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The Roots of Autism and ADHD Twin Study in Sweden (RATSS)

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Abstract

Neurodevelopmental disorders affect a substantial minority of the general population. Their origins are still largely unknown, but a complex interplay of genetic and environmental factors causing disturbances of the central nervous system's maturation and a variety of higher cognitive skills is presumed. Only limited research of rather small sample size and narrow scope has been conducted in neurodevelopmental disorders using a twin-differences design. The Roots of Autism and ADHD Twin Study in Sweden (RATSS) is an ongoing project targeting monozygotic twins discordant for categorical or dimensional autistic and inattentive/hyperactive-impulsive phenotypes as well as other neurodevelopmental disorders and typically developing twin controls. Included pairs are 9 years of age or older, and comprehensively assessed for psychopathology, medical history, neuropsychology, dysmorphology, as well as structural, functional, and molecular neurobiology. Specimens are collected for induced pluripotent stem (iPS) cells, neuroepithelial stem cells, genetic, gut bacteria, protein-/monoamine, and electron microscopy analyses. RATSS's objective is to generate a launch pad for novel surveys to understand the complexity of genotype-environment-phenotype interactions in autism spectrum disorder and ADHD. By October 2013, RATSS had collected data from $N = 55$ twin pairs, among them 10 monozygotic pairs discordant for autism spectrum disorder, 7 for ADHD, and 4 for other neurodevelopmental disorders. This article describes the design, recruitment, data collection, measures, collected pairs' characteristics as well as ongoing and planned analyses in RATSS. Potential gains of the study comprise the identification environmentally-mediated biomarkers, the emergence of candidates for drug development, translational modeling, and new leads for prevention of incapacitating outcomes.

Key words: psychiatry, etiology, brain, treatment, intervention, drugs.

Neurodevelopmental disorders are disturbances of the central nervous system's architecture, processes, and maturation that affect a person's behavior (e.g., social-communication and interaction skills, language, attention, emotions, motor performance, learning, memory) and significantly reduce functional adaptive skills in everyday life. DSM-5 (APA, 2013) acknowledges the significance of aberrant neurodevelopment for psychiatry by introducing the umbrella concept of neurodevelopmental disorders for the first time (Regier et al., 2009). No good prevalence estimates are available for neurodevelopmental disorders in total, but preliminary survey data from the USA indicates a frequency of 12% in the general population (Pastor & Reuben, 2009). Currently, increasingly accepted estimates for two of the most prominent neurodevelopmental disorders, autism spectrum disorder (ASD) and attention-deficit hyperactivity disorder (ADHD), in school-aged children are ~1-2% (Idring et al., 2012; Kim et al., 2011) and 5% (Polanczyk et al., 2007), respectively. Males are affected 2-4 times more often than females. ASD and ADHD (Rutter, 2005; Froehlich et al., 2007) have been increasingly diagnosed in the last decade. Improved knowledge, increased awareness, and better services, as well as reduced stigmatization, changes in and a more sensitive interpretation of diagnostic criteria probably account for a significant proportion of these apparent time trends (Wazana et al., 2007), but if they can fully explain the increase is debated. Comorbidity is high in ASD and ADHD individuals. For example, the overlap of ASD and intellectual developmental disorders is in the range of 50% (Fombonne, 2009). Simonoff et al. (2008) found that ~28% of school-aged children with ASD also fulfill DSM-IV criteria for ADHD, and 9% for chronic tic disorder. A meta-analysis of ADHD yielded a 10.7-fold risk for conduct disorder, 5.5-fold for depression, and 3.0-fold for anxiety disorder (Angold et al., 1999).

ASD and ADHD have many causes, but most evidence suggests that they are highly heritable and show modest nonshared environmental influence (environmental influences that

make children growing up in the same family different) (Lichtenstein et al., 2010; Chang et al., 2013). Aside from clinically relevant phenotypes qualifying for a categorical diagnosis, data increasingly support the notion of broader/extended phenotypes and traits of ASD and ADHD that are continuously distributed in the general population (Constantino & Todd, 2003; Normand et al., 2012), with their variation determined by factors overlapping with clinical phenotypes (Robinson et al., 2011; Lundström et al., 2012; Larsson et al., 2012). Heritability estimates from behavior genetic studies, predominantly twin research, indicate that genetic factors account for ~80% of symptom variation in ASD and ADHD (Ronald & Hoekstra, 2011; Wadsworth et al., 2007; Wood & Neale, 2010; Taylor et al., 2013), as well as ASD-like and ADHD-like traits (e.g. Constantino et al., 2003). In addition, behavior and molecular studies indicate partly overlapping genetic components for both disorders (e.g. Heiser et al., 2004; Reiersen et al., 2008; Ronald et al., 2008; Moreno-De-Luca et al., 2013; Taylor et al., 2013).

