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Neurodevelopmental Exposure to Pyrethroid Insecticides and Stress Affects Dopaminergic Pathways Relevant to Attention-Deficit Hyperactivity Disorder

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Doctor of Philosophy

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Abstract

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By Aimée I-Hsuan Maria Vester

Attention-Deficit Hyperactivity Disorder (ADHD) affects 7% of children and presents with inattention, hyperactivity, and impulsivity. Most children are treated with stimulants, but long-term use is associated with stunted growth, cognitive effects, and decreased quality of life. Understanding ADHD etiopathogenesis is thus imperative. Studies suggest altered dopamine signaling plays a major role in ADHD. Known dopaminergic genetic factors contribute to ADHD, but do not wholly explain its pathogenesis. This indicates a role for environmental risk factors.

Independently, pyrethroid insecticides and chronic stress are associated with dopaminergic dysfunction and ADHD. Pyrethroids are used residentially and agriculturally, and indoor pyrethroid use is elevated in poor housing conditions. This increases the vulnerability of children living in poorer housing, who are often of low socioeconomic status (SES), to pyrethroids and subsequent neurodevelopmental alterations. Children of low SES also experience higher levels of chronic stress and are at greater risk of developing ADHD. Chronic stress alters key dopaminergic components, and thus may also contribute to dopamine-related ADHD pathophysiology.

We hypothesized these two exposures together would lead to synergistic effects in the dopamine system and ADHD. To test this, we assessed dopaminergic consequences of combined exposure to deltamethrin and the major stress hormone, corticosterone, in a neurodevelopmental mouse model. In males exposed to deltamethrin, we observed decreased midbrain *Pitx3* RNA expression, decreased tyrosine hydroxylase in the frontal cortex, impaired dopamine uptake, and hyperactivity. We also observed hypermethylation at a CpG site of the *Nr3c1* promoter in males exposed to deltamethrin and corticosterone. Thus, we saw sex-specific dopaminergic alterations that contribute to our understanding of dopaminergic neurodevelopment and ADHD.

Next, we assessed the relationship between pyrethroids, chronic stress, and ADHD in the National Health and Nutrition Examination Survey (NHANES). We developed a pediatric model of allostatic load to better parameterize sociodemographic and biological stressors and demonstrated significant multiplicative interaction between urinary pyrethroid metabolites and allostatic load in the prevalence of ADHD. This research reflects exposure scenarios that target dopaminergic systems and could disproportionately affect low SES populations. Understanding these mechanisms can help inform initiatives to reduce the disease burden of ADHD.

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CHAPTER 1: INTRODUCTION

ATTENTION-DEFICIT HYPERACTIVITY DISORDER

Attention-Deficit Hyperactivity Disorder (ADHD) is one of the most common neurodevelopmental disorders of childhood, with a prevalence around 7% in the United States [1]. Characteristics include symptoms within three domains: inattention, hyperactivity, and impulsivity, that manifest by age 12 and can persist into adulthood. First-line treatment with methylphenidate, a stimulant, resolves symptoms of the three domains for the majority of children with ADHD, but treatment response is variable and use is associated with stunted growth [2], neural plasticity effects [3], and substance abuse [4]. Studies also indicate that children with ADHD report deficits in psychosocial well-being and family life [5]. Thus, ADHD continues to present a public health burden and further study of predisposing mechanisms and pathophysiology is necessary.

To date, no singular pathogenic mechanism of ADHD is known, and ADHD is likely to be multifactorial, involving genetic, epigenetic, and environmental factors. Several monoaminergic neurotransmitter circuits have been implicated in ADHD. Notably, genetic studies reveal variants in several genes related to the dopamine system are associated with ADHD, including dopaminergic receptors, enzymes, and transporters [6]. Variants in the dopamine receptor 5 gene (*DRD5*) modulate age of ADHD onset, while variants in the dopamine transporter (*DAT1*) gene predict severity of hyperactivity and impulsivity symptoms [7, 8]. Lower DNA methylation of *DRD4* is also associated with an increase in ADHD symptoms in children at age 6 [9]. Patient cohort studies associate altered DAT levels with ADHD as well [10-12]. Additionally, a *Dat1* knockout mouse displays hyperactivity, while a *Dat1* overexpressing mouse model shows impulsivity behaviors [13-17]. Finally, methylphenidate targets the dopamine and norepinephrine reuptake inhibitors and differential drug response is

associated with *Dat1* genotype [18]. Based on these genetic, mechanistic, and pharmacologic data, there is strong evidence for a role of dopaminergic signaling in ADHD pathogenesis.

THE DOPAMINE SYSTEM

The dopamine system has diverse roles, including coordination of motor function, motivation, reward, and reinforcement, as well as more peripheral functions in sympathetic ganglia. Dopamine is a catecholamine neurotransmitter primarily produced by dopaminergic cell bodies in the midbrain, specifically in the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) [19]. There are four major and four minor dopaminergic pathways that originate in the SNpc or VTA and project throughout the brain and are associated with different functions. Perhaps most studied in the context of neurotoxicant exposures is the nigrostriatal pathway, which projects from the SNpc to the striatum – which consists of the caudate nucleus and putamen [20]. Functions associated with the nigrostriatal pathway include motor function, reward-related cognition, and associative learning. Next, the mesolimbic pathway involves dopaminergic projections from the VTA that project to the nucleus accumbens and contributes to reward-related and aversion-related cognition. Third, the mesocortical pathway contains projections from the VTA to the frontal cortex and is associated with cognitive control of behavior. The mesolimbic and mesocortical pathways have both been implicated in ADHD via clinical and genetic studies [20, 21]. The fourth major pathway is the tuberoinfundibular pathway and contains projections from the arcuate nucleus of the hypothalamus to the pituitary gland and is involved in prolactin secretion. Additionally, there are four minor dopamine pathways that all start in the VTA and then project to the amygdala, hippocampus, cingulate cortex, and olfactory bulb [19].

DOPAMINE SYNTHESIS AND METABOLISM

To synthesize dopamine, dietary tyrosine is actively transported to dopaminergic neurons in the brain and hydroxylated by tyrosine hydroxylase (TH) [20, 22]. This yields dihydroxyphenylalanine (L-dopa), which is then converted to dopamine by the enzyme L-Aromatic amino acid decarboxylase (AADC) [22]. Notably, TH is the rate-limiting enzyme in dopamine synthesis and can be regulated by various stimuli. Since TH requires Fe^{2+}, molecular oxygen, and tetrahydrobiopterin to function, stimuli can either target the TH enzyme or one of its co-factors [23]. Acute activation of TH occurs through phosphorylation of the enzyme by various protein kinases. The phosphorylation is thought to induce a conformational change that increases TH's affinity for the tetrahydrobiopterin co-factor [24]. Longer-term TH regulation can occur through transcriptional regulation of *TH* itself, and previous studies show that caffeine, nicotine, morphine, and other environmental exposures are all capable of increasing *TH* gene expression [20]. Dopamine is catabolized by the monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) enzymes. Oxidation of dopamine produces 3-Methoxytyramine (3-MT), 3,4-Dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) metabolites [20, 25, 26] (Figure 1.1A).

