Vitamin D hormone regulates serotonin synthesis. Part 1: relevance for autism

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ABSTRACT **Serotonin and vitamin D have been proposed to play a role in autism; however, no causal mechanism has been established. Here, we present evidence that vitamin D hormone (calcitriol) activates the transcription of the serotonin-synthesizing gene tryptophan hydroxylase 2 (*TPH2*) in the brain at a vitamin D response element (VDRE) and represses the transcription of *TPH1* in tissues outside the blood-brain barrier at a distinct VDRE. The proposed mechanism explains 4 major characteristics associated with autism: the low concentrations of serotonin in the brain and its elevated concentrations in tissues outside the blood-brain barrier; the low concentrations of the vitamin D hormone precursor 25-hydroxyvitamin D [25(OH)D_3]; the high male prevalence of autism; and the presence of maternal antibodies against fetal brain tissue. Two peptide hormones, oxytocin and vasopressin, are also associated with autism and genes encoding the oxytocin-neurophysin I preproprotein, the oxytocin receptor, and the arginine vasopressin receptor contain VDREs for activation. Supplementation with vitamin D and tryptophan is a practical and affordable solution to help prevent autism and possibly ameliorate some symptoms of the disorder.—Patrick, R. P., Ames, B. N. Vitamin D hormone regulates serotonin synthesis. Part 1: relevance for autism *FASEB J.* 28, 2398–2413 (2014). www.fasebj.org**

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Autism spectrum disorders (ASDs) cover a range of neurodevelopmental disorders affecting >1% of children born in the United States and are characterized by 3 primary behavioral symptoms: impaired reciprocal social interactions, communication deficits, and propensity for repetitive behaviors (1). Autism prevalence is currently 1 in 88, and the incidence has grown by 600% since the 1970s; however, the fundamental cause of this rapid growth is unknown (2, 3). Better diagnostic procedures and increased awareness have been suggested to explain the accelerating autism incidence, but the U.S. Centers for Disease Control and Prevention (CDC) reported an increase in autism incidence from 2006 to 2008, during which there were no diagnostic changes (2, 3). Most autism research has focused on investigating genetic changes as an underlying cause. However, known gene variants have been shown to only modestly affect autism risk and cannot account for such an increased incidence (4, 5). Despite early evidence for heritability of autism, the largest population-based study on twins with autism found that concordance rates for dizygotic twins were actually higher than previously reported and that shared prenatal environment accounted for the bulk of autism risk in twins, with the genetic contribution being only modest (6–8). ASD has been tentatively associated with >440 identified gene variants, and of those cases that can be clearly linked to genetic causes, 7–20% can be accounted for by copy number variants, 5–7% are attributed to polymorphisms in a single gene, and <5% are linked to genes involved in rare metabolic disorders (5). Thus, ~70% of cases have a cause that has not been linked to genetics (5). Therefore, it appears that autism is a multifactorial disorder involving both genetics and environment.

Four observations are consistently associated with ASD: tissue-specific aberrant serotonin concentrations; low plasma concentrations of the vitamin D hormone precursor 25-hydroxyvitamin D [25(OH)D_3]; high male incidence; and presence of maternal antibodies to fetal brain tissue. This report first presents a brief review of current scientific evidence relevant to the roles of serotonin and vitamin D in autism. The main body of the article presents a unifying mechanistic

Abbreviations: 25(OH)D_3, 25-hydroxyvitamin D; *AVPR1A*, arginine vasopressin receptor 1A; *AVPR1B*, arginine vasopressin receptor 1B; ASD, autism spectrum disorder; BH4, tetrahydrobiopterin; GI, gastrointestinal; 5-HTP, 5-hydroxytryptophan; IDO, indoleamine 2,3-dioxygenase; *OXT*, oxytocin/neurophysin I prepropeptide; *OXTR*, oxytocin receptor; RXR, retinoid X receptor; SSRIs, serotonin reuptake inhibitors; TPH, tryptophan hydroxylase; *TPH1*, tryptophan hydroxylase 1; *TPH2*, tryptophan hydroxylase 2; T_{reg}, regulatory T; UCSC, University of California–Santa Cruz; UV, ultraviolet; UVB, ultraviolet B; VDR, vitamin D receptor; VDRE, vitamin D response element

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hypothesis that links vitamin D and serotonin concentrations to these disparate observations and to the increased autism incidence. This hypothesis is based on the identification of vitamin D response elements (VDREs) on two different tryptophan hydroxylase (TPH) genes involved in serotonin synthesis that are functionally opposite to one another: one of them induces transcriptional activation of tryptophan hydroxylase 2 (*TPH2*) by vitamin D in the brain and the other induces repression of tryptophan hydroxylase 1 (*TPH1*) in tissues outside the blood-brain barrier or peripheral to the brain, herein referred to as peripheral tissues. Transcriptionally activating VDREs are also present on the oxytocin-neurophysin I preproprotein gene, the oxytocin receptor gene, and the arginine vasopressin receptor genes, suggesting that vitamin D hormone may also regulate the synthesis of, and response to, oxytocin as well as the response to vasopressin, all of which play a role in autism.

ROLE OF SEROTONIN IN AUTISM

Serotonin (5-hydroxytryptamine), a neurotransmitter and brain morphogen, has been proposed to play a major role in autism based primarily on physiological evidence, genetic polymorphisms, and animal models (9–24). Serotonin is synthesized in 2 steps from tryptophan, an essential amino acid present in small amounts in dietary protein. Step 1: TPH, the rate-limiting enzyme in serotonin synthesis, uses tetrahydrobiopterin (BH4) and iron as cofactors to hydroxylate tryptophan to 5-hydroxytryptophan. Step 2: 5-hydroxytryptophan is decarboxylated to serotonin by aromatic amino acid decarboxylase, a pyridoxal phosphate-requiring enzyme (25). There are two separate tryptophan hydroxylase enzymes that are produced from different genes, *TPH1* and *TPH2*, which are localized in different tissues. TPH1 is found in nonbrain tissues, including the gut enterochromaffin cells, pineal gland, placenta, and T cells, and it is responsible for producing most of the serotonin found in the body, including the blood (26–28). Almost all of the serotonin in the blood is located in platelets, which do not synthesize serotonin, but instead take it up from the gut pool (29). TPH2 is entirely restricted to neurons of the raphe nuclei and the enteric nervous system and is the enzyme responsible for producing all of the serotonin in the brain (26).

