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Sleep and Cortisol in Preschool-Aged Children with Autism and Typically Developing Children

by

Sharon Audrey Kidd

B.S. (University of Massachusetts, Amherst) 1984
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A dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Epidemiology

in the

Graduate Division

of the

University Of California, Berkeley

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Spring 2010
Sleep and Cortisol in Preschool-Aged Children with Autism and Typically Developing Children

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Sharon Audrey Kidd
Abstract

Sleep and Cortisol in Preschool-Aged Children with Autism and Typically Developing Children

by

Sharon Audrey Kidd

Doctor of Philosophy in Epidemiology

University of California, Berkeley

Professor Ira B. Tager, Chair

Research has suggested that daytime cortisol secretion levels in children with autism may be higher compared to typically developing children. In addition, there is some evidence for higher cortisol secretion levels as a result of poor sleep hygiene in young children.

Fifty-two subjects (26 children with autism (AUT) and 26 typically developing children (TYP)) from 2 to 5 ½ years of age were recruited from the Sacramento region. Cortisol was obtained from saliva at waking, midday, and bedtime on two consecutive days at three phases (baseline, 3 months later, 6 months later). Sleep measurement was acquired from actigraphy over 7 days and nights at each phase. G-computation estimation and linear mixed models were used for the primary analyses.

At waking, AUT had a mean level of cortisol of 8.29 (95%CI 6.99, 10.51) nmol/liter and TYP of 6.95 (95%CI 5.97, 8.49) nmol/liter. The variability in the slope of cortisol for AUT (0.0214, 95%CI 0.0066, 0.0362) was 3 ½ times that of TYP (0.0061, 95%CI -0.0041, 0.0164). The between-subject variance estimate for AUT (0.0663, 95%CI 0.0148, 0.1178) was 1 ½ times that of TYP (0.0388, 95%CI 0.0021, 0.0756). There was also a graded response among AUT by functional status - cortisol secretion levels increased as IQ decreased.

There were also differences for some of the sleep parameters. The difference at the morning or waking sampling was 3 nmol/liter for 100 minutes awake compared to no waking during the night (95% CI for difference: -0.23, 7.69). The cortisol mean at the waking sampling was higher (13.6 nmol/liter) for a child with a night-waking problem compared to a child without a night-waking problem (7.4 nmol/liter) (95% CI for difference: -0.55, 9.35).

The Sleep and Cortisol Study has contributed to the current literature by establishing average cortisol secretion values at waking, midday, and bedtime in preschool-aged AUT and TYP. Important differences were observed in cortisol variability in AUT compared to TYP and in cortisol secretion by functional status. Novel results for cortisol secretion at waking and the association with being awake and night-waking need replication in future studies.
DEDICATION

This dissertation is dedicated to my brilliant and beloved 9-year-old daughter, Grace Corrigan, who one day may undertake a dissertation of her own, and to my father, Harry Kidd, who died shortly after I started the doctoral program.
ACKNOWLEDGEMENTS

I would like to acknowledge equally my dissertation chair, Ira Tager, and my dissertation sleep mentor, Tom Anders, for their support for the dissertation. In particular, they had little to gain from taking on an underfunded student wanting to develop their own research idea, but nonetheless stuck with me. In addition, Tom Anders provided the opportunity to piggy-back onto the host Sleep in Autism Study, gain access to the study data, and incorporated me into his research group when no one understood what an epidemiologist was doing there.

My most heartfelt thanks go to the families and their children who generously participated in the study, completed the tasks admirably, and took on the burden WITHOUT compensation. Many thanks to the research staff on the Sleep in Autism Study (Karen Tang, Anny Wu, Stephanie Sitnick, Sara Waters, and Shacunda Burton) who graciously assisted me with drop-offs, pick-ups, and communications with the families. I also want to thank those giving generously of their time and advice: Blythe Corbett, Doug Granger, Gig Levine; and the other members of my Committee, Tom Boyce, Alan Hubbard, and Lance Kriegsfeld (Lance pinch-hit as the outside member at the last moment); Denise Zabkiewicz, a fellow “mature-age” student, for listening to my general whinging.

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The Medical Investigation of Neurodevelopmental Disorders (MIND) Institute in Sacramento supported me in a variety of ways, but in particular by allowing me to attend the incredible post-doctoral seminar run by some of the top autism experts in the country (especially Sally Rogers and Sally Ozonoff). I thank Laura Lacey for facilitating this experience.
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CHAPTER 1: SPECIFIC AIMS AND HYPOTHESES

Introduction
Sleep problems are common in children with autism, and are characterized by difficulty falling asleep, frequent and lengthy awakenings during the night, shortened total night sleep, and early morning waking [1]. In addition, researchers have proposed that the hypothalamic-pituitary-adrenocortical axis may be dysfunctional in children with autism [2, 3], and have suggested that the diurnal cortisol rhythm may be different as compared to typically developing children [4]. Therefore, both cortisol and sleep-wake dysregulation may reflect a disorganization in circadian timing and they may be interrelated [5].

The HPA system plays a major role in stress resistance, glucose regulation, immune function, bone and energy metabolism, learning and memory, and emotional response. The cortisol rhythm throughout the day may indicate the functionality of this system and predict the ability of the child to handle cognitive functioning, regulate emotion, and perform tasks of learning. Therefore, management of sleep disorders in children may have a beneficial impact on daily functioning by modifying cortisol output. There are virtually no published studies that examine the relationship between sleep measured by objective methods and cortisol in a population of children with autism. The proposed study will examine whether there are differences in daytime cortisol secretion between children with autism and typically developing children, and whether differences in sleep quality affect cortisol secretion the following day.

Specific aims and hypotheses
The following specific aims will be addressed:
1. To determine whether levels of daytime cortisol secretion, based on salivary cortisol sampling, differ between preschool-aged children with autism and typically developing children.
Hypothesis 1: There will be a circadian rhythm disruption in children with autism that will manifest itself as elevated levels of daytime cortisol secretion (i.e. a higher cortisol level at several time points throughout the day) compared to typically developing children.
2. To determine whether within-subject variability of daytime cortisol secretion, based on salivary cortisol sampling, differs between preschool-aged children with autism and typically developing children.
Hypothesis 2: There will be more within-subject variability in children with autism compared to typically developing children.
3. To determine whether sleep measures (total night sleep; minutes awake during the night; frequency of night wakings; and minutes to fall asleep at bedtime) the night before are associated with higher cortisol secretion the following day.
Hypothesis 3a: Poor sleep quality (shorter duration of sleep; frequent night wakings; a longer sleep latency; and more minutes awake during the night) will be associated with higher levels of cortisol secretion the following day.
Hypothesis 3b: Sleep fragmentation (number of wakings and/or minutes awake during the night) is the best predictor of higher cortisol levels the following day.
4. To determine whether a child with a sleep onset or night waking sleep problem by RDC criteria is associated with cortisol secretion the following day. Hypothesis 4a: A child with a sleep onset or night waking sleep problem by RDC criteria will have higher cortisol levels the following day compared to children without these respective sleep problems.

5. To determine whether autism is an effect modifier of the association between sleep and cortisol. Hypothesis 5a: Children with autism that have poor sleep quality will have higher cortisol secretion levels compared to typically developing children that have poor sleep quality.

References


CHAPTER 2: REVIEW OF AUTISM

Characteristics and classification of autism

Autism, also referred to as autistic disorder, is characterized by: deficits in reciprocal social interaction; deficits in communication; and restricted, repetitive behaviors, interests, and activities. Autism is part of a spectrum of disorders called the pervasive developmental disorders (PDD), which also is synonymous with autism spectrum disorders (ASD). The PDD includes Rett's disorder, childhood disintegrative disorder, Asperger's disorder, and PDD not otherwise specified. Autism is distinguished from the other PDD primarily by its presentation in terms of age at diagnosis (0-36 months of age), IQ range (severe mental retardation to normal intelligence), and outcome (poor to good) (see Table 1). Many of the characteristics of autism, as described by Leo Kanner in his seminal work in the first half of the 20th century, were robust and largely have endured over time. His accounts of social isolation, resistance to change, and communication dysfunction have remained as hallmarks of the diagnosis. He believed that autism was an innate and inborn constitutional disorder or failure of development, rather than caused directly by dysfunctional interpersonal relationships [1, 2]. It is of note that in the 1960s, the author and lay psychologist, Bruno Bettelheim, had a contrasting view of the origins of autism that has been repudiated completely by contemporary experts. Bettelheim is responsible for the so-called "refrigerator mother" hypothesis that impugns mothers and suggests that they had restricted contact with and failed to make an emotional connection with their children [3]. Kanner’s observations that the parents of his case series were not especially warm or caring, were high achievers often with some psychological or psychiatric background, and had failed or failing marriages would be considered today as due largely to selection bias of families who are more likely to seek assistance because of these characteristics.

Kanner coined the term “autistic disturbances of affective contact” to describe the disorder at the time of his first paper [1], and the term “early infantile autism” was added to the psychiatric nomenclature in 1944. At the time of follow-up (early 1970s) of Kanner’s case series, the Diagnostic and Statistical Manual of Mental Disorders (DSM-II in 1968 (as well as the earlier DSM-I in 1952) was classifying these children as having “schizophrenia, childhood type”. However, Kanner believed this disorder to be unique and different qualitatively from childhood schizophrenia [4]. Later research made it clear that the history and life course of subjects with autism did not suggest an early form of schizophrenia; consequently, autism was included into the DSM-III when it was revised in 1980 [5].

The DSM-III was a major improvement in diagnostic classification and gave official recognition to the disorder. DSM-III definitively separated autism from childhood schizophrenia (although schizophrenia could be co-morbid with autism in later classification systems) and provided a pragmatic definition. However, the official term of “infantile autism” left a suggestion of a focus on autism as a disorder of very young children [5]. The subsequent DSM-III-R of 1987 considered that there may be changes in the expression of autism with chronological age and development, but the elimination of age of onset as a feature of the diagnostic criteria in this version was seen as a disadvantage that allowed more misclassification due to the inclusion of distinctly
different disorders of older ages. In addition, the DSM-III-R definition of autism was considered to be too broad and resulted in over-diagnosis of autism in the intellectually disabled group and under-diagnosis and clinical inattention in the more intellectually able.

The International Classification of Diseases (ICD), a classification system that first came into use at the end of the 19th century, was considered to have very limited utility for psychiatric conditions up until the middle of the 20th century. Coincident in time with the appearance of the DSM-III, the ICD-9 also officially included infantile autism in its 1978 ninth revision. The ICD is considered to be more flexible for clinical use and separates research criteria from clinical guidelines. It is of particular use for international and cross-cultural purposes. The process of revision of both systems to the forms currently in use took place simultaneously and with a view to establishment of a common approach to diagnostic coding.

The DSM-IV [6] and ICD-10 [7] criteria are remarkably similar to each other (see Appendix 1 (DSM-IV) and Appendix 2 (ICD-10) for the exact criteria and descriptive details of the behaviors). Briefly, the following criteria are used for the diagnosis of autism in both systems:

1. Abnormal functioning in at least one of the following areas, with onset prior to age three years: (1) social interaction; (2) language used in social communication; (3) symbolic play.
2. Demonstration of at least six symptoms from (1), (2), and (3) above, with at least two from (1), and one each from (2) and (3).
3. Not better accounted for by other PDD (symptoms are not better represented by other PDD diagnoses).

Prototype three year-old child with autism:
- No interest in maintaining joint attention and gaze with parent
- Does not exhibit joy in parent-initiated play
- Does not have spoken language except for occasional babbling
- Does not engage in spontaneous pretend play
- Has obsessional interest in tractor wheels and exhibits flapping of hands when playing with tractor wheels

At the present time, the diagnosis of autism cannot be made by the presence of any biological or anatomical markers and thus must be determined by specialists’ knowledgeable about the developmental characteristics of typically developing children. An assessment of a child should involve a multidisciplinary team of specialists that might include a neurologist, psychologist, psychiatrist, pediatrician, and speech and occupational therapists where possible [2]. There are a variety of diagnostic assessment tools specific for autism that have good precision and reliability, with the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R) representing the gold standards of autism diagnosis in research settings (see Methods: Autism Diagnosis for review and critique of ADOS and ADI). The ADOS is a semistructured standardized assessment of social interaction and communication, play, and imaginative use of materials [9]. The ADI is a semistructured interview for caregivers by an investigator [10]. Briefly, the ADOS in the original research sample achieved 94% correct classification [11]. The ADI-R achieved a sensitivity of 98% when first reported in a sample of 51 preschoolers with and 43
preschoolers without autism, and had good interrater reliability with item Kappas of .62-.89. [12]. The gold standard consisted of independent and congruent diagnoses by a child psychiatrist and clinical psychologist blinded to ADI-R scores and using ICD-10/DSM-IV clinical criteria [12]. The diagnosis can be made very reliably from the age of three years; however, some children as young as 12-18 months old are diagnosed due to severe deficits in the social realm, such as a lack of smiling at a caregiver and vocalizing, and visual joint attention with a caregiver. Toddlers can demonstrate developmental disturbances via lack of skill progress, especially in speech, and the emergence of abnormal stereotyped behaviors and interests. However, the predictive validity of the ADI-R at 2 years was not as good as that of 3 years for autism outcomes at age 7[13].

It is common to subgroup individuals with autism by “functionality” – i.e. division by intelligence quotient [14]. Individuals with autism and an IQ less than 70 are considered low-functioning, and those with an IQ over 70 are considered high-functioning. Low-functioning individuals are classified as intellectually disabled by virtue of the IQ and may have symptoms severe enough to require constant observation to prevent injury and to manage activities of daily living. High-functioning individuals may have good capability with activities of daily living but with unusual behaviors/interests that stand out, and poor social communication skills and interaction that make seamless incorporation into social and academic settings difficult. Some individuals with autism may be very functional and able to undertake school lessons and work in the community. The difficulties for these individuals often lie in their lack of social skills for engagement in purposeful relationships and leisure activities. Outcomes generally improve with age and development, but improvements may be less likely at the severe end of the spectrum.

Epidemiology of autism

Estimates of incidence

There have been no published incidence studies that have been designed for the express purpose of longitudinal follow-up of unselected populations of children for the outcome of autism. The traditional prospective design for incidence is relatively inefficient given the rarity of the disorder.

Estimates of the occurrence of autism come from a number of population-based studies that have examined trends over time with data collected for other purposes [15-23] (see Table 2. Incidence studies of autism). Much of the work in this area comes from the United Kingdom (UK). Based on regional data from the West Midlands, Powell et al. examined clinical records from regional child development centers and a psychiatric referral center to estimate an overall annual incidence of 3.5 (95%CI 2.7, 4.4) per 10,000 person-years from 1991 to 1996 in children one to four years of age [15]. Several studies have used the UK General Practice Research Database (GPRD) to obtain incidence estimates. The GPRD consists of the electronic clinical records of patients registered with participating general practitioners, and is considered to be broadly representative of England and Wales. Kaye et al. [16] and Smeeth et al. [17] examined similar time periods for the whole UK. Smeeth et al. had no age restrictions for inclusion, and obtained estimates of 0.11 (1988) to 1.92 (2001) per 10,000 person-years[17]. Kaye et al., used an age restriction of less than 12 years of age and determined a similar incidence of 0.3 (1988) to 2.1 (1999) per 10,000 person-years [16].
Jick et al. extended the years of study from 1992 through 2004 and obtained annual estimates that ranged from 2.0 to 8.0 per 10,000 person-years for a younger sample of two to four year-old children[22]. Kaye et al. [16] used a subset of the GPRD database made available to researchers in the United States, which may account for the different results for 1988 between Smeeth and Kaye. Similar to the UK studies, the one American study had a medical record and school record ascertainment for residents of one county in Minnesota [21]. Cumulative incidence estimates ranged from 0.5 (95%CI 0.1, 0.9) in the earliest time period to 4.5 (95%CI 3.3, 5.7) in the last three years of the study. The UK studies are notable for their lack of information about the criteria for the autism diagnosis. Existing medical and school records make for ease of carrying out epidemiological studies, but these databases are burdened by biases related to how they got into the database (access to services and health-seeking behaviors), and whether diagnosis is standardized across individuals within the database.

In Denmark, a study used a combination of inpatient admissions to psychiatric hospitals and from 1995, outpatient visits for psychiatric disorders. The authors estimated an annual cumulative incidence of autism of 2.0 (95%CI 1.7, 2.4) per 10,000 for children under 10 years of age observed over a 29-year period [18]. Unfortunately, the mix of populations (outpatients added to inpatients near the end of the study) represented in this database makes this estimate difficult to interpret. The underlying assumption that inpatient diagnoses are representative of the population of children with autism also is untenable since these children largely do not come to the attention of specialists through the inpatient setting (as evidenced by the inclusion of outpatient diagnosis later in the study). In two states in Australia, cumulative incidence estimates were made in children less than 14 years old for the time period July 1999 to December 2000 [19]. Data obtained from Western Australia from a prospective register and from New South Wales by active surveillance resulted in annual estimates of 5.5 (95%CI 4.5, 6.7) and 4.3 (95%CI 3.8, 4.8) per 10,000, respectively, in children from birth to four years of age. This study relied on a passive surveillance system from a specialist community that the authors confirm does not have full participation.

Two studies from Asia had estimates of a much higher cumulative incidence of autism in their populations than all of the other studies. In Japan, Honda et al. used a system of early detection and early intervention for developmental disorders to address cumulative incidence up to five years of age [20]. Children were mass-screened initially at a routine health check-up for 18-month-old children, and children also attended a routine health check-up at three years of age. Cumulative incidence over the four years was calculated as 27.2 per 10,000. This unique 2-stage screening process stands out among the other studies for the advantages of standardization of diagnosis and completeness of ascertainment. In Taiwan, Chen et al. used a nationwide health insurance database to estimate the incidence of autism among young children, with a cumulative incidence over five years of 37 per 10,000 [23]. Existing records of services obtained for the purposes of insurance coverage may overestimate the incidence, since obtaining benefits may be related to the receipt of a specific diagnosis of autism over that of other developmental disabilities, that could inflate the number of reported cases of autism.

Although the incidence studies used methods to determine true incidence (i.e. children were under observation and at risk before a diagnosis of autism was made),
methodological problems were numerous. One of the biggest problems with trends over time is that the changing diagnostic criteria could have led to under- and over-estimates of incidence. The current diagnostic criteria for the ICD and DSM were developed in 1993 and 1994, respectively. Most of the studies overlapped different phases of diagnostic criteria development over time. In addition, some of the clinical databases had changes to the entry criteria within the study period. There was no standardization of examination for autism within (except for Honda et al.) or across studies, and because of the source of data (clinical and other service databases), there is always the possibility of inflation of case diagnosis so that families can obtain available services for children with developmental disabilities who would not meet true diagnostic criteria for autism. Estimates also may be unreliable due to in-and out-migration within population-based studies that could affect both the numerator and denominator for incidence measures. Fortunately, the study populations were reasonably large with confidence intervals sufficiently narrow to indicate good precision. Finally, the broad range of ages included in the studies belies current diagnostic criteria that limit diagnosis to children under 3 years of age at onset. The studies that allowed children 10 years and older had lower rates than studies that restricted entry to the younger ages where diagnosis is most common. The diluting effect on incidence estimates of the inclusion of older ages can clearly be seen in the Australian study; there was a decrease in incidence with increasing age strata. Where studies reported incidence over time, there was an increase in incidence over time in all of these studies. However, the artifactual issues discussed above cannot be ruled out when trying to explain the changes over time in terms of causation.

In summary, so-called “Western” countries had cumulative incidence estimates and incidence rates largely in the single digits per 10,000 population or person-years, respectively. The estimates ranged from 0.1 to 7.0 per 10,000 person-years, and the confidence intervals, when provided, were narrow. The two studies from Asia had cumulative incidence rates in the double digits that ranged from 27 to 37 per 10,000 population. Although both studies enrolled young children (less than or equal to eight years of age), it is unlikely that this feature explains the magnitude of the difference between Japan and Taiwan, and the other countries. Further research on between-country differences in the incidence of autism might provide some insight. However, the use of standardized and strict diagnostic criteria, similar aged populations, and non-biased samples are the minimal criteria necessary for informative comparison data.

Prevalence estimates are used generally to estimate the burden of autism in a population [24, 25]. Studies on the prevalence of autism have been conducted since the 1960s, but different sets of diagnostic criteria have been used for diagnosis in the early studies. I undertook a comprehensive review of all prevalence studies of autism that used either ICD-10 or DSM-IV diagnostic criteria (the criteria in current use). There were 13 studies published since 1996 in a variety of countries, with nearly half having examined data from the UK (see Table 3. Selected prevalence studies of autism). Unlike the incidence studies of autism, several of the prevalence studies had very small study populations [26, 27], which makes for poor precision of the estimate. In the studies that have used review of disability registers or medical or psychiatric records [28-33], complete case identification rests on the belief that the entire population of
families with children with autism is seeking services. Several studies used school and special education records [27, 28, 31] that also would depend on children presenting for diagnosis. This method has some advantages for ascertainment in older school-age children, since eventually all of these children should be diagnosed. More commonly, whole population surveys or a multi-tiered approach to screening have been undertaken for complete case identification [26, 34-38]. Population screening of all residents has appeal for completeness of case ascertainment and standardization of diagnosis. At issue methodologically in all study designs is whether the level of health services seeking and survey participation influence the estimates. Families of low socioeconomic status and/or different ethnic or racial background may not have the knowledge base to seek services, and low participation in surveys/screening of families with children with autism would both tend to underestimate prevalence. Referral practices, access to specialists, and improvements in diagnosis are likely to have changed over time. These artifactual and/or methodological problems may also lead to an under- or over-estimate of the prevalence.

In an earlier comprehensive review of the literature, Fombonne suggested that current prevalence is estimated most accurately from 28 studies published since 1987 (first use of DSM criteria) [39]. The prevalence estimates ranged from 2.5 to 72.6 per 10,000 (average 95% confidence interval width: 14.1), with an average of 16.2 per 10,000 and a median prevalence of 11.3 per 10,000. In the subset of 13 studies reviewed for this dissertation (highly overlapping with the Fombonne review), the prevalence estimates ranged from 3.8 to 40.0 per 10,000, with an average of 17.9 per 10,000 and a median prevalence of 16.8 per 10,000. The confidence intervals were broader than those from the incidence studies, and reflect the smaller populations under study. When this review is restricted to the six studies with study populations over 40,000, the estimates have a narrower range from 3.8 to 13.0, and similarly the confidence limits range from 2.3 (lower) to 14.7 (upper) per 10,000.

There is a view by experts and lay people alike that autism is increasing over time and that the estimates from the literature are lower than what would be expected today. The prevalence studies from Table 3 included data through 1998 only, so there is a data lag of nearly 10 years. Recently, estimates from the Centers for Disease Control have been widely quoted and have become the de facto current prevalence estimates for ASD (autism was not reported separately) in the United States (US) [40, 41]. A population-based multi-site surveillance system was used to determine prevalence of ASD in six areas of the US in 2000 and 14 areas in 2002. Children aged eight years old were identified through screening, health record abstraction, education records (2002 only), and clinical review of records to determine case status consistent with DSM-IV-TR criteria. The overall prevalence of ASD across all six areas in 2000 was 67 (ranging from 45 to 99 in individual areas) per 10,000 children. The overall prevalence of ASD across all 14 areas in 2002 was 66 (ranging from 33 to 106 in individual areas) per 10,000 children. Fombonne reviewed a series of studies examining both autism and other PDD and determined that the ratio of the prevalence of other PDD to the prevalence of autism was on average 1.6 [39]. Applying this value to the averaged prevalence of the above studies would suggest a prevalence of autism of 41.6 per 10,000 children (or 1 in 240 children).
In summary, the prevalence studies may give a more accurate estimate of the burden of autism today than the incidence studies. Autism is a disorder that is incurable and not lethal, therefore, there is no concern over missing cases due to recovery and survival bias. The restriction of this review to diagnostic criteria currently in use suggests prevalence to be in the mid-teens per 10,000 population of children aged three to 18 years. Better estimates of the prevalence of autism may be gained from studies in well-defined populations of a narrower age range based on current diagnostic criteria and in more recent time periods.

Descriptive epidemiology

The descriptive epidemiology of autism is notable for its occurrence throughout all socioeconomic strata and racial and ethnic groups around the world. Given the behavioral aspects of the autism phenotype, it may be difficult to compare the occurrence of autism between countries, because some cultures may be more accepting of oddities in behavior or do not perceive these behaviors as “problems”. Nonetheless, diagnoses of autism have been made in various countries, including China, Brazil, India, and Zimbabwe [42-45]. Although the earliest findings of Kanner suggested a positive association with higher SES, this likely was due to better access to diagnosis in his case series of eleven children [1]. More recently, in studies from Scandinavian countries that have population-based ascertainment and good access to services countrywide, there were no associations between SES and risk for autism [46, 47]. In a study in South East London, no significant differences were found between the social class distribution of the fathers of children with autism compared to fathers of children with other mental retardation, childhood psychoses, or children from the general population of the area [48]. In the United States, a state-supported case-finding program in Iowa found the social class distribution of fathers of children with autism was similar to the fathers of children with other psychological disorders and children in the general population [49]. In metropolitan Atlanta, there was an association between autism and higher family income, but associations with social class varied by ascertainment source in secondary analyses [50]. Children from higher income families were more likely to be diagnosed at younger ages and from non-school ascertainment sources, which suggests a higher knowledge base in higher income families. In conclusion, positive associations between autism and higher SES are likely to be due to a bias in case ascertainment in the subject populations under study.

In contrast to SES, there is a preponderance of autism in males compared to females. The sex ratio averaged across studies in the Fombonne review was 4.3:1 males to females (median= 3.6, range = 1.8 to 15.7) [39], and in the dissertation review (see Table 2), the mean sex ratio was 4.7:1 males to females (median = 3.8, range= 2.1 to 15.7). There have been several theoretical mechanisms put forward for the excess of males with autism, that include the possibility of X-linked and autosomal transmission [51]. However, one study that examined a series of 16 highly polymorphic loci on the X chromosome found no genes that contributed to an increased susceptibility to autism [52]. The exception to this increased sex ratio is when samples are stratified by intellectual impairment. Among individuals with autism with intellectual impairment (i.e. mental retardation), the sex ratio is decreased, an observation that suggests that girls with autism are more likely to be severely affected [39].
However, there are some caveats with respect to the assessment of intelligence. Intelligence can be assessed with a variety of instruments, a situation that leads to the potential for differential classification issues when comparisons are made across studies that used different assessment instruments. There are only a few instruments used to assess intelligence in children with autism, with the Wechsler Preschool and Primary Scale of Intelligence [53] the preferred battery [54]. The concern with the choice of instrument relates to the communication problems inherent in autism and the limitations in testing with profoundly and severely affected children who cannot attempt the tasks involved. The cut-point used for the definition of intellectual impairment or mental retardation is standardized across assessment instruments. From ages 3 through 6.5 years, the reliabilities for performance, verbal, and full scale IQ range from .90 to .97 (The Psychological Corporation (website), 2007). A value of 70 for the full-scale IQ, which represents approximately two standard deviations below the mean, is used to separate those with normal intellectual functioning from those with intellectual impairment. In the Fombonne review the mean proportion of subjects with autism without intellectual impairment (intelligence quotient (IQ) greater than 70) is estimated at 34.1% (median= 33.6%, range=0 to 62.8%) [39]. In the dissertation review, the mean proportion of subjects with autism without intellectual impairment is estimated at 34.9% (median= 37.6%, range=0 to 60.0%) (see Table 2).

There are very few medical disorders that have strong associations with autism, with epilepsy and fragile X syndrome being the most frequent co-morbid conditions. In a review of the literature on the incidence of epilepsy among subjects over 10 years of age with autism, Kawasaki et al. found that a mean of 30% (range=18.2 to 42.0%) of individuals with autism have seizures [55]. The prevalence of epilepsy among all children is only two to three percent [56]. There appears to be a bimodal age distribution in the peak frequency of seizures in autism. One peak occurs before the age of five years and a second occurs in adolescence after the age of 10 years [57]. The risk of epilepsy also rises with evidence of diffuse underlying brain dysfunction from severe mental retardation. The cumulative probability of the development of epilepsy with autism alone compared to autism with severe mental retardation, is 0.08 versus 0.27, respectively [56]. The treatment of seizures in autism is not different from that of other children with epilepsy.

Fragile X syndrome (FXS) is the most common inherited cause of mental retardation and is characterized by a phenotype of communication and stereotypical disturbances similar to autism [58]. This genetic disorder occurs in approximately 1 in every 2500-4000 individuals or 0.03-0.04% in the general population [59]. Among children with autism, the prevalence of FXS ranges from 0-16% with a median of 4% [60]. Interestingly, in a study of behavioral phenotype in FXS, the prevalence of autism by strict diagnostic criteria was 33% of a group of 24 children with FXS [61].

Analytical epidemiology

Prenatal/perinatal period

The strongest evidence for a causal role for the prenatal environment in autism comes from the study of congenital rubella and thalidomide exposure, agents largely eliminated from the population today [62]. More recently, evidence of potential insults that occur in the prenatal period comes from epidemiological studies that have evaluated a battery of obstetrical and perinatal risk factors during pregnancy. These studies have found
varying and inconsistent findings and do not provide conclusive evidence for perinatal complications as markers of the risk of autism [58, 63]. The most robust finding in these studies has been an association between bleeding during pregnancy and autism [63-65]. Bleeding can be due to a variety of circumstances, including vaginal tears or more serious events that involve the position and attachment of the placenta. Generally, and in the following studies, the cause of the bleeding has not been reported or recorded. Juul-Dam et al. compared three studies of perinatal factors and autism and found the relative risk of prenatal bleeding in the pregnancies with children with autism to be one and a half to five times that of pregnancies with control children [65]. In their own study, 14% of 51 children with autism were from a pregnancy with uterine bleeding, as compared to one percent of a general population control group. Similarly, Brimacombe et al. found a higher prevalence of vaginal bleeding (16.7%) in mothers in an autism cohort compared to mothers in a general population in New Jersey and a national population of births in 2000 (both 6.6%, p<.001) [64]. In the study of Glasson et al., children with autism were at nearly two and a half times (OR 2.41, 95%CI 1.56 - 3.73) the odds of threatened abortion at less than 20 weeks than general population controls matched by gender [63]. There appears to be no pathological process or pathway for autism that could be supported by the combination of the myriad factors studied that encompass the antenatal and intrapartum time periods [63]. The obstetric abnormalities have not been of the type that are considered to carry a high risk for a condition such as cerebral palsy or mental retardation [66]. A possible explanation for an association between pregnancy complications and autism is that these complications (e.g. fetal distress, low Apgar score) reflect the phenotypic expression of a genetic causal component [63]. In effect, pregnancy complications are not likely to “cause” autism, but rather are a consequence of an already present disorder and in fact may be an expression of a particular gene or set of genes.

Prenatal psychological stressors are established as having a role in the causation of some adverse pregnancy and child outcomes [67]. Recent negative life events [68] and work-related stress [69] have been associated with spontaneous abortion and life event stress [70] has been linked to preterm delivery. With respect to autism, one retrospective study found a higher prevalence of reported prenatal life event stressors at mid-trimester in pregnancies of women who delivered a child with autism (32.4 per 100 women), compared to women who delivered a child with Down syndrome (21.7 per 100 women), and women who delivered a child with no developmental disabilities (18.9 per 100 women) [71]. In an examination of data collected prospectively from prenatal records, Ward et al. found that only 3.4% of 59 mothers with a normal or control pregnancy compared to 32.2% of 59 mothers with an autism-affected pregnancy disclosed histories of family discord [72]. Family discord was loosely defined from an open-ended question regarding family problems during pregnancy that included items such as interference in family functioning and frequent arguments. Abnormalities in the cerebellum seen in autism are hypothesized to be related to psychological stressors that have occurred during gestation [73]. The authors suggested that the timing of the stress coincides with the point in gestation (mid-trimester) that is consistent with the occurrence of neuroanatomical abnormalities in the cerebellum of the fetus [73, 74]. The strongest evidence of the consequences of a stress response comes from studies in rats where the risk of damage to cerebellar
granule cells increased after prenatal exposure to glucocorticoids [75]. In addition, the lateral nucleus of the amygdala was enlarged in adult rats (formerly prenatally stressed offspring) due to the presence of more neurons and glia [76]. Larger amygdala volume and abnormalities of the cerebellum have been a consistent finding in individuals in autism, but the literature has not demonstrated any direct causal link between prenatal psychological stress and autism.

It has been hypothesized that genetic susceptibilities in the mother may modify the intrauterine environment by the alteration of levels of neurotransmitters that in turn affect the fetal brain [77, 78]. Polymorphisms at the MAO-A (monoamine oxidase) and dopamine β-hydroxylase (DBH) loci affect neurotransmitter metabolism, and neurotransmitters act to stimulate receptors in the fetal brain [79]. In a study by Jones et al., the IQs of affected children with autism were lower based on the mother’s genotype at the MAO-A locus and not that of the genotype of the child or the father [77]. Robinson et al. suggested that lowered maternal serum DβH, that catalyzes the conversion of dopamine to norepinephrine, results in an imbalanced uterine environment with respect to dopamine and norepinephrine [78]. They found that in the mothers, the DBH- allele that contains the 19-bp deletion responsible for lowered enzyme activity was associated with having a child with autism.

Summary
Examination of obstetrical and perinatal factors and report of psychosocial stress during pregnancy have resulted in inconsistent findings that are complicated by the lack of knowledge regarding temporal ordering of the exposure and outcome and the potential for recall bias, respectively. There appeared to be consensus among authors studying obstetrical and perinatal complications that it is difficult to separate out whether the complications were causing, or reflecting the pathological consequences of, an adverse outcome. The reports of psychosocial stress by mothers were primarily retrospective and it is thus suspect as to whether knowledge of case status informed their recall of stress during pregnancy. Finally, the hypothesis of whether genetic susceptibility in the mother might modify the intrauterine environment has not been tested directly, for example, by examining neurotransmitters in amniotic fluid and/or the placenta. Only very recently have studies been funded to follow mothers from pre-pregnancy to diagnosis, and these studies should become the best available sources of data to disentangle the issues described above.

Postnatal period
It is hypothesized that environmental triggers during postnatal development may impact the disorder and influence its progress or manifestation [80, 81]. However, examinations of potential postnatal causes of autism have not been very fruitful so far. Mercury compounds are neurotoxic at high levels, but the research-to-date on typical or likely postnatal exposures to mercury and the occurrence of autism is not promising [82]. There have been some adverse neurodevelopmental outcomes in children due to methyl mercury exposure through the food chain in communities which eat large quantities of seafood, but autism has not been associated with such exposure [83].

A consecutive series of children with chronic enterocolitis and regressive developmental disorder suggested an association between measles, mumps, and rubella (MMR) vaccine and autism [84]. A hypothesis was further advanced that vaccines with thimerosal, a vaccine preservative made from ethyl mercury, may be the
causal factor [85]. The Environmental Protection Agency (EPA) guidelines on safe exposure to thimerosal have been extrapolated from data on methyl mercury [82]. However, ethyl mercury is metabolized and excreted more rapidly than methyl mercury and there have been only a few reported cases of ethyl mercury poisoning [86]. Pichichero et al. found that the levels of mercury in the blood of infants exposed to thimerosal in vaccines are well below concentrations associated with toxicity [86]. Nonetheless, thimerosal has now been eliminated from vaccines of childhood, and currently, only certain influenza vaccines contain thimerosal.

Following the earlier claims of a causal relationship between the MMR vaccine and autism, no well-conducted large population-based epidemiological studies have found evidence for this link [16, 20, 28, 32, 87]. The more direct subhypothesis of a causal link between the preservative thimerosal in vaccines and autism has been investigated in individual studies and a review paper has summarized the negative results. This review of epidemiological studies of thimerosal exposure through immunization found three ecological and seven cohort studies (which included two combined retrospective cohort/ecological studies) [88]. Three of the cohort studies did not meet adequate criteria for sound methodology (inclusion/exclusion criteria described, control for bias and confounding, and diagnosis precisely defined). In addition, Heron et al. did not have diagnoses made by health care providers, but relied on parental response to questionnaires related to concerns about speech, tics, and special needs, and behavior ratings [89]. The three remaining retrospective cohort studies had specific dose information on thimerosal exposure and autism outcomes in population-based studies. Hviid et al. compared children vaccinated with a thimerosal-containing pertussis vaccine with children with a thimerosal-free formulation of the same vaccine in Denmark from 1990-1996 [90]. The relative risk (RR) of autism (ICD-8 defined) in children exposed to at least one thimerosal-containing vaccine dose was 0.85 (95%CI 0.60-1.20) and the increase in RR of autism per 25 µg of ethylmercury was 0.98 (95%CI 0.60-1.20). One caveat is that there is some confounding by time period in this study since thimerosal-containing vaccination was used in the early years of the study and thimerosal-free vaccination in the latter years. Given temporal trends in the increase in autism over time, this would tend to dilute a difference between the two groups in the direction of a protective effect of thimerosal. In the study of Andrews et al., using the GPRD in the UK, hazard ratios (HR) were calculated for risk of autism (ICD-9 criteria) for doses of diphtheria-tetanus-pertussis (DPT) vaccine (25 µg of mercury per dose) at three months of age, four months of age, and cumulative exposure, compared to no vaccination [91]. The HR per dose at three months was 0.89 (95%CI 0.65, 1.21), at four months 0.94 (95%CI 0.73, 1.21), and cumulative exposure 0.99 (95%CI 0.88, 1.12). These data do not support an association between thimerosal exposure and autism. Finally, in the study by Verstraeten et al., computerized data were reviewed from 1995 to 2000 in a health maintenance organization, where diagnoses of autism (ICD-9 criteria) were linked to records of all vaccinations [92]. The RR for every increase of 12.5 µg of mercury exposure at seven months of age was 1.00 (95%CI 0.90, 1.09). Cumulative exposures of 87-162.5 µg and ≥ 175 µg compared to a baseline of 0-75 µg exposure, were associated with RRs of 0.95 (95%CI 0.62, 1.46) and 0.65 (95%CI 0.27, 1.52), respectively.
In summary, the cumulative exposure to ethylmercury via vaccination was much higher in the United States versus England or Denmark. Nonetheless, the risk estimates from all three countries were not statistically significant, and were not substantially different between countries. There is some controversy with regard to the Verstraeten study, in that anti-vaccine lobbyists suggested that a conflict of interest impacted the results, given that the author was hired by a pharmaceutical company that manufactures DPT vaccine following publication of the results [92].

The one animal study that examined the neurotoxic effects of postnatal thimerosal administered as challenges that mimic routine immunization, found that mouse strains resistant to autoimmunity were not susceptible but mice with autoimmune propensity showed outcomes consistent with neurotoxicity [93]. The immune system alterations in autism raise the possibility that exposures in the environment may have direct or indirect effects on the immune system that contribute to the etiology of autism [94]. Environmental exposures may act synergistically with genes as primary triggers of autism and/or modifiers of the phenotypic expression of the disorder [94].

Attempts to modify the phenotypic characteristics of autism through many complementary and alternative therapies have not demonstrated any evidence-based effects [95]. Dietary change through gluten- and/or casein-free diets has been studied primarily in uncontrolled trials with reports of positive changes in behavior [96, 97]. In a single-blind controlled trial [98], there were improvements in multiple domains of autism symptoms but results in a double-blind crossover design suggested no significant findings on improvement of autism symptoms [99]. Secretin, a hormone used in the diagnosis of gastrointestinal dysfunction, has been used widely in the absence of experimental evidence of its efficacy [100, 101]. Twelve of 13 placebo-controlled trials failed to demonstrate any affect on a battery of symptoms of autism that were studied [100].

**Neuroanatomy and neurobiology of autism**

The neuroanatomical aspects of autism have been characterized by several methods. Postmortem evaluation or autopsy has been carried out in instances where histology and microscopic examination of the brain has been the object of study. Computerized axial tomography (CAT), positron emission tomography (PET), and magnetic resonance imaging (MRI) are now being used for non-invasive examination of the brain. Functional MRI (fMRI) often is used to image a region of the brain when it is activated (e.g. during mental tasks). Abnormalities have been investigated by autopsy and/or imaging most frequently in the following areas: brain volume or size, gray and white matter, the cerebellum, and the limbic system.

Head circumference is considered to be an accurate index of brain volume in the first two years of life [102]. In a study by Courchesne et al., average head circumference in children with autism was at approximately the 25th percentile at birth and increased rapidly over two years to nearly the 90th percentile [103]. This is one of the few studies with prospective measurements in the first year of life prior to any behavioral manifestations or diagnostic hallmarks of autism. These findings have been replicated in samples of older 2-4 year-old children, with the additional features of enlargement of gray and white matter volume in the frontal, parietal, temporal, and occipital lobes as compared to typically developing children [104, 105].
It is believed that enlargement or increases in the size of gray and white matter are a result of proliferation of neural cell bodies, axons, and dendrites without appropriate pruning of abnormal connections [106]. This proliferation of abnormal neural material would likely interfere with information delivery, if it is obstructing normal connectivity by forming unusable connections and interfering with synchronization [107]. However, white matter abnormalities have not been consistently identified across studies. One study using MRI in nine-year-old children with autism found an increase in white matter in the outer radiate zone responsible for intra-hemispheric and cortico-cortical connections [108]. The most striking amount of volume increase is seen in the white matter that underlies the prefrontal cortex in the autism group (36% larger than healthy controls). This volume increase is most likely part of a pathogenic process of retarded development of later-myelinating white matter that may contribute to underlying pervasive core information processing deficits - a key deficit in autism [108]. Several studies have found reductions in the corpus callosum (i.e. a reduction of white matter), an area of the brain that is responsible for inter-hemispheric connectivity. In a study that used MRI imaging of the corpus callosum in ten-year-old boys with and without autism, the authors found that total corpus callosum size, after control for total brain size, was significantly smaller in boys with autism [109]. Just et al. in their fMRI study of high functioning adults with autism and healthy volunteer adults who were undergoing a puzzle test of executive function, found under-connectivity between frontal and parietal areas and a smaller corpus callosum in the autism group [110]. Abnormalities in the inter-hemispheric tracts (e.g. corpus callosum) may contribute to dysfunctional connectivity that in turn may result in the executive function (goal-oriented behaviors such as planning) problems observed so widely in autism.

Purkinje cells are a class of GABAergic inhibitory neurons in the cerebellum. They are some of the largest neurons in the brain and are characterized by a large number of dendritic spines. One of the most reproducible pathological observations in autopsied brains of individuals with autism across age and sex have been a significant decrease in the number of Purkinje cells [111-114]. An absence of glial hyperplasia suggests that the lesions have occurred early in development [111, 114]. Animal studies have shown a decrease in glial response after cerebellar lesions at early ages [114]. Additionally, olivary neurons in the brainstem appear to be preserved in the face of the loss of Purkinje cell number. This suggests an insult prior to the mid-trimester of pregnancy, since a tight bond between these two later in gestation would imply an obligatory loss of the other if there were damage to one [114]. Decreased Purkinje cells may impact on the appropriate balance of excitation and inhibition, as firing of a neuron results from an approximate balance between its excitatory and inhibitory inputs, and fewer inhibitory neurons could create a higher ratio of excitatory-to-inhibitory synaptic contacts.

Postmortem studies have also implicated the involvement of abnormalities in structures of the limbic system. On autopsy, the hippocampus has shown decreased complexity of dendritic branching in pyramidal cells [115], and unusually small neurons closely packed together in this and other parts of the limbic system [113]. Researchers suggest that these findings are consistent with curtailment of maturation some time in very early childhood [113-115]. Findings in the amygdala have also been of interest. The amygdala is responsible for processing related to fear and anxiety, and generalized
fearfulness and anxiety are commonly found in autism. On autopsy of adults with autism, abnormally small and densely packed cells in the amygdala have been found in a single study [116]. MRI studies measuring amygdala volume have not produced consistent findings [105, 117, 118]. High-functioning adults with ASD were examined in one study with no group differences in amygdala volume between these subjects and healthy controls [117]. The other study enrolled 3-4-year-old children with ASD and found enlargement of the amygdala bilaterally compared to age-matched typically developing children [105]. Most recently in a cross-sectional study, younger children with autism had larger amygdala volumes than age-matched control children, but there was no difference in volume between matched adolescent groups [118]. These findings suggest that the younger children with autism do not undergo the age-related increase in volume over childhood that is observed in typically developing children.

The area that appears to hold the most promise in terms of advancing the etiology of autism is that of neurons, synapses, and neurotransmitters. Disturbances in neuronal excitability, especially the balance of excitation and inhibition, may lead to disorders of cognition (schizophrenia and autism) caused by disruption of information processing [119]. Excitatory (glutamatergic) projection neurons and inhibitory (GABAergic) interneurons in the neocortex regulate the quality of information processing across the cortices. It is during prenatal and early postnatal development that interneuron development is most susceptible to disruption and its consequences [119]. Dysregulation of patterning of excitatory and inhibitory neurons in the cortex is an attractive hypothesis for the pathogenesis of autism [120]. Casanova et al. found abnormal patterning of minicolumns, the functional unit of excitatory and inhibitory neurons, in the frontal and temporal lobes of individuals with autism using a computerized imaging program [121]. N-acetylaspartate, a neurochemical involved in neuronal-glial homeostasis is responsible for maintenance of neuron and/or synaptic density [122]. Friedman et al. found reduced N-acetylaspartate in 3-4 year-old children with ASD, and suggest that this is consistent with a decrease in neuron and/or synaptic density [123]. The developing serotonergic (5-HT) system may be dysregulated in autism with changes in serotonin levels with increasing age showing a reverse trend as compared to typically developing children – i.e. neurotransmitter levels are initially low and then increase beyond adult levels in children and adolescents with autism [124, 125]. These data suggest that the developmental process of serotonin synthesis is disrupted in autism, with potential impacts on serotonin regulation of cell division and differentiation, and synaptogenesis.

**Future directions for imaging**

Before the advent of non-invasive brain imaging, autopsy was the sole source of data on the brain in individuals with autism. Due to very limited mortality in the first years of life, autopsy often is performed on adult individuals with autism. This has been an unsatisfactory approach to the examination of processes that have occurred most certainly before three years of age. Non-invasive imaging by CAT and MRI scans also are characterized by a static representation of the brain at one time point. Longitudinal studies of brain imaging are needed to examine critical developmental periods for within-group and between-group changes over time. Unfortunately, lying still up to one hour is a prerequisite for a good image, and most young children and many autistic children and adolescents find this intolerable and therefore brain imaging in children is
largely infeasible without sedation. Very recently, diffuse optical imaging (DOI), a mobile system that uses a flexible imaging cap and infrared light for imaging has been developed. The DOI detects blood movement similar to fMRI, but has been limited by low resolution and the lack of ability to measure area volumes (http://www.nih.gov/news/research_matters/july2007/07162007brain.htm). However, Zeff et al. set out to develop a high-performance, high-density diffuse optical tomography [126] system to enable a more superior image quality than DOI [127]. The researchers mapped the visual cortex with this method and achieved results that were consistent with previous mappings of the structure and function of this brain region by fMRI and PET.

**Genetic aspects of autism**

**Population genetics**

A heritability index of at least 90% has been widely quoted for autism [128]. An estimate derived from the four twin studies carried out to date suggests that there is a large disparity in concordance rates in the diagnosis of autism between monozygotic (MZ) and dizygotic [75] twin pairs with a heritability index ranging from 36-96% [129-132]. The earliest study, using Kanner and Rutter diagnostic criteria, found only moderate concordance in MZ pairs [130]. With a later extension of the dataset and diagnosis by ICD-10 criteria, concordance in MZ pairs nearly doubled [129]. DSM-III and DSM-III-R diagnostic criteria were used in the remaining two studies, which found similar high concordance rates of at least 90% in the MZ pairs [131, 132]. Only one study determined fragile-X (a single-gene disorder) status in the study subjects [132], and all of the studies had some uncertainty with respect to zygosity, although blood typing and other biological methods (versus examination of physical appearance) were used where possible. Information on specific heritability models tested within these papers was incomplete, but a Mendelian model was considered less likely, and a multifactorial model more likely, to explain the heritability of the liability to autism.

Family studies with an index child with autism further advance the case of autism as a potentially heritable disorder. In a pooled analysis of very early studies of sibship and autism, a frequency of 2% in siblings was obtained when both possible and definite cases were included [133]. In a more recent case-control study of family history of autism, data on siblings was collected for 99 probands with autism and 36 probands with Down syndrome [134]. Four (2.9%) of the 137 siblings of the probands with autism and none (0%) of the 64 siblings of the probands with Down syndrome met ICD-10 criteria for autism. However, 5.8% of the siblings of probands with autism and none of the siblings of the probands with Down syndrome met a broader PDD diagnosis. In a study of families with multiple members with autism (multiplex families), the authors noted that affected siblings were not significantly concordant for IQ or verbal ability, but were concordant on ritual behaviors, repetitive play, and social impairments [135]. The authors suggest that intrafamilial concordances for characteristic behaviors associated with autism may lead to the identification of behaviorally defined subgroups within

<table>
<thead>
<tr>
<th>Pairwise concordance by zygosity</th>
<th>MZ pairs</th>
<th>DZ pairs</th>
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<tbody>
<tr>
<td>Folstein</td>
<td>36% (4/11)</td>
<td>0% (0/10)</td>
</tr>
<tr>
<td>Ritvo*</td>
<td>96% (22/23)</td>
<td>23% (4/17)</td>
</tr>
<tr>
<td>Bailey</td>
<td>60% (15/25)</td>
<td>0% (0/20)</td>
</tr>
<tr>
<td>Steffenburg</td>
<td>90% (9/10)</td>
<td>0% (0/10)</td>
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*included opposite sex pairs
multiplex families and to more information on genetic heterogeneity. The rate of autism among siblings is higher than for the general public, but lower than for a hereditary disorder [133]. Rutter posited in the 1960s (but is still supported today) that in autism there may be low penetrance of the gene, that genetically determined autism may represent only a small fraction of cases, or that non-genetic factors exert a strong modifying impact [133].

**Molecular genetics**

Cytogenetic studies search for inherited or spontaneous abnormalities on an individual case basis, which can help narrow down regions for mapping susceptible genes [136, 137]. The prevalence of cytogenetic abnormalities in autism is estimated at 3-5% [138]. Cytogenetic abnormalities at the 15q11-q13 locus are reported most commonly, with a frequency of 1-4%, with inherited duplications of maternal origin common at this locus [137, 138]. In this chromosomal region lies a γ-amino butyric acid (GABA) gene cluster. Polymorphisms in these genes has implications for the inhibition of excitatory neural pathways and expression during early brain development [136, 137]. The phenotype for chromosome 15 duplication is characterized by ataxia, language delay, epilepsy, and mental retardation which overlap with the phenotype for autism [137, 138]. A recent study has used specific probe and marker information to delineate optimally the boundaries of cytogenetic abnormalities in order to better define candidate gene regions [139].

In whole genome screening, regions with putative susceptibility genes are defined by searching for common genetic markers in populations of families with multiple affected members [136, 137]. This can be achieved by covering all chromosomes, or by searching locally at certain chromosomal areas of interest [138]. Narrowing the search region generally involves the use of linkage disequilibrium and association studies. Linkage disequilibrium refers to the inheritance of a specific allele at a rate much greater than chance in affected family members. DNA sequencing by microsatellite marker is used to assess the frequency of this specific susceptibility allele [137]. Many research groups have performed genome-wide linkage studies and examined linkage to a specific gene(s) in more depth, but only a few have gone beyond a qualitative phenotype to confirm autism by diagnostic criteria currently in use. In these latter studies, linkage analysis was used and findings were reported for chromosomal regions 3q [140], 7q [141], and 3p [142]. The locus on chromosome 7 was further supported by a regional meta-analysis of four studies [143], and a genome search meta-analysis supported region 7q22-q32 in studies with strictly defined autism [144]. It is of note that results from linkage studies have often been inconsistent, with studies using different methods of analysis and samples with genetic heterogeneity [130].

The genome-wide association study is a design that involves genotyping cases and controls at a large number of markers throughout the genome, and identifying associations between the genotypes at each locus and disease status ([www.stats.ox.ac.uk/~mcvean/gwa4.pdf](http://www.stats.ox.ac.uk/~mcvean/gwa4.pdf)). Recently, the first genome-wide association study in autism was performed in a population of the Faroe Islands [145]. Twelve males with autism spectrum disorders and a randomly selected comparison group of 22 pairs of parents (presumably unrelated) were genotyped for polymorphic markers throughout the genome in a search for allele and haplotype association. Their data suggested that
microsatellite markers in regions on 2q, 3p, 6q, 15q, 16p, and 18q are in linkage disequilibrium with genes involved in autism; that is, the alleles are non-randomly associated at these loci and may lie close to susceptibility genes localized within these regions.

Finally, hypothesis-driven research designates a biologically plausible pathogenetic pathway in which several candidate genes are chosen \textit{a priori} [137]. The most promising candidate genes for autism currently fall into two categories: genes involved in patterning of the different regions of the central nervous system and neurons; and genes that are involved in the control of synapses [106]. The reelin gene (RELN) on chromosome 7q is an example from the first category and has had some of the most interesting findings to date. Reelin is a large secreted glycoprotein involved in neural migration. The 7q region has been under study because of the finding of a phenotype of delayed onset of speech associated with a paracentric inversion in the 7q22-q31.2 region [141]. On post-mortem examination of cortices and cerebella from brains of age- and gender-matched adults with autism versus adult controls, there were reductions in Reelin protein and mRNA, demonstrating impairments in the Reelin signaling system and transmission of signals downstream [146]. Interference with the Reelin signaling system may affect long-term potentiation, synaptic plasticity, cognition and memory [146]. Alterations in Reelin affect cortical and cerebellar development, and cerebellar neuronal abnormalities are one of the more robust pathological findings in autism [113, 137].

The neuroligin gene family (NLGN3 and NLGN4) is reflective of the second category. These genes are a component of postsynaptic glutamatergic synapses which play a role in the \textit{trans}-neuronal signaling that controls synapse differentiation [106]. Chih \textit{et al.} found that point mutations in NLGN3 and NLGN4 created \textit{in vitro} resulted in intracellular retention of mutant proteins [147]. Mutations in neuroligin genes are likely to be important with respect to neurodevelopmental defects in autism, since they impair the function of synaptic cell adhesion molecules [147]. Cell adhesion molecules are essential for the identification of the appropriate partner cell and forming a functional synapse [148]. Jamain \textit{et al.} have found mutations in X-linked genes encoding NLGN3 and NLGN4 in 2 of 158 subjects with autism and no mutations in the 200 subjects without autism, and suggest that these defects may affect the synapses essential for the communication processes that are deficient in autism [148].

\textbf{Summary of genetics}

Twin and family studies support the heritability of autism, although the mode of inheritance is not known. The high heritability indices found for MZ twins indicates genetic factors in the etiology of at least some autism, but the 10-60\% of MZ twins that are discordant suggest that there are influences from the environment that have yet to be determined. The issue of shared environment for the MZ twins has not been addressed in the study designs to date. Due to the rarity of the disorder, it is unlikely that studies can be carried out with a sufficient number of MZ twin with autism reared separately.

