




# DNA Methylation of the Oxytocin Receptor Across Neurodevelopmental Disorders

Michelle T. Siu<sup>1,2,3</sup> · Sarah J. Goodman<sup>3</sup> · Isaac Yellan<sup>3,4</sup> · Darci T. Butcher<sup>3,5</sup> · Maryam Jangjoo<sup>3</sup> · Daria Grafodatskaya<sup>5</sup> · Rageen Rajendram<sup>3</sup> · Youliang Lou<sup>3</sup> · Rujun Zhang<sup>3</sup> · Chunhua Zhao<sup>3</sup> · Rob Nicolson<sup>6</sup> · Stelios Georgiades<sup>7</sup> · Peter Szatmari<sup>8,9</sup> · Stephen W. Scherer<sup>3,4,10,11</sup> · Wendy Roberts<sup>3</sup> · Evdokia Anagnostou<sup>12,13</sup> · Rosanna Weksberg<sup>3,4,14,15</sup> 

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## Abstract

Many neurodevelopmental disorders (NDDs) share common learning and behavioural impairments, as well as features such as dysregulation of the oxytocin hormone. Here, we examined DNA methylation (DNAm) in the 1st intron of the oxytocin receptor gene, *OXTR*, in patients with autism spectrum (ASD), attention deficit and hyperactivity (ADHD) and obsessive compulsive (OCD) disorders. DNAm of *OXTR* was assessed for cohorts of ASD (blood), ADHD (saliva), OCD (saliva), which uncovered sex-specific DNAm differences compared to neurotypical, tissue-matched controls. Individuals with ASD or ADHD exhibiting extreme DNAm values had lower IQ and more social problems, respectively, than those with DNAm within normative ranges. This suggests that *OXTR* DNAm patterns are altered across NDDs and may be correlated with common clinical outcomes.

**Keywords** ASD · ADHD · OCD · DNA methylation · *OXTR*

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✉ Rosanna Weksberg  
rweksb@sickkids.ca

Michelle T. Siu  
michelle.siu@alum.utoronto.ca

Sarah J. Goodman  
sarah.goodman@sickkids.ca

Isaac Yellan  
isaac.yellan@mail.utoronto.ca

Darci T. Butcher  
butcherd@mcmaster.ca

Maryam Jangjoo  
maryam.jangjoo@sickkids.ca

Daria Grafodatskaya  
grafodatd@hhsc.ca

Rageen Rajendram  
rageen.rajendram@sickkids.ca

Youliang Lou  
youliang.lou@sickkids.ca

Rujun Zhang  
henryzhang7@gmail.com

Chunhua Zhao  
chunhua.zhao@sickkids.ca

Rob Nicolson  
robert.j.nicolson@gmail.com

Stelios Georgiades  
georgis@mcmaster.ca

Peter Szatmari  
peter.szatmari@sickkids.ca

Stephen W. Scherer  
stephen.scherer@sickkids.ca

Wendy Roberts  
wendy.roberts@sickkids.ca

Evdokia Anagnostou  
eanagnostou@hollandbloorview.ca

<sup>1</sup> Present Address: Department of Medical Genetics, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada

<sup>2</sup> Present Address: Alberta Children's Hospital Research Institute, Cumming School of Medicine, University of Calgary, Calgary, AB, Canada

Autism spectrum (ASD), attention deficit and hyperactivity (ADHD) and obsessive compulsive disorder (OCD) represent common childhood onset neurodevelopmental disorders (NDDs). ASD is characterized by dysfunction in three core domains: communication, social interaction, and a preference for repetitive behaviours rather than imaginative play; ADHD symptoms include inattention, hyperactivity and impulsivity; obsessions, compulsions, or both are behaviours that define OCD. However, there is marked comorbidity of features across all three disorders, highlighting the complexity of these NDDs and the challenges of clinical diagnostics. ASD is known to have a 4:1 male to female sex ratio, whereas in ADHD it varies from 3 to 6:1 across studies. OCD affects males and females equally (Loomes et al. 2017; Ramtekkar et al. 2010). Whether there is an underlying genetic and/or molecular basis for these observed sex differences has yet to be clarified. These NDDs are known to be multifactorial, heterogeneous disorders; it is likely that several factors including genetics and environment contribute to their underlying etiologies (Almandil et al. 2019; Nestadt et al. 2010).

The diagnostic guidelines for NDDs have become increasingly blurred as we discover more about the overlapping pathophysiology and underlying molecular etiologies across NDDs and other neuropsychiatric diseases such as schizophrenia (Carroll and Owen 2009; Kroon et al. 2013). Currently, all of the available pharmacological treatments in ASD target comorbid symptoms, such as seizures or sleep dysfunction, rather than core symptoms (Cohen et al. 2014; Frye et al. 2013; Relia and Ekambaram 2018). One promising treatment that may address a core feature of ASD is oxytocin (OXT). OXT is a peptide hormone synthesized in the hypothalamus and released into the bloodstream. OXT has been studied extensively in the context of labour and milk let-down (Leng et al. 2008; Smith and Merrill 2006;

Uvnäs-Moberg et al. 1990). A number of animal models have been employed to study the association between oxytocin and behaviour and cognition (Ferguson et al. 2000; Insel and Shapiro 1992; Sala et al. 2011). Mice carrying a targeted deletion of the *Oxtr* gene have been shown to have deficits in social recognition that were ameliorated by intraventricular oxytocin administration (Ferguson et al. 2000). In a study of NDDs, OXT has been identified as a potential therapy due to its role in regulating social behaviours as well as emotional and stress response, domains that are affected to different degrees across individuals diagnosed with ASD, ADHD, and OCD (Guastella et al. 2010; Guastella and Hickie 2016; Kalyoncu et al. 2019; Leckman et al. 1994; Park et al. 2010; Ross and Young 2009; Sasaki et al. 2015). It has also been reported, although inconsistently, that OXT, which has known anxiolytic effects, is elevated in the ventricular cerebrospinal fluid (CSF) of adult OCD patients, suggesting that this pathway is dysregulated (Altemus et al. 1999; Leckman et al. 1994). OXT has been tested in human clinical trials for individuals (children and adults) with ASD and has been found to be generally safe, but its efficacy in ameliorating core symptoms of ASD is mixed (Anagnostou et al. 2012; Dadds et al. 2014; Guastella and Hickie 2016; Tachibana et al. 2013). However, challenges in interpreting OXT findings include varying methods for measuring peripheral OXT across studies.