The level of serotonin in the brain depends on the blood levels of tryptophan, which, unlike serotonin, crosses the blood-brain barrier (30, 31). Tryptophan is a rare amino acid that competes for transport into the brain with the branched chain amino acids, which are more abundant and are preferentially transported into the brain (31, 32). Excess serotonin synthesis in peripheral tissues could result in most of the dietary tryptophan being consumed, which could further lower its availability to be transported into the brain.

The disruption of the serotonergic system is one of the most consistent observations associated with autism (9–16). Serotonin in the brain promotes prosocial behavior and correct assessment of emotional social cues (33). Brains of individuals with ASD display significantly lower concentrations of serotonin compared with the brains of nonautistic individuals (34, 35). Low serotonin during early brain development in rats can lead to neuroanatomical defects such as fewer dendritic spines, abnormally small dendritic arbors and somatosensory barrels, and reduced synaptic density (36–38). Furthermore, depletion of serotonin in neonate mice causes larger than normal cortical brain growth and behavioral characteristics, which are similar to autism (15, 23). Such neurodevelopmental defects have been observed in individuals with autism, suggesting that inadequate concentrations of serotonin in the brain prevent normal brain development (36–38). A developmental peak in serotonin synthesis occurs in the brain before puberty and is thought to play a role in growth and differentiation of neurons during brain development. This peak fails to occur in children with autism (35). On the other hand, elevated concentrations of serotonin have been found in the blood in 25–50% of children with autism (10–14). An inverse correlation between high serotonin concentrations in the blood and low serotonergic neurotransmission has been demonstrated in young male adults with ASD, a phenomenon we refer to as the serotonin anomaly (34). The high serotonin concentrations in peripheral blood cells from individuals with autism have been suggested to be the result of increased serotonin synthesis in the gut; however, the cause of the elevated serotonin production in the gut has not been explained (39). Additionally, transformed lymphoblastoid cells from individuals with autism have altered tryptophan metabolism as measured by decreased NADH production (24). As tryptophan is a precursor to serotonin, it may provide a partial explanation for the disruption in the serotonin pathway; however, it still fails to provide a molecular mechanism for the cause or which pathway of tryptophan metabolism is aberrant.

Polymorphisms in a wide range of serotonin-related genes have been examined in individuals with autism as a possible underlying mechanism to explain the physiological aberrations in serotonin concentrations observed in individuals with autism (17–21). For example, polymorphisms in the serotonin transporter have been correlated with autism; however, they are associated with a modification of the severity of autistic behavior rather than with risk of autism (19). A small percentage of the population with autism has been shown to harbor polymorphisms in the *TPH2* gene that are known to cause low concentrations of serotonin in the brain (20, 21). In accordance with the causal role of low serotonin in autism, mice lacking TPH2 are defective in brain serotonin synthesis and display behavioral symptoms of autism, including impaired social interaction and communication and the propensity for repetitive behaviors (22). These data suggest that disruption in serotonin levels are linked to autism, although no underlying mechanism has been identified.

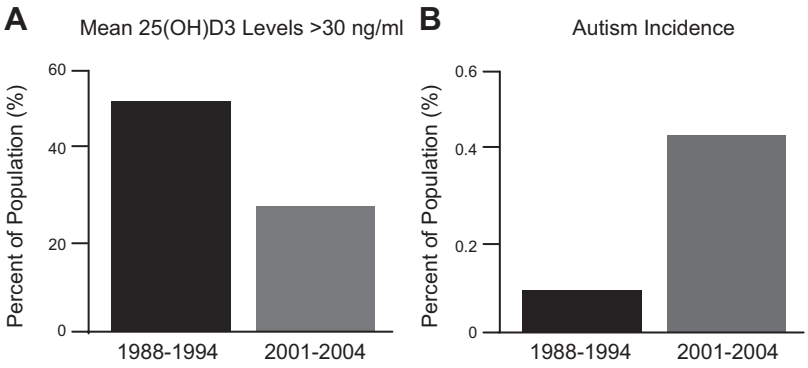

Figure 1. Inverse correlation between $25(OH)D_3$ levels > 30 ng/ml (vitamin D sufficient) and autism incidence. *A*) Percentage of non-Hispanic white males and females of all ages combined (95% confidence interval) who have mean levels of $25(OH)D_3$ > 30 ng/ml. To convert nanograms per milliliter to nanomolar, multiply by 2.5. *B*) Average incidence of autism spectrum disorders among surveyed states as reported by the U.S. Centers for Disease Control and Prevention (2, 49).

ROLE OF VITAMIN D IN AUTISM

Vitamin D hormone has been proposed to play a role in autism based primarily on a correlation between autism incidence in populations with low levels of vitamin D. Vitamin D is a fat-soluble vitamin that is converted to its biologically active form 1,25-dihydroxyvitamin D (calcitriol), herein referred to as vitamin D hormone, a steroid hormone that appears to regulate the expression of ~900 different genes, a large number of which impact brain development and function (40, 41). The primary source of vitamin D is from skin exposure to ultraviolet B (UVB) radiation emitted from the sun, which induces the epidermal synthesis of vitamin D from endogenous 7-dehydrocholesterol (41, 42). Vitamin D is first converted to $25(OH)D_3$, which is the major stable circulating form of vitamin D (42). $25(OH)D_3$ then is converted to the active vitamin D hormone, 1,25-dihydroxyvitamin D (42). Both sunscreen lotion and melanin, the brown pigment found in skin, block UVB radiation and, thus impair the ability of the skin to synthesize vitamin D (43). A modest amount of vitamin D can be obtained through dietary sources, such as seafood, which is its relatively richest dietary source (44).

