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Structural brain analysis in autistic compared to non-autistic adults and its relationship to autistic traits and the oxytocin system

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Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental condition defined by qualitative impairments of social interaction and communication and stereotyped repetitive behaviors. In light of the great challenges associated with ASD, it is of great importance to better understand the condition. Although progress has been made in characterizing the neuroanatomical underpinnings of ASD in children, the brain structural correlates in autistic adults and its relationship to prosocial neuropeptides such as oxytocin (OXT) remain poorly understood. Here we used voxel-based morphometry (VBM) to investigate brain structure and its relationship autistic traits in autistic and non-autistic adults. Furthermore a region of interest (ROI) -based VBM approach was used to study the link between OXT levels and hypothalamic grey matter volume (GMV), as OXT is produced in the hypothalamus (HTH). T1-weighted MRI images were obtained from autistic adults without intellectual impairment (n=29, age 36.03 ± 11.0) and matched non-autistic controls (n=27, age 30.96 ± 11.2). Peripheral plasma OXT levels and the autism spectrum quotient (AQ) for autistic traits were used for correlation analyses. Our results demonstrate that although hypothalamic GMV did not differ between groups, the groups showed significant differences in the association between hypothalamic GMV and peripheral OXT levels, such that a positive correlation was found for the ASD group only. In addition, hypothalamic GMV showed a positive correlation with autistic traits in the ASD group, while no such correlation was observed in the comparison group. These results point towards the important role of the HTH in ASD and its relationship to the OXT system, but demonstrate the importance of interindividual differences.

Abstract (deutsch)

Die Autismus-Spektrum-Störung (ASS) zählt zu den tiefgreifenden Entwicklungsstörungen, und ist durch qualitative Beeinträchtigungen der sozialen Interaktion und Kommunikation sowie stereotype Verhaltensweisen gekennzeichnet. Obwohl die letzten Jahrzehnte einige Fortschritte in der Erforschung der hirnstrukturellen Grundlagen der ASS bei Kindern hervorgebracht haben, sind diese bei Erwachsenen noch kaum verstanden. Forschungsergebnisse der letzten Jahre deuten darauf hin, dass dem prosozialen Neuropeptid Oxytocin (OXT) sowohl bei der Ätiopathogenese der ASS als auch bei therapeutischen Ansätzen eine wichtige Rolle zukommen könnte. Insbesondere der Zusammenhang zwischen Hirnstruktur und Veränderungen des OXT-Systems sind dabei noch wenig erforscht. Tatsächlich deuten jedoch bisherige Forschungsarbeiten auf strukturelle Veränderungen im Hypothalamus, dem Ort der zentralen der OXT Produktion, bei Autisten hin. Um unser Verständnis der hirnstrukturellen Unterschiede bei Erwachsenen mit und ohne ASS einerseits und deren Zusammenhang mit autistischen Merkmalen und dem OXT-System andererseits zu erweitern, wurden in der vorliegenden Arbeit T1-gewichtete MRT-Bilder von autistischen Erwachsenen ohne intellektuelle Beeinträchtigung (n=29, Alter 36,03 ± 11,0) und nicht-autistischen Kontrollen (n=27, Alter 30,96 ± 11,2) analysiert. Die Analyse allgemeiner struktureller Unterschiede im ganzen Gehirn erfolgte mittels voxelbasierter Morphometrie (VBM). Darüber hinaus wurde ein Region of Interest (ROI)-basierter VBM-Ansatz verwendet, um den Zusammenhang zwischen peripheren OXT-Spiegeln und dem Volumen der grauen Substanz (GMV) im Hypothalamus zu untersuchen. Für Korrelationsanalysen wurden die peripheren OXT-Plasmaspiegel unter Ruhebedingungen und der Autismus-Spektrum-Quotient (AQ) für autistische Merkmale erhoben. Als Hauptergebnis dieser Arbeit berichten wir, dass, obwohl sich das hypothalamische GMV zwischen den Gruppen nicht unterschied, die Gruppen signifikante Unterschiede in der Assoziation zwischen hypothalamischem GMV und peripheren OXT-Spiegeln aufwiesen, so dass nur für die ASS-Gruppe eine positive Korrelation gefunden wurde. Darüber hinaus zeigte das hypothalamische GMV in der ASD-Gruppe eine positive Korrelation mit autistischen Merkmalen, während in der Vergleichsgruppe keine solche Korrelation beobachtet wurde. Diese Ergebnisse sind ein weiterer Hinweis für eine potentiell wichtige Rolle des Hypothalamus bei ASD und seiner Beziehung zum OXT-System. Sie zeigen aber auch die Bedeutung von interindividuellen Unterschieden in diesem Zusammenhang auf.

List of abbreviations

IQR ..... interquartile range
ADH ..... Antidiuretic hormone
ADHD ..... Attention-Deficit / Hyperactivity Disorder
ALE ..... Anatomical Likelihood Estimation
ANCOVA ..... Analysis of Covariance
APA ..... American Psychological Association
AQ ..... Autism Spectrum Quotient
ASD ..... Autism Spectrum Disorder
BMI ..... Body Mass Index
Cat12 ..... Computational Anatomy Toolbox for SPM, Version 12
CSF ..... Cerebro Spinal Fluid
DALY ..... Disability-adjusted life year
DARTEL ..... Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra
DSM V ..... Diagnostic and Statistical Manual of Mental Disorders, Version 5
EQ ..... Empathy Quotient
fMRI ..... Functional magnetic resonance imaging
FWE ..... Family-wise-Error
GABA ..... Gamma-aminobutyric acid
GLM ..... General Linear Model
GM ..... Gray Matter
GMV ..... Gray Matter Volume
HC ..... Healthy controls
HTH ..... Hypothalamus
HTH-ROI ..... Hypothalamus region of interest analysis
ICD-10 ..... International Statistical Classification of Diseases and Related Health Problems, Version 10
IQR ..... Image Quality Rating
MANCOVA ..... multivariate analysis of covariance
MNI ..... Montreal Neurlogical Instiute
MRI ..... Magnetic Resonance Imaging
mRNA ..... Messenger ribonucleuc acid
OXT ..... Oxytocin

OXTR ..... oxytocin receptor
PFC ..... Prefrontal Cortex
PVN ..... Paraventricular Nucleus
ROI ..... Region of Interest
SBM ..... Surface Based Morphometry
SNP ..... single nucleotide polymorphism
SON ..... Supraoptic nucleus
SPM ..... Statistical Parametric Mapping/ Statistical Parametric Map
SPSS ..... Statistical Package for the Social Sciences
TIV ..... Total Intracranial Volume
ToM ..... Theory of Mind
TPM ..... Tissue probability Map
VBM ..... Voxel Based Morphometry
WM ..... White Matter
WMV ..... White Matter Volume
WST ..... Wortschatztest (Vocabulary Scale)
Δ%-OT ..... Percentage difference between baseline and stimulated oxytocin levels

1 Introduction

1.1 Introductory considerations

“The neurodiversity framework conceptualizes autism as a natural form of human variation, inseparable from individuals’ identity, and not in need of a cure or normalization”(Kapp, 2020; Bottema-Beutel *et al.*, 2021).

Many of the problems autistic people face today, such as stigmatization, underemployment, lack of autonomy, and mental illnesses such as depression, are not just the product of a single "disordered" brain, but arise from a “*dynamic interpersonal mismatch*” (Bolis, 2021) between autistic and non-autistic people. However, in the past the use of a binary distinction between "diseased" and "healthy" in medical research on autism often meant that problematic behaviors were identified and exclusively attributed to the autistic group. In contrast, within a neurodiversity framework, advocated by many in the autistic community, ASD is understood not as a disease but as part of identity. Recognizing this perspective, research such as that presented here, which seeks to investigate neurobiological differences in ASD in comparison to a neurotypical control group, can therefore be in danger of falsely pathologizing potential differences. Moreover, research that explores the neurobiological underpinnings of ASD with the goal of understanding so called deficits and seeking ways to intervene to "make people less autistic", could be seen as morally troubling (Pellicano and Houting, 2021). At the time of writing, this thesis was guided by the prevailing paradigm of ASD as a neurodevelopmental disorder defined by persistent deficits (ICD-10 and DMS-V). Therefore, this work reflects this zeitgeist and the related concept of ASD to a great extent. A paradigm shift away from deficit-oriented to strength-oriented research is increasingly called for (Pellicano and Houting, 2021). Such a shift could enrich future research in this field and provide new perspectives. In recognition of this positive development - with the help of relevant publications (Bottema-Beutel *et al.*, 2021) and to the best of my knowledge - I have tried to use language that is inclusive and non-stigmatizing and non-hierarchical. This included avoiding - as far as possible - imprecise, hierarchizing classifications and the use of terms such as "comparison group" instead of "healthy control group." Furthermore, this thesis uses identity-first language ("autistic person") instead of the person-first form ("person with autism"), as this is reportedly preferred by autistic people (Bottema-Beutel *et al.*, 2021). However, other points that were criticized in this context were retained because they were part of the original study design. Here, the reader is asked to be appropriately critical him-/herself. An example of this is the

use of the autism spectrum quotient (AQ) as an indicator of autistic traits that extend to the general population. This has been criticized as an inaccurate generalization because the generic term "autistic traits" in this context hardly reflects the very heterogeneous manifestations of autism. Furthermore, it was pointed out that autistic traits are rarely reported in a positive or neutral context (Bottema-Beutel *et al.*, 2021). A finer linguistic and scientific differentiation will be sought in future studies. An overall goal of this work is to contribute to a better mutual and appreciative understanding between autistic and non-autistic people through a better understanding of neurobiological differences and commonalities. Optimally, this will support autistic people to have a higher degree of autonomy in developing their own concept of life as equal members of society.

1.2 An overview of Autism Spectrum Disorder

1.2.1 Current diagnostic criteria and classifications

The two predominant diagnostic manuals used in psychiatry, the ICD-10 and the DSM V, define autism as a set of profound neurodevelopmental disorders that manifest in early childhood. As such autism is defined as a disorder, which is characterized by functional impairment and not as a desease, which is defined as a distinct and measurable pathological process. The characteristic behaviors observed in autistic individuals usually manifest within the first five years of life and persist throughout life. Although the disorder has a wide range of severity and manifestations, there are common features in three areas, also referred to as the autistic triad (Kamp-Becker and Bölte, 2014) (**Figure 1**).

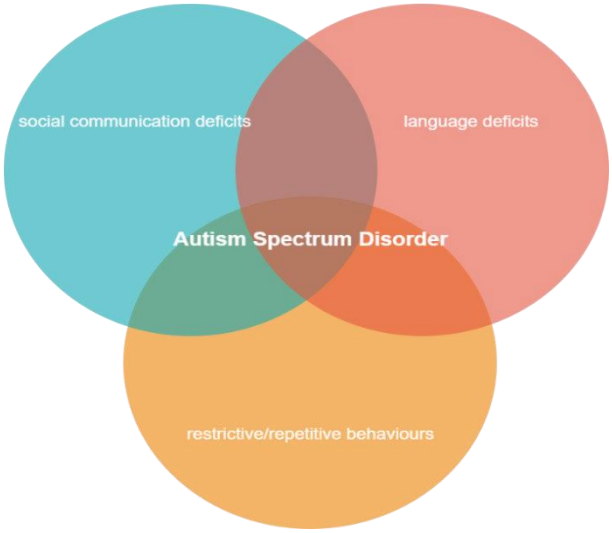


Figure 1 Simplified schematic diagram of the three main clinical characteristics of austimus spectrum disorder. Adapted from (Devlin and Scherer 2012, created with the freely available software 'draw.io'.

(1) Qualitative impairment in social communication. This core feature of ASD is characterized by impaired understanding and interpretation of social situations. The resulting behavior is often perceived as inappropriate by others, which leads to misunderstandings and difficulties in communication. An early manifestation of these altered communication behaviors in autistic children is, for example, a diminished social smile when smiled at by their caregiver. Another manifestation is the difficulty in establishing so-called " joint attention," i.e., coordinating the attention of two people toward a common goal (APA, 2013; Kamp-Becker & Bölte, 2014; ICD-10).

(2) Qualitative impairments in language. Autistic individuals often display a delay or a lack of development of communicative speech and language. The symptomatology can vary greatly from child to child. For example, autistic children without intellectual disability learn to speak relatively early and stand out sometimes because of unusual linguistic expressions. Another characteristic here is the lack of facial expressions, abnormal eye contact, and difficulties understanding or using gestures (APA, 2013; Kamp-Becker & Bölte, 2014; ICD-10).

(3) Restricted and repetitive behaviours or special interests. Autistic individuals often display a distinct fear of any change in their environment, resulting in very structured daily routines and ritualized behavior in daily activities. Interests are often monomaniacal and extraordinary in terms of subject matter (e.g. church towers, melting points of various metals), but also extraordinary in the intensity with which they are pursued. Repetitive movements such as slapping the hands in front of the eyes or rolling the head are also common (APA, 2013; Kamp-Becker & Bölte, 2014; ICD-10).

In the autistic individual, the symptoms of the classic autistic triad can manifest in various forms and degrees of severity. A conclusive assignment of a certain behavior to a superordinate symptom complex is not always possible and consequently both symptomatic categorizations and the corresponding diagnostic classifications are continuously discussed and changed. In the DSM-V for example, the main defining symptom complexes are grouped into only two domains with (1) persistent deficits in social communication and social interaction and (2) restricted, repetitive patterns of behavior, interests, or activities (American Psychiatric Association, 2013).Various attempts have been made to divide the very heterogeneous clinical picture of ASD into sub-groups. The main sub-classifications in ICD - 10 are: Childhood Autism (F84.0), which is defined by the onset of symptoms in the first 3 years of life and a delay in language development. Asperger syndrome (F84.5), which shares

the core features of childhood autism, but occurs without cognitive impairments and shows no delay in language development. Atypical autism (F84.1), which is defined by abnormalities not manifesting in all areas typical for autism or not occurring until after the age of three. Rett syndrome (F84.2) is a rare genetic neurodevelopmental disorder that causes a progressive loss of motor skills and language. Rett syndrome primarily affects females.

Contrastingly, in recognition of recent research showing that a valid and reliable subtype classification is often not possible or helpful, the DSM V has abandoned the previous DSM IV classification (into Childhood Autism, Asperger's Syndrome, Pervasive Developmental Disorder - Not Otherwise Specified (PDD-NOS) and Disintegrative Disorders) in favour of a dimensional system. With the objective of permitting more differentiated and individualised diagnoses, the DSM V merges the previous classifications into one category: Autism Spectrum Disorder (ASD). Within this dimensional system, diagnostic specifications allow for the consideration of individual characteristics - for example ASD with or without accompanying intellectual impairment or language impairment. In addition, the DSM V allows ASD to be specified by concomitant disorders such as attention deficit hyperactivity disorder (ADHD) (American Psychiatric Association, 2013; Lord *et al.*, 2018). The diagnostic classification, based on a spectrum concept, is widely accepted as it allows for a better representation of individual phenotypic heterogeneity and the different symptoms that people with ASD may display (Carrascosa-Romero and De Cabo-De La Vega, 2017). Another advantage of this classification system is that from a spectrum perspective, autistic traits are viewed as a continuum in which the actual diagnosis of ASD represents one extreme end of the spectrum. In this respect, the spectrum perspective can help to avoid a strict categorization and thus may avoid stigmatization (Kamp-Becker and Bölte, 2014). A further general classification is often made based on the presence or absence of intellectual impairments. It is estimated that about 32% of children with ASD have an intellectual disability whereas in the general population it is estimated to be about 1.1% of children (National Survey of Children's Health 2012, Centers for Disease Control and Prevention 2018). Here, the terms "low-functioning autism" and "high-functioning autism" (IQ > 70) are used to define groups within the autism spectrum based on their level of intelligence - though the usefulness of these terms have been called into question (Bottema-Beutel *et al.*, 2021).

1.2.2 Co-occurring conditions

The core symptoms of ASD rarely manifest solitarily. Estimates suggest that approximately 70% of ASD patients may be diagnosed with at least one co-occurring psychiatric disorder

(Simonoff *et al.*, 2008; Kamp-Becker and Bölte, 2014). The most common psychiatric co-diagnoses are attention deficit hyperactivity disorder (ADHD), social anxiety disorder as well as intellectual disability and depression (Simonoff *et al.*, 2008; Noterdaeme and Hutzelmeyer-Nickels, 2010). Other commonly reported co-occurring disorders include epilepsy, motor control difficulties, tics, sleep disturbances, gastrointestinal problems and metabolic disorders (Bauman, 2010; Noterdaeme and Wriedt, 2010). Genetic disorders such as Fragile X-Syndrome, tuberous sclerosis, Rett-syndrome and neurofibromatosis (Devlin and Scherer, 2012) or genetic metabolic disorders such as Phenylketonuria and Smith-Lemli Opitz syndrome are also found and considered in terms of ethiopathogenesis (Zafeiriou *et al.*, 2007). Furthermore, estimates suggest that 1 in 10 individuals diagnosed with ASD have exceptional, isolated abilities in disciplines such as art, arithmetic, music which are also referred to as savant skills. Although the link between ASD and savant abilities is not fully understood, it has been suggested, that these special abilities may be based on an extraordinary memory (Treffert, 2009).

1.2.3 Epidemiology

1.2.3.1 Prevalence

The prevalence of ASD is the subject of ongoing debate. In recent years since 2000, increased rates of diagnoses have been observed (Croen *et al.*, 2002; Fombonne *et al.*, 2020). Improved detection tools, greater awareness of the disorder, and changes in diagnostic criteria are discussed as major factors contributing to the observed increase in rates The APA (American Psychological Association) gives an approximate prevalence of 1% in the global population in the DSM V. Recent systematic reviews on the global point prevalence of ASD have reported a higher rate of 1.5% in developed countries (Lyall *et al.*, 2017) and slightly varying rates for the global prevalence of 1/150 children (Fombonne *et al.*, 2020), 62/10000 (Elsabbagh *et al.*, 2012), 1/86 (Wingate *et al.*, 2014). A systematic review by Baxter et. al, analyzing data from the Global Burden of Disease (GBD) initiative in 2010, estimated that there are approximately 52 million people on the autism spectrum worldwide, which corresponds to a population prevalence of 7.6/1000 or 1/132. Countering the notion of increased diagnosis rate, they reported stable rates within the last 20 years and no major regional differences in prevalence worldwide (Baxter *et al.*, 2015).

1.2.3.2 Sex distribution

Significant sex-dependent discrepancies in ASD have been consistently reported (Elsabbagh *et al.*, 2012; Baxter *et al.*, 2015; Lyall *et al.*, 2017; Fombonne *et al.*, 2020). The average male-to-female ratio of ASD is reported to be 4.3:1 (Fombonne *et al.*, 2020). When referring to Aspergers syndrome only, the ratio is reported to be as high as 10.8:1 (Gillberg *et al.*, 2006). It should be noted, however, that the gender ratio in ASD is subject of ongoing discussion and research in recent years points to a diagnostic gender bias resulting in lower diagnosis rates in women (Loomes *et al.*, 2017).

1.2.3.3 Economic impact

One DALY represents the loss of the equivalent of one year of full health. The age-standardized DALY rate for ASD per 100,000 persons worldwide in the year 2017 was estimated to be 62.4. This compares to eating disorders with 43.3, ADHD with 11.9 and major depressive disorder with 413.0 (Kyu *et al.*, 2018). Thus, the health loss in the general population caused by ASD is substantial. Consequently, ASD also poses important public health, policy and economic challenges. In the United States, for example, costs for supporting an autistic individual with intellectual disability over a lifetime have been estimated at $2.4 million and the mean annual costs associated with ASD in the United States have been estimated at $250 billion (Buescher *et al.*, 2014). By 2025, costs in the United States will reach $461 billion. This estimate takes into account, among other things, the rising prevalence, medical care and non-medical care expenditures, and costs associated with lost productivity of autistic persons and their caregivers (Leigh and Du, 2015). The substantial economic impact is particularly evident when compared to cost estimates of other common conditions. For example, in 2015, the annual cost of ASD in the US was estimated at $268 billion. In their 2015 publication Leigh and Du compare this estimate to annual cost estimates for diabetes $245 billion (2012), ADHD $205 billion (2010), stroke $36.5 billion (2010), and hypertension $46.4 billion (2010) (Doshi *et al.*, 2012; Leigh and Du, 2015; Yang *et al.*, 2018).

1.2.4 Diagnostic procedure and tools

It is generally agreed that a diagnosis of ASD should be made as early as possible in order to have the greatest benefit from supportive interventions (Kamp-Becker and Bölte, 2014; Reichow *et al.*, 2018). The usual diagnostic process involves initial reasonable suspicion by a caregiver, teacher, partner, or the individual him/herself, followed by a clinical screening with

established self-report questionnaires, scales, and checklists. If the screening confirms the suspicion, these diagnostic steps are followed by extensive standardized interviews focusing on early childhood development and a physical examination that also considers differential and co-occurring diagnoses. The final diagnosis is made after multiple interviews and interactions with multiple clinicians, reviewing all data (Kamp-Becker and Bölte, 2014). This makes the diagnostic process highly complex and multidisciplinary and involves data from anamnestic interviews with caregivers, interviews and interactions with the individual, collection of information from other sources such as school reports, cognitive assessments via standardized tests, and technical examinations including neuroimaging, electroencephalography, and genetic analyses (Lai *et al.*, 2014). There is a range of standardized diagnostic tools to evaluate whether the diagnostic criteria of the ICD-10 or DSM-V are met. Diagnostic tools aim to quantify impairments, particularly in the three main symptom complexes of ASD, and are based on behavioral observations, parents' reports of their children's early development, and neuropsychological testing. Established diagnostic instruments, such as those used in this study, include self-report questionnaires such as the Autism Spectrum Quotient (AQ) (Baron-Cohen *et al.*, 2001) and the Empathy Quotient (EQ) (Baron-Cohen and Wheelwright, 2004). Other more time-consuming methods include standardized interviews such as the semi-structured Autism Diagnostic Interview-Revised (ADI-R) (Lord *et al.*, 1994) and standardized behavioral observation protocols such as the Autism Diagnostic Observation Schedule (ADOS), which is considered the ‘gold standard’ for an autism diagnosis in childhood and adolescence (Lord *et al.*, 1989). Adaptations and recommendations for diagnostic procedures in adulthood are increasingly being established (S3 guideline of the DGKJP and the DGPPN, 2016)

1.2.5 Biomarkers of autism

Biomarkers are defined in medicine as measurable, objective parameters associated with a specific condition in an individual, which can thus serve as indicators for the presence or severity of a condition. As described above, diagnoses of ASD still rely mainly on subjective observation of behaviour by trained clinicians. In particular, autistic persons without intellectual impairments or obvious developmental delays are often not diagnosed until adulthood or may never receive a diagnosis. This is mainly due to the high complexity of the diagnostic process and “camouflage strategies” these individuals may have developed (Fombonne, 2020). Further complicating the diagnostic process in adults is the fact that there may be no one left to provide information about the patient's developmental history.

Moreover, common psychiatric comorbidities (e.g. anxiety disorders, depression) can further complicate the diagnosis of ASD (Lai and Baron-Cohen, 2015). Observer-independent diagnostic tools would thus be particularly helpful for the diagnosis of autism in adults. The identification of biomarkers, is therefore an important goal in ASD research and beyond (Bridgemohan *et al.*, 2019). Biomarkers could help to detect the disorder earlier, to make diagnoses more precise. Furthermore, it is hoped that the establishment of biomarkers will help to better identify individual factors with increased likelihood for ASD and to identify subgroups within the heterogeneous autism spectrum and ultimately to individualize supportive interventions. Yet, to date, there are no established reliable biomarkers for ASD (Lord *et al.*, 2018). Proposed markers include biochemical parameters (such as melatonin and serotonin in urine or plasma), mitochondrial and immunological alterations, as well as genetic and behavioral characteristics (Tordjman *et al.*, 2005; Wang *et al.*, 2011; Goldani *et al.*, 2014; Loth *et al.*, 2016; Varcin and Nelson, 2016; Bridgemohan *et al.*, 2019). Neuroimaging studies have proposed several different biomarkers using structural imaging methods, diffusion tensor imaging, resting-state functional MRI, and magnetic resonance spectroscopy (reviewed in Li, Karnath and Xu, 2017). Structural neuroimaging studies for example have suggested increased total WM and/or GM volume, decreased cerebellar volume, unilateral and/or bilateral amygdala enlargement, decreased corpus callosum volume, and hippocampal volume as brain structural biomarkers in autistic children (Li *et al.*, 2017). Studies that have used structural parameters in machine learning approaches to distinguish autistic from neurotypical brains in children have reported classification performances of up to 80% (Gori *et al.*, 2015) and 97.8% (Vidhusha and Anandhan, 2015) and positive predictive values of 81% in 6-12 month old children (Hazlett *et al.*, 2017). Despite these successes, studies have also shown that the transferability of biomarkers to routine clinical use is still very limited. One of the main criticisms here is the small sample size of the individual studies, given the high interindividual heterogeneity of ASD (Ecker, 2017; Pagnozzi *et al.*, 2018). Another problem is, given the high rate of co-occurring disorders in ASD, it is difficult to determine whether the potential markers are actually specific to ASD or whether they result from some other underlying alteration (Li *et al.*, 2017). Nevertheless, there is hope that imaging techniques may in future provide suitable biomarkers that can be used to identify clinical subgroups that can then ideally be supported with a more targeted therapy (**Figure 2**). There is general consensus that multimodal studies combining several parameters simultaneously have the best potential to establish an effective biomarker (Ecker *et al.*, 2010; Ecker, 2017; Li *et al.*, 2017; Pagnozzi *et al.*, 2018). Here, recent developments have for example indicated that an

observer-independent assessment of social behavior – by means of non-invasive motion tracking – could represent an important addition to quantitatively assess differences in social behavior (Lahnakoski *et al.*, 2020; Lahnakoski *et al.*, 2022).

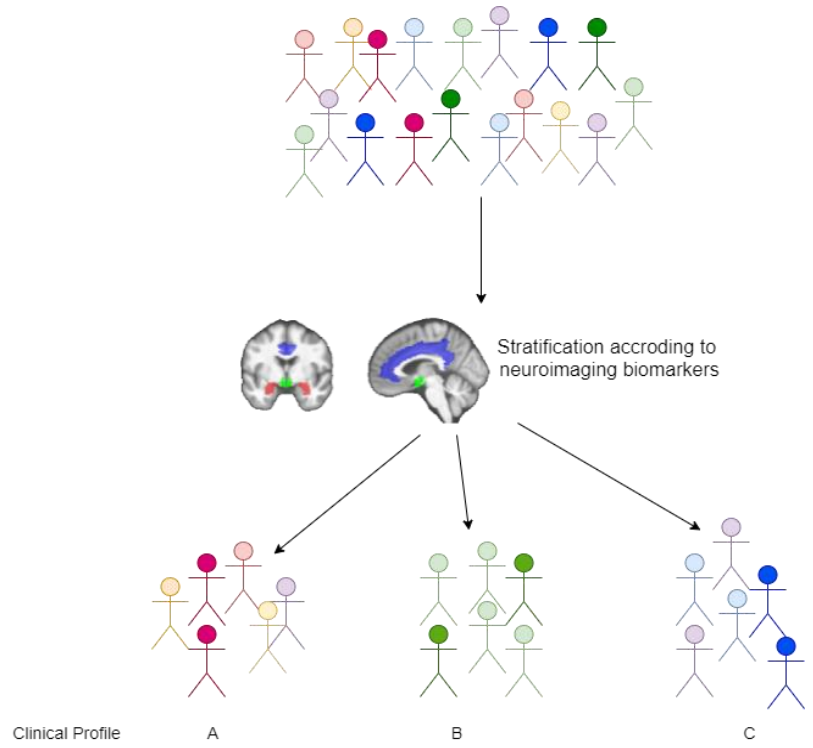


Figure 2 Schematic representation of a stratification of a heterogeneous patient population using for example neuroimaging biomarkers. In future, stratification based on such biomarkers and their combination could lead to a better definition of a clinical profile within the autism spectrum and, and optimally to treatment approaches adapted to these groups. Adapted from (Loth et al. 2016), created with the freely available software 'draw.io'.

1.2.6 Aetiology of ASD

To date ASD is considered a neurodevelopmental disorder that is accompanied by alterations in brain structure and function that manifest during early brain development. The aetiology underlying these alterations is heterogeneous and multifactorial and far from being fully elucidated. Underlying factors in the development of ASD have been identified primarily in genetics. However environmental factors, physical diseases, alterations in neuronal function, and biochemical alterations have also been identified as important influencing factors.

Although a final theory summarizing all influencing components is still lacking, a multifactorial concept (**Figure 3**) may help to get a better understanding of the neurobiology underlying ASD (Kamp-Becker and Bölte, 2014).