Studies are being carried out to identify the susceptibility genes for autism, and although studies have identified possible linkages, confirmatory studies are needed, and only a few gene linkages have been replicated. The modest nature of the gene effects for complex human diseases like autism likely contribute to the contradictory and
inconclusive results [149]. Quite often, further study has not borne out confirmatory findings for the myriad genes that have been studied [150]. Some of the reasons for unconfirmed findings across studies may be due to: heterogeneous populations within and between studies; small numbers of subjects within studies reducing the power to detect associations and/or allowing imprecise but statistically significant results to occur by chance; and the use of different genotyping and laboratory methods, as well as statistical analytical methods. Publication bias toward the publication of positive results might also be problematic when determining whether results are truly in the same direction. Large-scale collaborative projects, such as the Autism Genome Project (AGP) Consortium, which includes more than 1500 multiplex families in a whole genome screen approach, and the Autism Genetic Resource Exchange (AGRE), which is a shared genetic and clinical database of multiplex families may be the most promising in elucidating the genetics of autism given their size and scope.

There are a number of approaches to examine genes and their effects in ways that go beyond susceptibility genes and heritability. For example, the modifying effects of the environment on genes are likely to be important in the disorder. The classic example of this gene-environment interaction is phenylketonuria, wherein a diet low in phenylalanine can suppress the effects of a gene which causes severe mental retardation. A model has been developed to examine gene-environment interactions involving reelin and paraoxinase genes and prenatal exposure to organophosphates [62]. Reelin exerts a proteolytic activity on extracellular matrix proteins, which is crucial for neuronal migration. This proteolytic activity is inhibited by difluorophosphate, an organophosphate used as an agricultural pesticide and household insecticide [151]. Persico et al. hypothesize that if individuals with reduced reelin are exposed prenatally to organophosphate during periods of neuronal migration and have low paraoxinase activity (paraoxinase inactivates organophosphates), there may be altered neuronal migration resulting in autism [62].

Another innovative area in genetic epidemiology and its application to autism research is that of epigenetics – heritable genetic factors that don’t involve changes in the DNA sequence [152]. One example of this mode is that of genomic imprinting, where a parental allele is preferentially expressed in somatic cells of the offspring because of DNA methylation [152]. Ashley-Koch et al. found parent-of-origin effects in their studies of autism and chromosome 7 [141]. The authors observed sex-specific recombination and suggest that this might be a consequence of maternal imprinting (paternal expression) in the 7q region [141].
Table 1. Differential Diagnostic Features of Autism and Nonautistic Pervasive Developmental Disorders*

<table>
<thead>
<tr>
<th>Feature</th>
<th>Autistic Disorder</th>
<th>Asperger’s Rett’s</th>
<th>Childhood Disintegrative Disorder</th>
<th>Pervasive Developmental Disorder-NOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age to recognition (months)</td>
<td>0-36</td>
<td>Usually &gt;36</td>
<td>5-30</td>
<td>&gt;24</td>
</tr>
<tr>
<td>Sex ratio</td>
<td>M &gt; F</td>
<td>M&gt;F</td>
<td>F (?M)</td>
<td>M&gt;F</td>
</tr>
<tr>
<td>Loss of skills</td>
<td>Variable</td>
<td>Usually not</td>
<td>Marked</td>
<td>Marked</td>
</tr>
<tr>
<td>Social Skills</td>
<td>Very poor</td>
<td>Poor</td>
<td>Varies with age</td>
<td>Very poor</td>
</tr>
<tr>
<td>Communication skills</td>
<td>Usually poor</td>
<td>Fair</td>
<td>Very poor</td>
<td>Very poor</td>
</tr>
<tr>
<td>Circumscribed interests</td>
<td>Variable (mechanical)</td>
<td>Marked (facts)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Family history-Similar problems</td>
<td>Sometimes</td>
<td>Frequent</td>
<td>Not usually</td>
<td>No</td>
</tr>
<tr>
<td>Seizure disorder</td>
<td>Common</td>
<td>Uncommon</td>
<td>Frequent</td>
<td>Common</td>
</tr>
<tr>
<td>Head growth Decelerates</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>IQ range</td>
<td>Severe MR to normal</td>
<td>Mild MR to normal</td>
<td>Severe MR</td>
<td>Severe MR</td>
</tr>
<tr>
<td>Outcome</td>
<td>Poor to good</td>
<td>Fair to good</td>
<td>Very poor</td>
<td>Very poor</td>
</tr>
</tbody>
</table>

*Adapted from [153]; NA = Not Applicable.
<table>
<thead>
<tr>
<th>Authors, year</th>
<th>Study population</th>
<th>Number of subjects in population</th>
<th>Cases of Autism</th>
<th>Ages at enrollment</th>
<th>Results (incidence measures)</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powell, 2000[15]</td>
<td>West Midlands, UK, 1991-1996</td>
<td>178,484 person-years</td>
<td>62 cases</td>
<td>1-4 years, 11 months</td>
<td>Incidence rates per year: Overall 3.5 (95% CI 2.7, 4.4) per 10,000 person-years 91-92: 2.7 per 10,000 person-years 93-94: 3.5 per 10,000 person-years 95-96: 4.3 per 10,000 person-years p&lt; 0.03 Poisson regression trend</td>
<td>• Clinical Records and education database to establish children receiving services for “childhood autism” • DSM-III-R, DSM-IV, ICD-10</td>
</tr>
<tr>
<td>Kaye, 2001[16]</td>
<td>GPRD, UK, 1988-1999</td>
<td>3,092,742 person-years</td>
<td>305 cases</td>
<td>&lt;12 years old</td>
<td>Incidence rates per year: Overall 1.0 per 10,000 person-years Ranged from 0.3 (1988) to 2.1 (1999) per 10,000 person-years</td>
<td>• “Autism” not further defined from UK GP database</td>
</tr>
<tr>
<td>Lauritsen, 2004[18]</td>
<td>Denmark, 1971-2000</td>
<td>682,397</td>
<td>759 cases</td>
<td>&lt;10 years old</td>
<td>Annual cumulative incidence estimated at 2.0 per 10,000 (95% CI 1.7, 2.4)</td>
<td>• Includes ICD-8 to ICD-10 • Missing 20% of cases from non-participating hospital • All inpatient admissions to psychiatric hospital; from 1995, out-patient activities were included (Danish Psychiatric Central Register)</td>
</tr>
<tr>
<td>Smeeth, 2004[17]</td>
<td>GPRD, UK, 1988-2001</td>
<td>14,231,526</td>
<td>1097 cases</td>
<td>&lt;28 years old</td>
<td>Overall 0.8 per 10,000 person-years Ranged from 0.1 (1988) to 1.9 (2001) per 10,000 person-years</td>
<td>• Autism defined as those with “autism and similar presentations”</td>
</tr>
</tbody>
</table>

**Abbreviations:** GPRD = General Practices Research Databases
Table 2 (cont.): Incidence studies of autism

<table>
<thead>
<tr>
<th>Authors, year</th>
<th>Study population</th>
<th>Number of subjects in population</th>
<th>Cases of Autism</th>
<th>Ages at enrollment</th>
<th>Results (incidence measures)</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honda, 2005[20]</td>
<td>Yokohoma, Japan, 1988-1991</td>
<td>35,716</td>
<td>97</td>
<td>Up to 5 years old</td>
<td>Cumulative incidence of 27.2 per 10,000 over 1988-1991</td>
<td>• ICD-10 from health check screening at 18 months and 3 years</td>
</tr>
<tr>
<td>Williams, 2005[19]</td>
<td>Western Australia (WA) &amp; New South Wales (NSW), Australia, July 1999-December 2000</td>
<td>Not noted in paper (approximately 2,051,00 (WA) and 6,817,00 (NSW) in 2006)</td>
<td>169 (WA) 400 (NSW)</td>
<td>5-year age bands 0-4, 5-9, 10-14</td>
<td>Age band: Rate(95%CI)* (State): WA 0-4: 5.5 (4.5, 6.7); 4.3 (3.8, 4.8) 5-9: 2.4 (1.8, 3.2); 1.6 (1.3, 1.9) 10-14: 0.8 (0.0, 1.3); 0.3 (0.0, 0.4) *Annual cumulative incidence per 10,000</td>
<td>• Case ascertainment relied on reporting by professionals and a checklist of DSM-IV criteria</td>
</tr>
<tr>
<td>Barbaresi, 2005[21]</td>
<td>Olmstead County, Minnesota, 1976-1997</td>
<td>Ranged from 34,944 in 1976 to 37,726 in 1997</td>
<td>112 cases</td>
<td>≤ 21 years old</td>
<td>Cumulative incidence (per 10,000) ranged from 0.5 (95%CI 0.1, 0.9) over 1980-1983 to 4.5 (95%CI 3.3, 5.7) over 1995-1997</td>
<td>• DSM-IV  • Medical and school record ascertainment for virtually all county residents</td>
</tr>
<tr>
<td>Jick, 2006[22]</td>
<td>GPRD, UK, 1992-2004</td>
<td>440,332</td>
<td>308 males</td>
<td>2-4 years old</td>
<td>Incidence rates per year: Overall: 7.0 per 10,000 person-years From 2000-2004: 1.0 (95%CI 0.9, 1.1) per 10,000 person-years</td>
<td>• Computer-recorded diagnoses of autism</td>
</tr>
<tr>
<td>Chen, 2007[23]</td>
<td>National Health Insurance Database, Taiwan, 1996-2001</td>
<td>1,784,293</td>
<td>6673 cases</td>
<td>3-8 years old</td>
<td>Cumulative incidence over period 1996-2001 was 37 per 10,000</td>
<td>• ICD-9 (infantile autism) from medical record diagnoses</td>
</tr>
</tbody>
</table>

Abbreviations: GPRD= General Practices Research Databases
<table>
<thead>
<tr>
<th>Authors, year</th>
<th>Area, country, time period</th>
<th>Ages of children enrolled</th>
<th>Size of target population</th>
<th>Ascertainment methods</th>
<th>Diagnostic criteria</th>
<th>Number of cases of autism</th>
<th>Per-cent with IQ &gt;70 points‡</th>
<th>Gender ratio (M : F)</th>
<th>Prevalence rate per 10,000</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honda et al., 1996[34]</td>
<td>Yokohama, Japan, 1988-1994</td>
<td>5</td>
<td>8,537</td>
<td>Screening of resident children</td>
<td>ICD-10</td>
<td>18</td>
<td>50.0</td>
<td>2.6</td>
<td>21.1</td>
<td>11.4, 30.8</td>
</tr>
<tr>
<td>Arvidsson et al., 1997[26]</td>
<td>Molnlycke, Sweden, 1988-1991</td>
<td>3-6</td>
<td>1,941</td>
<td>Screening of resident children</td>
<td>ICD-10</td>
<td>6</td>
<td>0.0</td>
<td>5.0</td>
<td>31.0</td>
<td>6.3, 55.7</td>
</tr>
<tr>
<td>Taylor et al., 1999[28]</td>
<td>North Thames, United Kingdom, not defined</td>
<td>&lt;16</td>
<td>492,453</td>
<td>Review of disability registers and school records</td>
<td>ICD-10</td>
<td>214</td>
<td>4.0</td>
<td>----------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baird et al., 2000[36]</td>
<td>South-East Thames, United Kingdom, not defined</td>
<td>1.5-8</td>
<td>16,235</td>
<td>Screening of resident children</td>
<td>ICD-10</td>
<td>50</td>
<td>60</td>
<td>15.7</td>
<td>30.8</td>
<td>22.9, 40.6</td>
</tr>
</tbody>
</table>

† Selection based on diagnosis of autism made by ICD-10 and/or DSM-IV criteria.
‡ Intelligence quotient [14] scores greater than 70 regarded as normal intelligence/without mental retardation
Table 3 (cont.): Selected prevalence studies of autism†

<table>
<thead>
<tr>
<th>Authors, year</th>
<th>Area, country, time period</th>
<th>Ages of children enrolled</th>
<th>Size of target population</th>
<th>Ascertainment methods</th>
<th>Diagnostic criteria</th>
<th>Number of cases of autism</th>
<th>Per-cent with IQ &gt;70 points‡</th>
<th>Gender ratio (M : F)</th>
<th>Prevalence rate per 10,000</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bertrand et al., 2001[31]</td>
<td>Brick Township, New Jersey, United States, 1998</td>
<td>3-10</td>
<td>8,896</td>
<td>Active case finding from medical/school records and advertisement</td>
<td>DSM-IV</td>
<td>36</td>
<td>42.0</td>
<td>2.2</td>
<td>40.0</td>
<td>28.0, 56.0</td>
</tr>
<tr>
<td>Chakrabarti et al., 2001[37]</td>
<td>Midlands, United Kingdom, 1992-1995</td>
<td>2.5-6.5</td>
<td>15,500</td>
<td>Multi-tier screening of resident children</td>
<td>DSM-IV</td>
<td>26</td>
<td>30.8</td>
<td>3.3</td>
<td>16.8</td>
<td>11.0, 24.6</td>
</tr>
<tr>
<td>Tebruegge et al., 2004[27]</td>
<td>Kent, United Kingdom, 1991-1992</td>
<td>8-9</td>
<td>2,536</td>
<td>Review of school records</td>
<td>ICD-10</td>
<td>6</td>
<td>23.7</td>
<td>---</td>
<td>4.7, 42.7</td>
<td></td>
</tr>
<tr>
<td>Chakrabarti et al., 2005[38]</td>
<td>Midlands, United Kingdom, 1996-1998</td>
<td>4-6</td>
<td>10,903</td>
<td>Multi-tier screening of resident children</td>
<td>DSM-IV</td>
<td>24</td>
<td>33.3</td>
<td>4.0</td>
<td>22.0</td>
<td>14.1, 32.7</td>
</tr>
</tbody>
</table>

† Selection based on diagnosis of autism made by ICD-10 and/or DSM-IV criteria.
‡ Intelligence quotient [14] scores greater than 70 regarded as normal intelligence/without mental retardation
Appendix 1: Diagnostic and Statistical Manual of Mental Disorders – IV [6]

Diagnostic criteria for 299.00 Autistic Disorder

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

(1) qualitative impairment in social interaction, as manifested by at least two of the following:

(a) marked impairment in the use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction

(b) failure to develop peer relationships appropriate to developmental level

(c) a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest)

(d) lack of social or emotional reciprocity

(2) qualitative impairments in communication as manifested by at least one of the following:

(a) delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)

(b) in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others

(c) stereotyped and repetitive use of language or idiosyncratic language

(d) lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level

(3) restricted repetitive and stereotyped patterns of behavior, interests, and activities, as manifested by at least one of the following:

(a) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus

(b) apparently inflexible adherence to specific, nonfunctional routines or rituals
Appendix 1 (continued): Diagnostic and Statistical Manual of Mental Disorders – IV [6]

Diagnostic criteria for 299.00 Autistic Disorder

(c) stereotyped and repetitive motor mannerisms (e.g., hand or finger flapping or twisting, or complex whole-body movements)

(d) persistent preoccupation with parts of objects

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

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

Diagnostic Criteria For Autism Disorder

At least 8 of the 16 specified items must be fulfilled.

a. Qualitative impairments in reciprocal social interaction, as manifested by at least three of the following five:

1. failure adequately to use eye-to-eye gaze, facial expression, body posture and gesture to regulate social interaction.

2. failure to develop peer relationships.

3. rarely seeking and using other people for comfort and affection at times of stress or distress and/or offering comfort and affection to others when they are showing distress or unhappiness.

4. lack of shared enjoyment in terms of vicarious pleasure in other peoples' happiness and/or spontaneous seeking to share their own enjoyment through joint involvement with others.

5. lack of socio-emotional reciprocity.

b. Qualitative impairments in communication:

1. lack of social usage of whatever language skills are present.

2. impairment in make-believe and social imitative play.

3. poor synchrony and lack of reciprocity in conversational interchange.

4. poor flexibility in language expression and a relative lack of creativity and fantasy in thought processes.

5. lack of emotional response to other peoples' verbal and non-verbal overtures.

6. impaired use of variations in cadence or emphasis to reflect communicative modulation.

7. lack of accompanying gesture to provide emphasis or aid meaning in spoken communication.

Diagnostic Criteria For Autism Disorder

c. Restricted, repetitive and stereotyped patterns of behaviour, interests and activities, as manifested by at least two of the following six:

1. encompassing preoccupation with stereotyped and restricted patterns of interest.

2. specific attachments to unusual objects.

3. apparently compulsive adherence to specific, non-functional routines or rituals.

4. stereotyped and repetitive motor mannerisms.

5. preoccupations with part-objects or non-functional elements of play material.

6. distress over changes in small, non-functional details of the environment.

d. Developmental abnormalities must have been present in the first three years for the diagnosis to be made.
References


128. Minshew, N., et al., Neurologic aspects of autism, in Handbook of autism and pervasive developmental disorders, Vol. 1: Diagnosis, development,


CHAPTER 3: REVIEW OF THE LIMBIC-HYPOTHALAMIC-PITUITARY-
ADRENOCORTICAL (L-HPA) AXIS

Introduction
The L-HPA axis refers to the role of the limbic system, hypothalamus, anterior pituitary, and adrenal cortex as a pathway that maintains circadian rhythm of cortisol and controls reactions to stress. Although autism is of unknown etiology, there are many symptoms and behaviors that suggest the potential involvement of the L-HPA axis. The L-HPA axis is involved in homeostatic regulation involving many bodily processes working in conjunction with each other to maintain constant temperature, blood pressure, and neuroendocrine secretion. Thus, maintenance of appropriate L-HPA responsiveness is crucial for the adaptive functioning of the human body, and cortisol regulation is a central part of this process. Cortisol increases amino acid uptake and gluconeogenesis in the liver, has an anti-insulin effect, mobilizes serum lipids and cholesterol, has anti-inflammatory activity, and promotes appetite and the reabsorption of bone by affecting calcium metabolism. Several disease states that include Cushing’s syndrome and Addison’s disease, have cortisol dysregulation as a feature of the disease. Cushing’s syndrome is caused most often by a pituitary tumor and is characterized by prolonged excess exposure to cortisol that results in high blood pressure, high blood sugar, and mood disorders [1]. Addison’s disease is caused by an adrenal insufficiency that results in glucocorticoid deficiency and orthostatic hypotension and hypoglycemia [2]. The phenomenon of hypocortisolism - low and/or flattening of the daytime cortisol rhythm [3] - may impact brain development and functioning and stress-related disorders [3] [4]. Hypercortisolism as seen in major depression may create a hormonal environment similar to early Cushing’s syndrome [5]. Individuals with neuropsychiatric disorders that include affective [6], anxiety [7], and bipolar [8] disorders, have been found to have different cortisol rhythms and/or levels from normal control subjects. There is a body of literature that examines the shape of the cortisol rhythm and/or diurnal cortisol secretion in autism [9-14], and more recently increased variability in cortisol rhythm between subjects with and without autism has been observed [12]. Whereas Cushing’s syndrome and Addison’s disease are the end-states of chronic over- and under-exposure of tissues to cortisol, hypercortisolism and hypocortisolism from daily elevations or deficits in the cortisol diurnal rhythm may also pose a challenge to allostasis. That is, maintaining stability or homeostasis in the face of daily abnormal cortisol levels may pose a serious challenge to regulation. This hyperactivity/hyperreactivity of the stress system contributes to an excess exposure of tissues to glucocorticoids and their negative consequences as described earlier [15].

The hypothalamus and neuroendocrine function
The hypothalamus located in the central nervous system (CNS), (see Figure 1) is the key structure for the L-HPA axis that controls neuroendocrine secretion. The hypothalamus can be divided into regions with differing anatomical and functional qualities. The medial region of the hypothalamus contains most of the nuclei, including the suprachiasmatic nucleus (SCN) responsible for circadian timing and the paraventricular nucleus (PVN). The periventricular region contains many of the parvicellular neurons involved in production of corticotropin releasing hormone (CRH) that controls the release of the anterior pituitary hormones [16]. CRH is the primary
secretagogue for adrenocorticotropic hormone (ACTH), but arginine vasopressin (AVP) cells in the PVN are also of importance in stimulating ACTH release. It is ACTH that directly targets the adrenal cortex to release cortisol.

The lateral region of the hypothalamus has long fibers projecting below to the spinal cord and above to the cortex. Many aminergic neurons in the brain stem project to neocortical regions by way of these fibers, with the hypothalamus acting partly as a way-station [17]. There are reciprocal connections between the hypothalamus and the limbic forebrain structures and lower brain stem, and the pathways of the hypothalamus appear to reciprocate as efferent and afferent connections [17]. Neuronal circuits are partly excitatory and partly inhibitory, and the aminergic neurons and their neurotransmitters, such as gamma-butyric acid (GABA), act as chemical messengers between neurons to transmit information along neuronal networks that may be far-reaching. CRH neurons in the cortex may be involved in cognitive processing and those in the brainstem regulate autonomic nervous system function [18]. Although most of the fiber projections are bidirectional going to and from areas caudal to the hypothalamus, unidirectional connections from the retina terminate primarily in the suprachiasmatic nucleus, which generates circadian rhythms from light-dark cycles. Thus, the hypothalamus functions as both nervous tissue and as an endocrine organ. Some neurons release their peptides into synaptic clefts to act as neurotransmitters, and others release their peptides into the blood circulation to act on distant cells [16]. ACTH and the other pituitary tropic hormones ultimately target endocrine glands, such as the adrenal glands to secrete cortisol.

**Circadian Rhythmicity**

The physiological synchronization and timing of internal processes in the body with the environment is known as the circadian timing system [19]. The term “circadian” describes the 24-hour cycles or physiological rhythms of an organism [20]. It is now generally accepted that circadian rhythms persist even in the absence of environmental or exogenous cues and that there is a genetic basis for this periodicity. This element of “free-running”, a rhythm in the absence of exogenous stimuli, corresponds with a period of approximately 24-25 hours. Circadian rhythms can be sensitive to the environment and responsive to time cues called zeitgebers [21], such as light and dark for the sleep-wake cycle. However, circadian rhythms are self-sustaining and cycle under laboratory conditions of constant light as they would in the natural environment for a period of time before becoming dissociated and free-running. From an evolutionary standpoint, the light-dark zeitgeber may have been important for mammals in preparation for the coming light of day, a time when potential predators are active.

Circadian rhythms in mammals are governed by a “biological clock” mechanism located in the clusters of nerve cells of the SCN in the anterior hypothalamus [22]. Studies in the 1970s demonstrated the clock features of the SCN by creating lesions and by transplanting the SCN [22]. By creating lesions in the SCN in rats, circadian rhythms were disrupted and/or ameliorated [23]. By transplanting undamaged SCN into animals with lesioned SCN, the circadian rhythms could be restored [17]. In fact, the receiving host would have the circadian timekeeping properties of the donor. This clock in the SCN ensures that the multitude of physiological rhythms work in an internal temporal ordering in coordination with each other. There are various biological processes with circadian rhythms (e.g. cortisol and temperature) that have a temporal
order and reach their peaks and troughs at different times of the day [19]. This multiplicity of circadian rhythms indicates that there are multiple oscillators.

The properties of a circadian clock in mammals have been linked only recently to a genetic source [24]. The first mammalian circadian mutation, tau, was discovered in the golden hamster in 1988 and resulted in a shortened period (20-22 hours) [25]. There has been little further elucidation of circadian genes in the golden hamster because the genome has not been well studied. However, in 1994 [26], the first mouse circadian mutation, Clock, was described and soon after was cloned. The Clock gene has approximately 100,000 DNA base pairs and the protein product CLOCK is a “transcription factor” that allows it to bind to DNA and regulate other genes [27]. The mutant Clock affected the free-running period by lengthening it up to 27 hours and caused a loss of persistence of rhythm under constant conditions (after weeks in darkness). No anatomical defects in the SCN have been found to accompany a Clock mutation, and it appears to be a behavioral or functional mutation related to circadian rhythm [26]. Following this discovery, many more gene mutations have been identified that result generally in a loss of persistent rhythm and shortening or lengthening of the period [24].

The circadian clock mechanism can be outlined as follows: the SCN receives photic information from photoreceptors in the retina and this information is transmitted via the retinohypothalamic tract (RHT) [28]. Neurotransmitters that include glutamate, released at the RHT synapses that are in contact with the SCN, trigger an influx of calcium to activate protein kinases and stimulate gene activity [29, 30]. The CLOCK proteins promote activation of Per genes, and the PER proteins inhibit activation of the Per gene and the Clock gene in a negative feedback loop [22]. This gene activation is part of an auto-regulatory feedback loop lasting 24 hours for each cycle. The positive expression elements activate other clock-controlled genes transmitting time-sensitive information to other organs and tissues of the body [28].

Fig. 2.a. is a top-down diagram of the generic timekeeping system. Transducers are receptors that convert environmental signals (i.e., zeitgebers) into biological signals that are meaningful to the time-keeping system (e.g. light-dark cycle). They may also alter the waveform of the signal to transmit information about changes in the input rather than steady-state information. Mediators transmit temporal information from one place in the body to another through rhythmic activity (e.g. neural systems and neuroendocrine rhythms). Mediators play an important role in time-keeping by conveying information at all levels of the system. Pacemakers are the primary oscillators that entrain the organism. The two main functions of a pacemaker are to receive information from the environment and provide timing signals irrespective of cues from a transducer regarding the environment. Currently, it is thought that the SCN acts as the primary circadian pacemaker, although there may be more than one circadian pacemaker [19]. Other oscillators (e.g. secondary oscillators), such as the adrenal cortex, may be independent and capable of generating their own self-sustained rhythm. They may be synchronized whether or not the animal is in a periodic environment, and each oscillator is synchronized to specific environmental cycles [19]. Oscillators can also act on mediators to influence other tissues and organs (passive elements) to drive an overt rhythm. Overt rhythms are the periodic outputs of the circadian system, such as circulating hormones [19].
A good example of this mediator role is the activity of neural and endocrine systems in the form of hormonal circadian rhythms. An illustration of this cascade effect is described in Fig. 2.b. based on the light-dark cycle and its affect on cortisol rhythm [19]. Following exposure to the beginning of the light cycle, the retina transfers this information via the RHT to the SCN. The SCN activates ACTH release to stimulate the adrenal cortex. Cortisol acts as an effective mediator between the adrenal cortex and renal tubular sites that regulate the rhythm of urinary potassium excretion. In Fig. 2.c., ACTH levels may be viewed as a mediator between the anterior pituitary and adrenal cortex in one context (as a feedback mechanism) and as an overt rhythm (as a circadian rhythm) in another context.

**Feedback mechanisms of the L-HPA axis**

Again, the L-HPA axis refers to the role of the limbic system, hypothalamus, anterior pituitary, and adrenal cortex in the feedback pathway for cortisol (see Figure 3), although there are many other functions of the L-HPA axis. The key elements are hypothalamic nuclei that produce CRH, that then stimulate the secretion of ACTH from the anterior pituitary, that then target the release of cortisol from the adrenal cortices. The maintenance of homeostasis of this so-called “stress” pathway is due to various feedback mechanisms controlling the amount of hormone stimulated and released into this interactive network.

Starting with the parvocellular neurons of the PVN and other nuclei throughout the hypothalamus, CRH is synthesized and secreted in amounts determined by ultrashort, short, and target gland [31] feedback [17, 32]. The feedback alerts the system to either stimulate release or inhibit release that depends on acute need or the phase of the diurnal rhythm. There are basically two sites that receive hormonal feedback: the CNS areas containing CRH and other neurons and the anterior pituitary gland. Ultrashort feedback refers to direct feedback to the CRH via neuronal or humoral pathways. There is a close anatomical relationship between CRH-releasing nuclei in the PVN and the medial eminence that lies atop the anterior pituitary gland, but the feedback to the hypothalamic CRH neurons may also come from CRH neurons in the hypothalamus or collateral areas of the CNS [33]. The humoral pathway could be exerted via the hypophysial portal system and the basal area of the hypothalamus. This ultrashort feedback may exist in the form of positive or negative feedback and may depend on the nature of the release. Ono, et al. suggest that negative ultrashort feedback does not occur under basal conditions, but both positive and negative feedback may be possible following stress [33]. Short feedback refers to feedback of ACTH release from the anterior pituitary to the hypothalamic nuclei that produce CRH. AVP and oxytocin (from the short portal veins of the posterior pituitary and the hypothalamus) are also found in high concentrations in long portal blood of the anterior pituitary and potentiate the effect of CRH on ACTH secretion [15, 34]. It is of note that pituitary ACTH is unable to cross the blood-brain barrier and therefore would suppress hypothalamic CRH at the median eminence only [32]. Finally, target gland feedback refers to feedback from the adrenal cortex via glucocorticoids (cortisol) that impact on the anterior pituitary and release of ACTH and further up the pathway to the hypothalamic nuclei, the limbic system, and other sources of CRH. Neurotransmitter stimulation of hypothalamic CRH is strongly inhibited (negative feedback) by ACTH and glucocorticoids [32]. In addition, glucocorticoids reach the limbic system and other CNS
structures, where they also have effects on the nervous system. Glucocorticoid excess may cause damage to the hippocampus with resulting memory impairment, cognitive impairment [35] and inhibition of glucose transport [36]. Glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) have differing affinities for glucocorticoids [23, 37]. For example, although GR are important in mediating glucocorticoid feedback during stress, MR are highly bound during basal secretion [23]. Outside of this loop, corticosteroids may inhibit AVP release from the neurohypophysis of the posterior pituitary [34]. Since pituitary and tropic hormone receptors are widely distributed in the brain, the hormones themselves influence both their own secretion by feedback, but also other brain functions [17], such as appetite suppression for CRH [36].

**Pulsatile secretion and the normal cortisol circadian rhythm**

Recurrent secretory pulses of hormones throughout the 24-hour cycle (termed “ultradian”) maintain physiological concentrations of cortisol [17, 38]. This intermittent secretory activity involves communication via neurotransmitter or chemical signaling, to stimulate secretion or to enhance acquiescence or rest [17]. Although most anterior pituitary hormones have interpulse intervals (time between pulses) ranging from 45 minutes to four hours, ACTH has a pulsatile frequency of 2-5 pulses per hour [17]. The secretory pulses from pituitary hormones and pituitary hormone-driven hormones (e.g. CRH and cortisol) are considered episodic and less predictable than other hormones, but nonetheless they exhibit a pulse-like pattern that probably represents pulsatility throughout the HPA axis [38, 39]. However, there is a peak and trough at specific times of the day that characterize circadian rhythm (see Figure 4). Cortisol has a high-amplitude rhythm with peak and trough deviating greatly from the 24-hour mean [19].

The first few hours of sleep are associated with a quiescent period of suppression of cortisol secretion, with most cortisol secretion becoming active in the latter half of the early morning [19, 40]. That is, less than 5% of the total daily secretion of cortisol occurs in the hours near nocturnal sleep onset and approximately 40% takes place in the early morning hours near waking [41]. The hypothalamus serves as a master controller of neurotransmitter inputs specific to each neuroendocrine axis, but there also can be interactions between different axes (e.g. gonadotropic, somatotropic, and corticotropic) in response to specific stressors (e.g. starvation) [17]. The sequence of episodic cortisol secretion is programmed in concert with the other biological rhythms, such as the sleep-wake cycle [41].

Variations across 24 hours in serum hormones could arise from three dynamics: 1) secretory burst; 2) secretory pulse frequency; and 3) basal or nonpulsatile hormone release [17]. The magnitude of the pulses or bursts are the major variable in the circadian pattern of corticosterone release in rats [39]. Pulse frequency of corticosterone has a constant periodicity throughout the 24-hour cycle that indicates that the HPA axis is actively driven over this period [39]. Regulation of glucocorticoid pulsatility appears to be the result of rapidly alternating periods of activity and inhibition [38].

**Acute stress and cortisol**

Aside from regulation of a diurnal rhythm, the L-HPA axis is responsive to real and perceived threats to the body, such as, physical (e.g. heat, pain) and psychological stressors (e.g. fear, anxiety). These stressors activate the L-HPA axis to respond with glucocorticoids in amounts and timing to mobilize energy stores and to undertake other
metabolic actions. Given that some of these actions are catabolic in nature, it is important for the body to limit the strength and length of glucocorticoid release and restore homeostasis. It is the negative feedback mechanism of the L-HPA axis that keeps the release of glucocorticoids in check. The limbic system plays a prominent role in stress-induced glucocorticoid release. The hippocampus and prefrontal cortex are generally inhibitory to HPA axis secretion, whereas the amygdala are more likely to activate glucocorticoid secretion [23, 36].

Although stress is a strong stimulus for corticosteroid secretion, the underlying HPA activity or basal tone and time of day are predictive of the magnitude of the response [19, 39]. The rats of Windle, et al. were only responsive when noise stress coincided with a rising secretory phase [39]. When the noise stress fell during the falling phase when HPA inhibition is likely occurring, no response was seen. The sensitivity of animals and humans to glucocorticoid feedback varies by situation. There may be altered glucocorticoid receptivity depending on the nature of the stressor [23, 42, 43]. When an organism is exposed to chronic stress, the response may be one of “habituation” or “facilitation” [23]. In habituation, the intensity and predictability of the stressor is important, but if the same stressor is delivered again and again, there is a decreasing glucocorticoid response [43]. In facilitation, chronic stressed animals presented with a novel stressor have an overreaction in glucocorticoid rise [23].

**Epigenetics and L-HPA expression**

There has been an increasing amount of research on the area of epigenetics (environmental effects or other cues) and changes in gene expression (not DNA) in the L-HPA pathway in response to early postnatal life. Animal research on mother-offspring interaction and its effects on early development and behavior in the offspring serves as a useful context to review this topic. There is a body of work examining changes in both functional (e.g. hormonal endpoints and activation of the L-HPA) and gene expression (e.g. neural systems and CRH) [44, 45]. In particular, maternal care and environmental enrichment among rats appear to mediate L-HPA responses, not only in the index offspring, but intergenerationally in later offspring of the index offspring [46-48]. Liu et al. observed that adult offspring of mother rats that performed more licking and grooming in early postnatal life showed decreased plasma ACTH and corticosterone responses to restraint stress [48]. In addition, increased hippocampal glucocorticoid receptor messenger RNA (GRmRNA) expression, enhanced glucocorticoid feedback sensitivity, and decreased levels of hypothalamic corticotrophin-releasing hormone messenger RNA (CRHmRNA) – all considered positive mediators of L-HPA activity – were present in the adult offspring. The intergenerational effects are such that offspring reared by high licking and grooming mothers, even non-biological offspring, continued this maternal behavior into their own grooming practices with subsequent stress reactivity results mirroring those in Liu et al. [46, 48]. Finally, can negative stress reactivity responses as a result of low licking and grooming maternal behavior be reversed through a targeted intervention. Rats that were exposed either to maternal separation or handling during the first two weeks of life were transferred to standard social housing or an enrichment environment (larger cages, burrow system, and toys) after weaning [47]. Plasma corticosterone responses to stress were greater in the maternal separation group, but the differences were eliminated if this group was exposed to the environmental enrichment. Although CRFmRNA levels were higher in
the maternal separation group, the environmental enrichment did not reverse the CRFmRNA expression, suggesting that cellular mechanisms may be resistant to later intervention.

The understanding of processes initiated early in life has substantial ramifications for the developing child. Observational research in the area of history of child abuse or maltreatment has found lasting effects on stress mechanisms into adulthood [49, 50]. Variation in the CRH Type 1 receptor (CRHR1) gene appears to moderate the effect of child abuse on the risk for adult depressive symptoms [50] and for elevated cortisol on the dexamethasone/CHR test [49]. A more proximal link between prenatal depression and outcomes in the neonate was observed by examining methylation status, gene silencing through effects on chromatin structure [45], of the NR3C1 gene [51]. Third trimester depressed mood was associated with increased methylation of the NR3C1 gene, which may have implications for linking antenatal mood and altered stress reactivity. More work on epigenetic regulation of gene expression and lasting adverse effects need to be delineated to determine whether many of the findings in rats can be replicated in humans.

**Establishment of cortisol rhythm**

The cortisol and other circadian rhythms have been studied from late gestation/birth in humans. There is a body of literature that has attempted to determine when a cortisol circadian rhythm can be demonstrated in the human. It is of note that most human studies have not found a cortisol circadian rhythm at birth, but several have concluded that a rhythm is established at about three months of age, with an increasingly earlier demonstration of a rhythm with increasing recency of the publications. This corresponds generally to an improved methodological approach of more frequent collections during the day and longitudinal designs (see table below).

**Table of studies examining the establishment of cortisol circadian rhythm**

<table>
<thead>
<tr>
<th>Author, year</th>
<th>24 months</th>
<th>12 months</th>
<th>6 months</th>
<th>3 months</th>
<th>Prenatal to birth</th>
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<tr>
<td>Zurbrugg, 1976</td>
<td>X</td>
<td></td>
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<tr>
<td>Vermes, 1980</td>
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<td>X</td>
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<tr>
<td>Onishi, 1983</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
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</tr>
<tr>
<td>Price, 1983</td>
<td></td>
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<td>X</td>
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<tr>
<td>Spangler, 1991</td>
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<td>X</td>
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<tr>
<td>Mantagos, 1998</td>
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<td></td>
<td></td>
<td>X</td>
<td></td>
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<tr>
<td>Antonini, 2000</td>
<td></td>
<td></td>
<td></td>
<td>X</td>
<td></td>
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<tr>
<td>Serón-Ferré, 2001</td>
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<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Silva, 2007</td>
<td></td>
<td></td>
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<td>X</td>
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</table>

Zurbrügg took plasma cortisol measurements at four-hour intervals in neonates and children up to 11 years of age in a cross-sectional design and deduced that circadian rhythm activity is possible from two years of age [52]. Silva, et al. discerned circadian patterns in some infants at 45 days of life (when the first observations were made), but felt that statistically significant differences between morning and afternoon
samples (only two collection times per day) representing full maturity only occurred from 12 months of age [53]. Onishi, et al. collected blood for cortisol at four times of the day in a cross-sectional sample of hospitalized infants [54]. In 17 percent of the infants the reason for admission was abnormal growth development, although they were described as in apparent good health at the time of sampling. Nearly all of the infants under six months showed an irregular pattern, but a typical circadian pattern was observed in 70% of infants aged six to twelve months of age.

Vermes, et al. had improved on the previous estimates by measuring cortisol levels in blood every four hours in neonates over the first few days and then regularly up to three years of age [55]. Typical diurnal rhythms were observed from three months of age and were similar to one- and three-year-olds. Price, et al. in their longitudinal study of eight term infants with saliva sampled monthly up to six months of age also found a diurnal pattern from three months of age [56]. The characteristic adult pattern of the highest value in the morning followed by decline throughout the day emerged in the total group at three months. A pattern of sleep consolidation in the night (unbroken night sleep) coincided with the emergence of the cortisol rhythm in six of the infants. Three more studies followed chronologically that were supportive of a circadian rhythm being established by three months of age [57-59]. It is of note that similar to Price et al. sleep patterns were examined and found to parallel the timing of the development of the circadian pattern in cortisol in the longitudinal study of healthy newborns followed up to seven months by Spangler, et al. [57], and in the longitudinal study of preterm infants followed up to six months of age by Antonini, et al. [59].

One study has looked at the fetus at term to see if a clock mechanism exists before birth [60]. The authors suggest that at term there is a 24-hour rhythm of cortisol concentration in the umbilical artery, and that this cortisol was produced in the fetal compartment. However, it remains unclear whether maternal signaling via the maternal SCN is entraining the fetal rhythm or whether the fetal HPA-axis is driving the rhythm. It is intriguing that there is evidence for the clock mechanism functioning during fetal life in many mammal species [61]. In the human, the evidence increasingly points to earlier establishment of rhythms than were previously believed, and more sophisticated study designs starting in pregnancy should bear findings of some import. However, cortisol is not solely derived from fetal adrenal secretion and the maternal contribution during pregnancy to measurement of fetal levels of cortisol is unclear [60]. As well, the role of breastfeeding and maternal entrainment with bodily closeness may complicate the interpretation of cortisol rhythm in early postnatal life.

Developmental changes in the L-HPA

In the previous section I addressed the timing of the establishment of a cortisol rhythm in infancy. However, there are other developmental changes in cortisol with growth and development from the neonatal period through early childhood. The newborn period is characterized by low levels of corticosteroid-binding globulin (CBG), a measure of binding capacity, which increases over the first six months of life [62]. However, other authors have found high levels of serum cortisol in the first two weeks post-partum compared to later ages (through adolescence) [63], and Klug et al. observed that salivary cortisol levels were substantially higher on the first day of life compared to the second day [64]. Klug et al. hypothesize that the stress of labor and delivery might contribute to high cortisol production on the first day of life, although it is
uncertain how much the maternal to fetal transfer of cortisol via the placenta might be a factor in these higher levels soon after delivery.

In terms of stress reactivity, the newborn period is characterized by physiologically important cortisol responses to blood draws, physical exams, and circumcision [65]. This responsiveness has been demonstrated up to about three months of age, but thereafter a diminishing of the L-HPA response to such stressors is observed [66]. Physiological changes due to improved negative feedback and decreased sensitivity in the L-HPA system may account for these changes [67], but critical buffers begin to play a mediating role as well. Again, as in studies with rats reviewed in a previous section, the quality of care of children can produce differences in cortisol secretion in young children [68]. Stress reactivity is buffered by quality contact with the mother [69], although there is a dampening or hypocortisolism associated with chronically poor care as for institutionally-reared infants and young children [3].

As infants age the importance of coping to establish control over situations figures prominently in regulating the L-HPA response [68]. There is a reliable dampening of response given an adequate amount of time post-stressor (e.g. 30 minutes after inoculation) [70], as well as decreased cortisol levels and response with increasing age [71]. These results from the studies described above indicate a developmental trend toward a decline in stress reactivity to perturbations common to infancy and in the toddler period, including novel events and approaches by strangers [72].

This period of lesser stress reactivity extends throughout most of early childhood [73]. Most of the studies of stress reactivity in preschool-aged children examine transitions from the home to a preschool setting. De Haan et al. observed that mid-morning cortisol levels at home and during preschool and later transitions did not differ in two-year-old children [74]. Gunnar et al. also observed no differences in median cortisol at mid-morning in the initial weeks of the preschool year compared to several weeks later in the year [75]. However, for transitions from the home/preschool to kindergarten, Quas et al. observed increases in waking cortisol after kindergarten entry for those children with infrequent preschool experience and those who experienced a greater degree of change in the transition [76]. It is of note that there were, in general, positive associations between internalizing behaviors (inhibition and withdrawal), and negative associations between externalizing behaviors (aggression and assertion), and cortisol levels in the studies that examined these behaviors [74, 75, 77]. The literature in later childhood extends these findings to examine behavior and mood disorders and effects on cortisol levels and diurnal rhythm [6, 78].

**Autism and Cortisol**

**Background**

There are two related hypotheses that might explain how the diurnal or circadian rhythm of cortisol could be affected in autism. The first hypothesis is whether dysregulation of the L-HPA axis in autism is a result of neurobiological or neuroanatomical defects or deficits. This might be envisioned as a defect, similar to a congenital malformation or birth defect that is specific to causing L-HPA dysregulation. For example, it has been suggested that autism could result from a neurointegrative defect in the pathways of the CNS [79]. It is of note that in several of the psychiatric disorders of neurotransmitter (e.g. serotonin) imbalance, such as depression and
anxiety, researchers generally have found abnormal cortisol rhythms in adults [5, 15, 80]. In children with major affective disorder, cortisol hypersecretion following dexamethasone administration suggests a problem with negative feedback of the L-HPA axis and failure to suppress cortisol [81]. There is some support for this association in experimental animal research. Keen-Rheinehart et al. experimentally increased CRF expression in the amygdale of female rats which decreased the glucocorticoid negative feedback in the rats and resulted in depression-like behaviors [82]. There is good support for the hypothesis that serotonin synthesis capacity is disrupted in early childhood in autism [83]. Other forms of circadian regulatory control – feeding, temperature, and sleep - also can be found to function abnormally in autism [10, 84]. It also might be posited under this general hypothesis that there is a genetic defect(s) in autism that causes dysregulation of the L-HPA axis, but to-date there is no research to support this genetic hypothesis.

The second hypothesis suggests that there is a neurointegrative defect, but that the L-HPA axis of individuals with autism becomes dysregulated as a result of environmental triggers, such as novelty or change in routine that directly affect the L-HPA axis. These inputs might be considered chronic stressors that alter the regulation of the diurnal rhythm over time to achieve a hypocortisolism [3, 4] or hypercortisolism [85]. The neurobiology of chronic stress is distinctly different from acute stress [86]. In adults, post traumatic stress disorder (PTSD) shows the strongest association with altered basal cortisol levels, which supports hypocortisolism as a consequence [4]. However, in children with maltreatment or long-term abuse, mixed results with respect to cortisol are seen. De Bellis, et al. in their study of maltreated children with PTSD following early abuse observed elevated free cortisol excretion in urine collected over 24 hours [87]. Cicchetti, et al. in their study of maltreated children with internalizing problems (associated with depression) observed higher morning, afternoon, and average daily cortisol levels in the children while attending a week-long camp [85]. In contrast, two other studies of maltreated children with depression found lower morning cortisol levels [88, 89]. These four studies of maltreated children had different measures of psychiatric morbidity and different sampling settings that might account for differences in the results. Early exposure to stressors may induce persistent alterations of the L-HPA axis which have consequences for the developing organism [4]. Meaney, et al. exposed infant rats to daily handling and small periods of separation from their mothers [90]. As adults, the handled rats had better negative feedback control with less pronounced increase in glucocorticoids and better suppression of dexamethasone in response to stressors.

Children with autism may be more sensitive to zeitgebers which may result in greater variability in measurements of cortisol during the day due to circadian dysregulation [12]; thus, higher cortisol standard deviation ratios comparing children with autism to typically developing children. Yehuda et al. in their chronobiology work on cortisol regulation found a relatively poor fit of their data from depressed subjects to cosinor or multicosinor modeling compared to the healthy control group [91]. The authors suggest that a chaotic pattern of cortisol secretion rather than an increased cortisol secretion may be a better representation of cortisol dysregulation in depressed adults. Peeters et al. did not find differences in variability at a given sampling time between the major depressive disorder (MDD) and control groups, but they did find
statistically significant autocorrelation differences between the MDD (0.31) and control (0.50) groups (p<.0001) [92]. That is, there were lower associations between successive cortisol measurements in the MDD group suggesting a greater sample-to-succeeding sample variability. The authors believe that erratic activity rather than hypersecretion may be more intrinsic to HPA axis dysregulation. Few studies have examined variability directly, and this may explain why there is some heterogeneity or mixed results with respect to the comparison of cortisol secretion levels in major depression. Studies which have a multitude of collections relatively close in time across the day (and night) are more likely to have the power to detect autocorrelation and variability differences between selected groups.

Review of the literature

It is of note that the above hypotheses rely on other psychiatric disorders as templates for possible defects in autism. However, there is a body of published data that has encompassed the study of baseline and stimulated hormone levels as a “window” to the neurochemistry of autism [93]. A review of studies of cortisol diurnal rhythm among children and adults with autism presents a heterogeneous picture of neuroendocrine function. Most studies were limited by small numbers, since they generally enrolled less than 15 children with autism. The diagnosis of autism in some of the studies was also unsatisfactory by today’s standards, although diagnosis of autism by World Health Organization (WHO) International Classification of Disease (ICD) standards and by Diagnostic and Statistical Manual (DSM) criteria were the most common methods used. The quantification of cortisol also may have been affected by collection in a laboratory setting with venipuncture for plasma cortisol and by the presence of medications (e.g. antipsychotics and antiepileptics). Table 1 presents all of the epidemiological studies on cortisol levels in individuals with autism regardless of the strengths and limitations of the research. Variance estimates were noted when available, and methodological issues are detailed in the final column of the Table. For the purposes of the dissertation, studies of children with autism living in the community and their internal control groups offer the most useful data. Studies on adults were excluded because adults are sufficiently different biologically and developmentally from children so that comparisons may not be meaningful. Children who were hospitalized or institutionalized are excluded because of the representativeness of these samples and because hospitalization or institutionalization itself may in fact affect cortisol. The terms “highly developed” and “poorly developed” are used in earlier papers to reflect higher versus lower IQ, respectively. These terms have been supplanted by “high functioning” and “low functioning” in more recent papers. The terms “AUT” and “CONT” are abbreviations for individuals with autism and their typically developing or healthy control or comparison group. It should be noted that other comparison groups will be labeled as designated in the papers (e.g. MR for mentally retarded) and may have different purposes. The use of a comparison group of mentally-retarded subjects is designed to match more closely the intellectual and developmental level of the autism group. The typically-developing comparison group serves as a reference group for what is expected to be normal secretion of cortisol.
The methodological criteria for the review are outlined below:

<table>
<thead>
<tr>
<th>Classification</th>
<th>Methodological Criteria</th>
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<tbody>
<tr>
<td>Excluded</td>
<td>Adults Institutionized or hospitalized children</td>
</tr>
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<td></td>
<td>Diagnostic criteria for autism other than DSM or WHO ICD</td>
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<td></td>
<td>Inclusion of medication use without adjustment</td>
</tr>
<tr>
<td></td>
<td>Children with epilepsy without matching in control group</td>
</tr>
<tr>
<td>Poor to fair quality</td>
<td>Examination of potential confounding or use of matching</td>
</tr>
<tr>
<td></td>
<td>DSM or WHO ICD diagnostic criteria for autism</td>
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<tr>
<td>Good to excellent quality</td>
<td>Assessment of cortisol at equivalent times for comparison</td>
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</table>

The first group of studies to be discussed met the general entry criteria, but the quality of the methodology was poor to fair. Three of the earliest studies from the 1970s were carried out in laboratory settings and measured plasma cortisol on community-dwelling school-aged children with autism. All of these studies used a laboratory setting for blood collection with the attendant issue of heightened anticipation in a non-naturalistic environment that may have affected cortisol measurement. Two of these studies [94, 95] reported that AUT had the highest cortisol levels versus CONT at any given measurement time, but the methodological problems were difficult to overcome. In Goodwin, et al., any gains from using siblings to attain environmental similarities that might reduce confounding are outweighed by the potential influence of genetics on cortisol [94]. That is, there may be genetic factors related to autism in common between siblings and it may be more difficult to see a difference in cortisol. In Maher, et al., it is likely that sedation of the subjects may affect the cortisol results and the representativeness of the sample is certainly compromised due to the sedation [95]. In Yamazaki et al., the diagnostic criteria and sampling were unspecified, and a lack of control group made it difficult to interpret whether rhythms were “normal” or “abnormal” as claimed [96].

Two studies in the 1980s [81, 97] investigated the response of cortisol to the dexamethasone suppression test (DST), which is a functional test of the L-HPA axis [98]. Dexamethasone is a steroid that binds to glucocorticoid receptors in the hypothalamus to provide negative feedback to the anterior pituitary to suppress secretion of ACTH. A proper functioning L-HPA system would ensure that cortisol is thus suppressed following the administration of dexamethasone. These studies enrolled school-aged to teen-aged children with a wide range of ages, and blood was collected for plasma cortisol. The AUT were more likely to be nonsuppressors, especially those AUT with poor development as defined by a low IQ. It has been proposed that children with poor development may have a serotonin regulation disorder, leading to a less refined ability to suppress cortisol following dexamethasone [97]. In Hoshino, et al., all children in the study were taking antipsychotics, also known as neuroleptics; thus the representativeness of the sample is questionable [99]. Although previous studies suggest that antipsychotics don’t affect suppression [98, 100], cortisol diurnal secretion appears to be lowered [101, 102]. In Jensen, et al. there was no
internal control group, so no comparison can be made to a control or reference group [81].

More recently, in Ćurin, et al. there was a statistically significantly lower cortisol level for AUT versus CONT at one point in time (waking), but many subjects in the AUT group were taking antiepileptics and antipsychotics [103]. In Naber et al. salivary cortisol was collected from preschoolers before/after the Strange Situation Procedure (SSP), but the collection times were variable, thus the pre- SSP results cannot be interpreted as being from a homogeneous collection time [104]. Croonenberghs et al. in two associated papers studied cortisol before/after administration of 5-HTP, a precursor to serotonin. Unfortunately, there was no information on time of plasma collection so that again the baseline results cannot be interpreted [105, 106].

The remaining six papers [10-14, 97] are of good to excellent methodological quality and used collection procedures (saliva, except for one study using urine), populations (healthy or typically-developing school-aged children), and settings (the community) that are most relevant to the population and study question of interest for the dissertation. In the only study that used urinary cortisol measurement over 24 hours, Richdale, et al. compared 18 children with autism from the community compared to 19 control children from public schools, matched on age, sex, and IQ. The cortisol diurnal curves were elevated for AUT overall compared to CONT and for AUT greater than eight versus younger than eight years of age (from figures presented). There were no differences by high- and low-functioning status for AUT and the temporal placement was not shifted [10]. In Hoshino, et al. in 1987, the methods were markedly improved over an earlier paper with the collection of saliva for cortisol, the exclusion of medications, and the utilization of DSM-III diagnostic criteria [97]. The authors compared 22 children with autism (15 highly developed (HD); 7 poorly developed (PD)) to 27 healthy children based on salivary cortisol measurement. Pre-DST, the diurnal rhythm of cortisol (in µg/dl) for the children with autism remained high in the afternoon, from 10:00am (0.50) to 4:00pm (0.50) (variance estimates not given) compared to the healthy children (0.59 (± 0.08) at 10:00am; 0.33 (± 0.03) at 4:00pm). Among the HD children, six of ten were nonsuppressors, and among the PD children four of five were nonsuppressors. Abnormal rhythms were described for three of twelve HD and two of five PD, with four of five abnormal rhythms higher as compared to the healthy children. The following papers all have examined diurnal rhythm periods, and three of the four have pre-post test cortisol levels for investigating stress-reactivity. Jansen, et al. studied three groups of school-age children with AUT, multiple complex disability disorder (MCDD), and healthy controls [13]. Although AUT had higher cortisol levels during the latter half of a control period that ranged from 30 minutes before to 90 minutes after a control time marker, these levels were not higher before/after a public speaking or a physical exercise stressor period of the same duration. This is virtually a cross-sectional examination of diurnal rhythm on one day and over a short period of time (two hours between 10 am and 4 pm). Corbett, et al. also studied school-age typically developing children and AUT in two studies of diurnal rhythm and stress-reactivity following mock or sham magnetic resonance imaging (MRI) [11, 12]. The first study found that cortisol levels at waking, afternoon, and evening over two contiguous days were not different between the groups but were higher for AUT following the mock MRI [11]. The second study found evening cortisol values averaged over six days to be
higher in AUT (2.9 nmol/L) versus CONT (1.4 nmol/L), and morning values for AUT to trend lower over the six days [12]. This study is the only study to report on variability of cortisol in AUT versus CONT, with greater variability in AUT cortisol levels for afternoon (sd ratio of AUT:CONT 1.48) and evening (sd ratio of AUT:CONT 2.48). Marinović-Ćurin, et al. investigated diurnal cortisol levels across the day in an older age group of middle-school children and observed that all afternoon cortisol levels were slightly higher in AUT versus CONT [14]. The authors also found the biggest difference in cortisol to be at bedtime between AUT (0.21 ug/dL) versus CONT (0.09 ug/dL).