The current guidelines for vitamin D sufficiency are based on serum concentrations of $25(OH)D_3$ required to maintain bone health, the classical vitamin D function, which is considered to be >30 ng/ml (45). It is unclear whether these guidelines are sufficient to maintain nonclassical functions of vitamin D hormone in other tissues. In addition, the importance of free $25(OH)D_3$ compared with bound $25(OH)D_3$, which is determined by different levels of vitamin D-binding protein, a globulin protein that binds to various forms of vitamin D, may also be important for the classical and nonclassical functions of vitamin D (46). Indeed, the biological responses to vitamin D hormone have been shown to vary according to the fraction of $25(OH)D_3$ that is bioavailable, which is linked to different isoforms of the vitamin D-binding protein (46, 47). Vitamin D insufficiency (<30 ng/ml) has been increasing in recent decades. The National Health and Nutrition Examination Survey (NHANES) reported that vitamin D sufficiency (30–80 ng/ml) decreased between 1994 and 2004 from ~60 to 30% in Caucasians, from 10 to 5% in African Americans, and from 24 to 6% in Latinos, indicating that more than half of the U.S. population may have insufficient levels of this critical vitamin D hormone (48, 49). While a recent report indicated that as much as 80% of the African American population have a polymorphism in vitamin D-binding protein that may result in greater bioavailability of $25(OH)D_3$ than in Caucasians, other reports have associated this polymorphism with less bioavailability of $25(OH)D_3$ (46, 47, 50, 51). According to a 2006 NHANES report, 96% of Americans not taking vitamin and mineral supplements have insufficient vitamin D levels, whereas only 25% using supplements containing vitamin D have insufficient levels (52).

Evidence of increased autism prevalence in regions with lower sun exposure in the general population has been available for some time (2, 53–55). In the United States, there is an inverse correlation between autism incidence and exposure to UVB, as measured by the level of ultraviolet (UV) radiation in the child's state of birth (53). Children born in overcast and rainy counties of Oregon, Washington, and California are twice as likely to be diagnosed with autism as children born in sunnier parts of these states (56). Accordingly, there is an inverse correlation between the rapid rise in autism incidence and the percentage of the U.S. population with plasma concentrations of $25(OH)D_3$ considered sufficient by current guidelines (**Fig. 1** and refs. 2, 49).

Autism incidence has also been linked to maternal vitamin D insufficiency in dark-skinned mothers living in northern latitudes. Reports have correlated low concentrations of maternal $25(OH)D_3$ with increased risk of having a child with behavioral problems associated with autism, such as language impairment and attention-switching difficulties (57–59). In addition, Somali mothers who moved to Stockholm have been shown to be severely vitamin D deficient (<20 ng/ml) and have approximately a 4.5 times higher risk of having a child with autism, as compared with native Swedes (60–62). This is in contrast to African individuals living in East Africa, who have mean $25(OH)D_3$ concentrations of 48 ng/ml (63). Autism incidence in the Somali population living in Minneapolis also appears to be high. In 2008, the Minneapolis school district enrolled 0.94% of Somali children, 0.52% of non-Somali African American children, 0.38% of Hispanic children, and 0.20% of Caucasian children in

their citywide ASD preschool program (64). It has been proposed that the stress of migration may play a role in autism risk in offspring; however, no association between stressful events during pregnancy and children with ASD could be identified in 2 large population cohorts from Sweden and England (65). In Stockholm, it has been shown that children from migrant parents are also at an increased risk for low-functioning ASD; however, this risk is highest for children from mothers that migrated from equatorial regions, including Africa and the Caribbean, to Stockholm or England and have darker skin pigmentation (66, 67). A common denominator between Minneapolis and Stockholm with respect to the increased incidence of autism in Somali immigrants is that both of these regions are at much higher northern latitudes relative to Somalia and thus have lower levels of sun exposure. Since Somalis have high levels of melanin, migration to northern latitudes, such as Sweden and Minnesota, they would require 5–10 times more UVB exposure than light-skinned individuals or an alternative vitamin D source, such as from the diet or supplementation (43, 68).

Seemingly counter to the above observation that autism incidence should be higher among populations that have a higher incidence in vitamin D deficiency, the Autism and Developmental Disabilities Monitoring (ADDM) network reported that autism incidence is slightly lower in darker-skinned *vs.* lighter-skinned Americans (2). However, a positive correlation between socioeconomic status and autism prevalence has been identified (69–74). Within the context of these communities, a number of studies demonstrate that minority populations from a lower socioeconomic status are markedly underdiagnosed with ASD (69–74). This is likely due to the fact that these populations use mental health and healthcare services significantly less frequently than individuals from a higher socioeconomic status (75–77). Therefore, when the confounding factor of socioeconomic status is controlled for, individuals with darker skin, and belonging to a higher socioeconomic status, are twice as likely to have a child with autism, as compared with lighter-skinned individuals from the same socioeconomic status (78, 79). Therefore, to accurately ascertain autism prevalence between different racial and ethnic groups, one must account for socioeconomic status (2, 78).

A UNIFYING MECHANISM LINKING SEROTONIN AND VITAMIN D HORMONE TO AUTISM

Vitamin D hormone-regulated transcription occurs both by gene activation and repression (80). On binding of vitamin D hormone to the vitamin D receptor (VDR), the VDR heterodimerizes with the retinoid X receptor (RXR), and triggers the VDR to recognize VDREs in DNA sequences of vitamin D-regulated genes (81). It has been demonstrated that the VDRE sequence alone can determine whether the VDR-RXR heterodimer activates or represses transcription, possibly by inducing a conformational change that favors recruitment of either coactivators or corepressors; however, the exact mechanism is unclear (80–82). Multiple regulatory VDREs can be present in proximal and distal regions of a gene and have been shown to represent more than one way to modulate gene transcription in different tissues (81, 83). Communication between distal regulatory elements and the promoter is achieved through looping of the chromatin resulting in the juxtaposition and physical interaction of multiple regulatory elements to either activate or repress transcription (84). The most common VDREs are composed of 2 hexanucleotide direct repeats consisting of (A/G)G(G/T)TCA separated by a 3-nt space, called the DR3 subtype (**Table 1** and ref. 81).

The optimal VDRE for transcriptional activation is (A/G)GGTCA for the 5′ half-site and (A/G)GTTCA for the 3′ half-site (80). Variations in the sequence of the DR3 subtype of activating VDREs are common, with 1 to 3 base substitutions usually occurring in purines in either half-site (80, 85, 86). Multiple distal activating VDREs in a gene can synergize to up-regulate gene transcription, which is thought to occur through chromatin looping, thereby inducing a conformational change replacing bound corepressors with coactivators (81, 87).

Transcriptionally repressing VDREs consist of distinct base substitutions that differ from substitutions that are present in activating VDREs (81, 88). Repressing VDREs consist of substitutions in either the 5′ or 3′ repeat, or both, and typically occur in pyrimidines (Table 1 and ref. 80). VDRE-mediated repression may occur by multiple mechanisms that are less defined than activation (80, 89). Many genes that are transcriptionally repressed by vitamin D have multiple repressing and activating VDREs (90, 91). It has been shown that the activating VDRE, but not the repressing VDRE, binds to the VDR-RXR heterodimer and loops around to the repressing VDRE to replace coactivators with corepressors (89, 90).