The genetics of ASD and ADHD are complex. In 10% of individuals with ASD an identifiable single gene disorder, such as fragile X, tuberous sclerosis or neurofibromatosis exists, which may account for the phenotype. In addition, 5-10% have known chromosomal rearrangements such as maternally inherited duplications of 15q11-q13 or rare de novo or inherited copy number variations (CNVs), predominantly in genes with synaptic functions (e.g., *NLGN3, NLGN4, NEUREXIN1, SHANK3, and CNTNAP2*) (Persico & Napolioni, 2013). These findings, coupled with genome sequencing data, suggest the existence of hundreds of ASD risk genes (Devlin & Scherer, 2012). To date, ADHD studies have reported substantial evidence implicating several genes, including those in the dopaminergic (*DRD4, DAT1, DRD5, COMT*), noradrenergic (*DBH, ADRA2A*), serotonergic (*5-HTT, HTR1B, HTR2A*), cholinergic (*CHRNA4*), and central nervous system development (*SNAP25, BDNF*) pathways (Caylak, 2012).

Even though ASD and ADHD are highly genetic, the causes are multifactorial and environmental influences also play an important role in susceptibility, with nonshared environment consistently reported in twin studies of ASD, ADHD and their related traits (Ronald & Hoekstra, 2011). Environmental factors may act via epigenetic mechanisms, i.e. modifications of gene expression controlled by something other than the DNA sequence that are potentially reversible. These factors are influenced by the maturational stage, tissue type, and environment. Evidence suggests that dysregulation of epigenetic markers or mechanisms, such as DNA methylation, play an important role in ASD and ADHD and integrate genetic and environmental influences to dysregulate neurodevelopmental processes (Grafodatskaya et al., 2010; Smith et al., 2009). Environmental factors may play a role in the inception and lifelong modulation of ASD (Herbert, 2010). Some gene variants in ASD confer altered vulnerability to environmental stressors and exposures (Levy et al., 2009). Random *de novo* mutations increasing with advanced parental age are stochastic risk factors for ASD, suggesting a role of the environment (Parner et al., 2012). The systemic and central nervous system pathophysiology in ASD, including oxidative stress, neuroinflammation, and mitochondrial dysfunction, can be consistent with environmental influence (e.g., air pollution, organophosphates, or heavy metals) (e.g., Chauhan & Chauhan, 2006; Jung, Lin, & Hwang, 2013), and some of the underlying biochemical disturbances (e.g., abnormalities in glutathione, a critical antioxidant and detoxifier) might be reversed by targeted nutritional interventions (Geraghty et al., 2010). Dietary factors and food contaminants may contribute to the risk. Similarly, the environmental risk factors for ADHD include prenatal exposure to substances, exposure to heavy metals and chemicals, nutritional factors, and lifestyle/psychosocial factors (Froehlich et al., 2011).

As indicated by the designation neurodevelopmental disorder, genetic, epigenetic, and environmental factors detrimentally affect neurobiological and neurocognitive development

and maturation. In ASD, macrocephaly is noted by age 12 to 24 months in a substantial proportion of children (Courchesne, Campbell, & Solso, 2011). Structural neuroimaging studies demonstrate structural alterations in both cortical and white matter regions, together with abnormal patterns of growth mainly localized in the frontal lobe, temporal lobes, and limbic structures, such as the amygdala. Diffusion tensor imaging has demonstrated the disruption of white matter fiber bundle connecting brain regions associated with various high level cognitive functions (e.g. social functioning, language). Functional neuroimaging has found alterations in activation and synchronicity across cortical networks, with reduced functional connectivity relating to a variety of cognitive functions, including language, working memory, social cognition or perception, and problem solving (Vissers, Cohen, & Geurts, 2012). Neurochemical investigations with animal models and empirical drug studies remain inconclusive. Genetic differences in serotonin transport seem to have the most empirical evidence for a role in ASD, whereas data lending support to a role of the dopaminergic and glutaminergic systems are presently less robust, but evolving (Geschwind & Levitt, 2007).

Taken together, the results thus far indicate that ASD is characterized by a neuronal-cortical organization impacting on developmental trajectory of social cognition (including social attention and social orientation) (Senju, 2013), executive function and problem-solving mindset (Levy et al., 2009), and top-down vs. bottom-up information processing with the integration of information into meaningful gestalt (Bölte et al., 2007; Dziobek & Bölte, 2011). These findings tie in with behaviour genetic research that suggests that the triad of impairments that characterize ASD may have partly different causal pathways (e.g. Happé & Ronald, 2008; Ronald et al., 2011). Neurobiological alterations are widespread in ADHD with the most significant and consistent structural imaging findings including smaller total brain volumes and reduced volumes in the right frontal lobe, right parietal cortex, caudate nucleus,

cerebellar hemispheres, and posterior-inferior lobules of the cerebellar vermis (Tripp & Wickens, 2009). Neurochemically, ADHD involves hypofunctioning of catecholaminergic circuits, particularly those that project to the prefrontal cortex (Durston, 2008). On the cognitive level, a triple pathway model of ADHD is favored, with impairment in executive control/inhibition, motivation (delay-aversion), and timing as dissociable neuropsychological components (Sonuga-Barke et al., 2010).