DOPAMINE TRANSPORT AND SIGNALING

Once dopamine is synthesized in the neuronal cytosol, it is sequestered into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2) [27, 28]. VMAT2 acts as a H^+-ATPase antiporter, using the synaptic vesicle's electrochemical gradient to transport dopamine molecules into the synaptic vesicle [29-31]. Sequestration of dopamine is critical for neuronal

survival since free-floating dopamine is toxic to the cell. Not surprisingly, impairments of VMAT2 function, studied by our lab and others, are associated with increased susceptibility to disease outcomes such as Parkinson's disease [29, 32-34]. After dopamine is packaged into synaptic vesicles, the dopaminergic neuron can then release dopamine in response to a stimulus. Synaptic vesicles are exocytosed at the pre-synaptic terminal [35]. Dopamine diffuses across the synaptic cleft and binds post-synaptic dopamine receptors to enact downstream signaling.

Clearance of dopamine from the synapse occurs through three main mechanisms. The primary mechanism is through re-uptake from the terminal by the dopamine transporter (DAT) [36]. DAT is an integral membrane protein that resides in the plasma membrane of synaptic terminals in the striatum. DAT acts as a symporter, binding 2 Na+ ions and 1 Cl- ion along with dopamine substrate [37]. It utilizes energy from the ion gradient set up in the neuron by the Na+/K+ ATPase to transport dopamine [36]. DAT is targeted pharmacologically and by drugs of abuse, including methylphenidate – a first line drug for ADHD treatment. Alterations in DAT function are associated with neuropsychiatric disease and addiction [38-40]. Dopamine that remains in the synaptic cleft either binds postsynaptic dopamine receptors or diffuses away from the synaptic cleft (Figure 1.1).

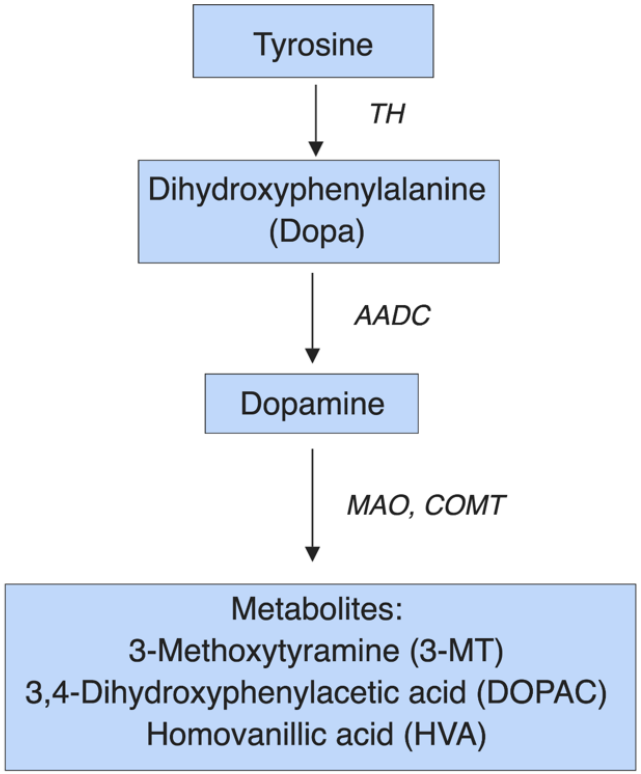
Figure 1.1: Dopamine metabolism and signaling overview.

A) Dopamine synthesis and metabolism. Tyrosine is hydroxylated by TH to form L-dopa, which is then converted to dopamine by AADC. Dopamine is metabolized by catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) [20].

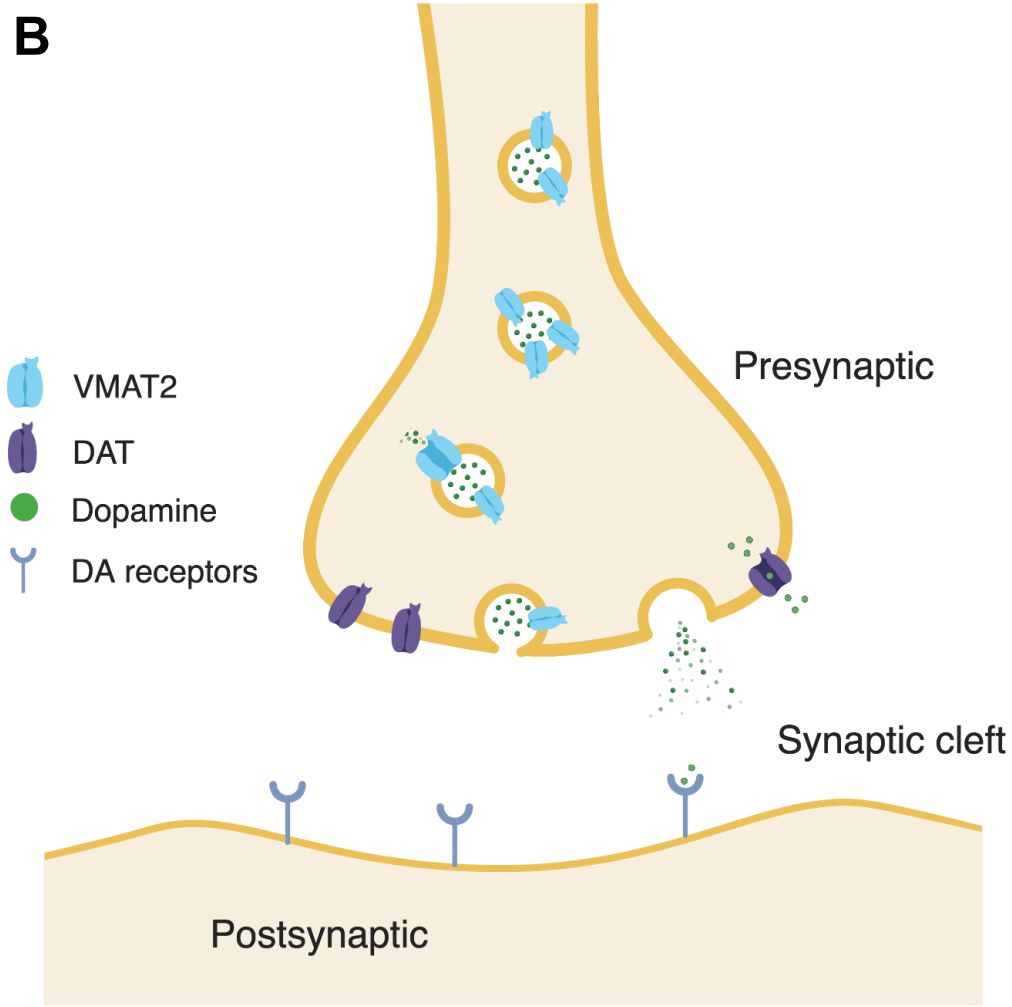
B) Dopamine signaling. Dopamine is sequestered out of the cytosol and into synaptic vesicles by VMAT2. A stimulus leads to release of dopamine through vesicular exocytosis at the presynaptic terminal. Dopamine diffuses across the synaptic cleft to bind postsynaptic dopaminergic receptors. DAT is the primary mechanism of dopamine clearance and is responsible for re-uptake of dopamine into the presynaptic terminal.

