Clinical Psychopharmacology and Neuroscience - Manuscript Submission

- **Manuscript ID**: CPN-20-842
- **Title**: Investigation of Relaxin-3 Serum Levels in Terms of Social Interaction, Communication, and Appetite as a Biomarker in Children with Autism
- **Running Title**: Autism and Relaxin-3
- **Article Type**: Original Article
- **KeyWords**: autism, communication, appetite, relaxin-3

Investigation of Relaxin-3 Serum Levels in terms of Social Interaction, Communication, and Appetite as a Biomarker in Children with Autism

ABSTRACT

Objective: To investigate the possible relationship between relaxin-3 and autism spectrum disorder (ASD).

Methods: Serum relaxin-3 was measured in 80 children (50 children diagnosed with ASD and 30 controls). Symptom severity in the ASD group was evaluated by the Childhood Autism Rating Scale (CARS). Behavioral and nutritional problems in the groups were evaluated using the Abnormal Behavior Checklist (ABC) and the Children's Eating Behavior Questionnaire (CEBQ).

Results: Our findings showed that serum relaxin-3 levels were higher in children with ASD than in the controls. The listening response sub-scale score of the CARS scale was found to decrease as the level of relaxin-3 increased. However, as relaxin-3 levels increased in children with ASD, it was found that the speech problem sub-scale score on the ABC scale and the desire to drink score on the CEBQ scale increased, but the satiety responsiveness and food fussiness scores decreased.

Conclusion: This study the first to investigate serum levels of relaxin-3, which has a role in regulating social behavior and nutritional behavior in children with ASD.

Keywords: autism; communication; appetite; relaxin-3

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by a restricted and repetitive pattern of behaviors, interests, or activities, as well as limitations in mutual communication and social interaction (1). ASD prevalence has been reported to have increased, especially in the last 30 years, affecting approximately 2% of children (2). Despite the increasing number of studies that investigate this disorder, which negatively affects a significant part of society, its etiology remains unclear, and there is no definitive treatment for ASD yet. In addition, one of the important limitations that we face when diagnosing ASD is the fact that the diagnostic process is based on observations and the history provided by the caregiver. Therefore, objective measurements (blood test or radiologic screening) are of importance for elucidating the etiology of ASD, as well as its diagnosis, and/or follow-up. Yet, numerous studies report that there are biologic abnormalities associated with ASD (3). Biomarkers to be developed to accurately measure these abnormal biological processes may be of importance in the diagnosis, follow-up, and treatment of ASD.

Especially in the study of psychiatric disorders, there is increasing interest in the study of neuropeptides such as orexin, neuropeptide Y, and substance P. In this context, a review of studies investigating relaxin-3, which is a neuropeptide, highlights that relaxin-3 may be associated with depression, schizophrenia, eating disorders, and ASD, and that it offers hope in the treatment of these disorders (4). Relaxin-3 was found to be mostly expressed from GABAergic (γ-aminobutyric acid) neurons in the nucleus incertus (NI) (5) of the brain stem in mammals, and transmitted to the midbrain, hypothalamus, amygdala, basal forebrain, hippocampus, and prefrontal cortex, which contains neurons that express the peptide receptor 3 (RXFP3), and that this signalization was found to promote excitation (6). Existing studies have shown that relaxin-3 plays a role in the integration of sensory inputs and regulation of behavioral responses to environmental and physiologic stimuli (7), memory and learning processes associated with the hippocampus (8), feeding, and control of the neuroendocrine axis (9).

Restrictions in social behavior are the main symptoms of ASD. The roles of the hippocampus and amygdala in ASD are often investigated because they are involved in the basic functions of the social brain. Human imaging studies in this area have shown that there are structural abnormalities in ASD in limbic structures, such as the hippocampus and amygdala (10). In addition, decreased activity of the anterior cingulate cortex (control of attention and executive functions) was also found in individuals with ASD (11). The paraventricular nucleus (PVN),

which plays a role in regulating social behavior, is another important limbic structure frequently investigated in ASD due to the presence of oxytocin neurons (12). Moreover, oxytocin neuron loss in PVN has been reported in animal models of ASD (13). Considering the animal studies investigating the effect of the relaxin-3/RXFP3 system on social behavior, one study found that chronic activation of RXFP3 in the ventral hippocampus increased social avoidance (14). In another study, it was stated that central relaxin-3/RXFP3 activation disrupted social recognition, and that as a result of this activation there was a functional interaction between RXFP3 and oxytocin receptors in the amygdala, where this interaction might be effective in modulating social memory (15). These results, showing the link between relaxin-3 and oxytocin, suggest that relaxin-3 may also play a role in ASD.

