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Research report

Prenatal vitamin D deficiency does not exacerbate behavioural impairments associated with prenatal ethanol exposure in juvenile male mice

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ABSTRACT

There is a high prevalence of vitamin D deficiency and exposure to low levels of ethanol in pregnant women. However, there are a paucity of studies that have addressed the impact of both vitamin D deficiency and ethanol exposure on the offspring's vulnerability to neurodevelopmental disorders later in life. The aim of this study was to examine whether the absence of vitamin D during gestation in mice would alter the effects of prenatal exposure to low dose ethanol on the behaviour and dopaminergic gene expression patterns of juvenile mice. Four-week old female C57BL/6J mice were placed on a prenatal vitamin D deficient (PVD) or standard diet for 6 weeks and mated at 10 weeks of age. Females were exposed to either 10%(v/v) ethanol or water between gestational days 0–8 and all were offered water thereafter. We found that blood ethanol concentration in the dams was not affected by maternal diet. Behavioural analyses of the offspring included ultrasonic vocalizations (USV) at postnatal day (P) 7, locomotion and social interaction at P21. The main findings were increased USV calling rate and impaired social interaction in males with prenatal ethanol exposure (PrEE). Gene expression analysis of transcripts involved in dopamine regulation revealed a main effect of ethanol exposure on *dopamine- and cyclic adenosine monophosphate- regulated neuronal phosphoprotein* (*Darpp-32*), a main effect of vitamin D diet on *Dopamine 2 Receptors* (*D2R*) and a main effect of Sex on *Tyrosine Hydroxylase* (*TH*) expression. The combination of PVD-PrEE did not exacerbate the alterations resulting from PVD or PrEE. Despite the limited evidence to support the interaction of PVD and PrEE during the postnatal period, males were more vulnerable than female offspring to the detrimental effects of PrEE. Therefore, based on these studies in mice we suggest that maintenance of optimal vitamin D levels and abstinence from ethanol during pregnancy would reduce risk of later disruption to brain function and behaviour in the offspring.

1. Introduction

Vitamin D deficiency is common in Australian adults, with recent population-based studies indicating that 35% of Australians over 25 years of age have suboptimal levels of vitamin D (< 50 nM) [1]. In addition, Australian women of childbearing age have reported ethanol consumption to be as high as 85%. Moreover, with over 50% of pregnancies unplanned [2], this may allow at least a four-week gap during which pregnancy might be unknown coincident with ethanol consumption, suggesting exposure to ethanol is a common exposure that may occur concurrently with maternal vitamin D deficiency. However, to our knowledge there are no studies that have measured the co-occurrence of vitamin D deficiency and ethanol exposure in clinical populations, and no direct causality between combined vitamin D deficiency and ethanol exposure, and neurodevelopmental disorders has been identified.

Autism spectrum disorder (ASD) is a neurodevelopmental syndrome, with a prevalence of approximately 1% of the population [3,4].

Abbreviations: 25(OH)D, 25-hydroxyvitamin D (calcidiol); *Aadc*, aromatic L-amino acid decarboxylase; ADHD, attention deficit hyperactivity disorder; ASD, Autism spectrum disorder; COMT, comethyl transferase; *D1R*, dopamine 1 receptor; *D2R*, dopamine 2 receptor; DA, dopamine; *Darpp-32*, dopamine- and cyclic adenosine monophosphate- regulated neuronal phosphoprotein; DOPAC, 3,4-dihydroxyphenylacetic acid; GABA, γ-aminobutyric acid; GD, gestational day; *Hprt1*, hypoxanthine guanine phosphoribosyl transferase; HVA, homovanillic acid; IU, international units; *Maoa*, monoamine oxidase A; Nurr1, nuclear receptor related-1 protein; P57kip2, cyclin-dependent kinase inhibitor 1C; PrEE, prenatal ethanol exposure; PVD, prenatal vitamin D; *TH*, tyrosine hydroxylase; USV, ultrasonic vocalizations; UVB, ultraviolet B; *Vmat2*, vesicular monoamine transporter 2; VTA, ventral tegmental area; WHO, World Health Organization