The role that genetics plays in the etiologies of these NDDs is well accepted, but genetics alone cannot account for all cases described. Approximately 25–40% of ASD cases have an identifiable genetic risk variant (Ropers 2010; Schaefer and Mendelsohn 2013), and the genetic heterogeneity of the disorder is underscored by > 200 ASD-risk genes that have been identified. The knowledge base for risk genes is much different for ADHD and OCD, both of which have had far fewer high confidence risk genes identified using

<sup>3</sup> Genetics and Genome Biology, Hospital for Sick Children, Toronto, ON, Canada

<sup>4</sup> Present Address: Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada

<sup>5</sup> Present Address: Department of Pathology and Molecular Medicine, McMaster University, Hamilton, ON, Canada

<sup>6</sup> Department of Psychiatry, University of Western Ontario, London, ON, Canada

<sup>7</sup> Department of Psychiatry and Behavioural Neurosciences, McMaster University, Hamilton, ON, Canada

<sup>8</sup> The Margaret and Wallace McCain Centre for Child, Youth & Family Mental Health and Campbell Family Mental Health Research Institute, Centre for Addiction and Mental Health, Toronto, ON, Canada

<sup>9</sup> Department of Psychiatry, University of Toronto, Toronto, ON, Canada

<sup>10</sup> The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, ON, Canada

<sup>11</sup> McLaughlin Centre, University of Toronto, Toronto, ON, Canada

<sup>12</sup> Holland Bloorview Kids Rehabilitation Hospital, Toronto, ON, Canada

<sup>13</sup> Department of Pediatrics, University of Toronto, Toronto, ON, Canada

<sup>14</sup> Division of Clinical and Metabolic Genetics, The Hospital for Sick Children, 555 University Ave, Toronto, ON M5G 1X8, Canada

<sup>15</sup> Institute of Medical Science, School of Graduate Studies, University of Toronto, Toronto, ON, Canada

next-generation sequencing (NGS) techniques (Stewart et al. 2013). With regards to genetic variation in oxytocin receptor (*OXTR*) gene, a meta-analysis of *OXTR* variation and ASD data from 8 studies found consistent associations between variation at five *OXTR* SNPs, rs7632287, rs237887, rs2268491 and rs2254298, and ASD (LoParo and Waldman 2015). Recently, one of these SNPs, rs2254298, was found to associate with increased social deficits in ASD but decreased social deficits in ADHD (Baribeau et al. 2017). Further, particular polymorphisms in *OXTR* have been shown to have a modulating effect on the age of onset for some individuals with OCD (Kang et al. 2017). It has also been reported that genetic variants in *OXTR*, including the AA genotype in rs53576, are associated with improved social cognition in a subset of individuals with ADHD (Park et al. 2010). These findings are not limited to individuals with NDDs; in individuals with no known diagnosis, associations between *OXTR* genetic variation and social behaviour have been identified in addition to associations between *OXTR* variation and limbic system structure and function, including amygdala volume (Reviewed in Kumsta and Heinrichs 2013).

A role for epigenetics in the etiology of NDDs is newly emerging through the identification of many ASD, intellectual disability (ID), and neurodevelopmental risk genes known to play a role in epigenetic regulation, such as chromatin remodeling (Iwase et al. 2017; Kleefstra et al. 2014; Kochinke et al. 2016). Further, dysregulation of epigenetic marks, such as DNAm and histone acetylation, has been demonstrated in multiple tissues, both targeted and genome-wide, primarily in association with ASD (Grafodatskaya et al. 2010; Gregory et al. 2009; Nagarajan et al. 2006). However, there have yet to be consistent findings of specific epigenetically dysregulated genes with high sensitivity and specificity for any one NDD. This is likely due, in part, to the heterogeneity of cohorts examined in past studies, small sample sizes, and the fact that there may be shared biological pathways involved in the pathogenesis across disorders (Butcher et al. 2017; Subramanian et al. 2015).

Epigenetic studies of the OXT pathway could be helpful in understanding the heterogeneous response to OXT therapy. One early report, in particular, has acted as a catalyst for our studies, identifying a region of the *OXTR* gene as the focus of our investigation. Gregory et al. (2009) demonstrated increased DNAm in the 5' untranslated region (UTR) and 1st intron of *OXTR* in a sex- and CpG site-specific manner, in a small, targeted study (20 ASD cases, 20 controls; 10 males and 10 females in each group) of post-mortem brain cortex and also from blood of ASD cases (Gregory et al. 2009). Similar to reports for ASD, DNAm in exon 3 of *OXTR* was found to be elevated in blood collected from OCD patients compared with controls, and such increased DNAm was associated with OCD severity (Cappi et al. 2016). In “neurotypical” subjects *OXTR* methylation at the 1st intron,

specifically CpG site –934 (also assayed in this study), has been associated with numerous social behavioural phenotypes including perception of emotional faces and response to displays of living vs. inanimate stimuli, as measured by fMRI (Jack et al. 2012; Puglia et al. 2015). This suggests that the relationship between DNAm of *OXTR* and phenotypic outcomes related to social behaviour likely extends across the spectrum of neurodevelopmental outcomes.

The goals of this study were to extend the Gregory et al. (2009) study with a larger ASD cohort, examining overlapping CpG sites with the Gregory et al. (2009) study as well as additional CpG sites, and to examine *OXTR* DNAm as an epigenetic biomarker of NDDs across diagnostic categories. We hypothesized that, given the suggested role of epigenetics and OXT in NDD molecular etiology, epigenetic modifications to the *OXTR* gene may act as a useful biomarker and/or contribute to the underlying etiology of subsets of patients across NDDs. We examined epigenetic marks, specifically DNAm in the 1st intron of the *OXTR* gene in the context of disorder status and found that CpG sites exhibiting differential methylation tended to be unique to disorder and sex.