As described above, the understanding of the potential causes of neurodevelopmental disorders, most notably ASD and ADHD, has significantly increased. Still, knowledge regarding their etiology and pathophysiology is far from complete. The genetic, epigenetic, and environmental mechanisms driving neurodevelopmental phenotypes remain poorly understood. Outcome variability, evidence of a treatment response to a variety of interventions, and the possibility of improvement in ASD and ADHD suggests that the brain circuitry is amenable to epigenetic/environmental modulation. Thus, targets need to be sought for early biopsychosocial prevention and treatment. However, the ASD and ADHD research so far has several limitations. First, most studies are based on cases versus typical controls only, which does not allow conclusions about causality. Second, many studies are one-dimensional, focusing on the associations between narrow aspects of psychopathology and (epi-)genetics, environment, neurobiology, or neuropsychology. Third, studies have mostly looked at behavioral ASD and ADHD phenotypes categorically (as clinical diagnoses), rather than dimensionally (as traits), and almost never jointly. Fourth, DSM-IV and ICD-10 include mutual diagnostic exclusion criteria for ASD and ADHD, which hampered appropriate research on conditions with high comorbidity.

With this project, we seek to overcome many of these shortcomings in order to generate a launch pad for pioneering, novel evidence in understanding the complexity of ASD and ADHD. For this purpose, the largest cohort ever of monozygotic (MZ) twins discordant

or concordant for carefully characterized clinical and subthreshold ASD and ADHD phenotypes, as well as typically developed controls, will be investigated in concert with multidimensional, simultaneous assessments, and combined analytical approaches comprising medical history, comorbidity, neuropsychology, dysmorphology, collection of neuroepithelial stem cells and specimens for (epi-)genetic, neurotoxic, gut bacteria, protein-/monoamine, electron microscopy analyses, and multimodal neuroimaging (e.g. structural, functional, molecular investigations) measures will be collected. We hypothesize subtle, but specific, differences within discordant monozygotic ASD and ADHD pairs on multiple biological and behavioral levels of assessment that will be informative for deciphering the complexity of genotype-environment-phenotype correlations, especially those related to non-shared environment.

Materials and Methods

Design

RATSS uses the co-twin control or twin-differences design (Martin et al., 1997). Only a moderate number of studies have specifically addressed discordant MZ twin pairs for ASD (e.g. Kates et al., 1998, 2004, 2009; Hu et al., 2006; Mitchell et al., 2009; Wong et al., in press) and ADHD (e.g. Lehn et al., 2007; Pearsall-Jones et al., 2008, 2009; Castellanos et al., 2003; Sharp et al., 2003; van't Ent et al., 2009) and the MZ difference has also been used to study traits of ASD and ADHD (e.g. Ronald et al., 2010; Groen-Blokhuis et al., 2011). The findings have been promising, indicating medical history (e.g., ADHD: low birth weight and delayed physical growth/motor development in cases), neuromorphological differences (e.g., ASD: reduced caudate, amygdala, hippocampus, and cerebellar volumes in cases; ADHD: reduced caudate nucleus and prefrontal lobe volumes in cases), and differential gene expression patterns on DNA microarrays (e.g., ASD: *ASS, NAGLU, FLAP,* and *ROBO I)* in

the affected twin. The current project will elaborate upon these works using an integrated, comprehensive assessment approach in a significantly larger cohort, providing the option of cross-disciplinary analyses.

In RATSS, we predict that the affected co-twins, compared to the unaffected co-twins, in MZ pairs discordant for ASD and ADHD will exhibit genetic differences (de novo), such as single nucleotide variations (SNVs) or copy number variants (CNVs) in genes crucial for neurodevelopment, epigenetic differences in selected genes or overall epigenome, and environmental factors as a possible source of epigenetic and phenotypic variation. In addition, we expect these differences to be associated with functional and structural neurobiology, particularly hyper- and hypoconnectivity in ASD, and eventually ADHD (Konrad & Eickhoff, 2010), and molecular brain characteristics (e.g., GABA, glutamate dysregulation). We also hypothesize that these mechanisms correlate with cognitive phenotypes (e.g., ASD: social cognition, attention to detail, flexibility, planning; ADHD: working memory, inhibition, timing, reward dependence), categorically and dimensionally defined ASD/ADHD and ASD/ADHD symptoms, and functional impairment. Finally, we predict that these findings will emerge as specific compared to typical controls, and partly specific to ASD or ADHD.

