PLASMATIC LEVELS OF NEUROPEPTIDES, INCLUDING OXYTOCIN, IN CHILDREN WITH AUTISM SPECTRUM DISORDER, CORRELATE WITH THE DISORDER SEVERITY

L. Kobylinska^{1,2}, A.M. Panaitescu^{4,*}, G. Gabreanu⁵, C.G. Anghel^{1,3}, I. Mihailescu¹, F. Rad^{1,3}, C. Nedelcu¹, I. Mocanu¹, C. Constantin⁶, S.V. Badescu², I. Dobrescu^{1,3}, M. Neagu⁶, O.I. Geicu⁷, L. Zagrean², A.M. Zagrean²

¹*“Prof. Dr. Al. Obregia” Clinical Psychiatry Hospital - Child and Adolescent Psychiatry*, ²*“Carol Davila” University of Medicine and Pharmacy* - ³*Division of Physiology and Fundamental Neuroscience*, ⁴*Dept. of Child and Adolescent Psychiatry*, ⁵*Filantropia Hospital*, ⁶*“Victor Babes” National Institute of Research-Development in the Pathology Domain and Biomedical Sciences* - ⁷*Dept. of Immunohistochemistry*, ⁶*Dept. of Immunology*, ⁷*University of Bucharest, Faculty of Biology, Dept. of Biochemistry and Molecular Biology, Bucharest, Romania*

Abstract

Context. Oxytocin has been investigated as a potential medication for psychiatric disorders.

Objective and design. This study prospectively investigates correlations between oxytocin and other neuropeptides plasma levels in patients with autism spectrum disorders (ASD) according to severity and treatment, as compared to controls.

Subjects and methods. Thirty-one children (6 neurotypical as control) participated in this study. The patients were classified into mildly and severely-affected, according to Autism Diagnostic Observation Schedule (ADOS) scores. Oxytocin, orexin A and B, α -MSH, β -endorphins, neurotensin and substance P were investigated using a quantitative multiplex assay or a competitive-ELISA method.

Results. Plasma oxytocin levels differed between the groups ($F(2, 24) = 6.48$, $p = 0.006$, $\eta^2 = 0.35$, observed power = 86%): patients with the mild ASD had higher values of plasma oxytocin than those with the severe form (average difference = 74.56 ± 20.74 pg/mL, $p = 0.004$).

Conclusions. These results show a negative correlation between plasma levels of oxytocin and the severity of ASD and support the involvement of oxytocinergic mechanisms in ASD.

Key words: oxytocin, autism, orexin, endorphin.

INTRODUCTION

In the past twenty years, oxytocin, previously known for its core functions in labour and lactation, and widely used in clinical practice in obstetrics and gynaecology, has been extensively studied for its

role in regulating social behaviour (1). Oxytocin has an important role in modulating maternal behaviour and mother-baby bonding (2) and it is one of the few substances that can be found, translationally, in very similar forms, in all the vertebrates and most invertebrates (3). The break-through in the field of behavioural effects of oxytocin came from animal studies, in which the investigators focused on differences between the monogamous and polygamous voles (4-9). The results have been translated into human research, thus proving that oxytocin is involved in modulating the amygdalian response to stress, through regulating the hypothalamic release of corticotrophin releasing hormone (10-11). Therefore, it acts as a central modulator of anxious behaviour, particularly in social circumstances (12). Most probably due to its regulation of social anxiety, oxytocin increases the ability to read emotions based on eye expression, to depict positive facial emotions and to focus the gaze on the eye region of another person. Moreover, it increases self-trust and peer-trust, generosity towards strangers, as well as the self-opinion over one's perceived attractiveness (13-18).

Interestingly, at genetic level, single nucleotide polymorphisms in the oxytocin receptor gene are associated with a lack of relationship contempt, conjugal infidelity and overall social interaction deficiency (18-23).

Given its pro-social effects and its potential to increase social behaviour in patients with schizophrenia and autism spectrum disorders (ASD), several clinical trials are currently under way, researching the potential effect of long-term oxytocin administration in patients with schizophrenia or ASD (24-26).

*Correspondence to: Anca Maria Panaitescu MD, Filantropia Hospital, Bld. Ion Mihalache, no. 11, Bucharest, 011161, Romania, E-mail: panaitescu.anca@yahoo.com

Autism spectrum disorders are characterized by stereotypical movements or behaviours, expressive language disorders (ranging from a monotonous prosody, to language development delays, with some severely affected patients being non-verbal) and social interaction deficits. A restrained area of interests, or peculiar, repetitive interests may also be encountered. The social interaction deficits of patients with ASD have been so far the main focus of oxytocin research in the field.