Tyrosine hydroxylase (TH), L-Aromatic amino acid decarboxylase (AADC), monoamine oxidase (MAO), catechol-O-methyltransferase (COMT), Dopamine transporter (DAT), vesicular monoamine transporter 2 (VMAT2), dopamine (DA)

A


Tyrosine
TH
Dihydroxyphenylalanine (Dopa)
AADC
Dopamine
MAO, COMT
Metabolites:
3-Methoxytyramine (3-MT)
3,4-Dihydroxyphenylacetic acid (DOPAC)
Homovanillic acid (HVA)

B


VMAT2
DAT
Dopamine
DA receptors
Presynaptic
Synaptic cleft
Postsynaptic

DEVELOPMENT OF THE DOPAMINE SYSTEM

General neurodevelopment

Neurodevelopment is comprised of a highly orchestrated sequence of biological processes, including proliferation, migration, differentiation, and synaptogenesis, which must occur for the normal development and function of the nervous system. *In utero*, the human nervous system arises from the neuroepithelium of the neural tube around day 18. The ventricular zone contains progenitors that form all the cell types that comprise the central nervous system (CNS). These cell types include glia, which have various supportive roles such as the protection and nourishment of neurons. Neurons are the major signaling cells of the nervous system and they generally consist of a cell body with a single, outgoing axon and numerous projecting dendrites. Once neurons differentiate in the ventricular zone they then migrate to specific regions throughout the brain, using radial glia as their guides to this localization. In humans, the majority of neuronal migration is completed around month five *in utero* and neuronal axons further refine the formation of neural circuits by extending their processes along highly regulated pathways to facilitate communication with proximal or distal neurons. To guide this process, growth cones form at the end of developing axons with long, thin filopodia that respond to chemotactic signals serving to either attract or repel the advancing growth cone [41, 42].

Crucial to the establishment of neural circuits are neuronal synapses, which allow for communication between neurons. When an axonal growth cone comes into contact with another neuron to form a synapse, the filopodia retract and intercellular signals from surrounding glia, the extracellular matrix of the neuron, and other nearby neurons initiate development of the synapse. Once synapses are formed, their connectivity and function are continuously refined

during adulthood, leading to a unique ability to form new neural networks throughout life. Thus, while the nervous system structure is largely in place early in life, refinement of neural circuits can continue well into adolescence and early adulthood [43, 44].

The development of the dopamine system

Given the complexity of dopaminergic neurodevelopment, environmental perturbations that occur during this process could act at diverse targets. Dopaminergic neurodevelopment can be broadly divided into four main steps: induction, specification, terminal differentiation, and maturation. First, induction prepares the midbrain territory for the development of dopamine neurons. Transcriptional regulation during this phase helps define boundaries for development of dopaminergic brain regions. *Otx2* expression helps define the forebrain and midbrain area along the anterior-posterior axis of the neural tube [45, 46]. The mid-hindbrain boundary is the area distinguished by *Otx2* and *Gbx2* transcription factor expression [47]. In addition, canonical *Wnt*-mediated repression of *Shh* expressed along the dorsal-ventral axis of the neural tube helps start neurogenesis from the midbrain floor plate [48].

Secondly, specification of dopaminergic precursors occurs and can be subdivided into transcription factors that specify a general neuronal phenotype and transcription factors that induce transcription of dopaminergic enzymes and transporters necessary for proper dopaminergic function. Rodent knockout models illustrate that general neuron properties are conferred by *Foxa1* and *Foxa2* [49] as well as *Lmx1a* and *Msx1* together [50]. *Otx2* expression is also induced by *Foxa1/2*, *Lmx1a*, and *Msx1* at this stage [45, 46], as well as *Ngn2* expression [51]. Additional gene studies show that dopaminergic properties are specified by three additional transcription factors. *Nurr1* directly activates the *Th* promoter and is also necessary for *Vmat2*

and *Dat1* expression [52]. *Lmx1b* plays a role in fate specification, though this role is likely different than the role of *Nurr1* in regulation of the expression of dopaminergic components [53]. Finally, *Pitx3* expression is highly specific for meso-diencephalic dopaminergic populations and also regulates *Th*, *Vmat2*, and *Dat1* expression [54]. Dopaminergic precursors arise from the medial and lateral zones of the floor plate after the induction phase. In mice, meso-diencephalic dopamine neurons arise around embryonic day (E) 10.5-14.5. Most of the SNpc meso-diencephalic dopamine neurons arise around E10.5, whereas those in the VTA arise around E11.5 [48, 55]. The aforementioned specification phase transcription factors help determine the dopaminergic fate of these meso-diencephalic dopamine precursors.

Third, the terminal differentiation phase allows for maturation of precursors to an irreversibly dopaminergic state as dopaminergic neurons continue to migrate to their target locations. While many of the transcription factors important for the specification phase are also expressed at this time, additional transcription factors help promote further differentiation. *Wnt5* helps promote differentiation of *Nurr1*-positive meso-diencephalic dopaminergic precursors into *Th*-positive dopaminergic neurons [56]. Transcription factors in the Transforming growth factors (*Tgf*) superfamily are also important in terminal differentiation – *Tgf* -/- mice show 50% reduction in SNpc *Th*-positive neurons, though not in the VTA [57]. Post-mitotic meso-diencephalic dopamine neurons migrate radially from the floor plate. Then, the SNpc dopaminergic neurons migrate tangentially while the VTA dopaminergic neurons continue to migrate radially [55]. The striatum is a prominent target of dopaminergic neurons, for instance, and dopaminergic innervation initiates around E13.5 in mice [55].

Fourth, the maturation phase is important for survival of dopaminergic neurons into adulthood. Several transcription factors expressed in earlier phases are also important here. For

instance, *Foxa2* plays a role in controlling survival of SNpc dopaminergic neurons in adulthood [58]. *Nurr1* also plays a role in survival of SNpc dopaminergic neurons, as evidenced by the motor deficits, reduced number of dopamine neurons, and reduced *Dat1* expression displayed by *Nurr1* +/- mice at 15 months of age [59]. The engrailed transcription factors (*En1*, *En2*) also promote dopamine neuron survival in a gene dosage-dependent manner [60]. Thus, development and maintenance of a fully-functioning dopamine system entails a multitude of factors expressed at specific timepoints. Given the wide range of neuropsychiatric disorders associated with neurodevelopmental and synaptic disruptions, we developed a new neurodevelopmental exposure paradigm to better understand the role of environmental perturbances in the development of the dopamine system and in the pathogenesis of ADHD.

ENVIRONMENTAL EXPOSURES IN ADHD

While extensive genetic studies suggest that ADHD is familial, this does not preclude exogenous risk factors that further modulate risk of the disease [61]. Studies of various perinatal and postnatal environmental exposures associate maternal smoking, early childhood adversity, and exposure to environmental toxicants with ADHD [61]. The neurodevelopmental process is especially vulnerable to environmental disruption because environmental contaminants may cross the placental barrier, pass through the immature blood-brain barrier, or transfer into breast milk [62-65]. ADHD prevalence is higher in low-income children [66], suggesting that environmental risk factors affect certain socioeconomic groups differentially and contribute to this health disparity. Two environmental risk factors linked to ADHD and low socioeconomic status are pyrethroids and chronic psychosocial stress [67-71].

PYRETHROID INSECTICIDES

Pyrethroids are insecticides derived from synthetic analogues of pyrethrins, compounds derived from *Chrysanthemum cinerariaefolium*, that bind the α subunit of voltage-gated Na^+ channels and hold them open to cause neuronal hyperexcitability [72]. Both type I and II pyrethroids bind voltage-gated Na^+ channels, but Type II pyrethroids such as deltamethrin can also inhibit $GABA_A$ receptors and voltage-gated chloride channels to potentiate neuronal excitability [73]. Off-target effects on the Ca^{2+} ATPase and voltage-gated Ca^{2+} channels have also been reported [74]. While actions at these channels do not appear to explain the acute intoxication syndromes, actions at these channels could explain some neurodevelopmental effects [75].