Participants

The long-term objective of the study is to collect 50 MZ pairs qualitatively or markedly quantitatively discordant for ASD or broader autism phenotype/autism traits, as well as 50 MZ pairs qualitatively or markedly quantitatively discordant for either ADHD or broader ADHD phenotype/symptoms aged 9 to 50 years. In addition, the studies aims to include 30 MZ pairs concordant for each ASD and ADHD, and 40 MZ typically developed control pairs, and 40 MZ pairs discordant for other neurodevelopmental disorders. Twin zygosity is determined on a panel of 48 SNPs (Hannelius et al., 2007). A more balanced gender

distribution than in the general population is aimed to be accomplished in order to allow gender specific research. Exclusion criteria for all participants are profound intellectual disability IQ<35, serious psychiatric (e.g., paranoid schizophrenia) or neurological (intractable epilepsy) conditions, or any well-defined genetic syndrome (e.g., fragile X). Additional exclusion criteria will apply for certain assessments, such as metallic implants, or claustrophobia for MR neuroimaging. Inclusion criteria for ASD will be an ICD-10-defined diagnosis of autism (F84.0), Asperger syndrome (F84.5), or atypical autism/PDD-NOS (F84.1, F84.8, F84.9), or a DSM-5 diagnosis of autism spectrum disorder. For ADHD, the inclusion criteria will be any ICD-10-defined diagnosis of hyperkinetic disorder (F90.0-F90.9) or DSM-5 diagnosis of ADHD (combined, inattentive, or hyperactive-impulsive subtype). Qualitative discordancy is defined as a main diagnosis of ASD or ADHD in twin 1, and either no diagnosis (typical developmental) or a different main diagnosis in the co-twin. Marked quantitative discordancy is defined by (i) both twins having a diagnosis in the same diagnostic group but the diagnosis differ in severity (e.g. autism vs. PDD NOS [ICD-10]; ASD with different severity specifiers [DSM-5]), (ii) twins scoring with ≥ 1 SD difference on dimensional ASD (e.g. SRS and/or AQ) or ADHD (Conners-3 and/or CBCL) scales. In addition, not stressing the concept of phenotypic discordancy, exploratory ASD-like and ADHD-like trait analyses are conducted using even smaller symptom differences.

Twins are currently being recruited from four sources. (1) The primary resource is the Child and Adolescent Twin Study in Sweden (CATSS) (Anckarsäter et al., 2011) targeting all twins born in Sweden since 1994. The response rate for CATSS is approximately 80%, with >25,000 twins born between 1993 and 2004 (2) The patient registry of the Swedish Board of Health and Welfare, (3) the clinical registries of the Division of Child and Adolescent Psychiatry, the Habilitation and Health centers, and pediatric units in Stockholm County, (4) summons in the journals of the National Society for neurodevelopmental disorders

("Riksförbundet Attention"; 13,000 members), the National Society for Autism and Asperger syndrome ("Autism och Aspergerföreningen"; 12,000 members), and the national twin organization ("Tvillingklubben"), exhibitions at their (bi-) annual meetings, and other forms of media (e.g., reports on the project in newspapers and on television).

Procedure and measures

Assessments within RATSS are comprehensive; lasting up to 3 days and with participants recruited from all over Sweden. Participants are continuously accompanied by an experienced research nurse from the first contact to the end of the assessments. In case of the identification of yet undetected psychiatric or medical problems or the emergence of adverse effects connected to the assessments, the project team can adequately and swiftly provide or to refer services. Assessments are carried-out in Stockholm, Sweden, by experienced clinical staff (nurses, psychologists and physicians) at Astrid Lindgren Children's Hospital, the Center of Neurodevelopmental Disorders at Karolinska Institutet, the Karolinska Institutet neuroimaging center, and St:Görans Hospital. Questionnaires are collected by mail before face-to-face assessments in order to limit the burden of the study. Specimens are primarily collected from participants during the visits, but also via express service (e.g., saliva, feces).

Diagnosis, general psychopathology. MZ twins with suspicion of or a previous clinically confirmed diagnosis of ASD or ADHD are followed-up to corroborate the suspicion/diagnosis and create comparable clinical data. For ASD, the Swedish forms of Autism Diagnostic Interview-Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS)-2 are used (note that the first author is a certified ADOS and ADI-R trainer). A diagnosis of ADHD is established using the Kiddie Schedule for Affective Disorders and Schizophrenia (K-SADS) or the Diagnostic Interview for ADHD in adults (DIVA). The K-SADS is also used to examine psychiatric comorbidity in both ASD and

ADHD. In adults, the Structured Clinical Interview for DSM-IV (SCID, axis I) is collected to assess comorbidity. Additional ASD measures collected in all twins are the (parent report) Social Responsiveness Scale (SRS; or adult version, SRS-A) and the Autism Spectrum Quotient (AQ) (or AQ-children). The parent report Conners 3 and the Adult ADHD Self Report Scale are used to quantify ADHD. To assess and quantify general psychopathology, age-appropriate versions of the Achenbach Scales (e.g., Child Behavior Checklist, Young Adult Behavior Checklist) are collected. The Children's Communication Checklist (or Communication Checklist for Adults), Sensory Profile for children or adults, and Adaptive Behavior Assessment Scale-II are obtained for additional psychopathological and functional phenotypic assessment.

