Published in final edited form as:
J Am Acad Child Adolesc Psychiatry. 2012 July ; 51(7): 712–721.e1. doi:10.1016/j.jaac.2012.04.010.

Examining Autism Spectrum Disorders by Biomarkers: Example From the Oxytocin and Serotonin Systems

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Abstract

Objective—Autism Spectrum Disorder (ASD) is a heritable but highly heterogeneous neuropsychiatric syndrome, which poses challenges for research relying solely on behavioral symptoms or diagnosis. Examining biomarkers may give us ways to identify individuals who demonstrate specific developmental trajectories and etiological factors related to ASD. Plasma oxytocin (OT) and whole blood serotonin (5-HT) levels are consistently altered in some individuals with ASD. Reciprocal relationships have been described between brain oxytocin and serotonin systems during development. We therefore investigated the relationship between these peripheral biomarkers as well as their relationships with age.

Method—In our first study, we analyzed correlations between these two biomarkers in 31 children and adolescents who were diagnosed with autism and were not on medications. In our second study, we explored whether whole blood 5-HT levels are altered in mice lacking the oxytocin receptor gene, *Oxtr*.

Results—In humans, OT and 5-HT were negatively correlated with each other ($p<0.05$) and this relationship was most prominent in children under 11 years old. Paralleling human findings, mice lacking *Oxtr* showed increased whole blood 5-HT levels ($p=0.05$), with this effect driven exclusively by mice younger than 4 months of age ($p< 0.01$).

Conclusions—Identifying relationships between identified ASD biomarkers may be a useful approach to connect otherwise disparate findings that span multiple systems in this heterogeneous disorder. Using neurochemical biomarkers to do parallel studies in animal and human populations

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Supplemental material cited in this article is available online.

Disclosure: Dr. Veenstra-VanderWeele has received funding from Seaside Therapeutics, Novartis, and Roche Pharmaceuticals, and has served as a consultant for Novartis. Dr. Cook has served as a consultant for and has received funding from Seaside Therapeutics.

Drs. Hammock, Yan, Morris, Anderson, Carter, and Jacob, and Mr. Kerr report no biomedical financial interests or potential conflicts of interest.

within a developmental context is a plausible approach to probe the root causes of ASD and identify potential interventions.

Keywords

autism; biomarker; serotonin; oxytocin; development

Introduction

The pattern of behavioral symptoms now described as Autism Spectrum Disorder (ASD) was first recognized by Leo Kanner in 1943 [1]. ASD symptoms have been broken down into various numbers of subcategories by clinical consensus [2] and by statistical analysis [3–6]. Current consensus is coalescing around two symptom domains: social communication impairment and fixed interests / repetitive behavior, with some support from factor analyses [7–9]. Beyond these diagnostic domains, comorbid developmental, cognitive, and behavioral symptoms are common and diverse in ASD, including intellectual disability [10], sensory dysfunction [11–16], and hyperactivity [17,18]. A number of comorbid neurological and medical conditions are also common in ASD, including epilepsy [19], sleep problems [20], and constipation [21,22]. Overall, the heterogeneity of diagnostic and associated symptoms suggests that diverse susceptibility factors may lead to social dysfunction and repetitive behaviors as a common final outcome. Biomarkers could potentially be used to parse this heterogeneity, which may ultimately lead to individualized interventions.

An ideal biomarker should be highly reproducible, as well as straightforward and economical to measure. The latter criteria can be achieved over time as technology improves. While we might hope for a diagnostic biomarker, this goal has proven elusive in behaviorally defined disorders. Alternatively, a biomarker could represent an intermediate phenotype that arises more directly from a particular genetic or environmental susceptibility factor and mediates the risk of the disorder itself [23]. A biomarker could also be an endophenotype, a heritable trait that arises from an underlying genetic factor and could be separately mapped in genetic studies [24,25]. We must also be vigilant for the possibility that a biomarker might be a secondary result of ASD symptoms or treatment, thereby being less useful for the identification of susceptibility factors. A number of biomarkers have been identified in ASD, including brain growth patterns and metabolic or immune markers [26–32]. The present study focuses on the relationship between blood measures of serotonin and oxytocin, two peripheral biomarkers that correspond to systems that interact in the brain.