Pyrethroids account for approximately 20% of global insecticide use and exposure has been characterized worldwide. Pyrethroid insecticide contact occurs via agricultural application, household use, and dietary ingestion [72, 76-81]. In the past few years, several epidemiologic studies have started to uncover associations between early pyrethroid insecticide exposure and adverse neurodevelopmental outcomes. A few studies of prenatal pyrethroid exposure measured urinary pyrethroid metabolites in mothers during the third trimester and offspring neurodevelopmental outcomes. Prenatal pyrethroid exposure is associated with lower mental development scores at 3 months [82], poorer social-emotional development at 1 year [83], poorer language development and lower Mental Development Index scores at 2 years [83, 84], as well as executive function and behavioral deficits from 4-9 years [85]. Additional studies have also examined urinary pyrethroid metabolites in children and assessed neurodevelopment. In the United States, children in the CDC's National Health and Nutrition Assessment Study (NHANES) with pyrethroid metabolites detectable in urine have an increased risk of ADHD

[67], and this association appears to be higher in boys and with hyperactive-impulsive symptoms [86]. Proximity to agricultural pyrethroid insecticide use was also negatively associated with IQ at 7 years of age [87]. Furthermore, there is an association between urinary pyrethroid metabolites and parent-reported behavioral problems [88]. In France, the PELAGIE study observed an association between urinary pyrethroid metabolites, internalizing and externalizing behavioral difficulties, higher odds for abnormal borderline social behavior, verbal comprehension, and working memory in children at 6 years of age [89, 90]. A similar cohort of children ages 6-9 years in Costa Rica observed poorer attention in children with detectable urinary pyrethroid metabolite levels [91]. Furthermore, prenatal exposure to piperonyl butoxide, an additive that potentiates the action of pyrethroids, is negatively associated with cognitive and motor development at 36 months of age in a cohort of black, Dominican mothers [92]. These results suggest there may be cognitive and motor sequelae associated with pyrethroid exposure. Conversely, a Thai case-control study saw no statistically significant differences in urinary pyrethroid metabolites and neurobehavioral testing at six-month intervals [93]. Others saw a negative association between *cis* isomers of pyrethroid metabolites and neurodevelopment, but a positive association between *trans* isomers and neurodevelopmental scores indicating there may be differential toxicities of various forms of pyrethroids as well [82]. As such, there is not yet an overwhelmingly definitive body of work indicating pyrethroids are causally linked to neurodevelopmental or neurodegenerative outcomes in humans. Taken together, however, the newer use of pyrethroids may be contributing to the lack of definitive epidemiologic data and further study of longitudinal, neurodevelopmental exposure effects are still of considerable interest.

NEURONAL EFFECTS OF PYRETHROID EXPOSURE

Alterations in neurotransmitter systems and synaptic structure in response to pyrethroid exposures have been illustrated in various animal studies. To begin, pyrethroid exposure alters the balance of inhibitory and excitatory signaling. This could potentially affect neuronal signaling and synaptic activity, and the related processes of synaptic plasticity and refinement. In the hippocampus, for example, the balance of inhibitory GABAergic and excitatory glutamatergic signaling affects learning and memory. Indeed, exposure to allethrin, deltamethrin, and cyhalothrin in a rat exposure model leads to compound-specific alterations to GABA and glutamate release in the hippocampus, suggesting that these pyrethroids could play a role in cognitive outcomes observed in additional studies [94]. Cypermethrin exposure in the neonatal period led to alterations in the glutamate receptor 1 (GluR1) in the hippocampus and tau in the frontal cortex. GluR1 and tau both play important roles in synaptic plasticity and scaffolding, respectively. These structural changes likely contribute to the observed behavioral outcomes: decreased rearing, locomotion, and habituation in the exposed mice [95].

Beyond effects on inhibitory and excitatory signaling, pyrethroid insecticides also affect monoaminergic systems. For instance, administration of allethrin, cyhalothrin and deltamethrin into the rat striatum causes changes in extracellular serotonin release [96, 97]. The striatum is highly innervated with serotonergic neurons, and earlier studies have shown that a reduction of serotonin leads to increased aggressive and locomotor behaviors, while a rise in serotonin leads to the opposite behavioral effects [98]. These serotonergic changes could contribute to the motor and cognitive behaviors observed in epidemiologic studies and animal behavior models of pyrethroid exposure.

DOPAMINERGIC EFFECTS OF PYRETHROIDS IN ADULTHOOD

In addition to serotonergic effects, dopaminergic effects of pyrethroid administration have also been studied. Prior studies of pyrethroid insecticides illustrate that the dopamine system is actually uniquely vulnerable to pyrethroid exposure. Treatment of adult mice with deltamethrin and permethrin increased maximal dopamine uptake [99, 100]. This increase in dopamine uptake is mediated by increases in DAT expression [99-103].

Pyrethroid insecticides impact other components of dopaminergic signaling besides dopamine uptake as well. Treatment of adult male rats with cypermethrin led to decreased levels of VMAT2 [104], decreased dopamine content in the substantia nigra [105], and reduced locomotor activity [105]. Changes in mRNA expression [106] and protein expression [107] in key components of microglial activation and mitochondrial function pathways are associated with the neurodegenerative effects of cypermethrin in the substantia nigra. Alterations in dopamine efflux were also observed in experiments that directly injected allethrin, deltamethrin, and cyhalothrin into the rat striatum [108]. In conclusion, adult animal models of pyrethroid exposure exhibit many different alterations in the dopamine system.

DOPAMINERGIC EFFECTS OF PYRETHROIDS IN DEVELOPMENT

While adult pyrethroid exposure models predominate the literature, some studies indicate that exposure to pyrethroids during development can also influence the dopamine system. Recently, we discovered that exposure to deltamethrin during critical periods of neurodevelopment results in similar alterations in the dopamine system. Male mice exposed to deltamethrin from gestation through weaning exhibit increased DAT and dopamine receptor 1 (DRD1) expression, causing hyperactivity and impulsivity, as well as deficient memory and

attention behaviors that reflect clinical symptoms of ADHD. Furthermore, the hyperactive and inattentive behaviors were attenuated by administration of methylphenidate, which is a first-line ADHD treatment [67]. Additionally, developmental deltamethrin exposure in male mice led to increased mRNA expression of sodium channels and brain-derived neurotrophic factor (BDNF) in the highly-dopaminergic striatum, but not the frontal cortex [109]. Lastly, in a zebrafish model of developmental deltamethrin exposure, mRNA expression of D1DR and D2DR decreased and TH protein expression increased. Similar to the hyperactivity observed in the mouse model, zebrafish developmentally exposed to deltamethrin displayed increased swim activity. This increase in activity was attenuated by administration of methylphenidate [110]. Thus, pyrethroid exposure during development has important consequences for proper dopamine function as well.