Cognition. Cognitive testing comprises different versions of the children's or adult Wechsler IQ (WISC-IV, WAIS-IV) or Leiter scales, and the Peabody Picture Vocabulary Test (in the case of low/borderline intellectual functioning) for general and verbal abilities in all participants. The Reading Mind in the Eyes Test and Movie for the Assessment of Social Cognition are collected for social cognition in ASD and ADHD. The Fragmented Picture test and (Children's) Embedded Figures Test is administered for attention to detail/central coherence, and the Tower test (in D-KEFS) and Wisconsin Card Sorting Test for executive problems in terms of foresighted planning and cognitive flexibility in ASD. The Corsi Block Test for visuo-spatial working memory (also Digit Span of the Wechsler will be available for verbal working memory), the Conner's Continuous Performance Test for sustained and selective attention and inhibition, a modified go/no-go task for timing, and the choice delay task for delay aversion will be used to test executive problems in ADHD.

Medical history. A comprehensive anamnesis and socio-demographic information is collected, including details on familial burden, pregnancy, mother's health and habits, obstetric complications, early nutrition/breastfeeding, viral infections, allergies, food

intolerances, medication, parents' socio-economic status and education, school problems, and diagnostic and therapeutic procedures. Existing clinical records are also reviewed.

Dysmorphology, specimens. Signs of dysmorphology are examined thoroughly by two experienced clinical geneticists, and photographs of the face, hands, feet, and habitus will be taken by a professional clinical photographer for quantitative dysmorphology analyses. Samples of blood, saliva, skin, cerebrospinal fluid (CSF), urine, baby teeth (if available, for toxicity, e.g., metals analysis; not described in detail in this proposal), and stool are collected.

Neuroimaging. A ~50 minutes session in a 3 Tesla MR750 GE scanner at the Karolinska Institutet MR center is conducted. A 32-channel coil array is used and the participants' head position fixed with foam pads to minimize movement. This MR session includes a T1-weighted Inversion Recovery Fast Spoiled Gradient Echo (IR-FSPGR) 3D-volume, three 60-direction diffusion weighted imaging sequences (b-value of 1000 and 3000 s/mm^2), and a resting-state session including 200 T2-weighted Echo Planar Image (EPIs) volumes. In addition, a T2-weighted Fluid Attenuated Inversion Recovery (FLAIR) volume is acquired and checked by an experienced neuroradiologist. With regards to metabolic imaging (MR Spectroscopy), we will add a second session using the same 3T GE scanner with an 8-channel coil array. A Mescher-Garwood point-resolved spectroscopy (MEGA-PRESS) sequence will be used with an 8-channel coil array. Each volume of interest (VOI – see Metabolic biomarkers/magnetic resonance spectroscopy below) is accurately positioned using a T1-weighted IR-FSPGR volume (see above for details). The voxel size, and the number of averages (NEX) are adjusted for each VOI, which ensures a SNR of ca. 15 which is sufficient for reliable quantification. A VOI volume of 8 to 15 mL, TR/TE of 2500/68 ms, and NEX of 192 were applied that resulted in experimental time of 7 min per VOI.

Analyses

We predict differences between affected MZ twins with ASD or ADHD (and pairs discordant for lesser phenotypes) and their unaffected, less affected or differently affected co-twins with regard to medical history, signs of dysmorphology, psychiatric comorbidity, and neurocognition. Those characteristics are not present in the concordant MZ pairs and other typically developing MZ pairs, hence indicating non-shared risks. In addition, we hypothesize additional differences with regard to these parameters between concordant ASD and ADHD MZ pairs and MZ typically developed pairs, in turn (indicating shared risks for neurodevelopmental disorders). Results from these analyses will help us understand the epi-(genetic), CSF, microbiota, and neurobiological findings (see below), as well as generate more phenotype-based, hypotheses-driven studies.

The primary objective of the brain imaging part of RATSS is to examine the "brain connectome" in ASD and ADHD. This model allows us to map and understand *in vivo* the structural and functional neural connections forming the brain, at the network level (Sporns et al., 2005). As for the ASD affected individuals, overall, we expect either a mixed pattern of local over-connectivity and long-distance under-connectivity (of the frontal lobe mainly; Courchesne & Pierce, 2005), or a different network topology. For ADHD, a hypotheses-free exploratory approach will be applied. The secondary aim of the neurobiological investigations is to complete the brain connectome approach with morphometric and further functional (e.g. MRS) investigations. With regards to morphometry, RATSS will analyze, on a voxel-based basis, gray/white matter volumetry, gyrification patterns, cortical thickness, and sulcal morphometry index to provide a characterization of the ASD and ADHD brain properties, which can be used as biomarkers in individuals with neurodevelopmental disorders (e.g., Shokouhi et al, 2012). 1H-magnetic resonance spectroscopy (MRS) is a promising tool for identifying metabolic biomarkers in neurodevelopmental disorders. Several MRS voxels will be positioned on the network nodes suspected in abnormal neuronal processing (from under or