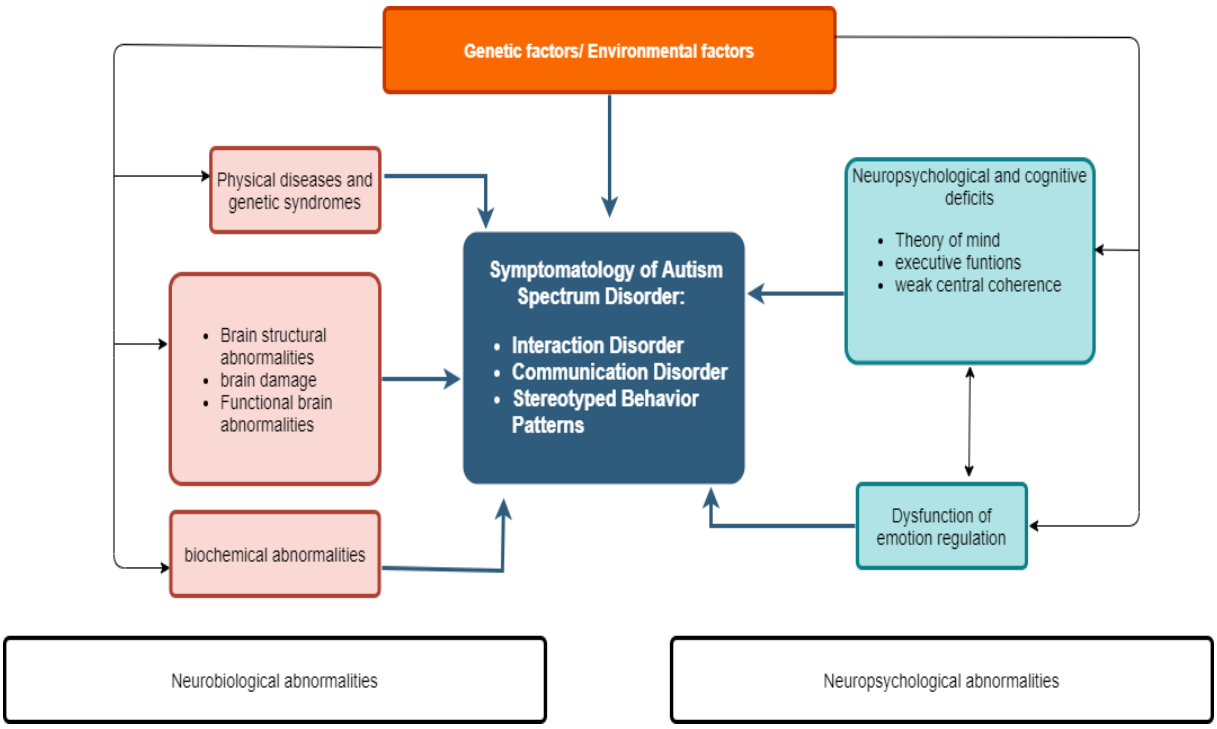


Figure 3 Schematic representation of the concept of a multifactorial etiology of ASD. Adapted from (Kamp-Becker and Bölte 2014), created with the freely available software 'draw.io'.

The genetic contribution to ASD has been strongly demonstrated in a multitude of studies. Twin studies, for example, showed a concordance of over 90% in monozygotic twins versus 10% in dizygotic twins, indicating a strong genetic component (Le Couteur *et al.*, 1995; Devlin and Scherer, 2012; Carrascosa-Romero and De Cabo-De La Vega, 2017). The overall heritability has been estimated to be about 50%. Family studies confirm that the higher the relatedness to an autistic individual in the family, the higher the individual likelihood for ASD (Sandin *et al.*, 2014). Reported recurrence rates for siblings are as high as 18.7% (Ozonoff *et al.*, 2011), which indicates an almost 20-fold increase in risk compared to the general population. However, it has been estimated that only in about 5-15% of autistic individuals can the aetiopathogenesis be attributed to known and circumscribed genetic alterations such as chromosomal rearrangements or monogenetic disorders such as the fragile X syndrome, tuberous sclerosis, Rett syndrome, and neurofibromatosis (Devlin and Scherer, 2012). De novo or inherited copy number variations (CNVs) of known genes associated with an

increased likelihood for ASD can be identified in 5-10% of the non-syndromic, idiopathic cases of ASD (Devlin and Scherer, 2012). Identified genes with an increased likelihood for autism are numerous and can be modulated by all kinds of genetic and epigenetic variations. Studies report a growing number of 103 genes and 44 genomic loci associated with autism, supporting the view that autism is a behavioral correlate of many different genetic disorders rather than a single entity (Betancur, 2011). Important genes have been identified, for example, in neuroligin genes 3 and 4 (NLGN3 and NLGN4), neurexin 1 (NRXN1), and SHANK3, which are known to be involved in synaptogenesis and the regulation of brain connectivity and development. Other genetic variations that have been linked to an increased likelihood for ASD include, for example, SNPs (single nucleotide polymorphisms) identified for oxytocin receptor (OXTR) genes, which are known to be involved in in reproduction, and social and emotional behaviors (LoParo and Waldman, 2015; Jurek and Neumann, 2018) . However, none of the individual identified loci appear to be responsible for more than 1% of cases (State and Šestan, 2012; Carrascosa-Romero and De Cabo-De La Vega, 2017). For the vast majority of idiopathic ASD cases, the genesis can be considered in terms of a multifactorial threshold model that includes multiple genetic variations and environmental factors (Carrascosa-Romero and De Cabo-De La Vega, 2017). Environmental factors in the context of ASD that have been discussed in recent years are for example advanced parental age, altered foetal milieu due to hormonal changes during pregnancy, medication during pregnancy, maternal obesity or/and diabetes, arterial hypertension as well as infections and subsequent immune activity but also various toxic exposures including smoking including the use of cannabis, alcohol and various metals (Bölte *et al.*, 2019; Sajdeya *et al.*, 2021). However, current evidence is insufficient to conclude that perinatal factors causally contribute to ASD in the newborn. Evidence for a potential role of alterations of the OXT system in the genesis of ASD is reported separately below.

1.2.6.1 Neuropsychological etiological concepts

Various neuropsychological concepts have been put forward to explain the underlying mechanisms of the symptomatology in ASD. Although a comprehensive description of all the theories and the often very sophisticated experiments that have contributed to their refinement would go beyond the scope set here, some of the most influential concepts will be briefly described. Three important and influential neuropsychological concepts regarding the understanding of the "autistic phenotype" are the (1) Theory of Mind concept , the (2) Theory

of Executive Dysfunction, and the (3) Theory of Weak Central Coherence (Rajendran and Mitchell, 2007).

(1) The term Theory of Mind (ToM) describes the ability to attribute mental states or conscious processes (e.g., desires, intentions, beliefs, opinions) to oneself and to other persons. Assumptions about a certain mental state of another person are essential to understand and predict that person's behavior (Novotný and Polonský, 2011). A seminal study that led to the assumption that ToM may in fact be central to understanding of autism used Wimmer and Perner's puppetry paradigm (Baron-Cohen *et al.*, 1985). In this paradigm a story is presented about a puppet that has a belief about the location of a marble, but that belief does not match the actual location. The participant is then asked to make a judgment about where the doll is likely to look. To give the correct answer, the participant must infer the mental state of the puppet (I think it thinks). In their study from 1985 Baron-Cohen and colleagues found that 80 % of the autistic children failed to make this transfer (Baron-Cohen *et al.*, 1985). Difficulties with tasks involving ToM have since been suggested to underlie the social impairment in autistic individuals (Baron-Cohen *et al.*, 1985; Baron-Cohen, 2000; Rajendran and Mitchell, 2007).

(2) Another theory is that autism could be explained as a deficit in so-called executive functions (Ozonoff *et al.*, 1991; Ozonoff and Jensen, 1999). The term "executive functions" is used to refer to a number of complex different cognitive functions, which underly the ability to plan actions, develop strategies, and respond flexibly to external circumstances including those of a social nature. Anatomically, these functions have been closely associated with the frontal lobe and in particular the prefrontal cortex PFC (Barkley, 2001). It has been suggested that characteristic symptoms of ASD such as problems with shifting attention, repetitive behaviors and lack of impulse control could be attributed to a dysfunction of these executive functions. In this regard, one of the strengths of this theory has been seen in its ability to explain the non-social motor symptoms such as hand flapping and chair rocking (Rajendran and Mitchell, 2007).

(3) A third important neuropsychological concept, the Weak Central Coherence theory, has been attributed the strength to also explain some of the perceptual abilities found in autism, such as the unusual attention to detail (Frith and Happé, 1994; Rajendran and Mitchell, 2007). In its basic outline, this theory assumes that in neurotypical individuals, information is processed in such a way that the overall meaning can be grasped quickly. This tendency to interpret information in a context-bound way is also

called "central coherence". Correct interpretation of a facial expression, for example, requires forming a "coherent picture" of the different parts of the face (i.e., eyes, mouth, forehead), but also of the situation in which this facial expression is shown (Kamp-Becker and Bölte, 2014). Autistic individuals, on the other hand, according to the theory, would process information in a more detail-oriented manner. In the case of facial expression interpretation, for example, an autistic individual would perceive the individual regions of the face (eyes, mouth) in isolation, which makes the correct interpretation of a facial expression difficult. A weaker central coherence, on the other hand, allows one to perform well on tasks where the "big picture" is more distracting than helpful. Evidence for this theory came primarily from perceptual studies using the Embedded Figures test (Shah and Frith, 1983) or Block Design paradigm (Shah and Frith, 1993), in which autistic individuals generally performed better than non-autistic individuals. This is particularly interesting because this theory highlights not only the deficits but also the strengths of the "autistic way of thinking." Since both, strong and weak central coherence have their advantages and disadvantages, Uta Frith stated that the tendency towards one of the two types of perception can be regarded as a "cognitive style", which occurs to varying degrees in the population (Frith and Happé, 1994). Closely related to the concept of weak central coherence is the theory of "context blindness" of ASD. This theory could explain phenomena such as difficulty in understanding figurative language or irony that are highly contextualized. It has also been suggested that the lack of top-down modulation and filtering of perceptions through contextualization eventually leads to stimulus overload (Vermeulen, 2015).

1.2.7 Supportive interventions

Although there have been occasional reports of individuals who no longer meet ASD criteria over time (Helt *et al.*, 2008) it is usually considered as a life-long persistent condition and no causally efficacious “cure” is available to date. As mentioned at the beginning, the concept of “curing” ASD has been increasingly criticized because it implies that autism is not part of normal human variation, but considered as a disease (Broderick and Ne’eman, 2008). However, therapeutic interventions to support autistic individuals exist and play an important role. An ASD diagnosis does not entail a standard therapy; rather, the identification of concrete individual everyday difficulties is central to the development of therapy goals. General therapy aims are the better individual independence and live quality through improvement of social skills and communication, treatment of comorbidities, support of the

individual and their families in daily live and generally creating an autism-friendly environment (Lai *et al.*, 2014; Lord *et al.*, 2020). Given the appropriate environment, some individuals even manage completely without further interventions (Freitag, 2022). In particular there is growing evidence that early intervention can lead to favorable outcomes and reduce further progression of symptoms (Fein *et al.*, 2013; Zwaigenbaum *et al.*, 2021). Supportive interventions are usually multidisciplinary and include mainly behavioural and educational training, while drugs play a minor role so far (Lai *et al.*, 2014). In particular the active involvement of families and/or caregivers and the treatment of accompanying illnesses such as epilepsy, gastrointestinal symptoms and sleep disorders are considered to be essential (Zwaigenbaum *et al.*, 2021). Pharmaceutical therapies including antidepressants and atypical antipsychotics, mood stabilizers have so far focused on repetitive behaviors and comorbidities like depression and hyperactivity (Siegel and Beaulieu, 2012; Farmer *et al.*, 2013; Sharma *et al.*, 2018). Importantly, there is now growing evidence for OXT application as a supportive agent for the qualitative social impairments in autistic persons (Young and Barrett, 2015). Since the role of OXT and its potential benefits in ASD is one of the central starting points for this thesis, the current state of research on this topic is reported in more detail separately below (section 1.4.4).

1.3 Neuroanatomy of Autism

With increasing evidence of diverse factors influencing the development of ASD and the heterogeneous manifestation of symptoms, it is also becoming clear that ASD cannot be understood as a focal impairment of one single brain region, but must be seen as the result of a complex reorganization of the brain during development (Lord *et al.*, 2018). Since the heterogeneity of ASD is evident not only between individuals but also within an individual across the lifespan, the identification of a neuroanatomical profile of autism is particularly difficult and complex. It is therefore hardly surprising that this complexity is also mirrored in highly diverse and sometimes inconsistent results that neuroanatomical research has presented in recent years. Furthermore, it is still unclear how the reported differences in brain structure are linked to the behavioural differences (Ecker *et al.*, 2012). Nevertheless, especially MRI studies of the brain in vivo have greatly advanced our understanding of neuroanatomical basis of the disorder (Ecker, 2017). Structural differences in the brains of autistic individuals could in some cases be demonstrated already during and even before the initial onset of autistic behavior, which is typically around the second year of life (Ecker *et al.*, 2015; Hazlett *et al.*, 2017). It is therefore thought that the neural alterations underlying these early changes

may also underlie ASD (Courchesne *et al.*, 2011). This notion also explains why most brain structural studies tend to focus primarily on childhood and studies focusing on adults tend to be underrepresented (Murphy *et al.*, 2011). Importantly, however, it was also pointed out that studies in adults are essential to better understand the time course of neuroanatomical changes and how best to support autistic adults (Courchesne *et al.*, 2011).

Since the work reported in this thesis builds on the current understanding of neuroanatomy of ASD, some of the more consistent brain morphological findings are summarised in the following sections.

1.3.1 Where would one expect to find neuroanatomical differences?

First, in order to locate and interpret possible brain anomalies in autism, it may be helpful to ask where one would expect to find differences based on the symptomatology of ASD. Based on lesion studies, animal studies, and structural and functional MRI studies, the basic neural systems and brain regions likely to be most involved in the core autistic symptoms have already been fairly well described (Adolphs, 2001; Amaral *et al.*, 2008; Blakemore, 2008; Ecker, 2017). A summary of the brain regions most likely to be involved in social impairment, differences in communication, and repetitive behavior was presented by Amaral and colleagues (2008) (**Figure 4**).

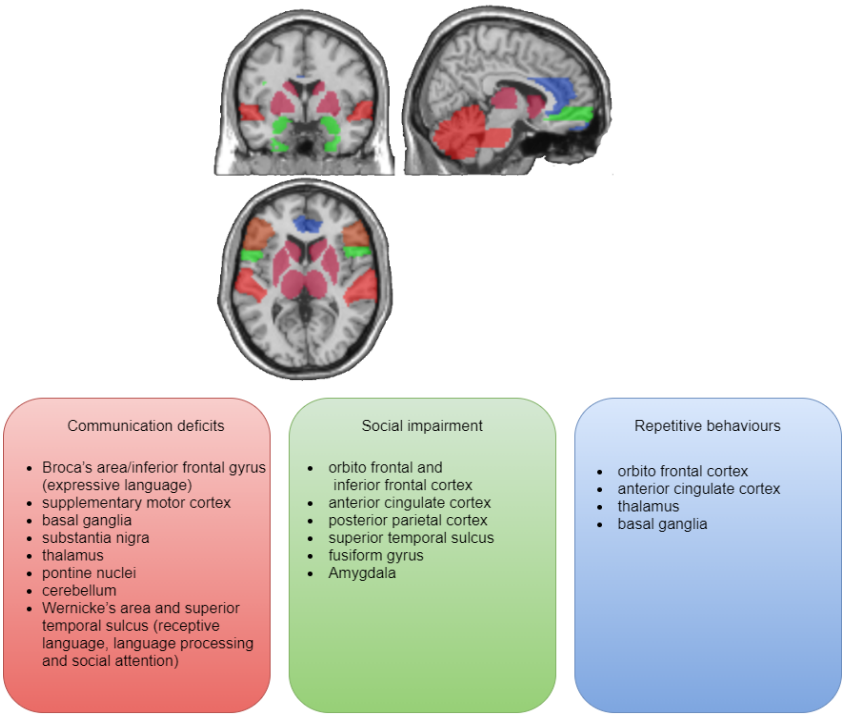


Figure 4 Brain areas (according to Amaral et al. 2008) involved in processing the three core symptoms of ASD: differences in language and communication, social impairment and repetitive and stereotypical behaviours. Note the colour coding of the individual symptom complexes (red, green, blue) as in the schematic brain image above. Areas that are mentioned in more than one symptom complex are colour overlapped in the brain image. Due to the sectioning, not all listed

1.3.2 Evidence for brain structural differences in ASD

A wide range of brain regions have been reported as structurally different in ASD, but there are often inconsistencies between studies. It is assumed that the discrepant findings between studies are partly linked to differences in age and IQ of the samples in the individual studies (Stanfield *et al.*, 2008), but also due to the heterogeneity of ASD itself (DeRamus and Kana, 2015). However, some findings at the brain structural level have been found to be more robust than others and are summarised briefly in the following sections.

Whole brain volumes

An often reported phenomenon at the structural level in ASD is an overgrowth of whole brain volume in early brain development (Courchesne *et al.*, 2001; Sparks *et al.*, 2002; Schumann *et al.*, 2010; Ecker *et al.*, 2015; Ecker, 2017; Hazlett *et al.*, 2017). This phenomenon seems to be limited to childhood and has been observed up to the age of 12 (Aylward *et al.*, 2002). Overall, studies suggest that the total brain volume is reduced immediately after birth, increases excessively in early childhood and reaches about normal size in adolescence/adulthood (Redcay and Courchesne, 2005). Furthermore, a large longitudinal study by Lange et al. (2015), who examined 100 autistic persons aged 6 to 35 years, suggested that there is an atypical decrease in brain volume, which continues into adulthood and leads to an overall lower brain capacity around the age of thirty. The authors concluded that this supports the hypothesis that late neurodevelopment in autism is also atypical (Lange *et al.*, 2015). A similar pattern has been shown for head circumferences, with smaller than typical circumferences at birth, a rapid increase to the 95% percentile at 6-14 months of age, followed by slower than typical growth (Courchesne *et al.*, 2003). Increased head circumferences have also been reported to be present into adulthood, which has been interpreted as a remnant of infantile overgrowth of the brain (Aylward *et al.*, 2002). Overall, a growth pattern characterized by early enlargement, slower growth in adolescence, and speculatively an accelerated decline potentially reflecting a general process of degeneration, loss of neurons and connections is hypothesized (Courchesne *et al.*, 2007).

While there is growing evidence of overall atypical brain development in autistic children and adults, the region- and tissue-specificity of this phenomenon is less clear. Differences in total brain volume, for example, were attributed to disproportionate WMV in a group of autistic boys aged 7-11 years (Herbert *et al.*, 2003), while others found increased mean GMV and ventricular volume in a sample of 7-15 year olds (Palmen *et al.*, 2005). While Carper and colleagues reported localized enlargement of large brain areas in the frontal cortex (Carper

and Courchesne, 2005), others reported no site specificity (Riddle *et al.*, 2017). Lange et al. (2015) reported a persistent increase in ventricular volume across all age groups (6-35 years) and a significant decrease in mean cortical WMV in their longitudinal study (Lange *et al.*, 2015). One of the largest morphometric studies in recent years examining 1.571 autistic persons (age range 2–64 years) found larger total intracranial volumes, and larger total GMV (Van Rooij *et al.*, 2018).

Cortex

The frontal and prefrontal cortices are the most anterior parts of the brain and have been reported to be strongly involved in higher functions such as decision making, emotion, social behaviour, communication, moral and beliefs (Donovan and Basson, 2017; Huggenberger *et al.*, 2019). As such, the frontal cortex is another focus of ASD research. Indeed, a wide range of differences have been reported in this region in autistic compared to non-autistic individuals. Observations include atypical growth patterns, cortical thickness, and changes in connectivity (Donovan and Basson, 2017). The current assumption is that certain areas in the frontal cortex increase in size early in brain development, followed by slower growth compared to non-autists with intersecting growth curves at 5-9 years (Carper *et al.*, 2002; Carper and Courchesne, 2005; Courchesne *et al.*, 2011; Ecker *et al.*, 2015). It has also been reported that hyperplasia in the cortex of autistic children appears to follow an anterior to posterior gradient, with the frontal lobe enlarged by up to 20% and the occipital lobe approximately typical in size (Carper *et al.*, 2002), however competing findings exists (Palmen *et al.*, 2005). Meta-analyses using, for example, anatomical likelihood estimation (ALE) have attempted to identify commonalities among the multitude of structural findings of recent years. They have reported decreases in GM and WM in temporal regions and GM increases in frontal and anterior-temporal regions (Deramus and Kana 2015) but also differences in occipital regions as well as subcortical structures such as the basal ganglia (Nickl-jockschat et al. 2012). Reviewing structural MRI studies from 1966 until 2003 Brambilla et al. reported increased total brain volume, increased volume of the parieto-temporal lobe and cerebellar hemisphere volumes to be the best replicated findings (Brambilla *et al.*, 2003).

With regard to the adult brain only, there are even fewer consistent findings. An accelerated reduction of cortical matter from adolescence to late middle age is discussed (Courchesne *et al.*, 2011; Ecker *et al.*, 2015). Hadjikhani et al. (2006) reported cortical atrophy in adults and adolescents, which was associated with symptom severity. More specifically, the group

reported that this atrophy was most evident in the inferior frontal cortex, inferior parietal lobule, and superior temporal sulcus, which according to the authors may point to a alterations of the mirror neuron system (MNS) (Hadjikhani *et al.*, 2006). Other VBM studies in adults have reported similar results of reduced volume in the frontal lobe and altered overall connectivity (Schmitz *et al.*, 2007; Murphy *et al.*, 2011; Ecker *et al.*, 2012).

Cerebellum

Cerebellar differences were among the first anatomical regions found to be structurally different in autism (Donovan and Basson, 2017). While the cerebellum was long thought to be primarily involved in motor processing and coordination, studies in recent years have highlighted its role in cognitive and affective processes (Stoodley, 2012; Becker and Stoodley, 2013). The most consistent findings reported in a meta-analysis of 17 VBM studies by Stoodley at al. (2014), include reduced GMV in the inferior cerebellar vermis (lobulus IX), left lobulus VIIIB, and right crus I. A more recent meta analysis however reported no significant difference in cerebellar volume between autistic and non-autistic persons (Traut *et al.*, 2018). Yet another meta-analysis which was conducted using only studies with high functioning ASD patients reported a decrease in volume with focus on cerebellar vermis IX/VIIIb (DeRamus and Kana, 2015). In contrast to the reported decreases in regional cerebellar subregions, the overall size of the cerebellum was reported to be increased in the majority of autists, although cerebellar hypoplasia has also been reported in some cases (Courchesne *et al.*, 1994; Amaral *et al.*, 2008; Stanfield *et al.*, 2008).

Another interesting comparably well-replicated finding in the cerebellum has been reported in regard to Purkinje cells. Purkinje cells are GABAergic cells in the cerebellar cortex and the major output neurons (Huggenberger *et al.*, 2019). A reduction in number and size of these cells has been reported in multiple histopathological post-mortem studies. However, it was pointed out that despite the seemingly consistent results from several studies, comorbidities such as seizures and medication need to be taken into account, especially in post-mortem studies, and therefore a final evaluation of this phenomenon is still pending (Amaral *et al.*, 2008; Donovan and Basson, 2017).

Summarising the most consistent findings, it can be said that the cerebellum in ASD mainly shows an increase in volume, a decrease in grey matter, especially in crus cerebellaris I, and a decrease in Purkinje cells. It has been pointed out however, that there are still great differences and many discrepancies in the studies on the role of the cerebellum in ASD, which

again may reflect the great interindividual heterogeneity in ASD and, thus, requires further investigation (Donovan and Basson, 2017).

Amygdala

The amygdala is an almond-shaped group of cells deep in the temporal lobe that can be anatomically divided into several subregions (Huggenberger *et al.*, 2019). In particular, the basolateral portion of the amygdala plays an important role in emotional processing and is well documented as one of the key regions of the so-called "social brain" (Baron-Cohen *et al.*, 2000; Davis and Whalen, 2001; Blakemore, 2008). Using fMRI, Baron-Cohen et al. (2000) found reduced activation of the amygdalae in autistic individuals compared to controls when asked to look into the eyes of another person's image. Recognizing the potentially important role of the amygdala in social processing in ASD, they proposed an "amygdala theory of autism" (Baron-Cohen *et al.*, 2000). At the structural level, amygdalean volume has been associated with anxiety/depression scores in autistic children (Juranek *et al.*, 2006). Although still not conclusive, a commonly reported finding in studies and reviews of structural studies in autistic people is an early enlargement of the amygdala followed by a decrease. In turn, studies focusing on older subjects have found no difference or even smaller volumes of the amygdala (Abell *et al.*, 1999; Schumann *et al.*, 2004; Schumann and Amaral, 2006; Amaral *et al.*, 2008; Donovan and Basson, 2017). It has been pointed out that the conflicting results regarding amygdalae volume may be due to rather poorly characterized samples in most studies, which have both children and adults and individuals with and without intellectual disability in their cohorts (Brambilla *et al.*, 2003). At the microscopic level, decreased cell size and increased cell packing density, as well as a decrease in the number of neurons throughout the amygdala have been discussed (Bauman and Kemper, 2004; Schumann and Amaral, 2006).

1.4 Oxytocin

OXT is a cyclic nonapeptide (i.e. consisting of nine amino acids) with the chemical formula $C_{43}H_{66}N_{12}O_{12}S_2$. In recent years, research on OXT has grown exponentially. It owes this popularity to its various important functions as a neurotransmitter and neuromodulator, which have been demonstrated in humans and animals (Landgraf and Neumann, 2004; Bakermans-Kranenburg and van IJzendoorn, 2013).

1.4.1 Neuroanatomy of the oxytocin system

OXT and the related hormone vasopressin (VP) are synthesized in the hypothalamus (HTH). The HTH/neuropituitary system represents the largest source of OXT. Other peripheral sources of OXT in the body are the ovaries, corpus luteum, and testes (Gimpl and Fahrenholz, 2001). In the HTH, OXT is synthesized in the magnocellular and parvocellular neurons of the supraoptic nucleus (SON) and the paraventricular nucleus (PVN). OXT synthesized in the magnocellular neurons is transported axonally via the hypothalamo-hypophysial tract to the posterior lobe of the pituitary gland, where it is stored and released into the bloodstream. The OXT-producing parvocellular divisions of the two nuclei (mainly PVN) also project to other parts of the brain such as the hippocampus, amygdala, and nucleus accumbens, to which OXT is directly axonally transported (Huggenberger et al. 2019). In addition, release occurs also in the form of neurosecretion directly from dendrites and somata (Swanson and Sawchenko, 1980; Sabatier *et al.*, 2003; Landgraf and Neumann, 2004) (**Figure 5**). It has further been reported that OXT neurons are particularly abundant in the immediate vicinity of the third ventricle and are sometimes in direct contact with CSF. OXT is therefore thought to be not only axonally transported but also distributed throughout the CNS by direct secretion into the cerebrospinal fluid of the third ventricle (Gimpl and Fahrenholz, 2001; Landgraf and Neumann, 2004; Jurek and Neumann, 2018). Using immunohistochemical techniques, optogenetics, and viral vectors, experiments in human post-mortem studies and animals have shown that the OXT system projects extensively to a variety of different brain areas. Most notably, in humans, in addition to projections to the neurohypophysis, projections from both nuclei have been reported to various forebrain and prefrontal regions, the anterior olfactory nucleus, the nucleus accumbens, the septal nuclei, the hippocampus, and the medial and central amygdala (Jurek and Neumann, 2018). Another technique to identify the neuroanatomy of the OXT system is to detect and quantify regions of high oxytocin receptor (OXTR) concentration. Using OXTR antibodies, quantification of mRNA, reporter genes and advanced autoradiographic methods, the OXTR has been detected throughout the brain. Interestingly, OXTR distribution has been shown to be not only region- and tissue-specific, but also species- and sex-specific (Gimpl and Fahrenholz, 2001; Jurek and Neumann, 2018). The most prominent regions in rodent brains that express OXTR are the hypothalamus, prefrontal cortex, hippocampus, and amygdala (Jurek and Neumann, 2018). In humans, in addition to these regions, OXTR has been found to be expressed primarily in regions of the limbic system (Insel and Young, 2001; Jurek and Neumann, 2018).