## Methods

### Research Subjects

Cohorts for this study were collected from the Autism Research Unit at the Hospital for Sick Children, Toronto, Canada and the Province of Ontario Neurodevelopmental Disorders (POND) Network. The subjects aged 2–18 years, with a primary diagnosis of ASD, ADHD or OCD in POND were included in the study. The clinical diagnosis was confirmed using Autism Diagnostic Interview-Revised (ADI-R; Lord et al. 1994) and autism diagnostic observation schedule (ADOS; Lord et al. 2000) by clinical staff formally trained on all measures. The final inclusion criteria for the study was meeting ASD diagnosis cut off on ADI-R and ASD or autism cut off in ADOS. Similarly, primary clinical diagnoses of ADHD or OCD, were confirmed using the Parent Interview for Child Symptoms and the CY-BOCS, respectively. Informed consent was taken from a parent or guardian. Participants were enrolled and consented in studies approved by the Research Ethics Boards of the respective institutions (Holland Bloorview Kids Rehabilitation Hospital, Toronto; The Hospital for Sick Children, Toronto; McMaster Children’s Hospital, Hamilton; and Lawson Health Research Institute, London); the experiments and analyses described below were performed in accordance with relevant guidelines and regulations.

Whole blood samples were collected from the ASD cohorts, whereas saliva samples were collected from the ADHD and OCD cohorts for DNA extraction. Blood was

collected from an antecubital vein into an EDTA (lavender) Vacutainer. Saliva was collected using Oragene OG-500 (DNA Genotek, Ottawa, ON) collection kits and stored at room temperature as per manufacturer's instructions. Samples from neurotypical control individuals matched for age and tissue were selected from a collection banked in our laboratory (blood), POND neurotypical controls (blood) and the Thoughts Actions Genes (TAG) study (saliva; details published in Crosbie et al. 2013). For a subset of ASD cases and controls, precise ages were not known, only that these individuals were between 4 and 12 years old. Calculations of age differences between groups were made using only cases with known ages and were not significantly different (Mann–Whitney U tests,  $p$ -values > 0.05).

### DNA Processing and Pyrosequencing of *OXTR*

DNA was extracted from whole blood or saliva using phenol–chloroform extraction and prepIT C2D Genomic DNA MiniPrep Kit (Oragene), respectively. DNAm analysis was performed using pyrosequencing of sodium bisulfite converted DNA as described by Tost and Gut (2007). Samples were bisulfite converted in batches of approx. 10–20 samples using the EpiTect Bisulfite Kit (QIAGEN). Three pyrosequencing assays were designed to target 9 CpG sites in the 1st intron of *OXTR* using PyroMark Assay Design Software (Qiagen, Germantown, MD) (Supplementary Table 1). Supplementary Fig. 1 displays a standard curve of each CpG, generated by pyrosequencing mixtures of Qiagen EpiTect PCR Control DNA Set (0%, 25%, 50%, 75, 100% methylated samples); DNAm values were not normalized to these standard curves. Pyrosequencing assays also measured allelic variation in the following SNPs: rs53576, rs2254298, rs237887 and rs13316193. Sodium

bisulfite converted DNA was amplified using Hot-Start Taq-polymerase (Qiagen, Germantown, MD). Amplicons were analyzed on either the Q96 or Q24 pyrosequencer as specified by manufacturer (Qiagen). Samples were pyrosequenced in six batches on the Q96 platform, and five batches on the Q24; batches were stratified for cases (from one or more NDDs) and controls, and % methylation was quantified as the ratio of C (methylated cytosines) to C + T (total methylated and unmethylated cytosines) using PyroMark Software (Qiagen).

A total of 248 ASD and 151 control blood samples and 38 ADHD, 38 OCD, and 65 control saliva samples were analyzed via pyrosequencing, although not all assays were run on each sample due to sample quantity restrictions. Following pyrosequencing, PyroMark software was run to exclude reference peaks with low quality using the default analysis perimeters of the Q24 or Q96 PyroMark software. CpG sites that “failed” due to low quality, including a peak intensity < 10 or > 7% unconverted sequence, were not included in further analysis. As a result, some statistical comparisons have distinct sample sizes.

### Statistical Analysis of Differential DNA Methylation

All data were analyzed using GraphPad Prism 5.0. DNAm data were analyzed at each CpG site for all cases and controls combined (i.e. mixed sex), as well as by sex-specific comparisons. For sample sizes used in each comparison, see Supplementary Table 2. Data for each group comparison were tested for normality using the D'Agostino-Pearson and Shapiro–Wilk normality tests. Significance was evaluated by t-test with Welch's correction for normally distributed data or by Mann–Whitney U test for or non-normally distributed data. As each comparison was run across 9 CpGs sites, we chose to correct for multiple testing using Bonferroni correction. As such, significance was achieved if the

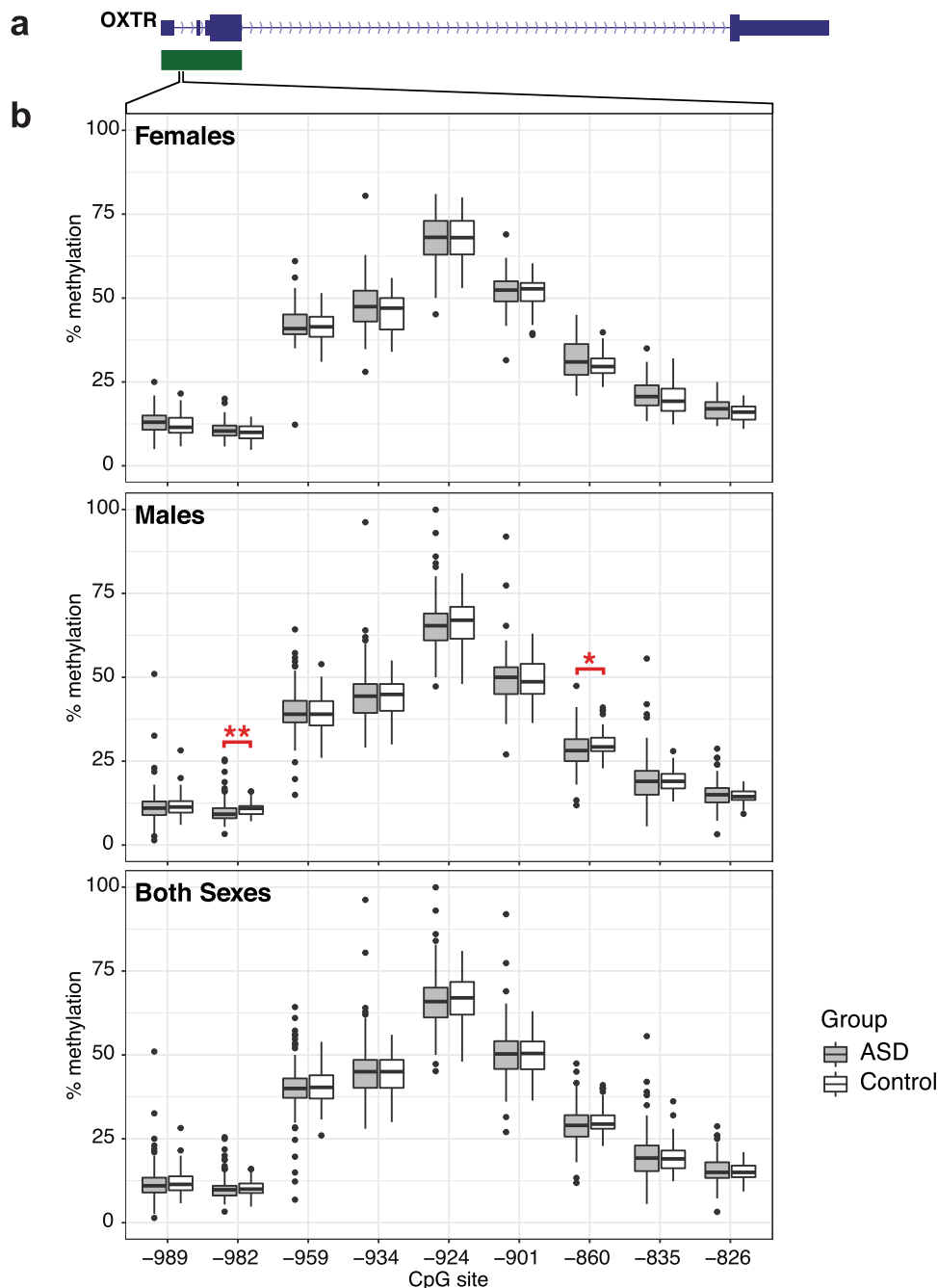
**Table 1** Clinical measures analyzed against DNAm