RXFP3 is also found in hypothalamic regions closely related to appetite control and hormonal balance (16). Acute RXFP3 activation has been shown to consistently increase food intake in satiated adult male rats (17). Sub-chronic intracerebroventricular (ICV) administration of relaxin-3 resulted in a significant increase in average daily food intake and a cumulative increase in body weight (18). Oxytocin and arginine vasopressin (AVP) are the hormones that strongly affect nutritional behavior. In both animals and humans, oxytocin has been shown to act as an anorexigenic signal (19, 20) and deficiencies in oxytocin synthesis have been shown to lead to hyperphagia and obesity (21).

Given that relaxin-3 projections reach out RXFP3 in the limbic hippocampus, amygdala, anterior cingulate cortex, and PVN, and considering that these areas modulate social interaction and communication (17), it is clear that the possible relationship between ASD and relaxin-3/RXFP3 systems should be investigated in more detail. According to a literature review of this association, no study was found investigating the relationship between ASD and relaxin-3 in humans. Given all these facts, the aim of this study was to investigate the possible relationship between relaxin-3 and ASD. In this study, differences between serum relaxin-3 levels were investigated between children diagnosed as having ASD and control children, matched in terms of age, sex, and socioeconomic level. A nutritional scale was also given to investigate the effects of relaxin-3 on nutrition. Given the animal studies that reported that chronic activation of relaxin-3 disrupted social behavior, it was assumed that the serum relaxin-3 levels of children with ASD in our study would be higher than those of the control group.

MATERIAL and METHOD

Study center and sample

This study was conducted at the Necmettin Erbakan University Meram Faculty of Medicine, Department of Child and Adolescent Psychiatry Department, between June and December 2019. Diagnoses of ASD were made through clinical psychiatric evaluations made by a child and adolescent psychiatrist based on the ASD diagnostic criteria of the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5). Of the children aged 2-8 years who were diagnosed as having ASD, only those whose parents agreed to participate were included in the study. To control confounding variables, children who took medicine, those diagnosed as having a gastrointestinal disease (e.g. Crohn's, ulcerative colitis), chronic disease (e.g. diabetes mellitus, hypertension, epilepsy, cerebral palsy), and children with infection or obesity were excluded from the study.

Patients were accepted for the control group who presented to our institution's outpatient clinics in the 2-8 years' age group (30 children), who had their laboratory tests (full blood count, erythrocyte sedimentation rate, C-reactive protein (CRP) and examinations resulting as normal, who were not on medication, who had no chronic disease (e.g. diabetes mellitus, hypertension, epilepsy, cerebral palsy), and no infectious disease and gastrointestinal disease. Children without any psychiatric disease, according to the semi-structured psychiatric interview form of the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL) were included in the study. Written consent was obtained from the parents of all children included in the study. A Sociodemographic Information Form and the Turkish version of the Childhood Autism Rating Scale (CARS) were completed by the researcher, and the Aberrant Behavior Checklist (ABC) and the Children's Eating Behavior Questionnaire (CEBQ) were completed by the parents of the children.

The study was approved by Necmettin Erbakan University Meram Faculty of the Medicine Non-Pharmaceutical and Medical Device Research Ethics Committee on February 8th, 2019 (Internal Review Board: 2019/1712).

Instruments and measures

Demographic data and clinical history

A 'Sociodemographic and Clinical Information Form' that was prepared by the researchers was used to obtain information about sociodemographic characteristics, along with developmental, mental disease, and medical history.

The Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version / Diagnostic and Statistical Manual of Mental Disorders, 5th (K-SADS-PL / DSM-5)

The K-SADS-PL is a semi-structured clinical interview for determining the present and lifetime psychopathologies of children and adolescents (22, 23). A reliability and validity study of K-SADS-PL / DSM-5 for the Turkish population was conducted by Unal et al. (24).

Childhood Autism Rating Scale (CARS)

The validity and reliability study of CARS was conducted by Schopler et al. between 1970 and 1980 within the scope of the TEACCH Autism Program (25). CARS is a 15-item behavioral rating scale, which was designed to distinguish children with mental disabilities, but without autism, from children with autistic symptoms. It is particularly effective in separating children with autism from children with educable mental disabilities. It also allows to determine the severity of autism as mild-moderate and moderate-severe. Sucuoğlu et al. (1996), who developed the Turkish form of the scale by the translation and back-translation studies in Turkey, evaluated the validity and reliability of the scale through internal consistency and item analysis methods (26, 27).