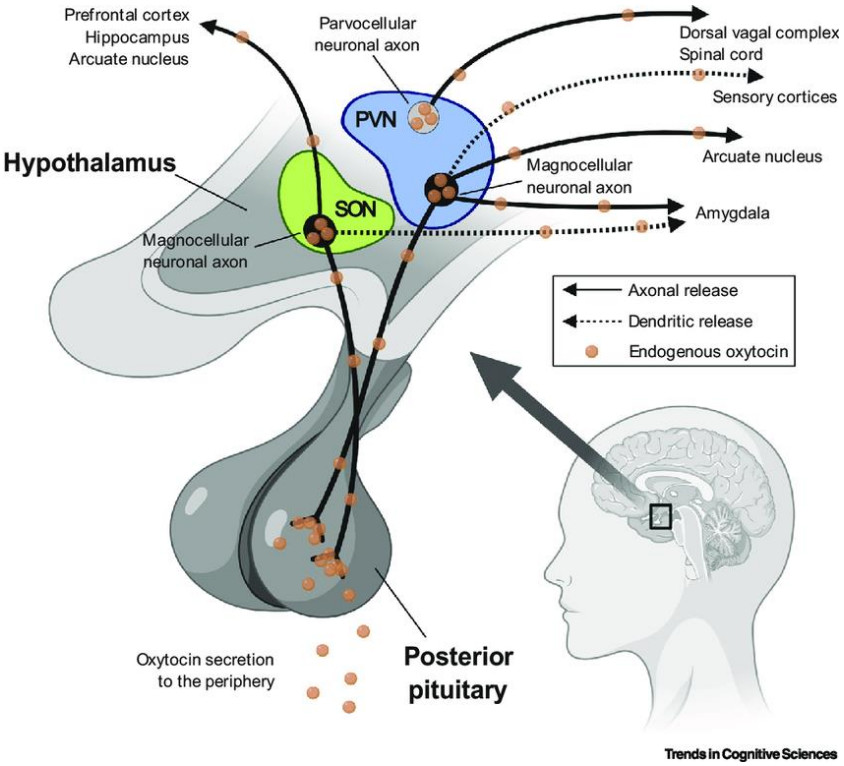


Figure 5 Oxytocin Production and Secretion within the Brain. In the HTH, OXT is synthesized in the magnocellular and parvocellular neurons of the supraoptic nucleus (SON) and the paraventricular nucleus (PVN). OXT synthesized in the magnocellular neurons is transported axonally via the hypothalamo-hypophysial tract to the posterior lobe of the pituitary gland, where it is stored and released into the bloodstream. Dendritically released oxytocin (dashed arrows) and the The OXT-producing parvocellular divisions of the two nuclei (mainly PVN) also project to other parts of the brain such as the hippocampus, amygdala, and nucleus accumbens and arcuatus. This figure was taken from (Quintana and Guastella, 2020) *with permission.*

1.4.2 OXT function

Taking its name from the Greek words ‘oxys’ and ‘tokos’, the meaning of which is ‘rapid birth’, OXT is known for its uterotonic activity and has long been used as a therapeutic agent during labor and delivery and to prevent post partum hemorrhage (Dale, 1906; Magon and Kalra, 2011). It has also been shown to be the responsible hormon for the milk-ejecting activity of the posterior pituitary gland during lactation (Ott and Scott, 1910). Current research further recognises OXT for a wide range of effects in the peripheral as well as in the central nervous system. Since the sequencing of the hormone by Nobel Prize-winning Vincent du Vigneaud 1955, OXT research has revealed many functions of the peptide as a peripheral and central neurohormone, neurotransmitter, and neuromodulator (Kiss and Mikkelsen, 2005). Among the best replicated phenomena are its antihypertensive, as well as hypertensive effects, inhibition of urinary excretion in humans, antidiuretic effects, and function in orgasm (Magon and Kalra, 2011). More recent research has also suggested a role for OXT in the

regulation of appetite and weight gain (Blevins and Baskin, 2015). However, the still growing popularity of OXT in research and an exponential increase in animal and human studies in recent decades is mainly due to its important function in social behavior (**Figure 6**) (Bakermans-Kranenburg and van IJzendoorn, 2013).

1.4.3 OXT and social behaviour

Animals

Homologs of OXT are also found in various species such as worms, insects, and vertebrates, indicating a high conservation of the corresponding genes for at least 700 million years. Interestingly, the homologs of OXT in these species are produced in brain areas that are similar in their neurosecretory function to the human HTH and appear to have a comparable general role in facilitating and modulating species-specific social and reproductive behaviors (Donaldson and Young, 2008). Animal studies in voles, sheep and chicks have shown regulatory effects of OXT in control of stress and anxiety (Windle *et al.*, 1997; Reed *et al.*, 2013), in pair bonding between mothers and infants (Francis *et al.*, 2000; Donaldson and Young, 2008), in mating pairs (Insel *et al.*, 1995; Insel and Young, 2001) and in general affiliative behaviour like trust (Baumgartner *et al.*, 2008) but also in protective aggression (Bosch, 2013). In regard to the many different functions of OXT, early animal studies of monogamous prairie voles and polygamous mountain voles highlighted the importance of the species-specific distribution of OXT receptors in specific brain regions (Insel and Shapiro, 1992; Insel and Hulihan, 1995) and species specific genetic variation in the OXT system (Donaldson and Young, 2008). This is one of the reasons why a direct transfer of results from animal studies to humans is only possible to a limited extent. While a variety of sophisticated experimental approaches, such as intracerebroventricular infusions, have been used in animal studies, intranasal administration of OXT is a common approach in human studies. Here, in regard to OXT application studies in humans, it should be noted that the distribution mechanism of intranasal OXT application, especially with regard to the blood-brain barrier, is still not fully elucidated (Striepens *et al.*, 2013; Leng and Ludwig, 2016).

Humans

Following a seminal study in humans showing that externally applied OXT combined with social support reduced anxiety and stress levels (Heinrichs *et al.*, 2003), a variety of studies have demonstrated behavioral effects of OXT in adult individuals (Kendrick *et al.*, 2017). For example, studies have shown increased trust even in a more complex economic environment

after OXT application (Kosfeld *et al.*, 2005) and increased levels of trust especially towards group members and improved emotional recognition (Van IJzendoorn and Bakermans-Kranenburg, 2012). Likewise, subjects receiving intranasal administration of OXT have shown more positive judgments toward others and more positive communication behaviors the higher the OXT concentration (Ditzen *et al.*, 2009; Theodoridou *et al.*, 2009) as well as improved mentalizing abilities (Feeser *et al.*, 2015). Neuroimaging studies, especially functional MRI studies, have also found evidence for the involvement of OXT in the "social brain" in humans. For example, the anxiolytic effects of OXT have been associated with decreased activation of the amygdala (Kirsch *et al.*, 2005). Other studies have shown that when OXT is administered, people judge the perceived touch of their romantic partner as more pleasurable than that of a stranger, which was also reflected in the activation level of the nucleus accumbens and the anterior cingulate cortex (Kreuder *et al.*, 2017). Genetic research has identified genes in the OXT system associated with increased likelihood for social dysfunction. Certain polymorphisms in the OXTR, such as rs2254298 and rs1042778, were associated with lower OXT levels and less affiliative behavior by parents towards their children (Feldman *et al.*, 2012) and between couples (Schneiderman *et al.*, 2014). Similarly, the CD38 allele rs3796863, which encodes an enzyme involved in OXT release in the HTH (Jin *et al.*, 2007), has been associated with reduced parent-child interactions (Feldman *et al.*, 2012).

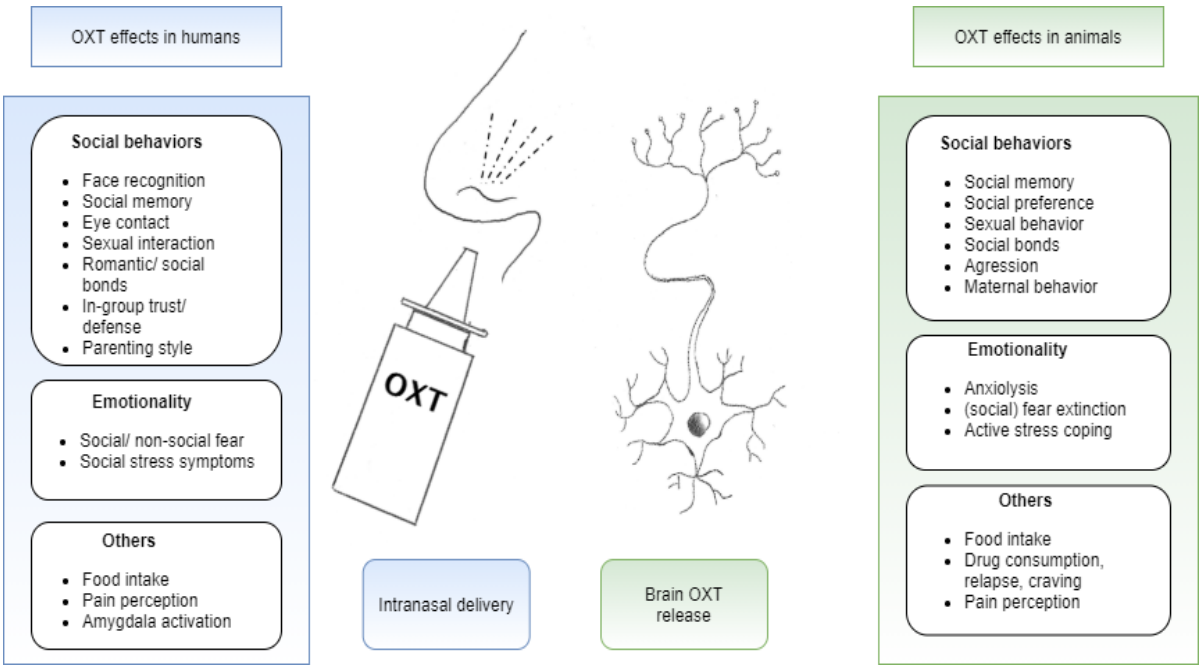


Figure 6 Overview of reported effects of OXT on social behaviour, emotionality and other functions in humans and animals. Figure adapted from (Jurek and Neumann, 2018)*, created with the free software 'draw.io' and 'Gimp'.*

1.4.4 OXT and Autism spectrum disorder

The effects of OXT in non-autistic persons, particularly in terms of improving social skills, have made the neuropeptide interesting for autism research. In some of the first studies to apply OXT to autistic individuals, OXT was shown to have beneficial effects on repetitive behavior (Hollander *et al.*, 2003) and on social cognition (Hollander *et al.*, 2007). Subsequently, a number of studies have used single-dose OXT administration in autistic persons, showing improved performance in the Reading the Mind in the Eyes Task (Guastella *et al.*, 2010), increased feeling of trust and more social interaction and prolonged gazing time into the eyes of a fictive game partner (Andari *et al.*, 2010), which has also been shown using eye-tracking paradigms (Auyeung *et al.*, 2015; Kanat *et al.*, 2017). These findings are also in line with a study showing significant improvement in social skills (measured by the Social Responsiveness Scale (SRS)) in autistic children receiving OXT compared to a placebo group (Parker *et al.*, 2017). Interestingly, in this study, pre-treatment OXT levels could also be used as a predictor of treatment outcome with intranasal OXT. Children with the lowest pre-treatment OXT levels showed the greatest social improvement (Parker *et al.*, 2017). Placebo-controlled studies have also examined the effects of long-term OXT use and have reported significant, yet modest, improvements in emotion recognition and social interaction (Tachibana *et al.*, 2013; Yatawara *et al.*, 2016), while others have reported no significant changes compared to placebo (Dadds *et al.*, 2014; Guastella *et al.*, 2015). The results of a large randomized, double-blind, placebo-controlled clinical trial (ClinicalTrials.gov Identifier: NCT01944046) remain to be seen. As mentioned above VP is a neuropeptide closely related to OXT and produced in the same nuclei. Similar to OXT, VP is thought to be involved in the regulation of social behavior, partly through concerted effects and partly through opposite effects compared to OXT (Stoop, 2012; Borie *et al.*, 2021). In this context, balovaptan, an orally administered selective vasopressin 1a receptor antagonist, was tested as a therapeutic agent to improve socialization and communication in autistic children and adolescents, but without significant effects compared to a placebo group (Hollander *et al.*, 2022).

Functional MRI studies that used intranasal OXT application in autistic individuals reported that improvements in facial emotion recognition were associated with changes in amygdala activation (Domes *et al.*, 2013; Domes *et al.*, 2014). Increased brain activity in limbic areas during social but not non-social judgments (Gordon *et al.*, 2013) and context-specific behavioral improvements associated with changes in amygdala and hippocampal activation have also been reported (Andari *et al.*, 2016). Overall, the amygdala in particular and areas

within the prefrontal and temporal cortex appear to be most consistently modulated by OXT application in autistic individuals (Domes *et al.*, 2013; Domes *et al.*, 2014; Kanat *et al.*, 2014). Of note, typical neuroimaging studies report group-specific mean differences in activity and due to small sample sizes often cannot inform us about interindividual differences and their relationship to intervention responses (Bartz *et al.*, 2011).

Based on these observations, research in recent years has also sought to determine whether and how an differences in the OXT system could be a factor in the pathogenesis of ASD (Modahl *et al.*, 1992; Quattrocki and Friston, 2014; Borie *et al.*, 2021). Several SNPs of OXTR have been associated with ASD (LoParo and Waldman, 2015), as have certain OXTR gene methylation patterns (Kumsta *et al.*, 2013), suggesting a genetic or epigenetic link between OXT and ASD. Peripheral OXT level measurements in autistic compared to non-autistic individuals have yielded inconsistent results, with OXT levels being lower (Modahl *et al.*, 1998), higher (Jansen *et al.*, 2006) or indifferent (Taurines *et al.*, 2014), while the latter study also reported a correlation of OXT plasma levels with autistic symptomatology (Taurines *et al.*, 2014). A meta-analysis from 2016 did not confirm OXT and VP levels to be significantly altered in ASD (Rutigliano *et al.*, 2016), while another more recent meta-analysis reported OXT levels compared to neurotypical controls to be significantly lower in autistic children but not in adults (John and Jaeggi, 2021).

Criticisms of research on OXT in ASD have been primarily that findings are often very inconsistent and that studies are underpowered, and finally that expectations for the neuropeptide in general may be too high (Preti *et al.*, 2014; Leng and Ludwig, 2016; Lord *et al.*, 2020). In conclusion, the role of OXT both as a therapeutic and as a pathogenetic factor in ASD has not yet been conclusively determined and needs further investigation.

1.4.5 Where would one expect structural abnormalities related to the OXT system in ASD

Few studies have directly investigated the relationship between OXT and the brain at a structural level, let alone in the brains of autistic individuals. Given the rather limited literature, it seems helpful to compare structural findings from ASD research on the one hand and OXT research on the other for commonalities. In this context, three regions stand out: the hypothalamus, the amygdala, and the anterior cingulate cortex (**Figure 7**).

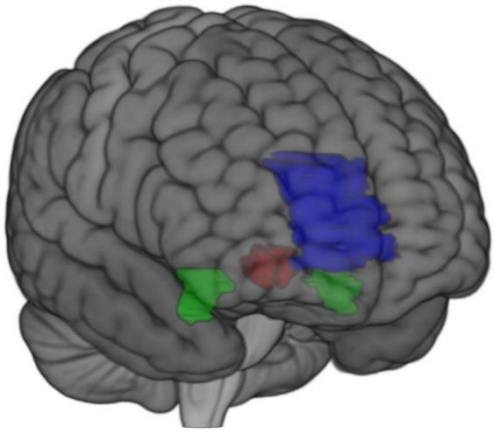


Figure 7 Brain regions that had overlap in our literature review between ASD and OXT research. Blue:anterior cingulate cortex, red: hypothalamus, green: amygdala. Image created with MRcroGL

Hypothalamus (HTH)

The HTH as the region of OXT production seems the most obvious brain region that can be considered a structural correlate of the OXT system. In addition to OXT production itself, it is not surprising that OXT binding sites have also been described in the HTH - mainly in the preoptic/anterior region (Loup *et al.*, 1991; Boccia *et al.*, 2013). Lesions of the HTH have been associated with a range of behavioral and emotional symptoms such as aggressiveness, depression, and social withdrawal (Giustina and Braunstein, 2016). For example, craniopharyngeoma patients, who frequently suffer from a lesion of the HTH caused either directly by the tumor or indirectly by therapeutic resection of the tumor, were found to have a high prevalence of socio-behavioral impairments (Zada *et al.*, 2013; Müller *et al.*, 2017). Consistent with the hypothesis that OXT regulation may be altered in these patients, Gebert et al. reported a significant association of decreased OXT levels and hypothalamic injury (Gebert *et al.*, 2018). Notably, the same sample of patients also showed significantly higher levels of autistic traits and more difficulties in rapid emotion recognition compared to controls (Brandi *et al.*, 2020). Three studies which have reported structural findings of the HTH in ASD, have concordantly reported a reduction grey matter volume (GMV) or concentration in autistic children, adolescents and young adults (Kurth *et al.*, 2011; Wolfe *et al.*, 2015; Shou *et al.*, 2017) as well as an associated enlargement of the 3rd ventricle (Wolfe *et al.*, 2015). Shou et al. (2017) further reported a positive association of bilateral hypothalamic volume with peripheral VP concentrations in autistic children (n = 14, mean age = 4.5 years), while they did not measure VP in the control group. Shou et al. (2017) thus hypothesized that impairment of the VP/OXT system is associated with changes in brain morphology and function in ASD. However, they did not examine OXT levels in this context. In line with structural HTH alterations in autistic individuals, studies in healthy carriers of OXTR variants associated with an increased likelihood of ASD have found a significant decrease in GM volume in the HTH in healthy carriers of the rs53576 and rs2254298A alleles (Tost *et al.*, 2010; Tost *et al.*, 2011), for review see (Caria *et al.*, 2020).

Amygdala

Another structure which shows overlap between the two fields of OXT and ASD research is the amygdala. As described earlier, the amygdala is strongly involved in social and emotional functions, and altered activation patterns in ASD patients have been widely reported (Baron-Cohen *et al.*, 2000; Ashwin *et al.*, 2007). Altered activation has also been reported in response to intranasal OXT application in non-autistic persons (Domes *et al.*, 2013; Domes *et al.*, 2014) and in neurophysiological experiments (Huber *et al.*, 2005). It therefore seems reasonable to assume that the social and emotional effects of OXT occur via modulation of amygdala activity. In accordance with this, OXT binding sites have been abundantly observed in the peri-amygdaloid region but also in central and basolateral regions of the amygdala in cell bodies as well as in fibres (Loup *et al.*, 1991; Boccia *et al.*, 2013). Inoue et al. (2010) reported an association of OXTR alleles and amygdala volume in non-autistic adults. They reported that an OXTR allele (rs2254298A), which is associated with an increased likelihood for ASD, was associated with larger bilateral amygdala volumes in an allele dose-dependent manner, whereas hippocampal and whole brain volumes were not significantly affected by genotype. Other SNPs were associated with smaller amygdala volumes (Inoue *et al.*, 2010).

Anterior cingulate cortex (ACC)

Like the amygdala, the anterior cingulate cortex (ACC) is part of the limbic system and is known to be involved in the regulation of cognitive and emotional processes (Bush *et al.*, 2000). Functional MRI studies in ASD patients have linked altered cingulate activity and functional connectivity to impairment in introspection (Chiu *et al.*, 2008) and repetitive behavior (Agam *et al.*, 2010) with focus on the dorsal anterior cingulate cortex (dACC). Structural abnormality in the cingulate cortex in ASD patients has also been reported (Nickl-Jockschat *et al.*, 2012). This region too was reported to have a high density of OXTR (Boccia *et al.*, 2013).

Other neuronal areas that, to the best of our knowledge, have not been reported as structurally altered in ASD, that should nevertheless be mentioned here, because of their reported functional but involvement in OXT-mediated modulation of social behavior, are the nucleus accumbents (Borie *et al.*, 2022) and the hippocampus (Riem *et al.*, 2019).

1.5 Morphometric methods in neuroimaging

The term "morphometry" describes a method of quantitative analysis of objects. In the case of neuroimaging this object is the brain. Morphometry of the brain experienced a major breakthrough with the invention of MRI technology and other newly developed applications that not only offer much higher spatial resolution than, for example, X-ray techniques, but also allow quantification of local changes and differences in structure and volume - in vivo. (Gaser, 2016). Modern methods of computational image analysis can be used to identify statistically significant structural differences between two or more groups, for example, between a group of patients and a control group. In addition, statistical correlations can be made between morphometric properties such as grey matter volume and parameters such as a clinical score. To compare individual brains of several subjects, these analysis methods usually rely on some kind of normalisation, i.e. registering the image data in the same stereotactic space so that gross interindividual differences such as the general size of the brain are removed. The regional differences of such a comparison of brains between different populations are then usually presented in the form of a statistical parametric map (SPM). As far as modern methods of analysis of MRI images are concerned, Karl Friston and John Ashburner have defined three main principles for comparing brains among multiple groups (Ashburner and Friston, 2003):

(1) Deformation-based morphometry (DBM) focuses on macroscopic distinguishable deformations in the brain. (2) Tensor-based morphometry (TBM) further allows for quantification of dimensions such as length and volume of specific regions by using gradients of deformation fields (the so-called Jacobian determinants). (3) Voxel-based morphometry (VBM) describes a voxel-wise comparison of entire brains across different subjects and makes use of a process called segmentation, which allows the analysis of specific tissue types such as gray matter (GM), white matter (WM), and cerebrospinal fluid (CSF) (Ashburner and Friston, 2003). In recent years, a fourth method has been established, referred to as (4) surface-based morphometry (SBM), which compares surface features such as gyrification patterns and complex surface deformations (Shi and Wang, 2015).

1.5.1 VBM

In the present work VBM was used and is therefore described in more detail. Essentially, VBM is a voxel-by-voxel comparison of the volumes or concentrations of, for example, GM between two groups of subjects. In other words, the method can be used to determine

statistical differences in the concentrations or volumes of tissue types within each voxel throughout the brain between multiple subjects or groups (Ashburner and Friston, 2000). Since the introduction of VBM in the field of neuroimaging by Friston and Ashburner (Ashburner and Friston, 2000) the method has been applied to a variety of research questions and has since been further refined and improved (Takao *et al.*, 2010; Gaser *et al.*, 2022). The method owes its popularity not least to the availability of freely downloadable and largely automated toolboxes that have made it accessible to a wider range of the scientific community, such as SPM, Cat12, FSL or Freesurfer ((SPM: http://www.fil.ion.ucl.ac.uk/spm), (FSL: http://www.fmrib.ox.ac.uk/fsl),(FreeSurfer: http://surfer.nmr.mgh.harvard.edu), (CAT12: http://dbm.neuro.uni-jena.de/cat/)). While all available tools use slightly different protocols and algorithms to solve specific problems, the basic steps of image processing are always the same. It was pointed out that, especially since the availability of automated and increasingly sophisticated algorithms, it is important to understand how and why images are processed in order to interpret the results correctly (Takao *et al.*, 2010). The following sections give a brief overview of the basic steps of a VBM analysis and describe important points in more detail.

The general steps for a VBM analysis include (1) segmentation, which divides the image into subunits according to certain image attributes and usually also includes steps to denoise the image from artifacts. (2) spatial normalization, which aligns the image to a template, making it comparable across subjects. (3) spatial smoothing, which describes a step of blurring the image to distribute remaining inaccuracies from the previous steps more evenly across the image, which has been shown to improve the (4) statistical model, which is then applied. An overview of the basic steps can be found in (**Figure 8**).

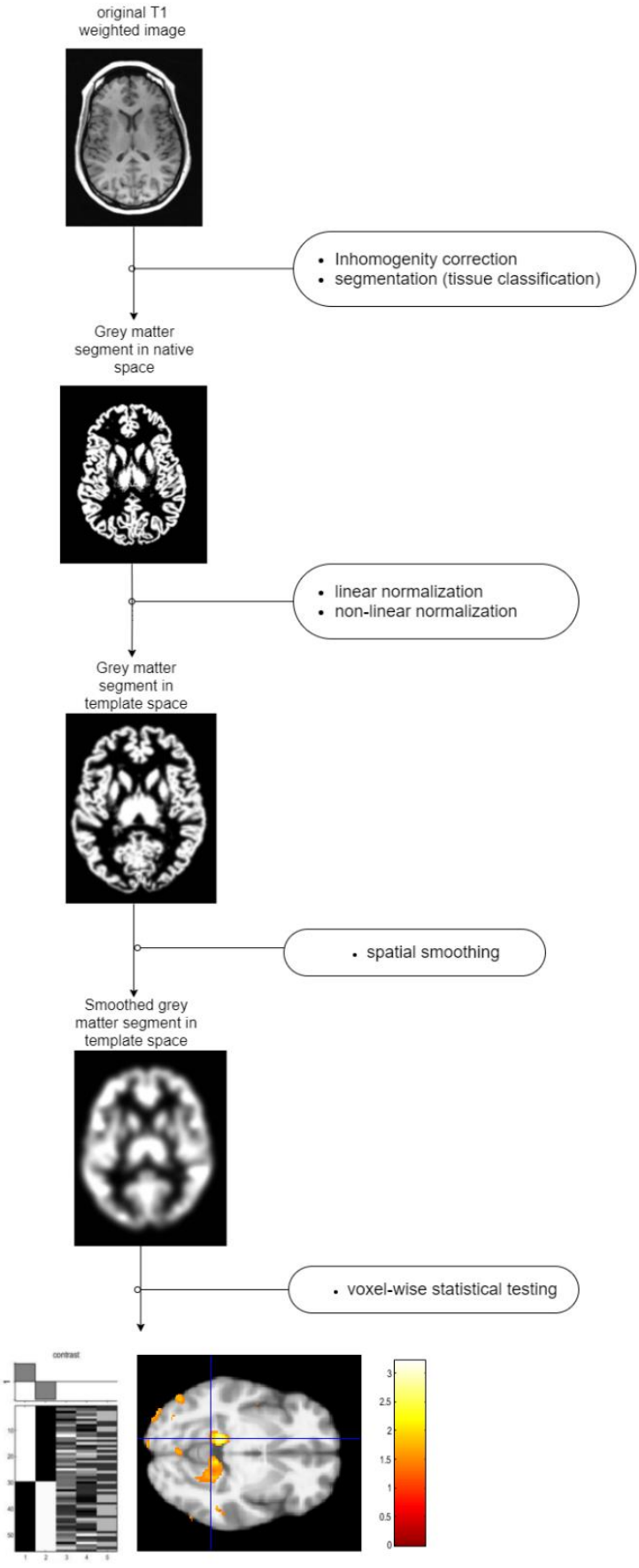


Figure 8 Overview of the basic steps of a VBM analysis. At the beginning, there are a number of pre-processing steps. These include removing inhomogeneities and noise from the brain image and segmenting it into GM, WM, CSF and other tissue types such as bone or vessels. The segments are then normalized, i.e., matched to a template, to make brain images from multiple individuals comparable. This is followed by the optional step of modulating the images (not shown here). The images are then smoothed with a Gaussian kernel. Finally, the normalized, modulated, and smoothed images are entered into a statistical model to perform a voxel-wise analysis. Significant regions can then for example be projected onto a mean brain image. Figure adapted from Kurth et al. (2015). Brain images are original images from a participant of this study. created with the freely available software 'draw.io'

1.5.1.1 Segmentation

At the end of the segmentation step, the image is divided into gray matter (GM), white matter (WM) and cerebrospinal fluid (CSF), allowing conclusions to be drawn about specific properties of the brain tissue of interest (**Figure 10**). This step typically also involves the removal of non-brain tissue such as the skull bone, a process known as skull stripping. In their 2005 publication, Ashburner and Friston describe two basic segmentation strategies. (1) One is to use a template brain into which the subject's brain is warped, thus matching the predefined regions and tissue classes in the template brain. (2) Another method is to classify the tissue classes by using the intensity properties of a tissue class (**Figure 9**). This is achieved by selecting voxels representing a particular tissue class and using their intensity features to classify tissue classes in other voxels. The selection of representative voxels can be done, for example, using the former method, i.e., a template, so that the two methods described are used complementarily (Ashburner and Friston, 2005). For modelling the intensity distributions of a tissue class, the Gaussian mixture model in particular has become very popular because it addresses the problem that intensities are not uniform throughout and within brain tissue. This non-uniformity is due to inhomogeneities of the magnetic field during scanning, which are more pronounced the higher the magnetic field is (Kurth *et al.*, 2015). Another reason is the different properties of the same tissue class in different areas, e.g., high iron concentration is found not only in brains of patients suffering from Huntington's disease, Parkinson's disease, or Alzheimer's disease, but also physiologically in the occipital lobe (Haacke *et al.*, 2005). A further step that leads to more accurate segmentation results is a general noise reduction and equalization of the tissue, although misclassifications may still occur (Dahnke *et al.*, 2012; Kurth *et al.*, 2015). These misclassifications are due, among other things, to an overlap of the tissue classes with respect to their intensity distribution (**Figure 9**) (Kurth *et al.*, 2015). This overlap arises primarily from the co-occurrence of multiple tissue classes in a single voxel, particularly in GM regions adjacent to CSF or in GM regions densely interspersed with WM fibers. This mixing of tissues in voxels is also referred to as partial volume effect (PVE) and can be addressed by targeted remodelling of tissue transition zones at the local level, a process that has been named local adaptive segmentation (Tohka *et al.*, 2004; Dahnke *et al.*, 2012). Further improvement in segmentation accuracy can be achieved by combining this process with

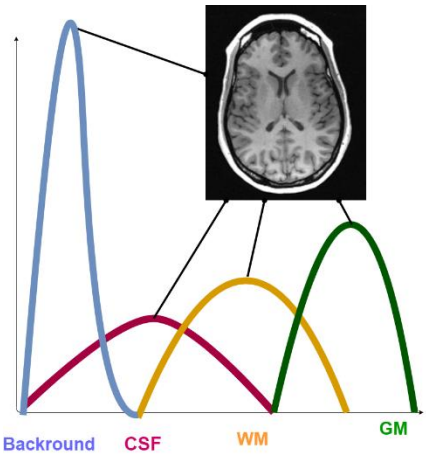


Figure 9 The classification tissue types can be achieved based on the different intensities. It should be noted that the intensity distributions overlap. Figure based on Kurth et al (2015).

additional prior information about the probability that a particular voxel actually belongs to a particular tissue type. This is achieved, for example, by using a hidden Markov random field (HMRF) model, which makes the assumption that adjacent voxels are likely to belong to the same tissue class (Ashburner and Friston, 2005). Another important method to use such prior probabilities is the use of so-called tissue probability maps (TPMs). TPMs, in simple terms, are maps that assign a probability to certain regions to belong to one tissue type or another, and are created based on images that have already been correctly segmented (Ashburner and Friston, 1997). This prior probability from the TPMs can then be combined with the probability based on intensities. In other words, in accordance with Bayes' rule, the probability of a voxel belonging to a certain tissue class based on intensities can be combined with the obtained prior probability of already correctly segmented images to a joint probability, with much more accurate results (Rajapakse *et al.*, 1997; Ashburner and Friston, 2005).