DIFFERENTIAL REGULATION OF *TPH1* AND *TPH2* BY VITAMIN D

A large *in silico* and microarray-based study previously identified >900 different genes, many in the brain, that contained putative DR3 VDREs upstream of the promoter regions, including human *TPH1* and *TPH2* (40). However, there has been no investigation of whether the VDREs present on *TPH1* and *TPH2* are associated with activation or repression and whether such an association has any functional significance. We scanned *in silico* the proximal 5′ 10 kb of *TPH1* and *TPH2* using the University of California–Santa Cruz (UCSC; Santa Cruz, CA, USA) genome browser (http://www.genome.ucsc.edu) and confirmed that *TPH1* and *TPH2* contain multiple putative VDREs. By examination of the specific sequences in all the putative VDREs of both *TPH1* and *TPH2*, we determined that *TPH2* has 2 distal

TABLE 1. *Activating and repressing DR3 VDREs in* TPH1, TPH2, OXT, OXTR, AVPR1A, *and* AVPR1B

Human gene	DR3 VDRE type	VDRE location	5′ half	Spacer	3′ half	Refs.
	Activation					
	Common		(A/G)G(G/T)TCA	nnn	(A/G)G(T/T)TCA	80, 81
	Known substitutions		T _ _ _ _ _		_ A _ _ _ _	83, 157
			_ A _ _ _ _		_ _ C _ _ _	80
			_ _ A _ _ _			81
			_ _ _ _ A _			
	Repression: known substitutions		_ _ _ C _ _		_ _ _ A _ _	80, 81
			_ _ _ _ T _		_ _ _ _ _ T	80, 81
			_ _ _ _ _ T			80
TPH1 distal	Repression	−4755	GGGTTA	gca	AGTTCA	40, 80, 81
TPH1 proximal	Activation	−915	AATTCA	ttg	GGTTCA	40, 80
TPH2 distal	Activation	−9771	TGGTCA	att	AGTTCA	40, 83
TPH2 distal	Activation	−7059	AGGTCA	att	TGGTCA[a]	40
OXT proximal	Activation	1759	GGTTCA	agc	GATTCA	40, 158
OXT distal	Repression	−2371	GGGCCA	agc	AGGTCA	40, 80
OXT distal	Activation	−2380	AGGTCA	cag	AGCTCA	40, 80
OXT distal	Activation	4971	GGTTCA	ggc	AATTCA	40, 157
OXTR proximal	Activation	−1940	AGTTCA	gtg	GATTCA	40, 157
AVPR1A distal	Activation	−3890	AGTTAA	gga	AGTTCA	40, 81
AVPR1B distal	Activation	3648	GCTTCA	tcc	AGGTCA	40, 81

The most common DR3 vVDRE for activation is represented as a 5′- and 3′-hexamer separated by 3 nt (spacer). Known substitutions in either the 5′ or 3′ half-sites associated with transcriptional activation or repression are underscored. Substitutions in purines are commonly associated with activation and substitutions in pyrimidines are repressing. The activating or repressing VDREs for *TPH1*, *TPH2*, *OXT*, *OXTR*, *AVPR1A*, and *AVPR1B* are shown with base substitutions underscored (40, 80–81, 83, 157). [a]This substitution occurs in an existing purine and is most likely associated with activation.

activating VDRE sequences that are associated with transcriptional activation (Table 1). Thus, *TPH2* is likely to be transcriptionally activated by vitamin D hormone (Table 1 and refs. 80, 81, 83). In contrast, *TPH1* contains a distal repressing VDRE that is only associated with gene repression and is identical to that of rat parathyroid hormone-related peptide, which is downregulated by vitamin D hormone (80–82, 90, 91). *TPH1* also has a proximal VDRE with variations that have been observed in activating VDREs (Table 1). The repressing VDRE in *TPH1* likely indicates transcriptional repression despite also possessing an activating VDRE in the promoter region (80–82, 90, 91). Thus, *TPH2* and *TPH1* may be differentially regulated by vitamin D hormone through transcriptional activation of *TPH2* and repression of *TPH1* thereby causing the production of serotonin by these 2 enzyme isoforms to be controlled in opposite directions. Future studies testing the functional significance of the transcriptionally activating and repressing VDREs in *TPH2* and *TPH1* will shed light on precisely how vitamin D hormone regulates both tryptophan hydroxylase genes.

There are 6 lines of evidence supporting the differential regulation of *TPH1* and *TPH2* by vitamin D hormone on serotonin production. *1*) Examination of DNA-transcription factor interactions using the UCSC ENCODE browser (http://genome.ucsc.edu/encode) revealed that RXR has been found to be associated with *TPH2* by whole-genome chromatin immunoprecipitation combined with DNA sequencing data. Since the RXR heterodimerizes with the VDR in the presence of vitamin D hormone, this suggests that *TPH2* is transcriptionally activated by vitamin D. *2*) It is well known that vitamin D deficiency increases the rate of bone turnover (osteoclastogenesis), and this is partly mediated through elevated levels of parathyroid hormone (PTH), a negative transcriptional target of vitamin D (42, 81, 92). Strikingly, TPH1-mediated serotonin production also induces osteoclast formation and causes bone loss, whereas mice lacking TPH1 display decreased osteoclastogenesis and increased bone mass (93). Furthermore, pharmacological inhibition of TPH1 in mice promotes osteoblast formation and increases bone mass in an osteoporosis mouse model (94). Indeed, boys with autism have decreased bone mineral density compared with nonautistic boys, suggesting that TPH1-mediated serotonin production may be may be elevated in boys with autism (95, 96). These data suggest that vitamin D hormone may regulate bone mass by a novel mechanism through *TPH1* gene repression, thus decreasing the production of serotonin from the gut enterochromaffin cells and increasing osteoblast formation. *3*) Genes that are transcriptionally repressed by vitamin D hormone have a high basal mRNA expression in the absence of vitamin D; however, on vitamin D hormone binding, mRNA expression is downregulated (90). This is in agreement with the high mRNA expression levels of *TPH1* in human pineal gland, which is 150 times higher than *TPH2* in the brainstem (97). *4*) Serotonin in the blood, which is produced from TPH1, is lowest in summer months and highest in winter, whereas brain serotonin, which is generated from TPH2, is highest in summer months and lowest in winter months (98–100). These data are in agreement