In summary, none of the six methodologically good to excellent studies found levels in AUT that were significantly lower than their respective control groups. Three of these studies [10, 14, 97] reported afternoon levels and/or rhythms that were higher for AUT versus CONT, and two [12, 14] reported evening cortisol levels that were higher for AUT versus CONT. In Marinović-Ćurin et al. the age range would have combined pre- and post-pubertal children; but Tordjman, et al. found no differences in cortisol levels by pubertal status [107]. In concert, these studies suggest an excess secretion or hypercortisolism occurring at one or several times during the day, particularly from the afternoon through bedtime. However, this summary is relying on only a small number of studies and the variability in the time of day that this excess secretion has been reported to occur may be more reflective of an erratic pattern of secretion rather than hypersecretion.

In most of the studies reviewed in the text, there was no discussion of an examination of potential confounders other than age and gender. Given that the age of most children in the review (six years of age or older), it is plausible that a variety of antipsychotics and other medications may have been used. Some studies reported this in the methods section, but it is possible that data on medication use, as well as other covariates may have gone uncollected or unreported. It is also unclear from these studies whether epilepsy, relatively common in autism, was present. This condition often necessitates antiepileptic medications with sedating and other properties. Increased cortisol levels are predictably found following seizures [108] and during antiepileptic therapy [109]. In addition, the earliest studies may have included PDDNOS and/or Asperger syndrome that are on the autism spectrum but not strictly defined as autistic disorder or autism. Contemporary diagnostic methods by DSM-IV criteria can reliably exclude these diagnoses from autistic disorder. It is unknown how other diagnoses on the spectrum might differ from autism in terms of diurnal cortisol secretion. However, the restriction to autism alone allows a more homogeneous diagnostic group presumably with a more similar underlying neurobiology. Finally, the small sample sizes of most of the studies to-date can not exclude random chance as an explanation for findings, nor do they allow sufficient precision of the estimates. Only one study [12] used a longitudinal design to study diurnal cortisol rhythm. A repeated measures approach is crucial when examining a biomarker like cortisol, with high day-to-day variability.

Several of the studies considered in this review have proposed that a dysfunction in the negative feedback mechanism of the L-HPA axis is responsible for abnormal diurnal cortisol rhythm and/or the ineffectiveness of shutting off cortisol following dexamethasone stimulation [81, 97, 99]. Hill, et al. have suggested that the lack of temperature variation in a customary temporal pattern along with cortisol dysregulation
are indicators of a neurointegrative defect of the HPA axis [84]. The authors posit that among the children with autism, a developing desynchrony is occurring in the two systems. In the more recent papers, a focus on social and environmental stressors may assist in elucidating whether these inputs have a role in influencing cortisol secretion during the day. The state of the research on autism and cortisol suggests that there are differences in L-HPA axis function between children with autism versus other comparison children. However, due to lack of precision in study estimates, study design issues, and the lack of an examination of potential confounding, this research question has not been resolved.
Figure 1. Mid-sagittal view of the central nervous system (Gray's Anatomy public access drawing).
Figure 2. The clock mechanism: 2A. Generic timekeeping system; 2B. Light-dark cycle and its affect on cortisol rhythm; 2C. ACTH as mediator and as an overt rhythm.
Figure 3. This figure shows the sites of feedback action of target endocrine gland hormones, anterior pituitary hormones (short feedback), and of hypophysiotrophic neurohormones (ultrashort feedback). Humoral pathway of ultrashort feedback is not indicated [17].
Figure 4. Figure of normal diurnal cortisol secretion. Permission for the use of the data was given by Douglas Granger of Salimetrics, Inc.
Table 1: Review of studies of cortisol levels between non-institutionalized children with autism and comparison groups

<table>
<thead>
<tr>
<th>Authors, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Type of cortisol measurement</th>
<th>Ages</th>
<th>Results</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goodwin, 1971, United States[94]</td>
<td>Community sample of children with autism, Sibling children as controls, Study of gliadin (a dietary peptide) compared to placebo</td>
<td>15 AUT 14 CONT</td>
<td>Plasma cortisol pre- and post-placebo</td>
<td>6-13 AUT 6-13 CONT</td>
<td>AUT CONT Average cortisol levels (µg%) pre-placebo: 13.9 (7.2-40.2) 13.0 (6.3-31.4) Post-placebo: a.m. 15.2 13.9 p.m. 13.0 10.8 Total 14.5 12.9</td>
<td>Diagnosis of autism by specialists at diagnostic centers, Exact times of sampling unknown, Sampling following potential stressor exams and testing, Laboratory setting</td>
</tr>
<tr>
<td>Maher, 1975, Australia [95]</td>
<td>Community sample of children with autism, Children with intellectual disability as controls, Study of insulin-induced hypoglycemia</td>
<td>11 AUT 11 CONT</td>
<td>Plasma cortisol Median (range) 11 (4-13)</td>
<td>AUT CONT Average cortisol levels (µg/100ml) pre-insulin: 12.6 9.2 Post-insulin: AUT with more sustained secretion of cortisol, whereas CONT returned to baseline faster</td>
<td>Diagnosis of autism by WHO standards, All subjects sedated, Laboratory setting, Unclear where subjects came from, Matching on age, sex, height, weight, IQ, SES, to control for confounding</td>
<td></td>
</tr>
<tr>
<td>Yamazaki, 1975, Japan[96]</td>
<td>Community sample of children with autism, No controls</td>
<td>7 AUT (infantile)</td>
<td>Plasma 11-hydroxycorticosteroids</td>
<td>6-10</td>
<td>Figures for each subject shown separately. Appears to be abnormal rhythm in 5 of 7.</td>
<td>Diagnosis of autism unspecified, Unclear where subjects came from, No internal control group</td>
</tr>
</tbody>
</table>

Abbreviations: AUT=children with autism; CONT=typically developing children as controls; HD=highly developed children with autism (with high intellectual development or level); PD (poor intellectual development or level); DST=dexamethasone suppression test
Table 1 (cont.): Review of studies of cortisol levels between non-institutionalized children with autism and comparison groups

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</tr>
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<tbody>
<tr>
<td>Hoshino, 1984, Japan [99]</td>
<td>• Children with autism referred to Medical Center  • Children with other brain dysfunction or mental retardation as controls</td>
<td>11 AUT HD (highly-devel.) 8 AUT PD (poorly-devel.) 10 MR (mental retardation) 5 MBD (minimal brain dysf.)</td>
<td>Plasma cortisol Dexamethasone suppression test</td>
<td>Mean (range) 11.5 (5-20) AUT 9.0 MR/MBD</td>
<td>Mean (± standard error) cortisol in µg/dl: pre-DST post-DST AUT(HD) 5.1 ± 0.7 2.9± 0.8 AUT (PD) 11.6 ± 1.7 9.8 ± 1.4 MR 6.3 ± 0.5 2.3 ± 0.7 MBD 4.1 ± 1.0 2.7 ± 0.3 2 of 11 AUT(HD) were nonsuppressors 8 of 8 AUT(PD) were nonsuppressors</td>
<td>• Diagnosis of autism by WHO standards  • All subjects taking neuroleptic or other medications</td>
</tr>
<tr>
<td>Jensen, 1985, United States [81]</td>
<td>• Children with autism seen on outpatient basis • No controls</td>
<td>13 AUT</td>
<td>Plasma cortisol Dexamethasone suppression test</td>
<td>Mean (range) 11 (2-17)</td>
<td>Mean and median cortisol in µg/dl at baseline (8 am): Mean: 17.5 Median: 19 11 of 13 were nonsuppressors</td>
<td>• Diagnosis of autism by Developmental Inventory (Ornitz) and DSM-III criteria  • No internal control group</td>
</tr>
<tr>
<td>Hoshino, 1987, Japan [97]</td>
<td>• Children with autism referred to Medical Center  • Healthy children as controls</td>
<td>22 AUT 27 CONT</td>
<td>Salivary cortisol Dexamethasone suppression test</td>
<td>Mean 9 AUT CONT</td>
<td>Average ± se cortisol levels (µg/dl) pre-DST: 10:00a.m. 0.50 0.59 ± 0.08 4:00p.m. 0.50 0.33 ± 0.03  • AUT non-suppressors tended to have elevated afternoon values  • 4 of 5 AUT(PD) were nonsuppressors  • 9 of 12 AUT(HD) w normal rhythm  • 4 of 5 abnormal rhythms elevated</td>
<td>• Diagnosis of autism by DSM-III criteria  • Selected rhythms presented in paper  • No medications at time of sampling</td>
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<tr>
<td>Aihara, 1989, Japan [110] (English abstract only)</td>
<td>• No information</td>
<td>30 AUT 16 MR 16 ADD</td>
<td>Plasma cortisol? (methods unspecified)</td>
<td>Range 1.8 to 9.8 (MR and ADD age-matched to AUT)</td>
<td>11 of 14 AUT with abnormal 24-hour hormone secretion rhythm</td>
<td>• Diagnosis of autism unspecified • Unclear where subjects came from • No data shown • Matching on age • Results refer to growth hormone and prolactin, too</td>
</tr>
<tr>
<td>Richdale, 1992, Australia [10]</td>
<td>• Children with autism recruited from special and public schools, and by advertisement • Control children from public schools</td>
<td>18 AUT 19 CONT</td>
<td>Urinary cortisol over 24 hours</td>
<td>Mean (range) 8.3 (4.6-14.3) AUT 7.5 (4.2-12.2) CONT</td>
<td>• Cortisol diurnal rhythm in nmol/hour elevated for AUT overall and when divided ± 8 years of age • No correlation between IQ and cortisol in AUT (r=-0.02, p&lt;.80) • Temporal placement normal</td>
<td>• Diagnosis of autism by DSM-III criteria • Matching on age, sex, IQ to control for confounding</td>
</tr>
<tr>
<td>Ćurin, 2003, Croatia [103]</td>
<td>• Teens with autism from centers for autism • Healthy teen controls</td>
<td>36 AUT 27 CONT</td>
<td>Plasma cortisol</td>
<td>Mean ± sd 15.6 ± 9.4 AUT 16.6 ± 10.3 CONT</td>
<td>Average ± sd waking cortisol levels (nmol/liter): AUT 335.8 ± 131.1 CONT 578.6 ± 116.7</td>
<td>• Diagnosis of autism by DSM-IV • Teens only • Matched on age and sex • Many subjects taking anti-epileptics or antipsychotics</td>
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Abbreviations: AUT=children with autism; CONT=typically developing children as controls; HD=highly developed children with autism (with high intellectual development or level); PD (poor intellectual development or level); DST=dexamethasone suppression test
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<tr>
<td>Jansen, 2003, the Netherlands [13]</td>
<td>• Children with autism and multiple complex develop-mental disorder (MCDD) recruited from outpatient clinic at University • Healthy control children recruited from primary schools</td>
<td>10 AUT 10 MCDD 10-15 CONT</td>
<td>Salivary cortisol (control session and pre/post stress testing)</td>
<td>Mean ± sd 9.4 ± 1.4 AUT (MCDD and CONT age-matched)</td>
<td>• AUT had statistically significantly (p&lt;.05) higher cortisol levels (60-80 nmol/L) compared to MCDD and CONT (both approx. 40-45 nmol/L) during a control period with salivary cortisol taken every 10 minutes • There were no differences between groups in public speaking or exercise stress testing</td>
<td>• Diagnosis of autism by DSM-IV criteria • Matched on age</td>
</tr>
<tr>
<td>Corbett, 2006, United States [11]</td>
<td>• Children with autism and healthy controls recruited from clinic, school, or community centers</td>
<td>12 AUT 10 CONT</td>
<td>Salivary cortisol (control period plus before/after mock MRI)</td>
<td>Mean 8.5 AUT 9.2 CONT</td>
<td>• Statistically significantly (p&lt;.05) higher cortisol levels for AUT versus CONT at 20, 40, and 120 minutes following mock MRI scan • Cortisol levels at home for waking, afternoon, and evening were not different by group (0.146 (±0.19) nmol/L higher for AUT (p=0.45))</td>
<td>• Diagnosis of autism by DSM-IV criteria • Matched on age and sex</td>
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<tr>
<td>Naber, 2007, the Netherlands [104]</td>
<td>• All children recruited from population-based sample of 30,000 at 14 months of age • Subjects were studied before/after Strange Situation Procedure</td>
<td>19 AUT 8 PDDNOS 12 MR 12 LD (language disability) 17 CONT</td>
<td>Salivary cortisol (before/after Strange Situation Procedure)</td>
<td>Mean ± sd (in months of age) 30.4 ± 5.0 AUT 27.5 ± 5.0 PDDNOS 24.5 ± 5.8 MR 25.1 ± 5.6 LD 28.1 ± 1.7 CONT</td>
<td>Baseline (±sd) AUT 9.6 (3.6) .18 (2.6) PDDNOS 8.2 (3.3) -.31 (1.9) MR 9.6 (5.1) .03 (5.0) LD 7.7 (2.8) .96 (3.9) CONT 7.1 (2.3) -.58 (1.8)</td>
<td>• Diagnosis of autism by DSM-IV criteria • Testing not at same times of the day • AUT slightly older than other groups</td>
</tr>
<tr>
<td>Croonenberghs, 2007, Belgium [106] abstract</td>
<td>• Teens with autism • Healthy teen volunteers</td>
<td>18 AUT 22 CONT</td>
<td>Plasma cortisol before 5-HTP administration</td>
<td>In baseline conditions there were no differences in cortisol between groups up to 45 minutes before 5-HTP</td>
<td></td>
<td>• Matching on age • No other information available from abstract</td>
</tr>
<tr>
<td>Corbett, 2008, United States [12]</td>
<td>• Children with autism and healthy controls recruited from clinic, schools, community centers, advertisement</td>
<td>22 AUT 22 CONT</td>
<td>Salivary cortisol (home and pre/post mock MRI #1 and #2)</td>
<td>Mean ± sd 8.8 ± 1.9 AUT 9.3 ± 1.7 CONT</td>
<td>• Evening cortisol values were higher in AUT versus CONT (p&lt;.04) • AUT with higher cortisol levels at all times pre/post mock MRI #1 Between-child variability (sd)</td>
<td>• Diagnosis of autism by DSM-IV criteria • Balanced on age</td>
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<td>Croonenberghs, 2008, Belgium</td>
<td>Teens with autism • Healthy teen volunteers</td>
<td>18 AUT 22 CONT</td>
<td>Plasma cortisol before 5-HTP</td>
<td>13-19 years of age (post-pubertal males)</td>
<td>In baseline conditions, the DHEA-S ratio significantly higher in AUT vs CONT up to 45 minutes before 5-HTP</td>
<td>• Matching on age • No other information available from abstract</td>
</tr>
<tr>
<td>Marinović-Čurin, 2008, Croatia</td>
<td>Children with autism attended autism center • Healthy control children</td>
<td>9 AUT 7 CONT</td>
<td>Salivary cortisol</td>
<td>Mean ± sd 11.9 ± 3.0 AUT 11.6 ± 4.3 CONT</td>
<td>• Cortisol levels (ug/dL) taken at: waking, waking + 30 minutes, right before lunch, lunch + 60 minutes, 6 pm, bedtime • All afternoon cortisol levels higher in AUT versus CONT. Biggest difference at bedtime: 0.21 (AUT) versus 0.09 (CONT) approximately</td>
<td>• Diagnosis of autism by DSM-IV criteria • Matched on age and sex • ACTH stimulation results not included because adults as controls</td>
</tr>
</tbody>
</table>

**Abbreviations:** AUT=children with autism; CONT=typically developing children as controls; HD=highly developed children with autism (with high intellectual development or level); PD (poor intellectual development or level); DST=dexamethasone suppression test.
References


CHAPTER 4: REVIEW OF SLEEP AND CHILDREN

Introduction
Sleep is a necessary process for the survival of the human, yet it is still unknown why it is essential [1]. It is extremely difficult to stay awake for more than a week and although sleep can be postponed, sleep cannot be eliminated entirely. Until the late 1950s, it was believed that the brain lapses into sleep when there is not enough sensory stimulation to remain awake [2]. Since that time, sleep has been viewed as an active process as measured by changes in brain activation, neurophysiology, heart rate, and metabolism. The stages of sleep can be represented as brain patterns on electroencephalogram, motor activity on electromyogram, eye movements on electrooculogram, heart rate, and the rate of respirations. Importantly, we know that there are consequences to poor sleep quality and/or lack of sleep. Sleep is important for the consolidation of memory and learning [6], enhancement of metabolic and inflammatory responses [7], and improvement in growth and development [8].

The implications for vulnerable children with atypical development, such as those with autism, are even more cogent as they tend to spend more time without sleep than typically developing children. Sleep disturbances among children with autism are thought to be relatively common. It is estimated that 44-83% of these children have been reported to have sleep problems that include longer sleep latencies (delayed sleep onset), lengthy night awakenings, shortened night sleep, and early morning waking [9-11]. This compares to approximately 15-20% of typically developing preschool-aged children being characterized as having difficulty falling asleep and/or frequent night awakenings [10, 12]. Circadian sleep-wake problems, such as the inability to fall asleep at the desired time, are of particular note because it has been suggested that they may reflect disorganization of the biological circadian timing system [13].

Sleep-wake circadian rhythm
There are two processes that describe the temporal organization of sleep. The first is the circadian rhythm of sleep-wake regulation and the second is the ultradian rhythm of rapid eye movement (REM) and non-REM (NREM) sleep stages [14]. The sleep-wake rhythm, similar to the circadian rhythms of temperature and cortisol, is affected by zeitgebers and internal body signals [14]. The sleep-wake rhythm is tightly coupled to temperature but only loosely coupled to the cortisol rhythm. Ambient temperature (too low or too high) can decrease sleep duration and it appears that there is an interaction between the circadian time-keeping mechanisms of temperature and sleep-wake rhythms [15]. The temporal establishment of the sleep-wake rhythm is similar to the temporal establishment of cortisol rhythm, with some circadian regularity at about three months of age [16-18]. There are two biobehavioral shifts in sleep-wake behavior in the infant: there is a marked increase in hours of wakefulness during the day from the newborn period to 2-3 months with a plateau following until four months; and to a lesser extent, a second shift to increased wakefulness from five to eight months, again followed by a plateau until the end of infancy [19]. The authors believe that development proceeds at uneven rates and that these two shifts represent major changes in developmental organization, heralding the onset of social smiling and stranger distress in the two shifts, respectively. The sleep-wake cycle is governed by a circadian timekeeping mechanism (see Circadian Rhythmicity section in Chapter 3 for...
overview) but its state organization may be modified by exogenous influences, such as light-dark exposure and timing of meals [15]. The average free-running period is approximately 25 hours, but with light-dark cycle entrainment via the suprachiasmatic nucleus (SCN), a 24-hour cycle is achieved. The SCN is reset daily by light to the retina in the daytime and by melatonin secretion from the pineal gland during the nighttime [20].

Briefly, the functional anatomy of the sleep-wake cycle can be described as primarily involving the following areas: the reticular formation (RF) (also called the reticular activating system) that runs longitudinally through the upper brainstem and connects with parts of the hypothalamus and thalamus [21]; the midbrain raphe (MR) which runs from the medulla to the midbrain; and the locus coeruleus [22] in the brainstem [15]. The MR nuclei contribute to sleep induction through the pathways of the neurotransmitter serotonin and by inhibiting the RF. The neurons of the RF and the adrenergic neural pathways with norepinephrine from the LC play an important role in the arousal from sleep. The pineal gland in the midbrain has connections to the light-dark cycle via the SCN and produces the hormone melatonin. There are many other nuclei in the RF, and two major branches in this area have been described that relay neural information to adjust the sleep-wake cycle [20]. The first branch ascends to the thalamus and activates relay neurons (primarily acetylcholinergic neurons) to transmit information to the cerebral cortex. The second branch bypasses the thalamus and activates neurons (including noradrenergic, serotonergic, dopaminergic, and histaminergic neurons) in the lateral hypothalamus, basal forebrain, and the cerebral cortex.

Sleep state ultradian rhythm

In sleep, there are the two distinctly different states called REM and NREM sleep. Eye movements, muscle tone, brain waves from EEG, and respiratory and heart rhythms are used to discriminate between REM and NREM sleep [3]. The neurobiology of REM and NREM sleep is somewhat different from that of the overt sleep-wake rhythm. While the neurons responsible for ultradian REM-NREM oscillation are located in the mesopontine brainstem, the modulation involves mechanisms in the forebrain [23]. At the neuronal level, the cholinergic or acetylcholine-containing neurons promote REM sleep and the monoaminergic (e.g. histamine, serotonin, and norepinephrine) neurons act to suppress most components of REM sleep. Orexin-containing neurons project widely and excite noradrenergic neurons of the LC and serotonergic neurons of the dorsal raphe (in vitro) to suppress REM sleep [24]. Cholinergic influences increase the excitability of brainstem RF neurons to initiate REM sleep by inhibiting GABAergic neurons which are inhibitory to RF neurons [25]. RF neurons are significantly involved in the different features of REM sleep. Pontine RF neurons rapidly increase their discharge as REM sleep is approached, dorsolateral pontine RF neurons become active to initiate muscle atonia, and midbrain RF neurons are important for EEG activation. Pontine RF neurons fire continuously through REM sleep maintaining membrane depolarization until an NREM sleep cycle; action potentials are almost silent during NREM sleep [25, 26]. Endogenous adenosine figures importantly in promoting the transition to slow wave NREM sleep by inhibiting the basal forebrain wake-promoting cholinergic neurons via the adenosine A1 receptors (A1R) located there [27]. Oishi et al. found that activation of A1R in the TMN of rats inhibited histaminergic arousal.
systems to increase NREM sleep duration without affecting REM sleep [28]. Other
differences between sleep stages are that neurons of the LC and DR fire most
intensively during waking, fire less in NREM sleep, and fire minimally or not at all in
REM sleep [26].

In the fetus and infant REM and NREM sleep are referred to as “active sleep”
(AS) and “quiet sleep” (QS), respectively. The emergence of sleep states starts in the
fetus with most of fetal life spent in cyclic patterns of AS and QS [18]. Although REM
sleep appears in the fetus at six or seven months gestation, NREM sleep appears from
seven to eight months gestation [5]. There is maturational development of sleep states
with increasing chronological age. In the newborn, there is no diurnal organization.
Although REM sleep occurs at sleep onset in the newborn, later in the first year of life
NREM sleep will start to occur at sleep onset [3] (see Figure 1). The duration of the first
QS episode increases over the first months and then plateaus at about 5 months of age
[19]. The organization of sleep states in the newborn is very different from that of older
children and adults. During the newborn period approximately 50% of sleep time is
spent in REM sleep compared to 33% by three years of age and 25% as an adult [5].
REM and NREM sleep alternate with each other through the night in an ultradian cycle
(within a 24-hour period) of approximately 50 minutes for infants and 90 minutes for
adults [3] (see Figure 1). Feinberg’s work on changes in sleep cycles with age (across
the life span) supports that there are discernible trends in NREM/REM sleep cycles
across the night [29]. There is an umbrella-shaped curve across the night in duration of
each REM sleep cycle, with the first and last cycles being the shortest (in all age groups
examined). The umbrella-shaped curve for NREM sleep cycles holds for older ages,
but is different for children in that the first cycle is lengthier. It is notable that young
children will continue to have some brief wake time during the night, especially from
NREM2 and REM sleep [4]. NREM sleep is characterized by basal regularity and REM
sleep is characterized by more irregularity in basal activity (see table below).

### Characteristics of sleep-wake of a child [3-5]

<table>
<thead>
<tr>
<th>Sleep stage</th>
<th>Heart patterns</th>
<th>Respiratory patterns</th>
<th>Eye movements</th>
<th>Brain waves on EEG</th>
<th>When during night</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>NREM 1</td>
<td>regular</td>
<td>regular</td>
<td>slow</td>
<td>theta waves</td>
<td>throughout the night</td>
<td>drowsiness</td>
</tr>
</tbody>
</table>
| NREM 2      | regular        | regular              | slow          | sleep spindles; k-
complexes       | throughout the night | beginning of deep sleep |
| NREM 3      | regular        | regular              | slow          | delta waves        | 1st third of the night | deep sleep; sweating |
| NREM 4      | regular        | regular              | slow          | delta waves        | 1st third of the night | dreaming; atonia; irregular temp. |
| REM         | irregular      | irregular            | rapid         | alpha waves; theta waves | most in last third of the night |       |

NREM sleep functions as restorative sleep. In contrast, the EEG, neuronal, and
metabolic activity of REM sleep is quite similar to waking patterns, which reflects a very
active CNS [3]. REM sleep is most notable for being the sleep state where true dreaming occurs, although hypnagogic or dream-like ideation can occur in any sleep stage. Dreaming allows the organism to process daytime experience and transfer information to long-term memory [5, 6]. Children as young as two years of age can report dreams following awakenings from REM sleep [5].

In infants, AS (REM sleep) functions as a mechanism for stimulation and development of the general CNS [30]. Although QS is largely restorative, it is actively involved in synaptic remodeling and information processing as well [18]. This endogenous stimulation may assist in differentiation, maturation, and maintenance of sensory and motor areas as they are not yet involved in externally derived sensory and motor reactions [30]. In essence, in early life REM sleep serves to stimulate the brain in the absence of and in preparation for future visual pattern stimulation and other exogenous stimuli from the postnatal environment [18].

**Characteristics of sleep in young children**

As discussed earlier, there is a progression of sleep consolidation confined primarily to the night with increasing chronological age. Whereas newborns spend half of their sleeping time during the night and half during the day, young children have consolidated all of their sleep time during the night except for daytime napping. Total sleep decreases from 16 hours per day in the newborn period to about 10-12 hours per day (depending on napping) in the preschool period [4]. Children in the United States generally give up napping at three to five years of age. Although there are differences in the absolute amount of time spent in REM versus NREM sleep for two year-old versus five year-old children, napping and total sleep time (which are longer in two year-olds) appear to be responsible for much of the differences, given that the proportions of REM and NREM sleep are similar [14].

Over time there has been a change in the type of procedures used to describe sleep-wake patterns in young children. In the earliest studies, polysomnography (a combination of EEG, EOG, and EMG) was used in a laboratory setting to describe the parameters of the sleep stages, sleep duration, and number and length of awakenings during the night. Although polysomnography is the gold standard for measurement of sleep, this is a less than satisfactory approach to describe sleep in a community-based sample of young children. Parents are reluctant to take a young child out of the home environment over several nights and days (if sleep from napping will be of interest), and sleep may be directly affected by the laboratory setting that results in an artifactual representation of sleep.

Replacement of polysomnography with other methods for characterization of the sleep-wake cycle in the home is now the rule, although ambulatory polysomnography which still requires some instrumentation has made the study of sleep states more feasible in the home setting. Sleep questionnaire, sleep diary, videosomnography, and actigraphy can be used in research that is designed to discriminate sleep and wake time, whereas polysomnography will allow examination of REM and NREM sleep states. Sleep questionnaires may be standardized or developed by individual institutions and/or researchers. These include questions on usual sleep behaviors and may be guided by a time frame (e.g. in the last two weeks) and used in cross-sectional and other study designs. Sleep diaries are completed over a defined time period (often over seven days) to give direct information on that seven-day period regarding bedtimes, wake times, number of awakenings, etc. Both questionnaire and diary are dependent on
subjective parental report with the attendant potential for problems of reliability and validity. Reliability refers to whether the parent could replicate their reporting for a given time point or time period. Validity refers to whether they are obtaining true data at a given time point or time period. Items from sleep diaries tend to be omitted as the time period increases [31], and although parents are accurate reporters of sleep schedule items (e.g. duration of sleep), they underestimate night waking [31, 32]. In videosomnography, time-lapse and real-time video recording is used to characterize night sleep [33]. This method has qualitative aspects that are missing from other objective methods such as actigraphy. The bedtime routine, reunions during the night, and co-sleeping or bedsharing practices (if occurring in the room with video equipment) can all be described from videosomnography. This method can be equipment- and labor-intensive (for rating the night data) and generally precludes capturing nap time. Finally, a small solid-state computerized motion detector, the actigraph, was developed for use in the home [34]. The actigraph can be inserted into a watch-like accessory that children wear during the day and night. This has been a promising approach to sleep measurement given that it is relatively non-intrusive and does not rely on parent report. Because it is motion-sensitive, its weakness is that one is equating lack of movement with sleep, and one cannot characterize sleep stages. Although the sensitivity for detection of sleep is very high, sensitivity with respect to awakenings may be low [35].

Review of studies of sleep parameters in typically developing children

The literature on sleep measurement in typically developing preschoolers is presented in Table 1. Ten of the studies have examined sleep duration throughout the night with some variation between studies (range of 9.1 to 11.3 hours). There were no remarkable differences depending on whether questionnaire, sleep diary, or actigraphy measurement was used. Two [36, 37] of the three European studies had reported the longest night and 24-hour sleep durations, but the third [38] reported one of the shortest sleep durations. Two studies [36, 39] that enrolled only children in full-time child-care had very disparate results. Whereas the Ward et al. study [39] may have been seeing an influence of parenting in reducing night sleep duration because children may have been subject to waking times of the parent’s choosing, the relatively long sleep duration results from Koch et al. [36] do not support this explanation. Of the eight studies recording sleep in 24 hours, total sleep ranged from 10.2 to 12.2 hours. Generally, there was one additional hour gained when comparing 24-hour to night sleep duration where data was available for both. There were no remarkable differences in characteristics between studies nor in the method of sleep measurement.

In the following section on awakenings and sleep onset, there were only a few studies that reported on these measurements. The number of awakenings varied across studies, ranging from 1.6 to 4.3 awakenings per night. The number of awakenings may be underreported in the questionnaire study of Scher et al. [40] since it relies on parent recognition of all awakenings. The heterogeneity in average minutes awake suggests that there may be some methodological differences (e.g. sensitivity settings) in the actigraph algorithms used in the three studies. Finally, the variation in minutes to fall asleep may reflect the intrinsic measurement differences between interview/questionnaire (relying on parent observation of sleep onset) and actigraphy.
Sleep problems and disorders in young children

Consolidation and self-regulation are biopsychosocial processes that contribute to eventual sleep hygiene as the infant ages [41]. Consolidation allows the child to approach sleeping through the night, and self-regulation allows the child to fall asleep without help both at the beginning and during the night. Although there may be perturbations in normative sleep that are only occasional and self-limiting, longitudinal studies have demonstrated that early sleep problems, if they are not addressed immediately, may persist beyond infancy and develop into chronic sleep problems [42, 43].

Earlier versions of the ICD and DSM have classification schemes or nosologies for pediatric sleep disorders of preschool-aged children, but these were primarily reserved for the more serious sleep disorders of: dysomnias such as narcolepsy, obstructive sleep apnea, and primary insomnia; and parasomnias defined as intrusions into sleep that consist of nightmare, sleep terror and sleep walking disorders [3]. In 1981, Richman may have been the first researcher to develop criteria to distinguish children with night waking problems from good sleepers [44]. The DSM-IV published in 1994 [45] did have criteria for disorders of initiation or maintenance of sleep, but few young children met the impairment or severity criteria [46]. The Diagnostic Classification of Mental Health and Developmental Disorders of Infancy and Early Childhood (DC 0-3) also published in 1994 was developed by infant specialists for children from birth to three years of age (Zero to Three, 1994). Both of the above nosologies were not empirically derived from research on the population of interest (infants and young children) and the DC 0-3 did not have quantitative metrics [46]. In 2005, the second edition of the International Classification of Sleep Disorders (ICSD) developed diagnostic criteria for Behavioral Insomnia of Childhood (see Appendix 1). Difficulty falling and staying asleep are largely a function of “sleep-onset association” and “limit-setting” behaviors from a parent and/or child [47]. Sleep-onset association refers to the reliance on specific conditions or associations (e.g. rocking, feeding) related to falling asleep at bedtime and to resettle during the night. Limit-setting refers to difficulties in enforcing bedtime limits (e.g. requests for delay, requiring attention after bedtime). Whereas the ICSD outlines the type of behaviors that would fit under the criteria for sleep-onset association and limit-setting types of insomnia, it does not give any direction regarding quantifying the criteria. For example, “extended” and “delay” do not have explicit numeric values assigned (i.e. lacking a number of minutes for bedtime routine), and “demanding” or “difficulty” have a qualitative character rather than a quantifiable level of severity (i.e. lacking the number of reunions with parent during the night). The terms protodyssomnia or behavioral dysomnias of childhood are used to describe disorders of sleep onset and night waking which are peculiar to toddlers and preschoolers [48]. In an attempt to operationalize and standardize the definition of sleep problems in this group of children, attention has been given to inclusion of parameters related to some combination of frequency, severity, and chronicity [47]. Anders et al. have devised quantitative schema called research diagnostic criteria (RDC) to classify children with a sleep onset subtype and/or night waking subtype of protodyssomnia or behavioral insomnia [46, 49, 50]. The first version of the classification schema quantifies the symptoms by age, duration and frequency, and further defines severity into perturbation, disturbance, or disorder [46]. Perturbations
are part of normal development, disturbances relay a risk component, and disorders are more serious and require intervention to prevent other behavioral symptoms and diagnoses [46]. The criteria do not apply to children less than 12 months of age or to co-sleeping children by family preference, and the nosology does not require parental complaint. This first version of the RDC is a more detailed schema than the more streamlined second version published in 2005 [49], with some important changes with respect to persistence of the problem, stringency of criteria, and duration of night wakings. In the most recent version [50] published in 2008 which is used for the RDC in the dissertation, the use of perturbation and other severity classes has been dropped and the criteria address only children greater than 24 months because of the restricted age range of the study (2-5 years of age).

**Research Diagnostic Criteria for Night Waking and Sleep Onset Insomnias**
(adapted from Goodlin-Jones, 2008 #218)

<table>
<thead>
<tr>
<th>Behavioral Insomnia of Childhood</th>
<th>Criteria (for children older than 24 months; five to seven episodes per week for the week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Night-waking subtype</td>
<td>• ≥ 1 awakening per night totaling ≥ 20 minutes</td>
</tr>
</tbody>
</table>
| Sleep-onset subtype (meets two of three criteria) | • > 20 minutes to fall asleep  
• Parent remains in room for sleep onset  
• > 2 reunions |

**Review of the prevalence of sleep problems in typically developing children**

In the studies examining the prevalence (defined as a prevalence proportion) of sleep problems among preschoolers, there was no unifying definition of a sleep onset or a night waking problem (see Table 2). Although the age range was intended to restrict studies to those enrolling preschool-aged children, the lower age limit was relaxed (down to six months) for several studies that otherwise enrolled through most of the preschool years. Sleep onset problems were variously defined by length of time to fall asleep and difficulty settling, and quantified by number of nights it occurred, and for what duration prior to study entry. The prevalence of a sleep onset problem ranged from 8.0 to 59.7% in similar-aged children and is likely to be reflective of the myriad definitions used. The studies with the lowered age limits, that might have had a higher prevalence of sleep-onset problems given that age is negatively associated with sleep-onset problems, do not account for the variation. In addition, restricting the summary to studies with stricter criteria (i.e. > 30 minutes to fall asleep, multiple criteria to meet) does not explain the variation. Therefore, the subjectivity of parental report, the use of many different definitions of the problem, and different interviews/questionnaires across studies most likely explain the heterogeneity of the results.

Night waking problems were variously defined by number of nights per week awakenings occurred and/or number of times in the night and/or whether the time awake met a specific threshold. The prevalence of a night waking problem ranged from 1.8% to 39%. Only one study used actigraphy to measure sleep [50], and it is believed that night waking will be better measured with this method over questionnaire. This is because many night wakings will not be known to the parent if they do not require the attention of the parent. A combination of sleep diary and actigraphy would be preferred over questionnaire because the parent records the event closer to its occurrence and
the actigraph is more accurate in measuring absolute time. There appeared to be no consistent differences in results by size of study, type of study population, or strictness of definitional criteria.

It is difficult to come to any finite conclusion about the absolute prevalence of sleep disorders in preschool-aged children given the different definitions used across the studies and the lack of variance estimates reported in any of the studies. However, from 29.0% to 81.8% of preschool-aged children were reported in five of the studies to have either a sleep onset or night waking problem. Two other studies used more vague language such as “sleep problem” or “unrefreshing sleep” to calculate a prevalence of sleep problems. One can conclude from the prevalence range above that the proportion of very young children with a sleep onset or night waking problem is likely to be relatively common (at least 29%).

**Autism and sleep**

**Background**

Sleep disturbances among children with autism are considered to be even more problematic and prevalent than those among typically developing children in terms of the consequences. It can be prohibitive for children with autism to be unsupervised during the night if they have bouts of awakening. They may have self-injury behaviors that could be exacerbated by lack of sleep and the stress of being awake and alone during the night. They may also create damage to the household if they should get out of bed to wander without monitoring.

In terms of etiology, sleep problems in autism may be due to behavioral issues similar to the behavioral insomnias of childhood of typically developing children, may be due to neurobiological defects intrinsic to autism, or a combination of the two. I have discussed the issues with behavioral insomnias in a previous section, but there are behavioral issues peculiar to autism. Children with autism may have difficulty synchronizing their sleep/wake rhythm due to the lack of attunement to environmental and social cues. Children with autism, particularly those who are high functioning, may have sleep problems associated with general anxiety and arousal.

Several neurobiological pathways have been proposed to explain how sleep disorders may differ in autism. One hypothesized pathway involves melatonin dysfunction or abnormal synthesis. The pineal gland produces the hormone melatonin which is involved in the regulation of the sleep/wake cycle. It is produced from serotonin and is secreted almost entirely during the night. Tordjman *et al.* examined overnight urinary output for a metabolite of melatonin, and found that the excretion rate was statistically significantly lower in children and adolescents with autism [51]. Kulman *et al.* collected blood at 4-hour intervals for serum melatonin from children with autism and age-matched healthy children, with the children with autism showing a significantly lower mean melatonin primarily during the dark phase of the day [52]. Recently, Melke *et al.* demonstrated a significant decrease in activity of the gene for the last enzyme of melatonin synthesis in concert with a decreased serum melatonin level in the morning for individuals with ASD [53].

Reduction in the amount of sleep has also been suggested to be a consequence of abnormalities in brainstem and serotoninergic pathways [54]. Segawa reported that sleep disorders of autism had been improved by administration of a serotonin precursor called L-5-HTP [55]. Although one of the more reliable findings in about a quarter of
children with autism is an elevated level of serotonin in blood [56], earlier research
found no relationship between brain serotonin and autism [57].

Abnormalities in the EEG and rapid eye movements during sleep in autism may
reflect a disorganization or impairment of subcortical origin [58]. According to the
authors, some of these findings (presence of a frequency band found in REM sleep of
earlier developmental ages) in autism, but not in typically developing children, suggests
a lack of maturation. Tanguay et al. also found immaturity in the development of REM
sleep phasic activity in autism [59]. The mean eye movement (EM) burst length was
longer and the ratio of EMs within bursts to those outside of bursts was substantially
less than in age-matched controls [59]. Thirumalai et al. found a high proportion (5 of
11) of children with autism aged three to nine to have REM sleep behavior disorder
which is characterized by muscles that are no longer atonic during REM sleep [60].
This condition is usually linked with degenerative conditions in the elderly, but the
authors suggest that this lack of tonic control may represent neurological dysfunction in
the brainstem centers of children with autism.

Review of the literature

Although these pathways may be promising areas for further research, there is
no consensus as to why children with autism may have more sleep problems than
typically developing children. In addition, there is no consensus as to which sleep
problems or whether sleep problems are more common in children with autism versus
typically developing children. Most of the studies to date among children with autism
(AUT) have used sleep diaries or questionnaires for sleep measurement, but more
recently a few have used actigraphy. The review for the dissertation excludes adults
and children with ASD (Asperger syndrome, PDDNOS) unless preschool-aged children
were the focus. The results are restricted to healthy or typically developing children as
control or comparison groups (CONT). Results from studies in Table 3 are presented
as means and standard deviations (in parentheses following the means) where possible
and/or prevalence proportion (percent of children with the problem).

Two studies examined the overall prevalence proportion of sleep problems by
sleep diary for 30 days in relatively large study populations of AUT in the community (no
CONT). Taira et al. (N=89) and Hoshino et al. (N=75) reported problems with falling
asleep (26% versus 82%) and early morning arousals/awakenings (12.5% versus 24%),
respectively, to be the most common types of sleep problems in AUT [9, 11]. The study
populations were substantially different, with a comparison of three to five year-olds (an
expected higher prevalence proportion of sleep problems) referred to a medical center
to three to twenty year-olds living in the community. Two other studies used
questionnaires to elicit the prevalence proportion of settling problems and night waking.
For settling problems, Polimeni et al.[61] reported a prevalence proportion of 69.0%
(AUT) versus 72.0% (CONT) and Krakowiak et al. [62] reported a prevalence proportion
of 51.2% (AUT) versus 31.9% (CONT) for settling problems. For night waking,
Polimeni et al. [61] reported a prevalence proportion of 51.0% (AUT) versus 53.0%
(CONT) and Krakowiak et al. [62] reported a prevalence proportion of 9.9% (AUT)
versus 1.8% (CONT). It is of note that Krakowiak et al. [62] had lower prevalence
proportion figures for all measures, and this may be due to what could be construed as
more stringent criteria (use of the term “frequent” to describe the problem).
The hours of total night sleep (including time awake) in AUT ranged from 6.5 to 11.6 hours compared to a range of 6.2 to 10.9 in CONT. One very early and small study of toddlers and preschoolers in a laboratory setting [58] found remarkably short durations of sleep in both AUT and CONT (6.5 versus 6.2 hours) by EEG with great variability around the estimates. Hoshino et al. [9] also reported no difference between the diagnostic groups. The results of the study by Richdale et al. which found a longer duration of sleep for the AUT compared to CONT (11.6 versus 10.9 hours) were paradoxical since low functioning AUT are believed to have more severe brain dysfunction [63]. The remaining studies [50, 61, 64] had internal comparisons that indicated that AUT had a shorter duration of sleep than the respective CONT. Only one of these studies [50] had a statistically significantly decreased duration (30 minutes less) of night sleep between the AUT and the internal CONT group. Two studies that examined hours of total night sleep (excluding time awake) [54, 64] among older school-age children, reported similar night sleep durations for AUT (average of 7.3 hours) and shorter sleep durations for AUT compared to CONT. Only one [54] of these studies had a statistically significant difference between the AUT and CONT internal comparison groups, with over an hour less sleep for the AUT. It is of note that because age is strongly negatively associated with sleep duration, when the review is limited to studies of preschool-aged children, there are longer night sleep durations for both AUT and CONT as expected. Only two studies [50, 62] examined 24-hour sleep duration, and this was among preschool-aged AUT and CONT. Goodlin-Jones et al. [50] found a statistically significantly different, decreased in 24-hour sleep in the AUT (approximately 30 minutes less), and Krakowiak et al. [62] observed an hour less sleep (no variance estimate) in the AUT.

There were only four studies that examined sleep onset (bedtime) times. Two [9, 50] of the studies had slightly later sleep onset times (approximately 15 minutes) for AUT, and one [64] had an earlier sleep onset for AUT (approximately 35 minutes). The only statistically significant difference between AUT and CONT is in the remaining study [63] in which the low functioning AUT had nearly an hour earlier bedtime than the CONT. Two [50, 64] of the three studies that examined sleep offset (rising) times had earlier rising times for the AUT, but with increased variability about the estimates. Only one [64] of these studies had statistically significantly earlier rising times for AUT (51 minutes earlier) versus CONT. The remaining study [9] had a later rising time for AUT (approximately 15 minutes).

The review of studies that examined sleep latency suggests that there may not be a clinically important difference in internal comparisons between AUT and CONT, but only three studies [50, 54, 63] with varying types of sleep measurement were under review. The study [54] with the largest difference (26 minutes longer to fall asleep for AUT) had very small numbers, and the next longest time to fall asleep was 12 minutes in the study by Richdale et al. [63]. No summary can be made about the frequency of night awakenings from the data under review because different time frames were used (awakenings per night versus per week).

Most of the studies from the review that measured night sleep suggest that night sleep duration for children with autism is shorter than for healthy typically developing children. As regards the other sleep parameters and sleep problem prevalence proportions, no firm conclusions can be made from these results. There were also too
few studies with preschool-aged children to make any conclusions specific to very young children.

As happens in many systematic reviews, there was little homogeneity in the type of measures used across studies. Although most of the definitions of the sleep outcomes were uniform, the type of measure (use of parent report versus actigraphy) can affect the accuracy of measurement (e.g. sleep latency if child falls asleep before parent acknowledges). Parent report of sleep problems, as discussed in a previous section, may underestimate some measures of sleep (e.g. frequency of night awakenings if parent is unaware), or may not be able to capture others accurately (e.g. sleep offset times if the child wakes before the parent). There is some support in the literature for a reporting bias between parents of AUT versus parents of CONT. Hering et al. used questionnaire alongside actigraphy for some sleep measurements and found that parents reported AUT with more abnormal sleep parameters on questionnaires than were measured by actigraphy [64]. In Goodlin-Jones et al. parents at intake reported more sleep problems for AUT versus CONT, but actigraph and sleep diary RDC ratings were not associated with intake reports [50]. Parents of AUT also may be more sensitive or alert to sleep disruptions versus parents of CONT. Nearly all of the studies with internal comparison groups used strict diagnostic criteria (DSM III/IV, ADI-R, ADOS). The review is restricted to autism (except for two studies of preschool-aged children with ASD) to reduce any attributions of sleep problems to autism that may be more directly due to the neurobiology of one of the spectrum disorders, such as Asperger syndrome. Individuals with Asperger syndrome may have sleep problems specific to the disorder, including increased stage 1 sleep, the absence of dreaming, and periodic limb movements [61]. The AUT and CONT were volunteers, and volunteers may be more likely to participate because they have the outcome of interest. As is true for many observational studies, subjects may believe that they will receive some help for the problem, even when it is stipulated otherwise by the researchers. This participation bias would tend to exaggerate differences if AUT with sleep problems were more likely to participate, and tend to underestimate differences if CONT with sleep problems were more likely to participate. In addition, families with AUT that have more behavioral problems may be less likely to participate due to the sheer difficulties posed by participation. These AUT also may be more likely to have sleep problems since behavioral problems are associated with sleep problems [42, 65]. Although variance estimates were not provided for any of the prevalence proportions, standard deviations were provided for nearly all of the continuous measures. Increased variability around some of the means (e.g. sleep latency) may be due to small sample sizes in some instances and/or measurement error due to the bluntness of the instruments for the measurement of sleep parameters.
FIGURE 1. SLEEP-WAKE HISTOGRAM FOR YOUNG CHILDREN

Awakenings are common here in NREM 2 and REM sleep

*Modified from [3] with permission from Thomas F. Anders
<table>
<thead>
<tr>
<th>Author, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Type of sleep measure-ment</th>
<th>Age in years</th>
<th>Mean night sleep duration in hours (± sd)</th>
<th>Mean total sleep in 24 hours (± sd)</th>
<th>Mean number of wakings (± sd)</th>
<th>Mean minutes awake (± sd)</th>
<th>Mean minutes to fall asleep (± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoshino, 1984, Japan[9]</td>
<td>Cross-sectional study of healthy children</td>
<td>33</td>
<td>Sleep diary (x 30 days)</td>
<td>3-5</td>
<td>10.5 (0.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Koch, 1984, France[36]</td>
<td>Cross-sectional study of healthy children attending full-time care</td>
<td>107</td>
<td>Sleep diary (x 9 months)</td>
<td>2 ½ to 4 ½</td>
<td>11.1 (0.9)</td>
<td>12.2 (1.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scher, 1995, Israel[40]</td>
<td>Cross-sectional study of healthy children</td>
<td>218</td>
<td>Sleep Questionnaire (usual sleep) [44]</td>
<td>2 - 4</td>
<td>10.0</td>
<td>1.7 (0.8)</td>
<td>25.0 (24.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ottaviano, 1996, Italy[38]</td>
<td>Cross-sectional study of healthy children in attending pediatric offices</td>
<td>769</td>
<td>Short structured interview (usual sleep in last month)</td>
<td>2-4</td>
<td>9.3 (0.9)</td>
<td>10.5 (1.2)</td>
<td>17.0 (9.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lavigne, 1999, United States[66]</td>
<td>Cross-sectional study of healthy children in private pediatric offices</td>
<td>510</td>
<td>Questionnaire (usual sleep)</td>
<td>2 - 5</td>
<td>11.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thorleifsdottir, 2002, Iceland[67]</td>
<td>Longitudinal study of healthy children</td>
<td>~ 160</td>
<td>Sleep diary (x 7 days)</td>
<td>2 - 5</td>
<td>11.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crosby, 2005, United States[69]</td>
<td>Cross-sectional study of healthy children from community sample</td>
<td>783</td>
<td>Questionnaire (with 1-month reference period)</td>
<td>2 - 5</td>
<td>10.2 (approximate from figure)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1 (cont.): Review of studies of selected sleep parameters in typically developing preschool-aged children

<table>
<thead>
<tr>
<th>Author, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Type of sleep measurement</th>
<th>Age in years</th>
<th>Mean night sleep duration in hours (± sd)</th>
<th>Mean total sleep in 24 hours (± sd)</th>
<th>Mean number of wakings (± sd)</th>
<th>Mean minutes awake (± sd)</th>
<th>Mean minutes to fall asleep (± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acebo, 2005, United States[68]</td>
<td>Cross-sectional study of healthy children (excluding children with sleep problems)</td>
<td>169</td>
<td>Actigraphy (x 7 days)</td>
<td>2 - 5</td>
<td>9.9</td>
<td>10.6 (time in bed)</td>
<td>4.3</td>
<td>69.0</td>
<td></td>
</tr>
<tr>
<td>Ward, 2008, United States[39]</td>
<td>Cross-sectional study of healthy children attending full-time care</td>
<td>52</td>
<td>Actigraphy (x 3 days)</td>
<td>3 - 5</td>
<td>9.1 (0.7)</td>
<td>10.2 (0.6)</td>
<td>1.6 (1.0)</td>
<td>39.0 (22.0)</td>
<td></td>
</tr>
<tr>
<td>Iglowstein, 2008, Switzerland[37]</td>
<td>Longitudinal study of healthy children</td>
<td>~ 450</td>
<td>Structured interview (usual sleep in last 3 months)</td>
<td>2 - 5</td>
<td>11.3</td>
<td>12.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Krakowiak, 2008, United States[62]</td>
<td>Cross-sectional study of healthy children from stratified random sample</td>
<td>163</td>
<td>CHARGE Sleep History Questionnaire</td>
<td>2 - 5</td>
<td>12.0 (median)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goodlin-Jones, 2008, United States[50]</td>
<td>Cross-sectional data from longitudinal study of healthy children</td>
<td>69</td>
<td>Actigraphy (x 7 days)</td>
<td>2 - 5</td>
<td>10.4 (0.6)</td>
<td>11.2 (0.7)</td>
<td>3.1 (1.8)</td>
<td>18.0 (12.0)</td>
<td>35.0 (19.0)</td>
</tr>
<tr>
<td>Mindell, 2009, United States[70]</td>
<td>Cross-sectional national representative sample of children</td>
<td>387</td>
<td>Standardized questionnaire</td>
<td>3-6</td>
<td>9.6 (1.5)</td>
<td></td>
<td></td>
<td>17.4 (16.7)</td>
<td></td>
</tr>
<tr>
<td>Author, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Type of sleep measurement</td>
<td>Age in years</td>
<td>Percent with sleep onset problem</td>
<td>Sleep onset definition</td>
<td>Percent with night waking problem</td>
<td>Night waking definition</td>
<td>Percent w either sleep onset or night waking problem</td>
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<tr>
<td>Jenkins, 1980, England [71]</td>
<td>Cross-sectional study of all healthy children living in a North London borough</td>
<td>~172</td>
<td>Questionnaire</td>
<td>2 – 4 ½</td>
<td>8.0</td>
<td>Settling to sleep – difficult most nights</td>
<td>10.5</td>
<td>Night waking (4 or more nights per week)</td>
<td></td>
</tr>
<tr>
<td>Beltramini, 1983, United States [72]</td>
<td>Cross-sectional study of healthy children from longitudinal data in the 1950s</td>
<td>109</td>
<td>Semi-structured interview</td>
<td>2-5</td>
<td>59.7</td>
<td>Requires &gt;30 minutes to fall asleep</td>
<td>27.2</td>
<td>Awakens 1 or more times every night</td>
<td></td>
</tr>
<tr>
<td>Salzarulo, 1983, France [73]</td>
<td>Cross-sectional study of children attending psychiatric or pediatric consultation</td>
<td>84</td>
<td>Questionnaire (lasting ≥ 3 months before interview)</td>
<td>2-5</td>
<td>16.5</td>
<td>Falling asleep disturbance</td>
<td>39.0</td>
<td>Night waking</td>
<td></td>
</tr>
<tr>
<td>Jenkins, 1984, England [74]</td>
<td>See Jenkins, 1980 accumulation of more data</td>
<td>~303</td>
<td>Questionnaire</td>
<td>2 – 4 ½</td>
<td>10.3</td>
<td>Settling to sleep – difficult most nights</td>
<td>12.2</td>
<td>4 or more nights per week</td>
<td></td>
</tr>
<tr>
<td>Author, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Type of sleep measurement</td>
<td>Age in years</td>
<td>Percent with sleep onset problem</td>
<td>Sleep onset definition</td>
<td>Percent with night waking problem</td>
<td>Night waking definition</td>
<td>Percent w either sleep onset or night waking problem</td>
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<tr>
<td>Lozoff, 1985, United States [75]</td>
<td>Cross-sectional study of healthy children attending randomly sampled child clinics</td>
<td>64</td>
<td>Behavior Screening Questionnaire [44]</td>
<td>6 months – 4 years</td>
<td>50.0</td>
<td>Bedtime struggles (3 or more nights each week for preceding month)</td>
<td>7.8</td>
<td>Night waking that involved parents (3 or more nights each week for preceding month)</td>
<td>29.0</td>
</tr>
<tr>
<td>Kataria, 1987, United States [42]</td>
<td>Consecutive cross-sectional sample of healthy children attending pediatric clinics</td>
<td>60</td>
<td>Behavior Screening Questionnaire (symptoms ≥ 1 month)</td>
<td>1 - 4</td>
<td>20.0</td>
<td>Bedtime struggles (3 or more nights each week)</td>
<td>28.0</td>
<td>Night waking (3 or more nights each week)</td>
<td>42.0</td>
</tr>
<tr>
<td>Ottaviano, 1996, Italy [38]</td>
<td>Cross-sectional study of healthy children attending pediatric offices</td>
<td>769</td>
<td>Short structured interview (usual sleep in last month)</td>
<td>2-4</td>
<td>4.7</td>
<td>&gt;30 minutes to fall asleep</td>
<td>22.5</td>
<td>≥ 2 wakings or requiring parents to resettle at least 4 nights per week</td>
<td>81.8</td>
</tr>
<tr>
<td>Gaylor, 2001, United States [46]</td>
<td>Cross-sectional study of healthy children from longitudinal study</td>
<td>33</td>
<td>Modified Sleep Habits Questionnaire (over the past week)</td>
<td>2 – 4.9</td>
<td>48.0</td>
<td>&gt; 20 minutes to fall asleep, and/or parent remains in room for sleep onset, and/or &gt;1 reunion (2-7 episodes per week &gt; 1 month)</td>
<td>33.0</td>
<td>≥ 1 awakening and requiring parental intervention to return to sleep (2-7 episodes per week &gt; 1 month)</td>
<td>81.8</td>
</tr>
<tr>
<td>Author, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Type of sleep measurement</td>
<td>Age in years</td>
<td>Percent with sleep onset problem</td>
<td>Sleep onset definition</td>
<td>Percent with night waking problem</td>
<td>Night waking definition</td>
<td>Percent w either sleep onset or night waking problem</td>
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<tr>
<td>Archbold, 2002, United States[76]</td>
<td>Consecutive cross-sectional sample of healthy children attending pediatric clinics</td>
<td>399</td>
<td>Pediatric Sleep Questionnaire (symptoms currently)</td>
<td>2 – 4.9</td>
<td>≥ 2 symptoms of unrefreshing sleep or difficulty with sleep onset, sleep maintenance, or early morning awakening</td>
<td></td>
<td></td>
<td></td>
<td>20.6</td>
</tr>
<tr>
<td>McGreavey, 2005, Scotland [77]</td>
<td>Cross-sectional study of a random sample of healthy children attending general practice clinics</td>
<td>1023</td>
<td>Tayside Children's Sleep Questionnaire (over previous 3 months)</td>
<td>1 – 5</td>
<td>Score of ≥ 8 on questionnaire representing disorders of initiating sleep</td>
<td></td>
<td>Score of ≥ 8 on questionnaire representing disorders of maintaining sleep</td>
<td></td>
<td>35.0</td>
</tr>
<tr>
<td>Gaylor, 2005, United States[49]</td>
<td>Cross-sectional study of healthy children from longitudinal study</td>
<td>83</td>
<td>Modified Sleep Habits Questionnaire (over the past week)</td>
<td>2 - 4</td>
<td>Meets 2 of 3 criteria: &gt; 20 minutes to fall asleep, parent remains in room for sleep onset, &gt;1 reunion (5-7 episodes per week for at least 1 month)</td>
<td></td>
<td>9.6</td>
<td>≥ 1 awakening per night requiring parental intervention, totaling ≥ 20 minutes (5-7 episodes per week for at least 1 month)</td>
<td></td>
</tr>
<tr>
<td>Author, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Type of sleep measurement</td>
<td>Age in years</td>
<td>Percent with sleep onset problem</td>
<td>Sleep onset definition</td>
<td>Percent with night waking problem</td>
<td>Night waking definition</td>
<td>Percent w either sleep onset or night waking problem</td>
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</tr>
<tr>
<td>Martin, 2007, Australia [79]</td>
<td>Cross-sectional study of healthy children from population-based longitudinal study</td>
<td>4836</td>
<td>Interviewer-administered questionnaire</td>
<td>4-5</td>
<td>Parent reported sleep problem as moderate/large</td>
<td></td>
<td></td>
<td></td>
<td>13.7</td>
</tr>
<tr>
<td>Krakowiak, 2008, United States[62]</td>
<td>Cross-sectional study of healthy children from stratified random sample</td>
<td>163</td>
<td>CHARGE Sleep History Questionnaire</td>
<td>2 - 5</td>
<td>31.9</td>
<td>Any frequent sleep onset problem?</td>
<td>1.8</td>
<td>Any frequent night waking problem?</td>
<td></td>
</tr>
<tr>
<td>Goodlin-Jones, 2008, United States[50]</td>
<td>Cross-sectional study of healthy volunteer children from advertising and clinics</td>
<td>69</td>
<td>Actigraphy and sleep diary (x 7 days)</td>
<td>2 - 5</td>
<td>20.6</td>
<td>Meets 2 of 3 criteria: &gt; 20 minutes to fall asleep or parent remains in room for sleep onset or &gt; 2 reunions (for 5-7 nights per week)</td>
<td>26.1</td>
<td>≥ 1 waking per night totaling ≥ 20 minutes (for 5-7 nights per week)</td>
<td>46.7</td>
</tr>
<tr>
<td>Mindell, 2009, United States[70]</td>
<td>Cross-sectional national representative sample of children</td>
<td>387</td>
<td>Standardized questionnaire</td>
<td>3-6</td>
<td>35.6</td>
<td>At least 1 night waking per night (on typical night in past 2 wks)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Review of studies of sleep measurement between children with autism and preschool-aged children with ASD, and typically developing children