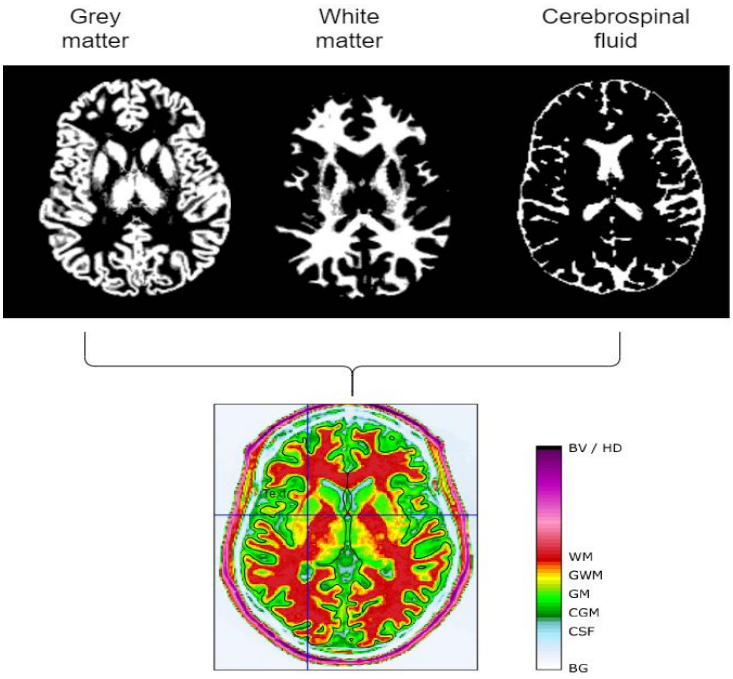


Figure 10 Classification of tissue types exemplarily shown for the three main tissue types GM, WM and CSF. The classification shown here is an example of the segmentation results of a randomly selected subject that was part of the present study and was performed with Cat12. Figure based on Kurth et al. (2015), arrangement created with the freely available software 'draw.io' and 'Gimp'.

1.5.1.2 Normalisation

Generally, human brains are very similar and have the same basic architecture. For example, all humans have a hippocampus and it would be located roughly in the same place if two brain images were superimposed. However, since all brains also differ in some basic macroscopic anatomical dimensions such as size and overall shape, it is important to make them comparable between subjects by removing these gross dissimilarities. This pre-processing step is called normalisation, in which the individual brains are computationally mapped to a standardised coordinate system so that a voxel in one subject corresponds to the voxel in another subject at the same location. This is achieved by transforming all individual brains to correspond to a template brain. One of the first stereotactic brain templates and once widely used is the one by Talairach et al. (1988), which makes use of a coordinate system based on the anterior and posterior commissures and the interhemispheric gap as defined anatomical landmarks. The space thus defined is then used to approximate the location of Brodman areas. An evolved approach that has largely replaced the Talairach-Tournoux space is the template created by the Montreal Neurological Institute. The so-called MNI coordinate system was created following and based on the system of Talairach, however, it has been pointed out that the two spaces still differ, which should be taken into account when comparing data based on different templates (Brett, 2002). Brain templates are created from an average of many hundreds of 'normal' brains. It is important for successful normalization that the template is as similar as possible to the subject's brain. For example, in addition to templates for the average European adult brain, there are also special templates for children. Various approaches exist to bring about this merging of the individual brain and the template, which have been much refined since the introduction of VBM. Generally speaking there are two basic methods of normalization: low -(linear) and high (non-linear)- dimensional (Kurth *et al.*, 2015). Linear transformations (**Figure 11**) such as rotations, scaling and translations, can account for interindividual brain sizes and rough overall differences in headpositioning for example. However since these transformations are applied to the entire image, they cannot

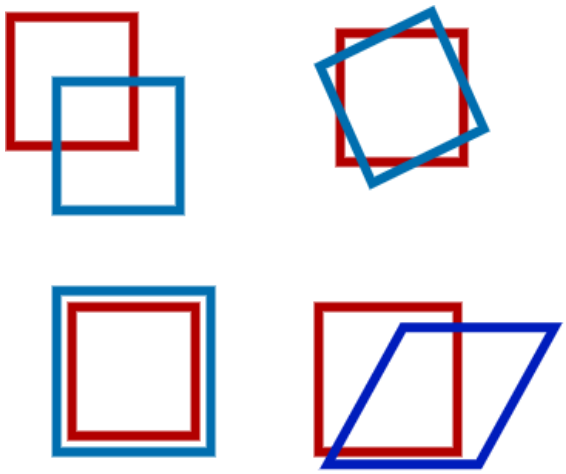


Figure 11 Examples of simple linear transformation operations that can be used to fit an image to a template. Based on Kurth et al .(2015)

account for individual local differences (Kurth *et al.*, 2015; Tohka, 2015). Normalisation at the local level requires non-linear transformations that adapt the image to a reference template in a voxel-wise manner (Ashburner and Friston, 2007). An important example of a non-linear form of transformation is the so-called high-dimensional DARTEL normalization (Ashburner, 2007), which was also used in the present work. DARTEL stands for "Diffeomorphic Anatomical Registration using Exponentiated Lie algebra" and essentially describes a computational method for generating a voxel-wise correspondence across individual brain images as well as across intraindividual hemispheres. In DARTEL, a mean reference template is formed from the average of all images and is thus study-specific. Subsequently, the individual images are registered to this newly created template. This process is repeated several times, resulting in a more detailed average template. This method has been shown to provide better overall normalization results (Klein *et al.*, 2009). However it should be noted, that a perfect match between individual image and template is still unlikely (Kurth *et al.*, 2015).

1.5.1.3 Modulation

All linear and non-linear transformations result in so-called deformation fields, which contain all the information about the operations (e.g. rotation, distortion) that were necessary to match the voxels of the subject to the voxels of the template. The gradients of these deformation fields, also called Jacobian determinants, can then be used to derive the original volume of the voxel. That is, although the brains have been spatially normalized, the information about the original state of the voxel is still contained. The direct analysis of these Jacobian determinants is what is called tensor-based morphometry and can be used to study, for example, shape and orientation differences in brains of a given population (Ashburner and Ridgway, 2015). An optional step, but nowadays performed by default, is the so-called modulation. In this step, the information contained in the Jacobian determinant is used to correct any volume changes during the normalisation process. For example: A GM area that was enlarged during normalisation and therefore appears to have a larger GM volume is scaled to match the original smaller volume in the new space, so that the original volumes within a voxel are preserved (Kurth *et al.*, 2015). This has primarily implications for the subsequent interpretation of the results, since modulated images are assumed to reflect volume, whereas non-modulated images are assumed to reflect the density or concentration of a particular tissue type (Good *et al.*, 2001).

1.5.1.4 Smoothing

Before statistical analysis the images are smoothed with an isotropic Gaussian kernel (Ashburner and Friston, 2000). Spatial smoothing involves averaging the surrounding voxels of each voxel, which results in a blurring of the image (**Figure 12**). Firstly, this process is an important prerequisite for the following statistical analysis, as in this way remaining noise tends to be normally distributed, which is a prerequisite for parametric tests. Secondly, as mentioned earlier, it is likely that the normalisation process will not match the template 100%. In these cases, smoothing can compensate for small local discrepancies by spreading them more evenly across the image. Simply put, the larger the smoothing kernel, the "blurrier" the image. Larger kernels result in more evenly distributed data, but obviously also reduce the spatial resolution. Since smoothing turns each voxel into a mean estimate of itself and the surrounding voxels, the used kernel size restricts the level at which possible effects can be found. It is therefore important to choose the kernel size according to the size of the expected local effects. This can be considered a balancing act between false positive singular effects which are most likely caused by noise and the detection of a true effect on a spatially detailed level i.e. in small brain structures like the hypothalamus for example (Ashburner and Friston, 2000; Kurth *et al.*, 2015).

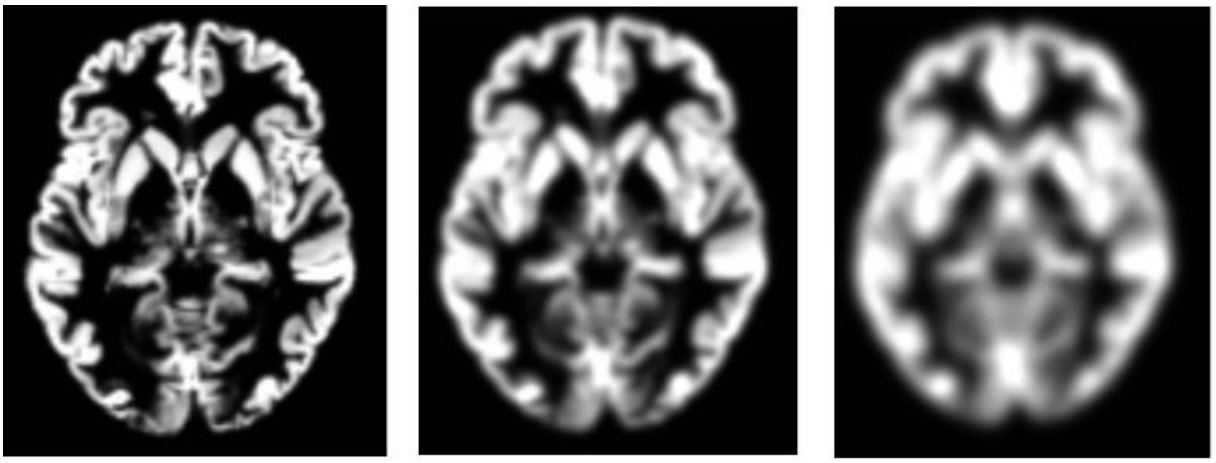


Figure 12 Examples of smoothed images of a normalized, modulated GM segment with a kernel of 4 and 8. The size of the selected kernel affects the size level at which possible effects can be detected.

1.5.1.5 Statistical testing

After the mentioned pre-processing steps, the segmented, normalized and modulated images i.e. usually the segments of either GM or WM, are entered into a statistical analysis. The by far most common approach to do this is by using a general linear model (GLM) (Ashburner and Friston, 2000). The GLM can be considered a basic framework for e.g. an ANCOVA or a correlation analysis and is therefore very flexible in terms of the questions for which it can be used (Friston *et al.*, 1994). It allows modelling a signal according to one or more variables or regressors, which can be categorical (e.g. group) or continuous (e.g. clinical score) (Pernet, 2018). The GLM approach is also the standard approach in SPM (the image processing software used in this work (https://www.fil.ion.ucl.ac.uk/spm/). Here, as part of a design matrix, all variables that are expected to have an influence on the image signal are added and modelled linearly to best fit the data. Common confounding variables include total intracranial volume, age or sex. Variables of interest can be the clinical group or a clinical score. Once the model is set up, it is estimated, i.e. the equation used to explain the actual signal is solved. This is followed by the statistical analysis of these models in the form of usually parametric tests, although non-parametric tests can also be used (Kurth *et al.*, 2015). A simple test could be, for example, a two-sample t-test to see if the signal in a particular region differs significantly between the groups. In other words, this test asks how likely it is to get a certain signal under the assumption that both groups are equal, which would be the corresponding null hypothesis H0. If the probability of obtaining a certain signal is very low, e.g., <0.05, the null hypothesis would be rejected, which is referred to as a statistically significant result that can be represented in a voxel-wise statistical parametric map (SPM) (Ashburner and Friston, 2000; Jenkinson and Chappell, 2017). An important issue here is that these parametric tests are usually applied in a mass-univariate approach, which means that the test is performed for each voxel simultaneously. Thus, an SPM is a result of a large number of statistical tests. This raises the problem of multiple comparisons. An example for better understanding: With a typical data set containing about 100,000 voxels, a statistical test would thus be performed 100,000 times. With the usual alpha significance level of 0.05, one would therefore risk that 5,000 voxels would yield false positive results (Nichols, 2012). Consequently, it is necessary to correct for multiple comparisons. It has been pointed out that a standard Bonferroni correction would be much too conservative, since this method of correction assumes independent tests. However, contiguous voxels in the brain are not independent but spatially correlated. The most common solution for this problem is to use

family-wise-error (FWE) correction, which is based on random field theory (RFT) (Ashburner and Friston, 2009; Kurth *et al.*, 2015). Correction for the Family-wise error can be applied at the voxel level or the cluster level (**Figure 13**). The latter method also takes into account the spatial extent of a cluster of voxels.

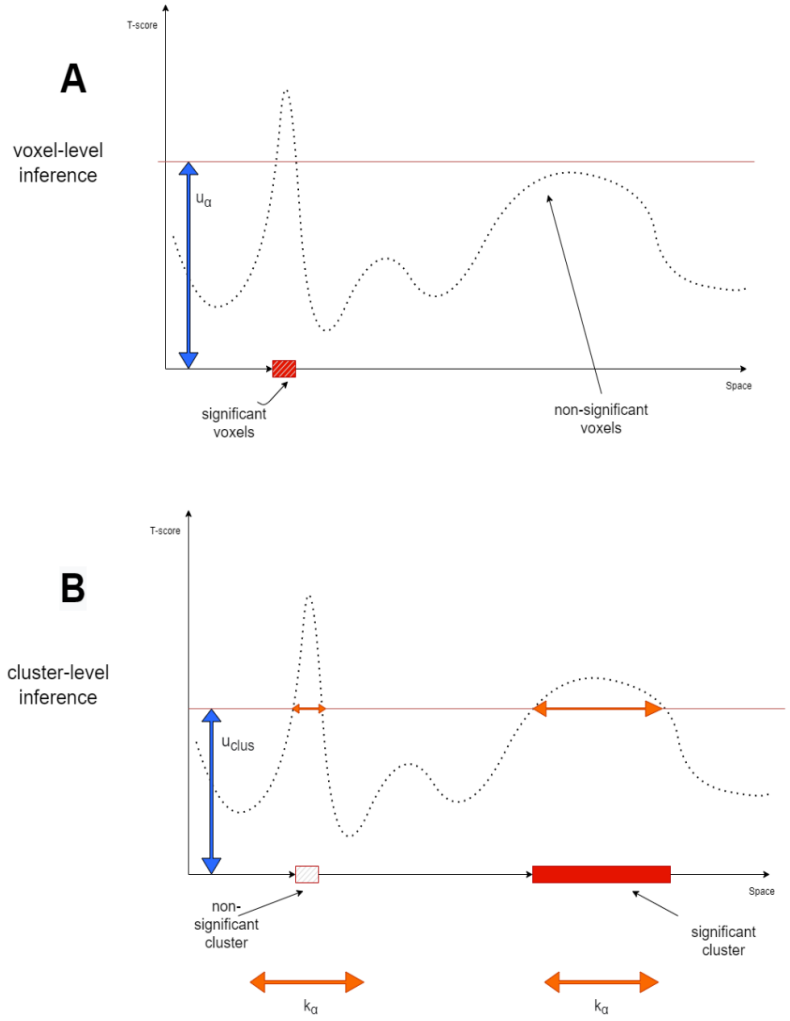


*Figure 13 Illustration of the two common methods of thresholding statistical brain maps. The dotted line represents the results of a statistical analysis, where each dot corresponds to a voxel. **A** shows thresholding at the voxel level. All voxels above an α-threshold u_α are retained. That is, the null hypothesis in these voxels can be rejected at the single voxel level. **B** shows thresholding at the cluster level. Clusters are first defined by an arbitrary threshold u_{clus}. Subsequently, those clusters larger than an α-threshold k_α are retained. Therefore, this method would reject the null hypothesis for the whole cluster. Illustration based on (Nichols, 2012), created with the freely available software 'draw.io'*

Regardless of the correction method, it should be noted that the arbitrary choice of the threshold has a strong influence on the results. For example, a high threshold value provides high specificity, but is associated with a higher risk of missing "actually significant" voxels,

i.e. the risk of false negative results is increased. The opposite is correspondingly observed with low thresholds (**Figure 14**).

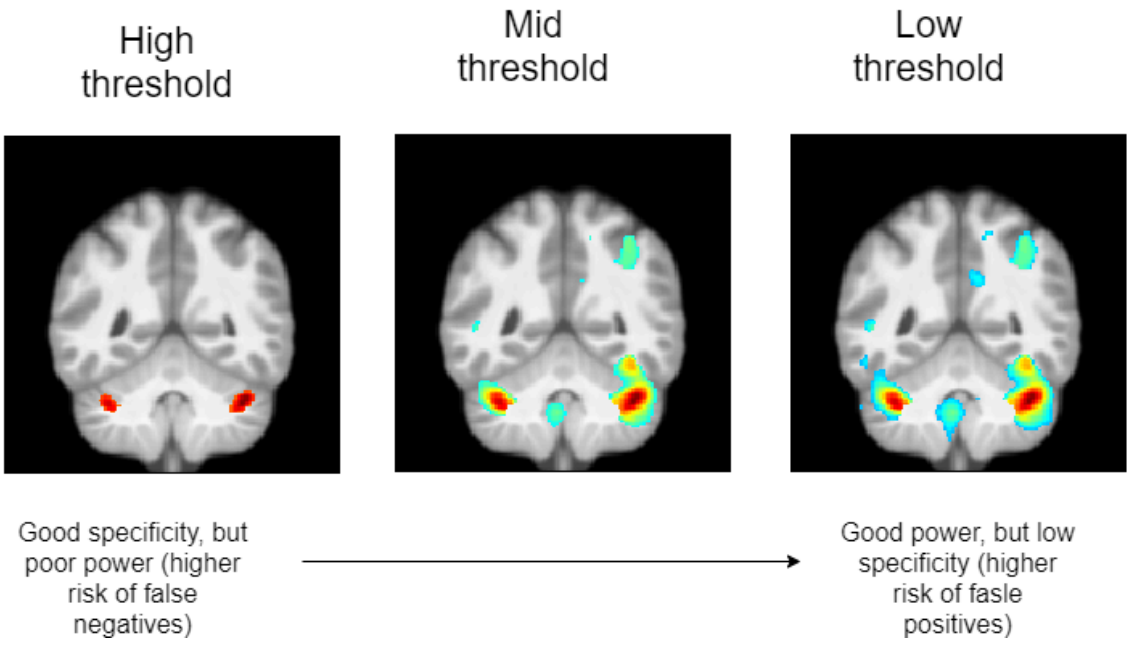


Figure 14 Influence of the selected threshold on the voxels identified as significant in the statistical analysis, created with the freely available software 'draw.io'

A further basic distinction must be made with respect to the extent of the brain regions considered in the analysis. While in so-called whole-brain analyses the entire brain is analysed, the so-called region-of-interest (ROI)-based analysis only focuses on distinctly predefined regions. In other words, whole-brain analyses are not based on prior spatial hypotheses about where in the brain differences are to be expected, while in a ROI based analysis an assumption is made in advance about which brain regions are expected to show an effect. This distinction between whole brain and ROI analysis has mainly statistical implications and both approaches have their own advantages and disadvantages (Giuliani *et al.*, 2005). In short, ROI analyses are a powerful tool for detecting local changes in predefined regions; whole-brain analyses, on the other hand, allow the investigator to search for arbitrary changes throughout the entire brain without making a priori spatial hypotheses about their location. The hypothesis- and region-independence of whole-brain analyses comes at the cost of comparatively nonspecific results and the possibility of overlooking small local changes. ROI analyses, on the other hand, are more sensitive to small local differences but may leave changes in other brain regions undetected (Kurth *et al.*, 2015).

2 Summary of backgrounds and objective

Background:

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by qualitative impairments of social interaction and communication and restricted and repetitive behaviors. In recent years, neuroimaging studies have reported a broad range of regional and global structural differences in the brains of autistic people. While it has become increasingly evident that there is no single defining neuroanatomical feature of ASD, meta-analyses and reviews have suggested that there may be neuroanatomical alterations that are, at least in large part, characteristic for ASD (Donovan and Basson, 2017; Ecker, 2017; Lord *et al.*, 2018). Consequently, there is hope that regional patterns of neuroanatomical differences could serve as diagnostic, prognostic, and treatment-determining markers (Ecker, 2017). Especially in the case of autistic adults, the possibilities of diagnosis, prognosis and possible beneficial interventions are still quite limited (Happé and Charlton, 2012; Howlin and Moss, 2012; Lai and Baron-Cohen, 2015) and neuroimaging studies in autistic adults tend to be underrepresented (Courchesne et al., 2011; Ecker et al., 2015). A better understanding of the brain structure in autistic adults, which could potentially lead to observer-independent markers would therefore potentially be of great use.

Oxytocin (OXT) is known to modulate social behavior and cognition and has, thus, been expected to be a potential therapeutic agent for autistic individuals (Kirsch and Meyer-Lindenberg, 2010). Based on this, research has also sought to determine whether and how an impairment of the OXT system could be a pathophysiological factor in ASD (Modahl *et al.*, 1992; Quattrocki and Friston, 2014; Borie *et al.*, 2021). However, the underlying neurobiology is far from clear and reviews and meta-analyses regarding the efficacy of treatment with OXT are mixed (Guastella and Hickie, 2016; Ooi *et al.*, 2016; Alvares *et al.*, 2017; Goel *et al.*, 2018; Huang *et al.*, 2021). A better understanding of the link between brain structure and peripheral OXT levels in ASD could provide important insights to better evaluate the therapeutic potential of OXT and to potentially develop appropriate stratification tools aiming for the identification of subgroups that could benefit from OXT-based therapy. With regard to a potential brain structural marker of the OXT system, the hypothalamus (HTH) is of particular interest. The three studies which have reported structural findings of the HTH in ASD, have concordantly reported a reduction grey matter volume (GMV) or concentration in autistic children, adolescents and young adults (Kurth *et al.*, 2011; Wolfe *et*

al., 2015; Shou *et al.*, 2017) as well as an associated enlargement of the 3rd ventricle (Wolfe *et al.*, 2015). Shou et al. (2017) reported decreased bilateral hypothalamic volume to be positively associated with peripheral VP concentrations. Further evidence for a structural and functional link between the HTH and ASD comes from studies with healthy carriers of OXTR variants associated with an increased likelihood of ASD (Tost *et al.*, 2010; Tost *et al.*, 2011), for review see (Caria *et al.*, 2020). To the best of our knowledge, the link between HTH structure and OXT plasma levels has not been investigated in adults with ASD.

Objective: As a first goal, we intended to compare global brain volumes as well as regional brain volumes in T1 structural MRI images of the brain of autistic and non-autistic adults. Expanding on this, we also sought to investigate the correlation of brain structure with autistic traits.

As a second goal of this study, we intended to explore a possible relationship between brain structure and OXT in autistic compared to non-autistic adults. Based on previous literature the focus here was set on the hypothalamus.

3 Methods

3.1 Participants

Participants were recruited as part of a larger study examining OXT and cortisol levels in autistic and non-autistic adults (Albantakis *et al.*, 2021). Recruitment sources included the "Outpatient and Day Clinic for Disorders of Social Interaction" at the Max Planck Institute of Psychiatry (MPIP) for ASD patients and an online study application system on the Institute's website, as well as public advertisements for participants in the control group. Of the sixty-four participants included in the larger project, fifty-nine underwent structural MRI. After exclusion of three scans following a quality check protocol (2 due to poor image quality, 1 due to structural abnormalities, described below in further detail), the final dataset included brain scans from fifty-six adults aged 18-60 years: twenty-nine autistic adults (17 men; mean age = 36.03 ± 11.0 years) and twenty-seven neurotypical adults (9 men; mean age = 30.96 ± 11.2 years). Patients met ICD-10 criteria for ASD and had been previously diagnosed in accordance with current guidelines (AWMF, 2015). Autistic participants had no intellectual impairment (IQ scores >70) and were thus regarded as persons with high-functioning autism (HFA). A detailed description of the diagnostic assessment is reported in (Albantakis *et al.*, 2021). Test for normal distribution of demographic data using a Shapiro-Wilk test revealed a non-normal distribution for age, handedness scores and verbal IQ (WST, Schmidt & Metzler, 1992). Group comparison for these variables was, therefore, performed with a Man-Whitney U test, whereas Chi^2 tests were used for categorical variables and t-tests for continuous variables. Lifestyle factors such as regular alcohol or nicotine consumption were defined as 'yes' for a reported frequency of consumption >1/week. Regular sport/physical activity was defined as 'yes' if participants reported a frequency of >1/week. There were no significant differences (all $p>0.05$) between groups in age, sex distribution, handedness score, BMI, verbal IQ, or lifestyle factors (smoking, alcohol, exercise) (**Table 1**). Thirteen subjects in the ASD group took psychiatric medication regularly (methylphenidate n=2, antipsychotics n=2, antidepressants n=9). These patients were asked not to take the medication on the morning before the OXT measurements, but only after participation in the study. General exclusion criteria were severe somatic illness, a current or previous schizophrenia diagnosis, breastfeeding, pregnancy, hormonal contraception, and a contraindication to MRI. The study protocol followed the guidelines of the Declaration of Helsinki and was approved by the ethics committee of the Ludwig-Maximilians-University of Munich. All participants gave

written informed consent before participating in the study and received fixed monetary compensation at the end of the experiment.

Inclusion criteria

- Age between 18 and 64

Exclusion criteria

- major somatic or psychiatric disorders
- current schizophrenia or anamnestic syndrome
- pregnancy
- use of hormonal contraception
- impairments of visual field (due to eye tracking experiments in a related study)
- contraindication for MRI
    - Metal implants
    - Cochlea implant
    - Cardiac pacemaker
    - Insulin pump
    - Claustrophobia
    - Metal splinter injuries
    - Employment in metalworking industry

All criteria were checked by a detailed interview, medical history questionnaires and a physical examination as well as an ECG (electrocardiogram), which was examined for abnormalities by a trained cardiologist, prior to the study. In accordance with the policy of the Max-Planck-Institute, the recorded MRI images were checked for abnormalities with pathological significance by a neuroradiologist.

	CG (n=27)	**ASD (n=29)**	**group comparison**	
	mean (± SD) / N (%)	mean (± SD) [range] / N (%)	*t/χ2/U-test*	*p*
Age	30.96 (11.3)	36.03 (11.2)	*275.5*	*0.06*
Sex (male:female)	9:18	17:12	*3.60*	*0.06*
BMI [b]	22.61 (4.1)	24.2 (4.3)	*-1.39*	*0.17*
Handedness score [b]	89.62	66.10	*281.50*	*0.13*
Smoker	2 (7.40%)	5 (17.24%)	*1.24*	*0.27*
Alcohol	10 (37.03%)	12 (41.37%)	*0.11*	*0.74*
Exercise	20 (74.07%)	17 (58.62%)	*1.49*	*0.22*
Psychiatric medication	0 (0%)	13 (44.82%)	*15.76*	*<0.001*
AQ-scores [a]	13.89 (5.47)	35.07 (10.27)	-9.6	$<10^{-11}$

Table 1 Demographic data.