SOCIOECONOMIC STATUS AND PSYCHOSOCIAL STRESS

Another environmental risk associated with ADHD is socioeconomic status [69, 111]. Socioeconomic status accounts for an individual's or caretaker's income, but also education, occupation, and other associated characteristics at an individual, family, and community level [112]. At the individual level, low socioeconomic status can be associated with financial, housing, and food insecurities, as well as psychological and behavioral influences [112, 113]. Therefore, since so many different factors are associated with poverty and can contribute to the experiences of underserved individuals, these factors are often studied under the broad definition of "low socioeconomic status". A large body of work has linked lower socioeconomic status to morbidity and mortality, as well as risk of developmental alterations in children and later chronic disease in adults [112-122]. Life experiences of low socioeconomic status individuals are thought to contribute to increased stress. Biological responses to activated stress pathways then

contribute to the augmented risk of disease [112, 113]. Thus, while a physiologic stress response may provide acute benefit for responding to a stressful event, cumulative increased stress can lead to over-taxed physiologic systems and altered development in children [123, 124]. A child's socioeconomic status, and the associated stress load, is therefore another important environmental exposure when studying the biological implications of altered development in the context of ADHD.

THE HPA AXIS AND STRESS RESPONSE

Environmental stressors activate the hypothalamic-pituitary adrenal (HPA) axis so that the body can respond to stress. Broadly, in normal HPA axis function an acute stressor will cause signaling from the paraventricular nucleus (PVN) in the hypothalamus via corticotrophin-releasing hormone (CRH), then release of adrenocorticotrophic hormone (ACTH) from the pituitary gland, and finally release of glucocorticoids from the adrenal glands [125] (Figure 1.2). To regulate the stress response, glucocorticoids inhibit the amygdala and pituitary gland via a negative feedback loop, leading to a reduction in cortisol production [126, 127].

The HPA axis' response to stress can be subdivided into a rising and falling phase [128]. In the rising phase, excitatory signaling to the PVN initiates the stress response. Several excitatory inputs can provide an initiation signal, depending on the characteristics of the stressor. First, noradrenergic neurons in the brainstem receive information from the vagus nerve, sympathetic nervous system, and local inflammatory signals. This population of neurons is primarily responsible for alerting the body to physiologic perturbations such as inflammation, pain, or hypovolemia [129, 130]. Secondly, neuropeptides also signal to the PVN in response to cardiovascular challenges, fluid or electrolyte abnormalities, or metabolic imbalances [129].

Thirdly, glutamatergic and serotonergic innervations provide additional excitatory signaling, as well as inflammatory cytokines that are thought to promote prostaglandin release that activates the PVN [131]. In addition to these inputs from peripheral signals, central limbic brain regions can also activate the PVN, mostly by disinhibiting tonic GABA signaling that keeps HPA activation at bay [128].

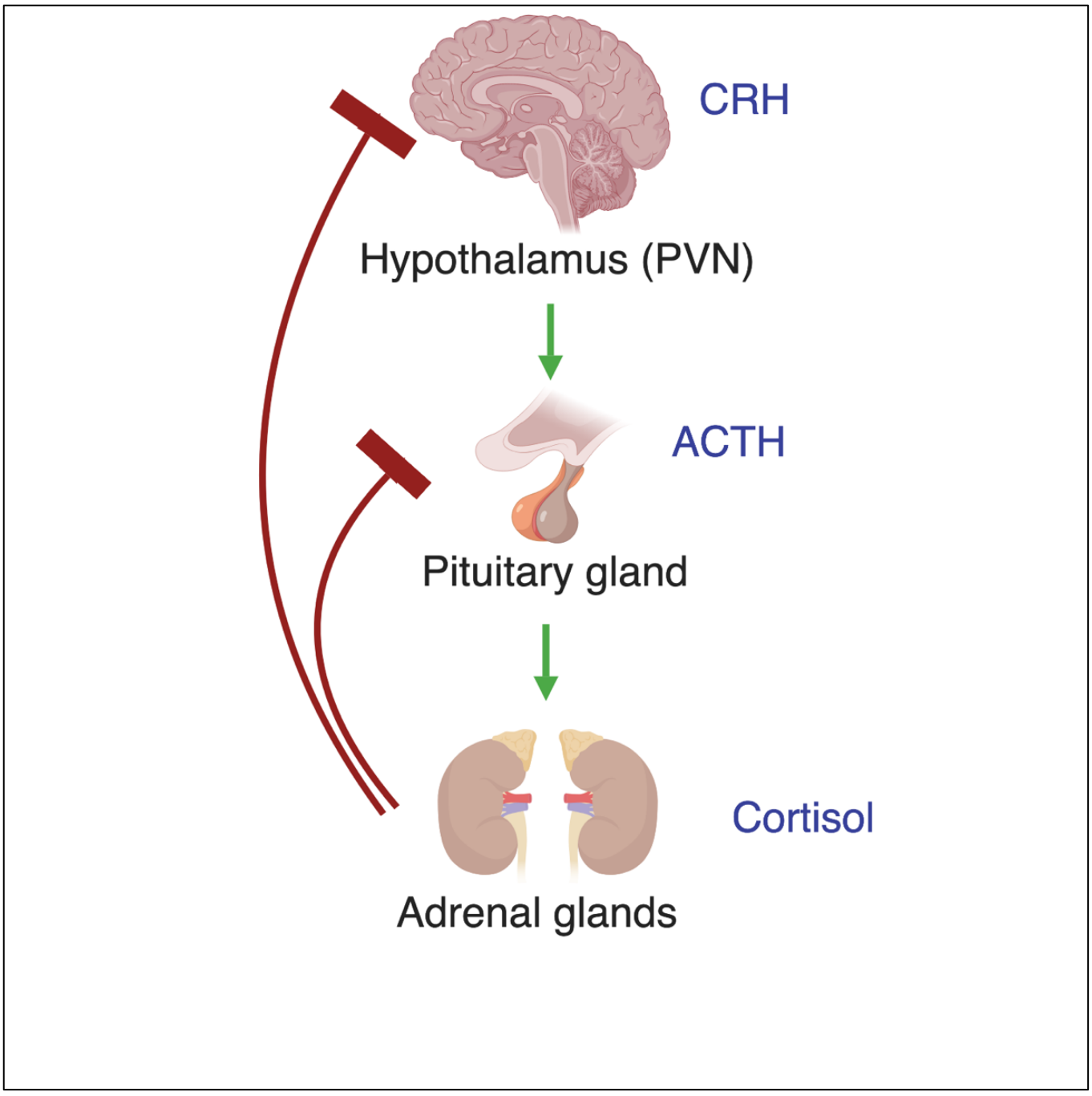
The subsequent falling phase is crucial for keeping the body's stress response advantageous, instead of harmful. ACTH release is rapidly stopped and glucocorticoids then return to their baseline levels more gradually [128]. The rapid response is mostly mediated by endocannabinoids that are released when glucocorticoids bind receptors in the PVN. The endocannabinoids inhibit excitatory glutamate release to CRH neurons of the PVN [132, 133]. Additionally, GABAergic inhibition from the hypothalamus and the subparaventricular nucleus right around the PVN control the HPA axis response [[134]].

Under chronic duress the HPA axis can become maladaptive. The degree of HPA axis dysfunction is dependent on the intensity, timing, and type of stressor but there are similarities in different rodent models of chronic stress [128]. To assess the effects of chronic stress on the HPA axis, one can think of the accumulation of repeated or persistent stressors that activate the stress response over and over [128]. Thus, the adrenal gland is repeatedly exposed to ACTH and often becomes more sensitive to ACTH and increases in size. The amount of glucocorticoids released basally often increases, though this can vary with circadian rhythm. The amount of negative feedback that glucocorticoids can provide centrally can also decrease [128]. All contribute to a physiologic stress response that becomes maladaptive over time.

Figure 1.2: Hypothalamic-pituitary-adrenal (HPA) axis.