Hyperserotonemia, or elevated blood serotonin (5-hydroxytryptamine; 5-HT) levels are found in 25–35% of individuals with ASD [33–38]. Whole blood 5-HT is contained almost entirely in platelets that take up 5-HT via the serotonin transporter as they pass through the enteric circulation, where 5-HT is synthesized and released by enterochromaffin cells. Blood 5-HT levels are correlated among children with ASD and their family members [33–36] and 5-HT levels were found to have a heritability of ~1.0 in a large inbred population study [39]. Abnormalities in the brain serotonin system are also reported in ASD, including evidence of altered serotonin synthesis [37,40] and receptor binding [41,42], as well as one report of dystrophic serotonergic axons [43]. Thus, the overall evidence implicates the 5-HT system in ASD, but attempts to link the robust and heritable whole blood 5-HT levels to clinical symptoms have been inconsistent, with isolated reports of association with stereotyped or self-injurious behavior [44–47].

Blood oxytocin (OT) levels are also a focus of ASD research. Children with autism have lower average levels of blood OT level in comparison to typically developing children matched for age [48], with a specific correlation with being rated as “aloof” using Wing’s

typology [49]. In contrast, a study of adults with ASD suggests that OT levels are higher at baseline in adults [50]. Interest in the oxytocin system in ASD is supported by its important role in mammalian social behaviors, including social recognition, parenting behavior, and attachment [51-57]. Mice lacking the oxytocin receptor gene (*Oxtr*) show reduced social recognition, increased susceptibility to seizures, and poor reversal learning, a laboratory proxy of cognitive flexibility [58,59]. Genetic studies suggest a modest association of oxytocin receptor gene (*OXTR*) alleles with ASD[60–68], but see also [69,70]. In addition to primary signaling molecules, allelic variation in *CD38*, a positive regulator of OT release [71,72], also shows association with ASD [73,74]. Based upon the biomarker and genetic data, as well as the effects of OT on affiliative behavior across species, considerable interest is focused on the potential use of OT in ASD. Initial studies with intravenous or intranasal OT are promising [75–79]; although sufficient data are not yet available to judge whether it leads to sustained benefit or is safe for long-term use in adults or in children.

The serotonin and oxytocin systems interact in the brain, both during development and in adulthood. In rats and voles, early exposure to a non-selective serotonin receptor agonist results in fewer oxytocin (OT) cells in the paraventricular nucleus of the hypothalamus (PVN), less affiliative behavior, and less social interaction [80,81]. Conversely, early exposure of vole pups to exogenous OT results in higher serotonin axon length densities in several brain regions [82]. In humans, the 5-HT system regulates OT release in adults, as evidenced by increased OT levels following treatment with 3,4-Methylenedioxymethamphetamine (MDMA, “Ecstasy”), a drug that causes release of 5-HT [83–88]. Further, OT acts as an anxiolytic in adult mice via oxytocin receptors expressed in serotonin neurons [89].

The separate lines of evidence for blood 5-HT and oxytocin measures as biomarkers in ASD, coupled with the evidence for intersection between the corresponding systems in the brain, led us to examine the relationship between peripheral 5-HT and OT biomarkers in children and adolescents with ASD. We complemented this observational biomarker study with an experimental paradigm in mice lacking the oxytocin receptor gene, assessing whether genetic manipulation of the oxytocin system can impact the peripheral biomarker of whole blood 5-HT.

Method

Human Participants

All participants were enrolled at the University of Illinois at Chicago Autism Center of Excellence and were recruited under a protocol studying rigid-compulsive features in ASD. They were assessed with ADI-R [90], the Autism Diagnostic Observation Schedule, ADOS [91] and clinical evaluation. Participants were included in this study if they were classified as strictly defined autism rather than autism spectrum disorder, because they met the classification criteria across diagnostic research instruments. Participants were screened to have no psychiatric medications and no medication use during the time the neurochemistry assays were completed for both plasma OT-RIA and whole blood 5-HT assays. In total, 31 unrelated probands were studied (mean age 8.8 ± 4.4 years; 28 male, 3 female; 27 reported to be of European ancestry).

Human Neurochemistry Biomarker Assays—OT concentrations in plasma were determined by a non-equilibrium plasma OT radioimmunoassay, as previously described [92]. Briefly, plasma samples (~1.0 ml) were extracted using the acetone/petroleum ether method, lyophilized and stored at −80 °C until analysis. ^{125}Iodine-labeled OT was purchased from PerkinElmer. OT was measured with antiserum (supplied by MM) [92]. Bound hormone was separated from unbound with a secondary antibody (goat anti-rabbit IgG) and anti-rabbit gamma globulin (ARGG).