Scale	Subscale	In text abbreviation	Trait/symptomology	Test cut-offs
WASI*, WASI II*, or WISC IV**	Full scale intelligence quotient	FSIQ	Intelligence	< 70 (composite score)
Adaptive Behavior Assessment System	General Adaptive Composite	ABAS II	Adaptive functioning	≤ 70 (composite score)
Adaptive Behavior Assessment System	Social adaptive domain	ABAS II	Social functioning	≤ 70 (composite score)
Child Behavior Checklist	Anxious/Depressed subscale	CBCL	Anxiety	> 67 (T-score)
Child Behavior Checklist	Social problems subscale	CBCL	Social problems	> 67 (T-score)
Social Communication Questionnaire	–	SCQ	ASD	1 (dichotomous diagnosis)
Strengths and Weaknesses of ADHD Symptoms and Normal Behavior Rating Scale	–	SWAN	ADHD	≥ 6 (number of symptoms)
Toronto Obsessive–Compulsive Scale	–	TOCS	OCD	> 8 (mean total score)

\*Wechsler Abbreviated Scale of Intelligence

\*\*Wechsler Intelligence Scale for Children

**Fig. 1** Site-specific blood DNA methylation differences in ASD cases and control. **a** Scheme of *OXTR* gene, promoter CpG island and assayed sites. **b** Tukey box plots of % DNAm for nine CpG sites in *OXTR*, in ASD samples and matched whole blood controls in females only (top panel), males only (middle panel), and both sexes (bottom panel). Red bars with one asterisk represent differences in median DNAm ( $p$ -value < 0.05, t-test or Mann Whitney U). Red bars with two asterisks represent significant differences in median DNAm following Bonferroni correction ( $p$ -value < 0.0055, t-test or Mann Whitney U). CpG sites are numbered according to translation start site. Black circles represent individuals that have outlier DNAm value beyond the Tukey fences (error bars) which represent the 75th percentile plus 1.5 times IQR and 25th percentile minus 1.5 IQR



$p$ -value < 0.0055 ( $p$ -value < 0.05/9 tests). Effect sizes are reported as median methylation differences ( $\Delta\beta$ ). All data were plotted as box plots with Tukey whiskers.

### Examination of Clinical Features with Outlier DNA Methylation Values

See Table 1 for descriptions of clinical measures.

The measures that we considered and their parameters included IQ measured using either WASI, WASI II, or WISC IV scores (FSIQ score used for each test), SWAN

scores (ADHD diagnosis score, within or below clinical range), SCQ diagnosis (score of 0 or 1, 1 indicative of ASD), TPOCS total score (> 8 was considered indicative of OCD), CBCL subscale scores for anxiety or social problems (> 67 was indicative of more anxiety or social problems), and ABAS II social or general adaptive composite scores ( $\leq 70$  was indicative of lower adaptive functioning). These clinical data were available for a subset of samples, who were recruited through the POND network. Tissue sample collection (saliva or blood) was performed in the same study visit as clinical feature testing, or within a 2-month period.