with the seasonal variation in serum vitamin D concentrations that have been observed (101). Our proposal explains the seasonal variation of serotonin concentrations in brain as compared with peripheral tissues. *5*) There is an inverse relationship between serum vitamin D concentrations and melatonin, which is made from TPH-1-mediated serotonin in the pineal gland (102). It has been demonstrated that with increasing doses of vitamin D supplementation there is a dose-dependent decrease in melatonin production (102). This suggests that the inverse relationship between vitamin D concentrations and melatonin may be due to vitamin D-mediated transcriptional repression of *TPH1*. *6*) *TPH1* mRNA expression is lowest during the day and highest during the night, whereas *TPH2* is highest in the day and lowest in the night (103–105). Together, all of this evidence points to a novel mechanism by which vitamin D transcriptionally represses *TPH1* and activates *TPH2,* thereby inversely affecting serotonin production in peripheral tissues relative to production in the brain.

ROLE OF ESTROGEN IN RESCUING THE AUTISM PHENOTYPE

Males have almost a 5-fold higher autism incidence than females, and yet no underlying mechanism for the sex-related discrepancy has been established (2, 106). We propose that during early brain development, low *TPH2* expression due to vitamin D inadequacy would be rescued by high levels of estrogen, because estrogen has been shown to have significant effects on boosting *TPH2* expression (107, 108). Female rats, mice, and humans have higher concentrations of serotonin in the brain compared with males, and this can be observed as early as neonatal day 2 and persist throughout adulthood (109–114). Furthermore, these gender differences in brain serotonin levels do not appear to be a consequence of varying concentrations of tryptophan substrate as female rats have higher tryptophan hydroxylase activity (115).

The increased serotonin concentrations in the female brain could be explained in terms of the known role of estrogen in regulating serotonin receptors, transporters, and *TPH2* expression (107, 108, 116–118). Estrogen increases the expression of serotonin transporters and receptors in the dorsal raphe and caudate putamen of the brain (119, 120); it also increases by 9-fold the mRNA levels of *TPH2* in the dorsal raphe of the brain in primates and mice (107, 108, 116–118). Since there is a significant similarity between the VDRE and the estrogen response element (ERE) sequences, which is an inverted repeat of AGGTCA with a spacer of 3 nt, we suggest that estrogen up-regulates *TPH2* mRNA expression, possibly through the putative VDRE in *TPH2* (121, 122). Indeed, using the UCSC ENCODE browser, we found evidence that the estrogen receptor α physically associates with *TPH2*.

In humans, fetal and neonatal estrogen appears to be higher in females than males, and this could account for the corresponding gender differences in serotonin levels. The developing fetal brain is dependent on its own production of estrogen *de novo* from cholesterol or from the adrenal gland, as maternal estrogen mostly does not reach the fetal brain (123). While it is less clear what the exact sex differences are in fetal brain estrogen levels, in humans, the female fetus has a higher concentration of estradiol in the amniotic fluid (123, 124). In support of this observation, the absence of testosterone prevents masculinization of the brain, whereas the absence of estrogen has no comparable effect suggesting that masculinization is not due to the conversion of testosterone into estrogen (123, 125, 126). Testosterone can be converted into estrogen by aromatase, which is present at various levels in the different brain regions (127–129). However, the conversion of testosterone into estrogen can boost serotonin concentrations only in the regions of the brain that express high levels of aromatase: in fact, males have higher levels of estrogen in the developing neonatal hypothalamus, where aromatase expression is highest, whereas females have higher estrogen levels in the neonatal hippocampus and prefrontal cortex (127–129). Therefore, the substantial role of *TPH2*-generated serotonin in brain development, together with the role of estrogen in promoting *TPH2* expression, could plausibly explain why males are 4 to 5 times more likely to be afflicted with ASDs.

MATERNAL AUTOIMMUNITY, VITAMIN D, AND AUTISM

Maternal autoimmunity has been strongly associated with the development of autism during pregnancy, although no satisfactory explanation of this phenomenon has been put forward (130–132). Here we suggest a plausible mechanism for how low maternal vitamin D hormone may result in maternal autoimmunity and autism incidence. Since the developing embryo is immunologically foreign, regulation of the autoimmune response through acquired immunological tolerance is critical to ensure that the mother does not generate autoantibodies that attack the fetus, including the fetal brain. It is known that mothers of children with ASD are 4 times more likely to have autoantibodies against fetal brain proteins in their blood compared with mothers of nonautistic children (130–132). It has been demonstrated that maternal autoantibodies target the fetal brain during pregnancy, thus playing a critical role in the pathology of autism (130, 132, 133). However, no mechanism has been identified for this autoimmune dysregulation.

We propose that the dysregulation of maternal immunity can be explained by altered tryptophan metabolism as a consequence of low vitamin D hormone. Tryptophan plays an important role in regulating the autoimmune response during pregnancy through its conversion to kynurenine in the placenta (134, 135). There are essentially 3 competing fates for tryptophan:

use in protein synthesis, metabolic conversion by TPH to serotonin, and metabolic conversion by the enzyme indoleamine 2,3-dioxygenase (IDO) to kynurenine. Kynurenine in the placenta is required during pregnancy to prevent a general autoimmune response by generating regulatory T (T_{reg}) cells, which maintain tolerance to self-antigens and keep autoimmunity under control by mediating maternal tolerance to the fetal-derived placenta (refs. 134–140 and **Fig. 2**, top panel).