Whole blood 5-HT (WB5-HT) is a reliable measure of platelet 5-HT because greater than 99% of whole blood 5-HT is in the platelet fraction [93]. Measurement of platelet 5-HT in platelet-rich plasma prepared by centrifugation can add variance due to 5-HT release during processing and variable platelet yield. Whole blood 5-HT was measured by HPLC with fluorometric detection, as described previously [94]. Intra-assay and interassay coefficients of variation were 1.7 and 6.2%, respectively.

Animals—*Oxtr* mice (*Oxtr*$^{tm1.1Knis}$) were a generous gift of Prof. Larry Young of Emory University. The mice were received on a mixed 129/C57 background [59] and fully backcrossed to C57BL/6J with confirmed backcrossing by strain-specific marker genotyping (Speed Congenics, The Jackson Laboratory, Bar Harbor, ME). Mice were maintained on corncob bedding on a 12:12 L/D cycle with *ad libitum* access to standard chow and water. All experiments were conducted with approval and oversight of the Vanderbilt University Institutional Animal Care and Use Committee following local, state and federal guidelines. Heterozygous breeder pairs were used to generate mixed litters containing wild type, heterozygous, and knockout animals. Co-housed littermate wild type and knockout males were used for experiments. Animals were weaned at the beginning of the fourth post-natal week and housed with same-sex littermates. PCR-based genotyping was performed on tail-samples with the following primers: *Oxtr*_common_For 5′-CTGGGGCTGAGTCTTGGAAG; *Oxtr*_WT_Rev 5′-CTCGATACTCCAGTTGGCTGC; *Oxtr*_KO_Rev 5′-GTTGGGAACAGCGGTGATTA.

Mouse whole blood 5-HT measurement—At harvest, 9 littermate pairs of animals (N = 18 mice) were scruffed, rapidly decapitated, and trunk blood was collected into a 1.5 mL microfuge tube containing 25 uL ACD Solution A (trisodium citrate, 22.0g / L; citric acid, 8.0 g / L; dextrose 24.5 g / L; Becton, Dickinson and Co., Franklin Lakes, NJ), mixed gently, immediately frozen on dry ice and stored at −80C until the time of analysis. 5-HT levels were measured in the Neurochemistry Core of Vanderbilt University by previously described HPLC electrochemical detection methods [95]. Briefly, 5-HT levels were determined by a specific HPLC assay using an Antec Decade (oxidation: 0.7) electrochemical detector. 5-HT was eluted with a mobile phase consisting of 89.5% 0.1M TCA, 10^{-2} M sodium acetate, 10^{-4} M EDTA and 10.5 % methanol (pH 3.8). Concentration was determined by comparison with injections of known standards.

Statistical analyses—Statistical analyses for the human data set were conducted using PSAW Statistic v. 18 (SPSS Inc, Chicago, IL). Shapiro-Wilk and Kolmogorov-Smirnov normality tests were used to examine deviations from a normal distribution. OT was converted to fg/ml and log-transformed in subsequent tests for normalization prior to using parametric statistics. T-test or Pearson's correlation was used to examine WB5-HT and log(OT) with respect to sex, ancestral background (European descent or other), and age in months. Pearson's correlation was used to examine the relationship between WB5-HT and log(OT) with age as a covariate. Exploratory unpaired t-test was used to compare the ratio of WB5-HT / log(OT) in participants less than 11 years old or greater than 11 years old.

Statistical analyses for the mouse data set were conducted using GraphPad Prism v. 5 (LaJolla, CA). An omnibus normality test was used to examine deviations from a normal distribution. Pearson's correlation was used to examine whether the dependent variable of mouse WB5-HT varied by age. Mouse WB5-HT levels were compared across genotype within littermate pairs using a paired t-test.

Results

We initially examined if human OT plasma levels and WB5-HT were normally distributed and whether they differed by sex, reported ancestral background, and age (Table S1, available online). Although WB5-HT approached a normal distribution, OT was positively skewed (skewness = 2.7, kurtosis = 10.3) and required log transformation in all further analyses.

There were no significant differences in log(OT) and WB5-HT across sex (all p values >0.30) or reported ancestral background (all p values >0.44). Although log(OT) levels were not significantly correlated with age (r=0.14, N= 31, $p = 0.22$), WB5-HT was negatively correlated with age (r =−0.32, N= 31, $p = 0.04$) and was lower in adolescence than childhood (see Figure 1).