Whether there is an actual difference in the plasma level of oxytocin between children with ASD and neurotypical children is still debatable, with studies in the field yielding conflicting results (27-28). Moreover, a meta-analysis on the subject published in 2016, and summarizing the results from 404 patients from 4 clinical trials found no overall differences in the levels of plasma oxytocin between the patients with ASD and the neurotypical controls (29). However, a major limitation of this meta-analysis consists in the fact that there are very few published studies that have measured the level of plasma oxytocin in patients with ASD.

The aim of the present study was to measure the levels of plasma oxytocin in a Romanian sample of children diagnosed with ASD and to compare the values with those obtained from neurotypical controls. We also evaluated the levels of oxytocin in correlation with other neuropeptides that indicate the functioning of the hypothalamus and might influence the pathogenesis and evolution of the disorder.

MATERIALS AND METHODS

Subjects and sample collection

Thirty-one children aged 3 to 12 years were included in this study. Six of the children were neurotypical (aged 5 to 8, 2 girls), the rest of 25 had been diagnosed with ASD (aged 3 to 12, 5 girls). The patients were recruited from the child and adolescent psychiatry department of the “Prof. Dr. Al. Obregia” Clinical Psychiatry Hospital. A certified child and adolescent psychiatrist established the diagnosis of ASD, based on clinical evaluation and diagnostic tests, among which ADOS (Autism Diagnostic Observation Schedule) (30).

The ADOS testing was performed by an experienced clinical psychologist, specifically trained for performing the ADOS and proficient in the assessment of children with neurodevelopment disorders. The children were tested using the appropriate module according to their language development. Based on the ADOS scoring cut-off point (12 points),

the children with ASD were divided into severe ASD (11 patients, ADOS score ≥ 12) and mild ASD (14 patients, ADOS score < 12). Venous blood was drawn by a certified nurse, through vacutainer veno-puncture. All the procedures had the approval of the local ethical committee.

Both parents of each child signed an informed consent, approved by the local ethics committee of the “Prof. Dr. Al. Obregia” Clinical Psychiatry Hospital in Bucharest, Romania.

As the children were randomly recruited from the patients of the department, some of them were receiving psychotropic medication, in accordance with their hyperactivity/ aggressiveness symptoms, in doses adjusted for their weight and the severity of the condition.

Sample processing and neuropeptides' level measurements

Six neuropeptides were simultaneously investigated using a quantitative multiplex assay based on magnetic beads (EMD Millipore's MILLIPLEX MAP Human Neuropeptide Magnetic Bead Panel) containing specific antibodies that depict α -Melanocyte Stimulating Hormone (α -MSH), β -Endorphin, Neurotensin, Orexin A, Oxytocin and Substance P.

According to the manufacture instructions and due to the low concentrations of the plasmatic neuropeptides, equal volumes of each sample were treated with acetonitrile in proportion of 1:1.5 and the supernatant obtained after centrifugation was dried over-night using a High-Speed Vacuum. Dried samples were stored at -20°C . Prior to the beginning of the immunoassay, the samples were reconstituted with the Assay Buffer provided by the kit. After a pre-incubation of the 96-well plate with assay buffer for 10 minutes, we treated the sample with the primary antibodies on a plate shaker for 2 hours at room temperature. Mixed beads (anti- α -MSH; anti- β -endorphin; anti-neurotensin; anti-orexin A; anti-oxytocin; anti-Substance P) were added and then incubated overnight at $2-8^{\circ}\text{C}$. The beads capture the neuropeptide of interest and the complexes are recognized by a biotinylated detection antibody. Streptavidin-Phycoerythrin was used for the completion of reaction. The data was acquired and analysed using the LUMINEX xMap technology MAGPIX.

The level of Orexin B neuropeptide was investigated using a competitive-ELISA method (Human Orexin B ELISA kit, Labscience). When adding the Biotinylated Detection Antibody, the Orexin B from the serum sample competed with the Orexin B pre-coated on

the wells provided by the kit. The unbound sample was washed away before adding the Horseradish Peroxidase Conjugate and the TMB substrate solution. The enzyme-substrate reaction was stopped by adding sulphuric acid solution provided in the kit. The colour change was measured using a spectrophotometer and the reading was done at 450 nm. Due to the quality of samples and to the processing, some neuropeptides were not measured for all the patients. For each neuropeptide mentioned, the non-accurate measurements were excluded from the report (31).