<table>
<thead>
<tr>
<th>Author, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Type of sleep measurement</th>
<th>Ages</th>
<th>Results</th>
<th>Methodological issues</th>
</tr>
</thead>
</table>
| Ornitz, 1969, United States [58] | • Children with autism  
• Normal children | 8 AUT  
6 CONT | EEG | Mean (range)  
3.0 (1.8-3.9)  
AUT  
2.8 (1.6-3.8)  
CONT | Total night sleep (hours)  
AUT mean±sd  
6.5 ± 2.1  
CONT mean±sd  
6.2 ± 1.0 | • Diagnosis of autism by Ornitz and Ritvo  
• No medication use  
• 1-2 nights in laboratory |
| Hoshino, 1984, Japan[9] | • Children with autism referred to Medical Center  
• Typically developing children as controls | 39 AUT  
33 CONT | Sleep diary (x 30 days) | 3-5 AUT  
3-5 CONT | Sleep onset (hr:min)  
8:58± 0:07  
8:44± 0:05  
Sleep offset (hr:min)  
7.05± 0:08  
6.48± 0:08  
Total night sleep (hours)  
10.5 ± 0.1  
10.5 ± 0.1  
Problems with falling asleep  
82%  
Early arousals  
24% | • Diagnosis of autism by WHO standards and Kanner's description  
• Unclear where controls came from  
• Medication use unknown |
| Richdale, 1995, Australia [63] | • Lower functioning (LF) children with autism associated with school for autism  
• Typically developing children as controls | 12 LF AUT  
35 CONT | Sleep diary (x 14 days) | Less than 8 years of age | Sleep latency (min)  
21.0± 12.0  
16.5± 12.0  
Sleep onset (hr:min)  
7.24± 0:27  
8.18± 0:42  
Total night sleep (hours)  
11.1 ± 0.5  
10.8 ± 0.7  
Total sleep (24 hrs)  
11.6 ±0.7  
10.9 ± 0.9 | • Diagnosis of autism by DSM-III or DSM-III-R criteria  
• Good control for potential confounders  
• Medication use similar between groups |
Table 3 (cont.): Review of studies of sleep measurement between children with autism and preschool-aged children with ASD, and typically developing children

<table>
<thead>
<tr>
<th>Author, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Type of sleep measurement</th>
<th>Ages</th>
<th>Results</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Takase, 1998[81]/ Taira, 1998, Japan [11]</td>
<td>• Children with autism living in community • No controls</td>
<td>89 AUT</td>
<td>Sleep diary (x 30 days)</td>
<td>Range 3-20 (only one subject was 20; the others were referred to as &quot;children&quot;)</td>
<td>Takase: AUT tended to have early rising and late retiring compared to the subject's mode or &quot;usual&quot; rising and retiring times (40% of subjects with CV ≥ 10%) Taira: Difficulty of falling asleep 26.1% Awakening during sleep time 21.6%</td>
<td>• No details regarding diagnosis of autism • No controls • Comparison to usual rising and retiring times problematic</td>
</tr>
<tr>
<td>Hering, 1999, Israel[64]</td>
<td>• Children with infantile autism randomly selected from special treatment center • Healthy children as controls</td>
<td>8 AUT with sleep problems 8 CONT without sleep problems</td>
<td>Actigraphy (x 3 days)</td>
<td>Mean ± sd 8.0 ± 3.0 AUT 8.0 ± 2.3 CONT</td>
<td>AUT CONT mean±sd mean±sd Sleep onset (hr:min) 10:35± 01:01 11:16± 1:14 Sleep offset (hr:min) 6:37± 00:52 7:28± 00:42 Total night sleep (hours) 8.0 ± 0.9 8.2 ± 1.1 Total night sleep minus time awake (hours) 7.1 ± 0.8 7.2 ± 1.3</td>
<td>• Diagnosis of autism by DSM-IV criteria • One child on carbamazapine medication • Good control for potential confounders by matching on age and sex</td>
</tr>
<tr>
<td>Elia, 2000, Italy[54]</td>
<td>• Children with autism • Healthy children as controls</td>
<td>13 AUT 5 CONT All males</td>
<td>Polysomno-gram (EOG, EEG, EMG) (x 2 days)</td>
<td>Mean (range) 8.7 (5-12) AUT 9.0 (7-11) CONT</td>
<td>AUT CONT mean±sd mean±sd Sleep latency (min) 36.0± 30.7 10.9± 7.4 Number of night Awakenings/night 0.53± 0.36 0.43± 0.35 Total night sleep minus time awake (hours) 7.5 ± 0.8 8.7 ± 0.4</td>
<td>• Diagnosis of autism by DSM-IV criteria and Childhood Autism Rating Scale • Small numbers • Laboratory setting</td>
</tr>
</tbody>
</table>
Table 3 (cont.): Review of studies of sleep measurement between children with autism and preschool-aged children with ASD, and typically developing children

<table>
<thead>
<tr>
<th>Author, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Type of sleep measurement</th>
<th>Ages</th>
<th>Results</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honomichl, 2002, United States [13]</td>
<td>• Children with autism (including PDD) living in community (ASD) • No controls</td>
<td>45 ASD</td>
<td>Sleep diary (x 4 weeks) a break, then sleep diary (x 2 weeks)</td>
<td>Mean ± sd 4.1 ± 0.9 Range 2-5</td>
<td>ASD mean±sd 25.5</td>
<td>• Diagnosis of autism by specialists • No controls • No mention of medication use</td>
</tr>
<tr>
<td>Polimeni, 2005, Australia [61]</td>
<td>• Children with autism recruited from special needs associations • Typically developing children recruited from advertisement</td>
<td>53 AUT 66 CONT</td>
<td>Behavioral Evaluation of Disorders of Sleep (BEDS) Questionnaire</td>
<td>6.5 ± 2.7 6.0 ± 3.1</td>
<td>AUT mean±sd 51.0 53.0</td>
<td>• Diagnosis reported by parent • Medication use was assessed and age was adjusted in analyses</td>
</tr>
<tr>
<td>Krakowiak, 2008, United States [62]</td>
<td>• Children with autism spectrum disorder (ASD) from Regional Centers • Typically developing children from stratified random sample from birth files</td>
<td>303 ASD 163 CONT</td>
<td>CHARGE Sleep History Questionnaire</td>
<td>3.7 3.3</td>
<td>ASD mean±sd 52.5 11.0</td>
<td>• Population-based sample • Diagnosis by ADI-R and ADOS • Narrow age range • Assessment of potential confounding</td>
</tr>
<tr>
<td>Author, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Type of sleep measurement</td>
<td>Ages</td>
<td>Results</td>
<td>Methodological issues</td>
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</tr>
<tr>
<td>Goodlin-Jones, 2008, United States[50]</td>
<td>• Children with autism from Regional Centers and clinics • Typically developing children recruited from advertisement and clinics</td>
<td>68 AUT 69 CONT</td>
<td>Actigraphy (x 7 days)</td>
<td>Mean ± sd 3.9 ± 0.9 AUT 3.4 ± 0.9 CONT</td>
<td>AUT CONT</td>
<td>• Diagnosis by ADI-R and ADOS • Narrow age range • Gender, maternal sociodemographics, and age were assessed and PEP-R developmental score was adjusted in analyses</td>
</tr>
</tbody>
</table>

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<tr>
<th></th>
<th></th>
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<th></th>
<th><strong>Mean ± sd</strong></th>
<th><strong>mean±sd</strong></th>
<th><strong>mean±sd</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Sleep latency (min)</strong></td>
<td>39.0 ± 28</td>
<td>35.0 ± 19</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Sleep onset (hr:min)</strong></td>
<td>9:36± 01:13</td>
<td>9:19± 0:53</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Sleep offset (hr:min)</strong></td>
<td>6:58± 00:57</td>
<td>7:11± 00:40</td>
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<tr>
<td></td>
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<td></td>
<td><strong>Total sleep (24 hrs)</strong></td>
<td>10.6 ± 0.8</td>
<td>11.2 ± 0.7</td>
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<tr>
<td></td>
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<td></td>
<td><strong>Total night sleep (hours)</strong></td>
<td>9.9 ± 0.9</td>
<td>10.4 ± 0.6</td>
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<tr>
<td></td>
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<td></td>
<td><strong>Average length of awakening (min)</strong></td>
<td>18 ± 16</td>
<td>18 ± 12</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Number of night awakenings/week</strong></td>
<td>2.5 ± 1.7</td>
<td>3.1 ± 1.8</td>
<td></td>
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</table>
Appendix 1. Diagnostic Criteria of Behavioral Insomnia of Childhood [82]

A. A child’s symptoms meet the criteria for insomnia based upon reports of parents or other adult caregivers.

B. The child shows a pattern consistent with either the sleep-onset association type or limit-setting type of insomnia described below:
   i. Sleep-onset association type includes each of the following:
      1. Falling asleep is an extended process that requires special conditions.
      2. Sleep-onset associations are highly problematic or demanding.
      3. In the absence of the associated conditions, sleep onset is significantly delayed or sleep is otherwise disrupted.
      4. Nighttime awakenings require caregiver intervention for the child to return to sleep.
   ii. Limit-setting type includes each of the following:
      1. The individual has difficulty initiating or maintaining sleep.
      2. The individual stalls or refuses to go to bed at an appropriate time or refuses to return to bed following a nighttime awakening.
      3. The caregiver demonstrates insufficient or inappropriate limit setting to establish appropriate sleeping behavior in the child.

C. The sleep disturbance is not better explained by another sleep disorder, medical or neurological disorder, mental disorder, or medication use.
References


CHAPTER 5: SLEEP AND CORTISOL IN TYPICALLY DEVELOPING INDIVIDUALS

Introduction

In this chapter, the interrelationship between sleep and cortisol will be discussed—both what is known physiologically and through the epidemiological literature. There is a natural bidirectional relationship between circadian measures, such that cortisol and its effect on sleep would be as legitimate a research question as sleep and its effect on cortisol. However, addressing the problems of sleep in young children is a more feasible approach to potentially resolving cortisol dysregulation. Whereas the use of medications to regulate or modify cortisol secretion is not an approach that would be embraced for young children, behavioral interventions to improve sleep quality have been acceptable and successful. For the purposes of the dissertation, sleep and the effect on cortisol is the research question under consideration and thus the literature review applies only to this temporal ordering.

Cortisol concentrations reach a diurnal minimum during the early part of nighttime sleep dominated by slow wave NREM sleep. Limbic-hippocampal structures appear to inhibit HPA activity during early sleep, that sleep which is essential for facilitating memory formation [1]. An increasing concentration of cortisol is secreted during the latter part of nighttime sleep when there is a decreasing amount of NREM sleep and when REM sleep predominates [2, 3]. A large proportion of total daily cortisol output occurs during the early morning hours between three and nine in the morning [2-4].

It has long been held that sleep serves a recovery function from the stress of being awake [3]. The inhibiting influence of sleep on the stimulation of cortisol is largely restricted to the beginning and early part of the sleep cycle (NREM sleep) [5]. This association between sleep start and cortisol secretion has led to the hypothesis that cortisol secretion is suppressed by sleep, particularly slow wave sleep [6], and this suppression is initiated with sleep onset and NREM sleep [7]. The trigger for the cortisol rise in the later hours of sleep is believed to be the demise of NREM sleep epochs, with the secretion of cortisol mainly during the NREM epochs of the latter part of the night [3, 8]. It may also be that frequent wakings which are more common during the latter part of the night are part of the stimulus to the L-HPA to secrete cortisol. These frequent wakings culminate in the final waking for the day with the accompanying peak in cortisol secretion soon after final waking [8, 9].

The effects of sleep on cortisol secretion are somewhat independent of the endogenous circadian rhythm of cortisol [3, 5], although the circadian cortisol pattern is relatively stable [10]. Studies of jet lag and shift work (night shift) indicate that there are exogenous influences that can affect circadian functioning, and transient chronobiological disturbances can result [11, 12]. For example, in night workers, instead of a quiescent period of cortisol secretion during sleep, a higher cortisol coincided during the sleep (in the daytime) [12].

In the review to follow, I will start with what is known following experimental periods of sleep manipulation. A typical experimental protocol involves a baseline period without manipulation, sleep deprivation in varying amounts, and finally a sleep recovery period. Observational studies that define poor sleep quality by sleep quantitation and/or enroll adults with some form of insomnia will follow. Finally,
observational studies of children with varying sleep parameters are examined with respect to cortisol.

**Sleep manipulation studies and nocturnal cortisol**

**Sleep deprivation night**

All of the studies reviewed in this section enrolled a small number of healthy adult volunteers, measured sleep with polysomnography, and sampled plasma cortisol from an indwelling catheter or similar apparatus approximately 10-30 minutes apart unless otherwise specified (see Table 1).

There have been three studies that have examined intermittent sleep deprivation (SD) during the night and observed reduced cortisol secretion over the same period [13, 14], or no differences in average cortisol across the night [15]. Spath-Schwalbe, *et al.* hypothesize that during the night, sleep attenuates negative feedback inhibition of the HPA axis, whereas in wakefulness, the HPA axis becomes more sensitive to negative feedback inhibition [15]. These results suggest that poor sleep quality may affect negative feedback of adrenocortical secretion and inhibit cortisol production and/or release at least during the night.

However, three other studies used plasma cortisol sampled every 10-60 minutes during the night and observed higher cortisol levels during an entire night of SD [16-18]. Both von Treuer and Chapotot observed statistically significant increases on the SD night by cortisol area under the curve (p<.005) [16] and levels in the early morning hours (p<.05) [18].

In a study of 24 hours SD in subjects with psychophysiological insomnia (also called primary insomnia) that were age-and gender-matched to healthy controls, cortisol levels were approximately 15-20% higher across the night in the insomnia group, but the levels were not statistically significantly different from the control group [19]. Davidson, *et al.* in a study with an extended period of total SD (40 hours) observed cortisol levels from 2 to 4 am that were markedly higher, although not statistically significantly higher than the baseline night [20]. Davidson, *et al.* reported that the nocturnal cortisol rise was one hour earlier in SD than a baseline night of normal sleep [20]. The authors suggest that the advance could reflect less inhibition of cortisol secretion at the close of an extended period of time awake during the night.

In summary, although there was a trend toward lower cortisol levels in studies of partial SD, there were higher levels in most studies with entire nights of SD, although results differed in the two studies with amounts of SD beyond an entire night. The higher cortisol may reflect arousal states that accompany being awake which alert an organism into a higher state of excitation and trigger a compensatory mechanism such as cortisol secretion [16, 21]. In the laboratory controlled settings of the SD studies there wasn’t strict attention to staying in bed during the SD night hours. Subjects were asked to undergo cognitive testing as part of a protocol, or were allowed to play cards, board games, undertake “recreation”, and even drink coffee and smoke tobacco in some studies. Only one study maintained subjects in a semirecumbent position under constant supervision during the SD period [19]. It may be argued that the activities associated with being awake (moving around, eating, etc.) may be responsible for the higher cortisol levels. In addition, the stress of sleeping in an unfamiliar environment and/or the physical intrusion of an indwelling catheter may act as stressors. Therefore, confounding from these nighttime activities and the laboratory setting is a concern.
**Sleep recovery night**

Several studies examined cortisol levels on a night or period of recovery sleep to determine whether there are differences between the baseline and recovery night(s) (see Table 2). Both the baseline and recovery periods took place in the laboratory ensuring comparability of setting. In four of the studies that had at least an entire night of SD, plasma cortisol levels in the sleep recovery [22] period were not different from the baseline night [16, 17, 20, 23]. However, two studies observed cortisol levels that were lower in the latter half of the SR night following partial SD compared to the latter half of the baseline night [14], or lower at nearly every time point across the entire night of SD [6]. Davidson, et al. also reported that the nocturnal cortisol rise was one hour later in SR than a baseline night of normal sleep [20]. The authors suggest the delayed rise in SR could suggest a stronger inhibition of cortisol secretion with a more intense sleep period. In studies of sleep deprivation, there is some rebound sleep that occurs on the night(s) of sleep recovery. The most consistent finding is that in recovery sleep there is an increase in slow-wave sleep. Several studies have observed that deep slow-wave sleep was highest during a partial sleep deprivation period of six nights [24] and on sleep recovery nights [6, 25] that suggests an increased pressure for slow-wave sleep. Given that there is an increase in rebound NREM sleep on SR nights, cortisol secretion may be expected to be suppressed resulting in lower cortisol levels, but it is also possible that the rebound is not sufficient to alter secretion compared to what is typical in a baseline night.

**Sleep manipulation studies and daytime cortisol**

Studies of SD with measurements of cortisol on the days following SD or SR nights have mixed results and varying degrees of SD manipulation (see Table 3). Three studies allowed partial SD with approximately four hours of sleep allowed during the night and observed statistically significant increases in plasma cortisol in the afternoon and/or evenings following SD compared to the baseline afternoon/evening [24, 26, 27]. Notably, this increase in evening cortisol levels was also substantially higher than the evening following a SR period of several nights [24, 27]. Speigel, et al. instituted three different sleep manipulation periods—four hours of sleep, eight hours of sleep, and twelve hours of sleep in their second study (data not in table) [27]. The authors observed mean cortisol levels that were statistically significantly higher in the late afternoon/evenings following the four-hour sleep period compared to the eight- and twelve-hour sleep periods.

In three of four studies that employed an entire night of SD there were similar cortisol levels across the day following SD and compared to baseline [6, 17, 28]. However, Chapotot, et al. observed statistically significantly higher levels of cortisol at nearly every time point during the day compared to baseline [18].

Several studies with increasing periods of SD have observed: higher levels in the late afternoon (1700), at bedtime (2300), and at waking following 26 hours of SD [19]; no change in cortisol circadian rhythm after 40 hours of SD [29]; no differences at 0800 following baseline, two different sessions of 24 and 48 hours of SD, and SR [30]; lower plasma cortisol at end (0800 hours) of 48 hours of SD [31]; and a slight decrease in cortisol in 24 hour urine after 72 hours SD [32].

This SD review section is the most relevant to the dissertation research question because it examines daytime cortisol secretion. Daytime cortisol secretion is important
because of the many tasks and challenges that children undertake during the day. Children need to have enough cortisol available for executive functioning and self-regulation of emotions [33] but not an excess that could contribute to negative externalizing or aggressive behaviors [34].

Unfortunately, there is no consensus on the effects of SD and daytime cortisol from the studies under review. The SD periods were highly variable, and contrary to what might be expected when ordering them by increasing duration, no consistent results were observed with increasing duration of SD. If anything, the results of lowered cortisol or no change in cortisol suggest that a compensatory mechanism may commence after extended SD to attempt to re-regulate cortisol secretion during the day, although this would be highly speculative. The study designs were dissimilar in terms of how the laboratory environment was maintained, which recreational activities were allowed, and some studies did not give any description of what transpired during SD hours. Only Leproult, et al. [26] enrolled a moderate number of subjects (N=33), leading to unstable estimates at best in the remaining studies of 12 or fewer subjects.

**Sleep manipulation studies in older children and teens (no cortisol)**

No studies have measured cortisol levels following SD in this age group. However, there have been five studies with varying sleep restrictions and behavioral outcomes measured. Sadeh, et al. asked children to restrict their sleep by an hour over three nights, with reduced alertness and effects on neurobehavioral functioning following the SD [35]. Fallone, et al. used a crossover design to study a comparison of self-selected or baseline sleep, approximately two hours of SD, and an optimized sleep schedule of no fewer than 10 hours of time in bed [36]. The authors reported that restricting sleep led to teacher ratings (blinded to sleep assignment) of increased academic problems and severity of attention problems relative to baseline. More extended SD of approximately four hours on a single night was associated with increased sleepiness and inattentive behaviors [37] and impairments in verbal creativity and abstract thinking [38]. Only one study has examined sleep stages following SD. Jenni, et al. have noted similar (to adults) increases in slow-wave sleep in teens following 36 hours of wakefulness [25]. They also reported that the increased sleep pressure during waking is slower in post-pubertal children as compared to pubertal or pre-pubertal children suggesting sleep maturational changes in adolescence.

It is unknown whether behaviors such as attention and impairments in cognition in children can be reliable and valid proxies for cortisol. Although there are findings of positive correlations between internalizing and externalizing behaviors and cortisol [34], less is known about more mild problems with cognitive function and behavior. Miller, et al. found that teacher ratings of overall learning behaviors in preschool-aged children were positively correlated with greater cortisol reactivity [39]. In another study of preschool-aged children, there was a positive association between cortisol reactivity and executive function [33]. A new avenue of research is needed to determine whether experimental sleep disruption or deprivation is associated with cortisol during the day in children. Importantly, studies that are sensitive to this study population will need to be carefully designed. In addition, an examination of sleep and cortisol concurrent with the assessment of cognitive and behavioral outcomes will be important to pursue.
Summary of sleep deprivation studies

None of the studies in adults measuring cortisol levels observed any difference in the basic cortisol circadian rhythm or pattern between the SD and baseline or SR nights. Circadian rhythmicity is preserved in these circumstances of short-term SD and doesn’t appear to be altered such as has been found in sleep-wake reversal studies [40]. SD studies always involve short-term manipulations and force an artificial sleep deficit that may not represent the natural occurrence of awakenings and insomnia in the general population. It is inexplicable why SD studies almost always enrolled only males, given that there may be differences between males and females in terms of the consequences of sleep. The two studies that did enroll females did not examine gender differences in the results. However, women with short sleep duration (less than five hours) were at higher risk of hypertension than women with longer sleep duration, but there was no risk for hypertension in males with this sleep classification [41]. Many studies took place in a sleep laboratory rather than the home and involved the placement of indwelling catheters and other procedures that might be experienced as physical stressors by the subject. The laboratory studies also provided stimuli during the night waking hours in an effort to maintain the SD. This may give an artifactual representation of the waking hours that would normally occur during the night in an unselected population, and more importantly alter the cortisol secretion so that a generalization to nights in the home with varying states of sleeplessness cannot be made. The lack of consensus in results may be due to the imprecision of the effect sizes due to small numbers (nearly all of the studies enrolled subjects in the single digits). It is of note that sleep deprivation studies are generally comprised of adults given the ethical issues of forced sleep deprivation on children. However, children are not small adults and the factors involved in sleep deficits and the physiological responses in adults are not likely to be the same in children. In fact, in the studies of children, only very limited SD was introduced and there were no physiological measurements. Indwelling catheters or serial plasma sampling are likely to be rejected by parents of young children. Therefore, observational studies of individuals following natural sleep episodes over time should prove more relevant to the research question under study.

Observational studies of sleep and cortisol in adults

The studies in this section either enrolled subjects with poor sleep quality defined by subjective and objective methods or subjects with primary or chronic insomnia (see Table 4). The studies of cortisol in urine examined the cortisol over 24 hours [42], or in pooled periods to get some breakdown of the time [43, 44], with all studies observing positive associations between measurements of poor sleep quality and cortisol. In the studies with pooling, the poor sleepers excreted more cortisol over the day and night compared to good sleepers. In Vgontzas, et al. there was higher cortisol in 24 hour urine for those subjects with higher total wake time [42].

In the studies with subjects who had a classification of short sleep duration, plasma cortisol was collected overnight [45, 46] or only during waking hours [42]. Nocturnal cortisol was higher for subjects with short sleep duration compared to those with long sleep duration [45, 46]. In addition, Vgontzas, et al. observed higher levels in the afternoon [46], although Spath-Schwalbe, et al. observed similar levels in the
morning hours [45]. In the earlier paper by Vgontzas, et al. there was no correlation between sleep and plasma cortisol during waking hours [42].

In the studies with strictly defined insomnia, all studies examined nocturnal plasma cortisol and, in addition, two studies investigated partial day/evening cortisol. Rodenbeck, et al. observed statistically significantly higher levels of cortisol in the evening and night [47]. Two other studies [48, 49], found no statistically significant differences during the night, although Backhaus, et al. [48] reported a crossover during the night, with lower cortisol levels in the first half, and higher levels in the second half of the night. In the two studies that examined night waking, Rodenbeck, et al. observed a positive association with number of wakings and evening cortisol, but Backhaus, et al. observed a statistically significant negative correlation between night waking and first morning cortisol [50]. Varkevisser, et al. studied a mixture of insomniacs from different clinic settings and found no differences in evening levels by groups that assessed themselves as having good, moderate, or poor sleep compared to controls [51]. This study may have been biased by the use of sleep and antidepressant medications in the insomniac groups (but not the control group) by masking the underlying sleep status, although the effects of this would be unpredictable.

Overall, the results were consistent in suggesting that there is more cortisol secreted in urine during the day and night and across studies of subjects with poor sleep quality. However, among the remaining studies of subjects with insomnia, the results were mixed with respect to insomnia and cortisol levels during the day and night, and the association between frequent wakings and cortisol. Although the studies had relatively small numbers of subjects, nearly all maintained good methodological quality to remove potential confounding effects. Nearly all of the studies had some matching criteria (most notably for age) to adjust for potential confounding. Importantly, subjects that used antidepressants, hypnotics, and other psychotropic medications, or had psychiatric co-morbidity (e.g. depression) that might affect cortisol secretion were largely excluded from the study populations. All of the studies comparing subjects with insomnia to controls used DSM-IV or ICSD diagnostic criteria to define insomnia, except for one. Studies enrolling subjects with insomnia and controls tended to have older study subjects than studies using less well-defined criteria, such as “poor sleepers”. It is not surprising that studies with well-defined insomnia have older populations since chronic insomnia is associated with age.

**Observational studies of sleep and cortisol in children and teens**

Some of the studies reviewed below were not designed to look at sleep as a predictor of cortisol as the primary hypothesis, but were adjunct or post-hoc examinations (see Table 5). Studies of naptime sleep alone are not included in the review. The relationship between adrenocortical and sleep-wake regulation has neither been studied extensively in infancy nor in childhood. Only since 2005 does there appear to be a more direct interest in the association between poor quality sleep and daytime secretion of cortisol in children.

Beginning with the studies of infants, two-month-old infants with colic on average slept two hours per day less than a comparably aged control group, and had both a dampened cortisol peak at wake-up and rhythm throughout the day [52]. Among healthy three-month-old infants, those infants who slept less than six hours continuously had lower peak morning cortisol levels versus those infants with more consolidated
sleep (≥ 6 hours continuously) [53]. For three- to seven-month-old infants studied longitudinally, a lower cortisol concentration resulted when a lower amount of sleep occurred in the preceding 4-hour period before sample collection [54]. In a sample that included toddlers as well as infants, Silva, et al. reported that fewer hours of sleep were statistically significantly correlated with higher morning cortisol [55].

Three studies examined sleep and cortisol in ways that are not directly relevant to the dissertation research question, because they examined nocturnal cortisol and cortisol reactivity. Lucas-Thompson, et al. examined pre- and post-inoculation changes in cortisol and observed that waking infants had greater increases in cortisol following inoculation than the non-waking infants [56]. In a study of sleep and nocturnal cortisol, Dahl, et al. observed that lower sleep maintenance (total sleep period spent asleep) was associated with higher nocturnal cortisol in school-aged children aged 6-13, but the authors did not look at waking or diurnal rhythm the following day [57]. Capaldi, et al. studied sleep and cortisol reactivity following three performance stressors in teens, and observed that poor sleep quality was associated with lower cortisol reactivity [58].

Finally, six studies reported on daytime cortisol values following night-time sleep measurements by actigraphy or sleep EEG. Scher, et al. examined sleep efficiency in toddlers attending daycare and observed higher waking cortisol levels for toddlers with low sleep efficiency compared to toddlers with high sleep efficiency [59]. Ward reported from dissertation research that nighttime sleep in preschoolers attending full-time daycare was not associated with morning or afternoon cortisol measurements (no data presented) [60]. Badanes, et al. also reported no association between nighttime sleep and cortisol patterning (no data presented) in a sample of predominantly preschool-aged children [61]. Hatzinger, et al. observed higher mean cortisol in the morning samples and in the mean of the area under the curve (AUC) in poor sleepers compared to good sleepers [62]. The authors also found a strong positive correlation between night wakings and the AUC stress basal values. In an earlier analysis, the authors also reported that children with severe sleep disturbances (no definition given) had higher morning cortisol levels compared to good sleepers [63]. El-Sheikh, et al. examined night sleep and a single afternoon cortisol and reported that poor sleep quality (lower total sleep time, lower sleep efficiency, and more minutes awake) was associated with higher afternoon cortisol [64].

In summary, the studies in infancy suggest that the consequences of shortened duration of and/or fragmented sleep result in hyposecretion, rather than hypersecretion that more commonly results as a physiologic response to stress. Alterations that lead to a deficiency in cortisol concentrations during the latter part of sleep, may involve decreased adrenocortical secretion, decreased adrenocortical reactivity, or increased negative feedback inhibition of the HPA axis [65]. The findings of studies on infants are disadvantaged by the uncertainties of circadian maturation. Most studies of cortisol circadian rhythm have been consistent in their reports that the rhythm is likely to be established no earlier than three months of age [54, 66-68]. In addition, between two and three months of age there is a consolidation of day-night organization of sleep [4, 69]. Therefore, a cause and effect relationship between nighttime sleep and cortisol is difficult to substantiate in early infancy. In addition, the use of parent report for sleep measurements was a limitation for all of the studies in infancy.
All of the studies in children of toddler-age and beyond, except one [58], used sleep EEG or actigraphy for sleep measurement. Actigraphy has now been developed to the point of providing wrist- or ankle-watch-sized equipment and algorithm programs for infants and ages beyond, so that the use of objective measurement of sleep should be the norm in sleep research in young children. Four of the studies that were closest in design to the dissertation (nighttime sleep and secretion of cortisol during the day) observed results that were all in the same direction – poor sleep quality and increased cortisol during the day. However, two other studies reported that there was no relationship between nighttime sleep and daytime cortisol but they did not report any data to support their thesis. It is of note that there were varying definitions of sleep quality in the studies in children and varying times and numbers of days of saliva collection for cortisol. The studies under review do not lead to a firm conclusion on the question of whether nighttime sleep is associated with daytime cortisol secretion.

**Overall summary of sleep and cortisol studies**

The results in older children are more akin to the results of the studies in adults with insomnia and/or poor sleep quality. However, the reasons for insomnia, and the consequences of insomnia, in adults may be behaviorally and physiologically different from children. It is difficult to disentangle the temporal ordering of the association between sleep and cortisol. That is, the cycling of the effects of sleep on cortisol versus the effects of cortisol on sleep are bound together. Studies of SD might offer the best opportunity to examine a causal effect of nighttime sleep on the following day of cortisol secretion. In general, the highest quality study populations for these studies are young adults unselected on sleep history to avoid any medical or sleep problems that might impede simple interpretation of the results. An amount of SD should be instituted that would produce a clinically important change in cortisol; however, there isn’t a consensus on the amount of SD to study - the most important parameters of timing and duration are unknown. For example, the amount of SD instituted ranged: from four hours to 72 hours; from intermittent during a single night to total SD over several days; and from interruptions during REM to interruptions during NREM sleep stages. Results were inconsistent across studies with similar designs and with ordering of SD duration. SD is also not a good representation of sleep in children, who generally do not have whole nights or long continuous periods of sleeplessness. As noted earlier, however, SD studies as currently implemented in adults are not a viable study design to use with young children, so future results from SD studies in young children are not likely.

The observational studies of sleep and cortisol in adults are also problematic when attempting to infer results to young children. The studies of younger adults, while unselected for insomnia, did enroll some subjects who complained of sleep problems and whom may have some of the underlying personality traits of subjects with insomnia. These studies were chronologically older than the studies with subjects with insomnia and used urine collection for cortisol which is not a state-of-the-art method for cortisol measurement today. Most of the remaining studies of insomnia and cortisol undertook plasma or salivary sampling schemes to assure frequent collections over the period of interest. The average age of the study subjects was approximately 40 years of age and the studies excluded subjects with concurrent medical problems that could conceivably interfere with sleep-wake and/or cortisol regulation. However, the DSM-IV diagnosis of primary insomnia is based on subjective reporting of sleep disturbance that causes
difficulty in a significant area of life (e.g. school, social, work life) [70]. The studies of subjects with insomnia used polysomnography not to define the current sleep problem, but to rule out disorders such as sleep apnea and restless leg syndrome.

**Discussion**

The evolving view of most primary insomnia is that it is fueled by the mutually reinforcing factors of tension-anxiety and negative conditioning [71]. The tension-anxiety is often somaticized into other bodily complaints which may cause sleeplessness in and of themselves. The negative conditioning factors may be reinforcers, such as the sleep environment or bedtime ritual that is associated with the sleeplessness, resulting in frustration and arousal. The ability to fall asleep or maintain sleep may be determined by this physiological arousal [72]. A vicious cycle of insomnia can be perpetuated long after the initial stressor has been discontinued [47, 71]. This residual tension-anxiety experienced by the subject with insomnia could contribute to chronically elevated physiological arousal [47]. The arousal that may have been initially caused by transient factors could become chronic with altered L-HPA axis function as a result.

These conditions while explanatory for adults may not have relevance for young children. For example, there are developmental differences in the maturity of cognitive skills, suggesting that cognitive arousal in adults is different than in children [73]. Although children may have anxiety and personality traits that could result in the arousal conditions above, there are other behaviors more salient to children that need to be considered. It is more likely that the stress of peer relationships, evening activities, electronic media, such as gaming and television, and bedtime habits and other sleep hygiene behaviors would cause arousal. Factors occurring during the day related to school/daycare, peers, and family may be stressors influencing sleep at night. This could include worrying, friendship tensions, and discord in family relationships. Participating in evening activities, particularly sports, is associated with higher bedtime cortisol levels in boys [74]. The use of electronic media has absorbed time that might be used for other solitary recreational pursuits, such as reading or imaginary play. Watching video games and television can be especially arousing for children and are related to later bedtimes, earlier waking times, and less time in bed [75]. Inappropriate sleep onset associations and limit-setting sleep disorders are behavioral aspects or learned behaviors of relevance to arousal [76]. Lack of a strict bedtime and/or routine does not give children a clear signal to enhance settling for sleep. Bedtime struggles between parents and children have known consequences of poor sleep quality. This reduces the probability of settling at an appropriate bedtime to gain sufficient sleep and may maintain the arousal into the sleep hours to cause frequent wakings and fragmented sleep. Naptimes can be a time of struggle if the child is resistant to napping or a time of rest with the additional sleep or stillness and minimal contact with others [77]. This constellation of factors could be important for both children with autism and typically developing children. However, in the case of children with autism, the stress of social relationships may be magnified, with a resulting chronicity that might be lacking in typically developing children.

It is of note that the study of the effects of sleep on cortisol (at least in adults) considers arousal the major causal factor affecting cortisol secretion with sleep disruption as a byproduct or secondary effect. However, there is little research on pre-
sleep arousal and sleep problems in children. Nicassio, et al. developed the Pre-sleep Arousal Scale (PSAS) to describe states of arousal as somatic or physiological arousal (e.g. cold feeling in hands, stomach upset) or cognitive or psychological arousal (e.g. worrying, ruminant thoughts) in adults [78]. Recently, Gregory, et al. used a modified version of the PSAS in children eight to ten years of age to determine whether cognitive and/or somatic components were associated with sleep disturbances [73]. The authors observed that cognitive pre-sleep arousal was associated to a greater extent than somatic pre-sleep arousal with sleep disturbance in children. Further investigation into what specific features of cognitive arousal are predictive in children is warranted.

In addition to arousal issues, there are circadian chronotypes that are characterized by later bedtimes (“owls” or eveningness) on the one hand and early morning waking (“larks” or morningness) on the other. The owls have difficulty falling asleep early in the night and sleep later in the morning, whereas the larks are early to bed for an earlier morning wake time [79]. In a study of the effect of morningness/eveningness on sleep in adults, Taillard, et al. reported that eveningness was associated with a greater need for sleep and more irregular sleep-wake habits than morningness [80]. These resulting sleep behaviors may contribute to cortisol changes due to their circadian nature.

**Future directions**

There are three areas that could be improved in future research: 1) using objective sleep measures to obtain a comprehensive measure of all aspects of nighttime sleep; 2) quantifying cortisol secretion during the day to determine effect sizes with sleep measurements; and 3) using analytic methods and study designs to assure the temporal ordering of sleep measurements before cortisol measurements.

The use of actigraphy has allowed a more objective and comprehensive measurement of sleep, but many studies are either not analyzing the spectrum of sleep measurements available or are not presenting the results for the reader to review (the latter is often referred to as a negative publishing bias). At least one measure from each of the spectra of sleep time, awake time, fragmentation, and time to fall asleep should be analyzed to obtain a comprehensive look at nighttime sleep. Consensus on the categorization of continuous variables would be helpful, or at least a justification of cutpoints (e.g. frequency of night waking often takes a poisson distribution, so dichotomization is often used). Actigraphy over at least five nights has been proven to be important for reliability of the estimate of sleep parameters in children [81]. Measurement of severity of sleep problems should be well-defined so that measures can be reproducible by other researchers and so that they can interpreted by the reader.

Cortisol secretion should be quantified so that the magnitude of effect of cortisol can be evaluated and be compared across studies. The use of the correlation coefficient is relatively unhelpful in this regard because it tells you nothing about the absolute magnitude of the daytime cortisol level(s). Calculating the mean with a variance estimate and/or the change in cortisol depending on the unit change in sleep would be more appropriate. It is understandable that due to the forced page or word limits from journals that the researcher may choose to focus on the positive or statistically significant results; however, a body of work is not only represented by positive results, but all findings related to the research question. It is recommended to
use an appendix format for work that you would like to see included, but that does not need to be included in the primary body of text.

The highest quality study designs include several days of measurement and use analytical methods that ensure that a temporal ordering of sleep before cortisol is maintained. Many studies used a cross-sectional approach to the data, associating sleep on any given night with cortisol on any given day. In the instance of a single night of sleep measurement and day of cortisol measurement, this is the only option available. However, in studies with multiple nights of sleep and/or cortisol, it appears that some studies have not used their data to advantage. They have used measurement in a replicate fashion (combining the several nights or days) obscuring which nights are related to which days. There are methodological strengths in having the individual days represented in terms of the precision of the estimate even at the expense of accounting for the correlation from repeated measures.

The current study

The current study attempts to fill-in-the-gaps in the current literature and further the study area of nighttime sleep and daytime secretion of cortisol in children. The Sleep and Cortisol Study (SACS) piggy-backed onto the Sleep in Autism Study, a larger sleep and daytime behavior study in the greater Sacramento, California, region. In SACS I added a saliva collection protocol for cortisol sampling and a questionnaire that addresses events from the 24-hour period before the day of collection.

Specifically, I have accomplished the following: enrolled a moderate number of children with autism (N=26) and typically developing children (N=26) in a narrow age range (2 to 5 ½ years of age); enrolled children with autism whom were determined to have a diagnosis of autism by the gold standard of DSM-IV criteria; focused on the preschool ages because the prevalence, perception, and consequence of sleep problems are expected to be greater; examined sleep-wake regulation with established valid and reproducible actigraphy methods; used salivary cortisol measurement that is known to have a high correlation with plasma cortisol; and took advantage of a longitudinal data collection over six months to attain precision around the estimates and to examine within-subject variability.

The dissertation improves upon the current literature by adding unique data on preschool-aged children followed longitudinally with sleep measurement on the nights immediately preceding cortisol sampling at several time points during the day. The dissertation moves the field of sleep quality and cortisol secretion forward by studying very young children who are most likely to have persistent sleep problems and also are acquiring rapidly developing cognitive and behavioral skills that might be affected by cortisol dysregulation.
<table>
<thead>
<tr>
<th>Authors, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Age in years</th>
<th>Type of cortisol measurement</th>
<th>Type of sleep deprivation (SD)</th>
<th>Results (Baseline night vs SD night)</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Born, 1988, Germany [13]</td>
<td>Healthy adult volunteers</td>
<td>10 males</td>
<td>18 - 29</td>
<td>Plasma cortisol by indwelling catheter every 15 minutes from 2300 to 0700</td>
<td>Partial REM and NREM SD using tone (polysomnography)</td>
<td>Partial NREM Baseline REM SD arousals 191.8(52.7) 157.5(42.5) 151.6(27.5) Mean (sd) in µg/dl across the night</td>
<td>• Excluding medication use during study, history of sleep disturbances • Laboratory setting</td>
</tr>
<tr>
<td>Spath-Schwalb, 1991, Germany [15]</td>
<td>Healthy adult volunteers</td>
<td>10 males</td>
<td>23 - 28</td>
<td>Plasma cortisol by indwelling catheter every 15 minutes from 2300 to 0700</td>
<td>Partial REM SD and continuous arousals (polysomnography)</td>
<td>• Increase in cortisol immediately following SD, but average cortisol not different across nights</td>
<td>• Excluding medication use during study, history of sleep disturbances • Subject awake after 2&lt;sup&gt;nd&lt;/sup&gt; REM period and allowed to talk and read in bed • Laboratory setting</td>
</tr>
<tr>
<td>Follenius, 1992, France [14]</td>
<td>Healthy adult volunteers</td>
<td>12 males</td>
<td>19 - 24</td>
<td>Plasma cortisol by indwelling catheter every 10 minutes from 2300 to 0700</td>
<td>4 hours of SD in first half of night x 4 nights</td>
<td>2300 - 0300 time period : Baseline SD 83 (17) 64 (17) 0300 - 0700 time period : Baseline SD 301 (33) 257 (22) Mean (se) in nmol/liter.</td>
<td>• Excluding medication use during study, history of sleep disturbances, smokers, underlying diseases • Laboratory setting</td>
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Table 1 (cont.): Review of sleep deprivation (SD) and cortisol levels on SD night compared to baseline night (sorted by amount of sleep deprivation)

<table>
<thead>
<tr>
<th>Authors, year, country</th>
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<th>Type of sleep deprivation (SD)</th>
<th>Results (Baseline night vs SD night)</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Von Treuer, 1996, Australia [16]</td>
<td>Healthy adult volunteers</td>
<td>9 males</td>
<td>17 - 29</td>
<td>Plasma cortisol by indwelling catheter every hour from 2000 to 0700</td>
<td>Entire night of SD</td>
<td>Baseline 2150 SD 3100</td>
<td>Area under the curve in nmol/liter, p&lt;.005 across groups</td>
</tr>
<tr>
<td>Von Treuer, 1996, Australia [16]</td>
<td>Healthy adult volunteers</td>
<td>9 males</td>
<td>17 - 29</td>
<td>Plasma cortisol by indwelling catheter every hour from 2000 to 0700</td>
<td>Entire night of SD</td>
<td>Baseline 2150 SD 3100</td>
<td>Area under the curve in nmol/liter, p&lt;.005 across groups</td>
</tr>
<tr>
<td>Brun, 1998, France [17]</td>
<td>Healthy adult volunteers in control group of double blind trial of modafinil</td>
<td>8 males</td>
<td>20 - 23</td>
<td>Plasma cortisol by indwelling catheter every 30 to 60 minutes from 2000 to 0800</td>
<td>Entire night of SD</td>
<td>Higher cortisol levels at nearly every sampling point in SD night versus baseline night among controls</td>
<td>Excluding medication use during study, history of sleep disturbances, psychiatric morbidity, smokers</td>
</tr>
<tr>
<td>Chapotot, 2001, France [18]</td>
<td>Healthy adult volunteers</td>
<td>10 males</td>
<td>21 - 27</td>
<td>Plasma cortisol by indwelling catheter every 10 minutes from 1800 to 1800</td>
<td>Entire night of SD (polysomnography)</td>
<td>Higher cortisol levels at nearly every sampling point in SD night versus baseline night, but statistically significant differences only in early morning (p&lt;.05)</td>
<td>Serial visual fixation task during day, Between tasks subjects could read, watch TV, listen to music, converse</td>
</tr>
<tr>
<td>Authors, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Age in years</td>
<td>Type of cortisol measurement</td>
<td>Type of sleep deprivation (SD)</td>
<td>Results (Baseline night vs SD night)</td>
<td>Methodological issues</td>
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<tr>
<td>Varkevisser, 2005, The Netherlands[19]</td>
<td>• Chronic insomniacs (CI) and healthy controls (HC)</td>
<td>11 CI 13 HC individually matched</td>
<td>31 – 54 CI 33– 53 HC</td>
<td>Salivary cortisol every 3 hours from 1100 to 1100 during SD</td>
<td>24 hours of SD</td>
<td>Cortisol levels approximately 15-20% higher across SD night compared to baseline, but not statistically significantly higher</td>
<td>• Excluding medication use, history of sleep disturbances, psychiatric morbidity, substance abuse • Laboratory setting</td>
</tr>
<tr>
<td>Davidson, 1991, Canada [20]</td>
<td>• Healthy adult volunteers</td>
<td>10 males</td>
<td>19 - 27</td>
<td>Plasma cortisol by indwelling catheter every 10 minutes from 1800 to 1800</td>
<td>40 hours of SD</td>
<td>Baseline 7.02 SD 7.12 Mean cortisol in µg/dl not different between nights</td>
<td>• Physically and psychologically healthy • Excluding smoking, alcohol, caffeine during study</td>
</tr>
</tbody>
</table>
### Table 2: Review of sleep deprivation (SD) and cortisol levels on recovery night after SD night compared to baseline night (sorted by amount of sleep deprivation)

<table>
<thead>
<tr>
<th>Authors, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Age in years</th>
<th>Type of cortisol measurement</th>
<th>Type of sleep deprivation (SD)</th>
<th>Results (Baseline night vs Recovery night after SD)</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follenius, 1992, France [14]</td>
<td>Healthy adult volunteers</td>
<td>12 males</td>
<td>19 - 24</td>
<td>Plasma cortisol by indwelling catheter every 10 minutes from 2300 to 0700</td>
<td>4 hours of SD in first half of night x 4 nights</td>
<td>2300 - 0300 time period: Baseline 83 (17) Recovery night 69 (11) 0300 - 0700 time period: Baseline 301 (33) Recovery night 232 (25)* Mean (se) in nmol/liter; *p&lt;.01 compared to baseline.</td>
<td>• Excluding medication use during study, history of sleep disturbances, smokers, underlying diseases • Laboratory setting</td>
</tr>
<tr>
<td>Brun, 1998, France [17]</td>
<td>Healthy adult volunteers in control group of double blind trial of placebo vs modafinil</td>
<td>8 males</td>
<td>20 - 23</td>
<td>Plasma cortisol by indwelling catheter every 30 to 60 minutes from 2000 to 0800</td>
<td>Entire night of SD</td>
<td>• Higher cortisol levels at nearly every sampling point in recovery night versus baseline night among controls</td>
<td>• Excluding medication use during study, history of sleep disturbances, psychiatric morbidity, smokers • Laboratory setting</td>
</tr>
<tr>
<td>Von Treuer, 1996, Australia [16]</td>
<td>Healthy adult volunteers</td>
<td>9 males</td>
<td>17 - 29</td>
<td>Plasma cortisol by indwelling catheter every hour from 2000 to 0700</td>
<td>Entire night of SD</td>
<td>Baseline 2100 (200) Recovery night 1950 (200) Area under the curve in nmol/liter (numbers approximate), p&lt;.005 across groups</td>
<td>• Excluding medication use during study, history of sleep disturbances, psychiatric morbidity, substance abuse • Laboratory setting</td>
</tr>
<tr>
<td>Authors, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Age in years</td>
<td>Type of cortisol measurement</td>
<td>Type of sleep deprivation (SD)</td>
<td>Results (Baseline night vs Recovery night after SD)</td>
<td>Methodological issues</td>
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<tr>
<td>Vgontzas, 1999, United States [6]</td>
<td>Healthy adult volunteers</td>
<td>10 males</td>
<td>20 - 29</td>
<td>Plasma cortisol by indwelling catheter every 30 minutes from 0800 to 0600</td>
<td>Entire night of SD (polysomnography)</td>
<td>Baseline: 176.6 (13.8) Recovery night: 157.3 (11.0) Mean (± se) in nmol/liter; p&lt;.05</td>
<td>Generally healthy Excluding current sleep disturbance and medications Laboratory setting</td>
</tr>
<tr>
<td>Salin-Pascual, 1988, Mexico [23]</td>
<td>Healthy adult volunteers</td>
<td>12 males and females</td>
<td>16 -26</td>
<td>Plasma cortisol by indwelling catheter every hour from 2200 to 0600</td>
<td>36 hours of SD (polysomnography)</td>
<td>Baseline: 945.4 (257.8) Recovery night: 963.7 (84.0) Area under the curve in mean (sd) dg/ml No differences in hourly levels across the night between baseline and recovery nights</td>
<td>Excluding history of sleep disturbances, psychiatric or physical morbidity, drug or alcohol abuse, smokers Laboratory setting</td>
</tr>
<tr>
<td>Davidson, 1991, Canada [20]</td>
<td>Healthy adult volunteers</td>
<td>10 males</td>
<td>19 - 27</td>
<td>Plasma cortisol by indwelling catheter every 10 minutes from 1800 to 1800</td>
<td>40 hours of SD</td>
<td>Baseline: 7.02 Recovery night: 6.48 Mean cortisol in µg/dl not different between nights</td>
<td>Physically and psychologically healthy Excluding smoking, alcohol, caffeine during study Laboratory setting</td>
</tr>
<tr>
<td>Authors, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Age in years</td>
<td>Type of cortisol measurement</td>
<td>Type of sleep deprivation (SD)</td>
<td>Results (Baseline day vs day after SD night)</td>
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<tr>
<td>Spiegel, 1999, United States [24]</td>
<td>Healthy adult volunteers</td>
<td>11 males</td>
<td>18-27</td>
<td>Salivary cortisol every 30 minutes from 1600 to 2100 day before and after SD</td>
<td>SD of 4 hours of sleep allowed 0100–0500 x 6 nights (polysomnography)</td>
<td>After partial SD period 4.2 (0.4) 4.8 (0.3) 3.9 (0.3) Mean (se) in nmol/l across evening (numbers approx); other groups versus baseline p&lt;.007</td>
<td>• Subjects allowed to watch TV, play board games, use computers, and read during SD • Laboratory setting</td>
</tr>
<tr>
<td>Spiegel, 2004, United States [27]</td>
<td>Healthy adult volunteers</td>
<td>11 males</td>
<td>Mean (sd) 22 (1)</td>
<td>Plasma cortisol by indwelling catheter every 10 - 30 minutes from 1600 to 2100</td>
<td>SD of 4 hours of sleep allowed 0100–0500 x 6 nights (polysomnography)</td>
<td>After partial SD period 5.3 (0.4) 6.1 (0.4) 4.4 (0.3) Mean (se) in µg/dl across the evening; other groups compared to baseline p&lt;.003</td>
<td>• Subjects allowed to watch TV, play board games, use computers, and read during SD • Laboratory setting</td>
</tr>
<tr>
<td>Leproult, 1997, United States [26]</td>
<td>Healthy adult volunteers</td>
<td>33 males</td>
<td>20 - 32</td>
<td>Plasma cortisol by indwelling catheter every 20 minutes from 1800 to 2300</td>
<td>SD from bedtime to 0400 and 32 hours of SD</td>
<td>After partial 32 hours of SD 130 (10) 155 (20)* 170 (25)** Mean (se) in nmol/l across the evening (numbers approximate); compared to baseline = *p&lt;.03, **p&lt;.003</td>
<td>• Excluding current sleep prob., physical morbidity, smokers • Subjects allowed to watch TV, read, and converse during SD • Laboratory setting</td>
</tr>
</tbody>
</table>
Table 3 (cont.): Review of sleep deprivation (SD) and cortisol levels on day after SD night compared to baseline day (sorted by amount of sleep deprivation)