An acute stressor signals the paraventricular nucleus (PVN) in the hypothalamus to release corticotrophin-releasing hormone (CRH). The pituitary gland then releases adrenocorticotrophic hormone (ACTH) and then the adrenal glands release of glucocorticoids [125]. To regulate the stress response, glucocorticoids inhibit the amygdala and pituitary gland via a negative feedback, leading to a reduction in cortisol production [126, 127].

Paraventricular nucleus (PVN), corticotrophin-releasing hormone (CRH), adrenocorticotrophic hormone (ACTH)


CRH
Hypothalamus (PVN)
ACTH
Pituitary gland
Cortisol
Adrenal glands

STRESS AND THE DOPAMINE SYSTEM

Various animal studies illustrate how glucocorticoids interact with the dopamine system [71, 135, 136]. Interestingly, glucocorticoid receptors – which potentiate the signaling of the stress hormone cortisol – are also present in the SNpc and VTA and co-localize with TH [137]. This suggests that glucocorticoid receptors are expressed on dopamine neurons and that stress may impact development and function of the dopamine system. Perhaps, some of the association between socioeconomic status and risk of ADHD could be explained by the increased exposure to cortisol. Providing support to this hypothesis are two studies measuring children's hair cortisol levels that show an inverse relationship between socioeconomic status and cortisol [116, 138].

A few animal studies have illustrated how corticosterone, the cortisol analog in rodents, interacts with the dopamine system. Acute stressors such as tail pinch induce dopamine efflux in the medial prefrontal cortex, and this is regulated by glucocorticoid action on dopamine neurons in the VTA [135]. More chronic stress exposures are also associated with dopaminergic alterations. Rodent pups separated from their mothers, a model of early life stress, exhibit hyperactivity and decreased attention that improves with methylphenidate [71]. Additionally, rats subjected to a visible burrow system, a chronic stress paradigm, exhibit decreased DAT and increased dopamine receptor 2 (DRD2) expression in the striatum [136]. Moreover, male offspring of stressed pregnant dams exhibit decreased DAT and TH protein expression in the SNpc and VTA, as well as increased dopamine turnover as measured by ratios of dopamine to dopamine metabolites 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) [143]. These results indicate that chronic stress influences dopamine signaling and neurologic alterations that may underlie ADHD.

In addition to experiencing greater amounts of psychosocial stress, low socioeconomic status groups are more likely to be exposed to environmental contaminants [139]. One such environmental contaminant is pyrethroid insecticides. High levels of pyrethroids occur in low-income, urban populations, likely due to poor housing conditions and increased need for pest control [140-142]. Certain populations may therefore be exposed to high amounts of both cortisol and pyrethroid insecticides that could modulate risk of dopaminergic dysfunction and development of ADHD.

EPIGENETICS AND ENVIRONMENTAL EXPOSURE

One mechanism by which environmental exposures could modulate the dopamine system and affect the risk of developing ADHD is through epigenetic regulation. Broadly, epigenetic changes can be defined as heritable alterations in gene expression not caused by changes in the DNA sequence [143, 144]. Epigenetic changes include DNA methylation, histone modification, and microRNA regulation. One of the most widely studied types of epigenetic modification is DNA methylation, which can repress or activate gene expression long-term [145-147]. In mammals, DNA methylation typically occurs at CpG islands, which occur when a cytosine residue is followed by a guanine residue [147, 148]. Work from our group and others shows DNA methylation plays an important role in proper neurodevelopment [149, 150]. For example, intrauterine growth-restricted mice display decreased methylation at a cyclin-dependent kinase promoter region and increased ADHD-like impulsiveness [149]. Our group showed that male infants with decreased DNA methylation of the leptin promoter were most likely to belong to a neurobehavioral profile with increased lethargy and hypotonicity [151]. In addition, methylation

of the glucocorticoid receptor, *NR3C1*, is associated with infant quality of movement and infant attention score [152].

Moreover, exposure to environmental toxicants, such as pesticides [153-162] and early life stress [163-171], are associated with epigenetic changes in humans and animal models. Low-dose exposure to organochlorine pesticides was associated with global hypomethylation [154]. Additionally, early life stressors such early childhood malnutrition, maternal depression, and distress during pregnancy were also associated with DNA methylation changes [163-171]. Recently, Frances Champagne and colleagues explored DNA methylation of *BDNF* as a potential biomarker of early-life adversity [166], suggesting that epigenetic mechanisms could provide valuable information for measuring early life stress and other environmental mediators in individuals' health outcomes.

Lastly, studies suggest environmentally-mediated epigenetic changes during early life contribute to risk and development of ADHD and influence the dopamine system [149]. For instance, DNA methylation of dopaminergic gene regions may correlate with clinical symptoms of ADHD [9, 172]. Furthermore, a mouse model used maternal separation to induce early-life stress and observed decreased histone acetylation of the DRD1 promoter in the hippocampus [173]. Another mouse model utilized maternal separation and social isolation to induce early-life stress and observed hypermethylation, decreased mRNA expression, and decreased protein expression of DRD1 in the VTA of stressed female offspring [174]. Thus, epigenetic regulation is responsive to environmental perturbation and plays an important role in neurodevelopment, including the dopamine system and ADHD pathogenesis.

SUMMARY

The etiology and pathogenesis of ADHD are still unclear, but ADHD continues to present a substantial public health problem. The existing data on ADHD support the scientific premise that alterations in dopaminergic signaling due to pyrethroid insecticide exposure influence ADHD pathogenesis and are important for studying ADHD risk, and that stress during neurodevelopment also alters the dopamine system and could further potentiate the risk of ADHD. We hypothesized that combined pyrethroid insecticide and stress hormone exposure would increase protein expression and function of key components of the dopamine system – thereby potentially increasing the risk of developing ADHD – and that altered DNA methylation may play a role in sustaining this prenatal effect throughout life. The proposed work addressed this hypothesis via three aims. The first aim focuses on neurobehavioral, neuropathologic, and functional responses in the dopamine system after a combined neurodevelopmental exposure to deltamethrin and CORT. The second aim studies molecular and epigenetic underpinnings of responses to neurodevelopmental deltamethrin and CORT responses in the dopamine and glucocorticoid systems. The third aim sought to explore the human relevance of this research question and utilized the CDC's NHANES (National Health and Nutrition Survey) data to determine whether pyrethroid insecticide and stress exposure interact to increase the risk of developing ADHD.

This research will contribute to overall understanding of ADHD risk and development. More specifically, these studies will yield insight into the behavioral and functional sequelae and molecular mechanisms that affect the dopamine circuit after developmental exposure to pyrethroids and the major stress hormone cortisol. Elucidating components of dopaminergic pathways involved in ADHD will inform development of more effective therapeutic

interventions and biomarkers for disease screening. Additionally, this research addresses an exposure scenario that is more likely to affect disadvantaged populations and provides both a novel mouse model exposure paradigm and a novel epidemiologic tool for studying the effects of chronic early life stress in children. This work will add to the development of public health interventions and regulatory initiatives to reduce the disparate environmental exposure burden that certain children face.