Dysregulation of tryptophan metabolism and thus of kynurenine could also result from low vitamin D hormone during pregnancy. The placenta expresses both TPH1 and IDO (28, 134). A normal concentration of TPH1-generated serotonin in the placenta is important for fetal brain development (28). However, low maternal vitamin D hormone levels may result in an aberrant increase in placental expression of *TPH1*. This proposal is supported by data from several studies demonstrating that mothers of children with autism also display abnormally high serotonin concentrations in peripheral white blood cells that express TPH1 (10–13). Since TPH1 protein has a 3-fold tighter tryptophan-binding affinity and a longer half-life than IDO, such increased *TPH1* expression would result in aberrant tryptophan catabolism (141–143). We suggest that elevated expression of *TPH1* as a consequence of low vitamin D hormone may cause TPH1 activity to act as a tryptophan trap, thus shunting tryptophan away from the kynurenine pathway and decreasing placental production of kynurenine and T_{reg} cells (Fig. 2, bottom panel). Indeed, vitamin D has been shown to increase T_{reg}-cell number *in vitro* and *in vivo*; however, a mechanism has remained unknown (144). Vitamin-D-mediated *TPH1* repression may provide such a mechanism. In summary, low maternal vitamin D during pregnancy

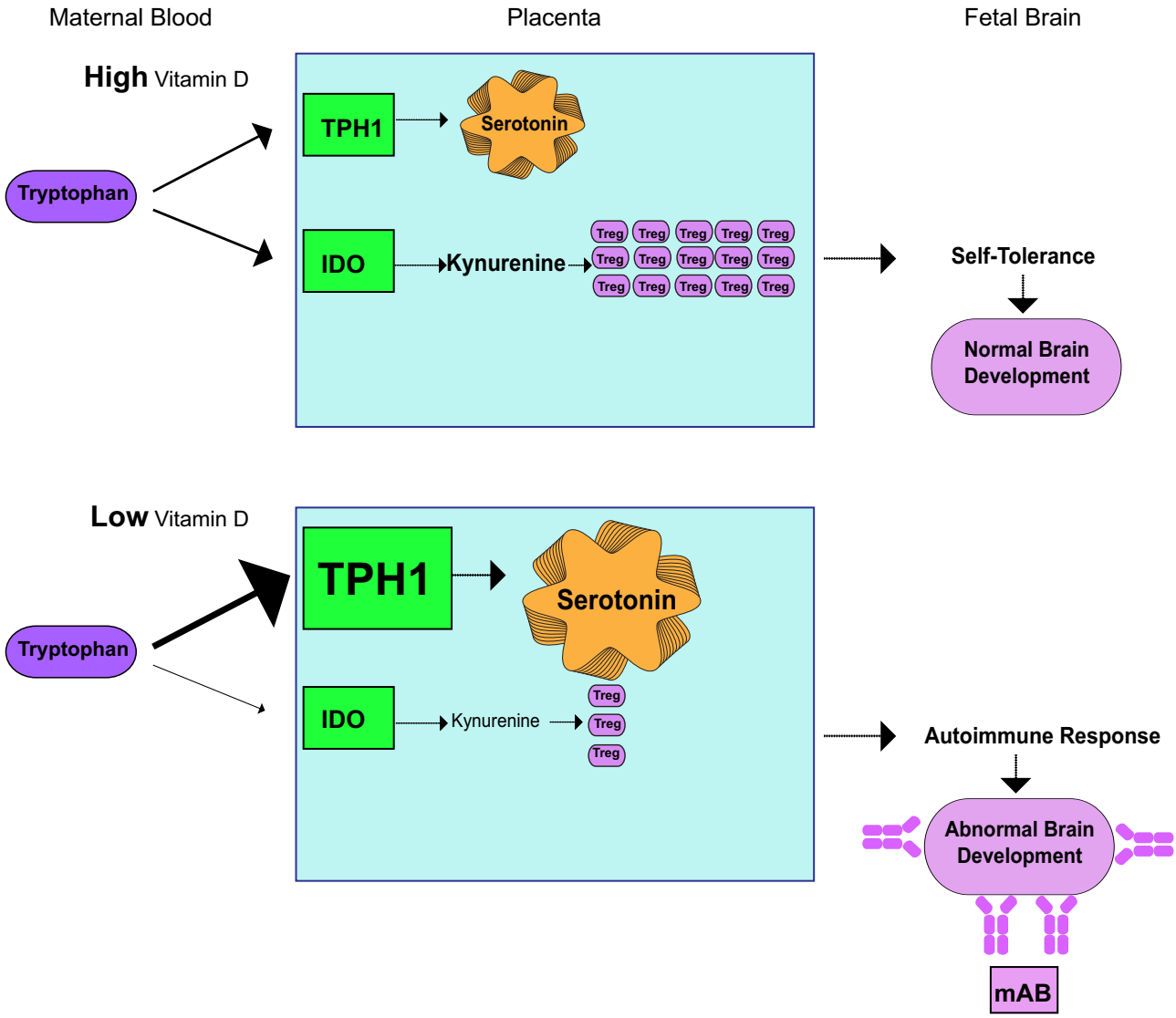

Figure 2. Model of the maternal contribution to autoimmune antibodies in the fetal brain. Top panel: vitamin D sufficiency (>30 ng/ml) during pregnancy allows normal tryptophan metabolism by TPH1 in the placenta, producing serotonin and kynurenines. Kynurenines generate T_{reg} cells, which allow self-tolerance and normal fetal brain development. Bottom panel: under vitamin D insufficiency (<30 ng/ml), TPH1 is overexpressed and shunts tryptophan away from IDO, thus blunting the production of T_{reg} cells and causing maternal autoantibodies (mAb) to attack the fetal brain tissue.

would cause an imbalance in tryptophan catabolism in the placenta resulting in too much serotonin and too little kynurenine, thus leading to an autoimmune response attacking the fetal brain, tipping the balance toward inflammation and autoimmunity.

OXYTOCIN, VASOPRESSIN, VITAMIN D, AND AUTISM

Oxytocin, a neuropeptide hormone, has been proposed to play a role in autism, particularly with respect to the social deficits associated with the disorder (145). Oxytocin markedly strengthens a wide variety of social behaviors including maternal care, pair bonding, social memory, social cooperation, social reward, social information processing, and others (146, 147). Notably, oxytocin is important for both aspects of socialization, including social comfort and social pain, and it works together with serotonin to reward social interactions, suggesting it may be important for reinforcing correct social behavior (148–150). Children with autism have been shown to have lower plasma concentrations of oxytocin as compared with nonautistic children (151, 152). In addition, variations in the oxytocin receptor have been associated with increased autism risk (153). Oxytocin administration in adults with ASD has been shown to decrease repetitive behaviors, enhance facial emotion recognition, increase eye-gaze, and promote emotional speech comprehension (152, 154–156). It has previously been shown that the genes encoding the oxytocin/neurophysin I prepropeptide (*OXT*) and the oxytocin receptor (*OXTR*) contain multiple putative VDREs (40). After examination of the VDRE sequences, we confirmed that *OXT* contains a proximal and 3 distal VDREs and *OXTR* has 1 distal VDRE (Table 1). Furthermore, these putative VDREs mostly appear to be consistent with transcriptional activation, suggesting that the vitamin D hormone would regulate both the production of the oxytocin hormone and the response to it (Table 1 and refs. 80, 157). The 4 different VDREs present in *OXT* likely modulate oxytocin production in different tissues. *OXTR* contains a putative VDRE that may be associated with activation (Table 1). Supporting evidence demonstrating that vitamin D regulates these oxytocin-related genes comes from data showing that the VDR colocalizes with oxytocin in hypothalamic neurons (158). Overall, these data suggest that vitamin D would modulate oxytocin synthesis as well as the response to the neuropeptide itself in different tissues, with important implications for benefiting social behaviors in ASD.