Aberrant Behavior Checklist (ABC)

The ABC is a test to identify behavior problems seen in children with autism. The ABC is a 58-item scale, rated from 0 (problem absent) to 3 (extreme problem) based on the severity of symptoms (over four different values) (28). The items are scored under five sub-scales. These sub-problem areas include (1) irritability, agitation, crying, (2) lethargy, social withdrawal, (3) stereotypical behavior, (4) excessive mobility, dissonance/opposition, and (5) speech problems. Its Turkish adaptation and validity and reliability study was conducted by Karabekiroğlu and Aman (29).

Children's Eating Behavior Questionnaire (CEBQ)

This questionnaire was first developed in 2001 by Wardle et al. to classify children's eating behaviors in order to be instrumental in the early identification of obesity and eating disorders (30). The questionnaire was subsequently translated into many languages and implemented in various countries. It was adopted to Turkish by Yılmaz et al., who conducted its validity and

reliability study in 2011. This study demonstrated the Turkish validity and reliability of the questionnaire (Cronbach's alpha coefficient: 0.69) (31). It is a 5-point Likert-type questionnaire consisting of 35 items (1= never, 2= rarely, 3= occasionally, 4= frequently, 5= always). In the original scale-development study, eight sub-scale factor structures were formed during the development of the questionnaire. These sub-scales are food responsiveness, emotional overeating, enjoyment of food, desire to drink, satiety responsiveness, slowness in eating, emotional undereating, and food fussiness. In addition, these sub-scales were collected in two groups under the headings 'food approach' and 'food avoidant.' The food approach group includes food responsiveness, enjoyment of food, emotional overeating, and desire to drink sub-scales, and the food avoidant group includes satiety responsiveness, slowness in eating, emotional undereating, and food fussiness sub-scales.

Blood sampling

Venous blood samples (5 mL) from the patient and control groups were taken in vacuum tubes under standard conditions (between 08:00 and 11:00 AM). The tubes were immediately centrifuged at 1500 *g* for 10 minutes. Serum samples were stored at -80°C until required for analysis. Serum relaxin-3 levels were analyzed using enzyme-linked immunosorbent assay (Human Relaxin-3 The Enzyme-Linked Immunosorbent Assay (ELISA) Kit, Catalog No: Abbkine KTE60790, USA). Results are given in pg/mL. Human relaxin-3 levels were measured using double-antibody sandwich ELISA according to the manufacturer's instructions. The sensitivity of the ELISA kit was 1 pg/mL, the measurement range was 25 - 400 pg/mL, the CV% values were inter-assay <9% and intra-assay <1%. No cross-reactions or interactions between human relaxin-3 and its analogues were observed.

Data evaluation and statistical analysis

Statistical analysis of the data was performed using the Statistical Package for the Social Sciences (SPSS) version 23.0. Skewness and Kurtosis statistics were used to evaluate the normality of the distribution of the data. Student's t-test was used to evaluate quantitative data. The Chi-square (χ^2) test or Fisher's exact Chi-square test was used for intergroup comparisons of quantitative data. Pearson's and Spearman correlation tests were used to investigate the relationship between two measured values in the groups. $P<0.05$ was accepted as the level of statistical significance in all analyses.

RESULTS

The study included 50 children with ASD and 30 children as a control group. There were no significant differences between the two groups in terms of age and sex. Sociodemographic data and clinical characteristics are presented in Table 1. Serum relaxin-3 levels were found to be statistically significantly higher in the group with ASD ($p<0.05$) **(Table 1).**

The correlations between serum relaxin-3 levels and the scale scores of the children in the patient group were also evaluated. There was no statistically significant positive correlation between serum relaxin-3 levels and CARS total scores. In contrast, a statistically significant negative correlation was found between serum relaxin-3 levels and scores of the 8th item of CARS, which refers to the listening response. A statistically significant correlation was found between serum relaxin-3 levels and only the speech-problem sub-scale score of the ABC ($r= 0.320$, $p<0.05$). A statistically significant correlation was found between the total score of CARS and all sub-scale scores of the ABC scale. A statistically significant positive correlation was only found between serum relaxin-3 levels and the 'desire to drink' sub-score of the CEBQ scale ($r= 0.356$, $p<0.05$), and a negative correlation was found between serum relaxin-3 levels and satiety responsiveness ($r= -0.381$, $p<0.01$) and food fussiness scores ($r= -0.354$, $p<0.05$). There was no statistically significant correlation between the sub-scale scores of CARS and the relaxin-3 **(Table 2).**