Statistical analysis

Microsoft Office Excel and SPSS 22.0 were used for the analysis. The distribution of the continuous variables was checked using Shapiro-Wilk's test, and parametric comparisons (one-way ANOVA with post-hoc Turk analysis) and correlations (Pearson's two-way correlation test) were run for p values >0.05 . For Shapiro-Wilk test $p < 0.05$ and for non-linear variables, non-parametric tests were used (Kruskal-Wallis, with standard post-hoc analysis and Spearman's correlation test). The results are presented as mean $\pm$ standard deviation, unless otherwise specified.

The data were analysed according to the diagnostic group (mild ASD, severe ASD, control), according to the neurotypical/non-neurotypical status of the patients and according to the medication received by the patients: control – healthy, no medication, ASD without medication, ASD with medication.

RESULTS

The plasma levels of α -MSH, β -endorphins and oxytocin were uniformly distributed in all three patient groups, as they were defined according to the diagnosis, whereas neurotensin, orexin A, orexin B and substance P had a non-Gaussian distribution.

The values of α -MSH did not vary according to the diagnosis group ($F(2, 28) = 2.21$, $p = 0.12$), and neither have those of β -endorphins ($F(2, 27) = 0.9$, $p = 0.41$). Plasma oxytocin levels were, significantly different between the three groups of diagnosis ($F(2, 24) = 6.48$, $p = 0.006$, $\eta^2 = 0.35$, observed power = 86%). Post-hoc Tukey analysis with Bonferroni adjustment of the confidence intervals revealed that these differences were due to the fact that patients with the mild form of ASD had higher values of plasma oxytocin than those with the severe form of ASD (average difference = 74.56 ± 20.74 pg/mL, $p = 0.004$) (Fig. 1).

To check whether there are differences in the

levels of neurotensin, a Kruskal-Wallis H test was employed. The distribution of the data was not similar in the three diagnosis groups, as assessed by the visual inspection of the box-plots of the data. The measurements varied significantly between the three groups ($H(2) = 10.58$, $p = 0.005$). Post-hoc paired comparisons using Dunn's procedure (1964) with Bonferroni correction for multiple comparisons (32, 33) were performed. The mentioned p -values are the adjusted values, and the values of the measurements are average ranks. This post-hoc analysis revealed significantly different results between plasma neurotensin detected in patients with mild forms of ASD (median value = 206.86 pg/mL, interquartile range = 16.79 pg/mL) and control group (median value = 178.25 pg/mL, interquartile range = 11.95 pg/mL), $p = 0.006$.

Kruskal-Wallis H testing, as described above, was also used for orexins A and B and substance P levels according to the diagnosis groups. There were no variations of the plasma values of either orexin B ($H(2) = 2.48$, $p = 0.28$), or substance P ($H(2) = 4.65$, $p = 0.09$) across the diagnosis groups.

For orexin A, the distributions of the data were similar across diagnosis groups, as assessed by the visual inspection of the box-plots. There were significant differences in the plasma values of orexin A according to the diagnosis group ($H(2) = 8.18$, $p = 0.017$). Post-hoc paired comparisons using Dunn's procedure (1964) with Bonferroni correction for multiple comparisons (32, 33) were performed. The mentioned p -values are the adjusted values, and the values of the measurements are median values. This post-hoc analysis revealed significantly different results between the values of plasma orexin A between the patients with mild forms of ASD (1343.7 pg/mL) and those with the severe form of ASD (1028.76 pg/mL), $p = 0.016$ (Fig. 2). When the data was analysed according to the administration of antipsychotic medication, there was a normal distribution of the values for all the measured substances, except for neurotensin, for which the data was slightly deviant from the Gauss curve in the control group. However, given the robustness of the ANOVA analysis and the small deviation from the normal distribution, it was decided upon its usage in this case, as well.

The plasma values of oxytocin did not vary according to the treatment group ($F(2, 24) = 0.35$, $p = 0.7$). Neither did those of α -MSH ($F(2, 28) = 2.21$, $p = 0.12$), β -endorphins ($F(2, 27) = 0.17$, $p = 0.83$), orexin A ($F(2, 27) = 0.59$, $p = 0.56$) and substance P ($F(2, 27) = 0.1$, $p = 0.9$).

