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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