[a] Data available for 55 participants .

[b] Data available for 54 participants.

3.2 Questionnaires

The following questionnaires were used in the present study.

- The Autism Quotient (AQ; (Baron-Cohen *et al.*, 2001)) is a well-established self-report questionnaire, which allows scaled measurement of autistic traits. The questionnaire consists of 50 statements covering the areas of social skill, attention switching, attention to detail, communication, and imagination. To each statement respondents are asked to choose one out of four possible answers to express their agree- or disagreement (definitely agree, slightly agree, slightly disagree, definitely disagree). For slight or definite agreement to autistic-like statements and slight or definite disagreement to non-autistic-like statements the scoring key assigns 1 point, which adds up to a possible overall scoring range from 0 to 50. Baron-Cohen et al. (2001) suggested a score of 32 or above as a clinical cut off. The autistic traits themselves are regarded as a dimensional construct, which reflects both the autistic and the non-autistic population (Austin, 2005; Ruzich *et al.*, 2015). In this study the German version of the AQ was used.
- The Empathy Quotient (EQ; (Baron-Cohen and Wheelwright, 2004)) is another self-report questionnaire with 60 items that focuses on measuring empathy as a key component of social functioning. Based on the answers, a score of 0 to 80 can be assigned, where 0 reflects no empathy and 80 the maximum empathy. The authors of the questionnaire report 30 points to be a useful cut-off for distinguishing individuals with high functioning autism from neurotypicals. In this study the German version of the EQ was used.
- The WST (Wortschatztest) (Schmidt and Metzler, 1992) is a measuring tool for the verbal IQ, which in turn has been shown to reflect the general intellectual status. In this test participants are asked to cross out real words in a list of similar looking pseudo-words, which can be assigned to a score from 0 to 42 corresponding to the correct answers.
- The Edinburgh handedness questionnaire (Oldfield, 1971) is a self-report questionnaire with 10 items, that can be scored to reflect left-handedness (-100) and right-handedness (+100).

All questionnaires except the WST questionnaire were filled in on a PC in a separate quiet room using the online-based software NetQ. The WST questionnaire was handed out on paper to make sure that no unknown words could be looked up online. The psychometric

questionnaires AQ and EQ were normally distributed within groups (Shapiro-Wilk test $p>0.05$), the WST showed non normal distribution (Shapiro-Wilk test $p< 0.05$). Statistical differences between groups were assessed using two sample t-tests for the AQ and EQ and a Man-Whitney U statistic for the WST in SPSS 27. Group comparisons of these questionnaires are reported in the results.

3.3 Oxytocin Data

For a detailed description of sample acquisition and OXT extraction please refer to the relevant publication (Albantakis *et al.*, 2021). In brief, the participants were asked to abstain from food (>12h), water (>1h) and sports the day before the study. After arriving at the outpatient unit of the MPIP at 8:30 am, blood samples were obtained during rest. OXT concentrations were quantified in an external laboratory (RIAgnosis, Sinzing, Germany) using radioimmunoassay (RIA) as previously described (De Jong *et al.*, 2015). For VBM correlation analyses, which included peripheral OXT baseline levels, the concentrations in pg/ml were used. To confirm that the present subsample of participants showed OXT characteristics similar to the sample included in Albantakis et al. 2021, univariate analyses were performed to test for effects of diagnostic group on baseline OXT concentrations, including age and sex as covariates. Data on baseline OXT levels were available for n=53 participants (ASD: n=26; HC: n=27).

3.4 MRI data

3.4.1 Image acquisition

Structural brain scans were obtained using a 3 Tesla MRI scanner by GE, model ‘Discovery MR750’. Before entering the MRI room, all participants were informed about the following procedure and the risks involved and were asked again about possible contraindications to MRI. In accordance with the policy of the MPI, participants were asked to fill in an additional medical history questionnaire. These questionnaires together with the obtained images were subsequently passed on to an external neuroradiologist for screening purposes. In the scanner room participants received earplugs and an emergency bell in their right hand, the head was then secured firmly in the head coil with inflatable cushions. 3D anatomical T1-weighted MPRAGE (magnetization-prepared rapid gradient echo) magnetic resonance images were acquired with the following parameters: Flip angle 12°, Prep Time 450, echo time (TE) 2.3 ms, frequency 256, voxel resolution 1×1×1 mm. Based on an initial overview image, the

recording area was centred on the brain and aligned with the anterior and posterior commissure (AC/PC) - a procedure that was checked again later before processing the data.

3.4.2 Image quality check

Prior to pre-processing, images were visually examined for scanner artefacts and correct alignment using the SPM display function. Hereupon one individual was excluded due to visible stripe artefacts in the image, probably due to head movement during recording. Another individual was excluded due to visible anomalies in the temporal lobe. In a further step, all scans underwent an automatic quality assessment in Cat12, based on quality parameters such as noise, image inhomogeneities and resolution, which resulted in an Image Quality Rating (IQR) from 0 (unacceptable quality) to 100 (excellent quality) (Appendix **Figure 25**). IQRs for each scan were extracted and implemented in SPSS for group comparison (ASD vs CG) using an independend two sample t-test to exclude significant group differences regarding image quality. After segmentation, but before smoothing, followed a series of quality checks in order to identify any inconsistencies that might have occurred during pre-processing. First, all GM and WM images were visually inspected individually for gross pre-processing errors. In a second step, one horizontal slice from the normalized, bias-corrected and modulated scans of each subject was visualized together on one page using Cat's function 'display slices'. This was done to facilitate the visual identification of potential gross segmentation errors (Appendix **Figure 24**). As a final step, Cat's function 'quality check' was carried out, which offers visualisation of the overall correlation of the images in the form of a violin plot (Appendix **Figure 26**) and a correlation matrix, thus facilitating the identification of potential outliers. Images with a low overall correlation were again inspected carefully. As an additional feature, the Mahalanobis distance between 'mean correlation' (reflecting the quality after pre-processing) and 'image quality' (reflecting the quality before pre-processing) was plotted as a measure of overall combined image quality before and after pre-processing (Appendix **Figure 27**). As a result of the quality check protocol, one outlier regarding overall correlation and image quality was identified, clearly distinguishable in the Mahalanobis distance plot (Appendix **Figure 27**). There are no clear criteria as to when a scan based on these quality measures should be excluded if no obvious artefacts are found. Although no obvious artefacts or anomalies could be identified visually, it was decided to exclude this scan from further analysis to avoid biased results.

In summary, three scans were excluded from further analysis in accordance with the quality check protocol. The remaining 56 images had a mean quality rating of 85.3 (with a range of 82.7 to 86.37), reflecting good overall image quality. The mean quality rating in the ASD group was 85.4 and mean quality rating in the HC group was 85.2. A two tailed two sample t-test for independent groups confirmed that the quality scores did not differ significantly between groups ($p= 0.62$).

3.4.3 Image Processing

First T1 images were converted from DICOM into NIfTI format. After checking for correct alignment to the AC/PC axis, images were pre-processed using the publicly available toolbox Cat12 Version 12.7 (1600) (http://www.neuro.uni-jena.de/cat/) implemented in SPM12 (Statistical Parametric Mapping software, http://www.fil.ion.ucl.ac.uk/spm/) using Matlab version R2019a (The MathWorks, Inc., Natick, Massachusetts, USA). Pre-processing was carried out using the standard pipeline and pre-set parameters as suggested in the Cat12 manual (http://www.neuro.uni-jena.de/cat12/CAT12-Manual.pdf). Pre-processing with Cat12 involved bias field inhomogeneity correction and denoising, using the Spatially Adaptive Non-Local Means (SANLM) Filter (Manjón *et al.*, 2010), segmentation into grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF) in accordance with the unified registration approach (Ashburner and Friston, 2005) and spatial normalization and affine registration to MNI space using a template for high-dimensional DARTEL registration derived from 555 healthy subjects of the IXI- database (http://brain-development.org/) with a final voxel size of 1.5 × 1.5 × 1.5 mm. In the version of CAT12 used here, this process is extended by refined voxel-based processing using adaptive maximum a posteriori (AMAP) estimation, Markov Random Field (MRF) (Rajapakse *et al.*, 1997) and accounting for partial volume effects (Tohka *et al.*, 2004). Finally, segmentations were modulated by multiplication with the Jacobian determinant derived from spatial registrations. This step preserves the original volumes within a voxel, which are altered during non-linear registration (Good *et al.*, 2001). For detailed description of the individual steps in Cat12 we refer to the publishers' website. Prior to smoothing, images were checked for correct pre-processing in accordance with the quality check protocol suggested in the CAT12 manual. For whole brain analyses images were then smoothed with a Gaussian kernel of 8 mm (FWHM). Following suggestions of applying comparably small kernels for analyses in the HTH due to its small size and size of expected effects (Wolfe *et al.*, 2015; Boes *et al.*, 2018) images for the ROI-based VBM analyses of the HTH were smoothed with a Gaussian kernel of 4 mm (FWHM). An absolute

grey matter threshold masking of 0.1 was applied to account for a possible misclassification of tissues.

3.5 Structural brain analyses

3.5.1 ASD-related differences in global brain volumes

Both, volumetric increases and decreases have been described in the literature for total grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF) as well as differences in total intracranial volume (TIV) in autistic persons. To exploratively check for global differences in these tissue types and TIV in our sample, the total volumes of GM, WM, CSF and TIV of all subjects were estimated in non-normalized space using Cat12. Volumes were extracted and implemented in SPSS for further analysis. The mean values of each tissue type are reported as absolute values in ml and in relative values as percentage of TIV. Based on the approach by Kurth and colleagues (Kurth *et al.*, 2011) group differences for absolute values were statistically assessed using a MANCOVA with GM, WM, CSF and TIV as dependent variables and diagnostic group as independent variable. Additionally, to control for differences in TIV, the MANCOVA was repeated with the relative values of GM, WM, CSF as percentage of TIV. Both analyses included sex and age as nuisance parameters

3.5.2 VBM analyses

3.5.2.1 Statistics

For VBM analyses, the standard general linear model (GLM) approach as implemented in SPM12 was used. Prior to analyses, the individual model designs (described in detail below) were checked for orthogonality of the variables in SPM. Clusters were regarded as significant when falling below an initial uncorrected voxel threshold of .001 and an FWE-corrected cluster threshold of .05 for whole brain analyses and inside the ROI respectively. For whole brain analyses a voxel cluster size of at least k = 100 voxels was set. Additionally, results are also reported at the FWE corrected peak-level. Differences of the two correction methods are described in (1.5.1.5). If no significant results were found under these conditions, we report results at an explorative, more liberal uncorrected threshold of $p < 0.001$, where appropriate. To account for variance due to differences in total intracranial volume (TIV), age and sex, these variables were included as nuisance parameters. Due to co-linearity between TIV and OXT ($\cos(\theta) = r = -0.35$) (see Appendix **Figure 29**), the correction for TIV in the analysis

including OXT levels was applied by global scaling with TIV, as is recommended (Gaser, 2017).

3.5.2.2 ASD-related structural brain differences: whole brain analysis

To investigate general structural differences between autistic and non-autistic adults, whole-brain analyses were performed in both the GM and the WM segments. Statistical significance was assessed using a t-statistic. Since both increases and decreases of these tissue types in ASD patients have been described in the literature, the differences between the groups were evaluated for both negative (ASD<CG) and positive contrasts (ASD>CG) (design matrix example in Appendix **Figure 28**). To identify brain regions associated with autistic traits across autistic and non-autistic adults, a regression analysis on all subjects was performed with AQ scores as covariate of interest, including age, sex and TIV as nuisance parameters. A t-statistic was used to test for significant positive and negative associations. Analyses were performed for both, GM- and WM- segments. AQ data were available for n=55 subjects (ASD: n=29; CG: n=26).

3.5.2.3 ROI-based analysis of the HTH

Smoothed and modulated GM images were entered into an ROI-based VBM analysis using a HTH mask derived from the subcortical brain nuclei atlas by Pauli et al. (2018) (https://neurovault.org/collections/3145/) (**Figure 15**). The mask was resliced to fit the template space in SPM12 and encompassed 1085 voxels. Voxel wise group comparison of hypothalamic GMV was conducted using a two-sample t-test for the contrasts ASD>CG and ASD<CG. Additionally, with regard to reported differences in GM density (instead of GM volume) in the HTH (Wolfe *et al.*, 2015), the group comparison was repeated with the unmodulated GM images. Unmodulated images are commonly assumed to reflect density or concentration rather than volume (Good *et al.*, 2001). To test for group differences in associations of GMV and OXT, i.e. interaction effects, OXT concentrations were included in a full-factorial model and tested for significant positive and negative contrasts inside the HTH mask, i.e. (GMV[ASD] × OXT > GMV[CG] × OXT) and the other way round. Data on OXT baseline levels were available for n=53 subjects (ASD: n=26; CG: n=27). Subsequent to the ROI-based analysis, we also performed an exploratory whole-brain analysis using the same model. To test if hypothalamic GMV is associated with autistic traits, a multiple regression analysis on all subjects was performed with AQ scores as covariate of interest and tested for

significant positive and negative associations. Since we did not find a significant association here, we subsequently tested associations of GMV and AQ scores in both groups separately. AQ scores were available for n=55 participants (ASD: n=28; CG: n=27).

To investigate whether significant results of the ROI-based analysis at the voxel level were also evident at the level of the overall mean hypothalamic GMV, significant statistical models were repeated using the mean hypothalamic GMV. To this end, the unadjusted eigenvariate within the HTH mask was extracted in SPM and implemented in SPSS for further analysis.

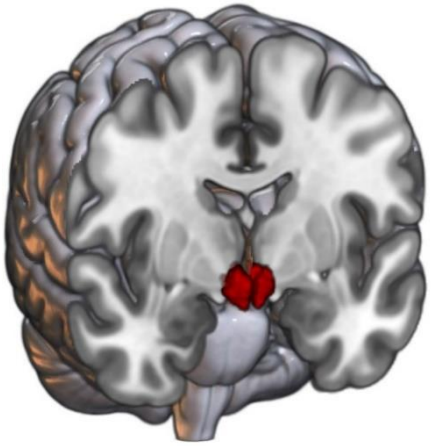


Figure 15 Resliced mask of the HTH (in red) used for ROI-based analyses. Image created with MRIcroGL.

3.5.3 Two alternative approaches to hypothalamic volumetry

3.5.3.1 3rd ventricular volume as indirect indicator for hypothalamic structural differences

In their publication from 2015 Wolfe and colleagues (Wolfe *et al.*, 2015) investigated CSF volumes of the 3rd ventricle (3rdVen) as an indirect indicator of altered hypothalamic volume, arguing that the structural properties of the 3rdVen strongly depend on the properties of the surrounding tissue, which is mainly the tissue of HTH. Aiming to replicate their findings of abnormally enlarged 3rdVen volume in autistic individuals, we adapted their approach by measuring the CSF volume of the entire 3rdVen as well as the volume of the lateral ventricle (LatVen) for scaling correction. Estimation of volumes in native space was achieved by a function of Cat12 of automatic volume estimation in predefined atlas regions based on the Neuromorphometrics atlas implemented in Cat12 (**Figure 16**). In short, estimations of volumes of defined anatomical regions in native space are achieved by inversing the non-linear deformations, which were needed to spatially normalize images to template space in the first place. Thus, the anatomical atlas labels of various regions, which are defined in template space, can be applied onto the native space images. Therefore the estimated volumes represent

the volumes before any normalization (Gaser and Dahnke, 2016). The extracted volumes in ml were implemented in SPSS for further analysis. One-way ANCOVA was conducted to determine a significant difference between the groups in total 3rdVen volume controlling for total LatVen volume, age and sex in SPSS27. The choice of total LatVen volume rather than TIV to correct for overall variance due to head size and ventricular system size is based on the approach by Wolfe and colleagues (Wolfe *et al.*, 2015). They argue, that due to observations of general larger brains in autistic people, TIV could be a misleading factor with regard to the ventricular system, whereas the LatVen as part of the continuous fluid CSF system would better reflect general volumetric differences due to bigger overall ventricular volume. They conclude that after correcting for LatVen volume, relative differences between the two ventricular volumes could only be explained by the surrounding brain tissue volumes, i.e. mainly the HTH in case of the 3rdVen. However, to avoid overlooking potential confounders with this correction method, the ANCOVA was repeated with TIV as nuisance covariate. As prerequisite for the ANCOVA, homogeneity of variance and homogeneity of regression slopes for all covariates across groups was tested in separate ANOVAs. The assumption of homogeneity of regression slopes was not violated. The assumption of homogeneity of variance was violated for the LatVen volume. A boxplot of the distribution of LatVen volume showed that non-homogeneity was mainly due to two extreme outliers in the ASD group with a distance to the median of 3*IQR (interquartile range). Repeating the test for the assumption of homogeneity of variance with exploratory removal of the two most extreme values resulted in non-violation of the assumption of homogeneity of variance. To ensure that the removal of outliers did not significantly alter the results of the ANCOVA, the analysis was performed twice - once with and once without inclusion of the outliers.

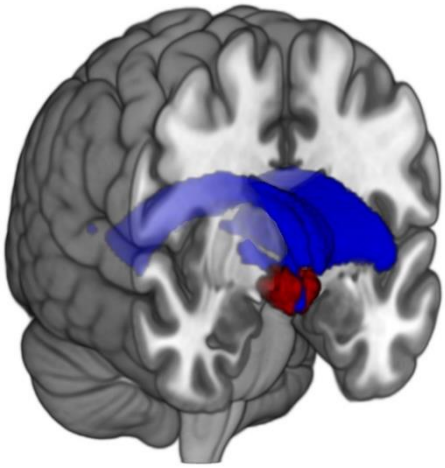


Figure 16 Masks of the lateral ventricles and 3rd ventricle from the Neuromorphometrics atlas (in blue) used for extraction of CSF volumes in the lateral and 3rd ventricle for each subject in native space in Cat12 for indirect volumetry of the HTH (red).

3.5.3.2 Automated machine learning approach to hypothalamus volumetry

In view of the difficulty of adequately identifying and measuring subregions of the HTH, i.e. single nuclear regions, with the VBM method, we sought for an alternative approach that would allow a finer differentiation and estimation of the hypothalamic subregions involved in the OXT production. While manual delineation is still considered the gold standard here, it is a very time consuming approach which demands a high level of expertise (Baroncini *et al.*, 2012). An automated alternative in this regard is image analysis using machine learning. Machine learning and particularly deep learning techniques have shown a great potential especially in the field of medical imaging (Ching *et al.*, 2018). In a recent publication Billot and colleagues (Billot *et al.*, 2020) presented a tool for HTH segmentation using a deep learning algorithm trained on 37 manually labelled T1-weighted scans extended by a data augmentation model. They demonstrated that the algorithm is capable of reliably identifying the HTH and hypothalamic subregions in unprocessed T1-weighted MRI images. After consulting the authors for further information about this tool, ensuring our data met the requirements to work properly with the algorithm, we used the publicly available code (https://github.com/BBillot/hypothalamus_seg) to automatically segment the unprocessed T1-weighted images. This resulted in segmentation maps with 10 labels of subregions of the HTH and a volume estimation of each subregion in ml for each subject. The subregions are named and defined according to the anatomic landmarks and boundaries reported by Makris and colleagues (Makris *et al.*, 2013) and encompass bilaterally the anterior-inferior HTH, anterior-superior HTH, superior tuberal HTH, inferior tuberal HTH and posterior HTH. Visual inspection for successful segmentation, revealed no obvious misclassifications (example in results section 4.5.3.2, **Figure 22**). Following the approach by Billot and colleagues (Billot *et al.*, 2020), who tested their algorithm in a study with Alzheimer patients, the hypothalamic volumes of each subregion were extracted for all subjects and implemented in SPSS. In SPSS an ANCOVA was used to determine the effect of the diagnostic group (ASD vs CG) on volumetric differences on total hypothalamic volume (sum of all subregions), including TIV, sex and age as nuisance parameters. In addition, since we were interested specifically in subregions of the HTH, known to be involved in OXT production, the bilateral volumes of the four subregions, which mainly include the SON and PVN were used in a MANCOVA to determine the influence of diagnostic group on the volume in these subregions, again including TIV, age and sex as nuisance parameters. The subregions containing the PVN are the bilateral anterior-superior and the superior tuberal HTH. Subregions including the SON

are the bilateral anterior-inferior and superior tuberal HTH (Makris *et al.*, 2013). Prior to the analysis, data were checked for outliers and assumptions of normality, homogeneity of variance and homogeneity of regression slopes, none of which were violated. Since Box's test of equality of covariance matrices was $p>0.001$, the overall significance for the MANCOVA is reported as Wilk's Lambda in addition to the individual p-values for each subregion.

4 Results

4.1 Psychometric data

The autism spectrum quotient-scores (AQ) and empathy quotient-scores (EQ) both differed significantly between groups with very small p-values of $p<10^{-11}$ for the AQ (where ASD>CG (comparison group)) (Boxplot in **Figure 17**) and $p<10^{-10}$ for the EQ (where ASD<CG) (for overview see **Table 2**). The WST-scores did not statistically differ between groups ($p=0.55$) with mean scores of 34 ±5 in the CG group and 34 ±3 in the ASD group. Psychometric data thus confirmed differences between groups in terms of autistic traits, but no differences in verbal IQ consistent with the diagnosis of HFA.

	total	**CG**	**ASD**	**Significance (*p*)**	
	mean (± SD) [range]	mean (± SD) [range]	mean (± SD) [range]	*t/U-test*	*p*
WST	34 (4) [16;40]	34 (5) [16;39]	34 (3) [24;40]	247	*0.55*
AQ	25 (13) [3;49]	14 (5) [3;26]	35 (10) [9;49]	40.55	***<0.001***
EQ	33 (18) [3;72]	46 (11) [19;56]	20 (13) [3;72]	8.17	***<0.001***

Table 2 Results table for psychometric data. Differences were statistically assessed using two sample t-tests for the AQ (autism spectrum quotient) and the EQ (empathy quotient) and a Man-Whitney U- test for the WST (Wortschatztest)

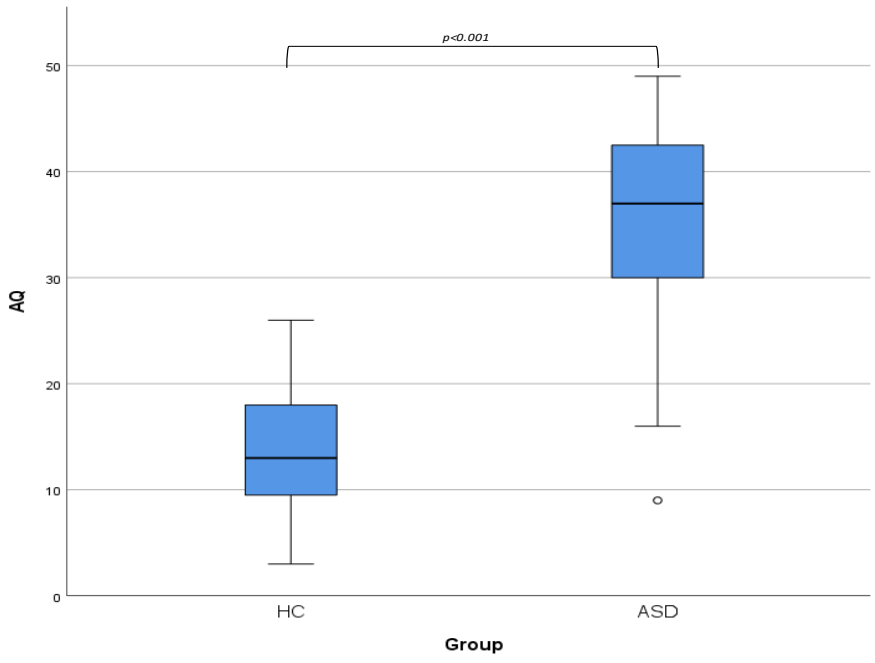


Figure 17 Boxplot displaying AQ scores for the ASD and CG group. Differences are statistically significant with very small p-values ($p< 10^{-12}$) (AQ>CG). The ASD group showed one outlier.

4.2 Peripheral OXT baseline concentrations

As in the original sample (Albantakis *et al.*, 2021), the present sub-sample showed no significant main effect of diagnostic group ($F(1,49) = 0.104$, $p = .749$), sex ($F(1,49) = 0.769$, $p = .385$), or age ($F(1,49) = 0.058$, $p = .810$) on plasma OXT baseline concentrations. Further analysis of OXT concentrations was not part of this thesis, but can be looked up in the relevant publication by Albantakis et al. (2021).

4.3 ASD related alterations in global brain volumes

Comparison of TIV, total GM, WM and CSF between groups using a MANCOVA including age and sex as nuisance parameters was not significant for any tissue type neither for absolute numbers (reported in ml) nor for relative numbers (as tissue type percentage of total intracranial volume) (**Table 3**).

	total mean (± SD)	CG mean (± SD)	ASD mean (± SD)	*Significance (p)*
TIV	1506.64 (±175.3)	1484,45 (±161.3)	1527,31 (±187.86)	*0.497*
GM absolute	699.3 (±74.1)	695.9 (±72.0)	702.5 (±77)	*0.233*
GM (%)	46.5 (±2.8)	47.0 (±2.5)	46.1 (±3.0)	*0.256*
WM absolut	528.6 (±70.4)	519.6 (±65.8)	537.0 (±74.6)	*0.489*
WM (%)	35.0 (±1.5)	35.0 (±1.5)	35.1 (±1.5)	*0.82*
CSF absolute	278.0 (±60.5)	266.0 (±47.6)	289.2 (±69.4)	*0.674*
CSF (%)	18.3 (±2.9)	17.9 (±2.3)	18.8 (±3.3)	*0.802*

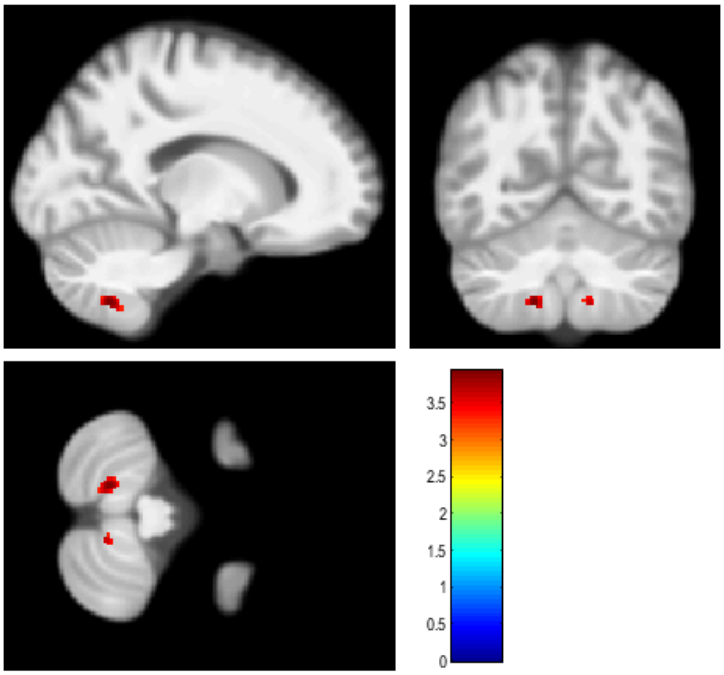
Table 3 Total intracranial volume and global tissue measurements on the whole brain. Means ± SD of absolute volumes of TIV, GM, WM, CSF are reported in ml. Relative volumes are reported as % of TIV. Significance (p) shows results of between groups test comparison using MANCOVA including age and sex as covariates of no interest.

4.4 VBM whole brain analysis

4.4.1 ASD related structural brain alterations

Voxel-wise whole brain group comparison of the GM and WM segments did not yield significant differences for either contrast. Uncorrected exploratory analyses revealed two symmetric clusters across hemispheres in the cerebellum of increased WM volume (contrast ASD>HC) (see **Table 4**) at peak-MNI coordinates [-15,-60,-50] (FWE corr. p=0.72, T= 3.93,

Z=3.66, k=84) and [-11,-62,-50] (FWE corr. p=0.84, T= 3.67, Z=3.44, k=22). None of the clusters survived FWE correction. However, since structural anomalies in the cerebellum are among the most frequently replicated findings in ASD, we nevertheless sought a more precise specification of the clusters to see if anomalies in these regions have been previously described in the literature. To determine a finer region within the cerebellum, automated labelling from the 'Cerebellar CoBra Atlas' (Park *et al.*, 2014) was used. The CoBra atlas assigned the two clusters to the left and right inferior posterior cerebellar lobule VIIIB with a probability of left (29.4%) and right (28.8%).