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ASD includes conditions such as autism, childhood disintegrative disorder and Asperger syndrome, and is diagnosed based on behavioural criteria [5–8]. There are no defined biological markers for ASD and the diagnostic manual of the World Health Organization (WHO) requires the presence of core elements in three specific categories for autism diagnosis: abnormal reciprocal social interaction, impaired communication and repetitive behaviours. These elements have been used as guidelines for the development of mouse models of autism, including behavioural tasks designed to maximize relevance to the types of deficits observed in autism patients, such as reciprocal social interaction, social approach, social preference, ultrasonic vocalizations (USV) and repetitive self-grooming [9].

Maternal vitamin D deficiency is a plausible risk factor for ASD [10–14], and vitamin D levels were significantly reduced in ASD children compared to healthy controls [13,15]. Vitamin D deficiency is associated with a number of maternal factors that are individually considered risk factors for ASD, including diabetes, preeclampsia, dysregulated steroidogenesis, depression and maternal infection [16,17]. Additionally, vitamin D supplementation during pregnancy has been shown to reduce the risk of pregnancy complications [18]. With respect to animal models, vitamin D-deficient rats had altered maternal retrieval behaviour during a maternal isolation test, but showed no changes in ultrasonic vocalizations [19]. Another study in mice reported no overall effect of prenatal vitamin D (PVD) deficiency on ultrasonic vocalizations, social interaction or repetitive grooming in the offspring from 4 inbred strains (C57BL/6J, BALB/c, BTBR or BTBRxC57BL/6J [20]). Overall, the relationship between vitamin D status and numerous maternal factors suggests that vitamin D deficiency may not be causal for ASD, but instead may modulate or exacerbate the effects of other ASD risk factors.

Prenatal ethanol exposure (PrEE) is also considered a risk factor for neurodevelopmental disorders. However, the literature is not clear on the relationship/causality between PrEE and ASD, although a number of studies have reported some of the effects of PrEE on rodent behaviours relevant to neurodevelopment, such as communication, and sociability. A study on PrEE rats showed reduced pup vocalizations and increased latency to call [21,22], which may then alter maternal care and have an impact on social behaviour. Previous studies using PrEE rats have found impairments in a number of sociability tasks, where PrEE males showed altered behaviours in the goal-box task, cagemate interaction and play-fight assessment [23]. Studies in rhesus monkeys have shown disrupted dopamine (DA)-dependent functions including orientation, motor maturity and state control neonatal scores in monkeys prenatally exposed to moderate ethanol during early gestation [24]. Additionally, moderate PrEE resulted in long-term increases in prefrontal DA 1 receptor (D1R) binding in male monkeys [25], and altered D2R levels in a time-dependent manner, where PrEE animals exposed during early gestation showed a reduction in D2R binding, while those exposed during mid to late gestation showed the opposite effect, reiterating the significance of timing of exposure in ethanol's long-term effects [24]. In rats, studies have shown that PrEE results in a reduction in dopaminergic neuron activity in the ventral tegmental area (VTA), correlated with hyperactivity and impulsivity [26]. Another study looking at the effects of PrEE on the interaction of the hypothalamic-pituitary-adrenal (HPA) and dopamine systems, which are known to have overlapping neurocircuits, found that PrEE alters both HPA and DA activity and regulation, resulting in increased HPA tone and an overall reduction in tonic DA activity in a sexually dimorphic pattern [27]. Therefore, PrEE dysregulates the expression of genes important for normal brain development, and dysregulation of dopaminergic signalling may be a plausible link between this exposure and risk of ASD.