<table>
<thead>
<tr>
<th>Authors, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Age in years</th>
<th>Type of cortisol measurement</th>
<th>Type of sleep deprivation (SD)</th>
<th>Results (Baseline day vs day after SD night)</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vgontzas, 1999, United States[6]</td>
<td>Healthy adult volunteers</td>
<td>10 males</td>
<td>20 - 29</td>
<td>Plasma cortisol by indwelling catheter every 30 minutes from 0800 to 0600</td>
<td>Entire night of SD (polysomnography)</td>
<td>Baseline day</td>
<td>Day after SD</td>
</tr>
<tr>
<td>Brun, 1998, France [17]</td>
<td>Healthy adult volunteers in control group of double blind trial of placebo vs modafinil</td>
<td>8 males</td>
<td>20 - 23</td>
<td>Plasma cortisol by indwelling catheter every 30 to 60 minutes from 2000 to 0800</td>
<td>Entire night of SD</td>
<td>Similar cortisol levels across the day after SD versus baseline day among controls</td>
<td>Excluding medication use during study, history of sleep disturbances, psychiatric morbidity, smokers</td>
</tr>
<tr>
<td>Chapotot, 2001, France [18]</td>
<td>Healthy adult volunteers</td>
<td>10 males</td>
<td>21 - 27</td>
<td>Plasma cortisol by indwelling catheter every 10 minutes from 1800 to 1800</td>
<td>Entire night of SD (polysomnography)</td>
<td>Higher cortisol levels at nearly every sampling point on day after SD versus baseline day</td>
<td>Serial visual fixation task during day</td>
</tr>
<tr>
<td>Authors, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Age in years</td>
<td>Type of cortisol measurement</td>
<td>Type of sleep deprivation (SD)</td>
<td>Results (Baseline day vs day after SD night)</td>
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<tr>
<td>Heiser, 2000, Germany [28]</td>
<td>Healthy adult volunteers</td>
<td>10 males</td>
<td>Mean (sd) 27.4 (2.8)</td>
<td>Salivary cortisol every 2 hours from 0700 to 1900 day before and after SD</td>
<td>Entire night of SD</td>
<td>Similar cortisol levels across the day after SD versus baseline day</td>
<td>Excluding history of sleep disturbances, psychiatric or physical morbidity, smokers</td>
</tr>
<tr>
<td>Gonzalez-Santos, 1989, Mexico [30]</td>
<td>Healthy young adult volunteers</td>
<td>8 males</td>
<td>21-23</td>
<td>Plasma cortisol by indwelling catheter every 20 minutes from 0800 to ? (during the days)</td>
<td>24 hours of SD and 48 hours of SD</td>
<td>After 24 hours of SD Baseline 99 (18) Mean (sd) in ng/ml across the day; no statistically significant differences</td>
<td>Clinically healthy Excluding smoking, alcohol, drug use prior to study</td>
</tr>
<tr>
<td>Moldofsky 1989, Canada [29]</td>
<td>Healthy adult volunteers</td>
<td>10 males</td>
<td>19-27</td>
<td>Plasma cortisol by indwelling catheter every 30 minutes to 2 hours from 0800 to 0730</td>
<td>40 hours of SD (polysomnography)</td>
<td>Similar cortisol levels across the day after SD versus baseline day</td>
<td>Physically and psychologically healthy Excluding smoking, alcohol, caffeine during study Could play games and read during SD</td>
</tr>
</tbody>
</table>

Table 3 (cont.): Review of sleep deprivation (SD) and cortisol levels on day after SD night compared to baseline day (sorted by amount of sleep deprivation)
Table 3 (continued): Review of sleep deprivation (SD) and cortisol levels on day after SD night compared to baseline day (sorted by amount of sleep deprivation)

<table>
<thead>
<tr>
<th>Authors, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Age in years</th>
<th>Type of cortisol measurement</th>
<th>Type of sleep deprivation (SD)</th>
<th>Results (Baseline day vs day after SD night)</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akerstedt, 1980, Sweden [31]</td>
<td>Healthy adult volunteers</td>
<td>12 males</td>
<td>19 -30</td>
<td>Plasma cortisol by venipuncture at 0800</td>
<td>48 hours of SD</td>
<td>After 48 hours SD 0.60 (0.02) 0.42 (0.02) Mean (se) in µmol/l at 0800; after 48 hours of SD compared to baseline p&lt;.001</td>
<td>Physically and psychologically healthy  Excluding current smoking during study  Laboratory setting</td>
</tr>
<tr>
<td>Kant, 1984, United States [32]</td>
<td>Healthy adult volunteers</td>
<td>6 males</td>
<td>18 -21</td>
<td>Urine cortisol from 0800 to 0800</td>
<td>72 hours of SD</td>
<td>After 72 hours SD 87 (27) 54 (5) Mean (se) in µg/l; no statistically significant differences</td>
<td>Subjects allowed to watch TV, eat, drink, smoke, drink coffee, and read during SD  Laboratory setting</td>
</tr>
<tr>
<td>Authors, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Type of sleep measurement</td>
<td>Type of cortisol measurement</td>
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<tr>
<td>Johns, 1971, Australia [44]</td>
<td>Medical student volunteers • 7 poor sleepers • 7 good sleepers</td>
<td>7 poor sleepers • 7 good sleepers</td>
<td>Questionnaire elicited sleep duration, sleep onset delay, duration of night wakings</td>
<td>Urine free 11-hydroxycorticosteroids over 72 hours; morning, afternoon, evening, overnight pooled periods</td>
<td>Mean (std) 22.4 (1.3) 21.9 (1.2)</td>
<td>Poor sleepers • Good sleepers 527.9 (118.6) 328.3 (57.1) p&lt;.01 Mean (std) cortisol in µg/hr</td>
<td>• Controls were age- and gender matched • No information on presence of psychiatric co-morbidity • No alcohol or drugs during study</td>
</tr>
<tr>
<td>Adam, 1986, Scotland [43]</td>
<td>Volunteers recruited from general practices, government, schools, and hospitals • 18 poor sleepers • 18 good sleepers</td>
<td>18 poor sleepers • 18 good sleepers</td>
<td>Based on self-report questionnaire</td>
<td>Urinary free cortisol (UFC) over 5 days; morning, afternoon, evening, overnight pooled periods</td>
<td>Mean (std) 51.9 (7.0) 51.4 (7.5) Range (40-66)</td>
<td>Poor sleepers • Good sleepers 9.0 (3.3) 8.1 (2.6) p=NS Mean (std) cortisol in nmol/hour</td>
<td>• Controls were age-, weight-, and height-matched • No use of medications • No presence of psychiatric co-morbidity • Laboratory setting</td>
</tr>
</tbody>
</table>
Table 4 (continued): Review of observational studies of sleep and cortisol levels in healthy adults (excluding elderly)

<table>
<thead>
<tr>
<th>Authors, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Type of sleep measurement</th>
<th>Type of cortisol measurement</th>
<th>Ages in years</th>
<th>Results</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spath-Schwalb, 1992, Germany [45]</td>
<td>Healthy men without sleep disturbance</td>
<td>7 with short sleep duration (&lt; 540 minutes) = SSG, 7 with long sleep duration (≥ 540 minutes) = LSG</td>
<td>Polysomnography x 1 night</td>
<td>Plasma cortisol every 15 minutes from 2300-1100</td>
<td>Mean (std) 24.9 Range (20-34)</td>
<td>SSG 210 (15) LSG 155 (9) p&lt;.01 Mean (se) cortisol in nmol/liter</td>
<td>• No use of medications • Presence of psychiatric co-morbidity not addressed • Laboratory setting</td>
</tr>
<tr>
<td>Vgontzas, 1998, United States [42]</td>
<td>Volunteers recruited from newspapers (history of difficulty falling asleep or staying asleep)</td>
<td>15 subjects with short sleep duration (&lt;6.5 hours) or increased sleep latency (&gt; 45 minutes) x 6 months</td>
<td>Polysomnography x 3 nights</td>
<td>24 hour UFC x 3 days; plasma cortisol in morning and evening (2 samples only)</td>
<td>Mean (std) 24.7 (1.5) Range (18-37)</td>
<td>・Higher total wake time was statistically significantly correlated with higher 3-day mean UFC (r = 0.53, p=.05) ・There was no correlation between sleep measures and plasma cortisol</td>
<td>• No use of medications • No presence of psychiatric co-morbidity • Laboratory setting</td>
</tr>
<tr>
<td>Vgontzas, 2001, United States [46]</td>
<td>Volunteers recruited from newspapers, community, and medical center (history of difficulty falling asleep or staying asleep)</td>
<td>11 insomniacs (sleep duration &lt;6.5 hours) or sleep latency (&gt; 45 minutes) x 6 months, and sleep efficiency &lt;80% on 1st night), 13 controls</td>
<td>Polysomnography x 3 nights</td>
<td>Plasma cortisol every 30 minutes x 24 hours</td>
<td>Mean (std) 31.4 (6.7) 27.7 (6.8)</td>
<td>Insomniacs Controls 218 (11.0) 190.4 (8.3) p=.07 Mean (se) cortisol in µg/dl ・There were higher cortisol levels in late afternoon and most of night in the insomniacs compared to controls</td>
<td>• Controls were age- and BMI-matched • No use of medications • No presence of psychiatric co-morbidity • Laboratory setting</td>
</tr>
</tbody>
</table>
Table 4 (continued): Review of observational studies of sleep and cortisol levels in healthy adults (excluding elderly)

<table>
<thead>
<tr>
<th>Authors, year, country</th>
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<th>Type of sleep measurement</th>
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<th>Results</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riemann, 2002, Germany [49]</td>
<td>• Insomniacs from outpatient sleep disorders clinic  • Healthy volunteers</td>
<td>• 10 with primary insomnia (average of 9 years)  • 10 healthy controls</td>
<td>Polysomnography x 3 nights</td>
<td>Plasma cortisol every 30 minutes from 1900 to 0900</td>
<td>Mean (std) 39.2 (9.1) 39.2 (10.1)</td>
<td>Insomniacs Controls 214.4 (62.5) 253.7 (75.9) p=.24 Mean (std) AUC (µg/dl x 30 min)</td>
<td>• Controls were age- and gender-matched  • DSM-IV sleep diagnosis  • No use of medications  • No presence of psychiatric co-morbidity  • Laboratory setting</td>
</tr>
<tr>
<td>Rodenbeck, 2002, Germany [82]</td>
<td>• Male volunteers from the community</td>
<td>• 7 with severe primary insomnia (average of 8 years)  • 7 healthy controls</td>
<td>Structured sleep interview</td>
<td>Plasma cortisol every hour from 4 hours before bedtime to 1 hour after waking</td>
<td>Mean (std) 40.0 (8.3) 35.9 (11.2)</td>
<td>Insomniacs Controls 79.9 (15.9) 49.6 (6.7) p&lt;.01 Mean (std) ng/ml per hour (AUC)  • There was a statistically significant correlation between the number of wakings and evening cortisol in both groups (r = .89, p&lt;.05)</td>
<td>• Controls were age-matched  • DSM-IV sleep diagnosis  • No use of medications  • No presence of psychiatric co-morbidity  • No alcohol or smoking during study  • Laboratory setting</td>
</tr>
<tr>
<td>Authors, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Type of sleep measurement</td>
<td>Type of cortisol measurement</td>
<td>Ages in years</td>
<td>Results</td>
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</tr>
<tr>
<td>Varkevisser, 2007, Netherlands [51]</td>
<td>Insomniacs from outpatient sleep disorders clinic &amp; family practice clinics, Healthy volunteers from advertisement</td>
<td>39 with chronic insomnia, 20 healthy controls</td>
<td>Groninger Sleep Quality Questionnaire &amp; sleep diary</td>
<td>Salivary cortisol at 2000, 1 ½ hours before bedtime, 15 minutes before bedtime, &amp; bedtime</td>
<td>Mean (std) ~41 (9.2) 42.6 (6.8)</td>
<td>Insomniacs assessed current sleep as poor, moderate, or good sleep</td>
<td>• ICSD sleep diagnosis • Use of sleep and anti-depressant medications in insomnia group but not in controls • No presence of psychiatric co-morbidity • Substantial missing data across sleep groups</td>
</tr>
<tr>
<td>Backhaus, 2004, Germany [50]</td>
<td>Insomniacs from outpatient sleep disorders clinic, Healthy volunteers from advertisement</td>
<td>14 with primary insomnia (average of 12 years), 15 healthy controls</td>
<td>Pittsburgh Sleep Quality Index</td>
<td>Salivary cortisol x 7 days at waking, 15 minutes later, &amp; bedtime</td>
<td>Mean (std) 47.1 (8.8) 46.8 (7.9)</td>
<td>Frequency of night awakenings were negatively correlated with waking cortisol ($r = -.50$, $p=.004$)</td>
<td>• Controls were age-, BMI-, and gender-matched • DSM-IV sleep diagnosis • No use of medications • No presence of psychiatric co-morbidity</td>
</tr>
<tr>
<td>Backhaus, 2006, Germany [48]</td>
<td>Insomniacs from outpatient sleep clinic, Healthy volunteers from advertisement</td>
<td>16 with primary insomnia (average of 9 yrs), 13 healthy controls</td>
<td>Polysomnography x 1 night</td>
<td>Plasma cortisol every 15 minutes during the night</td>
<td>Mean (std) 41.6 (1.2) 40.1 (11.3)</td>
<td>Insomniacs had a decrease in first half of night and increase in the second half of the night compared to controls, but the differences were not statistically significantly different.</td>
<td>• Controls were age, BMI, gender-matched • DSM-IV sleep diagnosis • No use of medications • No presence of psychiatric co-morbidity • Laboratory setting</td>
</tr>
<tr>
<td>Authors, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Type of sleep measurement</td>
<td>Type of cortisol measurement</td>
<td>Ages (in days, months, or years)</td>
<td>Results</td>
<td>Methodological issues</td>
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<tr>
<td>White, 2000, United States [52]</td>
<td>Infants with colic by targeted recruitment • Controls are a random sample from county births</td>
<td>20 infants with colic • 20 healthy control infants</td>
<td>Sleep diary x 3 days</td>
<td>Salivary cortisol x 2 days at waking, midmorning (10am), midafternoon (4pm), bedtime</td>
<td>Mean (std) 59.8 (8.9) days 60.9 (10.4) days</td>
<td>• Infants with colic had less sleep (mean =11.8 hours) and a flatter daytime cortisol rhythm than controls (mean=14 hours)</td>
<td>• Adjustment for crying</td>
</tr>
<tr>
<td>Larson, 1998, United States [53]</td>
<td>Healthy infants recruited by phone using birth lists</td>
<td>78 infants</td>
<td>Sleep-wake diary x 2 days</td>
<td>Salivary cortisol x 2 days at waking, 10am, and 4pm</td>
<td>Mean (std) 79.5 (15.0) days Range (55.5-106.5 days)</td>
<td>• Infants with &lt; 6 hours (versus ≥ 6 hours) continuous sleep had statistically significantly lower waking cortisol (p&lt;.05). • There were no differences for the other times</td>
<td>• Continuous sleep defined as no “signaling” to alert parents</td>
</tr>
<tr>
<td>Spangler, 1991, Germany [54]</td>
<td>Healthy infants drawn from local birth announcements</td>
<td>14 infants</td>
<td>Sleep diary x 5 days</td>
<td>Salivary cortisol x 5 days (4 samples every 4 hours from 0600 to 0600)</td>
<td>Range (3-7 months)</td>
<td>• Duration of sleep in preceding period before sample collection was positively correlated with cortisol level (r=.59)</td>
<td>• Substantial missing data</td>
</tr>
<tr>
<td>Lucas-Thompson, 2009, United States [56]</td>
<td>Sample from prospective study of prenatal stress and infant development</td>
<td>92 infants</td>
<td>Maternal report of sleep (night wakeings) in past month</td>
<td>Salivary cortisol at baseline before inoculation and 20 minutes after inoculation</td>
<td>6- and 12-months-old</td>
<td>Difference post-inoculation minus pre-inoculation in µg/dl No or rare 1+ wakeings per night 6 mos 1.02 1.77 12 mos 1.02 1.57</td>
<td>• Adjustment • Inoculation time of day included in models</td>
</tr>
</tbody>
</table>
Table 5 (continued): Review of observational studies of sleep and cortisol levels in healthy children (newborns to teens)

<table>
<thead>
<tr>
<th>Authors, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Type of sleep measurement</th>
<th>Type of cortisol measurement</th>
<th>Ages (in days, months, or years)</th>
<th>Results</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silva, 2007, Brazil [55]</td>
<td>Healthy children selected by review of medical records</td>
<td>91 children</td>
<td>Sleep in hours per day from questionnaire</td>
<td>Salivary cortisol x 1 day in morning (7-10am) and late afternoon (3-6pm)</td>
<td>Range (1 ½ - 36 months)</td>
<td>• There was a negative linear correlation between morning cortisol and hours of sleep (p=.03)</td>
<td>• Only one day of saliva collection</td>
</tr>
<tr>
<td>Scher, 2009, Canada [59]</td>
<td>Healthy children attending daycare</td>
<td>27 children</td>
<td>Actigraphy x 3 nights (sleep efficiency (SEF) dichotomized into high and low categories)</td>
<td>Salivary cortisol x 3 days at waking and bedtime</td>
<td>Mean (std) 24.5. (6.9) months</td>
<td>High SEF</td>
<td>• Cross-sectional approach to data?</td>
</tr>
<tr>
<td>Badanes, 2009, United States [61]</td>
<td>Healthy children</td>
<td>197 children</td>
<td>Sleep actigraphy x 7-9 nights</td>
<td>Salivary cortisol at waking, mid-morning, mid-afternoon, and bedtime x 2 weekend days</td>
<td>30- to 72-months-old</td>
<td>• Nighttime sleep was unrelated to cortisol patterning (no data presented)</td>
<td>• Control for confounding by age only</td>
</tr>
<tr>
<td>Ward, 2006, United States [60]</td>
<td>Cross-sectional study of healthy children attending full-time care</td>
<td>40 children</td>
<td>Actigraphy (x 3 days)</td>
<td>Salivary cortisol x 1 day in morning (10-11am) and late afternoon (2-4pm)</td>
<td>Mean (std) 45.9 (7.3) (Study A) to 47.7 (8.4) (Study B) months</td>
<td>• Nighttime sleep, awakenings, percent wake after sleep onset (by actigraphy) were unrelated to any of the cortisol measures (no data presented)</td>
<td>• Only one day of saliva collection</td>
</tr>
<tr>
<td>Authors, year, country</td>
<td>Study population</td>
<td>Number of subjects</td>
<td>Type of sleep measurement</td>
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<tr>
<td>Hatzinger, 2005, Switzerland[63]</td>
<td>• Same sample as Hatzinger, 2008 below</td>
<td>102 children</td>
<td>Sleep actigraphy x 7 days</td>
<td>Salivary cortisol x 1 day at waking, 10, 20, and 30 minutes after waking</td>
<td>Mean (std) 5.1 (1.1) years</td>
<td>• Children with severe sleep disturbance had increased morning cortisol compared to good sleepers (p&lt;.05)</td>
<td>• No definition given for “severe sleep disturbance”</td>
</tr>
<tr>
<td>El-Sheikh, 2008, United States[64]</td>
<td>• Children recruited from 3rd grade of local public school system in southeastern US</td>
<td>64 children</td>
<td>Sleep actigraphy x 7 days</td>
<td>Salivary cortisol x 1 day at approximately 3pm</td>
<td>Mean 8.7 (0.5) years Range (7-11 years)</td>
<td>• There were statistically significant (all at p&lt;.05) correlations between afternoon cortisol and total sleep minutes (r = -.29), sleep efficiency (r = -.30), and minutes awake after sleep onset (r = .30)</td>
<td>• No information on napping • Only one day and sampling time for cortisol</td>
</tr>
<tr>
<td>Hatzinger, 2008, Switzerland[62]</td>
<td>• Healthy children transitioning to school (from representative sample of Basel)</td>
<td>67 children</td>
<td>Sleep EEG x 2 nights at home (clustered by baseline EEG values for sleep onset, total sleep time, and waking after sleep onset into poor, normal, and good sleepers)</td>
<td>Salivary cortisol x 1 day at waking, 10, 20, and 30 minutes after waking</td>
<td>Mean (std) 5.3 (0.4) years</td>
<td><strong>Mean (std) AUC</strong> Good 1.19 (0.65) 36.8 (22.9) Normal 1.78 (0.89) 53.0 (27.3) Poor 2.15 (0.91) 65.2 (29.9) p&lt;-.001 p&lt;.01</td>
<td>• AUC stress basal values were statistically significantly associated with the number of awakenings after sleep onset (r=.36, p&lt;.01)</td>
</tr>
</tbody>
</table>
### Table 5 (continued): Review of observational studies of sleep and cortisol levels in healthy children (newborns to teens)

<table>
<thead>
<tr>
<th>Authors, year, country</th>
<th>Study population</th>
<th>Number of subjects</th>
<th>Type of sleep measurement</th>
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<th>Ages (in days, months, or years)</th>
<th>Results</th>
<th>Methodological issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dahl, 1992, United States [57]</td>
<td>Healthy children recruited from ads, health fairs, and personal contacts</td>
<td>25 children</td>
<td>Sleep EEG</td>
<td>Plasma cortisol every 20 minutes during the night</td>
<td>Mean (std) 10.3 (1.6) years Range (6-13 years)</td>
<td>• There was a strong negative correlation ($r = -0.55$, $p&lt;.005$) between sleep maintenance (time spent asleep during sleep period) and nocturnal cortisol</td>
<td>• No use of medications • No presence of psychiatric comorbidity • Laboratory setting</td>
</tr>
<tr>
<td>Capaldi, 2005, United States [58]</td>
<td>Healthy children recruited from community postings and direct mailings</td>
<td>31 children underwent 3 stressors: public speaking, mental arithmetic, and mirror tracing</td>
<td>Modified Sleep Habits Survey</td>
<td>Salivary cortisol at baseline, stress, and recovery periods</td>
<td>Mean (std) 13.1 (1.9) years Range (10-17 years)</td>
<td>• Child report of greater sleep-wake problems (partial $r = -.28$, $p&lt;.05$) and sleepiness (partial $r = -.28$, $p&lt;.05$) were associated with lower cortisol reactivity to stressors</td>
<td>• No use of medications • No presence of psychiatric comorbidity</td>
</tr>
</tbody>
</table>
References


60. Ward, T.M., Sleep patterns, cortisol levels, and behaviors in preschool children attending full-day child care centers, in Dissertations and Theses. 2006, University of California, San Francisco. p. 105.


CHAPTER 6: RESEARCH METHODS

Study population

The general flow of participants into the Sleep and Cortisol Study is described in the following diagram:

Participants for the larger Sleep Problems in Children with Autism Study (the Sleep Study) were in part recruited from a group of children who had just enrolled and been assessed in a National Institute of Environmental Health Sciences (NIH)-funded case-control study examining environmental risk factors for autism (hereafter referred to as the CHARGE Study).

The Sleep Study (P.I., Thomas F. Anders, M.D.; co-PI Beth Goodlin-Jones, Ph.D.) sample was also composed of other volunteers recruited to participate in a study of sleep and daytime behavior in the greater Sacramento, California, region but were not sampled on the basis of a current sleep problem.

The Sleep and Cortisol Study (SACS), which is the subject of this dissertation, included children with autism and typically developing children only (children with developmental delay without autism were excluded to maintain strictly defined diagnostic groups). The project was reviewed and approved by the Institutional Review Boards of the University of California, Davis and the University of California, Berkeley (see Appendix 1: Human Subjects). The SACS piggy-backed onto the Sleep Study (the host study) and began data collection in March 2005 (about midway through the host study) and was completed in May 2007. The final study sample consisted of 26 children with autism and 26 typically developing children (see Appendix 2: Enrollment Flow).

The SACS and the host study used a longitudinal study design with measurements
taken at enrollment (baseline), and three and six months following enrollment. Subjects could be enrolled into the SACS from the Sleep Study at any of the three phases (see Appendix 3: Enrollment of Subjects into SACS). The following table describes the inclusion and exclusion criteria:

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
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<tbody>
<tr>
<td>• 2 to 5 ½ years of age</td>
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<tr>
<td>• Children with autism met DSM-IV diagnostic criteria for autism</td>
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<table>
<thead>
<tr>
<th>Exclusion Criteria</th>
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<tbody>
<tr>
<td>• Down syndrome, fragile-X, or other chromosomal disorders</td>
</tr>
<tr>
<td>• Typically developing children with any evidence of cognitive impairment or developmental delay</td>
</tr>
<tr>
<td>• Presence of a chronic illness.</td>
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<tr>
<td>• Any evidence of significant visual impairment (over 20/200 in better eye)</td>
</tr>
<tr>
<td>• Tourette’s syndrome or seizure disorders</td>
</tr>
<tr>
<td>• Obsessive compulsive, anxiety, depression, or other psychiatric disorders</td>
</tr>
<tr>
<td>• Sought treatment for a sleep disorder</td>
</tr>
<tr>
<td>• Used oral or inhalant corticosteroids during the study for asthma or allergies</td>
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</table>

Pilot study

The objective of the pilot study was to test the saliva collection procedures among a few representative families. Specifically, the children were selected because they were very young children regardless of diagnosis and/or were low functioning children with autism. The collection procedures of interest were whether the children could accept the cotton roll and Kool-Aid and whether the families could obtain sufficient saliva in close concordance with target sample times. The families of 7 children with autism and 1 typically developing child were approached for participation. There were no refusals and no withdrawals. The ages of the children were: less than 3 years (2 children – 1 typically developing); 3-4 years (3 children); and 4-5 years (3 children). Six of the children with autism were considered low functioning (intelligence quotient less than 70). I requested that families complete a single phase of the proposed study (2 days of 3 collections per day to obtain 6 samples per family, to total 48 samples from the 8 families). All of the families were able to complete the protocol without incident or complaint, and with excellent compliance. Approximately 90% of afternoon samples were collected within 20 minutes of the 2:00pm target time. In 77% of all samples, at least 0.50 milliliters of saliva was obtained, and in 95%, at least 0.20 milliliters was obtained (0.20 milliliters is considered the minimum amount from which a cortisol analysis can be performed at the laboratory of choice).

Relevant study measurements

Diagnosis of autism:

Staff from the CHARGE Study was trained to the standards of Catherine Lord (the originator of the instruments) at her laboratory at the University of Michigan and maintained good reliability based on scoring 20% of tapes. In the instances where recruitment into the Sleep Study occurred through other sources, the staff of the Sleep Study conducted the assessments. Likewise, the Sleep Study research staff was
trained by the Sleep Study psychologist in the standards as above. Both the ADOS-G and ADI-R are videotaped for additional scoring at a later time. These measures are designed to differentiate children with autism from other children, primarily those with ASD or non-spectrum (NS) disorders, such as mental retardation and language disability. The typically developing children did not receive the ADI-R and ADOS-G but were screened clinically to confirm that they did not meet criteria for autism or ASD. The gold standard for the diagnosis of autism consists of an evaluation by a multidisciplinary team of specialists including a psychiatrist, psychologist, speech pathologist, and occupational therapist at clinics specializing in developmental disorders.

The Autism Diagnostic Observation Schedule- Generic (ADOS-G): This is a semi-structured, standardized assessment in which the researcher observes social interaction, skills in communication, play activities, and imaginative use of materials for children and adults suspected of having autism or ASD [2]. The ADOS was originally developed in the 1980s and was intended for administration to children between five and twelve years of age [3]. The ADOS-G was a modification of ADOS intended to extend the age and verbal limits to be used in children under five and non-verbal children [2]. There are four modules designed for use depending on the level of expressive language. There are also four domains from which a diagnostic algorithm is created and a threshold is specified for autism and separately for ASD: social; communication; social communication; and restricted repetitive.

The Autism Diagnostic Interview-Revised (ADI-R): This is a modified version of the Autism Diagnostic Interview developed in the 1980s [4]. The ADI-R is a standardized, semi-structured, investigator-based interview for the primary caregivers of individuals with autism or individuals with ASD [5]. As with the ADOS-G, the revision was designed to apply to children under five who constitute the bulk of cases needing an initial diagnosis [6]. Unlike the ADOS-G, the ADI-R takes into account the course of development and information on day-to-day behaviors of the child, rather than those only seen from observation in the novel research environment. There are three main areas used to provide a diagnostic algorithm and create a threshold cut-point for an autism diagnosis: qualities of reciprocal social interaction; communication and language; and restricted and repetitive interests and behaviors.

Evaluation of ADOS-G and ADI-R: There is no definitive method of diagnosis for autism. Clinical diagnosis by consensus using the DSM-IV criteria (see Chapter 2, Appendix 1 for detail) is the accepted gold standard. This type of diagnostic standard is the norm for nearly all neurodevelopmental and psychiatric disorders where the lack of a biological, pathological, or genetic marker has inhibited diagnostic accuracy. The experience, qualifications, and membership of the diagnostic evaluation team may vary substantially from one study to another. In addition, because autism is considered to be one of a number of so-called spectrum disorders reflecting differing severity of symptomatology, discriminating between autism and the autism spectrum disorders is difficult [7]. Currently, the accepted diagnostic standard in the field (the “field” standard) is the use of the ADOS-G and ADI-R measurement tools in conjunction with each other. One disadvantage of these tools is that they are related in content to the gold standard. That is, there is a lot of overlap between the DSM-IV criteria and the items on the ADOS-G and ADI-R. Agreement between the field standard and the gold standard is
likely to be positively biased in the direction of better agreement. This "incorporation bias" virtually always leads to the overestimation of predictive abilities [8, 9]. These tools are also costly, labor intensive, need extensive training, are not sensitive to IQs below 20 and best suited to confirm initial diagnosis only [10].

The results of validation studies for ADOS-G and ADI-R are presented in Table 1. There was only one validation study of the ADOS-G that measured inter-rater reliability. Reliability measured by intraclass correlation (ICC – unspecified type) across domains was 82% to 93% according to the original study paper [2]. The ICC is a measure of intra-rater and inter-rater reliability, but there are numerous versions that can give quite different results [11]. Mean weighted kappas (MWK) were used to examine inter-rater reliability for each item and were summarized (65% to 78%) over domains. For the ADI-R, the range over domains for inter-rater reliability by the ICC (93% to 97%) was higher than for the ADOS-G, but reliability by MWK was 73% to 78% for rater pairs in one of the original validation studies [6]. In a similarly aged sample from the same laboratory, the MWK indicated agreement of 62% to 89% across domains [5]. The reliability analyses used subsets of the overall data and multiple raters (> 2 raters). Even if the inter-rater reliability is high, one still needs good sensitivity and specificity. Otherwise, the reliability suggests systematic misclassification by the raters unrelated to the accuracy of the test.

The kappa results were described by the authors as "good" using rules-of-thumb quoted widely [12, 13]. However, kappa coefficients are greatly affected by the balance of marginal totals [14, 15]. In the situation of unbalanced marginals, where the prevalence of the positive and negative ratings are each far from 50%, the kappa can vary depending on whether they are symmetrically or asymmetrically unbalanced [14]. In the examples of Feinstein et al., symmetrical imbalance led to a reduced kappa, and asymmetrical imbalance led to a higher value of kappa. Weighted kappa is interpretable as the proportion of weighted agreement corrected for chance [16]. The weighting algorithms were unspecified in the text of the papers reporting on reliability. Weighted kappa allows the weights to be arbitrary in relative magnitude so that the weighted kappa may be arbitrary as a result [17]. However, if standard weights are used then weighted kappa become equivalent to the ICC. Kappa depends not only on the sensitivity and specificity but also on the prevalence of the disorder in the population [18]. The kappa for a rare disorder such as autism is especially likely to be lowered by the imperfect sensitivity and specificity of the ADOS-G and ADI-R.

Turning to the issue of validity, the ADOS-G had sensitivity ranging from 87% to 100% regardless of the referent group in these studies of highly selected samples which by design have a high prevalence of autism (see Table 1). However, specificity ranged from 93% to 100% for the NS comparison group, and 65% to 79% for the combined spectrum ASD/PDD/NS group. The ADI-R had sensitivity ranging from 96% to 98% and specificity ranging from 92% to 93% with the NS referent group, and sensitivity ranging from 75% to 77% and specificity ranging from 63% to 72% with the ASD/PDD/NS reference group.

In the evaluation of sensitivity and specificity, both the gold standard and comparison groups were different across studies. The composition of the gold standard teams differed in terms of expertise as well as in the number of examiners making the evaluations. The comparison groups generally consisted of a mix of spectrum disorders
and a variety of disorders that were distinct from autism but frequently co-morbid with autism (e.g. ADHD, MR, and OCD). If these disorders coexist with the disease under study, then false-negative results may occur [8]. Although the sensitivity and specificity of the tools were relatively high when using the non-spectrum comparison groups, the tools performed more variably in discriminating between autism and spectrum disorders. The specificity with the ASD/PDD/NS reference group suggests that there may be some children with ASD misclassified as having autism. The sensitivity results suggest that some children with high-functioning autism may be misclassified as having ASD. It is of concern that some of the subjects may not have been naïve or first attendees at a clinic. This may lead to differential information being obtained from parents on the ADI-R if their child has been evaluated before and would likely be in the direction of affirming an autism or ASD diagnosis. In addition, some gold standard examiners had access to the results of the instruments from which to make their “independent” diagnosis. This may have lead to a bias in the direction of greater agreement between the gold standard examiners and the instrument(s) under evaluation (again, a form of incorporation bias [8, 9]. With rare disorders such as autism, the predictive power is not only dependent on the sensitivity and specificity, but also on the rate of the disorder [19]. In the studies under review the prevalence of autism was very high by virtue of the sources of the subjects. Therefore, in the studies under review with a base rate of the disorder greater than or equal to 50%, the positive predictive values (PPV) and negative predictive values (NPV) were generally greater than 90%. However, in the de Bildt paper, the PPV was considerably lower (approximately 48%) in a study with a prevalence of autism of 26% [20]. The sensitivity, and especially the specificity, was lower than in the remaining studies. Clark et al. suggest that not until a base rate of a disorder is about 25% that a measure with sensitivity and specificity of 80% will predict a disorder more than half the time (PPV > 50%) [19]. Taking the average of the results from the studies under review and applying them to the general population, the PPV and NPV are considerably different from the results here. The estimated prevalence in the general population is approximately 17 per 10,000 for autism [21]. The average sensitivity and specificity was 95% and 70% for the studies under review, respectively. The resulting PPV would be 50% and the NPV would be 100%.

There is little research on the evaluation of the use of the combination of ADOS-G and ADI-R (see Table 2), but de Bildt et al. reported 83% overall agreement and a kappa (weighted for errors) of 67% for agreement between the two among children five to eight years of age [20]. Risi et al. examined the validity of the ADOS (the version prior to ADOS-G) and ADI-R together in children three years of age and older [22]. The authors reported a sensitivity of 82% and a specificity of 86% in a U.S. subsample, and a sensitivity of 77% and a specificity of 75% in a Canadian sample. It is somewhat surprising that the combination of the diagnostic instruments do not perform particularly better than either alone.

It is of note that the ADOS-G cannot be used alone to make a DSM-IV diagnosis of autism because evidence of abnormalities before three years of age are required (the ADOS-G does not elicit historical information as does the ADI-R) [2]. These instruments are considered the state-of-the-art for research purposes and perform better than other instruments evaluated concurrently, such as the Gilliam Autism Rating Scale [23]. In the current study, both the ADOS-G and ADI-R are used in conjunction
with each other to obtain a DSM-IV diagnosis of autism. However, the sensitivity and specificity of the ADOS-G and ADI-R suggest misclassification of autism and ASD so that a proportion of the children diagnosed with autism may actually be on the spectrum, and those with true autism may have been diagnosed as on the spectrum.

The typically developing children were not screened with the ADOS-G or the ADI-R instruments. The rarity of a diagnosis of autism suggests that the typically developing children are unlikely to be misclassified. If there were any suspicions of an incorrect diagnosis, there were three phases or opportunities to administer the ADOS-G and ADI-R if necessary to rule out an autism or ASD diagnosis. To date, there are no studies that have evaluated these instruments in children with autism compared to typically developing children alone.

Sleep Measures:
Actigraphy: Each child was recorded actigraphically with a “sleep watch”, the Mini-Mitter® Actiwatch Actigraph (AW64, Mini-Mitter, Inc., Bend, OR), for seven consecutive days and nights on three occasions over a 6-month period: phase 1 (at baseline), phase 2 (3 months following baseline) and phase 3 (6 months following baseline). All children wore the actigraph 24 hrs per day (except when bathing, swimming, or playing some selected sports) for the three seven-day phases. The actigraph weighs approximately two ounces and measures 1.75 inches by 1.3 inches by 0.38 inches. The actigraph generates voltage each time the unit is moved. The signal is then amplified, filtered, and compared with a reference signal. The values accumulated during a 1-minute period are then stored in the actigraph’s solid-state memory. A computer algorithm has been developed that automatically scores sleep-wake states based on this movement data [24], but it does not assess sleep stages. The actigraph was placed in a padded ankle band which attached with Velcro and had a neoprene cover with sensory-reducing foam to encourage better compliance. If preferred, subjects may have worn the actigraph as a wristwatch. Due to the small limb size of preschool-aged children, the ankle has proven a more feasible body part for attachment.

Evaluation of actigraphy: In Chapter 4, the various methods for measuring sleep were described. In the Sleep Study, actigraphy, the Children’s Sleep Habits Questionnaire, and sleep diary information were available for all subjects. In a validation subsample, approximately 30% of the subjects had two nights of videosomnography, which is considered the “gold standard” of the available measurements. For the purposes of the dissertation I chose to use the actigraphy data for sleep measurement. Given the small sample size of my study, I could not rely on whether videosomnography would be available in conjunction with the saliva collection, and it would have been very difficult to achieve 100% coverage for my sample. In fact, only 23% of the SACS subjects had at least one night of videosomnography. Videosomnography can also be limiting because the child must be captured on-camera, which is difficult if the child is sleeping off-camera in the child or parent bedroom. The lack of privacy for co-sleeping families presents another burden which may prevent the use of videosomnography. Actigraphy is used more frequently and is the current standard for sleep measurement in community samples.

In adults, the validity of actigraphy has been well-studied with the percent agreement between polysomnography (the original gold standard of sleep measurement) and actigraphy ranging from 79% to 100% for sleep time and 48% to
80% for wake time in a large review of studies [25]. Comparisons between actigraphy and polysomnography (PSG) can be difficult because the epochs of the PSG have to accurately time-lock with those of the actigraph to guard against inappropriate comparisons of different segments of sleep and waking [26]. Another issue is that in epoch-by-epoch comparisons with PSG, actigraphy is better at detecting sleep than waking [27]. Sleep episodes constitute more than 90% of sleep and may artifactually produce high agreement [27, 28]. In effect, measures of sleep schedule (e.g. sleep duration) are better detected with actigraphy than measures of sleep quality (e.g. wakings) [29]. It is of note that there has been little validation of actigraphy against PSG in young children, yet sleep schedule and quality are different from adults.

Table 3 summarizes the relevant studies examining actigraphy versus PSG in young children. Percent agreement is defined as the proportion of concordant results among all tested. The agreement and sensitivity for detection of sleep across the studies comparing actigraphy to PSG was 88% to 93%. Percent agreement is a simple method of assessing validity as it does not condition on the gold standard and can be calculated for many categories [30]. A significant limitation of percent agreement is that values tend to be high when there is a high prevalence of positive results (and a high proportion of positive-positive observations), such as epochs of sleep during the night, particularly when sensitivity is high [30]. The percent agreement for waking across studies comparing actigraphy to PSG was only 66% to 77% for children over 12 months of age [31, 32], and the specificity for the detection of sleep (i.e. the sensitivity for the detection of waking) was 31% in infants [33]. The comparison of the correlation between the two methods with respect to total sleep time (duration of night sleep) was 72% [32] and 87% [34] in the two studies which examined the data in this fashion. However, correlation is an inappropriate method to determine agreement. The correlation coefficient measures the strength of association between variables and is a measurement of linear association, not the agreement between the variables [30, 35]. You may have perfect agreement only if points are on the line of equality, but correlation can occur irrespective of the line. This is clearly demonstrated in the paper Insana, et al. in which a figure shows that PSG and actigraphy are highly correlated (r=0.87) [34]. However, the points are systematically clustered on a line below the line of equality indicating an underestimate of sleep time by actigraphy. In addition, information obtained from different sources may be correlated not out of independent evaluation of the truth, but because errors are correlated. Another limitation of the correlation coefficient is that it is very sensitive to the range of values – if the range is wide then the correlation will be higher than if the range is narrow [30, 35].

Table 3 also summarizes the only study examining actigraphy versus videosomnography in young children. The sensitivity for detection of sleep was approximately 98% with high agreement (95%; 89% by weighted agreement) driven by the high prevalence of sleep. However, the data corroborate the PSG validation studies in that there are general problems of specificity for detection of sleep (24%; 27% with adjustment) with actigraphy. The authors used prevalence-adjusted bias-adjusted kappa (PABAK) as a measure of observed agreement that takes into consideration bias between observers and the differences in marginal totals [36]. Again, the use of the correlation coefficient is not appropriate and suggests that in any case the variance explained is not very large in this study.
Infants and young children move around more frequently during sleep than adults and yet have more wakefulness than adults. It is important that actigraphy filters and algorithms take factors like this into account when establishing what defines waking in young children. The differing actigraphy brands and models also make cross-comparisons difficult. The Ambulatory Monitoring brand had better agreement rates for wake time than the Mini-Mitter brand, but there are too few studies with very small sample sizes to make any conclusions. There are many actigraphy scoring programs with different algorithms and not all of these have been validated, but sleep-scoring rules for PSG have been standardized [27, 29]. In addition, there are other sleep measures of interest besides total sleep and waking, such as sleep latency, and few validation studies have addressed these other measures.

The Sleep Study researchers conducted the validation study of actigraphy versus videosomnography presented in the table above. There were big differences in the number of wakings between both videosomnography (average of two to five per night) and sleep diary (average of two to five per night), and actigraphy (average of eight to ten per night). Readjustment of the factory calibration from medium sensitivity to high or low sensitivity did not make a significant difference in the number of wakings from actigraphy. Therefore, the data were recoded using a new “smoothing” algorithm to make the results more consistent with the diary and videosomnography. This new algorithm required that the length of a waking be a minimum of two minutes following the onset of sleep with activity counts greater than 100 in the two minutes [37]. Specifically, an awakening began at the first of at least two consecutive minutes awake and ended at the first of three consecutive periods (minutes) of no activity or sleep [37]. This secondary filter reduced the number of wakings so that they were more comparable to the videosomnography. Although adjustments were not made on an epoch-by-epoch basis, an incorporation bias is suggested by this strategy. In effect, the actigraphy results were modified downward to be closer to the results of the method that was being compared. It is surprising that this strategy still produced such poor results given that the aim was to improve the specificity. It is uncertain what affect this smoothing would have on the comparisons of these results to different studies that used only actigraphy, but it is likely that the number of wakings would be lower in the current study. This modification occurred across all subjects without regard to any particular characteristics. There were no differences in the actigraphy-videosomnography comparisons between diagnostic, gender, or age groups.

There has been only one study validating videosomnography against PSG. Anders, et al. compared these methods in six full term infants at two and eight weeks of age for the correlation of REM, NREM, and wakefulness [38]. The type of sleep being examined (sleep stages), the age group studied (very young infants), and the statistical method used (correlation) make application of the results to the current study difficult. In general, the use of ratings by people viewing time-lapse recordings of videosomnography of several nights is likely to have some human error incorporated. Fatigue, distraction, disinterest, or classification error of the rater is likely to affect videosomnography results. In fact, 20% of video recordings were scored by two raters and inter-rater correlations were greater than 85% (personal communication, Thomas F. Anders). The core classification of a waking in the current study as a child sitting up precludes identification of a waking that might occur in other positions (e.g. a child in a
reclining position on their side that may be awake and playing with a stuffed animal) Other positions are scored as waking only if the eyes are observed to be open or the child is crying (the videosomnography uses a microphone as well). If the child is facing away and is quiet then this epoch would not be observed and scored as waking. It will be important to have some validation of this method beyond infancy for the future potential of videosomnography in young children.

Sleep Diary: Parents kept a structured sleep diary during the three phases to complement actigraphy and to provide information when there were gaps due to unexpected removal of the actigraph. The sleep diary was a calendar-style weekly form which asked the parent to note specific sleep-related events and times on a daily basis. The times of note included: times of sleep onset, awakenings from sleep, subsequent returns to sleep, bedtimes and morning rise times, and times of daytime naps on days when they occurred. The parent usually completed this in the morning following the day and night that was observed. Completion of the sleep diary was intended to be done online using a website specifically for this purpose, but parents could have completed a paper form or responded to phone calls from the study staff.

Research Diagnostic Criteria (RDC): The background on the development of RDC over time has been addressed in Chapter 4. Briefly, these criteria have been developed to assign children with frequent night-waking and/or sleep-onset episodes as having a Behavioral Insomnia of Childhood (see table reproduced below). The night-waking subtype includes an algorithm that contains elements of frequency of waking and duration of the waking. The sleep-onset subtype includes an algorithm that contains elements of sleep latency and bedtime struggles and reunions. Both classifications necessitate having at least 5 episodes over the week of measurement with actigraphy.

### Research Diagnostic Criteria for Night Waking and Sleep Onset Insomnias (adapted from [1])

<table>
<thead>
<tr>
<th>Behavioral Insomnia of Childhood</th>
<th>Criteria (for children older than 24 months; five to seven episodes per week for the week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Night-waking subtype</td>
<td>• ≥ 1 awakening per night totaling ≥ 20 minutes</td>
</tr>
<tr>
<td>Sleep-onset subtype (meets two of three criteria)</td>
<td>• &gt; 20 minutes to fall asleep</td>
</tr>
<tr>
<td></td>
<td>• Parent remains in room for sleep onset</td>
</tr>
<tr>
<td></td>
<td>• &gt; 2 reunions</td>
</tr>
</tbody>
</table>

The Epworth Sleepiness Scale (ESS): This is an 8-item self-report scale [39] that has been adapted by the Sleep Study for reporting of daytime sleepiness in children by parents. The ESS measures the likelihood of falling asleep (sleep propensity) in specific situations. Daytime sleepiness is measured on a 4-point scale of 0 (would never sleep), 1 (slight chance of sleeping), 2 (quite likely to sleep) and 3 (very likely to sleep) producing a range of scores from 0 to 24. However, in children some situations were revised due to irrelevance (e.g. falling asleep behind the wheel of a car). The original scale correlates moderately ($r = -0.37$) with the results of the gold standard Multiple Sleep Latency Test [40]. For the purposes of the Sleep Study, a cutpoint of 11 or higher is being interpreted as indicative of excessive sleepiness during the day (scores up to 10 were considered “normal” in the original paper [39].
**Cortisol:**

**Collection:** Cortisol level was obtained by salivary sampling on 2 days of each of the three recording weeks of the SACS (at baseline, 3 months, and 6 months). There was a maximum of 18 samples on each child (3 per day x 2 days x 3 recording weeks). In the recording week, parents were given the materials and instruction sheet for the procedures to collect the six saliva samples (see Appendix 4: Saliva Packaging). Materials included 6 kits with the following in each kit: 1 needleless syringe, 1 plunger, two cotton rolls, 1 scooper with a few grains of Kool-Aid®, and 1 saliva collection tube. The instruction sheet had contact information and details about collection and storage of the samples. Two extra kits with collection materials were given in the case of loss of saliva or contamination. Parents were instructed to collect the samples as follows: 1) the morning sample was taken within 30 minutes of the child waking, but prior to the child eating and brushing his/her teeth (water alone was acceptable); 2) the mid-afternoon sample was taken with the instruction that the child have nothing to eat or drink (except water) in the 30 minutes before collection of the sample; and 3) the evening sample was taken within 30 minutes of the child being put to bed and prior to brushing teeth and at least 30 minutes after eating and drinking (except water). Parents were asked to pour the grains of Kool-Aid followed by the cotton roll into the child’s mouth, and to ask the child to roll the cotton roll around for a one to two minutes to absorb the saliva (if the 6-inch-long roll was used, an adult held one end). After a few minutes when the cotton roll was saturated, the parent was instructed to take the cotton roll out of the mouth of the child, put it into the syringe barrel, and squeeze out the saliva into the collection tube. Approximately 0.5-1.0 milliliters of saliva was requested to be collected for each sampling. The collection tube had volume marks from 0.25 ml to 1.25 ml (every 0.25 ml), and a permanent black marker was used to mark a line in advance at 0.50 ml for ease of reading by the parent at collection time. In addition, the TrackCap® system was used for checking compliance. The cap of the container housing the kits had a microchip to keep a record of when the container had been opened. The total time expected for collection and storage of each sample was between 2 to 5 minutes.

**Storage and analysis:** The saliva containers were stored in the refrigerator at the home until samples were picked up by a research assistant at the end of the recording week. The samples were then stored in a home freezer until they were sent by overnight delivery on dry ice to the research laboratory. Long-term storage was maintained at the laboratory in a freezer at -20°C (freezing precipitates mucins). For this study, the assays were conducted in the laboratories of Salimetrics, Inc. in State College, Pennsylvania. All samples were assayed for salivary cortisol in duplicate using a highly sensitive enzyme immunoassay. The cortisol assay was the Expanded Range Cortisol Determination assay from Salimetrics Inc. The assay uses 25 microliters (ul) of saliva per determination, has a lower limit of sensitivity of 0.003 ug/dl, standard curve range from 0.012 to 3.0 ug/dl, and average intra- and inter-assay coefficients of variation of 3.5 % and 5.1 %, respectively. Method accuracy, determined by spike and recovery is 100.8 %. Linearity determined by serial dilution is 91.7 %. Values from matched serum and saliva samples at the laboratory show a strong linear relationship (r = 0.91, p<0.0001) (personal communication, Mary Curran, Salimetrics, Inc.).
Evaluation of cortisol procedures: It has long been recognized that salivary cortisol has many advantages over serum or plasma cortisol, particularly in studies of young children. Although lower concentrations of cortisol are found in saliva as compared to serum [41], the correlation between saliva and serum ranges from approximately 0.76 to 0.90 [42-44]. In addition, saliva, as opposed to serum, contains most of the analyte in the active free form unbound to corticosteroid binding globulin [41, 45]. Salivary cortisol has a more well-defined diurnal rhythm and exhibits a stronger response to ACTH administration than serum cortisol (salivary cortisol concentration increased four-fold compared to two-fold for serum cortisol concentration) [43]. Therefore, salivary cortisol is considered to be more representative of dynamic HPA-axis activity [46]. Other advantages of salivary cortisol include avoiding a cortisol response related to the stress of the phlebotomy, capacity for home collection in a naturalistic environment, ease of implementation by parents in very young children, and feasibility of serial sampling.

I used a dental-quality cotton roll to absorb saliva for collection. Research on cotton-based sample collection methods indicates that although measurement of other hormones were compromised cortisol results were not affected by this method [47]. The use of non-cotton products, such as eye spears, is also considered to potentially interfere with cortisol determination when salivary stimulants are used [48].

The saliva collection used Kool-Aid as a saliva stimulant, although in older children sugar-free-gums can be used as a stimulant. Many preschool-aged children, especially those with autism, do not have the oromotor capabilities to chew gum. Moreover, there are concerns about swallowing gum and the attendant risk for choking. Other stimulants, such as citric acid, can be used with a salivette or cotton roll and has been shown to be highly correlated with plasma [49]. However, in some immunoassays a low PH may result giving falsely high cortisol results [41]. I chose to use cherry-flavored Kool-Aid based on my review of the methods of previous studies from the Gunnar Laboratory in Minnesota in toddlers and preschoolers [50-53]. Watamura, et al. conducted a comparison of dry cotton rolls and cotton rolls pretreated with Kool-Aid in three adults and sent the rolls to the Salimetrics Laboratory [53]. There was no significant difference between the mean cortisol extracted, nor were there any differences if an electroimmunoassay or radioimmunoassay was used. More recent work from the Gunnar Laboratory suggests that neither Sweet Tarts nor Kool-Aid affected the rank ordering of cortisol values, and that the treated samples were highly correlated with serially collected untreated samples (r=0.90) [54]. Due to some concerns when parental collection methods are used, I chose to make kits up in advance with all of the materials needed for each collection. In this way, a parent would not need to search for materials and I could also control the amount of Kool-Aid used by pre-measuring an amount well under the 1/16th of a teaspoon suggested as a cutpoint to avoid interference with assays [53, 55].

The sample tubes were refrigerated in the subjects’ homes until pickup two to five days later. They were then frozen in a home freezer for up to one month before sending them to a laboratory-quality freezer at the research laboratory. Several studies have examined the effects of temperature on
cortisol integrity in stored saliva. Although saliva samples should be stored frozen, they can be stored at 68 degrees Fahrenheit for four weeks without substantial reduction in cortisol extraction [56]. Clements, et al. split saliva samples in half, freezing one split sample within an hour and keeping the other split sample at a variety of non-frozen temperature conditions [57]. The mean (standard deviation) was 0.67(0.40) µg/dl for the frozen saliva samples and 0.62(0.43) µg/dl for the nonfrozen saliva samples. Aardal, et al. also examined saliva cortisol under different storage and temperature conditions, including storage at room temperature and refrigeration both for seven days [58]. The authors observed stable cortisol concentrations across different conditions, except that refrozen samples after thawing had significantly lower cortisol concentrations.

Other data (see Appendix 5: Table of Demographic and Other Data): Demographic and other characteristics of the child and family, aspects of the child’s health, and inventories regarding child behavior and stress were obtained through parent interview at entry into the Sleep Study. The Hollingshead Four Factor Index of Social Status was used to obtain an overall measure of socioeconomic status through an algorithm which combined education, occupation, gender, and marital status [59]. The Mullen Scales of Early Learning, a standardized developmental test of children 3 months to 60 months of age, was used at baseline to determine the intelligence quotient (IQ) [60]. A two-page questionnaire was self-administered by the parent on every day of the saliva collection (see Appendix 6: Daily Questionnaire). This questionnaire requested information on the child for any events which occurred in the last 24 hours (e.g. use of medications or presence of a cold or fever).