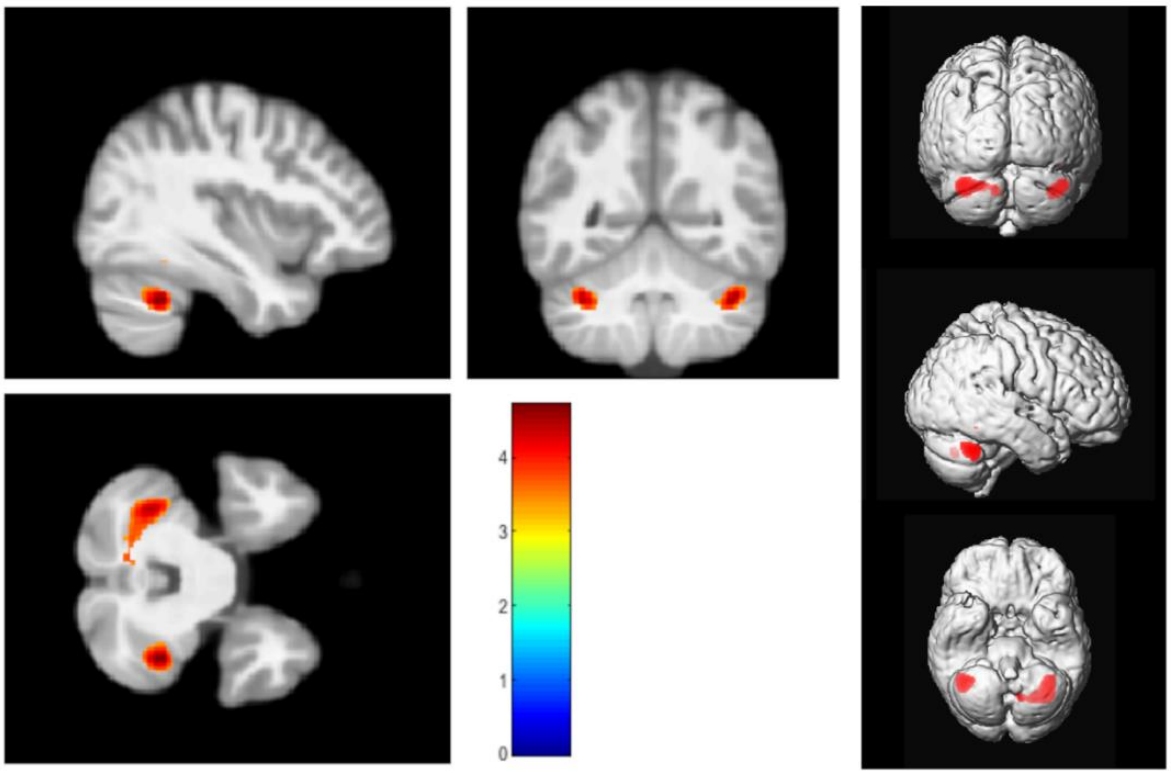
cluster		peak				coordinates	assigned structure
p(FWE)	*k*	*p(FWE)*	*T*	*Z*	*p(unc)*	*x,y,z*	
0.72	84	*0.38*	3.93	3.66	<0.001	-15,-60,-50	Left inferior posterior cerebellar lobule VIIIB
0.84	22	*0.60*	3.67	3.44	<0.001	-11,-62,-50	Right inferior posterior cerebellar lobule VIIIB

Table 4 Results of whole brain group comparison of WM volume with the contrast ASD > HC (including the nuisance covariates: TIV, age, sex). Results are reported at an exploratory uncorrected thresholds p<0.001 and no cluster-extent-threshold. The two detected symmetric clusters were not significant when corrected for FWE. Clusters were assigned to the left and right inferior posterior cerebellar lobule VIIIB by the Cerebellar CoBraAtlas. Pictures display T-score overlay at uncorrected p<0.001 on the mean structural normalized image of all participants in section view.

4.4.2 Correlation between GMV and autistic traits (AQ scores)

Regression analysis of whole brain GMV with AQ scores revealed a cluster in the cerebellum which was positively correlated with AQ scores at peak-MNI coordinates [-35,-57,-32] (FWE corr. p=0.037, T= 4.40, Z=4.02, k=837). Uncorrected exploratory analyses revealed a second

symmetric cluster in the contralateral cerebellum at peak-MNI coordinates [39,-51,-33] (FWE corr. p=0.193, T= 4.71, Z=4.26, k=456), which did not survive FWE correction. Automated labelling using the 'Cerebellar CoBra Atlas' (Park *et al.*, 2014) assigned the clusters to the 'right superior posterior cerebellar lobule crus I (with a probability of 61%) and 'left superior posterior cerebellar lobule crus I' (with a probability of 58%) (**Table 5**). In order to get a better impression of the association, GMV within the clusters was extracted for each subject using Marsbar (Brett *et al.*, 2002) and implemented in SPSS for plotting. The scatterplot (**Figure 18**) illustrates the positive association of AQ scores and GMV in this cerebellar region.



cluster		peak				coordinates	assigned structure (probability%)
p(FWE)	*k*	*p(FWE)*	*T*	*Z*	*p(unc)*	*x,y,z*	
0.193	456	*0.172*	4.71	4.26	*<0.001*	39,-51,-33	Right superior posterior cerebellar lobule crus I (61%)
0.037	837	*0.357*	4.40	4.02	*<0.001*	-35,-57,-32	Left superior posterior cerebellar lobule crus I (58%)

Table 5 Results for positive correlation between GMV and AQ scores at uncorrected p<0.001. Structures were assigned to the left and right superior posterior cerebellar lobule crus I by the 'Cerebellar CoBra Atlas'. The left cluster (k=837) was significant at the FWE corrected cluster level (p=0.037). Pictures display T-score overlay at uncorrected p<0.001 on the mean structural normalized image of all participants in section view (top left) and on a 3D render of one randomly selected, normalized brain of one participant (top right).

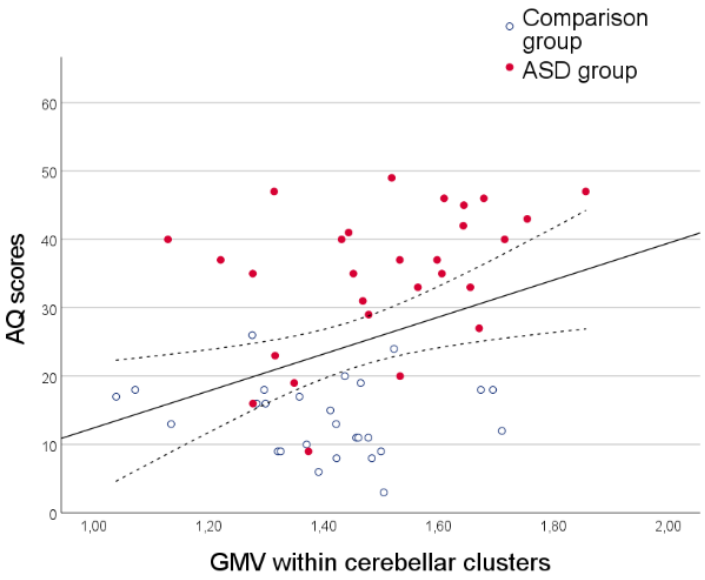
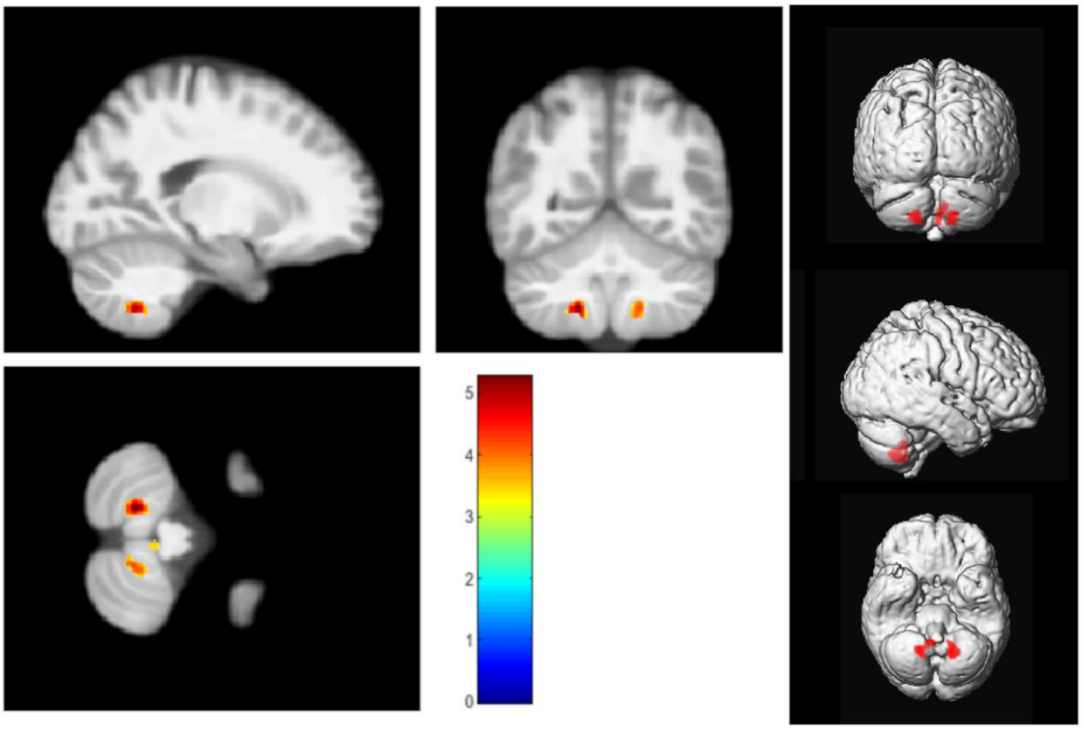


Figure 18 Scatterplot of extracted GMV clusters and AQ scores. The linear regression line illustrates a significant positive correlation of GMV with AQ scores in this region (R^2=0.125, p=0.008). Dashed lines indicate the 95% confidence intervals for the regression line

4.4.3 Correlation between WMV and AQ scores

Whole brain correlation analysis between WMV and AQ scores revealed a positively correlated peak which was significant only at the peak level but not the cluster level in the left cerebellum at peak-MNI coordinates [39,-51,-33] (*peak-FWE-corr. p=0.027*, T= 5.26, Z=4.67, k=248). The cluster was assigned to the 'left inferior posterior cerebellar lobule VIIIB' by the 'Cerebellar CoBra Atlas' (Park *et al.*, 2014). Uncorrected exploratory analyses revealed another symmetric cluster in the contralateral cerebellar hemisphere, which was assigned to the 'right inferior posterior cerebellar lobule VIIIB' and a third cluster assigned to the 'right inferior posterior cerebellar lobule IX'. These additional clusters were not significant when corrected for FWE (**Table 6**).



cluster		peak				coordinates	assigned structure (probability%)
p(FWE)	*k*	*p(FWE)*	*T*	*Z*	*p(unc)*	*x,y,z*	
0.392	248	**0.027**	5.26	4.67	<0.001	-17,-59,-48	Left inferior posterior cerebellar lobule VIIIB (33.1%)
0.519	189	0.442	4.17	3.85	<0.001	15,-59,-50	Right inferior posterior cerebellar lobule VIIIB (39.9%)
0.639	143	0.642	3.950	3.670	<0.001	6,-48,-42	Right inferior posterior cerebellar lobule IX (48.5%)

Table 6 Results for positive correlation of WMV with AQ scores at an uncorrected peak threshold of p<0.001. One peak, which was assigned to 'left inferior posterior cerebellar lobule VIIIB', survived peak-FWE correction (p=0.027). Clusters were not significant when corrected for FWE at the cluster level. Pictures display T-score overlay at uncorrected p<0.001 on the mean structural normalized image of all participants in section view (top left) and on a 3D render of one randomly selected, normalized brain of one participant (top right).

4.5 ASD-related structural brain differences with regard to the OXT system: ROI-based HTH approach

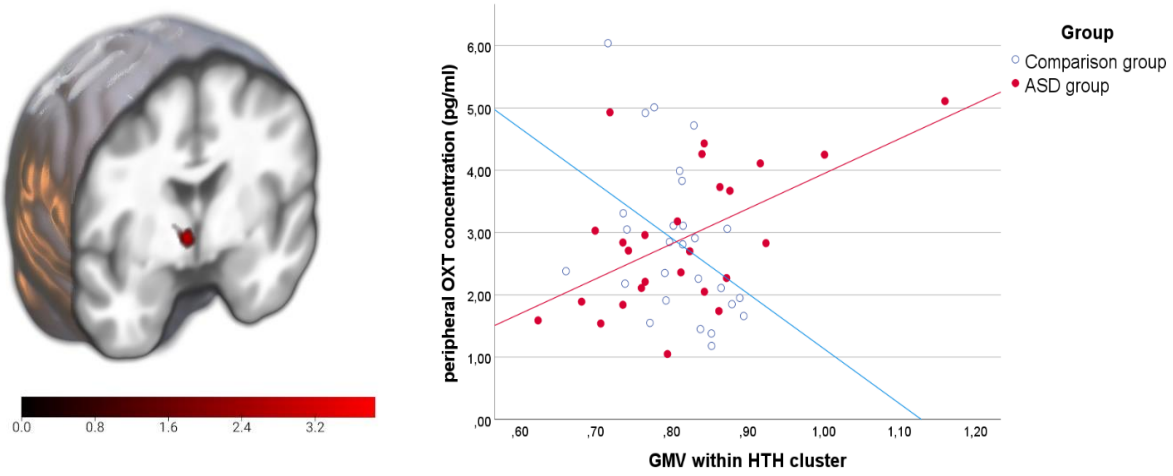
4.5.1 ASD related structural brain alterations in the HTH

The hypothesis-driven ROI-based VBM analysis did not yield significant differences in hypothalamic GMV between the groups for either contrast (ASD>CG and ASD<CG) neither using the modulated nor the unmodulated GM segments.

4.5.2 Associations of brain structure and OXT in autistic and non-autistic adults

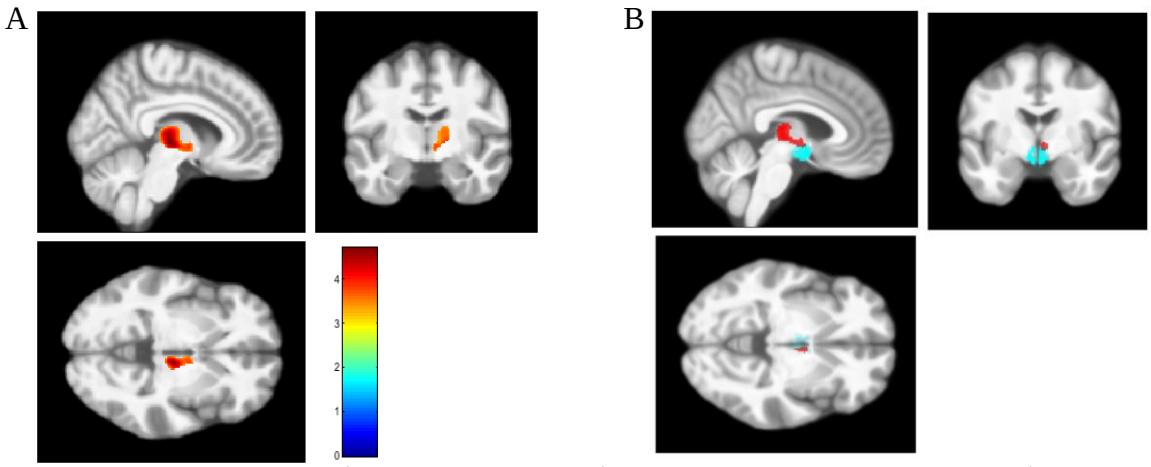
The hypothesis driven ROI-based VBM analysis of the HTH revealed differences in associations between GMV and OXT for the contrast (GMV[ASD] × OXT > GMV[CG] ×

OXT) in a cluster at peak-MNI coordinates [5,-9,-2] (FWE corr. p=0.017, T=3.85, Z=3.57, k=46). The cluster was extracted with marsbar (Brett, Anton, Valabregue, & Poline, 2002) and plotted for better illustration. Plotting in SPSS illustrates that the significance in this region is due to a positive association of GMV and OXT in the autistic group as compared to a negative association in the comparison group (Fehler! Verweisquelle konnte nicht gefunden werden. **14**). Likewise repetition of the statistical model using the mean hypothalamic GMV was significant for the interaction term of group and OXT ($F(1, 46) = 5.675$, $p = 0.021$, $\eta^2 = 0.110$) indicating a positive correlation in the ASD group ($R = 0.326$) opposed to a negative correlation in the CG group ($R = 0.211$). Exploratory whole brain analysis using the same model revealed that the cluster of the ROI-based analysis further extended from the HTH to the Thalamus with peak-MNI coordinates [5,-21,9] (FWE corr. p=0.005 , T= 4.69, Z=4.23, k=1373) (**Figure 19**). No other region reached significance here.



contrast	cluster		peak				coordinates
	p(FWE-corr)	*k*	*p(FWE-corr)*	*T*	*Z*	*p(unc)*	*x,y,z*
GM [ASD] × OXT > GM [CG] × OXT	***0.017***	46	*0.051*	3.85	3.57	<0.001	5,-9,-2
	0.129	3	*0.130*	3.45	3.24	<0.001	-5,-5,-2

Table 14 Association between GMV and OXT within the HTH mask (at peak-MNI coordinates [5,-9,-2], FWE corr. p=0.017, T=3.85, Z=3.57, k=46) positive for autistic adults and negative for non-autistic adults. Left: T-score overlay on the mean structural normalized image of all participants. Right: Scatterplot of the extracted GMV cluster illustrates the association with peripheral OXT in this region. Regression lines show a negative association of GMV and OXT in the CG (R=0.403) opposed to a positive association in the ASD group (R=0.558)



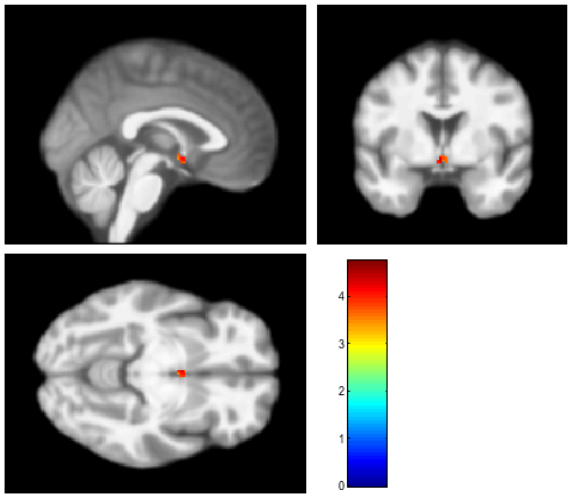
contrast	cluster		peak				coordinates
	p(FWE-corr)	*k*	*p(FWE-corr)*	*T*	*Z*	*p(unc)*	*x,y,z*
GM [ASD] × OXT > GM [CG] × OXT	***0.005***	1373	*0.194*	4.69	4.23	<0.001	5,-21,9

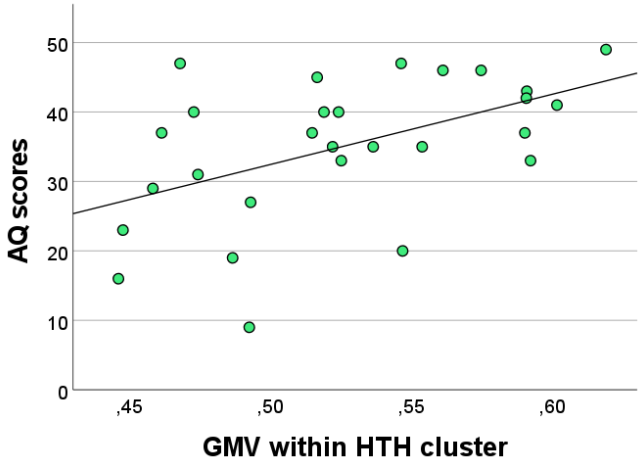
Figure 19 (A) Exploratory whole brain analysis for the contrast (GMV[ASD] × OXT > GMV[CG] × OXT) revealed that the HTH cluster is part of a larger cluster (FWE corr. p=0.005 , T= 4.69, Z=4.23, k=1373) extending from mainly the right thalamus to the HTH. (B) Synopsis of the larger cluster (red) and the HTH mask (cyan) at the section, where both overlap.

4.5.2.1 Association between hypothalamic GMV and autistic traits

There was no significant correlation between GMV and AQ scores within the HTH across the groups. Group-specific correlation analysis revealed a significant positively correlated cluster in the ASD group at peak MNI coordinates [-2,2,-9] (FWE corr. p=0.014, T= 4.74, Z=3.92, k=46), while there was no significant correlation in the CG (**Table 7**). Likewise, repetition of the model using mean hypothalamic GMV showed a significant association in the ASD group ($F(1, 23) = 12.78$, $p = 0.002$, $\eta^2 = 0.357$), while there was no significant correlation in the CG group ($F(1, 22) = 0.215$, $p = 0.648$). To determine if the cluster in the ASD group matched the cluster from the previous analysis including the OXT levels, the two clusters were plotted together on the structural mean image (**Table 7, Figure B**). Visual comparison showed only a marginal overlap between the two clusters. Explorative correlation of the cluster from AQ analysis with plasma OXT levels in the ASD group was not significant.

A





contrast	cluster		peak				coordinates
	p(FWE-corr)	*k*	*p(FWE-corr)*	*T*	*Z*	*p(unc)*	*x,y,z*
positive	***0.014***	46	**0.021**	4.74	3.92	<0.001	-2,2,-9

B

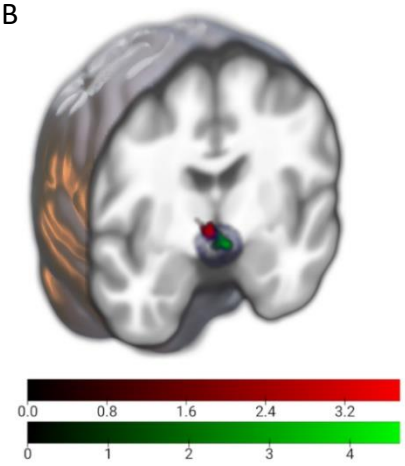


Table 7 ***A:*** *Results for positive correlation between GMV[ASD] and AQ scores inside the HTH mask. Both peak and cluster survived FWE correction. There was no correlation in the CG group. Left: Pictures display T-score overlay on the mean structural normalized image of all subjects from the ASD group in section view. Right: Scatterplot of the extracted GMV cluster illustrates the association with AQ scores in this region in the ASD group (R=0.504).* ***B:*** *Synopsis of clusters from OXT analysis and AQ analysis together with HTH outlines. Green: association between GMV and AQ scores in the ASD group. Red: Cluster from previous analysis using OXT levels*

4.5.3 Alternative approaches to hypothalamic volumetry

4.5.3.1 Indirect ventricular approach to hypothalamic volumetry

The indirect approach to HTH volumetry by means of 3rdVen volumes yielded mean volumes of 0.48 (± 0.17) in the HC group and 0.62 (± 0.25) in the ASD group. There was no significant effect of diagnostic group on 3rdVen volume after controlling for total LatVen volume, sex and age (F(1,51) = 0.19, p = .66). Boxplots visualizing the distribution of volumes are displayed in (Figure 20). As described in the methods (section 3.5.3.1), the analysis was repeated after exclusion of the two outliers with >3*IQR in the ASD group. Exclusion of outliers changed the results only marginally (*p= 0.651).* Likewise repetition of the ANCOVA while controlling for TIV instead of LatVen volume was not significant (F(1,51) = 1.6, p = .20). Total LatVen volume was closely related to 3rdVen volume (F(1,51) = 31.02, p = 9,6*10-7) with a large estimated effect size of ηp^2 = 0.38, whereas TIV (F(1,51) = 2.06, p = 0.16) showed only a small effect size of ηp^2 = 0.04, thus leaving much more unexplained variance. As a side finding, this may indicate that LatVen volume is indeed a more suitable proxy for 3rdVen volume (**Figure 21**).

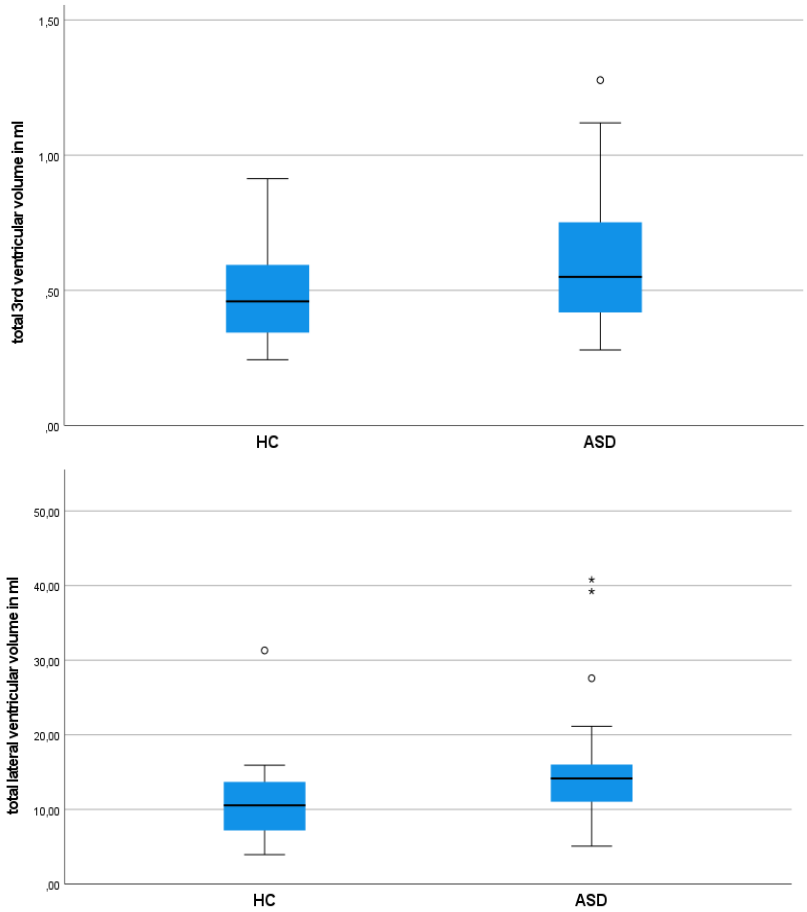


Figure 20 Boxplots displaying the volume of the summed 3rd ventricle (top) and lateral ventricle (bottom) for both groups.

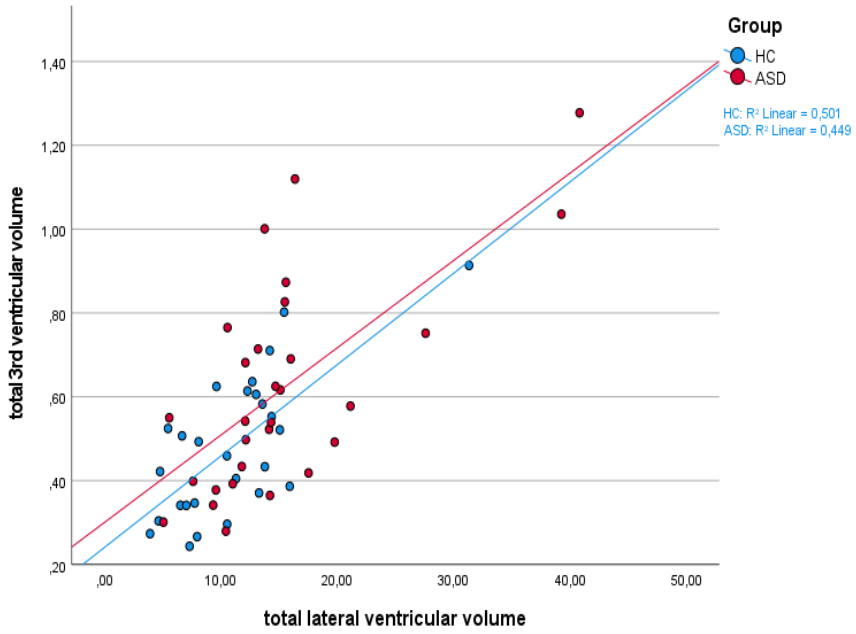


Figure 21 Scatterplot and linear regression lines for the association of lateral ventricular volume and 3rd ventricular volume for both groups. ASD group in red. CG in blue.