over functional or structural connectivity) or those brain regions demonstrated significant anatomical differences. Among the major hallmarks of 1H-MRS spectra in human brain the glutamate, glutamine (Glu, Gln, often Glu + Gln = Glx), and GABA reflect a excitatory-inhibitory balance that can be specific for certain network nodes. These metabolites are involved not only in signal transduction, but also brain maturation and cortical organization, especially neuronal migration and positioning. GABA and glutamate have been found to be involved in the pathophysiology of neurodevelopmental disorder, particularly ASD (e.g. Pardo & Eberhart, 2007; DeVito et al., 2007; Ecker, Spooren & Murphy, 2013).

Cerebrospinal fluid (CSF) is more likely than blood to contain potential markers of disorders in the central nervous system because the chemical properties of CSF more closely reflect the biochemistry of the brain than blood. CSF markers have the potential to act as diagnostics tools but can also function as predictors for treatment response, side effects, and/or disease progression (Perlis, 2011). CSF-based neurochemical diagnostics have been successfully developed for other neurological disorders (e.g., Alzheimer's disease), but not ASD or ADHD. In RATSS, established biomarkers of neurodegeneration, brain injury, synaptic function, inflammation, and monoamines will be assessed in CSF. The limitation with this candidate marker approach is, however, that we are limited to a set of tools that we already know are of some importance. We will therefore complement these analyses with an approach that is better suited to detect novel markers that may advance the field: hypothesis-free combined proteomics and peptidomics for the global analysis of proteins and endogenous peptides in CSF. New mass spectrometry-based proteomic techniques are now allowing researchers to detect and identify hundreds of biomolecules in a single analysis of minute biological samples. These techniques enable a more unprejudiced research approach that may lead to the discovery of novel molecules involved in disease, as well as disease mechanisms that involve co-action of several molecules. Several research articles in the last few years

indicate the feasibility to employ these techniques in the discovery of biomarkers in CSF. By subtracting the protein/peptide pattern of the typical co-twins from that of the affected ASD/ADHD twin, we hope to identify proteins that are only present in a clinical/subclinical neurodevelopmental disorder phenotype (see Kroksveen et al., 2011; Hölttä et al., 2012).

Induced pluripotent stem (IPS) cells and iPS-derived Neuroepithelial stem (NES) cells from patients are ideal building blocks for the development of disease-specific cellular models (Falk et al., 2012). Cellular models built on patient-specific iPS cells have successfully shown disease phenotypes, also when modeling psychiatric disorders (Christian et al., 2012; Park et al., 2008). The few reprogramming studies of cells from patients with psychiatric disorders have had encouraging results, proving the principle, as well as presenting possible molecular mechanisms. For example, calcium Ca(v)1.2 has been suggested to regulate the differentiation of cortical neurons in humans and offer new insights into the causes of autism in individuals with Timothy syndrome (Pasca et al., 2011). We will use the skin biopsies from MZ pairs discordant for ASD or ADHD, to derive iPS and NES cells that are further differentiated into disease-relevant neurons. The advantage of using NES cells instead of iPS cells as a starting cell line for directed differentiation is that NES cells only differentiate into neural cell types (neurons and glia), and no cells from other germ layers will disturb differentiation. In addition, the differentiation protocol will span two weeks instead of 5-6 weeks. Protocols will be developed for deriving neuronal subtypes known to be affected in neurodevelopmental disorders, such as cortical neurons and GABAergic interneurons. The literature provides good insight into signaling molecules important for these fates (Jagatha et al., 2009). To investigate the mechanisms causing neurodevelopmental disorders, mutated patient NES cells are studied side by side with healthy NES cells using assays reporting proliferation, differentiation, migration, maturation, and survival of neural cells.

Our primary hypothesis in RATSS is that the symptoms in the affected twin are caused by epigenetic and genetic differences between the twins. To identify the putative differences, a broad range of genetic analyses, including high-density DNA microarrays and whole exome and genome sequencing (WES and WGS, respectively) will be performed. First, the discordant twin pairs will be screened for their CNV status using high-resolution DNA microarrays. To identify putative pathogenic variants, the CNVs from twins with ASD or ADHD will be compared to control twins and other available controls. Then, WES and/or WGS of the twins and their parents will be used to pinpoint rare *de novo* mutations including mosaic mutations. To identify any epigenetic differences between the twins, a microarray based global methylation analysis will be performed using the collected blood samples. Any identified differentially methylated regions will be confirmed by pyrosequencing. Furthermore, we can correlate the methylation status to gene expression in blood derived RNA using qRT-PCR (selected genes) or RNA sequencing (global gene expression). For some of the twins, we will be able to measure the methylation status at birth from dried blood spots. For these twins, we can detect persistent methylation differences that could contribute to symptoms seen in the affected twin.