DISCUSSION

In this study, serum levels of relaxin-3 were compared between children with ASD and control group of similar age, sex, and socioeconomic level. In support of the hypothesis, our findings found that serum relaxin-3 levels were higher in children with ASD than in the controls. No statistically significant correlation was found between relaxin-3 levels and CARS total scores in the group with ASD, but the listening response sub-scale score of CARS was found to negatively correlated with the level of relaxin-3. Furthermore, as relaxin-3 levels increased in children with ASD, it was found that the speech problem sub-scale score on the ABC scale and the desire to drink score on the CEBQ scale increased, but the satiety responsiveness and food fussiness scores decreased. These results showed that levels of relaxin-3 were associated with listening, speech difficulties, and appetite problems in children with ASD as reported by their parents.

Relaxin-3 is a newly characterized neuropeptide belonging to the insulin-like peptide family, which plays a role in regulating stress, excitation, memory, feeding, and weight control (7-9, 32). In the up-to-date literature, no study has investigated the relationship between relaxin-3 and children with ASD. Considering that relaxin-3/RXFP3 is associated with social behavior due to its projections in the hippocampus, amygdala, anterior cingulate cortex, and PVN (33-36), our study sought answers to an important question in the literature. Moreover, strong links between relaxin-3 and oxytocin suggest that relaxin-3 might play a role in ASD (37). A large upregulation of oxytocin was observed in the rat hypothalamus, following the activation of RXFP3 by acute intracerebroventricular infusion of relaxin-3 (38). However, in a study with rodents, depletion of relaxin-3 in NI was found to increase oxytocin in the PVN (39). In our study, the relaxin-3 level in children with ASD was higher than in the control group. Given these findings, it may be considered that there is a difference in the production and/or destruction stage of relaxin-3 in children with ASD compared with controls.

We found no statistically significant correlation between serum relaxin-3 levels and CARS total scores, which indicates the severity of ASD. However, the level of relaxin-3 was found to increase with the decreased listening response score of the CARS scale. These results suggest that there may be more difficulties with listening skills in children with low levels of relaxin-3. Neuroanatomic evidence suggests that relaxin-3 should be considered as an arousal neurotransmitter. Relaxin-3 neurons have been shown to be distributed similarly to monoamine and other peptide stimulation systems. In this context, the listening response may be related to the arousal process (7, 40) Studies in rodents show that relaxin-3 modulators have a potential for the treatment of emotional disorders and social behavior deficiencies (7). Relaxin-3 neurons in the NI express corticotropin-releasing factor receptor 1 (CRF1), which is known to regulate stress responses (41). Although rodent studies on knockout of the relaxin-3 gene have reported an altered stress response and anxiety-related behaviors, the activation of central RXFP3 with selective agonists has been reported to reduce anxiety and depressive behaviors (42-44). It is not known if relaxin-3 plays its positive role in the listening response by regulating the processes of wakefulness and anxiety.

We determined no correlation between relaxin-3 levels and the total scores of the ABC scale, which evaluated inappropriate and maladaptive behavior. In contrast, the problem of speech was found to increase as the level of relaxin-3 increased, according to the ABC. The fact that the CARS scale's listening response sub-scale score and the ABC scale's speech problem sub-scale score were associated with relaxin-3 suggests that relaxin-3 may be associated with

communication skills. There is no study showing the relationship between relaxin-3 and communication skills. Understanding the connections between relaxin-3 and communication skills areas in the brain in future studies will enable us to better understand the role of relaxin-3.

Relaxin-3 serum levels, which are believed to function in stress, excitation, and memory, were investigated in our study (6). According to these results, relaxin-3 may have an effect on speaking from verbal communication skills and listening from non-verbal communication skills. In this study, communication skills were evaluated through clinical examinations, and the CARS and ABC scales, which were completed by the parents. In future studies, investigating the relationship between serum relaxin-3 levels and communication skills and social skills, through interviews such as the Autism Diagnostic Observation Schedule (ADOS) and the Autism Diagnostic Interview-Revised (ADI-R), will enable us to better understand the effect of relaxin-3 on communication skills. A prospective evaluation of the relationship between the change in communication skills and the relaxin-3 level will enable us to better understand the role of serum relaxin-3.