Vasopressin has also been proposed to play a role in autism, particularly because defects in the response to vasopressin have been linked to autism and these effects are specific to males (159). Vasopressin is another neuropeptide that regulates many different social and emotional behaviors including social recognition, social bonding, exploration, anxiety, and aggression (160). The social behavioral effects of vasopressin are mainly mediated through the arginine vasopressin receptor 1A (*AVPR1A*) and are more pronounced in males, which have a higher expression level of *AVPR1A* receptors (159–161). The *AVPR1A* gene has been identified as an autism susceptibility gene, and microsatellite variants have been linked to autism (162–164). Furthermore, it has been demonstrated that the common genetic variants of the *AVPR1A* gene linked to autism result in lower mRNA expression and are associated with hyperactivation of the amygdala, which is known to be connected with the diminished gaze fixation in individuals with autism (165–168). Putative VDREs have been identified in the genes encoding 2 receptors for the vasopressin peptide, *AVPR1A* and *AVPR1B* (40). Examination of the sequences of these 2 distal VDREs indicates that they are consistent with transcriptional activation (Table 1). Since the *AVPR1A* gene contains an activating VDRE, it is possible that vitamin D hormone may be important for normal expression of *AVPR1A* and, thus, the vasopressin receptor during brain development, which may be critical for normal social behavior particularly in males.

VITAMIN D, SEROTONIN, AND GASTROINTESTINAL ANOMALIES IN CHILDREN WITH ASD

Children with ASD commonly suffer from chronic gastrointestinal (GI) tract inflammation and digestive disorders (169–171). This phenotype may also be related to aberrant production of serotonin. While the majority of serotonin found in the gut is generated from TPH1 in the enterochromaffin cells, enteric neurons present in the gut express TPH2 and, thus, also produce a small amount of serotonin (26, 29). The serotonin produced from TPH2-expressing enteric neurons is required for gut motility, whereas the serotonin generated from TPH1-expressing gut enterochromaffin cells promotes inflammation (172). Excess serotonin in the gut is known to result in GI inflammation, possibly because TPH1-generated serotonin is required for T-cell activation and proliferation (27, 169). Furthermore, deletion of TPH1 protects the gut from inflammation in a colitis mouse model (173). We propose that this GI inflammation observed in individuals with autism may be a direct result of elevated serotonin in the GI tract due to increased *TPH1* expression as a consequence low vitamin D hormone levels. Therefore, we predict that raising vitamin D concentrations should help lower GI inflammation by decreasing serotonin concentrations in the enterochromaffin GI cells through transcriptional repression of *TPH1*. In total, vitamin D supplementation would increase *TPH2* and decrease *TPH1* expression because they contain VDREs consistent with transcriptional activation and repression, respectively. This would result in normalizing serotonin concentrations in the gut and concomitantly reducing GI inflammation and irritation while increasing gut motility.

DISCUSSION

We propose an underlying mechanism that reveals how the vitamin D hormone is a key regulator of brain serotonin synthesis through *TPH2,* which contains a VDRE consistent with activation. This mechanism explains how low vitamin D hormone levels result in aberrant serotonin synthesis, subsequently leading to abnormal brain development. It has been previously identified that brain tryptophan bioavailability is correlated with serotonin concentrations in the brain (25, 31). Vitamin D hormone levels may also be linked to serotonin concentrations in the brain. Low vitamin D hormone levels during fetal and neonatal development could result in poor *TPH2* expression and subsequently reduced serotonin concentrations in the developing brain. The important role of TPH2-mediated serotonin production in shaping brain structure and neural wiring during early neurodevelopment is well known (174). This mechanism suggests that adequate vitamin D hormone levels during pregnancy, as well as nutritional intake of tryptophan and vitamin D during early childhood, may have a critical influence on brain serotonin levels and, thus, on the structure and neural wiring of the brain.

The differential regulation of *TPH1* and *TPH2* by vitamin D hormone can explain some of the most prevalent phenotypes of ASD. Vitamin D-mediated differential regulation of *TPH1* and *TPH2* may also be an important clue in understanding the inverse relationship between serotonin concentrations in blood compared with the brain in children with autism. The significant positive effects of estrogen on increasing *TPH2* expression and serotonin in the female brain would explain why females have a lower susceptibility to developing ASD. Also, high *TPH1* expression due to low vitamin D hormone levels would explain the reduced bone density found in boys with autism, since TPH1-generated serotonin increases bone turnover (93, 95, 96). Furthermore, high *TPH1* expression would shift the equilibrium of tryptophan catabolism away from generating kynurenine and, subsequently T_{reg} cells, thereby activating an autoimmune attack toward the fetus during pregnancy.

In summary, we describe a mechanism by which vitamin D hormone activates *TPH2* and suppresses *TPH1* expression, thereby inversely controlling serotonin production in the brain relative to tissues outside the blood-brain barrier. Future studies directly testing vitamin D-mediated regulation of these 2 tryptophan hydroxylase genes will be important to understand precisely how this transcriptional regulation occurs and whether there are any other tissue-specific differences in regulation.