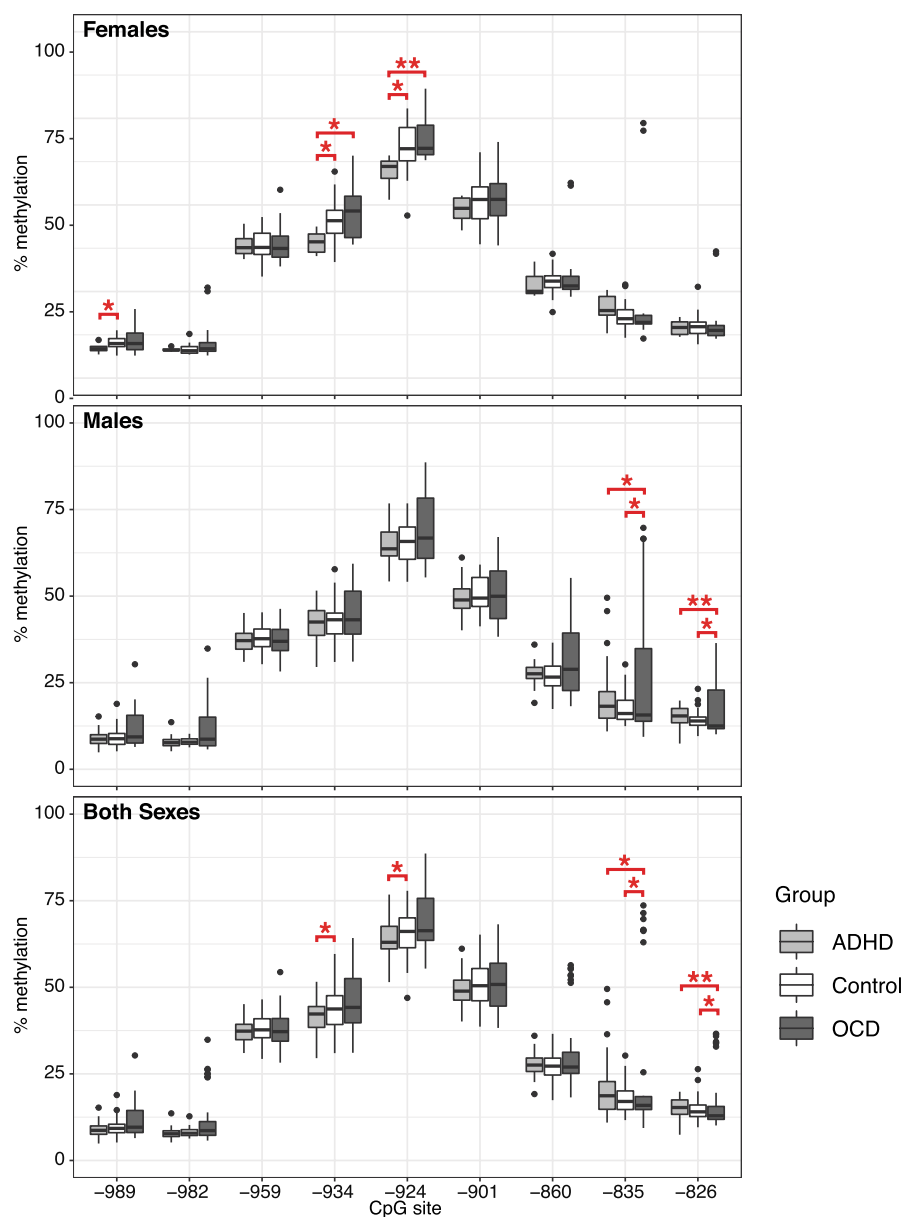
Individuals with outlier DNAm values, as determined by Tukey fences, were compared at each of these measures to a random selection of individuals of comparable sample size and with the same diagnosis who had DNAm values within Tukey fences. Differences in the frequency of non-standard scores for individuals with and without outlier DNAm values were tested using a Fisher’s exact test. As these tests were performed as an exploratory analysis, we opted to run a univariate analysis of each measure rather than a multivariate analysis despite the likely relationship between clinical features.

## Results

### Site-Specific Blood DNA Methylation Differences: ASD

For each of the 9 CpG in *OXTR*, measured over three assays, we ran two group comparisons of DNAm in (1) all ASD (both sexes) versus all neuro-typical control samples, (2) ASD males versus neuro-typical control males, and (3) ASD females versus neuro-typical control females (Fig. 1a; see Supplementary Table 2 for samples sizes used in each comparison). We defined neuro-typical controls as those with no features or behaviours suggestive of intellectual delay or any neurodevelopmental disorder, including ASD. T-tests

**Fig. 2** Site-specific blood DNA methylation differences in ADHD cases, OCD cases, and control. Tukey box plots of % DNAm for nine CpG sites in *OXTR*, in ADHD samples, OCD samples, and matched saliva controls in females only (top panel), males only (middle panel), and both sexes (bottom panel). Red bars with one asterisk represent differences in median DNAm ( $p$ -value < 0.05, t-test or Mann Whitney U). Red bars with two asterisks represent significant differences in median DNAm following Bonferroni correction ( $p$ -value < 0.0055, t-test or Mann Whitney U). CpG sites are numbered according to translation start site. Black circles represent individuals that have outlier DNAm value beyond the Tukey fences (error bars) which represent the 75th percentile plus 1.5 times IQR and 25th percentile minus 1.5 IQR





or Mann–Whitney U tests were used depending on the normality of inter-individual distribution for each CpG, and Bonferroni correction was applied. No tests were significant prior to or following Bonferroni correction when comparing all ASD samples to controls (sexes combined). Age was assessed in a post hoc approach, as a potential confounding factor of ASD-specific DNAm alterations; a linear regression of age vs. DNAm was run at each CpG site and no significant associations were identified following Bonferroni correction (Supplementary Fig. 2).

As an exploratory analysis, we then repeated these two group comparisons with samples separated by sex. One CpG site, –982, was found to be significantly hypomethylated in ASD males compared with control males ( $|\Delta\beta|$  1.73%), after Bonferroni correction (adjusted  $p$ -value  $< 0.0055$ ) (Fig. 1b). This was the only CpG to remain significant following  $p$ -value adjustments across all NDD comparisons with controls. One additional CpG, –860, was hypomethylated in ASD males compared with control males ( $\Delta\beta$  1.09%), but did not pass multiple test correction ( $p$ -value  $< 0.05$ ) (Fig. 1b). We did not find significant differential methylation at any *OXTR* sites in female only sample comparison.

#### Site-Specific Saliva DNA Methylation Differences: ADHD

Analyses identical to those run on ASD samples were applied to ADHD saliva samples and neuro-typical, tissue-matched controls. Two CpG sites, –934 and –924, were found to be hypomethylated in the mixed-sex comparison ( $\Delta\beta$  1.65% and 4.95%, respectively) and female-specific comparison ( $\Delta\beta$  8.35% and 7.37%, respectively) of ADHD versus controls at an uncorrected  $p$ -value  $< 0.05$  (Fig. 2). Additionally, CpG site –989 was hypomethylated in ADHD females compared to control females ( $\Delta\beta$  1.63%,  $p$ -value  $< 0.05$ ) (Fig. 2). Of the three NDDs assessed, hypomethylation in ADHD females was the most consistent finding, encompassing a total of three CpGs exhibiting this pattern compared to controls. No associations between DNAm and ADHD diagnosis were observed in males.

#### Site-Specific Saliva DNA Methylation Differences: OCD

Two CpG sites, –835 and –826, were found to be hypomethylated in the mixed-sex comparison ( $\Delta\beta$  1.37% and 1.62%, respectively) and male-specific comparisons ( $\Delta\beta$  1.45% and 1.83%, respectively) of OCD versus controls (all  $p$ -values  $< 0.05$ ) (Fig. 2). Differential methylation at these specific CpGs was unique to OCD and not observed in ADHD or ASD when compared to their respective control groups.