Data analysis

Independent and dependent variables:

The main effect variables for the specific aims related to diagnosis and cortisol were dichotomous: diagnosis (children with autism versus typically developing children); and functional status (children with autism with an IQ less than 55 (extremely low functional status), children with autism with an IQ less than 70 (low functional status) versus greater than or equal to 70 (high functional status). The main effect variables for the specific aims related to sleep and cortisol were both continuous and dichotomous: total sleep in the last 24 hours (in minutes); time awake during the night (in minutes); frequency of night awakenings (0-1 versus 2+ awakenings); time to fall asleep at bedtime (in minutes); presence of night-waking problem in child by Research Diagnostic Criteria (yes versus no); presence of sleep-onset problem in child by Research Diagnostic Criteria (yes versus no); Epworth Sleepiness Scale (ESS) score indicating excessive sleepiness (ESS score greater than or equal to 11 versus less than 11).

Time is a strong independent predictor of cortisol. There is a diurnal curve that starts with the highest or peak cortisol at waking and a nadir at bedtime or in the late evening (see figure re diurnal cortisol in Chapter 3). Therefore, time of day (waking, midday, bedtime) was an important variable to consider when determining the association of the independent variables with the outcome. Time was entered as an independent covariate and/or as an effect modifier depending on the analysis. Some analyses were stratified by time to get specific estimates at a given time of day (e.g., mean cortisol by diagnostic status at waking, midday, and bedtime).
The outcome variable was cortisol measured in nanomoles per liter (nmol/l, continuous) but was natural log transformed for the analyses. Because the standard deviation increased as the mean increased at each of the three times of day, the data was likely to be positively skewed, and a log transformation was performed [61].

**Potential confounders:**

The primary research questions are whether cortisol secretion levels differ between children with autism and typically developing children, and whether sleep quality is associated with levels of cortisol secretion. Covariates that were potential confounders for these research questions were chosen *a priori* by discussion with content experts and from reviews of the literature (See Appendix for description of the covariates). Bivariate analyses were performed with these covariates and the exposure and outcome variables. Using a cutpoint of alpha level 0.10, the remaining covariates that were associated with both the exposures and the outcome, or were strong predictors of the outcome were examined further.

In addition to the main effect variable, the following covariates were part of each dataset used in the DSA modeling process for choosing the best model by cross-validation.

<table>
<thead>
<tr>
<th>Potential covariates for further evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intrinsic biological factors</strong></td>
</tr>
<tr>
<td>• Diagnosis</td>
</tr>
<tr>
<td>• Child age in months</td>
</tr>
<tr>
<td><strong>Features of the longitudinal design</strong></td>
</tr>
<tr>
<td>• Phase</td>
</tr>
<tr>
<td>• Day</td>
</tr>
<tr>
<td>• Time</td>
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<tr>
<td><strong>Sociodemographic factors</strong></td>
</tr>
<tr>
<td>• Hollingshead 4-factor social index</td>
</tr>
<tr>
<td>• Race</td>
</tr>
<tr>
<td>• Mother works</td>
</tr>
<tr>
<td><strong>Direct effects on sleep</strong></td>
</tr>
<tr>
<td>• Nap on day of saliva collection</td>
</tr>
<tr>
<td><strong>“Stress” and other events of the day</strong></td>
</tr>
<tr>
<td>• Count of events during the day</td>
</tr>
<tr>
<td>• Hours of television during the day</td>
</tr>
<tr>
<td>• Total score on the child scale of the parenting stress index</td>
</tr>
<tr>
<td>• Total score on the child behavior checklist</td>
</tr>
<tr>
<td>• Hours of school/therapy per day</td>
</tr>
</tbody>
</table>

**Observational methods:**

The study design was a short longitudinal or prospective study design. There were a maximum of 18 saliva samples (records) for cortisol on each child: three per day (waking, midday, bedtime); on two consecutive days; at three recording phases (baseline, baseline plus three months, baseline plus six months). The GENMOD procedure in SAS (SAS version 9.1, SAS Inc., Cary, NC) was used to fit a generalized linear model to the data by maximum likelihood estimation. A generalized estimating
equations (GEE) approach that considers the repeated measurements was used to obtain variance estimates around the effect estimate. In longitudinal designs, the GEE approach yields robust standard errors for the regression coefficients [62]. The exchangeable or compound symmetry working correlation model was applied.

The coefficients and confidence intervals by GEE were given only to get approximate variability or precision around the effect estimates— they were not provided to determine statistical significance. Causal inference methods (see Causal inference section of chapter) were used for the purposes of addressing the causal research hypotheses. Although cortisol was transformed by the natural logarithm for analysis, the antilog of the standard deviation or standard error is not measured in nmol/l and cannot be back-transformed to the original scale [63]. Therefore, the 95% confidence intervals were back-transformed.

For exploring within-subject variability the linear mixed model approach was used because both fixed and/or random effects could be examined (the MIXED procedure in SAS, SAS version 9.1, SAS Inc., Cary, NC). The fixed effects portion of a “mixed” model represents expected differences within a population, given a change in all subjects’ $X$, (standard regression) and suggests that the effect is the same for all in the population. The random effects portion of a mixed model represents expected differences within a subject, given a change in their $X_i$, and allows random variation between subjects in their response. The following model represents these two aspects of the statistical analysis for the dissertation:

\[
Y_{ij} = \beta_0 + (\beta_{0i} + \beta_{0ij}) + (\beta_1 X_{ij}) + e_{ijk}
\]

$i =$ child; $j =$ time of collection
$Y =$ cortisol secretion level
$X =$ diagnosis (autism versus typically developing)

For example, one specific aim intends to examine whether diagnosis is associated with cortisol secretion. The assumption is that the effect of the diagnosis is universal to all in the population (a fixed effect). However, another specific aim is designed to examine whether there are differences in the within-subject variability between children with autism and typically developing children (a random effect).

Causal inference:

In an ideal situation – a situation that is not possible in the real world every subject would receive an “exposure” or “no exposure” under the exact same conditions. In effect, every outcome in each subject under both exposure regimens delivered at the same time could be observed. In observational studies only one exposure and corresponding outcome is observed for each subject. Thus, we have incomplete or missing data on other potential exposures/outcomes for the subject. Even in the realm of the experiment, such as a crossover trial with sequential treatments, it is impossible to observe an exposure/no exposure effect simultaneously. One approach to simulate the ideal situation from observational data is based on the concept of counterfactuals. The counterfactual outcome distribution represents the observed exposure and outcome as well as those outcomes that would have occurred if, “counter to fact”, the subject received an exposure other than the one actually observed [64]. The missing data problem alluded to earlier is resolved by creating a pseudopopulation of the full
data that would occur if all potential exposures/outcomes occurred [65]. To illustrate how the methods of causal inference are incorporated into my analysis, notation and an example are introduced. Let $Y$ be the outcome; $A$ the treatment; and $W$ the confounders. The counterfactual outcome ($Y_a$) under a binary treatment situation would take on the value $Y_1$ (if $a=1$) and $Y_0$ (if $a=0$). $W$ remains simply as a vector of covariates representing confounding. In the following example, the effects of the treatment (diagnosis) on the outcome (cortisol) are examined. These are the counterfactuals of interest: $Y_a$, outcome $Y$ (cortisol) measured under exposure value $A = a$ (diagnostic status), such that $Y_1 = $ cortisol of child with autism and $Y_0 = $ cortisol of typically developing child. To estimate the marginal causal effect, we also have to consider the set of potential confounders or covariates that might preclude direct examination of the causal effect [66]. The adjusted (pooled) causal effect of $A$ on $Y$ per stratum of $W$ is:

$$E(Y_{a}) = (\text{under assumptions}^1) E_{w} E[Y|A=a] = B_0 + B_1A + B_2W$$

$B_1$ is the causal (marginal) effect of $A$ (having autism) on $Y_{a}$ (cortisol level), after pooling over all strata of confounders, $W$. This is similar to the traditional approach to examining the effect of exposure on an outcome except that $a$ represents the counterfactual distribution of diagnosis (values of 1 and 0). The notation for examining the causal risk difference between diagnostic groups is simply:

$$E(Y_{1}) – E(Y_{0})$$

That is, the causal effect of diagnosis, conditional on the presence of confounders $W$ is the $\beta$ expressed here.

The Deletion/Substitution/Addition (DSA) algorithm:

The Deletion/Substitution/Addition (DSA) algorithm (the R Project for Statistical Computing, copyright © 2000-2009, R Development Core Team) is a data adaptive machine learning procedure designed to select best-fitting models for $E(Y|A,W)$. The algorithm builds a candidate model space through deletion, substitution, and addition of covariates for examination. In addition, the algorithm uses cross-validation to find the best user-defined model over the entire model space based on polynomial generalized linear models. By averaging over several partitions cross-validation is more robust than a single training/validation. The full data set is split into training data (data on which each model is trained) and validation data (each model’s accuracy is tested on this data) to calculate cross-validation risks for different training/validation dataset combinations. DSA uses a loss-function-based (the L2 loss function) estimation procedure to select the model with the best fit [67]. A final cross-validation risk is calculated from the empirical average of the loss to obtain the optimal model from among the best models trialed. In practice, the user supplies the program with specified characteristics to apply in the model-fitting process (see Appendix 7 for syntax example). The final model chosen based on the cross-validation results was used for the purposes of G-computation estimation.

---

1 Under assumptions of g-computation estimation noted later in the chapter
G-computation estimation:

G-computation is one type of estimator used in causal inference methods to examine a causal effect of interest in both point-treatment and time-varying exposure studies [68]. There are four key assumptions that should be met for G-computation techniques to be used [66, 68, 69]. The stable unit treatment value assumption states that the outcome of a subject is independent of the exposure, outcome, or counterfactual of any other subject. As with standard regression techniques, there should be no contamination between subjects that render their experiences non-independent. The consistency assumption states that the observed data on a subject is a member of the set of all potential counterfactual outcomes in the full data. The observed outcome for a subject may also be his/her counterfactual outcome under the same exposure. The sequential randomization assumption considers that there are no unmeasured confounders for the exposure and that, within strata of a covariate set, the exposure is randomized with respect to the set of counterfactual outcomes. Whereas a researcher is never certain that all confounders are measured, a thorough approach to obtaining a set of covariates with some breadth and with attention to a causal diagram is usually considered sufficient. The temporal ordering assumption is achieved by assuring that the confounders occurred before the exposure and exposure occurred before the outcome. In longitudinal studies this condition is more easily met by virtue of the prospective data collection than is the case for cross-sectional or retrospective designs. The experimental treatment assignment assumption states that all exposures, given covariates, are possible for all members of the target population. In practical terms, all subjects should have a probability between zero and one of being exposed or unexposed within subgroups of covariates. The implications of violation are that one may be making untenable assumptions by extrapolating beyond the range of the observed data. One other consideration is that because G-computation is a likelihood-based estimator, it is dependent on the accurate specification of the outcome model.

For the purposes of the dissertation, I was interested in the marginal estimates of cortisol for different levels of exposure that may be continuous or dichotomous. For example, I wanted to obtain the marginal estimates of cortisol at waking, midday, and bedtime for children with autism compared to typically developing children. The first step in G-computation is to estimate the association between the exposure of interest and the outcome adjusted for relevant covariates using a technique to formulate the best fitting model. For the purposes of the dissertation, I chose to use a DSA algorithm as described earlier in this chapter. The second step is to reassign the exposure to the counterfactual values leaving all other covariate values the same. The next step is to take the chosen model and use it for prediction of the counterfactual estimates that result from manipulation of the exposure assignment distribution, using maximum likelihood techniques (the R Project for Statistical Computing, copyright © 2000-2009, R Development Core Team). A cortisol level is obtained as if the subject experienced that imputed counterfactual exposure value. The observed study population is actually doubled to create the full pseudopopulation dataset. All subjects now have each exposure history – the observed and the counterfactual. Finally, an average of the imputed cortisol levels across the sample is taken to represent the predicted cortisol level if all subjects were similarly exposed to the counterfactual exposure (i.e. if counter-to-fact the set level of exposure had occurred in the whole sample). This gives a
marginal estimate for cortisol in the original observed exposure state and the counterfactual exposure state for each subject. This is also referred to as the marginal causal estimate - the marginal (unconditional population-level) estimate of exposure on outcome (see Appendix 7 for syntax example). Confidence intervals were obtained around the marginal estimate using cluster bootstrapping (N=1000 repetitions). All means were back-transformed from the natural log values to the original units of cortisol.
Table 1. Studies evaluating the ADOS-G and the ADI-R separately

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study size</th>
<th>Comparison group definitions</th>
<th>Age range</th>
<th>Inter-rater reliability</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lord, 2000[2] (original study)</td>
<td>N=20 (AUT) N=18 (PDD) N=16 (NS)</td>
<td>GS = consensus clinical diagnosis based on clinical impressions of child by clinical psychologist and child psychiatrist NS = MR, ADHD, ODD, OCD, anxiety, depression, TYP</td>
<td>15 months – 10 years</td>
<td>0.82 – 0.93 (range over domains)* 0.65 – 0.78 (range over domains)†</td>
<td>range over domains 0.93 – 1.0</td>
<td>range over domains 0.93 – 1.0</td>
<td>range over modules 0.87 – 1.0</td>
<td>range over modules 0.68 – 0.79</td>
</tr>
<tr>
<td>de Bildt, 2004[20]</td>
<td>N=48(AUT) N=136 (PDD/ ASD/NS)</td>
<td>GS = consensus clinical diagnosis based on clinical impressions of child by clinical psychologist and child psychiatrist PDD/ASD/NS = All MR (2/3 PDD; 1/3 MR only)</td>
<td>5 – 20 years</td>
<td></td>
<td>0.92</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mazefsky, 2006[23]</td>
<td>N=32(AUT) N=24(PDD) N=19(NS)</td>
<td>GS = evaluation team (as above + speech pathologist, education specialist, occupational therapist) PDD = PDD not otherwise specified, Asperger syndrome) NS = language disorders; disorders outside spectrum (anxiety, ADHD)</td>
<td>2 – 8 years</td>
<td>1.0</td>
<td>0.94</td>
<td>0.97</td>
<td>0.77</td>
<td></td>
</tr>
</tbody>
</table>

* Intraclass correlation coefficients
† mean weighted kappa coefficients
GS = gold standard; NS = non-spectrum; OTH = other comparison group; ASD = autism spectrum disorder; PDD = pervasive developmental disorder; MR = mentally retarded; ADHD = Attention deficit hyperactivity disorder; ODD = oppositional defiant disorder; OCD = obsessive compulsive disorder; TYP = no disorder
Table 1 (cont.). Studies evaluating the ADOS-G and the ADI-R separately

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study size</th>
<th>Comparison group definitions</th>
<th>Age range</th>
<th>Inter-rater reliability</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lord, 1993 [5] (original study)</td>
<td>N=51(AUT) N=30(NS)</td>
<td>GS = child psychologist or child psychiatrist NS = mentally handicapped, language impaired</td>
<td>2 – 6 years</td>
<td>0.62 – 0.89 (range over domains)**</td>
<td>0.98</td>
<td>0.93</td>
</tr>
<tr>
<td>Lord, 1994[6]</td>
<td>N=25(AUT) N=25(NS)</td>
<td>GS = child psychologist or child psychiatrist NS = mentally handicapped, language impaired</td>
<td>3 – 5 years</td>
<td>0.93 - 0.97 (range over domains)* 0.73 – 0.78 (range over domains)†</td>
<td>0.96</td>
<td>0.92</td>
</tr>
<tr>
<td>de Bildt, 2004[20]</td>
<td>See ADOS-G</td>
<td>See ADOS-G</td>
<td>See ADOS-G</td>
<td></td>
<td>0.77</td>
<td>0.63</td>
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<tr>
<td>Mazefsky, 2006[23]</td>
<td>See ADOS-G</td>
<td>See ADOS-G</td>
<td>See ADOS-G</td>
<td></td>
<td>0.75</td>
<td>0.72</td>
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</tbody>
</table>

*Intraclass correlation coefficients
†Mean weighted kappa coefficients

GS = gold standard; NS = non-spectrum; OTH = other comparison group; ASD = autism spectrum disorder; PDD = pervasive developmental disorder; MR = mentally retarded; ADHD = Attention deficit hyperactivity disorder; ODD = oppositional defiant disorder; OCD = obsessive compulsive disorder; TYP = no disorder
<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study size</th>
<th>Comparison group definitions</th>
<th>Age range</th>
<th>Percent agreement/ weighted kappa</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>de Bildt, 2004[20]</td>
<td>N=48(AUT) N=136(PDD/ ASD/NS)</td>
<td>GS = consensus clinical diagnosis based on clinical impressions of child by clinical psychologist and child psychiatrist PDD/ASD/NS = All MR (2/3 PDD; 1/3 MR only)</td>
<td>5 – 20 years</td>
<td>0.83/ 0.67</td>
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<td></td>
</tr>
<tr>
<td>Risi, 2006[22] (US sample)</td>
<td>N=443 (AUT) N=361 (PDD/ASD/NS)</td>
<td>GS = consensus best estimate diagnosis based on clinical impressions of child by clinical psychologist and child psychiatrist NS = language disorder or learning disability</td>
<td>3+ years</td>
<td>0.82</td>
<td>0.86</td>
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<tr>
<td>Risi, 2006[22] (Canadian sample)</td>
<td>N=142 (AUT) N=36 (PDD/ASD/NS)</td>
<td>GS = consensus best estimate diagnosis based on clinical impressions of child by clinical psychologist and child psychiatrist NS = language disorder or learning disability</td>
<td>3+ years</td>
<td>0.77</td>
<td>0.75</td>
<td></td>
</tr>
</tbody>
</table>

*weighted kappa coefficients (1 for exact agreement, 0.5 if one rater scored autism and the other PDD/ASD/NS, and 0 in all other cases GS = gold standard; NS = non-spectrum; ASD = autism spectrum disorder; PDD = pervasive developmental disorder; MR=mentally retarded
### Table 3. Studies evaluating actigraphy against other methods of objective sleep measurement

#### Actigraphy versus Polysomnography

<table>
<thead>
<tr>
<th>Author, year, study population</th>
<th>Actigraphy type</th>
<th>Age range (N)</th>
<th>Percent agreement for both sleep/wake</th>
<th>Percent agreement for sleep</th>
<th>Percent agreement for waking</th>
<th>Correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sadeh, 1989[31], healthy children</td>
<td>Ambulatory Monitoring, Inc. x 1 night</td>
<td>3 - 13 years (N=11)</td>
<td>89.9</td>
<td>92.9</td>
<td>66.0</td>
<td>r = 0.81 (sleep efficiency)</td>
</tr>
<tr>
<td>Sadeh, 1991[32], children referred for sleep problems</td>
<td>Ambulatory Monitoring, Inc. x 1 night</td>
<td>1 - 4 years (N=11)</td>
<td>85.3 (range = 75.7 to 92.5)</td>
<td>87.7 (range = 80.0 to 98.1)</td>
<td>76.9 (range = 53.9 to 86.0)</td>
<td>r = 0.72 (total sleep time); r = 0.56 (sleep efficiency)</td>
</tr>
<tr>
<td>So, 2005[33], term and preterm infants</td>
<td>AW-64 Mini-Mitter, Inc. x 1 episode of sleep (naptime or nighttime)</td>
<td>5 - 6 months (N=11)</td>
<td>90.9 (1.6)%</td>
<td>91.9 (1.9)%</td>
<td>30.7 (10.6)%</td>
<td>n/a</td>
</tr>
<tr>
<td>Insana, 2009[34], healthy infants</td>
<td>AW-64 Mini-Mitter, Inc. x 1 night</td>
<td>13 - 15 months (N=22)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>r = 0.87 (total sleep time); actigraph underestimated total sleep time by 27 minutes*</td>
</tr>
</tbody>
</table>

#### Actigraphy versus Videosomnography

<table>
<thead>
<tr>
<th>Author, year, study population</th>
<th>Actigraphy type</th>
<th>Age range (N)</th>
<th>Agreement rate for both sleep/wake</th>
<th>Sensitivity for detection of sleep</th>
<th>Specificity for detection of sleep</th>
<th>Correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sitnick, 2008[37], children with autism, children with developmental disabilities, typically developing children</td>
<td>AW-64 Mini-Mitter, Inc.</td>
<td>2 - 5 ½ years (N=58)</td>
<td>94.6 (5.3)% Weighted agreement† 89.4 (8.2)%</td>
<td>97.6 (3.1)%</td>
<td>24.3 (23.0)% Adjusted specificity‡ 27.3 (20.9)%</td>
<td>r = 0.50 (sleep latency) r = 0.67 (total sleep time) r = 0.25 (number of wakings) r = 0.43 (minutes awake after sleep onset)</td>
</tr>
</tbody>
</table>

* Bland-Altman concordance technique
† Weighted agreement by prevalence-adjusted bias-adjusted kappa (PABAK)
‡ Adjusted specificity by PABAK
Appendix 1: Human Subjects

Population Sample:
Subjects for this study are a total of 58 boys and girls 2 to 5 ½ years of age. These will be non-institutionalized children with autism and typically developing children, none of which will have any serious health problems precluding their involvement in this research. Children are the focus of this proposal because of the dearth of data available on correlates of sleep and neuroendocrine function in young children. Sleep problems are a major complaint amongst the parents of children with autism in this age group, and the solutions to these problems may lie in gaining a better understanding of the neurodevelopmental function of this population. However, the children with autism are especially vulnerable populations, and it is important to consider the burdens that might already be placed on families in terms of their time and activities related to clinical care of these children. With this in mind, recruitment, enrollment, data collection, and follow-up procedures have been tailored to obtain only the essential involvement and data to be addressed by the hypotheses.

Sources:
The sources of research material planned in the larger Sleep Disorders in Children with Autism Study are the following: demographic, sleep, and health characteristics from parent report; sleep behavior from actigraphy (a sleep watch) and videosomnography (videotaping of sleep); cognitive and developmental function measures on the children. I will have access to the material above for my research purposes, and in addition, I will be collecting saliva samples for cortisol assessment and questionnaire information on aspects of the child’s day or night that might influence their cortisol samples. Existing data will be used as part of the collaboration with the NIEHS Center project for recruitment of subjects, and informed consent will be obtained at entry to review pertinent clinical records and to share this existing data.

Exemption Categories:
Not applicable. No exemption is being sought.

Protection of Human Subjects:
1. Risks to the Subjects

There are minimal risks involved in participation in this study. Children with autism often have difficulty with novelty and new situations. Some of the testing, wearing an actigraph, and sampling saliva may represent such novel situations. Children will be unfamiliar until they become accustomed. In piloting the methods for this study, parents relayed that children became more accepting of the cotton roll as early as the 2nd or 3rd sampling. No families withdrew from piloting, and there were no concerns voiced about the safety of the procedures. Only one family has refused participation due to sensory defensiveness. All sampling will be carried out in the presence of parents and/or tutors, and these accompanying adults will assist with finding the approach to the child that is most comfortable for the child. If children strongly resist or reject the cotton roll at the demonstration, staff will not persist, regardless of the desire on the part of the parent to be helpful to the study. However, because of the young developmental
Appendix 1: Human Subjects (continued)

and chronological age of the children, staff will need to defer to the parent’s decision-making in most instances. The parent is their child’s best protector, the most capable in interpreting non-verbal cues indicating distress, and will be the saliva collector in the home setting, and need to feel that they and their child are comfortable with the procedures. To assuage any concerns about attempts to swallow the cotton roll, we will use a method involving a much longer cotton roll for collection from the youngest children in this study between 2 and 3 years of age. The method consists of using a 6-inch-long cotton roll, in which one end is chewed or rolled around in the mouth by the child, and the other end is held during sampling. Families may withdraw from this study at any time without compromising their participation in the Sleep Disorders in Children with Autism Studies or their care at UC Davis.

2. Adequacy of Protection Against Risks
Recruitment of subjects will occur following consent to the Sleep Disorders in Children with Autism Study. Once families are enrolled in that study, I will arrange to explain this study to families either in-person at one of the clinic visits or by telephone prior to the week of baseline data collection. If parent(s) agree(s) to enroll, I will describe my study in greater detail, outlining the procedures to be performed and training the parent(s) in the collection of saliva. Written informed consent will be obtained from both parents wherever possible. Parents will be provided with a subject’s bill of rights and a copy of the consent form. There is limited risk associated with the proposed study protocol. However, all procedures will be discontinued at the request of the child subject or the child’s care provider. In the unlikely event of an adverse event, the Principal Investigator (P.I.) will contact the Human Subjects Committee of UC Davis and follow standard procedures for the reporting, care and management of research participants.

All saliva specimens, and questionnaire and other data collected from children will be identified by a human subjects number (HSN) number and all personal identifiers will be removed. A separate list with HSN and identifying information linked will be kept under lock and key, as will all research materials, in the offices of Thomas Anders, MD (P.I., Sleep Disorders in Children with Autism Study) at the M.I.N.D. Institute at UC Davis. No information will be released about any subject without the explicit written consent of the subject or the parent(s).

3. Potential Benefits of the Proposed Research to the Subjects and Others
There are no immediate benefits from participation in this study. The results from this study should provide information that will assist in fine-tuning and targeting future research hypotheses with respect to sleep and cortisol diurnal rhythm, and may assist in identifying the most appropriate interventions.

4. Importance of the Knowledge to be Gained
This may be the first study to examine daytime cortisol secretion with respect to sleep onset delay. Children with autism may have an altered cortisol diurnal rhythm due to an underlying neurobiological defect, or it may be as a result of the sleep onset delay. This study will go far in describing sleep and cortisol secretion.
Appendix 1: Human Subjects (continued)

among children with autism, as well as that of typically developing children. The risks to subjects are minimal as compared to the gain expected by increasing the scientific knowledge base of sleep and cortisol dysregulation.

Collaborating Site(s):
The project has been reviewed and approved by the Institutional Review Boards (IRBs) of the University of California, Davis and the University of California, Berkeley.

Women and Minority Inclusion in Clinical Research:
This study will be enrolling children for the purposes of addressing the research question. There are no data to suggest that minorities are under- or over-represented in the population of children with autism. The Targeted/Planned Enrollment Table portrays the number of participants projected for this study as a subsample of a larger study which aims to reflect the demographic composition of the greater Sacramento region. If adequate ethnic representation is not attained by the Sleep Disorders in Children with Autism Study, additional children will be sought by the investigators. A 4-6:1 male ratio can be expected, given that autism affects males predominantly. Therefore, more males than females will be expected to be eligible for the study. Since children for this study are drawn initially from California Regional Centers, which have a legislative responsibility to identify all children with autism, most preschool-aged children with these diagnoses will be captured in the NIEHS study. If refusal to participate is rare, the current study will have the same characteristics proportionally as the NIEHS Study, which is designed to be a representative sample.
Appendix 2: Enrollment Flow

Number approached: 83
Number ineligible: 3 (1 AUT - no Spanish language consent for nanny; 1 AUT – seizure disorder; 1 DD – not AUT after testing)
Number refused: 10
• oral sensory probs (3 AUT, 1 TYP)
• too much of a burden (3 AUT, 3 TYP)
Number dropped-out (without data) after consent: 12
• child would not comply at demonstration visit (6 AUT, 6 TYP)
Number enrolled: 58 (28 AUT, 30 TYP)
• Number with 1 phase completed: 49 (25 AUT, 24 TYP)
• Number with 2 phases completed: 42 (21 AUT, 21 TYP)
• Number completed study (3 phases): 32 (17 AUT, 15 TYP)
• Number dropped out (with data): 6 [1 TYP withdrew from Sleep Study due to eczema and sleep watch irritation (only have phase 1 data); 1 TYP withdrew from Sleep Study due to chaotic family circumstances (only have cortisol from phase 2 – no sleep data); 1 AUT withdrew from Sleep and Cortisol Study – child wanted to eat cotton at phase 2 (only have phase 1 data); 1 TYP withdrew from Sleep Study due to burden of study requirements (only have phase 1 data); 1 AUT withdrew from Sleep Study due to child resisting wearing watch at phase 2 (only have phase 1 data); 1 TYP withdrew from Sleep Study due to chaotic family circumstances (only have phase 2 data)]
• Number withdrawn due to too much missing data: 3 (1 AUT, 2 TYP)
• Number withdrawn due to corticosteroid use: 3 (1 AUT; 2 TYP)
• Number in final analyses: 52 (26 AUT, 26 TYP)
Appendix 3: Enrollment of Subjects into SACS

Recruitment
1. Obtain names of potential subjects from the Timeline printout of the Sleep in Autism Study (the host study).
2. Discuss the contact of the subject’s family with the research assistant responsible for the host enrollment. Use this information to assist you with the contact. Contact ALL potential families regardless of the possible resistance of the subject and/or family. Do NOT contact subjects if the host study is worried about the family dropping out.
3. After subject has signed the consent forms for the host study (or in some rare instances before this), phone the subject’s family to discuss the study (can use Telephone Script) and to make an appointment for a demonstration of the procedures and consenting.
   - Start with a brief description of the purpose of the study
   - Explain how the saliva collection is done using the Kool-Aid and cotton roll
   - Describe the flow of the data collection (3 times a day over 2 consecutive days)
   - Let the family know that you will come to their home so that they don’t need to make a special trip in to the MIND Inst.
     - If the family lives far away or if it is difficult to see them outside of the Sleep Study appt., make an appt. to do the demonstration immediately following the scheduled Sleep Study appt.
   - Let the family know that the visit should not take more than 25 minutes, and that you only need 1 to 2 minutes of the child’s time at the end of the visit. The bulk of the time is spent going through the procedures and signing the consents.
   - Find out the important details for scheduling the collection:
     - Which parent would be doing the collections? Sometimes it is a shared responsibility, but you want to know who will be doing the mid-day collection.
     - Does this parent work outside the home? If so, is he/she home for the mid-day collection (anytime between 1:00 and 3:00pm, but 2:00pm preferred)?
     - Is the subject in preschool or out of the home during the day? Does this make the mid-day saliva collection impossible?
     - Weekdays are preferred over weekends. Try to secure a commitment to weekday collection rather than weekend, where possible.
     - Problem solve with the parent to determine the collection days and to discuss any other pertinent issues.
4. Once a commitment is made, schedule the family on the SACS timeline and on the month-at-a-glance calendar sheet.
Appendix 3: Enrollment of Subjects into SACS (continued)

Putting a kit together

5. Make up the kit a day or two in advance of the appointment (or for continuing subjects, in advance of the drop-off).
6. Use the "Kit Checklist" to tell you what you need in the kit. Here is the summary:
   • In a Ziploc gallon bag, you will put the following items:
     • 1 Parent Instructions brochure (parent_instructions_new)
     • 1 Daily Diary for recording of information on samples (daily_diary_clean). Record subject ID on both sides of form.
     • 1 Daily Questionnaire for recording illnesses and medications (daily_quaire_new). Record subject ID on both sides of form.
     • 2 permanent ink black freezer-resistant pens for writing on small collection tubes
     • 1 Ziploc snack bag labeled “Saliva Collection Tubes” to store samples in refrigerator
     • 1 Ziploc snack bag labeled “Extra Materials” with the following: 2 extra collection tubes, 2 extra cotton rolls, 2 extra needleless syringes with plungers, 2 scoopers with Kool-Aid
     • 1 TrackCap container (Do not open container until you are ready to get 1st sample) OR quart-sized bag labeled “TrackCap Container” with the 6 ziploc bags inside.
       o 6 Ziploc snack bags inside TrackCap container – each containing the following: 1 needleless syringe with plunger, one cotton roll, 1 scooper with Kool-Aid, 1 collection tube
       o Each collection tube should have the ID number recorded on it using a freezer-resistant pen. Also, the 0.50 mark on the tube should be highlighted with the pen.
   • In addition:
     o 2 sets of primary consent forms (2) and HIPAA consent forms (2) should be included in the gallon bag

The demonstration visit

7. At the time of the demonstration visit (or following the Sleep Study visit):
   o Remind the family how much time the visit will take
   o Give the parent one copy of the primary consent to look at, and you use the other copy, so that you can both go through the details of the study together. This allows you to go through the consent and tackle the explanation of the procedures at the same time.
   o Briefly address all of the sections of the primary consent before asking the parent to sign. Make sure that signatures and initials are complete for all pages of the consent AND for both copies. Briefly explain that
Appendix 3: Enrollment of Subjects into SACS (continued)

the HIPAA consent is exactly the same as the Sleep Study version, and that it allows the studies to share data with each other.

- At some point, go through and discuss all of the items in the kit bag.
  
  Begin the demonstration:
  
  - Make sure that you go through the consent and get signatures before demonstrating the saliva collection.
  
  - Remove one of the kits in the container (there will actually be 7 plastic bags because you will be using one for the demonstration). Open the bag and take out everything and get each piece ready to use (i.e. take the plunger out of the syringe, take the cap off of the microtube). Put these on a hard surface so that the microtube won’t fall over.
  
  - Obtain the cooperation of the child. Tell them that you have a little sweet cherry powder that tastes like candy and you want to sprinkle it into their mouth. After that you will tell them that you are putting the cotton roll into their mouth for them to chew or gently roll around to get very wet. I often go into what will happen next, even for those children who are lower functioning. I tell them that we will squeeze the spit out of the cotton into the tube and that they can help and/or watch. Make sure you are in control of this process, even if they help you hold something. Letting them help with the procedure can gain their interest and compliance.

  - Potential problems:
  
    - Children can be resistant at both the Kool-Aid and cotton roll insertions, although generally, when the Kool-Aid is tasted it tends to soften the resistance to the cotton roll.
    
    - Take the cue from the parent as to how hard they want to push getting the child to open his/her mouth. Some parents will be very forceful and others will be very passive. If everyone is getting stressed OR after a minute you are not making any progress on getting the cotton in OR the parent wants to stop, then let them know that this would be too difficult to try to do over several days and thank them for their time. Make sure you reassure them that this happens quite a bit and that all you needed to do was to give it a good try and that you are very grateful for their help. Parents tend to feel like failures when this happens and you need to make them feel OK about the refusal by the child.

After the demonstration:

- The child will either be able to do the collection or not – it is that simple.
- If you are successful, then summarize the important details and make sure you have decided on a start date before you leave.
Appendix 3: Enrollment of Subjects into SACS (continued)

- Be sure to take one set of the consent forms back with you to the office, and leave the other set. Remind the parent that they should KEEP their set and NOT return it in the kit. If they do return it, then I just file it with my set in the binder.

Data collection

Follow-up with the family:

- If the start date is not the next day, then let them know you will call them the late afternoon before the 1st day of collection to remind them to get started.
- Tell the parent that you will call them about a half hour before the planned mid-day collection on the 1st day to remind them and to check if they have any questions or are experiencing any problems. If they do not answer the phone, just leave a message and tell them that they don’t need to call you back unless they have any questions. Ask the parent at the demonstration whether they feel that they would need more than the afternoon reminder each day. Very rarely do people ask for more reminders, but I take their cue as to whether they need them. It is better to harass them with phone calls than to miss the collection times.
- Make a reminder phone call one half hour before the mid-day collection on the 2nd day of collection (and any other calls if agreed upon).
- Make one last phone call on the day before (or the day of) the PEP-R, if that is when they are bringing the actigraph back, to remind them to bring the cortisol kit and the tubes in the refrigerator back to the office when they come for the appt. They DO NOT need to put the tubes on ice or with cold packs – just ask them to put them in the gallon-sized bag. Thank them for their participation at this phase and tell them that you will be in touch at the next phase (if there is one).
## Appendix 4: Table of Demographic and Other Data

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Variable Name</th>
<th>Time of data collection</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of entry into study</td>
<td>TODAY</td>
<td>Baseline</td>
<td></td>
</tr>
<tr>
<td>Maternal age</td>
<td>MOTHAGE</td>
<td>Baseline</td>
<td></td>
</tr>
<tr>
<td>Paternal age</td>
<td>FATHAGE</td>
<td>Baseline</td>
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</tr>
<tr>
<td>Marital status</td>
<td>MARSTAT</td>
<td>Baseline</td>
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</tr>
<tr>
<td>Child DOB</td>
<td>DOB</td>
<td>Baseline</td>
<td></td>
</tr>
<tr>
<td>Child age</td>
<td>AGEMOS</td>
<td>Baseline</td>
<td>AUT 5 months older on average</td>
</tr>
<tr>
<td>IQ</td>
<td>IQ</td>
<td>Baseline</td>
<td></td>
</tr>
<tr>
<td>Intellectual impairment (yes/no)</td>
<td>FUNCT</td>
<td>Baseline</td>
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</tr>
<tr>
<td>GI problems</td>
<td>GIDXGI</td>
<td>Baseline</td>
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<td>Health status of child</td>
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<td>Baseline</td>
<td>All children described as in good/excellent health</td>
</tr>
<tr>
<td>Gender</td>
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<td>Baseline</td>
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<tr>
<td>Gestational age</td>
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<td>Adults in household</td>
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<tr>
<td>Mother employed (ft., pt., student, etc.)</td>
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<td>Baseline</td>
<td></td>
</tr>
<tr>
<td>Paternal occupation (ft., pt., student, etc.)</td>
<td>FATHEMP</td>
<td>Baseline</td>
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</tr>
<tr>
<td>Maternal/Paternal occupation</td>
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<td>By hand from CBCL</td>
<td></td>
</tr>
<tr>
<td>Ethnicity</td>
<td>ETHNICIT</td>
<td>Baseline</td>
<td></td>
</tr>
<tr>
<td>Maternal education</td>
<td>MOTHEDU</td>
<td>Baseline</td>
<td></td>
</tr>
<tr>
<td>Paternal education</td>
<td>FATHEDU</td>
<td>Baseline</td>
<td></td>
</tr>
<tr>
<td>Stress in last year</td>
<td>STRESS</td>
<td>Baseline</td>
<td>Very few positive responses, mostly death of family friend</td>
</tr>
<tr>
<td>Hollingshead 4-Factor Index of Social Status</td>
<td>HOLLSES</td>
<td>Baseline</td>
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</tr>
<tr>
<td>Income</td>
<td></td>
<td>From STS</td>
<td>Only 1/3 of families with data</td>
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</tbody>
</table>
Appendix 4: Table of Demographic and Other Data (continued)

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Variable Name</th>
<th>Time of data collection</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternative treatments</td>
<td>ALTP1D1-ALTP3D2</td>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>FEVP1D1-FEVP3D2</td>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>Special diet</td>
<td>DIETP1D1-DIETP3D2</td>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>Acute illnesses</td>
<td>COLDP1D1-COLDP3D2</td>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>Daycare/other care (yes/no)</td>
<td>CAREP1D1-P3D2</td>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>School/tutoring (yes/no)</td>
<td>SCHLP1D1-P3D2</td>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>Total hours per day of school/tutoring and daycare/other care</td>
<td>THRSP1D1-P3D2</td>
<td>Days</td>
<td>Combined to get more range, but dichotomous variable better</td>
</tr>
<tr>
<td>Total hours per day of care+school (&lt;=3 vs 4+)</td>
<td>THRDP1D1-P3D2</td>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>School (yes/no)</td>
<td>SHRSP1D1-P3D2</td>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>School (&lt;&lt;=3 vs 4+)</td>
<td>SHRDP1D1-SHRDP3D2</td>
<td>Days</td>
<td>Looks like a better separator of groups</td>
</tr>
<tr>
<td>Count of events per day (ordinal)</td>
<td>COUNP1D1-P3D2</td>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>Count of events per day (0-1 vs 2-5)</td>
<td>C1P1D1-P3D2</td>
<td>Days</td>
<td></td>
</tr>
<tr>
<td>Any medications</td>
<td>MEDSP1D1-MEDSP3D2</td>
<td>Days</td>
<td>No differences between groups</td>
</tr>
<tr>
<td>Asthma, allergies, cold, sleep meds</td>
<td>ASTHP1D1-ASTHP3D2; ALLEP1D1-ALLEP3D2; COLP1D1-COLP3D2; SLEEP1D-SLEEP3D2</td>
<td>Days</td>
<td>No differences between groups (roughly 2-3 subjects taking any specific medication on any data collection day)</td>
</tr>
<tr>
<td>Hours/day of TV</td>
<td>NEWTV1-3</td>
<td>Phases</td>
<td></td>
</tr>
<tr>
<td>Child Behavior Checklist (CBCL)</td>
<td>CBCLINT1-3</td>
<td>Phases</td>
<td>NB: Strong correlation with PSI</td>
</tr>
<tr>
<td>Parenting Stress Inventory (PSI)</td>
<td>PSICHIL1-3</td>
<td>Phases</td>
<td>NB: Strong correlation with CBCL</td>
</tr>
</tbody>
</table>
Appendix 5: Daily Questionnaire

ID Number: ____________

DAILY QUESTIONNAIRE (DAY 1)

QUESTIONS TO BE ANSWERED AT THE END OF EACH SAMPLING DAY:

Today’s date: _____/_____/______

month day year

In the last 24 hours, did your child have symptoms of a cold or flu?:

Yes _____ No _____

If YES: Could you describe what kind of illness and/or its symptoms?:
________________________________________________________________________
________________________________________________________________________

In the last 24 hours, did your child have a fever of 100 degrees or more as determined by a thermometer?:

Yes _____ No _____

In the last 24 hours, did your child take any medications? This includes pills, inhalers, and shots, whether prescribed by a doctor or purchased without prescription:

Yes _____ No _____

If YES: Could you give the name of the medication, and the amount and frequency?:

1. ______________________________________________________________________

2. ______________________________________________________________________

3. ______________________________________________________________________

In the last 24 hours, did your child take any alternative treatments, other than vitamins and minerals?:

Yes _____ No _____

If YES: Could you describe what kind of treatments, and the amount and frequency?:

1. ______________________________________________________________________

2. ______________________________________________________________________

In the last 24 hours, was your child on any special diet?:

Yes _____ No _____

If YES: Could you describe what kind of diet?:
________________________________________________________________________

Was your child in any CHILDCARE (other than your care or school/tutoring of some kind) TODAY? This would include home daycare, babysitter/nanny, or family outside the home.

Yes _____ No _____

If YES: How many hours was your child in another’s care today?: ______ hours

Was your child in any SCHOOL/TUTORING/Therapy TODAY? This would include preschool, home tutoring, behavioral or other therapy?

Yes _____ No _____

If YES: How many hours was your child in this type of setting today?: ______ hours
Appendix 5: Daily Questionnaire (continued)

Please tell me whether your child participated in any of the following activities TODAY?:

Shopping/errands: Yes _____ No_____ Recreation class (like tumbling, music, etc.): Yes _____ No_____ Visiting friends/playdate: Yes _____ No_____ Television/movie/video watching: Yes _____ No_____ Going to park/playground: Yes _____ No_____ Other activity: Yes _____ No_____ If YES: Please describe: __________________________

ID Number: ____________

DAILY QUESTIONNAIRE (DAY 2)

QUESTIONS TO BE ANSWERED AT THE END OF EACH SAMPLING DAY:

Today’s date: _____/_____/______ month day year

In the last 24 hours, did your child have symptoms of a cold or flu?:
Yes _____ No _____
If YES: Could you describe what kind of illness and/or its symptoms?:
________________________________________________________________________
________________________________________________________________________

In the last 24 hours, did your child have a fever of 100 degrees or more as determined by a thermometer?:
Yes _____ No _____

In the last 24 hours, did your child take any medications? This includes pills, inhalers, and shots, whether prescribed by a doctor or purchased without prescription:
Yes _____ No _____
If YES: Could you give the name of the medication, and the amount and frequency?:
1. _______________________________________________________________________
2. _______________________________________________________________________
3. _______________________________________________________________________ 

In the last 24 hours, did your child take any alternative treatments, other than vitamins and minerals?:
Yes _____ No _____
If YES: Could you describe what kind of treatments, and the amount and frequency?:
1. _______________________________________________________________________
2. _______________________________________________________________________

In the last 24 hours, was your child on any special diet?:
Yes _____ No _____
If YES: Could you describe what kind of diet?:
____________________________________________________________________
Appendix 5: Daily Questionnaire (continued)

Was your child in any CHILDCARE (other than your care or school/tutoring of some kind) TODAY? This would include home daycare, babysitter/nanny, or family outside the home.
Yes ______ No ______
   If YES: How many hours was your child in another's care today?: ______ hours

Was your child in any SCHOOL/TUTORING/Therapy TODAY? This would include preschool, home tutoring, behavioral or other therapy?
Yes ______ No ______
   If YES: How many hours was your child in this type of setting today?: ______ hours

Please tell me whether your child participated in any of the following activities TODAY?:
Shopping/errands: Yes _____ No _____  Recreation class (like tumbling, music, etc.): Yes _____ No _____
Visiting friends/playdate: Yes _____ No _____  Television/movie/video watching: Yes _____ No _____
Going to park/playground: Yes _____ No _____
Other activity: Yes _____ No _____  If YES: Please describe: __________________________
                                                   __________________________

Currently, how many children other than this child, live in your home?: ________ child(ren)

Currently, how many adults other than you, live in your home?: ________ adult(s)
Appendix 6: Saliva Packaging and Mailing Directions

Preparing for shipment:
1. Call or email contact at Salimetrics about a week ahead to determine if day you want to send samples is suitable. Plan for agreed-upon date.
2. Next, send a dual email to contacts at both UCB and at Salimetrics requesting that cortisol kits are ordered and charged to faculty advisor (to be equivalent to $3 per sample). Price will vary slightly because it is based on number of kits being purchased. Number of kits depends on number of samples being sent.

Obtaining boxes and dry ice:
1. Obtain styrofoam cases and cardboard boxes from outside VLSB.
2. Obtain dry ice from contact at local UCB-connected laboratory. Give contact a week’s notice.

Preparing for packaging:
At home:
1. Get FedEx slip in advance from contact in faculty advisor’s group. This is pre-made with billing information, etc. Make sure that “dry ice” is noted.
2. Fill in the Salimetrics order form (see photocopy of previously sent form).
3. Fill in the Dry Ice handling form and cut out the square and apply to the outside of the cardboard box (see specific instructions on form).
4. Determine which Sarstedt freezer boxes are being sent (e.g. #1 and #2?)
5. Make sure that samples and sample number are the same via checking the freezer box and the respective saliva log sheet
6. Tape closed or put rubber band around each freezer box to reduce juggling of samples.
7. Enclose the freezer box(es) in a Ziploc gallon-sized plastic bag (for waterproofing).
8. Insert the respective saliva log sheets and Salimetrics order form into another plastic bag (again, for water proofing).
9. Take off the Styrofoam lid on the container within the cardboard box and put the bag of freezer boxes into the Styrofoam box.
10. Put Cold-Packs on bottom, sides and top of freezer boxes to assure freezing conditions until arrival at UCB labs.
11. Bring along clear packing tape to seal outer box, rubberized gloves to handle dry ice, and a scissors.

At UCB:
1. Pack dry ice around the freezer boxes. Use gloves to handle the dry ice unless have cup or pitcher (dry ice will burn hands).
2. DO NOT use tape to seal the Styrofoam box after packing with dry ice (can explode).
3. Use tape to seal outside of cardboard box only.
4. The weight of the packed box with dry ice will be approximately 6 pounds.

Sending samples:
1. Address the slip for receipt at the cortisol laboratory.
2. Deliver prepared package to the FedEx store in Emeryville.
Appendix 7: Selected Analysis Code

Example of DSA statement in R language:
The following programming statements in R represent the steps described in the DSA algorithm section:

Library(DSA)

Modelname <- DSA (outcome~main effect,family=distribution,data=dataset name, maxsize=n,maxorderint=n,userseed=n,maxsumofpow=n,vfold=n, nsplits=n)
e.g.,
 dsadx1 <-DSA(lognm~dx1,family=gaussian,data=dsadxa,maxsize=6, maxorderint=2,userseed=100,maxsumofpow=2,vfold=5,nsplits=5)

- After calling up the DSA Library in R, the model statement consisted of (e.g., for the association of diagnosis and cortisol): the specified outcome (lognm), any main effect to remain in the model (dx1), the distribution family (Gaussian), and the dataset to be used (dsadxa).

- In all of the DSA modeling performed for the dissertation, the elements of maxsize through nsplits were the same (e.g., the size of the candidate variable set (n=6 variables) was the same in each model statement).

- Maxsize specified the maximum size of the variable set (excluding the intercept) examined simultaneously (n=6). Maxorderint specified the order of interaction – 2-way or 3-way (n=2). Userseed specified the integer used to set the seed to determine the data splits (n=100 to start, and then by hundreds to 500). Maxsumofpow specifies the power terms to be tried – square or cubic (n=2). V-fold and nsplits are specific to the cross-validation procedure regarding the number of partitions (vfold n=5; nsplits n=5).

Example of G-computation estimation for marginal estimates of cortisol:
The following programming statements in R (right-hand column) represent the steps described in the G-computation estimation section (as explained in left-hand column):

Create counterfactual dataset: dsadxa.0 <- dsadxa
( ~counterfactual dataset=old dataset)

Reassign all diagnosis values to 0: dsadxa.0$dx1 <- 0
(put all values to 0 so that counterfactual values are created for diagnosis=0)

Calculate the predicted values using the final DSA model:
(cortisol values for each subject are predicted by the DSA model (see DSA example above) as applied to the counterfactual dataset) predx0 <- predict(dsadx1,newdata=dsadxa.0)
Appendix 7: Selected Analysis Code (continued)

Average predicted values over sample: mean(preddxa.0,na.rm=TRUE)
(take the mean of all predicted cortisol values for diagnosis=0 in the counterfactual dataset)

e.g., syntax used to get marginal estimate for cortisol if diagnosis=0
dsadxa.0 <- dsadxa
dsadxa.0$dx1 <- 0
preddxa.0 <- predict(dsadx1,newdata=dsadxa.0)
mean(preddxa.0,na.rm=TRUE)
[1] 0.8957656 (results in units of log cortisol)

e.g., syntax used to get marginal estimate for cortisol if diagnosis=1
dsadxa.1 <- dsadxa
dsadxa.1$dx1 <- 1
preddxa.1 <- predict(dsadx1,newdata=dsadxa.1)
mean(preddxa.1,na.rm=TRUE)
[1] 1.073455 (results in units of log cortisol)
References


CHAPTER 7: RESULTS – DIAGNOSIS AND CORTISOL

Descriptive characteristics:
Characters of the study population at baseline are shown in Table 1. A total of 52 children (26 AUT and 26 TYP) were included in the analyses. Overall, there was a preponderance of males in the study population, largely due to the skewed distribution of autism cases in males versus females (roughly a 4:1 ratio). The children were mostly Caucasian from intact families with college-educated parents aged in their mid-30s on average. Socioeconomic status was very homogeneous as evidenced by the Hollingshead index scores, and a high proportion of parents were also employed at least part-time (approximately 90% of fathers and 33% of mothers).

There were few differences between the two diagnostic groups. Although all children were reported by the parents to be in good or excellent health, there were a greater proportion of TYP children reported by parents to be in excellent health (77% versus 58% for AUT). AUT were less likely to be only children but more likely to be born preterm, although the differences were small. By design, AUT were older (mean of 45 months) than TYP (mean of 39 months) because younger TYP were selectively recruited. This allowed the host study to compare children by developmental as well as chronological age. Baseline cognition or IQ was expected to be lower in AUT compared to TYP because of deficits in expressive and receptive language skills among AUT. Eighty percent of the AUT were considered low functioning (IQ<70) and 77% of the AUT were extremely low functioning. No TYP could meet this IQ criterion without having mental retardation (TYP with mental retardation are part of the exclusion criteria).

Table 2 displays data on two key covariates in the broader analyses of both diagnosis and cortisol and sleep and cortisol. These data were collected for the 24-hour period preceding the sleep period for each of the days of saliva collection (approximately six days of data). The results are presented as a proportion of the days with the factor for each diagnostic group and for the total sample. Due to the special needs of AUT for intensive therapy and education, there was a greater proportion of days in which AUT (56% of days) compared to TYP (9% of days) were in a longer day of school or therapy. In general, preschool programs for TYP are three hours or less per day. In addition, AUT attended fewer events during the day (greater than or equal to 2 events on 63% of days) compared to TYP (82% of days). These events or activities included playdates, running errands with parent(s), and enrichment classes (e.g., gymnastics). The difficulty of transporting and supervising AUT is likely responsible for the decrease in attending these events among AUT. The burden of a longer day of school/therapy and/or an increase in number of events attended in a day may contribute to differences in cortisol secretion; therefore it was important to collect information on days of saliva collection.

Table 3 presents data from the host study that were collected at the beginning of each phase (baseline, baseline plus three months, baseline plus six months). The Child Behavior Checklist (CBCL) reflects externalizing (e.g., aggression and tantrum) and internalizing (e.g., anxiety and depression) behaviors expressed by children and the Parenting Stress Index (PSI) – Child scale reflects the child characteristics that contribute to stress in the family. As expected, total scores on the CBCL and the PSI were higher for AUT versus TYP: for the CBCL, the scores for AUT were on average
approximately 14 points higher than for TYP; the scores for the PSI were on average approximately 34 points higher for AUT compared to TYP. However, there were no differences within groups by different phases. It is of note that television (TV) viewing likely exceeded the American Academy of Pediatrics recommended limit of one to two hours per day of total media time (which includes computer use and video games) for both diagnostic groups. I expected to see a significantly greater number of hours of TV for AUT, as TV might be used as a behavioral management tool by parents of AUT. However, this was not borne out by the findings, since there were approximately one and three-quarters hours of TV viewing per day for both groups.

Compliance:
The use of a TrackCap (MEMS 6, Aprex, Inc.) cap on a plastic bottle containing the sampling kits was used to check compliance with the sampling protocol. The cap contains a microchip which logs the absolute clock time at the opening of the bottle. The TrackCap time was checked against the sample time that was recorded directly on the sample tube (the “gold” standard) for the time of day associated with waking, midday, and bedtime saliva collection. There were 48 (92%) subjects that used the TrackCap for at least one phase of saliva collection. No subject refused to use the TrackCap, but there were a limited number available at any given time; subjects with only one phase of data were more likely to have missed using it. Although the design for checking compliance was envisioned as consecutive sampling, because of the shortage of TrackCaps, this could not be achieved. However, I do not have reason to think that there was any systematic bias associated with who received the TrackCap.

There was a very high degree of agreement (approximately \( r=0.98 \)) between TrackCap time and sample time for both AUT and TYP (Table 4). High correlation does not necessarily mean that the measurements are close, but that the measurements are a linear transformation of the other. It is of note that the data points from a plot of the difference in minutes between recording methods by clock time were clustered around the planned sampling times with no suggestion of systematic bias (Figure 1). As an additional descriptive measure, I examined the distribution of the difference in minutes between the two sample time recording methods by diagnosis (Table 4). The average difference in minutes between the TrackCap and sample times was two minutes or less and three minutes or less for AUT and TYP, respectively. Ninety percent of samples had a difference of 12 minutes or less and 16 minutes or less for AUT and TYP, respectively. All of these results on compliance indicate that the TrackCap system was a very good proxy or representative of sample time recorded. Presumably this was close in time to the actual sample time, but it is of note that a “true” measure of sampling time doesn’t exist. However, in order to “cheat the system” the parent would have to plan in advance to open the TrackCap at a preselected time close to the target, even though they did not intend to collect the sample at that time.