There were marginally significant differences

in the plasma levels of orexin B ($F(2,21)=3.23$, $p=0.06$), due to the fact that the patients with ASD that did not receive antipsychotic medication had slightly higher levels of orexin B than the subjects in the control group (average difference= 0.02 ± 0.008 pg/mL, 95%CI= $(-0.0004, 0.04$ pg/mL), $p=0.055$; these results were obtained by post-hoc Tuckey analysis, with Bonferroni adjustment of the confidence intervals) (Fig. 3).

The plasma values of neurotensin varied according to the medication group ($F(2,28)=3.52$, $p=0.04$). Post-hoc Tuckey analysis following the Bonferroni adjustment of the confidence intervals has revealed that the patients with ASD that did not receive psychotropic medication had higher plasma values of neurotensin than the neurotypical controls (the average difference is 25.06 ± 9.96 pg/mL $p=0.04$, 95% CI =

(0.41, 49.7 pg/mL)) (Fig. 4).

The correlations between the ADOS score and the plasma values of the measured neuropeptides were checked using Spearman's bivariate non-parametric correlation test. All the mentioned p-values are two-tailed, apart from the p-value for oxytocin, for which the one-tailed value was considered, given the existence of significant differences in the plasma values according to the severity of the diagnosis, as it was shown above. Specifically, the following reverse average (respectively strong for orexin A) correlations have been found between the ADOS score and the tested serum markers (Table 1).

The correlations between the values of the neuropeptides measured with the multiplex kit were checked through the Pearson bivariate parametric

Figure 1. Bar-graph of the plasma values of oxytocin according to the diagnosis group. The error bars represent the standard error. The average plasma values are: control group= 255.3 ± 19.37 pg/mL, mild-ASD group= 295.8 ± 14.3 pg/mL, severe-ASD group= 221.24 ± 15.01 pg/mL.

Figure 2. Box-plot of the values of orexin A, with significant outliers plotted on the graph as the points outside the box-plots.

Figure 3. Bar-graph of the plasma values of orexin B according to the treatment group. The error bars represent the standard error of the mean.

Figure 4. Bar-graph of the plasma values of neurotensin according to the medication group. The error bars represent the standard error of the mean.

correlation test for each of the diagnosis groups (Tables 2-4).

Table 1. Correlations between the ADOS score and the values of the measured neuropeptides. r = Spearman correlation coefficient (weak (0.1-0.3), average (0.3-0.5) and strong (>0.5) correlations), – for a negative correlation. Sig=p-value (bold for statistically significant values, *p - one-tailed). N=the number of children for whom the respective neuropeptide was measured

	r	sig	N
α -MSH	-0.45	0.024	25
neurotensin	-0.46	0.018	24
orexin A	-0.59	0.002	24
substance P	-0.41	0.042	24
oxytocin	-0.42	0.029*	21

Legend: ADOS =Autism Diagnostic Observation Schedule. MSH= Melanocyte Stimulating Hormone.

Table 2. Correlations between the measured neuropeptides in control children. Sig= p-value (bold for statistically significant values), N=the number of children for whom the respective neuropeptide was measured.

		α -MSH	β -endorphins	neurotensin	orexin A	oxytocin
β -endorphins	Pearson Correlation	.215				
	Sig. (2-tailed)	.682				
	N	6				
neurotensin	Pearson Correlation	.854*	.224			
	Sig. (2-tailed)	.030	.669			
	N	6	6			
orexin A	Pearson Correlation	.815*	-.275	.573		
	Sig. (2-tailed)	.048	.598	.235		
	N	6	6	6		
oxytocin	Pearson Correlation	.645	-.232	.756	.494	
	Sig. (2-tailed)	.167	.658	.082	.319	
	N	6	6	6	6	
substance P	Pearson Correlation	.575	-.665	.442	.857*	.618
	Sig. (2-tailed)	.232	.150	.380	.029	.191
	N	6	6	6	6	6

*. Correlation is significant at the 0.05 level (2-tailed). Legend: MSH= Melanocyte Stimulating Hormone.