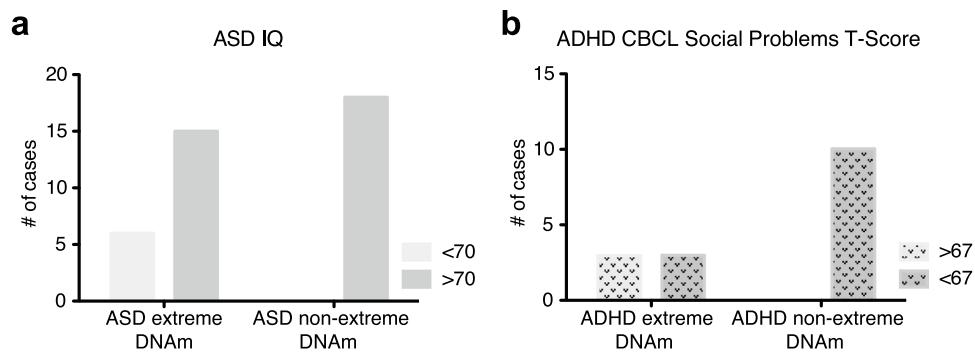
#### Cross-Disorder Comparisons of Differentially Methylated Sites in *OXTR*

As ADHD and OCD samples were both analyzed in saliva samples, we were able to make direct comparisons of DNAm across these two diagnostic groups. In females with ADHD, CpG site –924 was significantly hypomethylated as compared to females with OCD ( $\Delta\beta$  5.23%,  $p$ -value  $< 0.0017$ ); CpG, –934 followed the same trend and met the threshold of  $p$ -value  $< 0.05$  but did not reach significance after multiple test correction (Fig. 2). CpG sites –835 and –826 were both hypermethylated in individuals with ADHD in both the mixed sex and male-specific comparisons with OCD samples, with CpG –826 passing correction for multiple testing for both groups ( $\Delta\beta$  males only 2.9%,  $\Delta\beta$  both sexes 1.78%,  $p$ -values  $< 0.0017$ ; Fig. 2). In sum, differences in DNAm between OCD and ADHD were sex- and CpG-specific with two sites exhibiting hypomethylation in ADHD females versus OCD females and two sites exhibiting hypermethylation in ADHD males versus OCD males.

As ASD was measured in blood, not saliva, rather directly comparing DNAm values to that of ADHD and OCD, DNAm trends relative to controls were compared. Despite tissue specific DNAm difference, one CpG was found to be differentially methylated across independent disorder groups; in both ASD and ADHD males, site –982 was hypomethylated relative to controls.

#### Examination of Clinical Features and Genetic Variation in Individuals with Extreme DNAm Values

Tukey box plots of all samples in each disorder (male and female combined) at each assayed CpG revealed a number of individuals with extreme DNAm values (“outliers”), defined as those beyond the Tukey fences (error bars representing the 75th percentile plus 1.5 times IQR and 25th percentile minus 1.5 IQR). Six individuals with ADHD (all male) and six individuals with OCD (4 male) exhibited extreme methylation. In the ADHD samples, each individual displayed extreme methylation at only one CpG site, with five individuals displaying hypermethylation and one displaying hypomethylation. Similarly, in the OCD samples, all but one individual displayed extreme methylation at only one CpG site, with five individuals displaying hypermethylation and one displaying hypomethylation. A total of 35 individuals with ASD exhibited extreme DNAm at one or more CpGs, resulting in 63 CpGs extreme values (Supplementary Fig. 3). Twenty-four of the 34 individuals exhibited extreme values at more than one CpG. Of the 35 individuals, 23 were hypermethylated, as compared to the entire ASD sample set, six were hypomethylated, and six exhibited both hyper- and hypomethylation at different sites. Approximately 72% ( $n = 26$ ) of these individuals were male,



**Fig. 3** Clinical features enriched in outlier cases (ASD and ADHD). **a** Bar graph demonstrating significant differences in IQ scores between individuals with ASDs with outlier DNAm values compared with non-outlier DNAm values. Those individuals exhibiting outlier DNAm values consisted of a greater proportion of individuals with IQ < 70 than those with non-outlier DNAm values. **b** Bar graph dem-

onstrating significant differences in CBCL social problem subscale T-scores between individuals with ADHD with outlier DNAm values compared with non-outlier DNAm values. CBCL subscale score > 67 was indicative of more social problems. A subset of ADHD individuals exhibiting outlier DNAm values consisted of a greater proportion of individuals with higher CBCL social problem subscale T-scores

consistent with the sex proportion in the greater ASD sample of 77% male. These individuals ranged in age from 2 to 17 years (mean = 10).

Of the 63 extreme methylation values measure in our ASD cohort, 46 were hypermethylated as compared to the greater ASD cohort. This prompted us to test whether the proportion of hypo- and hypermethylated values at each CpG were significantly different from random outcomes generated from 50% probability, to assess if hypo- or hypermethylation was significantly more common than expected by chance. CpG -981, with 10 of 11 hypermethylated extreme values, and CpG -835, with five of five hypermethylated extreme values, were both significant ( $p$ -value < 0.05, two-tailed). By comparison, the control samples were also assessed for “extreme” methylation values using the same criteria and only five of nine CpG sites contained extreme values (-989 [3 hypermethylated samples], -982 [2 hypermethylated samples], -959 [1 hyper-, 1 hypomethylated sample], -860 [3 hypermethylated samples], -835 [2 hypermethylated samples]). At no CpG, were the occurrences of extreme hyper- vs. hypomethylated control samples significantly different from 50% probability suggesting that hypermethylation at *OXTR* may be specific to ASD.

Next, we examined several clinical feature measures within each disorder to investigate whether there were any commonalities amongst these individuals with extreme DNAm values that could distinguish them from those falling within the Tukey fences (Table 1). Of the individuals with these data available (POND participants only), comparisons were made for each NDD, i.e. ASD samples with outlier DNAm values versus ASD samples with DNAm within error bars; ADHD samples with outlier DNAm values versus ADHD samples with DNAm within error bars;

and OCD samples with outlier DNAm values versus OCD samples with DNAm within error bars. For each comparison, samples were stratified by clinical measure scores (see Table 1 for cut-offs), and Fisher’s exact tests were applied.