CHAPTER 2: A Neurodevelopmental Model of Combined Pyrethroid and Chronic Stress Exposure: Deltamethrin Causes Dopaminergic Alterations Independent of Corticosterone

ABSTRACT

Attention-Deficit Hyperactivity Disorder (ADHD) is one of the most common neurodevelopmental disorders of childhood and previous studies indicate the dopamine system plays a major role in ADHD pathogenesis. Two environmental exposures independently associated with dopaminergic dysfunction and ADHD risk include exposure to deltamethrin, a pyrethroid insecticide, and chronic stress. We hypothesized that combined neurodevelopmental exposure to both deltamethrin and corticosterone (CORT), the major stress hormone in rodents, would result in additive changes within the dopamine system. To study this, we developed a novel dual exposure paradigm and exposed pregnant C57BL/6 dams to 3 mg/kg deltamethrin through gestation and weaning, and their offspring to 25 μg/mL CORT dissolved in the drinking water through adulthood. Midbrain RNA expression as well as striatal and cortical protein expression of key dopaminergic components were investigated, in addition to ADHD-like behavioral tasks and electrochemical dopamine dynamics via fast-scan cyclic voltammetry. Given the well-described sexual dimorphism of ADHD, males and females were assessed separately. Males exposed to deltamethrin have significantly decreased midbrain *Pitx3* expression, decreased cortical tyrosine hydroxylase (TH) expression, increased activity in the Y maze, and increased dopamine uptake rate in the dorsal striatum. These effects do not occur in males exposed to CORT only, or in males exposed to both deltamethrin and CORT, suggesting that CORT may attenuate these effects. Additionally, deltamethrin and CORT exposed females do not display these dopaminergic features, which indicates these changes are sex-specific. Our results show dopaminergic changes from the RNA through the functional level. Moreover, these data illustrate the importance of testing multiple environmental exposures together to better understand how combined exposures that occur in certain vulnerable populations could affect

similar neurodevelopmental systems, as well as the importance of studying sex differences of these alterations.

INTRODUCTION

Attention-Deficit Hyperactivity Disorder (ADHD) is one of the most common neurodevelopmental disorders of childhood [1]. Characteristics include symptoms within three domains: inattention, hyperactivity, and impulsivity, that manifest by age 12 and can persist into adulthood. To date, no singular pathogenic mechanism of ADHD is known, and ADHD is likely to be multifactorial, involving genetic, epigenetic, and environmental components. Several monoaminergic neurotransmitter circuits have been implicated in ADHD. Notably, genetic studies reveal variants in several genes related to the dopamine system are associated with ADHD, including dopaminergic receptors, enzymes, and transporters [6]. Variants in the dopamine receptor 5 gene (*DRD5*) modulate age of ADHD onset, while variants in the dopamine transporter (*DAT1*) gene predict severity of hyperactivity and impulsivity symptoms [7, 8]. Lower DNA methylation of *DRD4* is also associated with an increase in ADHD symptoms in children at age 6 [9]. Cohort studies associate altered DAT levels with ADHD as well [10-12]. Additionally, a *Dat1* knockout mouse displays hyperactivity, while a *Dat1* overexpressing mouse model shows impulsivity behaviors [13-17]. Finally, methylphenidate targets the dopamine and norepinephrine reuptake inhibitors and differential drug response is associated with *Dat1* genotype [18]. Based on these genetic, mechanistic, and pharmacologic data, there is strong evidence for a role of dopaminergic signaling in ADHD pathogenesis.

Dopamine is a catecholamine neurotransmitter synthesized by tyrosine hydroxylase (TH) and metabolized by catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO)

enzymes, and subsequently transported into dopaminergic vesicles by the vesicular monoamine transporter 2 (VMAT2). Upon stimulation, vesicles release dopamine across the synapse. Dopamine interacts with postsynaptic dopamine receptors (DRD1-5) and is then recycled via DAT or metabolized after neurotransmission. Dopamine is primarily produced by dopaminergic cell bodies in the midbrain, specifically in the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) [19]. The SNpc sends projections to the dorsal striatum, where dopaminergic signaling plays a key role in learning and motor function [20]. The VTA sends projections to the nucleus accumbens, olfactory bulb, amygdala, hippocampus, prefrontal cortex, and cingulate cortex. In these areas, dopamine modulates motivation, reward-related behavior, attention, and memory [20]. Many of these dopaminergic networks are implicated in ADHD [21].

While extensive genetic studies suggest that ADHD is familial, this does not preclude exogenous risk factors that further modulate risk of the disease [61]. Studies of various perinatal and postnatal environmental exposures including maternal smoking, early childhood adversity, and exposure to environmental toxicants have been linked with ADHD [61]. The neurodevelopmental process is especially vulnerable to environmental disruption because environmental contaminants may cross the placental barrier, pass through the immature blood-brain barrier, or transfer into breast milk where additional exposure can occur [62-65]. ADHD prevalence is higher in low-income children [66], suggesting environmental risk factors affect certain socioeconomic groups differentially and contribute to this health disparity. Two environmental risk factors linked to ADHD and low socioeconomic status are exposure to pyrethroid insecticides and chronic psychosocial stress [67-71].

Pyrethroid insecticides are synthetic analogues of pyrethrins, compounds derived from *Chrysanthemum cinerariaefolium*, that bind the α subunit of voltage-gated Na^+ channels and hold them open to cause neuronal hyperexcitability [72]. Type II pyrethroids, such as deltamethrin, can also inhibit $GABA_A$ receptors and voltage-gated chloride channels to potentiate neuronal excitability [73]. Although pyrethroids are known to target neuronal channels present in most neurons, work from our group and others has shown that the dopamine system is uniquely vulnerable to pyrethroid exposure. Treatment of adult mice with pyrethroids increases maximal dopamine uptake and increases striatal DAT levels [99, 100]. This increase in dopamine uptake is mediated by DAT [101], and alterations in dopamine release were replicated in experiments directly injecting pyrethroids into the rat striatum [108]. More recently, we discovered that exposure to deltamethrin during critical periods of neurodevelopment results in similar alterations in the dopamine system. Specifically, we observed increased DAT and dopamine receptor 1 (DRD1) expression that was associated with hyperactivity and impulsivity, as well as deficient memory and attention behaviors that reflect clinical symptoms of ADHD [67]. In addition to animal studies, elevated levels of pyrethroids are associated with ADHD in humans. Notably, children in the National Health and Nutrition Examination Survey (NHANES) that have detectable pyrethroid metabolites in urine have an increased risk of ADHD [67]. This association appears to be higher in boys, and in children with hyperactive-impulsive symptoms [86]. Together, these results indicate pyrethroids alter dopamine activity, and pyrethroid exposure is an important ADHD risk factor to evaluate.

Another environmental risk associated with ADHD is socioeconomic status [69, 111]. At least part of this risk has been attributed to the higher levels of psychosocial stress experienced by individuals in these communities due to factors within their homes and communities [112,

121, 175]. This psychosocial stress activates the hypothalamic-pituitary adrenal axis to release the major stress hormone, cortisol in humans and corticosterone in rodents [125]. Studies measuring children's hair cortisol levels show an inverse relationship between socioeconomic status and cortisol [116, 138], suggesting effects of disproportionate psychosocial stress start early in life. In rodent models, corticosterone has been shown to interact with the dopamine system: acute stressors such as tail pinch induce dopamine release in the medial prefrontal cortex, and this is regulated by glucocorticoid action on dopamine neurons in the VTA [135]. Rodent pups separated from their mothers, a model of early life stress, exhibit hyperactivity and decreased attention that improves with methylphenidate [71]. Additionally, rats subjected to a visible burrow system, a chronic stress paradigm, exhibit decreased DAT and DRD2 expression in the striatum [136]. These results indicate that chronic stress influences dopamine signaling and neurologic alterations that may underlie ADHD. Interestingly, glucocorticoid receptors, which potentiate the signaling of the stress hormone cortisol, co-localize with dopamine neurons in the SNpc and VTA [137], suggesting that stress may impact development and function of the dopamine system. In addition to experiencing greater amounts of psychosocial stress, low socioeconomic status groups are more likely to be exposed to environmental contaminants [139], including pyrethroid insecticides. Elevated levels of pyrethroids have been observed in low-income, urban populations, likely due to poor housing conditions and increased need for pest control [140-142]. Certain populations may therefore be exposed to high amounts of both cortisol and pyrethroid insecticides that could modulate risk of dopaminergic dysfunction and development of ADHD.