Table 3. Correlations between the measured neuropeptides in children diagnosed with mild autism spectrum disorder (ASD). Sig= p-value (bold for statistically significant values), N=the number of children for whom the respective neuropeptide was measured

		α -MSH	β -endorphins	neurotensin	orexin A	oxytocin
β -endorphins	Pearson Correlation	.757**				
	Sig. (2-tailed)	.003				
	N	13				
neurotensin	Pearson Correlation	.813**	.482			
	Sig. (2-tailed)	.000	.095			
	N	14	13			
orexin A	Pearson Correlation	.781**	.736**	.770**		
	Sig. (2-tailed)	.002	.004	.002		
	N	13	13	13		
oxytocin	Pearson Correlation	.643*	.415	.487	.776**	
	Sig. (2-tailed)	.033	.204	.129	.005	
	N	11	11	11	11	
substance P	Pearson Correlation	.840**	.829**	.602*	.910**	.750**
	Sig. (2-tailed)	.000	.000	.029	.000	.008
	N	13	13	13	13	11

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed). Legend: MSH= Melanocyte Stimulating Hormone

DISCUSSION

The results of other studies in the field do not present the values of oxytocin according to the severity of the disorder (29), therefore the results of this study come as a relevant finding for the importance of oxytocin as a biomarker in ASDs. The analyses of plasma and salivary oxytocin in children diagnosed with ASD have generally been reported without a correlation with the severity of the disorder, and a meta-analysis performed on these findings in 2016 by Rutigliano and his collaborators did not find any differences in the values of oxytocin between the neurotypical controls and ASD patients (29). Our result suggests that this bulk-type of analysis might not yield a suggestive result, and

Table 4. Correlations between the measured neuropeptides in children diagnosed with severe autism spectrum disorder (ASD). r = Pearson correlation coefficient (weak (0.1-0.3), average (0.3-0.5) and strong (>0.5) correlations). Sig= p-value (bold for statistically significant values), N=the number of children for whom the respective neuropeptide was measured

		α -MSH	endorphins	neurotensin	orexinA	oxytocin
β -endorphins	r	.507				
	Sig. (2-tailed)	.112				
	N	11				
neurotensin	r	.731*	.245			
	Sig. (2-tailed)	.011	.468			
	N	11	11			
orexin A	r	.657*	.196	.367		
	Sig. (2-tailed)	.028	.563	.266		
	N	11	11	11		
oxytocin	r	.581	.675*	.435	.252	
	Sig. (2-tailed)	.078	.032	.209	.483	
	N	10	10	10	10	
substance P	r	.845**	.487	.604*	.723*	.578
	Sig. (2-tailed)	.001	.129	.049	.012	.080
	N	11	11	11	11	10

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed). Legend: MSH= Melanocyte Stimulating Hormone.

that the problem should be re-analysed starting from a stratified analysis of values, according to the severity of the diagnosis. Given the crucial differences between the prognosis of the patients according to the severity of the initial presentation and the core variations that induce a different score on the ADOS, we consider that a comparison between the patients with various degrees of severity is very important.

As ASD remain among the most prevalent neurodevelopmental disorders, with a growing incidence and subsequent economic consequences (34), studies in the field are searching for biological markers that could help with the earliest diagnosis profile and prognosis. Moreover, as the golden standard in the therapeutic management of these children remains age and pathology-adjusted behavioural therapy, pharmacological enhancers of the disrupted executive functions are much needed to amplify the effects of psychotherapy and to maximize the results. According to the associated comorbidities, stimulants, antipsychotics, antidepressants or benzodiazepines can be administered (35). However, in this context, our results suggest that oxytocin should be administered as adjuvant medication precisely to the ASD patients who are receiving other psychotropic drugs. Moreover, these results suggest that oxytocin could be investigated as a potential marker of response to behavioural therapy, in conjunction to the specific diagnosis tests, such as ADOS and ADI-R (Autism Diagnostic Interview, Revised).

Even if our results are promising in the described context, one question still needs to be

addressed: is plasma oxytocin measurement relevant for its central nervous system action? Oxytocin is produced in the hypothalamus but also in other central (36) and peripheral regions and these sources of oxytocin might influence its plasma concentration (37). It has been hypothesised that it is not only oxytocin secretion that is altered in children with ASD but the whole oxytocinergic system (38). This altering can be considered in the context of brain development in non-typical children with ASD, possibly associated with an excitation-inhibition imbalance correlated with alterations in oxytocin processing. Even if the permeability of the blood-brain barrier is rather limited for oxytocin, there have been other recent studies that have not only correlated the severity of ASD communication and social impairment with plasma oxytocin levels in children (39, 40), but have also shown that in children with anxiety disorders, oxytocin levels in plasma and the cerebrospinal fluid (CSF) are strongly positively correlated (41, 42). The findings reported by Taurines *et al.* (39) are similar to our findings of a strong negative correlation between plasma oxytocin and ADOS scores in children with ASD. Carson *et al.* (41) reported on the existence of very strong negative correlations between anxiety symptoms in children and both plasma and CSF oxytocin concentration. These findings are in accordance to our results and support the hypothesis that oxytocin plasma levels in ASD could predict social adaptability and children with higher plasma oxytocin concentrations might have a higher potential to respond to therapy.