Table 5 displays the differences between the sampling and target times by diagnosis. The target time for waking was within 30 minutes of waking, 2:00pm was intended to represent the midday collection, and the bedtime sample should have been collected within 30 minutes of sleep onset. The average time of sampling at waking was approximately 37.5 minutes after waking with very little difference (less than three minutes) between the AUT and TYP. It is of note that there was a different pattern of discrepancy between the sample time at midday for AUT versus TYP. AUT averaged a
collection time 13 minutes after, and TYP 17 minutes before, the 2:00pm target time.
There was a difference of 30 minutes between the diagnostic groups, with the
confidence interval around the difference that suggested a difference approaching an
hour, although the confidence interval was very wide. It is unknown why this pattern
emerged for the different diagnostic groups, but it is possible that napping preferences
that were different for AUT (less likely to nap) versus TYP influenced sample time.
Parents were asked to take the sample before naptime which is often mid-afternoon in
this age group and thus there may have been a push to earlier sampling for the TYP
group. At bedtime, sample times between the diagnostic groups were not very different,
although both were well before (a little over an hour) the target time of within 30 minutes
of sleep onset. This is likely a function of parents trying to achieve sampling in the
context of a lengthy bedtime routine for a preschooler. The most precipitous decline in
cortisol during the day is soon after waking (see Figure 4 in Chapter 3 for normal diurnal
cortisol). The close proximity to the waking target time is reassuring in that the average
collection was not substantially beyond the target window. The other target times have
a flatter cortisol slope surrounding them and thus are not as strongly affected by
differences in collection time.

**Diagnosis and cortisol:**

Figure 2 displays the relationship between cortisol and time across the day in the SACS
sample. The rhythm is as expected from what is known about children's diurnal
concentration of cortisol. There is a precipitous drop after waking (usually 30-60
minutes following waking) and then a flatter slope from midday to bedtime, when the
cortisol nadir approaches (this also depends on absolute time of day). Figure 3 displays
the cortisol secretion by age group (4+, 3-4, and 2-3 year-olds) and time of day.
Although the confidence limits were overlapping, the youngest age group has a pattern
of secretion that is less mature (flatter slope later in the day and cortisol levels of an
order of magnitude less at waking and midday) than the older children.

One of the first study challenges was to determine whether diagnosis interacted
with time before determining what effect diagnosis had on the level of cortisol secretion
across the day. Model 1 in Table 6 indicates that time was a strong independent
predictor of cortisol level (also see Figure 2). In the series of models in Table 6, I fit
interaction terms of diagnosis with different definitions of time. In Model 2 time was
defined as ordinal having values representing waking, midday, and bedtime. Although
time was a strong independent predictor of cortisol as expected, the interaction term
between time and diagnosis was not statistically significant and the model was not a
better fit (using log likelihood comparison) than Model 1. In Model 3 time was defined
hourly from approximately 05:00 to 23:00, and again, time was a strong independent
predictor of cortisol. Even allowing for more frequent data points to fill in any
informative gaps obscured by the ordinal definition of time, the interaction term for time
and diagnosis in Model 4 was not statistically significant. Again the model was not a
better fit than the model (Model 3) without the interaction term. Although subjects were
instructed to collect samples at the three specified times, there was some delayed
sample collection and samples collected earlier than requested (see Figure 5) - that is,
a waking sample may have been taken at 12:00, although it might better reflect cortisol
secretion at midday. Although the numbers were not large, some misclassification may
have occurred between the target times of the day. However, the use of continuous
time still confirmed the lack of an interaction between diagnosis and time on cortisol level. Based on the Akaike Information Criterion, Model 1 (with ordinal time) was a slightly better fit for the data than Model 3 (with continuous time). Figure 4 displays the slope of cortisol secretion across the day (i.e. from waking to bedtime) by diagnostic status. The slopes were not materially different from each other, especially later in the day, and did not cross over. Figure 5 displays cortisol by time (by hour) by diagnosis to illustrate the similarity in slopes when time was examined as a continuous variable. Thus, diagnosis was evaluated as an independent main effect.

Table 7 presents the model from the DSA for predicting cortisol by diagnostic group. The coefficient for the effect of diagnosis on cortisol suggested a small increase in cortisol for AUT compared to TYP. The unit change was an increase of 0.17 units of cortisol in nmol/liter adjusted for important covariates. Based on g-computation estimation and back-transformation to the original units of cortisol, an average of 2.92 and 2.45 nmol per liter of cortisol were secreted from waking to bedtime (i.e. the midday value is the daytime average) for AUT and TYP, respectively. Figure 4 displays the means by diagnostic group at waking, midday, and bedtime. The 95% confidence intervals indicate that there is some overlap with no meaningful differences in means between diagnostic groups at the later times of the day. The waking values, while 1.34 units higher for AUT versus TYP, had wider confidence intervals that overlapped more than at the other times of the day.

Table 8 displays the findings on the association between functional status in children with autism (restricted to this diagnostic group by definition) and cortisol secretion. Functional status is defined as high (HFA) if IQ is greater than or equal to 70 points (N=5) and low (LFA) if IQ is less than 70 points (N=21). The final model based on the DSA indicated that there was a 0.29 increase in cortisol in nmol/liter over the day if the child has LFA. The g-computation estimation produced an estimate of an average of 2.98 and 2.38 nmol per liter of cortisol over the day for LFA and HFA, respectively. When I stratified the LFA group further into extremely low functioning (ELFA) children with IQ<55 (N=17), then a similar association as above can be seen between this definition of functional status and cortisol. In fact, the increase in units of cortisol was higher (0.37) for ELFA compared to HFA. The sample for this analysis is reduced by 50% by design given that the functionality description applies only to children with autism. I was unable to obtain confidence intervals around the g-computation estimates from bootstrapping since there were not enough high-functioning subjects (N=5) to be re-sampled.

Figures 6 and 7 display the data for functional status by time of day. Although it appeared that there might have been a higher cortisol secretion level for LFA during the day, the small sample and lack of variance estimates precluded any definitive conclusion about the association between functional status and cortisol.

**Random effects analysis (within- and between-subject variability):**

One research question of interest was whether cortisol secretion was more variable for children with autism versus typically developing children. For example, children with autism may not have a fine-tuned pattern of cortisol secretion across the day, regardless of the mean level of cortisol, so that secretion might be more variable compared to typically developing children. In a series of linear mixed models, I allowed the intercept to vary to examine whether there were subject-specific effects on cortisol
(each child could have his/her own mean cortisol). I also entered a time variable to allow children to have his/her own cortisol slope across the day. The addition of a diagnostic group term produced variance estimates for comparing the intercepts and slopes for children with autism versus typically developing children.

The first half of Table 9 displays the findings from the linear mixed models allowing a random intercept. Each parameter line includes the variance estimate, the standard error of the estimate, the p-value indicating whether the variance components are statistically significantly different from zero (although the tests may not be reliable), and the Akaike Information Criterion (AIC), which is a measure of the goodness of fit. Model 1 examines all of the subjects without regard to diagnosis. The between-subject variance suggests that there is some variability in mean cortisol secretion between the study children, but that most of the variability can be explained by the within-subject variability. The intraclass correlation reflects that only 11% of the total variance occurs between subjects (between-subject variance/total variance) and the within-subject variance is about 7 ½ times that of the between-subject variance. In model 2 diagnostic group is added to examine within-subject variability within each diagnostic group. This examines whether the child’s own variability between repeated sample collections is different for children with autism versus typically developing children. The between-subject variance for all subjects (regardless of diagnostic group) changes very little, suggesting that diagnosis does not help to explain the between-subject variability in cortisol. In addition, the within-subject variance within each diagnostic group is not very different from the overall within-subject variance regardless of diagnostic group (Model 1). Model 3 examined the between-subject variability within each diagnostic group. Here, the research question is whether there is more variability between children with autism and each other versus between typically developing children and each other. The results suggested that children with autism had 1 ½ times more variable secretion than typically developing children. This between-subject variability was still substantially less than the within-subject variability, but the p-values suggest that these variance estimates were different from zero. The 95% confidence limits overlap and indicate that the variance estimates for AUT (0.0663, 95%CI = 0.0148, 0.1178) compared to TYP (0.0388, 95%CI = 0.0021, 0.0756) were not statistically significantly different from each other. The AIC value is the smallest in Model 1, but the AIC values are not substantially different from each other.

The second half of Table 9 displays the findings from the linear mixed models allowing the addition of a random slope term for time (i.e. waking, midday, and bedtime). In Model 4, the estimate for between-subject variability is zero given the relatively small sample size and the difficulty (relatively low power) in estimating variances. It must be noted that I have low power to estimate the relative contribution of various sources to the total variance-covariance in the outcomes. The slope for time indicates that a small proportion of the variability is explained by entering time (which allows a different slope for each subject) into the model. Model 5 introduces diagnostic group into the modeling. Again, the estimate for between-subject variability is zero due to the low statistical power and the slope term for all of the subjects doesn’t change. The within-subject variability is now broken down by diagnostic group with the within-subject variance estimate decreasing for AUT and increasing for TYP, from the within-subject variance estimate for all subjects combined (Model 4). Model 6 provides
between-subject within-group estimates by diagnostic group but now with the introduction of the slope term. Again, the between-subject variance within the group of children with autism is zero due to low statistical power. The variability in the slope of cortisol for children with autism is 3 ½ times that of typically developing children. The 95% confidence limits around the variance estimates for the slopes for AUT (0.0214, 95% CI = 0.0066, 0.0362) compared to TYP (0.0061, 95% CI = -0.0041, 0.0164) do not overlap, but the estimate for children with autism is not large in terms of absolute magnitude. The within-subject variance estimate for all subjects is similar to model 4. It is clear that the major portion of the variance in cortisol is represented by the within-subject variability. Using the AIC to determine the best fit of all the trialed models (Models 1-6) Model 4 had the lowest AIC. This model, which allows for both a random intercept and slope, did not retain diagnostic group as an explanatory variable. In conclusion, the results suggested that about 40% of the variance in cortisol secretion is explained by variability within the child over successive measurements. Variability between children and in terms of the slope across the day did not account for much of the variance. However, there were differences between AUT and TYP in terms of the between-subjects intercept and slope, although the size of the variance estimates was small.
Table 1. Baseline Demographic Characteristics By Diagnostic Group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Children with autism N=26</th>
<th>Typically developing children N=26</th>
<th>Total children N=52</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (percent)</td>
<td>N (percent)</td>
<td>N (percent)</td>
</tr>
<tr>
<td>Male gender</td>
<td>22 (84.6%)</td>
<td>23 (88.5%)</td>
<td>45 (86.5%)</td>
</tr>
<tr>
<td>Caucasian race</td>
<td>17 (65.4%)</td>
<td>19 (73.1%)</td>
<td>36 (69.2%)</td>
</tr>
<tr>
<td>Child’s age in months - mean (± std; range)</td>
<td>45.1 (± 8.9; 28-64)</td>
<td>39.4 (± 10.5; 24-61)</td>
<td>42.3 (± 10.1; 24-64)</td>
</tr>
<tr>
<td>Child in excellent health</td>
<td>15 (57.7%)</td>
<td>20 (76.9%)</td>
<td>35 (67.3%)</td>
</tr>
<tr>
<td>Baseline cognition (IQ*) - mean (± std; range)</td>
<td>57.5 (± 13.5; 49-94)</td>
<td>99.7 (± 18.0; 70-130)</td>
<td>78.6 (± 26.5; 49-130)</td>
</tr>
<tr>
<td>Low functional status (IQ*&lt; 70)</td>
<td>21 (80.8%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Extremely low functional status (IQ*&lt; 55)</td>
<td>17 (65.4%)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Index is only child</td>
<td>5 (19.2%)</td>
<td>8 (30.8%)</td>
<td>13 (25.0%)</td>
</tr>
<tr>
<td>Parent-level</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Married parents</td>
<td>25 (96.1%)</td>
<td>25 (96.1%)</td>
<td>50 (96.1%)</td>
</tr>
<tr>
<td>Parents with at least a bachelors degree</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mothers</td>
<td>22 (84.6%)</td>
<td>19 (73.1%)</td>
<td>41 (78.8%)</td>
</tr>
<tr>
<td>Fathers</td>
<td>16 (61.5%)</td>
<td>17 (65.4%)</td>
<td>33 (63.5%)</td>
</tr>
<tr>
<td>Parent’s age in years - mean (± std; range)</td>
<td>34.0 (± 7.0; 25-58)</td>
<td>34.3 (± 5.7; 22-46)</td>
<td>34.1 (± 6.3; 22-58)</td>
</tr>
<tr>
<td>Parents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mothers</td>
<td>35.9 (± 8.0; 25-60)</td>
<td>36.1 (± 6.0; 21-47)</td>
<td>36.0 (± 7.0; 21-60)</td>
</tr>
<tr>
<td>Fathers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent employed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>10 (38.5%)</td>
<td>7 (26.9%)</td>
<td>17 (32.7%)</td>
</tr>
<tr>
<td>Father</td>
<td>22 (84.6%)</td>
<td>25 (96.1%)</td>
<td>47 (90.4%)</td>
</tr>
<tr>
<td>Hollingshead Four Factor Index of Social Status - mean (± std; range)</td>
<td>49.4 (± 12.0; 27-66)</td>
<td>49.8 (± 9.9; 30-66)</td>
<td>49.6 (± 10.9; 27-66)</td>
</tr>
</tbody>
</table>

*IQ = intelligence quotient

Table 2. Average Activities Over The Data Collection Period* By Diagnostic Group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Children with autism N=156 days</th>
<th>Typically developing children N=156 days</th>
<th>Total children N=312 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent of days</td>
<td>N (percent)</td>
<td>N (percent)</td>
<td>N (percent)</td>
</tr>
<tr>
<td>School/therapy &gt; 3 hours per day</td>
<td>87 (56.0%)</td>
<td>15 (9.4%)</td>
<td>101 (32.3%)</td>
</tr>
<tr>
<td>≥ 2 events/activities per day attended by child</td>
<td>99 (63.4%)</td>
<td>128 (82.3%)</td>
<td>228 (73.0%)</td>
</tr>
</tbody>
</table>

*The number of days (N) represents the number of days with data on these characteristics (i.e. six days for each subject over the 6-month period of follow-up)
Table 3. Phase-level Characteristics By Diagnostic Group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Children with autism N=26</th>
<th>Typically developing children N=26</th>
<th>Total children N=52</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (± std)</td>
<td>Mean (± std)</td>
<td>Mean (± std)</td>
</tr>
<tr>
<td>Child Behavior Checklist (CBCL) total score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1</td>
<td>61.5 (10.0)</td>
<td>48.2 (10.8)</td>
<td>55.0 (12.3)</td>
</tr>
<tr>
<td>Phase 2</td>
<td>60.7 (7.6)</td>
<td>47.4 (11.9)</td>
<td>54.0 (12.0)</td>
</tr>
<tr>
<td>Phase 3</td>
<td>60.4 (9.3)</td>
<td>44.3 (12.7)</td>
<td>52.5 (13.7)</td>
</tr>
<tr>
<td>Parenting Stress Index (PSI) total score (Child)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1</td>
<td>131.1 (23.1)</td>
<td>95.6 (21.0)</td>
<td>113.0 (28.2)</td>
</tr>
<tr>
<td>Phase 2</td>
<td>133.9 (20.4)</td>
<td>98.0 (25.9)</td>
<td>115.9 (29.3)</td>
</tr>
<tr>
<td>Phase 3</td>
<td>130.5 (23.8)</td>
<td>100.2 (26.7)</td>
<td>116.0 (29.2)</td>
</tr>
<tr>
<td>Hours of television per day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phase 1</td>
<td>1.9 (1.6)</td>
<td>1.4 (0.9)</td>
<td>1.7 (1.3)</td>
</tr>
<tr>
<td>Phase 2</td>
<td>1.7 (1.0)</td>
<td>1.8 (1.1)</td>
<td>1.7 (1.0)</td>
</tr>
<tr>
<td>Phase 3</td>
<td>1.8 (0.9)</td>
<td>1.7 (1.1)</td>
<td>1.7 (1.0)</td>
</tr>
</tbody>
</table>

Table 4. Comparison of sample time with TrackCap bottle opening

<table>
<thead>
<tr>
<th>Number of samples*</th>
<th>Children with autism N=220</th>
<th>Typically developing children N=168</th>
<th>Total samples N=388</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation between TrackCap and sample times (± std)</td>
<td>0.98 (0.004)</td>
<td>0.99 (0.003)</td>
<td>0.98 (0.003)</td>
</tr>
<tr>
<td>Difference between TrackCap and sample times</td>
<td>Median 75th percentile 2 minutes or less</td>
<td>3 minutes or less</td>
<td>3 minutes or less</td>
</tr>
<tr>
<td>90th percentile</td>
<td>5 minutes or less</td>
<td>5 minutes or less</td>
<td>6 minutes or less</td>
</tr>
<tr>
<td></td>
<td>12 minutes or less</td>
<td>16 minutes or less</td>
<td>14 minutes or less</td>
</tr>
</tbody>
</table>

*Number of samples represents samples both logged in by TrackCap method and sample tube recording
Table 5. Comparison of actual sample time with target sample time

<table>
<thead>
<tr>
<th>Difference between actual sample and target sample times (mean ± std)</th>
<th>Number of samples for children with autism (N=100-131)*</th>
<th>Number of samples for typically developing children (N=108-134 )*</th>
<th>Difference between groups (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minutes after waking (waking)</td>
<td>36.0 (40.1)</td>
<td>39.2 (46.1)</td>
<td>-3.2 (-13.8, 7.5)</td>
</tr>
<tr>
<td>Minutes before/after 2:00pm (midday)</td>
<td>12.8 (78.3)</td>
<td>-17.0 (120.9)</td>
<td>29.8 (5.3, 54.4)</td>
</tr>
<tr>
<td>Minutes before sleep onset (bedtime)</td>
<td>-62.2 (66.9)</td>
<td>-69.4 (65.2)</td>
<td>7.1 (-10.8, 25.1)</td>
</tr>
</tbody>
</table>

*Varying number of samples due to missing information on waking and/or sleep-onset times

Table 6. Model of the association between diagnosis and cortisol secretion (including interaction terms for diagnosis and time)*

<table>
<thead>
<tr>
<th>Model</th>
<th>Coefficients in nmol/liter of cortisol (95% CI)*</th>
<th>p-value</th>
<th>Log likelihood</th>
<th>Comparison of models by log likelihood</th>
<th>Akaike information criterion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>Diagnosis 0.10 (-0.06, 0.28)</td>
<td>0.23</td>
<td>-827.9300</td>
<td>N/A</td>
<td>1661.86</td>
</tr>
<tr>
<td></td>
<td>Time (ordinal*) -0.64 (-0.67, -0.61)</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td>Diagnosis 0.11 (-0.13, 0.43)</td>
<td>0.40</td>
<td>-827.9300</td>
<td>for model 2 vs 1: chisq = 0, 1 df, p = 1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time (ordinal*) -0.64 (-0.67, -0.61)</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time x diagnosis -0.01 (-0.14, 0.15)</td>
<td>0.93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 3</td>
<td>Diagnosis 0.09 (-0.07, 0.27)</td>
<td>0.31</td>
<td>-832.1977</td>
<td>N/A</td>
<td>1670.39</td>
</tr>
<tr>
<td></td>
<td>Time (continuous§) -0.14 (-0.15, -0.13)</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 4</td>
<td>Diagnosis 0.05 (-0.20, 0.38)</td>
<td>0.74</td>
<td>-832.1948</td>
<td>for model 4 vs 3: chisq = 0.006, 1 df, p = 0.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time (continuous§) -0.14 (-0.16, -0.13)</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time x diagnosis -0.003 (-0.02, 0.02)</td>
<td>0.81</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Generalized estimating equation regression model adjusted for repeated measures
†Units were back-transformed from natural log-transformed units.
‡Time defined as waking, midday, and bedtime
§Time defined as hourly from 05:00 to 23:00
Table 7. Final model of the association between diagnosis and cortisol secretion

<table>
<thead>
<tr>
<th>DIAGNOSIS AND CORTISOL (AUT VS TYP)*</th>
<th>Coefficients in nmol/liter of cortisol (95% CI)†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis (dichotomous)</td>
<td>0.17 (-0.08, 0.49)</td>
</tr>
<tr>
<td></td>
<td>Predicted mean in nmol/liter of cortisol (95% CI)§</td>
</tr>
<tr>
<td>Waking</td>
<td>AUT = 8.29 (6.99, 10.51)</td>
</tr>
<tr>
<td></td>
<td>TYP = 6.95 (5.97, 8.49)</td>
</tr>
<tr>
<td>Midday</td>
<td>AUT = 2.92 (2.45, 3.76)</td>
</tr>
<tr>
<td></td>
<td>TYP = 2.45 (2.13, 2.97)</td>
</tr>
<tr>
<td>Bedtime</td>
<td>AUT = 1.03 (0.81, 1.38)</td>
</tr>
<tr>
<td></td>
<td>TYP = 0.86 (0.74, 1.08)</td>
</tr>
</tbody>
</table>

* AUT = children with autism (N=26); TYP = typically developing children (N=26); adjusted for time (ordinal), tv hours per day*§ (continuous), nap on day of saliva collection (dichotomous), CBCL total score (continuous), and hours of school/therapy per day (dichotomous)

† Units were back-transformed from natural log-transformed units.

‡ Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures

§ Marginal estimate obtained with g-computation estimation; 95% confidence interval based on 10000 bootstrap samples
Table 8. Final model of the association between functional status in autism and cortisol secretion*

<table>
<thead>
<tr>
<th>Model 1</th>
<th>Functional status and cortisol (low functioning (LF) versus high functioning (HF))†</th>
<th>Coefficients in nmol/liter of cortisol (95% CI)‡§</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Functional status (dichotomous)</td>
<td>Predicted mean in nmol/liter of cortisol</td>
</tr>
<tr>
<td>Waking</td>
<td></td>
<td>LF AUT = 8.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HF AUT = 6.91</td>
</tr>
<tr>
<td>Midday</td>
<td></td>
<td>LF AUT = 2.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HF AUT = 2.38</td>
</tr>
<tr>
<td>Bedtime</td>
<td></td>
<td>LF AUT = 1.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HF AUT = 0.82</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model 2</th>
<th>Functional status and cortisol (extremely low functioning (ELF) versus high functioning (HF))**</th>
<th>Coefficients in nmol/liter of cortisol §δ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extremely low functioning (dichotomous)</td>
<td>Predicted mean in nmol/liter of cortisol</td>
</tr>
<tr>
<td>Waking</td>
<td></td>
<td>ELF AUT = 9.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HF AUT = 6.61</td>
</tr>
<tr>
<td>Midday</td>
<td></td>
<td>ELF AUT = 3.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HF AUT = 2.27</td>
</tr>
<tr>
<td>Bedtime</td>
<td></td>
<td>ELF AUT = 1.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HF AUT = 0.78</td>
</tr>
</tbody>
</table>

*LF AUT = low functioning children with autism (N=21); HF AUT = high functioning children with autism (N=5); ELF-AUT = Extremely low functioning children with autism (N=17);
†Adjusted for time (ordinal), Hollingshead index (continuous), Hollingshead index^2 (continuous), hours per day of television^2 (continuous), count of events (dichotomous)
‡Units were back-transformed from natural log-transformed units.
§Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures
¶Marginal estimate obtained from causal modeling with g-computation estimation
δConfidence intervals were unable to be calculated due to insufficient sample size for bootstrapping
**Adjusted for time (ordinal), interaction of race (dichotomous) and hours per day of television (continuous), interaction of day of saliva collection (dichotomous) and hours of school/therapy per day (dichotomous)
Table 9: Linear mixed models using random effects for the comparison of the variability of cortisol secretion within- and between-subjects

<table>
<thead>
<tr>
<th>Covariance parameter</th>
<th>Variance Estimate</th>
<th>(Standard error)</th>
<th>p-value</th>
<th>Akaike information criterion</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1: Overall</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between-subject variability - ALL</td>
<td>0.0544 (0.0165)</td>
<td>0.4248 (0.0221)</td>
<td>0.0004 &lt;0.0001</td>
<td>1629.5</td>
</tr>
<tr>
<td>Within-subject variability - ALL</td>
<td>0.0528 (0.0160)</td>
<td>0.4229 (0.0313)</td>
<td>0.0005 &lt;0.0001</td>
<td>1632.1</td>
</tr>
<tr>
<td><strong>Model 2: By diagnostic group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between-subject variability - ALL</td>
<td>0.0528 (0.0160)</td>
<td>0.4229 (0.0313)</td>
<td>0.0005 &lt;0.0001</td>
<td>1632.1</td>
</tr>
<tr>
<td>Within-subject variability - AUT</td>
<td>0.0388 (0.0187)</td>
<td>0.4250 (0.0221)</td>
<td>0.0001 &lt;0.0001</td>
<td>1631.3</td>
</tr>
<tr>
<td>Within-subject variability - TYP</td>
<td>0.0463 (0.0263)</td>
<td>0.4250 (0.0221)</td>
<td>0.01 &lt;0.0001</td>
<td>1631.3</td>
</tr>
<tr>
<td><strong>Model 3: By diagnostic group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between-subject within-group variability – AUT</td>
<td>0.0663 (0.0263)</td>
<td>0.4250 (0.0221)</td>
<td>0.01 &lt;0.0001</td>
<td>1631.3</td>
</tr>
<tr>
<td>Between-subject within-group variability – TYP</td>
<td>0.0388 (0.0187)</td>
<td>0.4250 (0.0221)</td>
<td>0.02 &lt;0.0001</td>
<td>1631.3</td>
</tr>
<tr>
<td>Within-subject variability – ALL</td>
<td>0.0158 (0.0043)</td>
<td>0.4069 (0.0212)</td>
<td>0.0001 &lt;0.0001</td>
<td>1604.8</td>
</tr>
<tr>
<td><strong>Model 4: By time</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between-subject variability – ALL</td>
<td>0 (.)</td>
<td>0.0158 (0.0043)</td>
<td>0.0001 &lt;0.0001</td>
<td>1606.1</td>
</tr>
<tr>
<td>Time</td>
<td>0.0154 (0.0042)</td>
<td>0.4069 (0.0212)</td>
<td>0.0001 &lt;0.0001</td>
<td>1606.1</td>
</tr>
<tr>
<td>Within-subject variability - AUT</td>
<td>0.0386 (0.0288)</td>
<td>0.4239 (0.0310)</td>
<td>0.0001 &lt;0.0001</td>
<td>1605.8</td>
</tr>
<tr>
<td>Within-subject variability - TYP</td>
<td>0.0158 (0.0043)</td>
<td>0.4069 (0.0212)</td>
<td>0.0001 &lt;0.0001</td>
<td>1605.8</td>
</tr>
<tr>
<td><strong>Model 5: By diagnostic group by time</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between-subject variability - ALL</td>
<td>0 (.)</td>
<td>0.0158 (0.0043)</td>
<td>0.0001 &lt;0.0001</td>
<td>1606.1</td>
</tr>
<tr>
<td>Time - ALL</td>
<td>0.0154 (0.0042)</td>
<td>0.4069 (0.0212)</td>
<td>0.0001 &lt;0.0001</td>
<td>1606.1</td>
</tr>
<tr>
<td>Within-subject variability - AUT</td>
<td>0.0386 (0.0288)</td>
<td>0.4239 (0.0310)</td>
<td>0.0001 &lt;0.0001</td>
<td>1605.8</td>
</tr>
<tr>
<td>Within-subject variability - TYP</td>
<td>0.0158 (0.0043)</td>
<td>0.4069 (0.0212)</td>
<td>0.0001 &lt;0.0001</td>
<td>1605.8</td>
</tr>
<tr>
<td><strong>Model 6: By diagnostic group by time</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between-subject within-group variability – AUT</td>
<td>0 (.)</td>
<td>0.0158 (0.0043)</td>
<td>0.0001 &lt;0.0001</td>
<td>1606.1</td>
</tr>
<tr>
<td>Between-subject within-group variability – TYP</td>
<td>0.0158 (0.0043)</td>
<td>0.4069 (0.0212)</td>
<td>0.0001 &lt;0.0001</td>
<td>1606.1</td>
</tr>
<tr>
<td>Time – AUT</td>
<td>0.1680 (0.0228)</td>
<td>0.4250 (0.0212)</td>
<td>0.23 &lt;0.0001</td>
<td>1605.8</td>
</tr>
<tr>
<td>Time – TYP</td>
<td>0.0214 (0.0075)</td>
<td>0.4250 (0.0212)</td>
<td>0.02 &lt;0.0001</td>
<td>1605.8</td>
</tr>
<tr>
<td>Within-subject variability – ALL</td>
<td>0.0158 (0.0043)</td>
<td>0.4069 (0.0212)</td>
<td>0.0001 &lt;0.0001</td>
<td>1605.8</td>
</tr>
</tbody>
</table>

Model 1 includes random intercept for cortisol and time (ordinal) and day (dichotomous) as fixed effects.
Model 2 includes random intercept for cortisol and diagnostic group (dichotomous), time (ordinal) and day (dichotomous) as fixed effects.
Model 3 includes random intercept for cortisol and diagnostic group (dichotomous), time (ordinal) and day (dichotomous) as fixed effects.
Model 4 includes random slope for cortisol and time (ordinal) and day (dichotomous) as fixed effects.
Model 5 includes random slope for cortisol and diagnostic group (dichotomous), time (ordinal) and day (dichotomous) as fixed effects.
Model 6 includes random slope for cortisol and diagnostic group (dichotomous), time (ordinal) and day (dichotomous) as fixed effects.
Figure 1. Plot of absolute difference (minutes) between recording methods by mean time (clock time) of sampling.
Figure 2. Mean (95% confidence intervals) salivary cortisol levels in children from the SACS Study*.

*Generalized estimating equation linear regression model adjusted for repeated measures only
Figure 3. Mean (95% confidence intervals) salivary cortisol levels by age group*

*Generalized estimating equation linear regression model adjusted for repeated measures only; 2-3 y/o (N=12); 3-4 y/o (N=27); 4+ y/o (N=13)
Figure 4. Mean (95% confidence intervals) salivary cortisol levels in children with autism compared to typically developing children*†

*Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
†Adjusted for time (ordinal), hours per day of television² (continuous), nap on day of saliva collection (dichotomous), CBCL total score (continuous), and hours of school/therapy per day (dichotomous)
‡AUT = children with autism (N=26); TYP = typically developing children (N=26)
Figure 5. Mean (95% confidence bands) salivary cortisol in nmol/liter across sample time by diagnosis*

*Linear regression model adjusted for repeated measures only
Figure 6: Mean salivary cortisol levels in children with low functioning autism compared to children with high functioning autism*†‡

*Marginal estimate obtained from causal modeling with g-computation estimation
†Adjusted for time (ordinal), Hollingshead index (continuous), Hollingshead index² (continuous), hours per day of television² (continuous), count of events (dichotomous)
‡Confidence intervals were unable to be calculated due to insufficient sample size for bootstrapping with repeated sampling
§LF - AUT = low functioning children with autism (N=21); HF - AUT = high functioning children with autism (N=5)
Figure 7: Mean salivary cortisol levels in children with extremely low functioning autism compared to children with high functioning autism*†‡

*Marginal estimate obtained from causal modeling with g-computation estimation
†Adjusted for time (ordinal), interaction of race (dichotomous) and hours per day of television (continuous), interaction of day of saliva collection (dichotomous) and hours of school/therapy per day (dichotomous)
‡Confidence intervals were unable to be calculated due to insufficient sample size for bootstrapping with repeated sampling
§ ELF-AUT = Extremely low functioning children with autism (N=17); HF-AUT = high functioning children with autism (N=5)
CHAPTER 7: RESULTS – SLEEP AND CORTISOL

Descriptive sleep characteristics

Table 1 presents the primary sleep characteristics stratified by diagnostic group. Children with autism spent less time in bed and had a shorter duration of sleep during the night and in the last 24 hours. There was almost a 30 minute deficit in sleep in the previous 24 hours for children with autism. This was due to a combination of less nighttime sleep and less napping and nap time among children with autism (see Table 2). The sleep duration results were similar to the findings of my systematic review on children with autism in Chapter 4. Children with autism fell asleep at bedtime 15 minutes earlier on average at bedtime, but woke up in the morning 25 minutes earlier on average compared to typically developing children. There was little difference between the diagnostic groups in terms of the average minutes awake during the night and the minutes to fall asleep at bedtime. However, there were 1.6 times as many wakings during the night for typically developing children versus children with autism. Given that the minutes awake were very similar, the absolute time awake was not influenced by the increased number of awakenings.

In Table 2 miscellaneous sleep characteristics are described. The average total score on the Epworth Sleepiness Scale was similar in the two diagnostic groups. As described in the first paragraph of this section, nap time was shorter and the percent of days where napping occurred was less among children with autism compared to typically developing children. This is due to the older age on average of the AUT compared to the TYP. The findings on total nap time indicate that there was a little less than a 20-minute deficit in nap time for children with autism with fewer days of napping as well. The remaining three characteristics reflect habits of poor sleep hygiene. A recent paper by Mindell et al. found that preschool-aged children had a shorter night sleep duration if bedtime was later than 9:00pm. The authors also found that the average amount of night sleep was approximately 9 ½ hours among the preschool-aged children. In addition, the National Sleep Foundation recommends that children three to five years of age get 11-13 hours of sleep in a 24-hour period. In the SACS there was a higher percent of days with a late sleep onset, less than 9 ½ hours of night sleep, and less than 11 hours of 24-hour sleep in children with autism (percents ranging from approximately 9 to 12 percent) compared to typically developing children. In the total sample, approximately 50% and 80% of the children had deficits in night sleep (<9 ½ hours) and 24-hour sleep duration (<11 hours), respectively.

In Table 3 the presence of Research Diagnostic Criteria-defined sleep problems and excessive sleepiness were described. Although the numbers were small, the presence of a night-waking or sleep-onset problem was 3 and 2.7 times, respectively, as common among typically developing children compared to children with autism. Given that the frequency of night-wakings (more common in typically developing children) was one component of a night-waking problem, it was not surprising that typically developing children were more likely to have had a night-waking problem. However, sleep latency was only slightly longer for typically developing children, so bedtime reunions or struggles among the younger typically
developing children might have been driving the measurement of a sleep-onset problem. In terms of daytime sleepiness, children with autism were much more likely to have excessive sleepiness during the day compared to typically developing children (33% versus 7%, respectively), but again the numbers were small.

**Sleep measures for analysis**

There were many potential sleep measures that could have been used to examine sleep from actigraphy. I chose measures that are considered to be comprehensive and complementary, although there is some overlap. The sleep measures were forced in as main effects in all of the DSA models. Although one of the hypotheses was to determine whether autism was an effect modifier on the association between sleep and cortisol, none of the final sleep models chosen by the DSA algorithm included diagnosis as a covariate. All of the variables listed in the table of potential confounders on page 220 in Chapter 6 were available to be selected by the DSA algorithm. They could enter as single covariates, as part of a second order interaction term, or as a quadratic term. This chapter is organized so that each sleep measure has its own section, table and figure(s) for data display. Any adjustment for covariates and other relevant information are noted as a footnote(s) in the table and figure(s).

**Total night sleep and cortisol:**

Total night sleep is measured as the time from first falling asleep until final waking and excludes minutes awake during the night. This sleep measurement reflects the sleep period spent in sleep immediately preceding the day of saliva collection for cortisol. In Table 4, the coefficient for total night sleep indicated that there was no association between hours of night sleep and cortisol on the following day. Figure 1 displays total night sleep in minutes by the original units of cortisol (nmol/liter), with the slope of cortisol declining only slightly with increasing hours of sleep in the night. Table 4 also includes results of cortisol over the day by sleep in hours. There was very little difference in cortisol level from minimal sleep (8 hours) to maximal sleep (12 hours), although the confidence interval was wide for the estimate for 12 hours of night sleep. The difference in cortisol level between 8 and 12 hours was only 0.10 nmol per liter of cortisol. Using the study average of approximately 9 ½ hours of sleep (same as the Mindell study), it is clear that those children getting less than the average night sleep duration did not have substantially higher average cortisol secretion across the day than their peers with higher amounts of sleep.

**Total 24-hour night sleep and cortisol:**

Total 24-hour sleep is measured as the sum of total night sleep and total naptime for the daytime prior to the night sleep (Table 5). This measure represents sleep that occurs in the 24 hours before the day of saliva collection for cortisol. Analysis of this measure shows no association between resulted cortisol secretion by the hours of sleep in the past 24 hours, except that higher levels were observed at each end of the hour of sleep (see Figure 2). At each end the confidence intervals were very wide and suggested very few data points to represent these categories. A rug plot on the original log transformed data also demonstrates this for cortisol at the lower end of time (Figure 3). There was a large gap (approximately 40 minutes) between these few values at the extreme ends and the next closest values. If you examine the values from 8 to 13 hours of sleep, which are lengths of sleep that can
be reasonably expected in this population, Figure 10 shows a cortisol slope that
doesn’t deviate from horizontal with increasing amount of 24-hour sleep. This is
closer to the relationship between total night sleep and cortisol (Figure 1). This might
be expected given that total night sleep is 24-hour sleep minus naptime, and thus 24-
hour sleep is essentially an extension of time on the total night sleep measure.
Marginal estimates from both total night sleep and 24-hour sleep suggest no
association between measures of duration of sleep and cortisol secretion.

Sleep latency and cortisol:
Sleep latency is measured as the time from bedtime (as recorded by the sleep
diary) until first sleep occurs. Sleep latency captures a circadian feature of sleep –
time awaiting sleep-onset for the night. In Table 6, the coefficient was negative but
there was essentially no association between minutes to fall asleep and cortisol
secretion. Figure 4 displays the lack of association between cortisol and minutes to
fall asleep in terms of the slope, which had barely a decline as minutes to fall asleep
increase. The marginal estimates from Table 6 and in Figure 4 indicated a difference
of only 0.44 nmol per liter of cortisol between falling asleep immediately and being
awake for a very long amount of time (100 minutes). Given that the study average
was about 30 minutes to fall asleep, there was no difference between cortisol level if
falling asleep soon after bedtime compared to the study average.

Late sleep onset and cortisol:
As with sleep latency this measure is designed to capture another circadian
feature of sleep. The hypothesis is that later time at first sleep may delay the
initiation of cortisol suppression during the night and thus affect the secretion
potentially from that point on and through the day. In Table 7 the coefficient
suggested a small increase in average cortisol secretion over the day, but the
confidence interval indicated some variability. As can be seen in the Table and
Figure 5, there was a mean cortisol at waking for children with a sleep onset after
9:00pm that was 1.76 units higher compared to children without a late sleep onset.
However, the 95% confidence interval was very wide for the children with a late sleep
onset. The differences were not very different at the later times of the day. A late
sleep onset is often indicative of increased stress and/or activity in the home during
the evening, which may be reflected in the child by higher waking cortisol.

Frequency of night wakings and cortisol:
A waking is defined as having began at the first of at least two consecutive
minutes awake, lasted at least two minutes, and ended at the first of three
consecutive minutes of no activity or sleep. Wakings reflect the frequency of being
awakened (or aroused) during the night when cortisol-suppressing sleep should be
occurring. The night waking variable was heavily skewed to the left (no wakings). A
decision was made a priori to dichotomize the variable into 0-1 versus 2+ wakings.
Although this is not in keeping directly with the part of the night waking RDC criterion
that relates to waking frequency, that part insists on at least a single waking with total
wake time being greater than or equal to 20 minutes long. However, a single waking
could be only five minutes long and be counted under the definition of the wakings
variable. I hypothesized that there was little severity attached to a single waking and
that two or more wakings would be a better representation of waking as a problem.
Table 8 displays the results for 2 or more wakings compared to 1 or fewer wakings.
The coefficient suggested no change in cortisol secretion across the day if a child had 2 or more wakings compared to one or no wakings. The marginal estimates from Table 8 and Figure 6 also suggested no difference between the two classifications. The estimates at all three times of the day were very similar between the two classifications and varied at most by only 0.20 nmol per liter at waking.

Minutes awake during the night and cortisol:

Minutes awake during the night captures time spent without sleep during the sleep recording period. It is complementary to the frequency of night wakings by quantifying the amount of time awake resulting from a waking(s) during the night. It is also complementary to total night sleep as that measure excludes the amount of time awake during the night sleep period. Table 9 presents cortisol secretion results at waking, midday, and bedtime by minutes awake during the night. The data beyond 100 minutes were sparse and had large gaps between units of minutes awake. The coefficient for minutes awake suggested a small incremental increase for every minute awake. The midday values represent the average cortisol across the day and indicated a little over 1 nmol/liter of cortisol difference between 0 and 100 minutes awake. The largest difference was found at the sampling at waking with a little over 3 nmol/liter between no waking and 100 waking minutes (95% CI for difference: -0.23, 7.69). However, the average for the sample was 17 minutes awake during the night, and the difference between 0 and 20 minutes was only 0.56 nmol/liter of cortisol at waking (95% CI for difference: -0.33, 1.10). Figure 7 displays the data by increments of 20 minutes awake from 0 minutes up to 100 minutes awake during the night by waking cortisol secretion. The overall results suggested that for most children, those who are not awake for a long amount of time, the increase in cortisol was not sizeable at any time of the day. However, despite the lack of statistical power due to the sample size, the magnitude of the difference between no waking during the night and being awake for a substantial amount of time (60 minutes or more) may be important.

Miscellaneous sleep-related measures

The remaining sleep and sleep-related measures are not in the same temporal sequence as the previous measures of sleep the night before the day of cortisol secretion measurement. They are global in the sense of trying to capture the sleep period for the week (the RDC criteria), over the period of saliva for cortisol collection (the napping variable), or attempting to capture how the general feeling of sleepiness during the day (the ESS) may affect cortisol secretion. It is of interest that the RDC criteria for sleep problems were measured over the week of cortisol measurement with the measures incorporating data from all seven days of the week. The end measures implied that the sleep problems were pervasive over the whole week and preceded the cortisol measurement.

Research Diagnostic Criteria (RDC) for night-waking problem and cortisol:

The RDC criteria for night-waking and sleep-onset problems are defined across the 7-day period of sleep measurement to give a quantitative estimate of the chronicity or regularity of a sleep problem for the child (see Chapter 6, page 212 for definitions). The sleep problem is considered to be present over the week regardless of the nights of the week used to define the problem. In Table 10 and Figure 8, the results indicated that there was a large increase in cortisol if a child was classified as
having a night-waking problem. The marginal estimate for the predicted cortisol mean at waking was higher (13.6 nmol/liter) for a child with a night-waking problem compared to a child without a night-waking problem (7.4 nmol/liter) (difference = 6.2 nmol/liter; 95% CI for difference: -0.55, 9.35). The 95% confidence interval for the children with a night-waking problem was quite a bit wider than for the children without a night-waking problem due to the variability, especially at waking, for children classified in the former group. It is of note that the RDC criteria for night-waking are composed of night-waking frequency and time awake during the night. Although there was virtually no association between number of night-wakings and cortisol secretion the following day (Table 8), there was an increase in cortisol secretion following a large number of minutes awake the night before. It is likely that time awake is contributing most to the night-waking sleep problem.

**Research Diagnostic Criteria (RDC) for sleep-onset problem and cortisol:**

In Table 11 and Figure 9, the results of the association between sleep-onset problem classification and cortisol secretion are displayed. There were virtually no differences between the classification groups for mean cortisol at waking, midday, or bedtime. The 95% confidence intervals overlap greatly and were wider for the children with a sleep-onset problem compared to children without a sleep-onset problem. The RDC for having a sleep-onset problem was composed of sleep latency, parent struggles, and reunions. Only sleep latency was derived in part from actigraphy, but given the results from Table 6, this sleep latency component was not important in the association between having a sleep-onset problem and cortisol.

**Epworth Sleepiness Scale (ESS) and cortisol:**

The ESS is dichotomized to represent the more severe end of the symptoms of sleepiness (≥ 11 points) compared to less severe sleepiness (0-10 points). This measure is not part of the actigraph measurements but is in the form of a questionnaire given at the beginning of every phase to reflect recent experiences with sleepiness during the day (by parent report). In Table 12 and Figure 10, the results of the association between sleepiness during the day and cortisol secretion during the day are presented. This measure suggested that there was a lower mean cortisol at waking for children with excessive sleepiness (6.18 nmol/liter) compared to children without excessive sleepiness (7.73 nmol/liter) (difference = 1.55 nmol/liter; 95% CI for difference: -0.94, 2.51). It is of note that the effects of sleepiness did not appear to be related to mean cortisol at later times of the day which might coincide with the feelings of sleepiness. Given that cortisol secretion is usually suppressed during night sleep, it is possible that the feeling of sleepiness is also suppressive of cortisol secretion.

**Napping and cortisol:**

A new napping variable was created to reflect whether a child was a napper (on at least one of the days) during the two-day period of saliva collection for cortisol versus a child who did not nap on either of the days of the saliva collection for cortisol. Although there were variables to represent napping on the day before, and the day after, the saliva collection, they were viewed primarily as potential confounders. This new variable was intended to represent whether being a (regular) napper was different from a child who did not nap at all in relation to cortisol secretion during the day. In Table 13, the coefficient suggested no meaningful
change in cortisol secretion for a child that was a regular napper, with the variability suggesting some uncertainty. In Figure 11, the mean cortisol estimates and the overlapping confidence intervals suggested very similar results between nappers and non-nappers at waking, midday, and bedtime.
Table 1: Primary Sleep Characteristics By Diagnostic Group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Children with autism N=26</th>
<th>Typically developing children N=26</th>
<th>Total children N=52</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time from bedtime to wake time in minutes</td>
<td>606.1 (8.9)</td>
<td>621.1 (6.4)</td>
<td>613.7 (5.6)</td>
</tr>
<tr>
<td>Total sleep time during the night in minutes</td>
<td>557.3 (7.6)</td>
<td>570.0 (5.6)</td>
<td>563.7 (4.8)</td>
</tr>
<tr>
<td>Total sleep time in last 24 hours in minutes</td>
<td>590.9 (7.5)</td>
<td>618.5 (6.7)</td>
<td>604.6 (5.4)</td>
</tr>
<tr>
<td>Sleep onset time (time at falling asleep)</td>
<td>21:01 (00:09)</td>
<td>21:16 (00:08)</td>
<td>21:08 (00:06)</td>
</tr>
<tr>
<td>Sleep offset time (time at final waking)</td>
<td>06:40 (00:08)</td>
<td>07:04 (00:07)</td>
<td>06:52 (00:06)</td>
</tr>
<tr>
<td>Minutes awake during the night</td>
<td>17.2 (3.1)</td>
<td>17.1 (1.9)</td>
<td>17.1 (1.8)</td>
</tr>
<tr>
<td>Minutes to fall asleep at bedtime</td>
<td>32.1 (3.9)</td>
<td>36.1 (4.9)</td>
<td>34.0 (3.1)</td>
</tr>
<tr>
<td>Number of awakenings during the night</td>
<td>1.9 (0.3)</td>
<td>3.0 (0.3)</td>
<td>2.5 (0.2)</td>
</tr>
</tbody>
</table>

Table 2: Miscellaneous Sleep-Related Characteristics by Diagnostic Group

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Children with autism N=26</th>
<th>Typically developing children N=26</th>
<th>Total children N=52</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total score on Epworth Sleepiness Scale</td>
<td>6.6 (0.6)</td>
<td>5.6 (0.4)</td>
<td>6.1 (0.4)</td>
</tr>
<tr>
<td>Total nap time in minutes among nappers</td>
<td>80.3 (7.5)</td>
<td>95.1 (5.8)</td>
<td>88.6 (4.8)</td>
</tr>
<tr>
<td>Percent of days where napping occurred – N (percent)*</td>
<td>51(32.7)</td>
<td>68 (43.6)</td>
<td>119 (38.1)</td>
</tr>
<tr>
<td>Percent of days where sleep onset was after 9:00pm – N (percent)*</td>
<td>25 (19.4)</td>
<td>10 (7.6)</td>
<td>35 (13.5)</td>
</tr>
<tr>
<td>Percent of days where night sleep was &lt; 9 ½ hours – N (percent)*</td>
<td>74 (55.6)</td>
<td>63 (47.4)</td>
<td>137 (51.5)</td>
</tr>
<tr>
<td>Percent of days where 24 hour sleep was &lt; 11 hours – N (percent)*</td>
<td>115 (86.5)</td>
<td>101 (75.9)</td>
<td>216 (81.2)</td>
</tr>
</tbody>
</table>

*The percent reflects the proportion of days over the three phases

Table 3: Presence of Sleep Problems and Excessive Sleepiness during the Study Period

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Children with autism N=26</th>
<th>Typically developing children N=26</th>
<th>Total children N=52</th>
</tr>
</thead>
<tbody>
<tr>
<td>Night waking problem by RDC†</td>
<td>3 (11.5)</td>
<td>9 (34.6)</td>
<td>12 (23.1)</td>
</tr>
<tr>
<td>Sleep onset problem by RDC†</td>
<td>3 (11.5)</td>
<td>8 (30.8)</td>
<td>11 (21.1)</td>
</tr>
<tr>
<td>Epworth score ≥ 11 (indicating excessive sleepiness during the day)</td>
<td>9 (34.6)</td>
<td>2 (7.7)</td>
<td>11 (21.1)</td>
</tr>
</tbody>
</table>

*The percent reflects the proportion of children who had the characteristic at least once over the three phases
†RDC = Research Diagnostic Criteria
Table 4. Final model of the association between total night sleep and cortisol secretion

<table>
<thead>
<tr>
<th>Coefficients in nmol/liter of cortisol†‡</th>
<th>Predicted mean in nmol/liter of cortisol (95% CI)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total night sleep (cont.)*</td>
<td>-0.0001 (-0.0010, 0.0013)</td>
</tr>
<tr>
<td>Cortisol secretion over whole day</td>
<td></td>
</tr>
<tr>
<td>8 hours of sleep</td>
<td>2.78 (2.42, 3.16)</td>
</tr>
<tr>
<td>9 hours of sleep</td>
<td>2.75 (2.40, 2.96)</td>
</tr>
<tr>
<td>10 hours of sleep</td>
<td>2.73 (2.41, 2.95)</td>
</tr>
<tr>
<td>11 hours of sleep</td>
<td>2.71 (2.23, 3.53)</td>
</tr>
<tr>
<td>12 hours of sleep</td>
<td>2.68 (2.04, 5.37)</td>
</tr>
</tbody>
</table>

*A Adjusted for time (ordinal) and hours of TV per day (continuous)  
† Units were back-transformed from natural log-transformed units.  
‡ Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures  
§ Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling

Table 5. Final model of the association between total 24-hour sleep and cortisol secretion

<table>
<thead>
<tr>
<th>Coefficients in nmol/liter of cortisol†‡</th>
<th>Predicted mean in nmol/liter of cortisol (95% CI)§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total 24-hoursleep (cont.)*</td>
<td>-0.0125 (-0.0293, 0.0045)</td>
</tr>
<tr>
<td>Cortisol secretion over whole day</td>
<td></td>
</tr>
<tr>
<td>6 hours of sleep</td>
<td>7.08 (2.00, 31.89)</td>
</tr>
<tr>
<td>7 hours of sleep</td>
<td>4.19 (2.16, 8.70)</td>
</tr>
<tr>
<td>8 hours of sleep</td>
<td>3.18 (2.29, 4.45)</td>
</tr>
<tr>
<td>9 hours of sleep</td>
<td>2.71 (2.30, 3.13)</td>
</tr>
<tr>
<td>10 hours of sleep</td>
<td>2.58 (2.26, 2.98)</td>
</tr>
<tr>
<td>11 hours of sleep</td>
<td>2.71 (2.30, 3.15)</td>
</tr>
<tr>
<td>12 hours of sleep</td>
<td>3.14 (2.05, 4.43)</td>
</tr>
<tr>
<td>13 hours of sleep</td>
<td>3.52 (1.94, 5.88)</td>
</tr>
<tr>
<td>14 hours of sleep</td>
<td>5.44 (1.67, 17.14)</td>
</tr>
</tbody>
</table>

*A Adjusted for time (ordinal), hours per day of television, and total 24-hour sleep  
† Units were back-transformed from natural log-transformed units.  
‡ Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures  
§ Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval (Bonferroni-adjusted) obtained from bootstrapping with repeated sampling
Table 6. Final model of the association between minutes to fall asleep and cortisol secretion

<table>
<thead>
<tr>
<th>Minutes to fall asleep (cont.)*</th>
<th>Coefficients in nmol/liter of cortisol†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol secretion over whole day</td>
<td>Predicted mean in nmol/liter of cortisol (95% CI)§</td>
</tr>
<tr>
<td>0 minutes awake</td>
<td>2.85 (2.49, 3.39)</td>
</tr>
<tr>
<td>20 minutes awake</td>
<td>2.76 (2.49, 3.08)</td>
</tr>
<tr>
<td>40 minutes awake</td>
<td>2.67 (2.41, 2.92)</td>
</tr>
<tr>
<td>60 minutes awake</td>
<td>2.58 (2.27, 2.85)</td>
</tr>
<tr>
<td>80 minutes awake</td>
<td>2.49 (2.15, 2.83)</td>
</tr>
<tr>
<td>100 minutes awake</td>
<td>2.41 (1.98, 2.86)</td>
</tr>
</tbody>
</table>

*Adjusted for time (ordinal), interaction of mother working (dichotomous) and hours per day of television (continuous), interaction of nap on day of saliva collection (dichotomous) and time (ordinal), interaction of hours of school/therapy per day (dichotomous) and Hollingshead index (continuous), and interaction of hours of school/therapy per day (dichotomous) and count of events (dichotomous)
†Units were back-transformed from natural log-transformed units.
‡Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures
§Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling

Table 7. Final model of the association between late sleep onset and cortisol secretion

<table>
<thead>
<tr>
<th>Late sleep onset (&gt; 9pm versus ≤ 9pm) (dichot.)*</th>
<th>Coefficients in nmol/liter of cortisol†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol secretion by time of day</td>
<td>Predicted mean in nmol/liter of cortisol (95% CI)§</td>
</tr>
<tr>
<td>Waking</td>
<td>&gt; 9 = 9.38 (5.92, 14.30)</td>
</tr>
<tr>
<td></td>
<td>≤ 9 = 7.62 (6.98, 8.41)</td>
</tr>
<tr>
<td>Midday</td>
<td>&gt; 9 = 3.27 (2.42, 5.11)</td>
</tr>
<tr>
<td></td>
<td>≤ 9 = 2.66 (2.43, 2.91)</td>
</tr>
<tr>
<td>Bedtime</td>
<td>&gt; 9 = 1.14 (0.83, 2.16)</td>
</tr>
<tr>
<td></td>
<td>≤ 9 = 0.93 (0.78, 1.06)</td>
</tr>
</tbody>
</table>

*Adjusted for time (ordinal) and hours of TV per day* (continuous)
†Units were back-transformed from natural log-transformed units.
‡Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures
§Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
Table 8. Final model of the association between number of night-wakings and cortisol secretion

<table>
<thead>
<tr>
<th>Number of night-wakings (2+ versus 0-1) (dichot.)*</th>
<th>Coefficients in nmol/liter of cortisol†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>-0.0099 (-0.0984, 0.0786)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortisol secretion by time of day</th>
<th>Predicted mean in nmol/liter of cortisol (95% CI)§</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Waking</strong></td>
<td></td>
</tr>
<tr>
<td>2+ = 7.61 (6.94, 8.56)</td>
<td></td>
</tr>
<tr>
<td>0-1 = 7.81 (6.94, 8.55)</td>
<td></td>
</tr>
<tr>
<td><strong>Midday</strong></td>
<td></td>
</tr>
<tr>
<td>2+ = 2.69 (2.46, 3.00)</td>
<td></td>
</tr>
<tr>
<td>0-1 = 2.79 (2.43, 3.05)</td>
<td></td>
</tr>
<tr>
<td><strong>Bedtime</strong></td>
<td></td>
</tr>
<tr>
<td>2+ = 0.95 (0.83, 1.11)</td>
<td></td>
</tr>
<tr>
<td>0-1 = 0.98 (0.82, 1.13)</td>
<td></td>
</tr>
</tbody>
</table>

*Adjusted for time (ordinal) and hours of TV per day * (continuous)
†Units were back-transformed from natural log-transformed units.
‡Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures
§Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
Table 9. Final model of the association between minutes awake during the night and cortisol secretion

<table>
<thead>
<tr>
<th>Minutes awake during night (cont.)</th>
<th>Coefficients in nmol/liter of cortisol</th>
<th>Predicted mean in nmol/liter of cortisol (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0034 (0.0013, 0.0054)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cortisol secretion at waking

<table>
<thead>
<tr>
<th>Minutes awake</th>
<th>Cortisol secretion (nmol/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 minutes awake</td>
<td>7.23 (6.60, 8.10)</td>
</tr>
<tr>
<td>20 minutes awake</td>
<td>7.79 (7.04, 8.48)</td>
</tr>
<tr>
<td>40 minutes awake</td>
<td>8.38 (7.17, 9.52)</td>
</tr>
<tr>
<td>60 minutes awake</td>
<td>9.01 (7.32, 11.02)</td>
</tr>
<tr>
<td>80 minutes awake</td>
<td>9.70 (7.37, 12.71)</td>
</tr>
<tr>
<td>100 minutes awake</td>
<td>10.44 (7.43, 14.71)</td>
</tr>
</tbody>
</table>

Cortisol secretion at midday

<table>
<thead>
<tr>
<th>Minutes awake</th>
<th>Cortisol secretion (nmol/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 minutes awake</td>
<td>2.55 (2.35, 2.79)</td>
</tr>
<tr>
<td>20 minutes awake</td>
<td>2.75 (2.49, 3.01)</td>
</tr>
<tr>
<td>40 minutes awake</td>
<td>2.95 (2.53, 3.38)</td>
</tr>
<tr>
<td>60 minutes awake</td>
<td>3.18 (2.59, 3.88)</td>
</tr>
<tr>
<td>80 minutes awake</td>
<td>3.42 (2.65, 4.52)</td>
</tr>
<tr>
<td>100 minutes awake</td>
<td>3.68 (2.68, 5.25)</td>
</tr>
</tbody>
</table>

Cortisol secretion at bedtime

<table>
<thead>
<tr>
<th>Minutes awake</th>
<th>Cortisol secretion (nmol/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 minutes awake</td>
<td>0.90 (0.79, 1.04)</td>
</tr>
<tr>
<td>20 minutes awake</td>
<td>0.97 (0.84, 1.12)</td>
</tr>
<tr>
<td>40 minutes awake</td>
<td>1.04 (0.85, 1.26)</td>
</tr>
<tr>
<td>60 minutes awake</td>
<td>1.12 (0.88, 1.46)</td>
</tr>
<tr>
<td>80 minutes awake</td>
<td>1.21 (0.90, 1.70)</td>
</tr>
<tr>
<td>100 minutes awake</td>
<td>1.30 (0.91, 2.02)</td>
</tr>
</tbody>
</table>

*Adjusted for time (ordinal), hours of TV per day (continuous), nap on day of saliva collection (dichotomous), and hours of school/therapy per day (dichotomous)

†Units were back-transformed from natural log-transformed units.