Of all the measures examined (IQ, SWAN, SCQ and TOCS scores, CBCL subscale scores, ABAS II composite scores), only IQ and the CBCL social problems subscale measures exhibited significant differences in scores between individuals with and without “outlier” DNAm values in ASD and ADHD, respectively. There was a significantly greater proportion of individuals with ASD and outlier DNAm values with IQ scores < 70 (i.e. greater incidence of intellectual disability) than observed in individuals with DNAm values within the normal range (Tukey fences) ( $p$  < 0.05; Fig. 3). In the individuals with ADHD and outlier DNAm values, there were a greater proportion who exhibited CBCL social problem subscale scores > 67, i.e. more severe social problems (Fig. 3).

Previous findings of allelic variation in *OXTR* conferring high versus low risk for social deficits in individuals with ASD and ADHD, including some individuals present in this analysis, led us to test for epigenotype–genotype–phenotype associations (Baribeau et al. 2017). We examined SNPs previously shown to confer differential risk in ASD and ADHD populations, rs53576, rs2254298, rs237887 and rs13316193, and compared the frequency of alleles in our ASD and ADHD cases with outlier DNAm values compared to those exhibiting values within the normal range for individuals; this analysis was identical to that run on the clinical features. We did not observe any differences in frequency of any genotypes between the groups of ASD or ADHD cases versus controls ( $p$ -values > 0.05 Fisher’s exact test; data not shown).



## Discussion

Our results demonstrate that DNAm at specific sites in the 1st intron of *OXTR* are differentially methylated in the blood or saliva of individuals with ASD, ADHD or OCD. As well, individuals with ASD or ADHD exhibiting the most extreme DNAm values at specific sites were more likely to have lower IQ (ASD) or a higher score on the CBCL social problems subscale (ADHD), respectively, than those with DNAm within the normal ranges for each respective NDD group. Overall, our results reflect a complex but quantifiable relationship between *OXTR* DNAm and NDDs.

The causes of NDDs are multi-factorial and likely include not only genetic factors but epigenetic factors as well (Grafodatskaya et al. 2010), while the OXT pathway has been implicated in specific behavioural phenotypes of NDDs (Francis et al. 2014; Grinevich et al. 2014, 2016). From a functional point of view, it has been suggested that disease status (e.g. ASD diagnosis) may have a direct association with *OXTR* density in the brain itself (Freeman et al. 2018). Specific regions of interest in areas of the brain

(ventral pallidum, nucleus basalis of Meynert in the basal forebrain) that have functional relevance to the behavioural and cognitive features of ASD (reinforcement learning and visual attention, respectively) were found to have differential *OXTR* binding using competitive-binding receptor autoradiographical methods.

Genetic factors such as deletions, replications, and polymorphisms in genes in this pathway have been associated with ASD and ADHD outcomes (Ayaz et al. 2015; Baribeau et al. 2017; Park et al. 2010). Deletion of chromosome 3p25.3 has been found in ASD cases; however, partial deletions of *OXTR* have also been found in controls samples (Gregory et al. 2009; Shaikh et al. 2009). As well, linkage and association studies have identified SNPs in *OXTR* that are associated with ASD (Jacob et al. 2007; Lerer et al. 2008; Wu et al. 2005), although findings are inconsistent. The most interesting associations with *OXTR* gene variants and ASD are specific to phenotypic domains, including social domain dysfunction and social auditory processing, including rs1042778 associating with both social and communication cutoffs on the ADOS (Campbell et al. 2011) A smaller number of studies of *OXTR* SNPs have also been

**Table 2** Summary of DNA methylation changes in cases as compared to controls

		-989	-982	-959	-934	-924	-860	-835	-826
Gregory (2009)	ASD M				↑		↑		
	ASD F			↑					
	ASD M+F			↑	↑		↑		
Our data	ASD M		↓ ASD 10.1% control 10.7%				↓ ASD 28.3% control 30.3%		
	ADHD M+F				↓ ADHD 41.7% control 44.3%	↓ ADHD 64.2% control 66.8%			
	ADHD F	↓ ADHD 8.7% control 10.3%			↓ ADHD 39.2% control 46.5%	↓ ADHD 59.6% control 67.8%			
	OCD M							↓ OCD 14.9% control 17.6%	↓ OCD 12.6% control 14.2%
	OCD M+F							↓ OCD 15.5% control 17.9%	↓ OCD 13.1% control 14.7%

Direction of arrow = change in DNAm relative to controls; black arrow = p-value < 0.05; red arrow = passes Bonferroni correction. Values indicate mean DNAm in cases and controls

performed in children with ADHD, identifying various associations with specific behavioural domains. However, findings were not consistent across studies (Ayaz et al. 2015; Kalyoncu et al. 2019; Park et al. 2010). The contribution of OXT and its receptor OXTR to NDDs has gained more interest, given the therapeutic potential of OXT in the treatment of NDD symptoms. This is the first study to examine OXTR DNAm across ASD, ADHD and OCD, with the largest ASD cohort to date.

The majority of studies investigating altered DNAm of OXTR (various gene regions) reveal hypermethylation in ASD cases compared with controls, but results across studies are not entirely consistent (Maud et al. 2018). Gregory et al. (2009) showed that several CpGs tested within the region overlapping the 1st intron of OXTR in individuals with ASD have altered DNAm compared to controls (Table 2). DNAm at CpG sites -934 and -860 was significantly higher in blood (-934 also in brain) in ASD males compared to controls, where DNAm at site -959 was significantly higher in blood of ASD females than controls (Gregory et al. 2009). Our data did not replicate these findings, showing hypomethylation, rather than hypermethylation, in ASD males at site -860 (Table 2). As well, CpG site -934 was hypomethylated in our data, but hypermethylated in Gregory et al. (2009) in ASD females. However, within our ASD samples, the majority of outlying DNAm values (76%) exhibited hypermethylation. As such, these data support previous findings of hypermethylation of OXTR in some individuals with ASD, despite our trends of reduced mean methylation of the ASD cohort as compared to controls. We posit that the smaller Gregory et al. cohort ( $n = 20$ ) was either more homogenous, displaying hypermethylation with high frequency, or that a few individuals may have driven group trends. Our ASD cohort sample size ( $n > 100$ ) likely encompasses a much more varied patient composition, representative of the general ASD population, resulting in less consistent DNAm changes within the group. We also used targeted bisulfite pyrosequencing to assess for site-specific DNAm, which produced a percent methylation value for each CpG site amplified from the antisense strand, rather than sodium bisulfite sequencing (SBS), which gives absolute DNAm value for each CpG site as measured on the sense strand of DNA. This may also contribute to the reported differences between these studies. However, we expect no systematic difference in sense vs. anti-sense CpG methylation due to the symmetrical nature of the dinucleotide.