The main limitation of our study remains the

small control sample size, in comparison to the clinical population. The discussion in the field is far from over and more research on larger sample size still needs to be undertaken as there is conflicting evidence in the literature to whether plasma levels of oxytocin correlate with CSF levels and reflect the oxytocin action at the level of the central nervous system. In 2013, Kagerbaure *et al.* (43) reported not having found any correlation between plasma and CSF oxytocin levels in neurotypical adult subjects, whereas Carson *et al.* (41) reports positive strong correlation between the two. These findings also raise the question of whether it would be possible to have differences in the degrees of correlation between plasma and CSF oxytocin in typical and non-typical individuals as well as in children and adults. However, there are not, to our best knowledge, enough results reported so far to allow the drawing of a clear conclusion, therefore it may only be supposed for now that in children with ASD, plasma concentrations of oxytocin might predict CSF levels and that the correlation with symptoms substantiates this hypothesis.

Our paper comes in the middle of a revolution in psychiatry - from moving towards symptom rather than syndrome-based approach, to the DSM-5 introducing several new diagnostic categories that would place most of the population within a diagnosable pathology. Thus, we are witnessing a shift in mental health paradigm. Overlapping this, the incidence of neurodevelopmental disorders is steadily increasing, with several factors being investigated as possible culprits, but with no real clear answer in this sense. Where are we heading? How is the human brain changing? Will our oxytocinergic circuits - one of the oldest phylogenetic vestiges that we hold - withstand these society-imposed social-norm and social-communication altering? These questions will, most probably, not be answered any time soon. However, considering that social isolation behaviours occur in the animal kingdom in any population that is undergoing a period of excess of resources, as means of population control, we strongly believe that the investigation of oxytocin and oxytocinergic systems in social deficits disorders brings us one step closer to an inkling of an answer for this epidemic of social interaction deficits.

In conclusion, our study aimed to investigate plasma values of selected neuropeptides in children with ASD according to severity and treatment. These neuropeptides and their positive correlations with the disease could be corroborated in a wider panel of markers. The negative correlations between the ADOS score and the plasma levels of some of the investigated

substances raise a question upon the competence of the underlying circuits and this is an indirect inkling into the respective receptors's statuses, with a possible up-regulation of the involved receptor populations and their subsequent altered functioning with the disease. These aspects may support the theory of the excitation-inhibition imbalance in the cortical circuits of children with ASD (44, 45).

Given the higher plasma values observed in children with milder forms of ASDs, it may be hypothesised that their increased responsiveness to the behavioural therapy might be, at least partly, mediated through oxytocinergic mechanisms (46). Oxytocin's involvement in the GABA-switch from excitatory to inhibitory neurotransmitter at birth might substantiate the importance of its appropriate regulation for typical brain development (47, 48). The correlations observed with other neuropeptide levels suggest that these substances might be inter-connected in terms of potential effectiveness in the administration of adjuvant treatment. One important finding is the very strong correlation and similar trend in the plasma levels differences between oxytocin and orexin A across the diagnostic groups that were investigated. This finding fits in the context of current research of the orexinergic field, which places dysregulations of this system at the core of social interaction deficits and eating behaviour stereotypes in patients with ASDs (49). Moreover, given the proposed importance of oxytocin in influencing sleep architecture, dream content and dream awareness (50), this finding suggests that the interconnection between the two substances could be the one that explains this phenomenon, and it should be further investigated.

Another important observation is that the plasma oxytocin levels of children with the severe form of ASDs did not vary significantly from those of the neurotypical children. This raises the question of receptor expression and regulatory circuits activation. Therefore, oxytocin administration as adjuvant therapy should be considered according to its basal bio-availability, as the results of the present study suggest that it might be of more assistance in children with severer forms of ASD, rather than in those with milder forms. This might come as a counterintuitive observation, as the children with the milder forms of ASD tend to have more pronounced social deficits in comparison to the motor behaviour alterations and language impairments, whereas the children with the more severe forms tend to have a uniform pattern of deficits across all the symptomatic domains (49).

Conflict of interest

The authors declare that they have no conflict of interest.

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