4.5.3.2 Exploratory machine learning approach to hypothalamic volumetry

Visual inspection of the HTH segmentation performed with the automated segmentation algorithm (Billot *et al.*, 2020), yielded good results and no obvious misclassifications were visually identified. An example from a randomly selected subject is visualized in (**Figure 22**). The segmentation resulted in 10 distinguishable subregions of the HTH i.e. 5 regions each for the right and left hypothalamus.

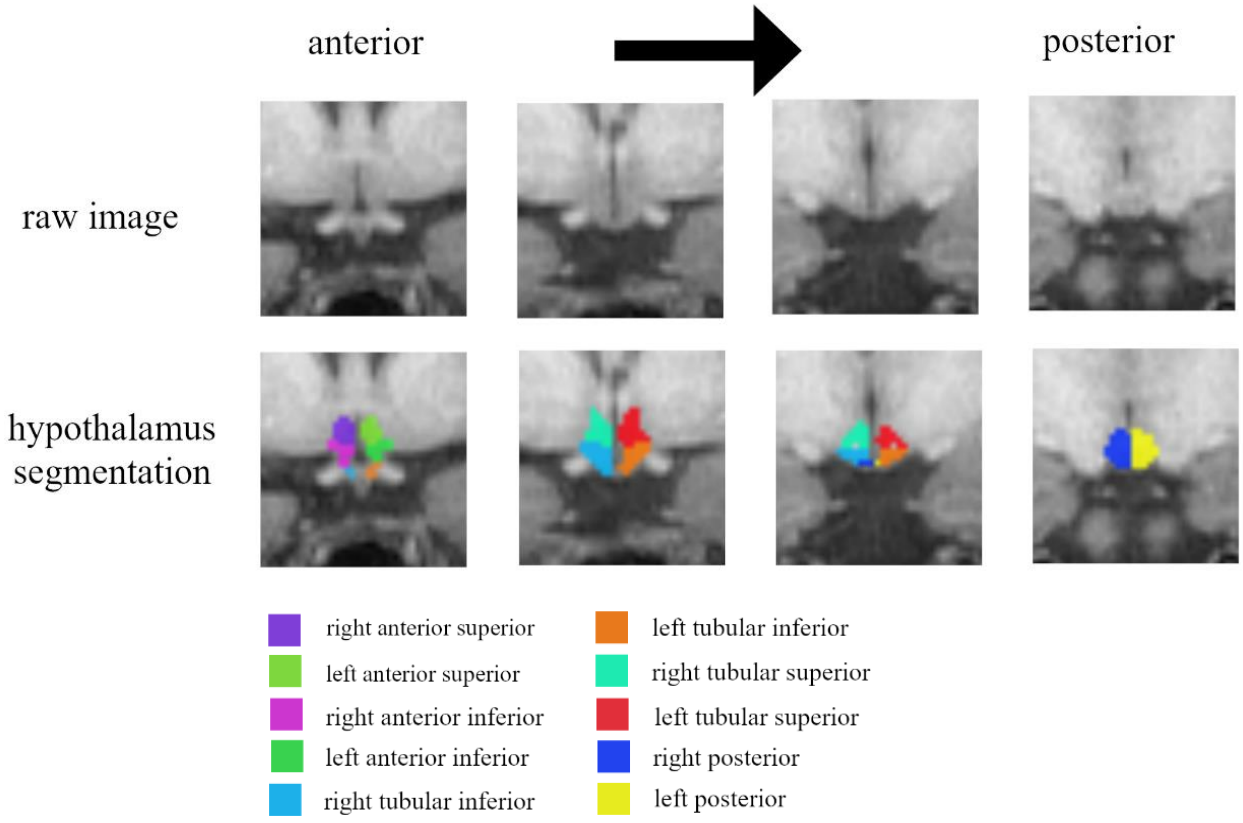


Figure 22 Automated HTH segmentation results of one randomly selected subject displayed as color coded overlay on the subjects unprocessed brain scan. Top row displays coronal zoom on the hypothalamic region in the unsegmented image. Bottom row shows the same image with the color coded segmentation overlay. The images are arranged anterior to posterior from left to right. Figure created with the freely available software 'Gimp'.

The mean hypothalamic volume according to the algorithm without controlling for nuisance parameters was 3.2 ±0.95 ml in the CG group, and 3.19 ±0.08 ml in the ASD group (**Figure 23**).

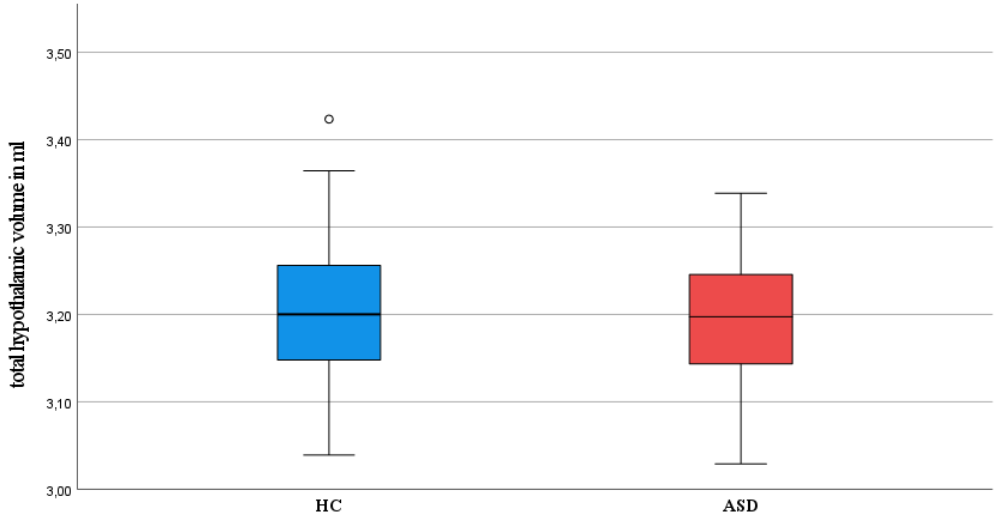


Figure 23 boxplots show the distribution of total hypothalamic volume obtained with the automated segmentation algorithm. For both groups. ASD group in red, comparison group in blue.

The ANCOVA to determine the influence of the factor group on total hypothalamic volume, when controlling for TIV, sex and age, was not significant (*p=0.496*). The MANCOVA to determine the influence of the factor group on the four hypothalamic subregions of interest containing the SON and PVN (described in section 3.5.3.2) was not overall significant (*Wilk's Lambda= 0.553*). The individual significances for each tested region are reported in (**Table 8**). No region reached significance.

bilateral hypothalamus subregion	Group				MANCOVA
	CG		ASD		
	Mean	(± SD)	Mean	(± SD)	*Significance (p)*
anterior-inferior	0.31	0.01	0.31	0.01	*0.11*
anterior-superior	0.34	0.01	0.33	0.01	*0.32*
tubular-inferior	0.96	0.03	0.96	0.03	*0.95*
tubular-superior	0.84	0.03	0.85	0.03	*0.73*

Table 8 Reported are the mean volumes in ml for the four hypothalamic subregions likely to be contain parts of the SON and PVN for both groups. Significance shows results for MANCOVA testing for the influence of the diagnostic group on volumetric differences, including TIV, sex and age as covariates of no interest.

4.6 Summary of results

First, we compared global brain volumes, that is total GMV, total WMV, and total CSF volume and TIV between groups. The analyses showed no significant differences in global brain volumes between autistic and non-autistic adults. To investigate ASD-related differences in brain structure in a voxel-wise manner, we then compared the groups in a whole brain VBM analysis using both the WM and GM segments. Voxel-wise comparison showed no significant differences in regional GM and WM volumes, but exploratory analysis using more liberal statistical thresholds revealed clusters of increased WMV in the cerebellum in autistic adults as compared to non-autistic adults. To investigate the association of brain structure and autistic traits, whole-brain regression analyses using AQ scores were performed across all participants (i.e. autistic and non-autistic adults). Analyses revealed positively correlated clusters of cerebellar GMV and WMV. We then sought to investigate ASD-related structural brain differences with respect to the OXT system. With regard to a potential structural marker of the OXT system in the brain, the hypothalamus (HTH) is of particular interest due to its role in the production of OXT. To replicate the results from previous studies reporting decreased hypothalamic GMV in autistic people, we performed a hypothesis-driven ROI-based VBM analysis in the HTH. There were no differences in the voxel-wise group comparison of the HTH neither using modulated nor unmodulated GM segments. To examine OXT-related variance in GMV in the HTH between autistic and non-autistic adults, we then tested for significant differences in the association of GMV and baseline OXT concentrations in the HTH and, exploratively, at the whole brain level. Here, a positive correlation of GMV and OXT levels was found in autistic adults as compared to a negative association in the comparison group. Explorative whole-brain analysis further revealed that the HTH cluster was part of a bigger significant cluster extending from the HTH to the thalamus. We then examined whether autistic traits (as measured by AQ scores) are associated with hypothalamic GMV. While there was no correlation between GMV and AQ scores across all subjects (i.e., autistic and non-autistic adults), there was a positive correlation in the autistic group only, but no correlation in the group of non-autistic adults. The significant results from the voxel-wise ROI-based VBM analysis were also significant at the level of mean hypothalamic GMV using the unadjusted eigenvariates within the HTH mask. Finally, we performed two additional alternative approaches to examine hypothalamic volumetry, first by comparing 3rdVen volumes between groups and, second, by using an automated deep-

learning algorithm. Consistent with the results of the ROI-based VBM analysis of the HTH, there were no significant differences between groups using these two alternative approaches.

5 Discussion

The aim of this work was to investigate structural brain differences in autistic compared to non-autistic adults and to explore a possible link to the OXT system. First, we aimed to identify and reproduce general ASD-related brain structural differences and to investigate the link with autistic traits in a whole brain analysis. Second, we aimed to explore brain structural associations of ASD in the HTH as a key region of the CNS OXT system. These aims were addressed by analysing T1-weighted MRI images using voxel-based morphometry (VBM) in a sample of autistic adults without intellectual disability and a matched comparison group. For correlation analyses, we used peripheral plasma OXT levels and the autism quotient (AQ) scores as a measure of autistic traits. We then also performed two alternative approaches to hypothalamic volumetry. In the following section, the results from whole brain and ROI-based analyses are discussed separately, followed by a discussion of the methods.

5.1 Discussion of results

5.1.1 Global brain volumes

Comparison of global brain volumes such as total GM, WM, CSF, and TIV showed no difference between the diagnostic groups. This is consistent with previous studies in adults with autism, which overall suggest that abnormalities in global brain volume observed in childhood are no longer present in adulthood but converge towards normal global brain volumes. Longitudinal studies as well as meta-analyses suggest that global brain volumes in ASD follow a sequence of slightly reduced volumes at birth, followed by a period of overgrowth in early childhood and a period of slowed growth in late childhood and preadolescence, eventually leading to global brain volumes in adulthood that are indistinguishable from those of non-autistic individuals (Redcay and Courchesne, 2005; Courchesne *et al.*, 2011; Sacco *et al.*, 2015; Haar *et al.*, 2016; Ecker, 2017). A prematurely accelerated decline of brain volume and cortical thinning are being discussed in autistic adults (Courchesne *et al.*, 2011; Lange *et al.*, 2015; Ecker, 2017), but this was not evident in our sample. Although the absence of differences in global brain volumes at the group level is well compatible with other studies in adults with autism, it cannot be ruled out that there may nevertheless be interindividual differences in this regard. For example, it should be noted that also among children the phenomenon of increased brain volume has not been observed in all but only in a subset of autistic children and that there are interindividual differences in the

extent and persistence of the phenomenon (Libero *et al.*, 2016). Interestingly, a meta-analysis also reported lower effect sizes regarding brain overgrowth in high-functioning compared to low-functioning autism (Sacco *et al.*, 2015). Whether there are differences in global brain volumes between clinical subgroups within the autism spectrum also in adults should be explored systematically in future studies using larger samples. In this regard, well-defined and homogeneous subgroups in terms of age, IQ and clinical symptomatology seem to be an important prerequisite for answering this question.

5.1.2 Whole brain VBM analysis of ASD-related structural brain differences and association of brain structure with autistic traits

We found no significant brain structural differences in the voxel-wise group comparison of GMV and WMV. Even with a more liberal statistical threshold of $p<0.001$ uncorrected and no cluster-extent threshold, there were no differences in GMV between the groups. For WMV, however, the uncorrected threshold revealed increased volumes in the ASD group compared to the comparison group symmetrically located in the left and right inferior posterior cerebellar lobules VIIIB. Interestingly, the same two regions in the cerebellum were also the only two WM regions, which were positively correlated with AQ scores in the subsequent whole brain correlation analysis across all participants, which could be taken to suggest that these regions are associated with the autism spectrum. However, only the peak in the left lobule reached significance and the clusters did not survive cluster-level FWE correction. In light of these findings, it is tempting to speculate that these results would show an effect in this region, which, in a larger sample, could also prove significant at the FWE corrected level, but to determine this will be a task for future research. Interestingly, cerebellar anomalies are among the first and most often reported brain structural findings in autism (Bauman and Kemper, 2004; Becker and Stoodley, 2013). However, with respect to the exact location of the findings within the cerebellum, studies vary considerably. A generally more consistent finding is early cerebellar overgrowth in childhood, which is suggested to be mainly driven by WM (Becker and Stoodley, 2013). In contrast, the cerebellar volume in adults has been reported to be decreased (McAlonan *et al.*, 2002; Hallahan *et al.*, 2009). Whether our results, in the light of these earlier findings, represent an independent phenomenon in adults or must rather be interpreted as a remnant of the increase in WMV in childhood cannot be conclusively clarified here. In this context, it would be interesting to investigate whether disproportionate cerebellar overgrowth in childhood is likewise associated with higher AQ scores. Accurate functional interpretation of this finding at the finer structural level in the

cerebellum is difficult. In general, the complex role of the cerebellum in ASD is subject of ongoing discussions (Becker and Stoodley, 2013). While the cerebellum is primarily associated with motor function, it also has an important regulatory role in cognitive and affective functions, mainly through connections to the thalamus and prefrontal cortex (Ramnani, 2006; Van Overwalle *et al.*, 2015). The posterior cerebellar lobule region in particular has been associated with language processing and working memory (Stoodley and Schmahmann, 2009) and communication impairment in autism (Hodge *et al.*, 2010). This is well in line with our findings of structural alteration and association with autistic traits in this region.

Another finding in the cerebellum in our analysis were clusters of positive correlation of GMV with AQ scores, which were symmetrically located in the right and left superior cerebellar lobe crus I. However only the left cluster survived FWE correction. Again, significant correlations were not observed in any other brain region. Crus I is involved in social cognition (Van Overwalle *et al.*, 2015) and has been reported to be enlarged in autistic children and adolescents (Sussman *et al.*, 2015) and to be smaller in young autistic adults (Rojas *et al.*, 2006). Interestingly, one study (Laidi *et al.*, 2017) reported a positive correlation of Crus I volume and the fixation time allocated to the eyes of a person's image, assessed in an eye-tracking experiment in adults with high functioning autism and controls. The latter would suggest that autistic adults, who tend to show stronger eye avoidance, are also more likely to have smaller Crus I volumes. This seems to contradict our finding of a positive correlation of GMV and AQ scores in this region. One possible explanation for the discrepant findings on Crus I, as with many structural findings in ASD, is certainly to be found in the heterogeneity of the samples of the individual studies. The heterogeneity of the samples between studies, but also the subjects within a study, may be largely due to the heterogeneous clinical manifestations of the disorder itself, once again highlighting the need for studies in more homogeneous subgroups of ASD. While we cannot provide a conclusive explanation for the structural relationship of the cerebellum and ASD at this stage, our results support the general notion of a potentially important role of the cerebellum in ASD. This may warrant future studies with a predefined ROI approach focusing on the cerebellum. Given the remarkable diversity of the cerebellum at the finer structural level, higher resolution MRI images may prove essential in this context.

5.1.3 Brain structural findings related to the OXT system: HTH ROI-based approach

Recognizing the role of the neuropeptide OXT in social behavior, research in recent years has sought to determine if and how OXT might be involved in the pathogenesis of ASD. The idea that differences in the OXT system may underlie some of the core symptoms of ASD has already been proposed more than two decades ago (Waterhouse *et al.*, 1996). Human research in recent years has since highlighted a wide range of potential differences in ASD related to the OXT system. These include the processing of OXT peptides (Green *et al.*, 2001), genetic variations in the oxytocin receptor gene (OXTR) (LoParo and Waldman, 2015), the structural gene for OXT (neurophysin-I) (Feldman *et al.*, 2016), and epigenetic alterations (Kumsta *et al.*, 2013; Andari *et al.*, 2020). Another often studied marker for an altered OXT system in ASD is peripheral OXT concentrations. Since the first report of decreased basal OXT levels in autistic children (Modahl *et al.*, 1998), there have been a range of studies in children with partly inconsistent results. The situation is particularly unclear in adults. While there are reports of lower (Andari *et al.*, 2010) and higher levels (Jansen *et al.*, 2006) in autistic adults, our group recently added to the existing literature by reporting no differences in basal OXT levels (Albantakis *et al.*, 2021), which was also true for the sample reported here. In accordance with our results and highlighting the effect of age in this context, a recent meta-analysis including 31 studies reported OXT baseline concentrations to be lower in autistic children but indifferent in adults (John and Jaeggi, 2021). This is suggestive for relevant developmental changes in the OXT system in ASD and possibly for a normalization of OXT levels in adulthood. John and Jaeggi (2021) suggest this to be in line with studies reporting improved symptomatology in adults as compared to children or adolescents with autism (Magiati *et al.*, 2014). The importance of developmental effects in this context has also recently been reported with regard to OXTR expression patterns (Rokicki *et al.*, 2022). Andari et al. (2014) have previously speculated on a relationship between the OXT system and social behavior, suggesting a reciprocal enhancement of OXT release and social contact during development. In light of findings, which demonstrate that social experiences such as social network size are reflected at the level of brain function and structure (Sallet *et al.*, 2011; Von der Heide *et al.*, 2014), they suggest that this developmental interplay of OXT and social experiences could modulate or bring about such structural changes in the brain. Taking into account previous findings on the HTH in autistic children, the results of our study including adults could be taken to suggest that there may be indeed developmental changes in the HTH brain structure related to the development of the OXT system in ASD. In contrast to previous structural studies reporting diminished hypothalamic GM volume or concentration in

autistic individuals, we found no differences in GMV in our adult sample. While previous studies examined children (Shou *et al.*, 2017) (mean age 4.1 ± 0.72; n=28), adolescents (Kurth *et al.*, 2011) (mean age 11.2±3.7; n= 104) and young male adults (Wolfe *et al.*, 2015) (mean age 21.0 ± 0.9; n= 20) in a narrow age range, in the present study we examined adult individuals with a wide age range (mean age 36.03 ± 11.0 range [18;60]; n=56). Consistent with the finding of no volumetric GM differences, 3rdVen volumes and automated HTH volumetry using deep-learning also did not reveal differences between groups. These results would be consistent with the idea that a hypothesized normalization of OXT levels in adulthood in ASD is associated with normalization of hypothalamic volume, but this question is clearly beyond the scope of our cross-sectional study. While, to the best of our knowledge, our study is the first to explicitly report no volumetric differences in the HTH between autistic adults and non-autistic adults, it should be noted that from a formal perspective whole-brain VBM is a method that does not depend on a prior spatial hypothesis, so that VBM studies in ASD that found no abnormalities in the HTH could be considered nonsignificant results with respect to this structure. However, as pointed out by Schindler et al. (2012), the sensitivity of this method to differences in brain structures as small as the HTH depends largely on the hypothesis-dependent parameter settings, so a lack of discussion of the HTH in these studies does not automatically imply a lack of effect in this region. Further supporting the notion of a potentially important role of HTH in ASD, but also indicating the importance of individual differences in this regard, we found a significant association between autistic traits and GMV in HTH in the ASD group, but no such correlation in the CG. Taken together these results may point to a link between the growth pattern of the HTH and ASD. While further, preferably longitudinal studies are needed to confirm this hypothesis, the concept of complex growth patterns of regional brain volumes in ASD that are strongly restricted to specific phases of neurodevelopment is not new (Courchesne *et al.*, 2011; Greimel *et al.*, 2013; Ecker *et al.*, 2015). A growth pattern of volume reduction and convergence to normal volume has for example been reported for the striatum (Langen *et al.*, 2014). A ‘reverse’ growth pattern of a decrease with age has been suggested for the amygdala and a range of other structures (Schumann *et al.*, 2004; Nacewicz *et al.*, 2006; Lange *et al.*, 2015). The mechanisms underlying these changes in ASD, however, are unclear. While an ASD inherent growth pattern is one possibility, these structural changes might also reflect compensatory adaptations. In the present study, we found evidence that the GMV in the HTH may be linked to peripheral OXT levels and that OXT may therefore be one of the factors underlying structural changes in this region. More precisely, we found a positive association of OXT with

hypothalamic GMV in autistic adults as compared to non-autistic adults. The exploratory whole-brain analysis further revealed that these differences were significant not only in the HTH but also in the thalamus. The latter observation is consistent with reports suggesting that the thalamus may be involved in OXT release in the HTH triggered by social stimuli (Dobolyi *et al.*, 2018). As an underlying mechanism for how OXT might be associated to brain structure, the regulatory function of OXT on neuronal plasticity and its role in inhibitory or proliferative cellular processes is well established (Theodosis and Poulain, 1987; Theodosis, 2002; Gimpl *et al.*, 2008; Lestanova *et al.*, 2016; Rajamani *et al.*, 2018; Falougy *et al.*, 2019). However, at this stage, it remains unclear whether the here observed differences in associations are directly attributable to OXT or mirror other factors related to both peripheral OXT concentrations and volume of the HTH. First, it should be noted that it remains controversial whether and to what extent peripheral OXT measurements can reflect central (dys-)regulation of the OXT system. In particular, a coordination of peripheral and central OXT levels at baseline conditions has been called into question (Valstad *et al.*, 2017). The inconclusive picture regarding the informativeness of OXT levels has motivated researchers to investigate genetic variation in the human OXTR gene, where several single nucleotide polymorphisms (SNPs) such as rs53576 and rs2254298 have been linked to ASD (LoParo and Waldman, 2015). In a VBM study with healthy carriers of the ASD associated OXTR allele rs53576 in 212 healthy subjects (103 males, 109 females, mean age = 29.9 ± 9.0 years), Tost et al. presented evidence for a significant allele load-dependent decrease of GMV in the HTH. Furthermore, they found that decreased hypothalamic volumes predicted lower prosocial temperament scores in males only (Tost et al., 2010). They reported similar results in a follow-up study for the OXTR variant rs2254298A, another ASD associated gene (Tost et al., 2011). However, the latter association could not be replicated in a Japanese sample (Yamasue et al., 2011). rs2254298, along with other OXTR variants with increased likelihood for ASD, was further reported to be closely linked to peripheral OXT levels (Feldman *et al.*, 2012). This raises the question of whether morphological changes in the HTH and OXT levels are modulated by common factors such as variations in OXTR. In this context, it also remains to be clarified to what extent other genetic variations of the OXT system such as the structural gene for OXT (oxytocin-neurophysin I) and CD38 (associated with OXT release) play a role (Quintana *et al.*, 2019). Another factor that could be involved here is Vassopressin (VP). VP is a neuropeptide closely related to OXT and produced in the same nuclei. Similar to OXT it has been suggested to be similarly involved in the regulation of social behavior (Borie *et al.*, 2021). Shou et al. (2017) reported decreased bilateral hypothalamic volume to be positively

associated with peripheral VP concentrations. Shou et al. (2017) thus hypothesized that impairment of the VP/OXT system is associated with changes in brain morphology and function in ASD. However, they did not examine OXT levels in this context. Here, in future studies, it could be interesting to examine both ADH and OXT in parallel and possibly uncover parallel effects or determine interactions between the two peptides in relation to ASD. As previously mentioned, the investigation of the selective vasopressin 1a receptor antagonist Balovaptan as a therapeutic agent to improve socialization and communication in autistic children and adolescents has so far remained without significant effects compared to a placebo group (Hollander *et al.*, 2022). Further investigation of a stratification of individuals who might benefit from a therapy with Balovaptan based on the volume of HTH might prove beneficial.

A better understanding of the structure and function of the HTH in ASD may also be relevant for other psychiatric disorders with similar socioemotional deficits as in ASD. For example, using an approach similar to the one used here, Mielke et al. 2018 conducted an analysis in women with early childhood maltreatment guided by the hypothesis that the deficits in reward processing in adults who were maltreated as children may be related to OXT and structural differences in the HTH. Interestingly, compared to our results, they found an opposite relationship between OXT and HTH volume in patients compared to controls (Mielke et al. 2018), possibly indicating a differential relationship of OXT and the HTH compared to ASD. Although it is too early at this stage to draw any conclusions here, comparative analyses of the relationship between OXT and the HTH in different psychiatric conditions could be a promising approach for future studies. In addition to the already mentioned craniopharyngioma patients (Fjalldal *et al.*, 2013; Gebert *et al.*, 2018; Brandi *et al.*, 2020), hypothalamic abnormalities have also been reported for schizophrenia (Bernstein *et al.*, 2021) and mood-disorders (Schindler *et al.*, 2012). Given the high prevalence of depression in ASD (Albantakis *et al.*, 2018), a comparative analysis in this regard would be particularly relevant to determine the specificity of hypothalamic abnormalities for ASD.

5.2 Discussion of the methods

5.2.1 General considerations of structural neuroimaging in ASD

In the present study, we used morphometric methods to extend our understanding of the neurobiology of ASD. However, it seems legitimate to ask what benefit brain structural

findings can have in a disorder that is mainly defined by clinical behavioral features. The situation is different, for example, for diseases clearly defined as ‘neurodegenerative’, such as Alzheimer's disease, where structural atrophy of brain tissue is part of the known neurobiological basis of the disease (e.g. Falkai *et al.*, 2018). The interpretation of structural findings is much more difficult in conditions like ASD, where the precise neurobiological basis is still only poorly understood. Moreover, it should be noted that structural brain differences in one population compared to another generally do not allow causal explanations for that condition. Rather, structural findings can be considered as an additional source of information that must be interpreted in conjunction with behavioral features and prior knowledge of region-specific functions. Overlap between structural and functional findings and similar symptoms in other disorders could thus lead to a better understanding of the underlying neural mechanisms. Ideally, these mechanistic insights can then guide the development of new therapeutic strategies (Ecker, 2017) or the information can help to stratify patients for different interventions. An example by Ecker et al. (2007) is the finding of an enlarged caudate nucleus, which is part of the cortico-striatal network, in young autistic persons. The cortico-striatal network has been associated with repetitive behavior in both autistic individuals and individuals diagnosed with obsessive-compulsive disorder. This may therefore be an indication that both groups may respond to similar treatment strategies. In addition, brain structural features could help to predict treatment courses and may potentially inform the selection of different treatment options.

Furthermore, it should be noted that while neuroanatomical findings using MRI have greatly improved our understanding of the brain structures involved in ASD, they represent only one of many levels at which relevant features can be observed. For example, it still remains unclear what tissue properties may underlie the altered GM signal in VBM studies including our own. In addition to physical cell volume, also nuclear volume, local cell number, and spatial cell grouping of neurons, glia, vessels, and neuropil could play a role (Asan et al., 2021). Remarkably, changes in all these morphological and cytoarchitectonic features have been described throughout the brain in ASD (Varghese et al., 2017). This highlights the importance of a multimodal investigation of structural alterations in ASD. Optimally, in the future, macroscopic structural abnormalities can thus also be understood at the level of the microarchitecture of cortical and subcortical structures and ultimately also at the cellular and genetic level. At the cellular level, for example, alterations in neurogenesis and synaptic pruning have been suggested to underly atypical neuroanatomy and connectivity in early brain development (Donovan and Basson, 2017). An example of how genetic variation can cause

synaptic dysfunction, which in turn is associated with changes in brain structure, is the Shank3 gene. Shank3, which codes for a postsynaptic protein at glutamatergic synapses, has been associated with autistic-like behavior in knockout mice lacking this gene (Peça *et al.*, 2011), causing structural changes in total brain volume and white matter (Golden *et al.*, 2021). A further example given by Ecker et al. (2013) are the genes encoding the two ASD-associated enzymes GAD65/67 (glutamic acid decarboxylase), which are involved in the synthesis of the neurotransmitter GABA and play an important role as trophic factor in both the developing and the mature brain (Ecker *et al.*, 2013). To what extend such genetic variations might be linked to the findings presented in this study remains to be seen. As pointed out above, genetic variation in the OXTR gene, the structural gene for OXT (oxytocin-neurophysin I) and CD38 (associated with OXT release) could be of particular importance here.