In addition to the clinical manifestations typically associated with CNS dysfunctions, ASD (and to a lesser extent ADHD) has been frequently linked to various gastrointestinal (GI) symptoms such as abdominal pain, constipation, diarrhea, enterocolitis, and abnormal intestinal permeability (Kral et al., 2013). GI functions are critically dependent on the integrity and diversity of the gut microbiota, i.e., the collection of commensal microorganisms that colonize the GI tract. It has therefore been suggested that at least parts of the GI symptoms in ASD may stem from alterations in gut microbiota, and such microbial changes may be amenable to therapeutic interventions that could improve the general life qualities of affected individuals (De Angelis et al., 2013). The gut microbiota has further emerged as an

important functional node within the gut-brain axis, which in turn functionally interconnects the viscera, including the GI tract, with the CNS (Mayer, 2012). One of the burning questions in this context is whether GI- and CNS-associated symptoms in neurodevelopmental disorders such as ASD are two separate pathological entities, or whether they are causally related. Owing to the constant bidirectional information flow between the gut and the brain, it is indeed feasible to assume that GI disturbances may causally contribute to at least some brain dysfunctions (Reichelt & Knivsberg, 2009). In RATSS, we test the hypothesis that primary alterations in the gut microbiota not only facilitates the emergence of GI dysfunction, but also impacts brain and behavioral functions via changes in afferent vagal signaling, gut immunity, and/or altered secretion of neuroactive substances. In the current project, the nature of microbiotic changes in fecal samples obtained from twins affected by ASD and ADHD are compared to the microflora of their unaffected co-twins. Potential differences in the microbiota between twins and co-twins are related to differences in psychopathology, cognition, and functional and structural neuroimaging findings, as a well as CSF biomarkers and (epi)- genetics.

Results

The RATSS data collection began in mid-2011. As of October 2013, *N* = 109 individuals (68 males, 41 females) from 54 twin-pairs and one triplet (Figure 1) have been assessed using the RATSS protocol, and additional 8 pairs are scheduled for 2013. Eighty-two are MZ, 20 dizygotic (DZ), and zygosity is pending in 8 individuals. In three twin pairs, twin-to-twin transfusion syndrome was reported. The average age is 15.0 years (*SD* = 3.2, *range* = 9-23), the average IQ is 93.3 (*SD* = 18.9, *range* = 39-138). Sixty-six percent of the sample has been recruited via the CATSS study, 28% via advertisements, 4% from clinical unit (e.g. child psychiatry) and 2% from the Swedish patient registry.

Ten MZ twin-pairs are qualitatively or markedly quantitatively discordant for ASD and 7 for ADHD. Please note that where one twin was diagnosed with ASD and the co-twin with ADHD, twin pairs were assigned to the ASD discordant pairs count. One additional pair pending for zygosity testing results, at the time of the writing up of this paper, was highly likely to be MZ and discordant for ASD (Table 1). Moreover, the sample includes 4 MZ pairs discordant for other neurodevelopmental disorders (intellectual disability, tic disorder, speech sound disorder), and 4 MZ pairs concordant for ASD or ADHD.

Overall, the completeness and usability of the collected data and specimens is adequate (Figure 2). Data for psychiatric and psychological assessments are almost complete, and so are samples of blood, saliva, hair, nails, urine (>99%). Coverage is somewhat lower for dysmorphology examination, extensive medical history taking (93%), and MRI (88%). Coverage is lowest for cerebrospinal fluid (63%) and skin biopsies (55%). However, the collection of skin samples was only introduced one year ago. With regards to parental data and specimens, RATSS has almost complete questionnaire data on mothers and fathers (89%), as well as a good coverage for mothers (93%) and fathers blood (59%) and saliva (98% & 83%) (Figure 3).

Current ongoing analyses focus on differences in executive functioning in MZ twins discordant for ADHD traits, including the role of age and gender effects in $n = 27$ pairs; a detailed descriptive comparison analysis of the nine MZ pairs discordant for ASD. Brain imaging data analyses focus on the structural connectome (mapping whole-brain white matter bundles) and volumetric properties observed in the most discordant ASD and ADHD MZ twin pairs, and CSF analysis on the inflammatory markers and markers of brain injury. All the collected twins will be genotyped using Infinium HumanCoreExome chip with additional 50K SNPs relevant for psychiatric disorders (PsychChip). This platform will allow detection of CNVs larger than 30 kb and enable us to explore the association of SNPs and different

behavioural, cognitive and neurobiological phenotypes of the twins. Exome sequencing is planned for the most clinically discordant pairs for ASD, ADHD, and other neurodevelopmental disorders together with their parents.

Conclusions

RATSS is the largest multilevel clinical study of ASD and ADHD, their broader phenotypes and traits using MZ twins. The project has already generated a rich collection of data to be used in a variety of analyses described in this paper. The project will within short get access to new options of recruitment via clinically based registries in Stockholm County (Division of Child and Adolescent Psychiatry, Habilitation and Health Centers, Pediatrics) to markedly increase the number and "clinicalness" of the twin pair collection. Overall, there is good likelihood that the project will be successful to double or triple the amount of twins and material in the next 2-3 years. For 2015, it is planned to start following-up on the collected pairs, starting with the most informative (discordant clinical ASD and ADHD) pairs, in order to establish the longitudinal arm of RATSS.