Implications for prevention of ASD

The vitamin D-dependent regulatory mechanisms of serotonin synthesis and their relationship to the underlying causes of ASD suggest that risk of ASD may be decreased by a practical and affordable solution: adequate vitamin D supplementation during pregnancy and early childhood. The vitamin D hormone levels of a developing embryo and neonate are completely dependent on those of the mother. It is known that 25(OH)D3 plasma concentrations in the fetus reflect those of the mother (175). In the United States, $25(OH)D_3$ concentrations are surprisingly low during pregnancy, despite some supplementation with prenatal vitamins (176, 177). One study of 400 pregnant females who were taking prenatal vitamins found that roughly 50% of mothers and their neonates had insufficient levels of vitamin D (25(OH)D3 <30 ng/ml), strongly suggesting that prenatal supplementation needs to be improved (177). The American College of Obstetricians and Gynecologists recommends that pregnant women use 1000–2000 IU of vitamin D, instead of the 400 IU currently present in prenatal vitamins (178). The National Institute of Medicine states that vitamin D doses up to 4000 IU/d are acceptable for pregnant and lactating women (179). It has been demonstrated that individuals deficient in vitamin D (<20 ng/ml) can achieve sufficient vitamin D concentrations (30–80 ng/ml) after 1 yr of supplementation with 4000 IU/d without any toxic side effects (180). It is also important to keep in mind that obesity lowers the lipophilic vitamin D bioavailability by 50% and that larger than usual doses may be required for obese individuals (181). Some foods have been fortified with vitamin D, including milk (100 IU/8 ounces) and orange juice (100 IU/8 ounces), but these foods do not contain adequate levels. Furthermore, dairy products are a suboptimal choice for fortification for the ~50 million Americans who are lactose intolerant, including 75% of African Americans (182).

Therapeutic intervention to treat some symptoms in individuals with autism

Understanding the mechanism by which vitamin D levels regulate serotonin synthesis in different tissues gives some insight into therapeutic treatment, with the goal of improving a wide-range of social behaviors. Low serum concentrations of $25(OH)D_3$ have been associated with autism severity (183). Individuals with ASD have a difficult time engaging in social interaction, understanding and processing facial expressions of others, and cooperating and working together (184, 185). Prosocial behavior, including assessment of emotional social cues, is largely associated with serotonin levels in the brain, which depend on blood levels of tryptophan (33, 186). Many individuals with ASD, as well as nonautistic individuals, are lacking appropriate levels of tryptophan (32, 187). It is known that depletion of tryptophan causes a rapid and temporary reduction in brain serotonin in normal individuals and has major effects on their social behavior (33, 186). On experimental tryptophan depletion, these otherwise healthy individuals become socially withdrawn, do not cooperate in social groups, and have difficulty process-

ing facial expressions of sadness and anger (33, 186). These data indicate that low levels of serotonin resulting from tryptophan depletion in the normal brain cause abnormal social behaviors, some of which are very similar to abnormal behaviors associated with autism. The reason that acute tryptophan depletion might not be expected to precipitate the full set of autistic characteristics is because these normal individuals have not built abnormal neural pathways during early brain development. In individuals with autism, further decreasing their brain serotonin by acute depletion of tryptophan exacerbates symptoms such as repetitive behaviors and facial recognition patterns revealing a continuing requirement for serotonin in modulating these behaviors (188, 189). Furthermore, tryptophan supplementation has been shown to reduce social anxiety, which could be relevant to individuals with ASD (190, 191). Together, these data provide strong and convincing evidence for a causal role of tryptophan-derived serotonin in regulating many social behaviors and support the proposal that supplemental interventions affecting the serotonin pathway may lead to improvements in a wide range of social behaviors in ASD.

Vitamin D and tryptophan supplementation may be a simple method of increasing brain serotonin without negative side effects. There are other common approaches to boosting concentrations of serotonin in the brain such as supplementation with 5-hydroxytryptophan (5-HTP), which crosses the blood-brain barrier (192). However, 5-HTP may be immediately converted into serotonin in the GI tract, which lowers the bioavailability of 5-HTP to be transported into the brain. Additionally, the conversion of 5-HTP into serotonin in the GI tract is known to cause inflammation, which has been associated with 5-HTP supplementation (169, 192, 193). Accordingly, vitamin D and tryptophan supplementation may be a better alternative to 5-HTP to boost brain serotonin. This is because, in addition to promoting serotonin synthesis in the brain, vitamin D would also transcriptionally suppress *TPH1*, which produces serotonin in the gut, thus increasing tryptophan bioavailability and reducing GI irritation, and would also provide the benefits of increased oxytocin and the other vitamin D hormone-controlled genes.

In recent years, serotonin reuptake inhibitors (SSRIs), which are thought to function by increasing the extracellular levels of serotonin, have been used to treat some autistic behaviors with both positive and negative results (194). However, the mechanism of action of SSRIs is still unclear, and it has been shown that SSRIs can cause the indirect activation of 5-$HT1_A$ and 5-$HT1_B$ autoreceptors, which has a negative effect on serotonin cell firing and release, and this may limit the ability of SSRIs to enhance serotonin transmission (195). For this reason, a more direct method of modulating the serotonergic system may be through increasing tryptophan and vitamin D hormone concentrations.

In summary, we propose that adequate levels of vitamin D hormone may be necessary for activation of *TPH2* and consequent elevation of serotonin levels in the brain. This vitamin D-mediated production of serotonin would be critical to produce serotonergic signals during neurodevelopment, thus shaping the developing brain, and throughout adulthood, where it plays a critical role in regulating a variety of brain functions including social behavior. In addition, adequate vitamin D hormone levels would suppress *TPH1* expression, which has important implications for lowering GI inflammation, increasing bone mineral density, and keeping autoimmunity at bay. Vitamin D could also regulate the synthesis and response to oxytocin, as well as the response to vasopressin, which could help improve social functioning in ASD, as well. In addition, ω-3 fatty acid supplementation from fish oil has been shown to improve some cognitive function and behaviors in individuals with autism (196–198). This may be due to the important role ω-3 fatty acids play during neurodevelopment, including serotonin production, neurogenesis, dendritic arborization, synaptogenesis, selective pruning, and myelination (199, 200). A few studies have found a correlation between ω-3 deficiency and autism (201–203). For these reasons, dietary intervention with vitamin D, tryptophan, and ω-3 fatty acids would boost brain serotonin concentrations and help prevent and possibly ameliorate some of the symptoms associated with ASD without side effects. In addition, vitamin B6, BH4, and iron are cofactors in the serotonin pathway and may also help modulate brain serotonin levels and facilitate moderate improvements in some autistic behaviors (204–208). Micronutrient nutrition is an important modulator not only of brain function but also of most physiological processes in the body (209–212). Notably, >900 genes contain VDREs, many of which are important for cognitive function, suggesting that vitamin D supplementation has additional benefits outside the scope of this article. FJ

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