‡Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures

§Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
### Table 10. Final model of the association between night-waking problem and cortisol secretion

<table>
<thead>
<tr>
<th>Night waking problem (Yes versus No) (dichot.)</th>
<th>Coefficients in nmol/liter of cortisol†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.8673 (4.4325, 5.3376)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortisol secretion by time of day</th>
<th>Predicted mean in nmol/liter of cortisol (95% CI)†§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waking</td>
<td>YES = 13.58 (7.04, 16.81) NO = 7.40 (6.84, 8.06)</td>
</tr>
<tr>
<td>Midday</td>
<td>YES = 4.79 (2.50, 5.90) NO = 2.61 (2.41, 2.90)</td>
</tr>
<tr>
<td>Bedtime</td>
<td>YES = 1.69 (0.87, 2.13) NO = 0.92 (0.81, 1.07)</td>
</tr>
</tbody>
</table>

*Adjusted for time (ordinal), interaction of hours of school/therapy per day (dichotomous) and hours per day of television (continuous), interaction of race (dichotomous) and night waking problem (dichotomous), interaction of time (ordinal) and Hollingshead index (continuous), and interaction of nap on day of saliva collection (dichotomous) and CBCL total score (continuous)*

†Units were back-transformed from natural log-transformed units.

‡Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures

§Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling

### Table 11. Final model of the association between sleep-onset problem and cortisol secretion

<table>
<thead>
<tr>
<th>Sleep onset problem (Yes versus No) (dichot.)</th>
<th>Coefficients in nmol/liter of cortisol†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-0.0984 (-0.3198, 0.1951)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortisol secretion by time of day</th>
<th>Predicted mean in nmol/liter of cortisol (95% CI)†§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waking</td>
<td>YES = 7.42 (5.75, 10.43) NO = 7.60 (7.01, 8.35)</td>
</tr>
<tr>
<td>Midday</td>
<td>YES = 2.61 (2.05, 3.70) NO = 2.68 (2.46, 2.99)</td>
</tr>
<tr>
<td>Bedtime</td>
<td>YES = 0.92 (0.70, 1.32) NO = 0.94 (0.82, 1.11)</td>
</tr>
</tbody>
</table>

*Adjusted for time (ordinal), hours of TV per day (continuous), nap on day of saliva collection (dichotomous), CBCL total score (continuous), and hours of school/therapy per day (dichotomous)*

†Units were back-transformed from natural log-transformed units.

‡Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures

§Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
Table 12. Final model of the association between Epworth Sleepiness Scale and cortisol secretion

<table>
<thead>
<tr>
<th>Epworth score (≥ 11 versus &lt; 11) (dichot.)</th>
<th>Coefficients in nmol/liter of cortisol†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>†Units were back-transformed from natural log-transformed units.</td>
<td></td>
</tr>
<tr>
<td>‡Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortisol secretion by time of day</th>
<th>Predicted mean in nmol/liter of cortisol (95% CI)†§</th>
</tr>
</thead>
<tbody>
<tr>
<td>†Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling.</td>
<td></td>
</tr>
<tr>
<td>§Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling.</td>
<td></td>
</tr>
</tbody>
</table>

- **Waking**
  - ≥ 11 = 6.18 (5.29, 8.75)
  - <11 = 7.73 (7.01, 8.35)

- **Midday**
  - ≥ 11 = 2.18 (1.93, 3.05)
  - <11 = 2.73 (2.48, 3.00)

- **Bedtime**
  - ≥ 11 = 0.77 (0.67, 1.08)
  - <11 = 0.96 (0.83, 1.11)

*Adjusted for time (ordinal), interaction of hours of TV per day (continuous) and nap on day of saliva collection (dichotomous), hours of school/therapy per day (dichotomous), interaction of CBCL total score (continuous) and hours of school/therapy per day (dichotomous) interaction of phase (ordinal) and hours of school/therapy per day (dichotomous).*

Table 13. Final model of the association between napping and cortisol secretion

<table>
<thead>
<tr>
<th>Napper (Yes versus No) (dichot.)</th>
<th>Coefficients in nmol/liter of cortisol†‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>†Units were back-transformed from natural log-transformed units.</td>
<td></td>
</tr>
<tr>
<td>‡Coefficient and 95% confidence interval obtained from generalized estimating equation regression model adjusted for repeated measures.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortisol secretion by time of day</th>
<th>Predicted mean in nmol/liter of cortisol (95% CI)†§</th>
</tr>
</thead>
<tbody>
<tr>
<td>†Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling.</td>
<td></td>
</tr>
<tr>
<td>§Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling.</td>
<td></td>
</tr>
</tbody>
</table>

- **Waking**
  - YES = 7.75 (6.90, 8.87)
  - NO = 7.57 (6.49, 8.41)

- **Midday**
  - YES = 2.74 (2.51, 3.09)
  - NO = 2.68 (2.26, 3.01)

- **Bedtime**
  - YES = 0.97 (0.85, 1.14)
  - NO = 0.95 (0.70, 1.11)

*Adjusted for time (ordinal) and interaction of hours of school/therapy per day (dichotomous) and hours of TV per day (continuous).*

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Figure 1. Mean (95% confidence intervals) salivary cortisol levels by total night sleep in hours*†‡

*Adjusted for time (ordinal) and hours of TV per day2 (continuous)
†Units were back-transformed from natural log-transformed units.
‡Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
Figure 2. Mean (95% confidence intervals) salivary cortisol levels by total 24-hour sleep in hours*†‡

*Adjusted for time (ordinal), total 24-hour sleep squared (continuous), and hours of TV per day squared (continuous)
†Units were back-transformed from natural log-transformed units.
‡Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval (Bonferroni-adjusted) obtained from bootstrapping with repeated sampling

Figure 3. Original log units of cortisol with rug (density) plot showing lack of data at low end of minutes of sleep
Mean log cortisol over the day by total 24-hour sleep

total 24-hour sleep in minutes

Log cortisol

0.5
1.0
1.5
2.0
2.5

Figure 4. Mean (95% confidence intervals) salivary cortisol levels by minutes to fall asleep*

*Adjusted for time (ordinal), interaction of mother working (dichotomous) and hours per day of television (continuous), interaction of nap on day of saliva collection (dichotomous) and time (ordinal), interaction of hours of school/therapy per day (dichotomous) and Hollingshead index, and interaction of hours of school/therapy per day (dichotomous) and count of events (dichotomous)

†Units were back-transformed from natural log-transformed units.

‡Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
Figure 5. Mean (95% confidence intervals) salivary cortisol levels in children with a sleep onset >9pm compared to children with a sleep onset ≤ 9pm*†§

*Adjusted for time (ordinal) and hours of TV per day2 (continuous)
†Units were back-transformed from natural log-transformed units.
§Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
Figure 6. Mean (95% confidence intervals) salivary cortisol levels in children with 2+ night-wakings compared to children with 0-1 night-waking*†§

*Adjusted for time (ordinal) and hours of TV per day2 (continuous)
†Units were back-transformed from natural log-transformed units.
§Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
Figure 7. Mean (95% confidence intervals) salivary cortisol levels at waking by minutes awake during the night * † ‡

*Adjusted for time (ordinal), hours of TV per day (continuous), nap on day of saliva collection (dichotomous), and hours of school/therapy per day (dichotomous)
†Units were back-transformed from natural log-transformed units.
‡Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
Figure 8. Mean (95% confidence intervals) salivary cortisol levels in children with a night-waking problem compared to children without a night-waking problem*†§

*Adjusted for time (ordinal), interaction of hours of school/therapy per day (dichotomous) and hours per day of television (continuous), interaction of race (dichotomous) and night waking problem (dichotomous), interaction of time (ordinal) and Hollingshead index (continuous), and interaction of nap on day of saliva collection (dichotomous) and CBCL total score (continuous)
†Units were back-transformed from natural log-transformed units.
§Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
Figure 9. Mean (95% confidence intervals) salivary cortisol levels in children with a sleep-onset problem compared to children without a sleep-onset problem*

*Adjusted for time (ordinal), hours of TV per day2 (continuous), nap on day of saliva collection (dichotomous), CBCL total score (continuous), and hours of school/therapy per day (dichotomous)

†Units were back-transformed from natural log-transformed units.

‡Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
Figure 10. Mean (95% confidence intervals) salivary cortisol levels in children with Epworth Sleepiness Scale (ESS) score ≥ 11 compared to children with ESS score < 11*

*Adjusted for time (ordinal), interaction of hours of TV per day (continuous) and nap on day of saliva collection (dichotomous), hours of school/therapy per day (dichotomous), interaction of CBCL total score (continuous) and hours of school/therapy per day (dichotomous) interaction of phase (ordinal) and hours of school/therapy per day (dichotomous)

†Units were back-transformed from natural log-transformed units.

‡Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
Figure 11. Mean (95% confidence intervals) salivary cortisol levels in napping children compared to non-napping children*†‡

*Adjusted for time (ordinal) and interaction of hours of school/therapy per day (dichotomous) and hours of tv per day (continuous)
†Units were back-transformed from natural log-transformed units.
‡Marginal estimate obtained from causal modeling with g-computation estimation; 95% confidence interval obtained from bootstrapping with repeated sampling
CHAPTER 8: CONCLUSIONS

Specific aims and hypotheses
1. To determine whether levels of daytime cortisol secretion, based on salivary cortisol sampling, differ between preschool-aged children with autism and typically developing children.

Hypothesis 1: There will be a circadian rhythm disruption in children with autism that will manifest itself as elevated levels of daytime cortisol secretion (i.e. a higher cortisol level at several time points throughout the day) compared to typically developing children.

Although there were higher levels of cortisol secretion at waking for AUT (1.34 nmol/liter higher), cortisol at midday and bedtime were not very different between the diagnostic groups. The increase in cortisol secretion across the day was 0.17 nmol/liter. There were no statistically significant differences between the diagnostic groups at any time of the day. The sample size of the AUT and TYP (26 children in each group) may have been too small to detect a difference between the groups, although this is the largest sample size to date with AUT and TYP to address this research question. At waking, when the largest difference in cortisol levels was seen, the confidence intervals were very wide. The summary in Chapter 3 of previous studies with acceptable methodology suggested higher levels of cortisol during the day, particularly at late afternoon or bedtime. These were studies with fewer subjects than SACS and enrolled primarily school-age children and teens. Only two of these studies used repeated measures across days and had a similar design to the SACS with waking, afternoon, and bedtime cortisol levels collected [1, 2]. Corbett et al. in the earlier paper observed an increase of 0.15 nmol/liter across the day for AUT compared to TYP [1]. This effect size was similar to the SACS results described above. However, the largest difference in cortisol in the Corbett study was observed at evening, whereas the SACS observed the largest difference at waking.

In addition to investigating AUT as a whole, I divided the AUT into high, low, and extremely low functioning groups. High functioning children with autism (IQ ≥70) were defined as having no intellectual disability (mental retardation). I used currently accepted cut points for intellectual disability (IQ<70) to define low functioning AUT and a lower cutpoint (IQ<55) to define extremely low functioning AUT (i.e. moderate intellectual disability). The results suggested that there was an increase in cortisol levels for low functioning AUT and extremely low functioning AUT compared to high functioning AUT. This response was graded – cortisol secretion levels increased as IQ decreased. The sample size was halved (N=26) to investigate functional status as all comparisons are among AUT. The statistical power was greatly diminished by the reduction of sample size, which precluded any variance estimates to determine the precision. The numbers were too small to obtain bootstrap confidence intervals around the marginal estimates, but the confidence interval around the effect estimate coefficient (using GEE) suggested an increase above zero. Previous research described in Chapter 3 had found that children with autism with an IQ under 60 (termed poorly developed) had higher plasma cortisol levels before and after the Dexamethasone suppression test [3]. In a later paper, these authors also observed that four of five poorly developed AUT were non-suppressors and that non-suppressors tended to have higher afternoon cortisol values [4]. These are the only other studies in AUT dividing
groups by functional status and observing cortisol levels, and they are over 20 years old. Many early studies may have only enrolled low functioning AUT given that diagnostic tools were not sensitive for ASD at that time, but IQ was not noted. Eighty percent of the AUT in the SACS were defined as low functioning. This higher enrollment of low functioning AUT was somewhat surprising because the heavier burden from saliva collection than for high functioning AUT might have precluded their participation. For a variety of reasons (lower level adaptive behavior skills and less expressive and receptive language abilities) the enrollment of low functioning AUT into studies is generally difficult. These results and those of previous studies suggest that there may be differences in L-HPA functioning between extremely low, low, and high functioning AUT that warrants further research. IQ is being used as a measure of ability and daily functioning in children with autism. Lower functioning children with autism may be particularly susceptible or sensitive to environmental stressors and sensory processing associated with difficulties in adaptability and functioning.

2. To determine whether within-subject variability of daytime cortisol secretion, based on salivary cortisol sampling, differs between preschool-aged children with autism and typically developing children.

Hypothesis 2: There will be more within-subject variability in children with autism compared to typically developing children.

Using a linear mixed models approach the within-subject, between-subject, and random slope differences in cortisol secretion were examined with respect to diagnostic group. For all subjects, the within-subject variance (42%) accounted for much of the total variance compared to the between-subject variance (5%). However, within-subject variability for AUT was not very different from TYP, even when accounting for the slope. The main findings were that the between-subject variance and the variance of the slope of cortisol across the day were greater for AUT compared to TYP. Although the variance estimates were small, there was a 1.5 fold greater variance for the between-subject variance and a 3.5 fold greater variance for the slope, for AUT compared to TYP. There have been only two other research papers that examined differences in cortisol variance estimates between AUT and TYP and used a similar study design to examine daytime cortisol secretion. The authors observed that between-subject variance for school-aged AUT was greater than for TYP in both papers [1, 2] and within-subject variance was greater for AUT than for TYP in the more recent paper [2]. A dysregulation in cortisol could be reflected in greater fluctuations in cortisol secretion during the day in AUT, hence the greater between-subject variability in AUT compared to TYP. Corbett, et. al., in a recent paper extending earlier findings displayed this variability between the diagnostic groups in a figure of individual cortisol secretion patterns across six days [5].

3. To determine whether sleep measures (e.g. total night sleep; minutes awake during the night; frequency of night wakings; and minutes to fall asleep at bedtime) the night before are associated with cortisol secretion the following day.

Hypothesis 3a: Poor sleep quality (shorter duration of sleep; frequent night wakings; a longer sleep latency; and more minutes awake during the night) will be associated with higher levels of cortisol secretion the following day.

Hypothesis 3b: Sleep fragmentation (number of wakings and/or minutes awake during the night) is the best predictor of higher cortisol levels the following day.
Most of the sleep measures from the night directly before the day of cortisol measurement were not associated with cortisol secretion. Duration of sleep (night or 24-hour) and sleep latency had virtually flat cortisol slopes by minutes of sleep and minutes to fall asleep, respectively. The cortisol slopes across the day for 0-1 wakings was virtually the same as that for 2+ wakings. It is of note that minutes awake during the night was associated with higher cortisol secretion as minutes awake increased. There was a large absolute difference in cortisol secretion at the waking sample between children awake for many minutes during the night (100 minutes) compared to those with no minutes awake. Although the confidence interval for the difference (3.0 nmol/liter) was not statistically significant, the upper limit suggests that a relative large difference (7.7 nmol/liter) cannot be ruled out. Therefore, with respect to Hypothesis 3b, sleep fragmentation as measured by minutes awake was the best predictor of higher cortisol levels the following day.

There was nearly uniform consensus from the review of studies of sleep and cortisol in children (excluding infants) that night sleep duration and daytime cortisol secretion were not associated (see Table 5 in Chapter 5). This was also the conclusion from the SACS. However, there were mixed findings with respect to waking after sleep onset, as some previous studies did not separate out the number of wakings from minutes awake during the night. Terms such as, “poor sleepers” and “severe sleep disturbances”, contained both components of waking. These studies did find higher cortisol levels at waking [6] and waking with serial measurements shortly after waking [7, 8]. Two studies that did not look at waking cortisol observed no association between being awake after sleep onset and late morning and late afternoon cortisol in one study [9], and observed a positive correlation between minutes awake and later afternoon cortisol secretion in the other study [10]. The lack of an association between night wakings and cortisol secretion in the SACS may be due to a blunt definition of increased night waking. The frequency of night waking was heavily skewed to the left with 75% of the SACS data classified as three or fewer wakings (range 0-18 wakings). There is no research on the predictive value of frequency of night wakings alone and the association with cortisol secretion and there is no consensus on how to categorize this variable. Studies of sleep and cortisol may need to oversample children with a greater frequency of night wakings in order to better examine this question, with the caveat that improvement in actigraphy for the detection of night waking should precede additional research.

4. To determine whether a child with a sleep-onset or night-waking sleep problem by RDC criteria is associated with cortisol secretion the following day.

Hypothesis 4a: A child with a sleep-onset or night-waking sleep problem by RDC criteria will have higher cortisol levels the following day compared to children without these respective sleep problems.

The results from the SACS indicate that there was no association between having a sleep-onset problem and daytime cortisol secretion at any time on the days of cortisol measurement. However, having a night-waking problem was associated with daytime cortisol secretion at waking, although the confidence interval was very wide about the estimate for mean cortisol. The difference between the two groups was large (6.2 nmol/liter) with an upper limit to the confidence interval around the difference of 9.3 nmol/liter. Only 12 children had a night-waking problem at any given phase, so the
statistical power was low for this research question. Minutes awake during the night and having a night-waking problem were likely reflecting the same underlying biological issue, and that being awake during the night rather than waking itself was driving an association.

One of the main hypotheses about the etiology of insomnia is that physiological arousal experienced in the day or evening is carried over to the night with difficulty falling asleep or difficulty maintaining sleep [11]. Sleep latency was not associated with cortisol secretion, so difficulty falling asleep does not appear to be an intermediate between arousal and cortisol. For the RDC criterion for night-waking, being awake during the night could be a continuation of arousal processes that started earlier in the day. Variables that were used to fit the final model included television viewing, socioeconomic status, total behavior score, and hours of preschool or therapy. These are all factors that are likely to influence the arousal process. However, having accounted for them through the modeling process, being awake during the night appears to be independently associated with increased cortisol at waking. In the summary section later in the chapter the associations between the sleep measures and cortisol and the RDC criteria and cortisol are discussed in detail.

5. To determine whether autism is an effect modifier of the association between sleep and cortisol.

Hypothesis 5a: Children with autism that have poor sleep quality will have higher cortisol secretion levels compared to typically developing children that have poor sleep quality.

Diagnostic group was never included as a main effect term, as part of an interaction term, or as a quadratic term in any of the final models chosen by the DSA algorithm. In effect, based on the results from the SACS, diagnosis did not modify the effect of any of the sleep measures on daytime cortisol secretion. The analysis involved using a cross-validation technique to examine several different models at one time with the final model chosen based on the lowest empirical risk (see Chapter 6 DSA section for more details). This method allowed a maximum of six covariates to be examined at a time. In none of the successive steps was diagnosis included as a potential final covariate suggesting that diagnosis was never considered to be important in the modeling process for any of the sleep measures. It is possible that if the number of covariates to be examined at a given step were less restrictive (greater than six) diagnosis may have entered the modeling process, but with the customary six iterative steps diagnosis would likely have been included in at least one of these steps if it were important.

There have been no previous studies to examine diagnostic group (AUT compared to TYP) as an effect modifier of comprehensive measures of sleep on daytime cortisol secretion. This aim was generated as a result of the review of some previous research that summarized that AUT had more sleep problems than TYP (not borne out by subsequent systematic review) and from the identification of studies that observed higher levels of cortisol during the day among AUT (equivocal from subsequent systematic review). The dataset was uniquely able to examine this aim because the SACS incorporated sleep measures and cortisol among AUT and TYP. Although the findings were negative in all of the sleep analyses this was a single study with a small sample size that was not designed to specifically address the research
hypothesis. Replication of these results in future studies is needed before concluding that there is no effect modification of autism on the association between sleep measures and cortisol secretion.

**Strengths and limitations**

**Sample size and study design**

This is one of the few studies in young children to collect saliva for cortisol and to measure sleep both in a longitudinal design. While I was limited in the number of subjects due to a fixed sample size from which to obtain specimens, I was able to gain some statistical precision from the repeated measures, given that repeat assessment reduces within-subject variability [12]. However, there is a “ceiling” on the benefits from repeated measures with a benefit up to six measures at a given time point [12]. It has also been recommended that at least six days of measurement are needed to assess cortisol awakening response reliability [13]. Phase (baseline, plus three months, or plus six months) and day (consecutive day one or two) were not associated with concentrations of cortisol in the SACS and were never chosen by the DSA in any of the final analyses of diagnosis and cortisol or sleep and cortisol. This consistency across the six months added some confidence to the findings of cortisol secretion in the SACS. The use of the prospective measurement and temporal ordering were also important in several other ways. I was able to use causal inference methods and examine within-subject variability because of the longitudinal design. The linear mixed models approach allows both fixed and random effects to be included in a model and can distinguish between-subject and within-subject sources of variability. These models are also useful for analyzing unbalanced longitudinal data as they do not require the same number of observations on each subject (can accommodate missing data) [14]. However, application of these models assumes that the model form chosen is the “correct” model, which is not very likely.

The SACS recruited subjects from the Sleep Study and depended on the enrollment progress of the host study. The SACS started about midway through the enrollment into the Sleep Study. Therefore, there was a fixed number of subjects from which to draw and the SACS ultimately included 26 AUT and 26 TYP into the study (the original goal was to enroll 30 subjects in each group). Even owing to the relatively large sample size compared to previous research and the benefits of repeated measures it remains unclear what would have been a sufficient sample size. No previous studies in preschool-aged children existed that compared cortisol secretion levels across the day in AUT and TYP. In the study that was closest in design among school-aged children [1], the results from which to base a sample size calculation were published following the initiation of the SACS. At that point the SACS was enrolling as many consecutive subjects that met the inclusion criteria as possible. In the end there were four more subjects in each diagnostic group than the Corbett study (the next largest study devoted to this research question).

**Inclusion/exclusion criteria**

The children enrolled were restricted to a narrow age range to avoid potential age affects on the circadian rhythms of sleep and cortisol secretion. A mature cortisol secretion during the day is developed largely by the end of the toddler period (equivalent to two to three years of age) and remains at a similar level to six years of age [15]. In addition to the exclusion criteria used by the host study, for the purposes of
the SACS, several other criteria were used. Any child that used corticosteroids during the study (regardless of the phase) was excluded. Cortisol levels are known to be affected by exogenous glucocorticoid use [16, 17], in particular, the 30-minute post-awakening cortisol level [17]. Any child with co-morbid disorders (e.g. chromosomal disorders, chronic illnesses) was excluded to maintain a strictly defined sample. In particular, children with anxiety or depression were excluded because of the known association between these psychiatric disorders and cortisol [18-20]. In a study of infants and mothers taking medications, there was a less pronounced cortisol reactivity to challenge tasks in infants taking acetaminophen compared to infants not taking any medications [16]. An examination of other medications (medications for sleep, colds, and asthma) resulted in no differences between diagnostic groups nor were these medications associated with cortisol secretion. Due to small numbers in each of these categories, a measure of “all medications” was examined and again there was no association between medications and the variables of interest. None of the children were on antipsychotics or neuroleptics possibly due to the young age of the children.

Compliance

There was very good compliance with the protocol among all families enrolled in the SACS as measured by the difference in minutes between the TrackCap and sample tube recording. For 90% of the samples, the difference between the TrackCap and the sample tube recording time was 14 minutes or less. Bedtime proved to be the most difficult time of the day for families to meet the target sampling time window (within 30 minutes before bedtime). Families, regardless of diagnostic group, obtained saliva about one hour before bedtime. There is usually little variation in cortisol secretion in the evening hours and this was supported by the SACS data. The waking cortisol collection was on average about 30 minutes after waking, which is preferred in order to determine the cortisol awakening response (CAR). The CAR is defined as the change in cortisol that occurs approximately 30 minutes after waking from sleep and is independent of time of awakening [21]. The SACS was able to examine the CAR, as there were objective measures of the sleep-offset (final waking) and good compliance with recording of the saliva collection time. Only one child was unable to continue after enrollment due to the saliva collection procedure (family described that the child was trying to eat the cotton roll). The study was well accepted with no other safety concerns or complaints.

Selection bias

The SACS enrolled a non-random selection of subjects (volunteers) and was unlikely to have been a representative sample [22]. Volunteers are self-selected and choose to participate based on a variety of reasons that make them different from those who do not volunteer [23]. Subjects in observational studies often participate in order to find out more about a problem they are experiencing (e.g. sleep). Families of children with autism who were behaviorally difficult and families of children with poor sleep hygiene both may have opted out of participation because of the burdens of participation on an already stressed family. Therefore, children at the less severe end of the spectrum (in terms of autism and sleep hygiene) may have been over-represented in the study. Eighty percent of the AUT were low functioning and would have had less adaptive functioning than high functioning AUT which suggests that there were actually more severe AUT enrolled. The Sleep Study excluded subjects who had
previously sought treatment for a sleep disorder. In terms of sleep hygiene, almost a third of the TYP currently had sleep-onset or night-waking problems – this was within the very wide range of proportions in other samples among TYP. The prospective design can also be used to avoid recall and other biases related to the collection of cross-sectional and retrospective data [24]. Therefore, selection bias due to knowledge of the outcome was not an issue.

Measurement error

Diagnosis: The diagnosis of autism was performed using two instruments that are the current standard in research studies. The ADOS-G and the ADI-R are used for observation and interview of AUT and their families. Although more stringent diagnostic techniques and a longer period of observation might be used in the individual diagnosis of autism in a child, the ADOS-G and ADI-R are the standard of the research tools available. The sensitivity results for determining autism by the ADOS-G (87-100%) and the ADI-R (96-98%) were very good for AUT and children not on the spectrum (mentally retarded and other learning disabilities) (see Table 1 in Chapter 6). However, the sensitivity and specificity of the ADOS-G and ADI-R among children with strict autism and children on the spectrum (ASD) suggest that a proportion of the children diagnosed with strict autism may actually be ASD, and those with strict autism may have been diagnosed as ASD. The typically developing children were not screened with the ADOS-G or the ADI-R instruments, but the rarity of a diagnosis of autism suggests that the typically developing children are unlikely to be misclassified. Although it is preferred to have all instruments used in all members of a study sample, there were three phases or opportunities to administer the ADOS-G and ADI-R to the TYP if necessary to rule out an autism or ASD diagnosis. The co-investigators that are expert in autism noted that any misclassification of ASD to autism would likely have been discovered over the follow-up period. However, any strict autism cases misclassified as ASD would have been lost, as they would have been excluded after initial assessment (see Chapter 6: Research Methods for an extensive review of the methodological issues with respect to the diagnostic criteria).

Sleep measurement: A well-controlled sleep laboratory setting with polysomnography would be preferred for the diagnosis of sleep problems in an individual child. However, actigraphy is arguably the best method of sleep measurement in a home setting and on very young children who are still napping. Neither polysomnography nor videosomnography are well-suited for capturing daytime sleep outside the home or when sleep occurs in a variety of rooms/locations within the home. There are serious problems with the specificity of the actigraph in relation to determining waking status and the amount of time awake. The specificity of the actigraph ranged from 31% to 77% for the detection of sleep in those studies evaluating actigraphy against polysomnography, and 27% in the only study evaluating actigraphy against videosomnography (see Table 3 in Chapter 6). In this work from the Sleep Study, the number of wakings with actigraphy was much higher than the number of wakings with videosomnography or sleep diary. Adjustment of the factory calibration did not make a significant difference in the number of wakings from actigraphy, so the data were recoded using a new “smoothing” algorithm to make the results more consistent with the sleep diary and videosomnography. This suggests that the number of wakings and minutes awake would be underestimated using this algorithm and that
the results would be more conservative. It is surprising that this strategy still produced such poor results (the 27% specificity noted above) given that the aim was to improve the specificity. Some of the sleep measures used subjective components in their definition (sleep latency, the RDC criterion for sleep-onset problem, and ESS). Parent report has been a less adequate source of information on the child owing to the demands of diary recording of sleep and/or remembering child behaviors as a proxy [25]. There is a potential for differential and non-differential misclassification. Sleep behaviors might be under- or over-reported depending on the attention of the parent during the night and/or the subjective perception of the problem in advance of reporting. The issue of differences in reporting by diagnosis suggests that there may be differences but not necessarily in the same direction. Parents of AUT reported more sleep problems by questionnaire in one study of both questionnaire and actigraphy [26], but when sleep measurements were compared side-by-side (diary versus actigraphy), there was an underestimate of sleep measurements by diary compared to actigraphy [27]. There may non-differential bias due to the general inaccuracies of recall when reporting events after the fact (in the morning for the sleep diary). Non-differential misclassification biases results toward the null (if the misclassification is independent of other errors), but differential misclassification may bias results toward or away from the null [28]. The data being used for sleep latency and the other two variables is not in lieu of actigraphy – there are simply portions of the measurements that depend on self-report. I think that non-differential misclassification is a more likely source of measurement error in the SACS given that sleep diaries are prone to general recall issues and inattentiveness [25], and compliance tends to drop over time [29].

Cortisol: For cortisol measurement, salivary cortisol is the definitive method in young children to avoid phlebotomy, especially with serial measurements, and the stress influences associated with phlebotomy. Although the product choice and the amount of Kool-Aid used was unlikely to interfere with the assay [30-32], avoiding stimulants is still the preferred mode of collection. All subjects were asked to use the Kool-Aid, and only six samples were collected without Kool-Aid due to parents forgetting or spilling the premeasured cap. Other variables that were evaluated were: saliva collection on same day as cognitive testing (2%), saliva collection on the weekend where bedtimes and wake times might have been different from weekdays (11%); eating food within 30 minutes of the target time (7%); and samples with saliva volume lower (< 0.25 µl) than recommended (8%). All samples were over the lower limit of sensitivity for distinguishing cortisol concentrations from zero (0.083 nmol/liter or 0.003 ug/dl) (Salimetrics, Inc., www.salimetrics.com). Every effort was made to conform to the recommendations of Salimetrics, Inc. for the collection, handling, and storage of the saliva. The individual effects of some factors (e.g. low saliva volume) on the precision of the measurement of cortisol are difficult to quantify, but the use of a cotton braid to collect saliva did result in an incomplete recovery of cortisol when low saliva volume was present [33]. Both brushing teeth (due to the possibility of cross-contamination with blood) and food intake (acidic or high sugar foods) can compromise assay performance by lowering the pH and contributing to bacterial growth (Salimetrics, Inc., www.salimetrics.com). There are mixed results with respect to the association between food and/or drink and cortisol levels. Within 20 to 40 minutes of consuming a noontime meal, levels of cortisol rose in two groups of lean and obese adult women [34]. In a
group of three- to seven-month-old infants, cortisol levels were low soon after feeding and became increasingly higher later on [35]. In both cases full meals or feedings were involved, whereas in the SACS the food during the period before the target was confirmed to be a snack or beverage (in only two cases there was a meal). In addition, none of the intended target times were close to meal times (waking sample was taken before breakfast) by design.

Due to limitations in funding and burdens on the family it was decided that only three time points would be used to reflect daytime secretion. Waking, midday, and bedtime samples were chosen to reflect the peak (waking), an interim point (midday), and nadir (bedtime) of secretion across the day (more akin to a curve) rather than only two collection points (a straight line). I opted to take advantage of the host study follow-up and collected repeated measurements on consecutive days and over six days total to obtain better precision for the estimates at the given time points during the day.

Missed data: The study suffered from large amounts of missing data due to: data collection midway through the host study (not all phases available); lapses in collection during a phase; and dropping out following the first phase. Almost all of the missing data were due to whole phases missing prior to SACS enrollment rather than lapses in collection during any given phase. Of the six children that dropped out with data following enrollment, only one dropped out due to problems with the SACS protocol. The others were drop-outs from the Sleep Study. Every attempt was made to obtain complete data by frequent and targeted contact with families at the most difficult collection times of the day (identified by the parent) and to assist with removing any burdens on participating and completing the study (I would come to the home to do the collection if necessary). The validity of any statistical analysis must take into consideration that assumptions are being made about the reasons for any missing data; that is, the assumptions regarding the missing data mechanism are tenable [14]. The missing data in the SACS was considered “missing at random” (MAR) rather than “missing completely at random” (MCAR). In MCAR, the observed data can be thought of as a random sample of the complete dataset. In MAR, the probability that responses are missing depends on the set of observed responses but is not related to specific missing values. MAR is essentially random missingness within strata of covariates. An important consequence of assuming MAR is that you cannot restrict an analysis to a fully complete dataset since this is considered a biased sample from the target population and the analysis is not a valid one. The problem is also not so much that you have a reduced sample size, although precision is an issue, but that the remaining dataset of non-missing values may also be a biased representation of the data. MAR is less restrictive than MCAR but necessitates a correctly specified model for the mean response and the covariance among the responses in order to use maximum likelihood estimation to get valid estimates of $\beta$ [14]. Although you cannot directly test MAR, I created a dichotomous variable reflecting whether cortisol data was missing (yes/no). I used this “missing” variable as the outcome in a series of logistic regression models to determine whether diagnosis or any of the sleep variables were related to missing cortisol data. Only the RDC for a sleep-onset problem was associated with the “missing” variable. In addition, I believe that all relevant covariates that predict the outcome are in the model, and I make the assumption that if there is any unmeasured confounding it is unrelated to missingness. Fitzmaurice, et. al, consider the MAR
assumption to be the default assumption for the analysis of partially missing longitudinal data [14].

Confounding

Potential confounders were identified through literature searches and through discussion with the co-investigators that were content experts. There were approximately 14 variables that were ultimately selected for possible inclusion in the DSA modeling. None of these variables were considered to be on the causal pathway. Other than the inclusion of time in every model, there was no single pattern of covariate inclusion, although hours of school/therapy during the day, hours of TV viewing, nap on day of saliva collection, and Hollingshead Index (measuring socioeconomic status) figured prominently. The level of unknown confounding was reduced as much as possible by covering all general areas that influence the exposures and cortisol – biological factors, socioeconomic factors, and stress and other events of the day. However, I cannot verify that all confounders were accounted for by the process described above.

The measurement of the potential confounders was variable depending on the class of variable. For example, child age and the longitudinal design parameters (phase, day, time) were considered highly accurately obtained. Race can be considered as a biological and/or sociodemographic characteristic in the SACS. Differences in race that have been observed in previous studies were primarily related to sleep schedule and napping, which may be due to differences in parenting styles or genetic differences in sleep needs [36]. The diagnosis and napping variables had all the attendant concerns outlined under the diagnosis and sleep strengths and limitations sections. The sociodemographic factors of mother working and the Hollingshead Index are based on self-report of employment status, occupation, and years of education. In terms of the measures of “stress” and other events of the day, three variables were obtained for the 24-hour period preceding the evening of the saliva collection day. The count of events, hours of television, and hours of school/therapy, were in fact asked for that given day so the recall period was short, but still relied on parent-report. For both the sociodemographic and 24-hour period variables, there may be errors related to recall or estimation, social approval-seeking, and fatigue or inattention of the respondent.

The Parenting Stress Index (PSI) [37] and the Child Behavior Checklist (CBCL) [38] are well-established instruments used over decades, designed specifically for the preschool-aged population and their families, and the CBCL has been validated in children with autism spectrum disorders [39]. These instruments were administered at the beginning of each phase. The CBCL had items that described the child “now or within the past two months” and the PSI had items that reflected how the parent was feeling currently. Although these are both parent report instruments, the CBCL reports about the child and the PSI reports about the parent. The CBCL relies on observations of the child by the parent, which may be modified by social approval-seeking behavior about the child or biased reporting based on diagnosis. The PSI self-report may also be influenced by social approval-seeking behavior of the parent. Both instruments have elements of recall – the CBCL more than the PSI – with reporting that may be blunted by time.
It is important to discuss how the misclassification of the potential confounders might impact the findings from the SACS. Child age, hours of school/therapy per day, the CBCL, and PSI were associated with diagnosis in bivariate analyses. However, these associations are most likely due to real differences between the groups (except for age which was by design) and not a systematic reporting bias. Therapy for children with autism is very intensive and may take up a full day, whereas a full day of preschool is less likely for the majority of typically developing children. The CBCL and PSI reflect stress and behavior in the family unit and previous research has shown differences between the groups [40-42]. To my knowledge, there is no work on whether social approval-seeking behavior is different for parents of a child with autism compared to parents of typically developing children. I believe that for all potential confounders, except for the CBCL and PSI, non-differential misclassification may be present, but not to any large degree. However, non-differential misclassification results in residual confounding due to an imperfect surrogate measure with proper adjustment more difficult to attain [43, 44]. Therefore, the overall results may be subject to some residual confounding. The PSI and CBCL are associated with autism, but likely due to true differences in children and families with autism compared to children and families of typically developing children. It is of note that in the host Sleep Study the results from a standardized questionnaire used to assess sleep problems (the CSHQ) were similar to the results from the more objective RDC criteria [45]. This suggests that there isn’t differential reporting by parents of children in the different diagnostic groups. The PSI was not chosen for any final models, but the CBCL was a covariate in the final model for the association between diagnosis and cortisol. It is difficult to assess whether any differential misclassification between the diagnostic groups occurred, given the strong exposure-disease relationship between the CBCL and diagnosis.

**Statistical analysis**

I used a loss-function-based (the L2 loss function) estimation procedure, the DSA algorithm to determine the model with the best fit or best estimate of the given data distribution [46, 47]. One of the strengths of the DSA algorithm is that it allows a candidate space to be developed for modeling with the user-specified options of simultaneous consideration of main effects, up to 3-way interaction terms, and up to cubic power terms for a set of variables deemed relevant based on a hypothesized causal model. This is an improvement over techniques that use a trial and error stepwise approach that is limited for comparing candidate models. In addition, in many standard regression procedures missing data can influence final model selection since these procedures exclude observations with any missing variable values from an analysis. By incorporating cross-validation the DSA avoids over-fitting the data and allows comparison of models with different numbers of observations without the loss of information [47].

I used G-computation estimation, one of several methods for causal statistical inference, to obtain marginal (unconditional or population-level) estimates of average cortisol secretion levels over a day. By imputing the counterfactual distribution for subjects observed in one exposure state, we create a pseudopopulation that allows inference for all possible exposure states. While not unique to G-computation estimation, the marginal estimates derived by this method are superior to observational techniques, such as standard regression. This is because the marginal estimates are
not constrained by stratification – the results are closer to a causal interpretation of an
effect. G-computation estimation is a link from the real (observational) world to the
ideal (counterfactual) world. The four key assumptions for the use of G-computation
estimation were met reasonably well by the data at hand. The sequential randomization
assumption implies no unmeasured confounding. It is difficult to assure no unmeasured
confounding in observational studies, but a comprehensive review and expert input was
persuasive. I am confident that the temporal ordering assumption was achieved for the
analyses related to diagnosis and those related to sleep. Since the G-computation
estimator is dependent on the accurate specification of the model, misspecification will
likely bias the estimates. This is can be of concern with a small sample size such as
that of the SACS.

Summary

Diagnosis and cortisol

In summary, there were only slightly higher cortisol secretion levels at waking
with wide and overlapping confidence intervals among children with autism compared to
typically developing children. A graded association between functional status and
cortisol secretion was observed although no variance estimates could be obtained due
to insufficient sample size for bootstrapping. There was greater between-subject within-
group variability and variability of the slope of cortisol secretion for children with autism
compared to typically developing children, suggesting that dysregulation may be
intrinsic to autism.

It has been suggested that autism could result from a neurointegrative defect in
the pathways of the CNS [48]. This defect could be represented as an inability to
integrate various pathways of the CNS so that neural processes are working to facilitate
normal brain function. It is of note that in several of the psychiatric disorders of
neurotransmitter imbalance, such as depression and anxiety, researchers generally
have found abnormal cortisol rhythms in adults [18-20]. It also might be posited that in
addition to a neurointegrative defect, the L-HPA axis of individuals with autism becomes
dysregulated as a result of environmental triggers, including stress, that directly affect
the L-HPA axis. These inputs might be considered chronic stressors that alter the
regulation of the diurnal secretion over time to achieve a hypercortisolism [49].
Researchers of depression and cortisol suggest that a chaotic pattern of cortisol
secretion rather than an increased cortisol secretion may be a better representation of
or more intrinsic to L-HPA axis dysregulation [50, 51]. Siever, et. al., suggest
that neurotransmitter activity in a variety of psychiatric syndromes may be better understood
as a relative failure in regulation instead of simple increases or decreases in secretion
[52]. Few studies of autism have examined cortisol variability directly, and this may
explain why there is some heterogeneity or mixed results with respect to the
comparison of cortisol secretion levels in selected groups. The findings from the
Corbett papers [1, 2] and those from the SACS suggest that although hypercortisolism
cannot be ruled out, a dysregulation for AUT may be a more likely characteristic of the
L-HPA system in these children. Some researchers posit that irregularities in more than
one pathway of the CNS are consistent with a neurointegrative defect in autism [48, 53].
Failure of circadian timing may also reflect environmental interaction with the biological
clock [54]. This has important implications for the regulation of sleep, which also relies
on a circadian feedback mechanism and environmental cues as part of its L-HPA pathway. The host Sleep Study observed that the RDC sleep problems were more intermittent in children with autism compared to typically developing children reflecting some variability for the presence of a sleep problem over the six months of observation [45]. There was greater night-to-night variability in sleep parameters according to sleep diaries for adults with Asperger syndrome compared to adult healthy controls [55].

Children with autism may be more sensitive to zeitgebers - exogenous cues that synchronize an individual’s time-keeping system. This may result in greater cortisol secretion and/or variability in measurements of cortisol during the day due to circadian dysregulation [2]. For example, measures of sensory sensitivity and stress were associated with cortisol secretion in school-age high-functioning children with autism [5]. Other authors have reported that abnormal sensory processing was correlated with a higher autism severity score (IQ was not examined) in 3- to 12-year-old children [56]. It is unknown whether functional status, as an indicator of autism severity, is associated with cortisol variability. Studies of individuals by IQ have shown more sensory symptoms in low IQ groups compared to higher IQ groups [56, 57] and this has been hypothesized as ineffective inhibitory control of sensory processing that may reflect an imbalance of neuronal excitation/inhibition in intellectually disabled children with autism [58]. Thus the increase in sensory symptomatology and difficulty processing sensory input may be reflected in cortisol secretion levels that may be higher than for higher functioning children with autism. However, among school-age high-functioning children with autism, sensory sensitivity and stress were associated with cortisol secretion [5] suggesting that sensory and/or stress symptoms may be a robust finding in all children with autism.

This study was not designed to investigate or address the underlying neurobiology of autism. Studies using neuroimaging or other techniques that aid in examining the brain may elucidate what is occurring in the anatomy and/or physiology of the CNS in individuals with autism. Given the lack of a large difference overall comparing strict autism to typically developing children, it is plausible that subgroup analyses may clarify what, if any, association there is between autism and cortisol secretion. In the future, enrollment of sufficient numbers of children with autism across the IQ spectrum to focus on functional status, sensory processing, and other pertinent subgroup characteristics, and their association with cortisol secretion and variability during the day, may be a promising avenue of research.

Sleep and cortisol

In summary, there were several findings that warrant further discussion. Minutes awake during the night and the RDC criterion for having a night-waking sleep problem were associated with higher concentrations of cortisol secretion at waking. As discussed in Chapter 5 on sleep and cortisol, physiological arousal can result in a difficulty in maintaining sleep [11, 59, 60]. Although there is a broad literature in adults, there are few studies in children, and they have been published only recently. These studies all observed that cognitive pre-sleep arousal (e.g. can’t shut off thoughts and worrying about falling asleep) was associated with sleep disturbances in school-age children. Gregory, et. al., observed that both child- and parent-report of insomnia that focused on difficulties falling asleep, night-waking, and trouble falling back to sleep, were associated with cognitive arousal [61]. A study of anxious children aged 7-14
years reported that pre-sleep cognitive arousal was associated with greater sleep problems (an array of problems including night wakings) [62]. A study of 3rd graders observed that low sleep efficiency (the proportion of time spent asleep during the sleep period) was positively related to pre-sleep arousal (and maternal psychological control) [63].

The link between arousal and cortisol secretion has been well established in adults with a recent systematic review concluding that the CAR was positively associated with job and general stress [64]. Studies in children have focused on stress and anxiety, as a proxy for arousal, in examining the association with cortisol. The results are consistent in school-age children and adolescents suggesting that there is: an increase in CAR with family conflicts and acute life events [65]; a stronger cortisol rise in morning with low level anxiety in girls [66]; higher CAR with persistent anxiety problems in young adolescents [67]; and a steep rise in morning cortisol in prepubertal children with anxiety [68].

Thus, the question of whether sleep quality or hygiene is an intermediary between pre-sleep arousal and cortisol secretion in the morning (exhibited as the CAR or early morning rise) is important to address. The aims of the SACS in relation to sleep were to determine the independent role of sleep quality in the association with cortisol secretion during the day. The potential covariates in the modeling procedure were expected to reflect general stress of the parent-child system (e.g. the PSI) as well as more proximal stress (e.g. count of events child participated in during the last 24 hours before the cortisol measurement). The models for minutes awake during the night, the RDC criteria for night-waking, and the ESS were further examined with weekend sampling (different stressors may occur on weekdays versus weekend days) and sleep-offset time (absolute time of waking may affect the cortisol level obtained). Later sleep starts and ends were found on weekends compared to weekdays in the Sleep Study [27]. However, weekend sampling and sleep-offset time did not confound the primary findings. Therefore, the findings should reflect the role of sleep independent of that of pre-sleep arousal and other covariates that were measured.

The mechanisms of the actions of poor sleep quality have not been determined unequivocally. Limbic-hippocampal structures appear to inhibit HPA activity and cortisol concentrations reach a diurnal minimum during the early part of nighttime sleep [69]. An increasing amount of cortisol is secreted during the latter part of nighttime sleep and a large proportion of total daily cortisol output occurs during the early morning hours (approximately three to four hours before final waking) [70-72]. This association between the beginning of sleep start and minimal cortisol secretion has led to the hypothesis that cortisol secretion is suppressed by sleep [73]. It may also be that frequent wakings which are more common during the latter part of the night (early morning hours) are part of the stimulus to the L-HPA to secrete cortisol. These frequent arousals with accompanying bursts of cortisol may culminate in the final waking for the day with the peak in cortisol secretion soon after final waking [74, 75]. A biologically plausible hypothesis to potentially explain the findings with minutes awake during the night may be that the accumulation of cortisol secretion from bursts during the time awake leads to the later peak reflecting the CAR. However, it would be surprising if the number of wakings would not lead to this effect as well, yet there was no association between frequency of waking and cortisol secretion. The salient causal factor is likely
absolute time awake with an inhibition of cortisol suppression for an increased amount of time to produce more cortisol secretion. For the measure of “excessive sleepiness”, there was a lower level of cortisol secretion at waking. Two studies measured daytime sleepiness and also measured cortisol secretion in the morning. Barcelo, et. al., studied patients with obstructive sleep apnea with and without excessive daytime sleepiness (by the ESS) and concluded that cortisol levels between 8:00 to 10:00am were lower for patients with excessive daytime sleepiness than for those without excessive daytime sleepiness [76]. Vgontzas, et. al., in a study of sleep restriction observed significant sleepiness by the multiple sleep latency test after one week of sleep restriction and observed a drop in peak values of cortisol [77]. Chida, et. al., in their systematic review found that CAR was negatively associated with fatigue, burnout, or exhaustion [64]. A plausible hypothesis would be that the subtle suppression of cortisol from the sleepiness would contribute to lower secretion at times of sleepiness. In fact, Larson, et. al., measured cortisol following napping in nine-month-old infants and observed lower cortisol values independent of the time of day or length of the nap [78]. However, in the SACS there was no association between being a napper and cortisol secretion during the day, although the midday target time was designed to come before a daytime nap. Some caveats with the ESS are that it is based on parent-report about “recent times”, and although temporally reported prior to the beginning of each phase is nonetheless a blunt instrument for measuring sleepiness or fatigue during a given day.

In conclusion, the magnitude of the marginal estimates, although with substantial variability around the estimates, suggested that being awake during the night was associated with higher cortisol secretion at waking the following day. This finding was independent of measures of stress and the burden of the child’s schedule, implying an effect of sleep disruption that does not depend on pre-sleep arousal. It is possible that measurement of pre-sleep arousal was insufficient (i.e. residual confounding) to ameliorate its effects or that in some instances sleep was intermediate to the effects of pre-sleep arousal.

In the future, more accurate measurement of minutes awake during the night should be pursued using videosomnography or ambulatory polysomnography (which can be used in the home setting), unless the sensitivity and specificity of the actigraph has been improved. The association between excessive sleepiness and waking cortisol was more tentative. The marginal estimates were lower, but not substantially lower, in children with excessive sleepiness compared to those without excessive sleepiness. The plausibility for the findings was not supported by the negative results in nappers. Measurement of excessive sleepiness could also be improved by its measurement more proximal to the cortisol collection times on a given day, rather than measured in a more generic time period prior to the day in question.

**Contribution to the current body of knowledge**
The SACS is one of the very few studies of cortisol secretion in preschool-aged children with autism. Just as children are not “little adults”, preschool-aged children are developmentally different from infants and school-age children. The study, by using prospective data collection in a short longitudinal design has established average cortisol secretion values at waking, midday, and bedtime over a six-month period in both children with autism and typically developing children.
The examination of functional status (defined as IQ by contemporary standards) in autism and cortisol secretion has generated a novel hypothesis about the importance of subgroups in describing differences in cortisol secretion. In addition, the SACS was uniquely able to examine variability in cortisol secretion in a population of very young children to determine whether variability might be an important descriptor of L-HPA function in autism, as it is believed to be in other psychiatric disorders. Although diagnosis was not an effect modifier of sleep on cortisol secretion in this study, the introduction of this hypothesis was informative to other studies that might proceed with this hypothesis in the future.

The SACS examined the most comprehensive measures of sleep and cortisol secretion over the largest number of days and times per day than any study in children to-date. There was a paucity of data on sleep measures other than night and 24-hour sleep duration in studies of preschool-aged children. Sleep latency, minutes awake, and frequency of night waking, were underrepresented and were measured in only three to four studies in this age group. The SACS is also one of the few studies to use actigraphy in this age group, although the results are chastened by the issues of the validity of actigraphy. The RDC criteria for sleep-onset and night-waking problems were developed over several study populations and designed to capture a combination of frequency, severity, and chronicity. The use of quantitative and qualitative RDC criteria is an improvement over the current literature which exists largely of questionnaire-based measures of sleep characteristics and sleep problems with little quantification. It will be important to use the RDC criteria in future studies to determine the predictive capabilities of the RDC criteria for daytime cortisol secretion.

As well as the results described above from original data collection, there were valuable findings from reviewing the literature in preparation for developing the research questions and hypotheses. I performed complete systematic reviews on sleep measurements and prevalence of sleep problems in preschool-aged typically developing children, on studies of cortisol secretion in non-institutionalized children with autism and comparison groups, and sleep differences between children with autism and typically developing children. Much of the literature prior to the dissertation quoted estimates and conclusions on these topics that were incomplete and/or inaccurate. I believe that the combination of literature review and original data from the SACS is an important contribution to the extant body of knowledge on sleep and cortisol in children with autism and typically developing children.
References