Log(OT) levels were significantly negatively correlated with WB5-HT (r=−0.37, N= 31, $p = 0.04$, Figure 2a) when controlling for age as a covariate with a partial correlation analysis. Exploratory analyses demonstrated that the ratio of WB5-HT to log(OT) was significantly different (t=3.0, p=.008, Figure 2b) in children under 11 years (M=91.2, SD=32.1) versus older children and young adults (M=61.7, SD=19.1) and similar to the pattern of differences in WB5-HT (t=2.4, p=.04, Figure 1b).

Paralleling the human sample, mouse WB5-HT levels did not deviate significantly from a normal distribution. In mice, WB5-HT levels showed a non-significant trend for a negative correlation with age (r=−0.40, N = 18, $p < 0.10$) when both genotypes were analyzed together (Figure 3A). In male *Oxtr*$^{-/-}$ mice, we observed higher concentrations of whole blood 5-HT compared to wild-type littermates (Figure 3B), (Paired student's t = 2.29, N = 18, $p = 0.05$). Interestingly, a post-hoc analysis showed that this difference was evident in younger animals (male littermates < 4 months of age: paired student's t= 8.82, N = 8, $p < 0.01$), but not in animals older than 4 months (male littermates > 4 months of age: paired student's t = 0.43, N = 10, $p = 0.69$; (see Figures 3C–3D).

Discussion

Both the human and mouse data are consistent with a relationship between peripheral OT and 5-HT biomarkers. In humans, we observed a negative correlation between whole blood serotonin levels and peripheral OT levels. In mice, we observed that mice lacking the *Oxtr* gene had lower concentrations of whole blood serotonin. In both humans and in mice, the relationships were strongest in younger individuals.

The negative correlation between OT and 5-HT levels in the human data can be interpreted in a number of ways, as this relationship could be mediated at multiple levels. Platelet-poor plasma ultrafiltrate serotonin levels are not elevated in subjects with autism[96–98], suggesting that peripheral OT could affect either increased gastrointestinal synthesis, increased platelet serotonin uptake, or decreased serotonergic release from platelets [99]. Conversely, peripheral 5-HT is unlikely to affect central OT release because it doesn't cross the blood-brain barrier, but it could plausibly affect peripheral metabolism of OT. Importantly, a direct relationship is not required to explain the correlation, since both biomarkers could also be regulated in opposite directions by a shared pathway.

The decrease in whole blood 5-HT across development is consistent with previous reports in human samples [100]. Although hyperserotonemia is not limited to prepubertal children[100], previous work has shown a decrease or stabilization around the age of 12 years [101]. This led to our selection of >11 years as a division point when considering the relationship between OT and WB5-HT. It is possible that the smaller dynamic range of WB5-HT in adolescents

prevents this small sample from demonstrating a strong relationship with OT. If we compare our results with this cut-off to pre-pubertal healthy control children of European origin determined in the same laboratory using the same HPLC methodology, we observed a marked elevation in whole blood 5-HT. Reported WB5-HT reference ranges (mean+/−SD) for prepubertal white healthy control children were 190+/−44 (N=35) (Pfeffer et al., 1998), and 187+/−50 ng/ml (N=21) (McBride et al., 1998), in comparison to 278.8+/−84.9 ng/ml (N=22) in our population of children with ASD of European origin ≤11 years old. It is possible that our recruitment for a study examining rigid-compulsive symptoms in ASD, resulted in more young children with hyperserotonemia, but it is not possible to make a true assessment without a direct ethnic and age-matched control group.

The mouse data show that experimental alteration of the oxytocin system can affect platelet 5-HT levels. This suggests that the OT system is likely to directly or indirectly regulate the peripheral 5-HT system at some level, but it does not rule out a bidirectional relationship or explain the mechanism of regulation. The influence of oxytocin receptor (OXTR) absence could be at the level of the intestinal lumen[102], where OXTR is expressed in the epithelium; although its co-expression in serotonin positive enterochromaffin cells is unknown. OXTR is unlikely to have a direct effect at the level of the platelet, where it does not appear to be expressed. The effects of OXTR could also be mediated through some intermediate system that in turn regulates the 5-HT system. Further, OXTR absence could affect the development of the peripheral 5-HT system or could have an ongoing effect in older animals. The stronger findings in the younger animals suggest that the relationship between these two systems in the periphery may, like the human correlation, be stronger earlier in development, thus highlighting the importance of patient age in the identification of biomarker interrelationships and subsequent reproducibility.