Dopamine modulates processes and behaviours that are altered in individuals with ASD, including cognitive processes [28], motor functions [29] and emotional regulation [30]. DA receptors are defined by their effect on adenylate cyclase activity [31]- D1 class encompasses D1 and D5 receptors, these are coupled to stimulatory G-proteins that increase adenylate cyclase activity, while D2 class of receptors includes D2, D3 and D4 receptors, which are coupled to G-proteins that decrease adenylate cyclase activity [32]. Furthermore, the opposing downstream effects of D1 and D2 receptor activation are mediated by dopamine- and cyclic adenosine monophosphate- regulated neuronal phosphoprotein (*Darpp-32*), which is a critical modulator of DA signal transmission and controls a range of downstream physiological effects [32]. Studies in rats have consistently shown that PVD deficiency alters dopamine signalling. For example, adult female PVD rats are more sensitive to the locomotor enhancing effects of amphetamine, a DA-releasing agent [33]. Adult PVD-deficient rats were also more sensitive to the DA receptor antagonist haloperidol [34]. In neonatal forebrain tissue, PVD deficiency has been shown to reduce levels of expression of the dopamine-metabolizing enzyme catechol-O-methyltransferase (COMT). Additionally, PVD deficiency was shown to alter the ratio of major DA metabolites dihydroxyphenylacetic acid/homovanillic acid (DOPAC/HVA), suggesting there is abnormal dopamine turnover in the neonatal forebrain [33]. Furthermore, factors involved in early post-mitotic specification of dopaminergic neurons, Nuclear receptor related-1 protein (Nurr 1) and Cyclin-dependent kinase inhibitor 1C (p57Kip2), were reduced as a result of PVD deficiency in the mesencephalon [35]. Overall, there is strong evidence to suggest vitamin D plays an important role in the maturation of dopaminergic systems.

There are very few epidemiological studies reporting alcohol intake and vitamin D deficiency in pregnant women, despite knowing separately the proportion of the child-bearing female adult population that have low vitamin D levels [1], and those that report alcohol intake [2]. Multiple studies in humans have reported that one of the side effects from chronic ethanol exposure is vitamin D deficiency, as the toxic effects of ethanol impairs vitamin D/calcium homeostasis and results in decreased bone mineral density [36–38]. We might speculate that high risk behaviours of excessive sun exposure and alcohol intake coincide. However, in one of the first studies to measure 25(OH)D in alcohol exposed mothers [39], the authors concluded that women consuming alcohol have an increased risk of vitamin D deficiency in winter, which could have a negative impact on pregnancy-related outcomes. The overarching hypothesis for these experiments was that the absence of vitamin D during development would exacerbate the deleterious effects of ethanol exposure. To test this hypothesis we placed female C57BL/6J mice on a vitamin D deficient or standard diet for 6 weeks and mated them at 10 weeks of age. To assess mild-moderate exposure to ethanol we selected an appropriate model in which females were exposed to either 10%(v/v) ethanol or water between gestational days (GD) 0–8 and all were offered water thereafter. We used a mouse model of chronic 10% ethanol exposure during the first 8 days of gestation that produces measurable changes in the epigenotype and phenotype of adults as previously described [40]. It should be noted that the rate of alcohol elimination of mice is 5 times faster than the rate in humans [41], and using this model overt signs of intoxication are not observed [40], with an approximate human equivalent blood alcohol concentration of $< 0.05\%$. We first analysed whether maternal ethanol metabolism was altered in vitamin D deficient and control dams. We then characterized the behavioural phenotype of juvenile offspring exposed to PVD deficiency and/or PrEE with a focus on autism-related behaviours starting with basic communication patterns at early developmental stages (P7), followed by locomotion and sociability at a prepubescent age (P21). Finally, we assessed the whole brain expression patterns of a number of genes involved in the regulation of the dopaminergic system including, *D1R, D2R, Darpp-32, TH, Monoamine oxidase A (Maoa)*, Vesicular monoamine transporter (*Vmat2)* and aromatic L-amino acid decarboxylase (*Aadc)*.