Another issue with regard to brain structural analyses in autistic adults is that we do not know whether the same mechanisms responsible for structural alterations observed in autistic children are actually linked to alterations in adulthood. Longitudinal studies suggest that not only early neurodevelopment but also late neurodevelopment, i.e., neurodevelopment from age three into adulthood, is atypical (Lange *et al.*, 2015). A question that arises here is whether atypical structural development must be viewed as a single continuous process or whether different processes with different onset points across the lifespan play a role. For example, it has been speculated that an increase in brain volume in childhood may be linked to premature atrophy in adulthood, but it is also thinkable that the two phenomena occur independently (Lange *et al.*, 2015).

Finally, the heterogeneity of the clinical picture in ASD patients is one of the major challenges for neuroanatomical research in this field. It is not surprising that no singular structure has yet been identified to uniquely define the disorder. However, this might also represent an opportunity, especially for novel structural imaging methods such as machine learning. Automated analysis of large patient groups using automated techniques could help to classify subgroups within the heterogeneous patient population based on structural and functional neuroimaging data (Dwyer and Koutsouleris, 2022). In this respect, functional and structural MRI data could be seen as complementary sources of information, the joint consideration of which allows a more accurate picture to be obtained (Mellema *et al.*, 2019). Despite the outlined limitations, structural imaging in ASD research may thus be of central importance in the future to improve diagnostic procedures, provide accurate prognoses and,

optimally, also provide the basis for subgroup-specific support. Not least, neuroanatomical insights into conditions such as ASD can also improve our understanding of the neurotypical brain (Donovan and Basson, 2017).

5.2.2 Strengths and limitations of the present study

The results presented here should be interpreted in the context of some methodological considerations and limitations. Given the important role of the HTH for the OXT system and the potentially important role of OXT in ASD, a surprisingly small number of studies have focused on this brain structure. This is certainly partly due to the methodological difficulties in the accurate identification of the HTH in MRI images. However, the advantages of magnetic resonance imaging (MRI) as a general method of choice for brain imaging are evident and are mainly due to the possibility to examine the brain anatomy in vivo, and to achieve higher resolution and better contrast without harmful radiation compared to Computer Tomography (CT). T1 weighted images are generally considered the best option for morphometric MRI studies (van der Kouwe and Fischl, 2015). A field strength of 3 Tesla that we used in our study provides detailed spatial resolution and is standard for most current structural MRI studies, although ultra-high field MRI (≥ 7T) has been shown to provide even better spatial resolution and contrast, especially for subcortical and microstructures (Maruyama *et al.*, 2019). Thus, while studies have also shown that delineation of the HTH can be achieved even at a lower field strength of 1.5T (Baroncini *et al.*, 2012), higher resolution may be a factor to consider in future studies focusing in the HTH.

Here, we took a relatively straightforward approach by using a HTH mask created on the basis of 168 typical adults (Pauli *et al.*, 2018). Since this mask was not created specifically for our sample, it can only be considered a rough regional reference. Manual delineation remains the gold standard here, but comes at the cost of a high degree of expertise and time investment (Bocchetta *et al.*, 2015; Boes *et al.*, 2018). Other studies have used a HTH mask based on the WFU pick atlas (Mielke *et al.*, 2018; Le *et al.*, 2020), which we decided against since it covered the HTH much less accurately in a visual comparison. Since we did not aim for an exact volumetry of the entire HTH but for a voxel-wise comparison between groups in this region, some degree of inaccuracy may not have had a large impact. Due to these methodological difficulties, another indirect attempt to detect structural changes in the HTH has been to investigate the adjacent 3rd ventricle. Limiting the informativeness of this indirect approach, it has previously been criticized that the volume of the entire 3rdVen could be a

misleading parameter since only its anterior portions would be actually adjacent to the HTH (Schindler *et al.*, 2012). Promising developments in terms of accurate and user-friendly volumetry in this region come from deep-learning approaches (Billot et al. 2020). While we exploratively used the latter method in our study, we did not systematically question or test the validity of the underlying algorithm.

Further limitations concern the sample studied here. First, although the groups were not significantly different, they were not optimally balanced in terms of age and sex distribution. It has previously been pointed out that even in very homogeneous groups, it would be wrong to assume that sex and age can therefore not have a significant influence on morphological differences between groups (Henley *et al.*, 2010). We therefore included these variables as nuisance parameters in all models as is recommended by default in VBM studies (Barnes *et al.*, 2010). While the large age range (18-60 years) of adults tested here may provide a good overview of robust structural effects, it risks overlooking age-specific effects given the apparent age dependency of morphological abnormalities in ASD. Moreover sex-dependent morphological differences of the HTH have been reported in neurotypicals (Makris et al. 2013), as have structural associations with OXTR (Tost *et al.*, 2010; Yamasue *et al.*, 2011). These notions may warrant sex- and age-specific analyses in future. Longitudinal study frameworks could be particularly helpful here to determine how structural characteristics of HTH manifest across the lifespan. Age related subgroup evaluation was not possible in our study because it would have made the resulting sample size too small.

Second, 45% of autistic participants in our study received psychotropic medication on a regular basis. This adequately represents the high degree of comorbidities in these patients in terms of a naturalistic study design (Lai and Baron-Cohen, 2015). However, this also carries the risk of bias in the results. Due to the various pharmaceutical substances (antispsychotics, antidepressants, stimulants) and different dosages, it did not seem helpful to include medication as confounding variable. While we cannot rule out the possibility that medication may have had an impact on brain structure, studies to date show no evidence of structural effects in the HTH in this regard (Scherk and Falkai, 2006; Bellani *et al.*, 2011). In an ideal study, it would be desirable to minimise confounding through medication through sample selection prior to the data collection, which, however, would be a major challenge in terms of participant recruitment.

Third, our analysis included only ASD patients diagnosed with high-functioning autism. This, naturally, limits the generalizability to the entire autistic spectrum. Confirming the previous

diagnosis of the participating autistic adults, regarded as high-functioning autism without intellectual impairment, there were no group differences in verbal IQ but significant differences in AQ scores. AQ scores were well in line with the corresponding reference norms for autistic and non-autistic individuals (Ruzich *et al.*, 2015). This seems to justify the use of AQ scores as a suitable discriminator between groups. However, given the large interindividual heterogeneity in symptoms and traits in ASD, which are also reflected at the structural level of the brain, it should be noted that this may be an imprecise overgeneralization and may not adequately reflect the characteristics of individual subjects. Instead of total-scores, a consideration for future studies would therefore be to use a more detailed analysis using the AQ subscale scores, which have been reported to provide a more accurate characterization (English *et al.*, 2020). In general, accurate and homogeneous characterization of autistic individuals is one of the major challenges in ASD research. Here, we have used a rough classification based on the absence of intellectual disability, also referred to as "high-functioning" autism. High-functioning autism in this context refers to a subgroup of autistic individuals which is characterized by an IQ of at least >70. With respect to HTH, this criterion may be advantageous in light of reports that hypothalamic abnormalities may be associated with intellectual disability (Swaab, 2004). Thus, attributing findings in the HTH to the autistic phenotype in individuals with intellectual disability would certainly be more problematic. It should be noted, however, that this differentiation based on IQ alone has been criticized as an inaccurate clinical descriptor (Alvares *et al.*, 2020). We did not distinguish between Asperger syndrome and HFA autism. Both subtypes show no learning disability, but a distinction between the two groups is made based on the age at which speech is acquired. Per definition speech development is slightly delayed in HFA, whereas in Asperger syndrome it occurs normally (ICD-10). However, it has been pointed out that this distinction is not useful clinically (Howlin, 2003) or in terms of brain morphological studies (Via *et al.*, 2011; Ecker *et al.*, 2012). In summary, our study, like most studies with autistic individuals, allows only very limited conclusions to be drawn about the autistic population as a whole.

6 Summary and outlook

In this study, we investigated brain structure in autistic and non-autistic adults in a VBM-based whole-brain analysis, as well as a possible association of brain volumetry with peripheral OXT levels in the HTH using a ROI-based analysis. In the whole-brain analysis using liberal thresholds, we found indications for increased local cerebellar WM volumes in autistic as compared to non-autistic adults. In addition, we found an association of regional cerebellar WMV and GMV with autistic traits across all participants. Although a conclusive interpretation of these results is still pending, this may represent further evidence that the cerebellum may be involved in ASD. Regarding the hypothesis-driven ROI-based analysis of the HTH, we observed an association between interindividual differences in autistic traits and GMV, but only in autistic adults. Furthermore, in autistic as compared to non-autistic adults, we observed a positive association between peripheral OXT concentration and GMV in the HTH and, exploratively, also in the thalamus. Although this study does not provide insight into a causal relationship of these associations, our results provide new evidence for a potentially important role of HTH in ASD and its relationship to the OXT system, but also point to the importance of interindividual differences. In conjunction with previous studies our results also raise new questions about possible developmental changes in brain structure and its relationship to OXT in ASD and motivate further investigations. A better understanding of the interrelationship between genetic variation of the OXT system, OXT levels, and brain structure may considerably enhance our understanding of the role of OXT in ASD both as pathophysiological factor and potential therapeutic avenue.

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8 Appendix

8.1 Quality check

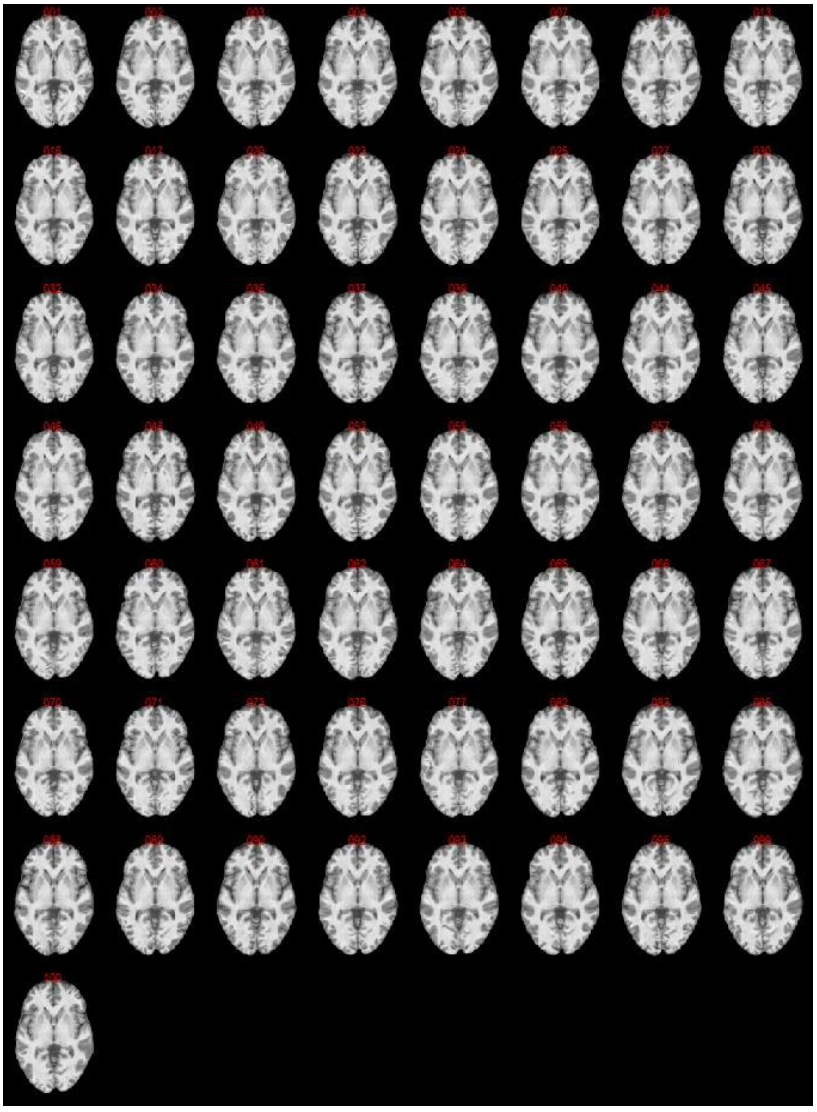
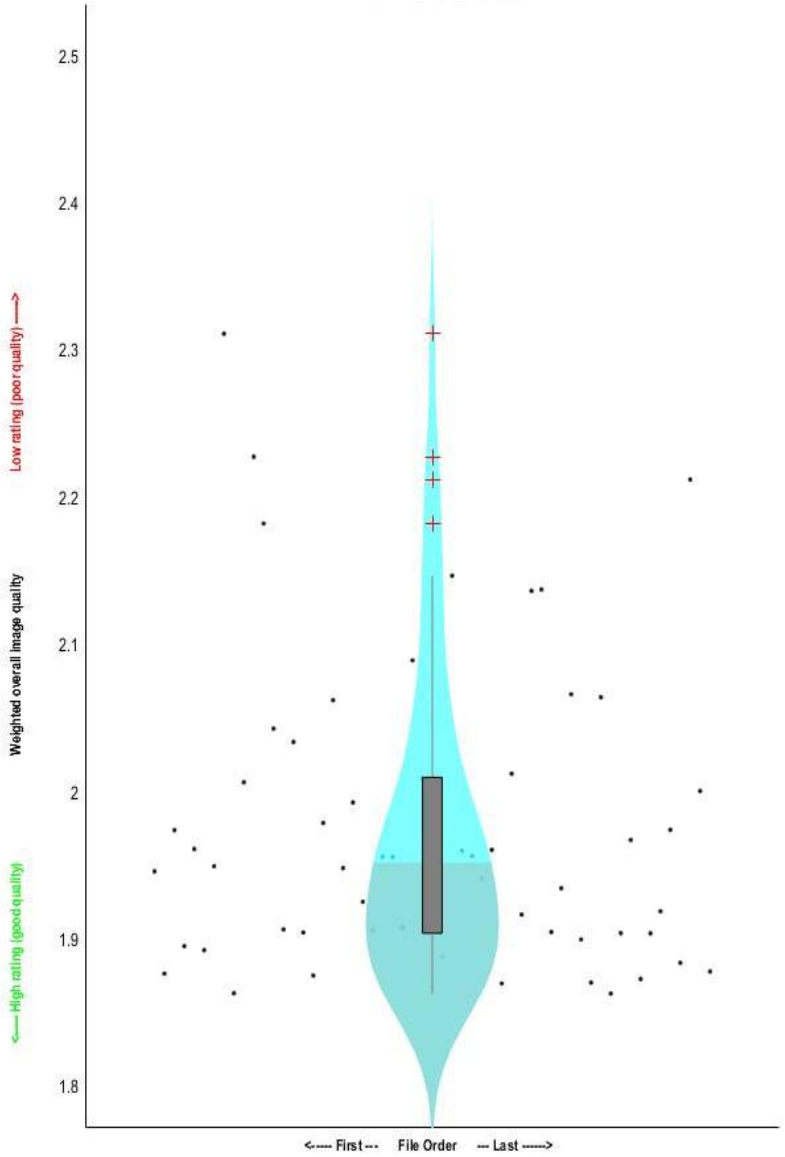


Figure 24 With CAT12's function 'display slices' one horizontal slice for each subject of the normalized bias-corrected images was displayed. This allowed for a gross overview of successful pre-processing. No images were removed due to visible abnormalities in this view.

Image quality definition	excellent			good			satisfactory			sufficient			critical			unacceptable / failed
BWP noise (in percent)	0	1	2	3	4	5	6	7	8	9	10	15	20			
BWP bias (in percent)	0	20	40	60	80	100	120	140	160	180	200	300	400			
resolution RES (mm)		0.5		1.0		1.5		2.0		2.5		4.0	5.5			
Quality ratings																
procentaged rating points (rps)	100	95	90	85	80	75	70	65	60	55	50	25	0			
linear rating scale	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	8	10.5			
nominal numbers	1+	**1**	1-	2+	**2**	2-	3+	**3**	3-	4+	**4**	4-	5+	**5**	5-	**6**
nominal letters	A+	**A**	A-	B+	**B**	B-	C+	**C**	C-	D+	**D**	D-	E+	**E**	E-	**F**
description	excellent			good			satisfactory			sufficient			critical			unacceptable / failed

Figure 25 Figure displaying quality rating from Cat12 manual (http://www.neuro.uni-jena.de/cat12/CAT12-Manual.pdf). Weighted image quality rating in CAT12 is based on quality parameters such as noise, image inhomogeneities and resolution. The 56 volumes, which were included in this study had a mean quality rating of 85.3 (range 82.7 - 86.37), which corresponds to good overall quality. The two diagnostic groups (ASD and HC) did not significantly differ regarding image quality.



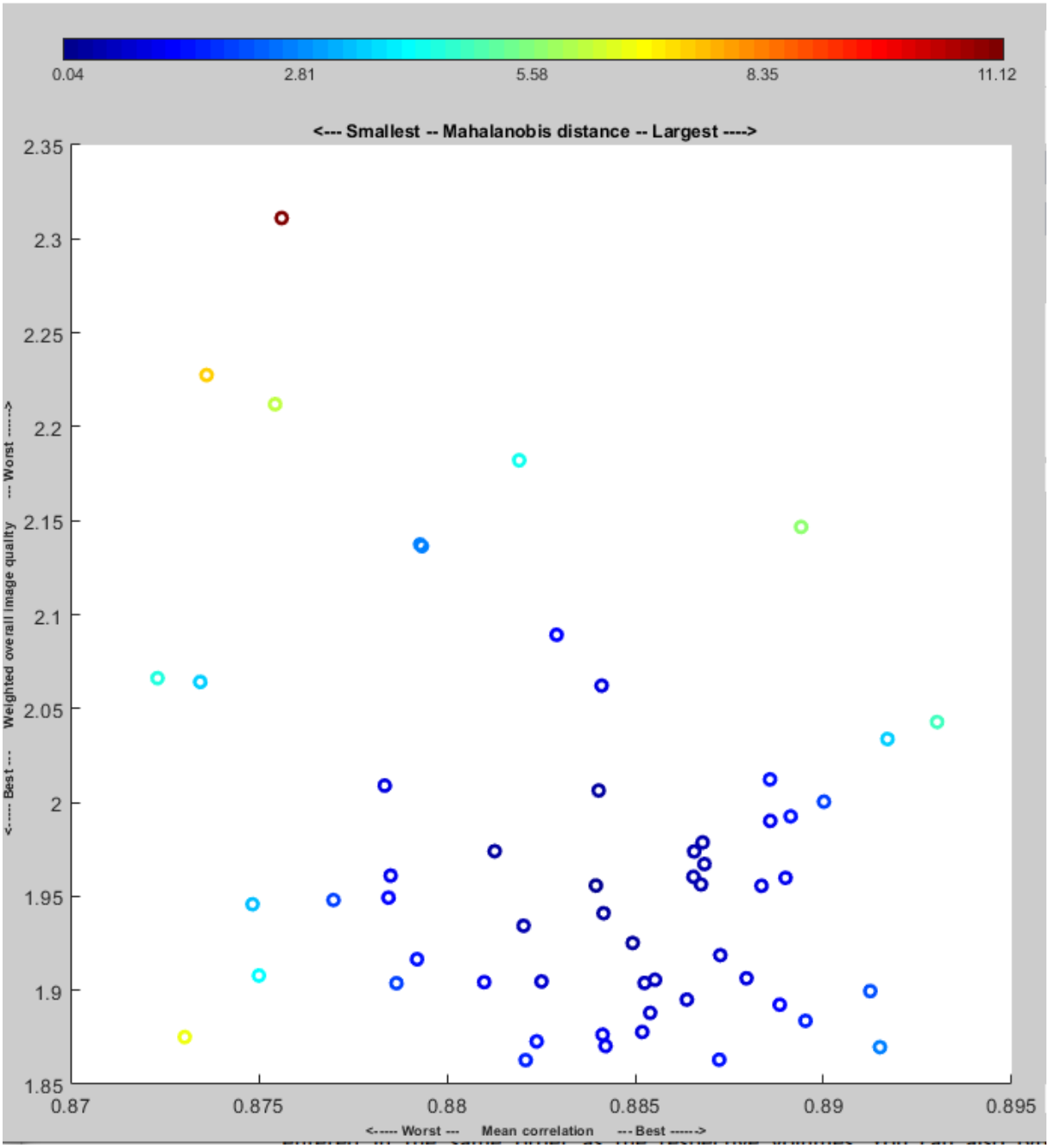


Figure 27 Color coded Mahalanobis distance between mean correlation (reflecting after pre-processing quality) and overall image quality (reflecting before pre-processing quality), as a measure of overall combined image quality before and after pre-processing. There are no clear criteria about when to exclude an image based on quality measures, if no artefacts are found. Although no obvious artefacts or abnormalities were found, it was decided to exclude the most deviating volume (displayed in dark red) from further analysis in order to avoid biased results.

8.2 VBM

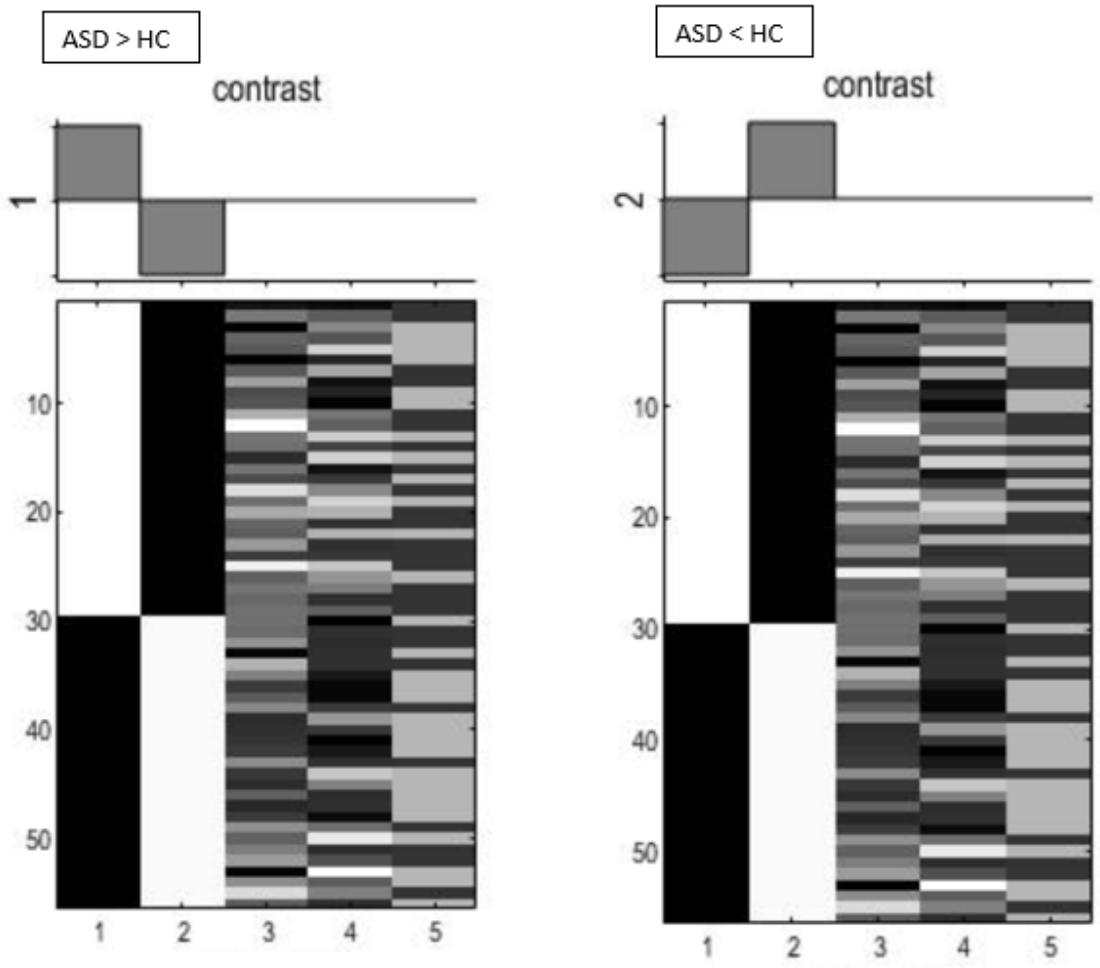


Figure 28 Exemplary illustration of design matrices of the two-sample-t-tests conducted for smoothed GM and smoothed WM images in SPM12. (columns are 1: ASD images 2: HC images, 3: TIV, 4: Age, 5: Sex)

8.3 Orthogonality

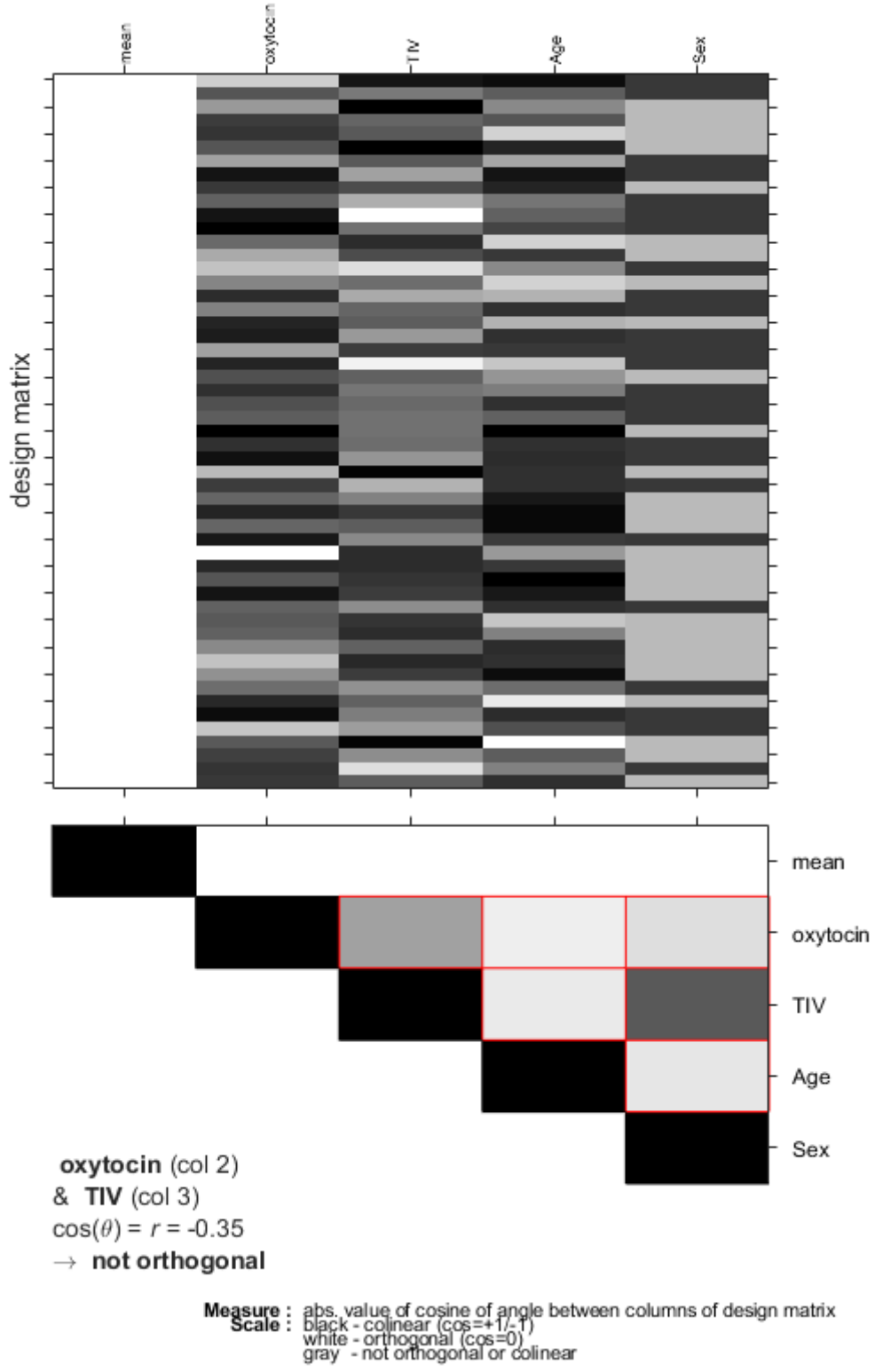


Figure 29 Color coded illustration of check for orthogonality in SPM12. Non-orthogonality between oxytocin baseline levels and TIV is illustrated by the darker grey colored square at the intersection of these two variables. Unlike the non-orthogonality between TIV and Sex, this is problematic, as parts of the variance explained by oxytocin would be removed as well. Therefore, instead of being modelled as a covariate, global scaling with TIV was used instead.

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