Notably, we identified new DNAm alterations at CpG sites in OXTR across disorders and tissues, observing decreased DNAm at various sites in both ADHD and OCD cases in saliva. OCD analysis revealed the fewest number of differentially methylated sites and smallest magnitude overall, with no significant sites overlapping with either another NDD or with the Gregory et al. (2009) sites. This finding

mirrors brain imaging findings in which OCD group was most similar to controls in terms of structural connectivity, as compared to ASD and ADHD groups (Ameis et al. 2016). ADHD analysis uncovered one CpG site (-982) hypomethylated in males, which was also hypomethylated within males with ASD. However, the analyses in ADHD and OCD could benefit from further increases in sample size for both the NDD and neurotypical control groups to evaluate the generalizability of our current findings, as well as parallel analysis in blood in order to make a direct comparison with our ASD data. The fact that we observed little overlap in differentially methylated CpG sites across ADHD/OCD and ASD is most likely due to the tissue-specific nature of DNAm patterning and were comparing blood and saliva. In fact, differences in tissue of origin is one of the strongest contributors to DNAm differences (Jaffe and Irizarry 2014; Ziller et al. 2014).

By assessing individuals with outlier DNAm values, we observed that DNAm may be more strongly associated with particular clinical features, such as anxiety, social problems or other cognitive/behavioural measures than the categorical disorder themselves. The strongest associations were identified for IQ in ASD cases and the social problems subscale on the CBCL in ADHD cases, where worse scores (i.e. lower IQ or indication of greater social problems, respectively) were significantly overrepresented in individuals with outlier DNAm when compared with scores from ASD or ADHD cases with normative DNAm values. The association with social problems in individuals with ADHD and OXTR DNAm is notable as OXT is the most widely used treatment for targeting social cognition deficits, often employed in schizophrenia and ASD (Fernandez-Sotos et al. 2018). Individuals with ASD treated with intranasal OXT have demonstrated altered cognitive and motor characteristics including improved face recognition memory, better ability to infer mental state of others, and more frequently fixed sight on the eye region of human faces (Guastella et al. 2008; Keech et al. 2018), all of which are associated with social behaviours. It is important to note that despite the promise that OXT holds potential as a therapy for social cognition deficits, results are mixed (Keech et al. 2018), and OXT has yet to be tested as a treatment in either ADHD or OCD patient cohorts. These inconsistencies may be due to the heterogeneity of patient cohorts tested that could potentially benefit from clinical and/or molecular pre-stratification, i.e. a precision medicine approach.

A study by Baribeau et al. (2017) investigated SNPs in OXTR conferring high versus low risk for social deficits in individuals with ASD and ADHD, confirming previously demonstrated genotype-phenotype associations between OXTR variation and social abilities. Some of these individuals overlapped the cases that were included in our current study. Baribeau et al. (2017) examined variants of the

SNPs rs53576, rs2254298, rs237887 and rs13316193 and their association with scores on the Social Communication Questionnaire (SCQ) and the Reading the Mind in the Eyes Test (RMET), two established quantitative measures of social behavioural phenotypes. It was found that significant diagnostic differences were strongly associated with specific SNP risk genotypes, demonstrating reciprocal associations between genotype and social abilities in ASD and ADHD. However, we did not observe any differences in frequency of any genotypes between groups of individuals with ASD or ADHD stratified by DNAm values, suggesting that our epigenotype–phenotype correlations were not driven by allelic variation in proximal SNPs. It would be valuable in future to further explore the genotype–epigenotype–phenotype relationships in NDDs and to better understand DNAm and *OXTR* genotype as risk modifiers.

It is worth noting that our study had a few inherent limitations with respect to potential variation in data output, secondary to batch effects and DNA availability for replicate analysis. Our bisulfite conversion was performed in batches of approximately 10–20 samples. While pyrosequencing is not as susceptible to batch effects as other methods of assaying DNAm, such as microarray technologies, quantifiable inter-batch variation does exist (Supplementary Fig. 1). Due to the limited quantities of DNA, we were only able to assay all samples a single time rather than in duplicate or triplicate. Of note, the pyrosequencing output consistently passed stringent quality control. PCR bias, especially in regions of high CpG density, can alter the precision of pyrosequencing. Standard curves of each assay generated by assaying various dilutions of artificially methylated and unmethylated DNA samples, illustrated some bias at high and low methylation extremes but suggest that our reported methylation differences between NDD cases and controls are likely underestimated (Supplementary Fig. 1). Beyond these limitations, we propose that present and future studies would benefit from phenotyping both neurotypical controls and individuals with the NDD of interest. To that end, DNAm patterns may reflect spectra of social cognition and related phenotypes within both groups, in addition to significant differences attributed to diagnosis.

Future research in the area of DNAm and NDDs could be extended to better understand individual responses to pharmaceutical and interventions in NDDs. It is known that many drugs can have strong, measurable epigenetic effects, resulting in changes in epigenetic marks such as DNAm and histone acetylation (Jessberger et al. 2007; Kubota et al. 2012). The possible efficacy of OXT as a treatment option for NDD patients is likely to be modified by *OXTR* expression and function. Understanding the role DNAm has in determining *OXTR* expression in clinically relevant cell types could be useful in stratifying the patient population for individuals who may respond more positively to treatment.

It could also be the case that particular *OXTR* DNAm associations may indicate whether an individual may be more likely to express particular NDD traits (e.g. social problems), which can help to individualize targeted therapies to particular cognitive/behavioural domains. Given our sex-specific DNAm results, it will be important in future to evaluate drug efficacy in a sex-specific manner. Although it has not been tested in this study, there is evidence to indicate that there is potential for DNAm to act as a therapeutic biomarker of drug efficacy (Bock 2009; Goud Alladi et al. 2018; Labermaier et al. 2013).

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**Author Contributions** MTS analyzed and interpreted the data, generated figures/tables, and wrote the manuscript. SJG and MJ helped with manuscript writing and editing, and data analysis. DTB and DG helped design the study, manage collaborations and assisted with data analysis and interpretation. IY, RR, YL, RZ and CZ prepared the samples, designed the assays and generated the DNA methylation data. RN, SG, PS, SWS, WR, and EA provided patient samples (or were involved in patient recruitment), phenotypic data, and assisted with study design, as well as secure funding and help prepare the manuscript. RW is the principal investigator and was involved in all aspects of the study. All authors have read and approved the manuscript.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare no competing interests.

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