Considering the studies that showed that relaxin-3/RXFP3 signaling was associated with a significant cumulative increase in food intake and body weight during both the acute and chronic phase (17, 18, 45), it was also found in our study that liquid food intake increased and satiety behavior and food fussiness behavior decreased as the serum relaxin-3 level increased in children with ASD. In the literature, RXFP3 blockage reversed the orexigenic effect of acute RXFP3 activation (17, 46). However, chronic hypothalamic RXFP3 signaling (viral-mediated hypothalamic delivery of an RXFP3 agonist for 3 months) has been reported to reduce hypothalamic oxytocin mRNA by ~50% (47). Another study using a rat model showed that central administration of peptide antagonists for RXFP3 reduced self-administration of alcohol (44). Given that relaxin-3 is involved in regulating appetite, according to the findings, it may regulate appetite directly or through a peptide/hormone, such as orexin or oxytocin. In future studies, investigation of the relationship between oxytocin and orexin levels and relaxin-3 will enable us to better understand the effect of relaxin-3 on appetite.

Our study is of importance because it is the first to investigate serum levels of relaxin-3, which has a role in regulating social behavior and nutritional behavior in children with ASD. In our study, the relaxin-3 levels in children with ASD were found to be higher than in the controls. Further investigation and verification of this difference between the groups with a wider sample

in future studies may enable relaxin-3 to be used as a biomarker in ASD. As the most important limitation, a causality relationship could not be established due to the cross-sectional nature of the study. Another limitation is that appetite and nutrition were evaluated only by parent reports. The use of appetite and nutrition-related biomarkers in future studies will help us better understand the relationship between relaxin-3 and appetite.

Finally, the findings of this study have important implications. Our findings suggest that additional research is needed to study the effects of serum relaxin-3 levels on both communication and appetite in children with ASD. In addition, the investigation of the mechanism underlying the different levels of relaxin-3 between ASD and the control group may be of importance in clarifying the etiology of ASD, as well as its follow-up and treatment.

Declaration of Conflicting Interest.

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding.

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Informed consent.

Informed consent was obtained from all individual participants included in the study.

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Table 1. Characteristics of patients and control groups and relaxin-3 level

	Patient group (n=50)	Control group (n=30)	t or x^2	p
Age (months), Mean(±SD)	55.64 (24.06)	64.23 (21.36)	1.608[a]	0.07
Gender, (female/male)	6/44	6/24	0.941[b]	0.33
Relaxin-3 (pg/mL),	43.14 (45.46)	23.20 (19.73)	2.992[a]	0.026
CARS	32.27 (6.77)	15	31.266[a]	0.000

CARS: Childhood Autism Rating Scale

a=t test b= x^2

Table 2. Correlation of clinical variables and serum values in children with autism

	Relaxin-3	CARS total score	Child age
CARS total score	r:-.112 p: 0.44	1	-
CARS listening response	r:**-.380**** p: 0.009	r:**.397**** p: 0.006	r:**.352*** p: 0.016
ABC			
Total score	r:.065 p: 0.66	r: **.441**** p: 0.002	r:.104 p: 0.49
Irritability	r:.087 p: 0.56	r:**.300*** p: 0.043	r:.153 p: 0.31
Somnolence	r:.110 p: 0.46	r:**.323*** p: 0.028	r:.001 p: 0.99
Stereotypy	r:-.067 p: 0.65	r: **.449**** p: 0.002	r:.114 p: 0.45
Hyperactivity	r:.007 p: 0.96	r: **.523**** p: 0.000	r:.122 p: 0.41
Speech problem	r:**.320*** p: 0.024	r: **.331**** p: 0.019	r:-.035 p: 0.80
CEBQ			
Food responsiveness	r: 0.81 p: 0.57	r:-.108 p: 0.45	r:105 p:0.46
Emotional overeatin	r:-.049 p: 0.73	r:.052 p: 0.71	r:**.281*** p: 0.048
Enjoyment of food	r:-.176 p: 0.22	r:-.132 p: 0.36	r:.123 p: 0.39
Desire to drink	r: **.356*** p: 0.011	r:-.141 p: 0.33	r:.070 p: 0.62
Satiety responsiveness	r: **-.381**** p: 0.006	r:-.098 p: 0.49	r:.100 p: 0.49
Slowness in eating	r:.223 p: 0.12	r:.251 p: 0.07	r:-.041 p: 0.77
Emotional undereating	r:-.209 p: 0.14	r:.101 p:0.48	r:-.057 p: 0.69
Food fussiness	r:-**.354*** p: 0.012	r:-.068 p: 0.63	r:.173 p: 0.22

*<0.05 **<0.01 CARS: Childhood Autism Rating Scale, ABC: Aberrant Behavior Checklist, CEBQ: Children's Eating Behavior Questionnaire