There are a number of limitations of these results. Both the human and the mouse sample sizes were small. This is particularly challenging with regard to sample sizes across development. The human sample was limited to children with ASD, so it is not possible to know whether the relationship between OT and 5-HT is specific to ASD or generally true regardless of diagnosis. With regard to the mouse data, elimination of the oxytocin receptor is a dramatic genetic change that should be expected to be extremely rare in humans, so it cannot be extended directly to the human data. Smaller perturbations in the oxytocin system may or may not parallel the effects seen with *Oxtr* deletion. Importantly, the human results appear to point to a change in the relationship between 5-HT and OT around the time of puberty, but Tanner staging data was not complete in this sample. In contrast, the mouse data are confined to post-pubertal animals beginning in adolescence and extending into adulthood. The parallels between human and mouse developmental processes are not clear with respect to the relationship between OT and 5-HT.

Future research should examine whether the relationship between blood OT and 5-HT levels is specific to children with ASD or extends into the general population. This work should also examine whether this relationship is particularly true for those individuals with hyperserotonemia relative to a control population or whether it corresponds to a quantitative relationship across the full range of 5-HT levels. The developmental course of OT and 5-HT levels within individuals also demands further study, both individually as well as in interaction with each other. In particular, Tanner staging or sex hormone status should be assessed in future work to examine whether 5-HT levels decline gradually over time or show a specific decrement at puberty. A larger sample size will be necessary to fully examine the impact of development, as well as other factors such as sex or ancestral background. Future work in the 5-HT system should also include considering whether peripheral levels correlate with brain measures (e.g. transporter binding, receptor binding, synthesis capacity). Further, it remains to be determined if these biomarkers relate to response to medications targeting

the serotonin system, such as serotonin reuptake inhibitors. Pharmacological approaches could also be used to examine 1) how serotonergic drugs in humans as well as animals modulate OT levels and function and 2) how OT drugs (intranasal as well as peripartum pitocin) influence serotonin levels both in the brain and in the periphery. Mouse studies offer the possibility of more targeted experimental intervention, including selective deletion of *Oxtr* from specific cell types at particular points in development. Finally, studies of susceptibility factors could focus on individuals with low OT and high 5-HT levels that may allow mapping of genes or environmental factors that impact both systems.

As quantitative measures reflecting multiple genetic or environmental effects within a single system, biomarker assessment is an appealing way to study individuals with ASD. Despite this appeal, it is important to note that previous work has not connected hyperserotonemia to specific clinical features of the disorder. Future ASD biomarker research, whether focusing on peripheral neurochemistry or targeted neuroimaging, should integrate measures of multiple biomarkers in the same set of individuals, rather than continue to focus on a single biomarker at a single point in development. It is especially important that peripheral biomarkers be evaluated in studies that focus on the same system in the brain, such as serotonin receptor binding studies. Finally, while additional relationships between biomarkers may emerge with further study, the mechanisms underlying these biomarkers and related systems in the brain will be difficult to dissect in humans, given the present limited availability of postmortem brain tissue from children with ASD. Hypotheses generated from human clinical research should therefore be carefully tested using experimental paradigms in animal models, with results then corroborated with human research and eventually translated into testing of novel treatments. The combination of human and rodent studies may allow us to relate peripheral biomarkers to genetic and neural circuit pathology in ASD. While preliminary, our findings point to the promise of connecting biomarker findings across systems and across development, rather than confining our focus to one system at one time point.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by a Vanderbilt Luton Society Research Award (J.V., E.H.), a National Alliance for Research on Schizophrenia and Depression (NARSAD) Young Investigator Award (E.H), a National Institutes of Health (NIH) grant K08MH081066 (J.V.), the National Institute of Child Health and Human Development (NICHD) grant P30HD15052 to the Vanderbilt Kennedy Center for Research on Human Development (E.H., J.V.), NIH Autism Center of Excellence grants P50 HD055751 (E.H.C.), and K23MH082121 (S.J.).

We extend our sincere gratitude to all of the families at our research clinics. We gratefully thank Tal Levin-Decannini and Sunday Francis of University of Illinois at Chicago for their assistance with samples, data processing, and their review of this manuscript.

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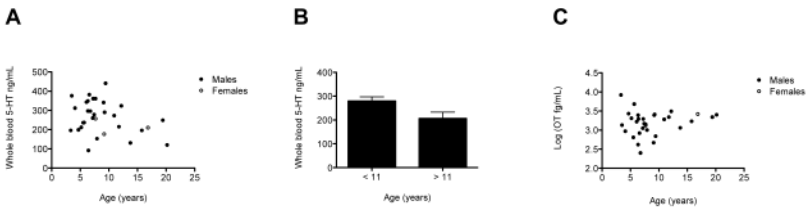

Figure 1.