Recently, parts of RATSS joined EU-AIMS: European Autism Interventions – a multicenter study for Developing New Medication. The involvement in EU-AIMS will create synergies, as well as higher research output, and visibility of RATSS. The engagement in EU-AIMS also means that additional assessment are integrated in RATSS, such as eye-tracking, questionnaires, cognitive tests, and brain imaging (Positron Emission Tomography - PET). As RATSS combines several strengths, particularly access to nationwide registry data on twins screened for neurodevelopmental disorder traits, multilevel assessments, and a multidisciplinary environment, there is a good chance for novel leads regarding pathogenic pathways, informative biomarkers, and options for treatment and prevention in ASD and ADHD. There are several techniques developed by collaborators in this project that have

never been used in ASD or ADHD or in twins discordant for these phenotypes, such as iPS and NES cells, mass spectrometry-based proteomic techniques analyzing the CSF, and brain connectome approaches.

Despite all of the advantages of twin studies, there are some potential limitations, making cross-validation of twin findings in singletons desirable for endorsing generalizability to the total population. Twins are often born premature, and are lighter at birth than singletons, which could compromise the ability of twins to represent the general population. Some authors have suggested twinning to be a risk factor for some neurodevelopmental disorders (e.g., ASD), but this has not been confirmed in large population-based studies (e.g., Hallmayer et al, 2002); in twin family studies including siblings that controlled for confounds (e.g., parental education, socioeconomic status), sibs and twins did not differ (e.g., for IQ, see Hoekstra et al, 2007).

In conclusion, RATSS provides some unique opportunities to examine altered development in ASD and ADHD, hazardous and protective environmental and genetic factors and their interplay, as well as their effects on neurobiology, and cognition. Finally, while RATSS focuses ASD, ADHD and other neurodevelopmental disorders, the twin collection offers many opportunities for spin-off research through collaboration with additional medical disciplines, as quite a few of the MZ twin-pairs show discordance for other medical conditions, such as epilepsy, deafness or asthma.

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Tables

Table 1. List of monozygotic twin pairs discordant for autism spectrum disorder, ADHD, and other neurodevelopmental disorders (DSM-5/ICD-10 diagnoses) in RATSS

Twin 1: primary diagnosis (DSM-5/ICD-10)	Twin 2: primary diagnosis (DSM-5/ICD-10)	Qualitative/ quantitative discordance
ASD / PPD NOS	none	ASD discordant pairs (N=20)
ASD / PDD NOS	none	
ASD / PDD NOS	none	
ASD / PDD NOS	none	
ASD / PDD NOS	none	
ASD / PDD NOS	none	
ASD / Autism	ASD / Asperger Syndrome	
ASD / Autism	ADHD / Hyperkinetic Disorder	
ASD / Asperger Syndrome	Social Anxiety Disorder / Social Phobia	
ASD / PDD NOS	Mild Mental Retardation / Mild Intellectual Disability	
ADHD hyperactive-impulsive / Hyperkinetic Disorder NOS	none	ADHD discordant pairs (N=14)
ADHD unspecified / Hyperkinetic Disorder NOS	none	
ADHD unspecified / Hyperkinetic Disorder NOS	Tic disorder, unspecified / Tic NOS	
ADHD unspecified / Hyperkinetic Disorder NOS	Specific Phobia / Specific Phobia	
ADHD combined / Hyperkinetic Syndrome	ADHD inattentive /Hyperkinetic Disorder NOS	
ADHD combined / Hyperkinetic Syndrome	ADHD inattentive / Hyperkinetic Disorder NOS	
ADHD combined / Hyperkinetic Syndrome	ADHD unspecified / Hyperkinetic Disorder NOS	
Mild Mental Retardation / Mild Intellectual Disability	none	Other NDD discordant pairs (N=8)
Speech Sound Disorder / Phonological Disorder	none	
Tic disorder, unspecified / Tic NOS	none	
Mild Mental Retardation / Mild Intellectual Disability	Speech Sound Disorder / Phonological Disorder	
ASD / PDD NOS	none	Pending for zygosity (N=2)

Note: *Including one trio of triplets; counted as two pairs. ASD=Autism Spectrum Disorder; ADHD=Attention Deficits Hyperactivity Disorders; DZ=dizygotic; MZ=monozygotic; NDD=Neurodevelopmental Disorders; TD=Typically Developed

Figure legends

Figure 1. Number and type of twin-pairs collected in RATSS on October 9, 2013.

Figure 2. Completeness of twin pair assessments and specimen collections in RATSS

Figure 3. Completeness of parents' assessments and specimen collections in RATSS