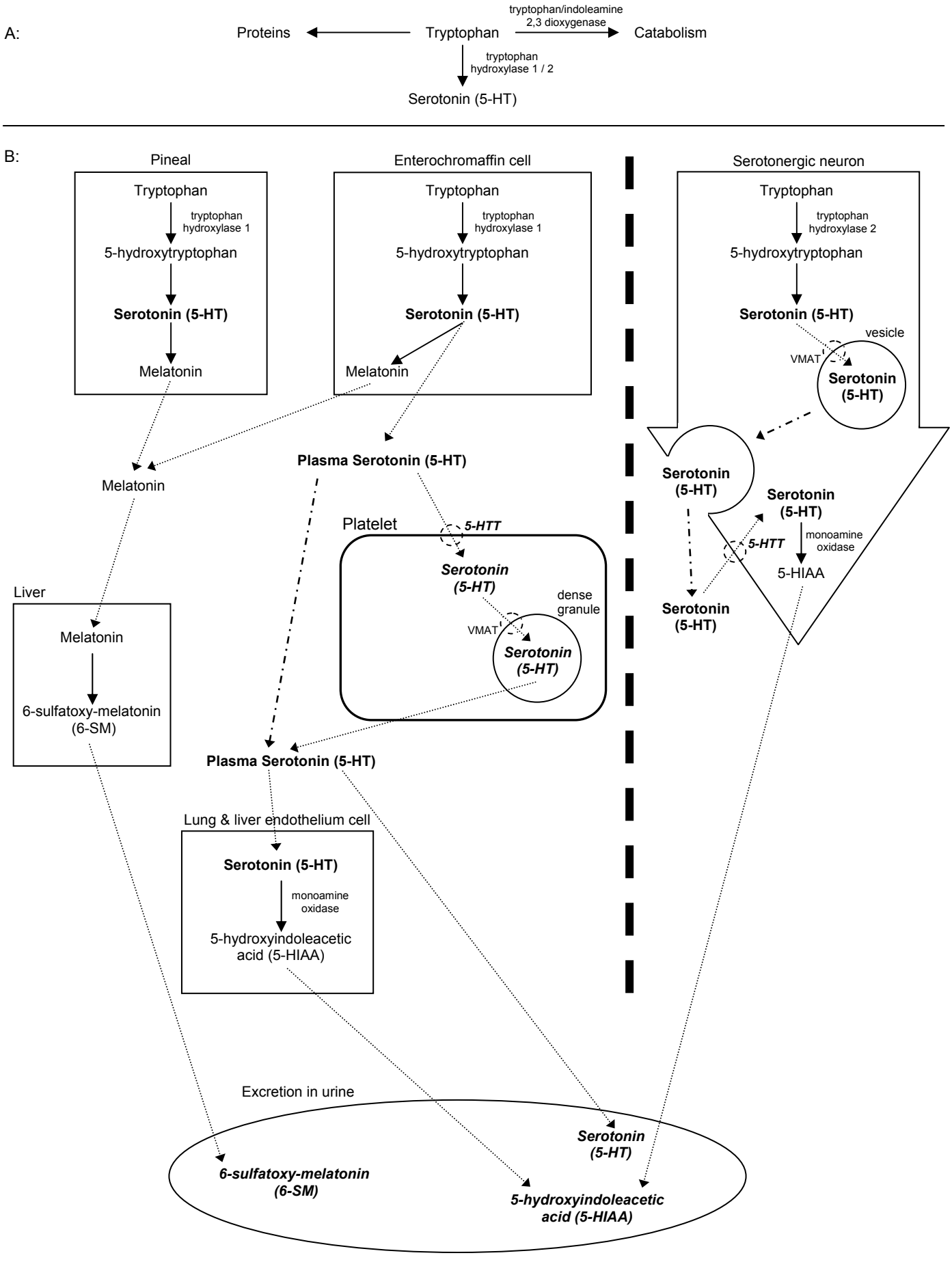

Figure 1.1: Schematic overview of tryptophan and serotonin metabolism. A: three metabolic routes of tryptophan. B: routes and compartments of serotonin metabolism. Normal arrows are used when chemical reactions are taking place, dotted arrows depict transport of a molecule over a cell membrane, dashed 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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