Whole blood serotonin (WB5-HT) levels in individuals with autism spectrum disorder (ASD) change across development. Note: (A) The level of WB5-HT negatively correlates with age in individuals with ASD (N=31). Males are shown with closed circles and females with open circles. There is a significant negative correlation (r=−0.32, p=0.04) between age and WB5-HT levels. (B) Individuals with ASD who are younger than 11 years, 0 months have higher average WB5-HT compared to those who are older than 11 years, 0 months. Error bars represent standard error of the mean (SEM). (C) The level of log-transformed oxytocin (log(OT)) does not significantly vary across age in individuals with ASD (N = 31). Males are shown with closed circles and females with open circles.

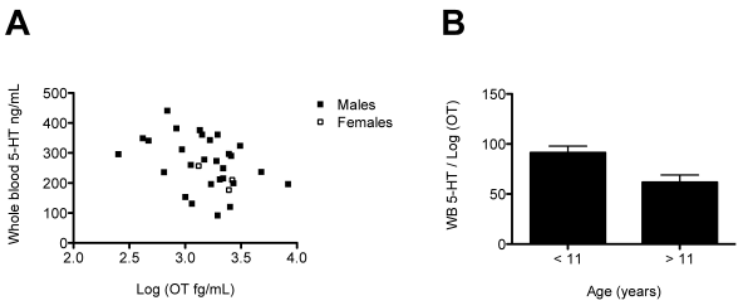

Figure 2.

A relationship exists between plasma levels of oxytocin (OT) and whole blood levels of serotonin (WB5-HT) in individuals with autism spectrum disorder (ASD). Note: (A) The level of WB5-HT across log-transformed oxytocin (log(OT)) levels in individuals with ASD (N=31). Males are shown with closed squares and females with open squares. There is a significant negative correlation (r=−0.326, $p = 0.040$) between WB5-HT and log(OT) levels when age is included as a covariate. (B) The ratio of WB5-HT / log(OT) for individuals who are younger than 11 years, 0 months is higher compared to those who are older than 11 years, 0 month. Error bars represent standard error of the mean (SEM).

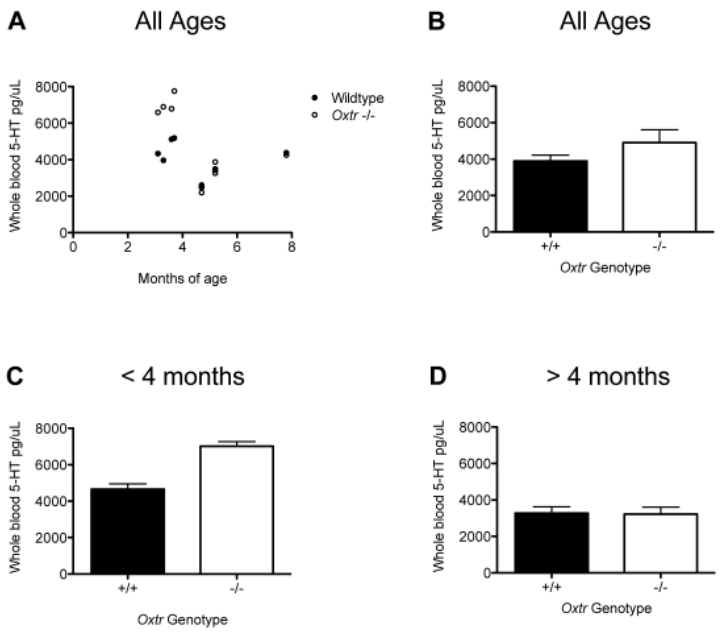

Figure 3.

Congenital loss of the oxytocin receptor gene (*Oxtr*) is associated with higher serotonin levels (WB5-HT). Note: (A) There is a non-significant trend for a correlation (r=−.40, N=18, p<0.10) between WB5-HT and age across all mice, including wildtype (closed circles) and *Oxtr* −/− littermates (open circles). (B) Increased whole blood serotonin is observed in *Oxtr*−/− mice (paired t=2.29, N=18, *p*=0.05 overall), with a highly significant (paired t=8.82, N = 8, *p*<0.01) difference in WB5-HT between the knock-out and wild-type mice younger than 4 months of age (C) but not after 4 months of age (paired t=0.43, N = 10, *p*=0.69) (D).