We hypothesized that combined pyrethroid insecticide and stress hormone exposure would lead to additive changes in RNA expression, protein expression, and function of key

components of the dopamine system, thereby potentially increasing the risk of developing ADHD in certain populations. While previous studies have investigated the effects of singular environmental exposures, we developed a neurodevelopmental mouse model of combined exposure to deltamethrin, a pyrethroid insecticide, and corticosterone (CORT), the major stress hormone in rodents, to test this hypothesis. We illustrate that there are effects of deltamethrin on dopaminergic gene expression, protein expression, behavior, and release kinetics, and examine these impacts separately in males and females, considering the well-described sexual dimorphism of ADHD.

METHODS

Animals

Eight-week-old C57BL/6NCrl wild-type mice (Charles River Labs) received food and water *ad libitum* and were maintained on a 12:12 dark/light cycle. Females were dosed with 3 mg/kg deltamethrin every three days for two weeks prior to breeding in triplicate. Deltamethrin dosing continued during breeding and gestation. One male and one female mouse per litter were utilized for each experiment to reduce confounding due to litter effects. Throughout, an independent litter represents one sample (n = 1). All experiments were approved by the Institutional Animal Care and Use Committee at Emory University and were conducted in accordance with the National Institutes of Health Guide of Care and Use of Laboratory Animals.

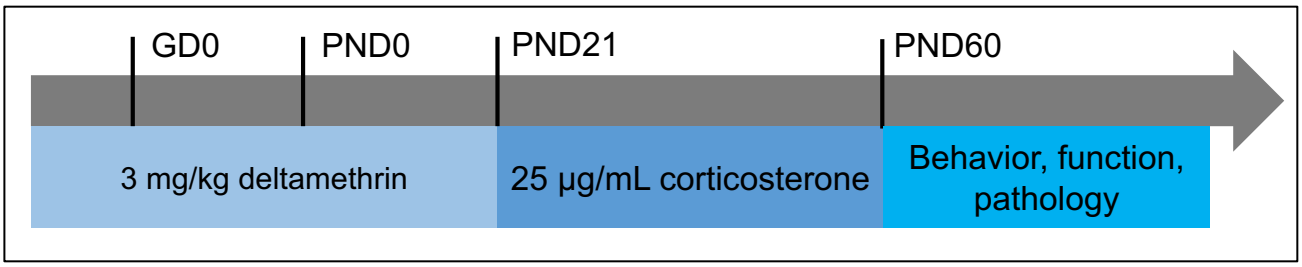
Exposure paradigm

We utilized a deltamethrin dose of 3 mg/kg based on our previous study showing dopaminergic effects upon neurodevelopmental exposure to 3 mg/kg deltamethrin in male

offspring. Additionally, this dose models a realistic exposure in the human population [76, 176-183] and is lower than the developmental no observable adverse effect limit (NOAEL). Adult C57BL/6J females were exposed to deltamethrin or vehicle every 3 days during gestation, lactation, and weaning at postnatal day (PND) 21. Deltamethrin was administered via corn oil dissolved in peanut butter to minimize trauma to pregnant mice. Corticosterone (CORT) was dissolved in the drinking water to minimize handling stress and reduce variation in CORT levels seen in behavioral chronic stress paradigms [184, 185]. CORT doses were prepared as previously described [186]. Offspring were continuously exposed to CORT or drinking water vehicle from adolescence through adulthood (PND21-60) (Figure 2.1). We chose our CORT exposure paradigm because we wanted to model a more persistent state of chronic stress than oft-used behavioral chronic mild stress paradigms [186]. Behavioral chronic mild stress paradigms involve randomization of stressful conditions such as damp bedding, a reversed dark/light cycle, and cage tilting; they are often highly variable in administration and outcomes [185]. Water bottles were weighed daily to assess intake and mice were weighed at weaning and at least once a week to assess proper weight gain. At PND60, offspring either underwent behavioral testing or were sacrificed for fast-scan cyclic voltammetry. All remaining offspring were sacrificed for molecular and pathologic studies.

Figure 2.1: Exposure paradigm timeline

Adult C57BL/6J females were exposed to deltamethrin or vehicle every 3 days during gestation, lactation, and weaning at postnatal day (PND) 21. Deltamethrin was administered via corn oil dissolved in peanut butter to minimize trauma to pregnant mice. Corticosterone (CORT) was dissolved in the drinking water to minimize handling stress and reduce variation in CORT levels seen in behavioral chronic stress paradigms [184, 185]


GD0
PND0
PND21
PND60
3 mg/kg deltamethrin
25 µg/mL corticosterone
Behavior, function, pathology

Behavioral analyses

One male and one female offspring from each litter were tested at 8-10 weeks of age. Mice were habituated to the testing room for at least one hour before testing. Behavioral tests were conducted during the same time window for each test by the same set of researchers to reduce confounding due to circadian and olfactory cues. For all animals, the order of behavioral testing was 1) locomotor activity, 2) Y maze, and 3) marble burying.

Locomotor activity

To assess locomotor activity, mice were placed in sound-attenuated chambers equipped with photo beams (Med Associates, St. Albans, VT, USA). Mice were habituated for 30 minutes and then observed for an additional 90 minutes. Number of ambulations, measured by the number of beam breaks, was quantified in 5-minute intervals after the habituation period.

Y Maze

Mice moved freely in the three-arm maze for eight minutes total and were recorded with a digital video camera. Noldus Ethovision software (Wageningen, The Netherlands) tracked alternation behavior and total distance traveled in the maze. One arm entry was scored whenever a mouse entered all four paws into a maze arm. An error consisted of an entry into a maze arm that was the same as one of the previous two arm entries. Percent error was calculated by dividing the number of errors by the total entries – 1.

Marble burying

Twenty marbles were placed in a 4 x 5 pattern on top of 2 inches of fresh bedding in a clean cage. Mice were placed at the lower right corner of the cage and allowed to explore freely for 30 minutes. After 30 minutes of exploration, mice were removed, and two independent observers scored the total number of marbles buried at least 50%. Any discrepancies greater than 2 marbles were discussed and scores were averaged for each animal.

Serum corticosterone ELISA

Following decapitation, trunk blood was collected from mice between 8-10 weeks of age and placed into 1.5 mL centrifuge tubes and allowed to clot at room temperature for one hour. Samples were centrifuged at 10,000 rpm for 10 minutes at 4°C. Serum was carefully pipetted off, placed into a clean 1.5 mL centrifuge tube, and stored at -80°C until ELISA analysis. ELISAs were performed per manufacturer guidelines (Enzo Life Sciences, Farmingdale, NY) and samples were run in triplicate and averaged for each animal.

mRNA expression analysis

Mice underwent rapid decapitation at 8-10 weeks of age and the midbrain was isolated and immediately flash frozen. Total RNA and DNA were extracted with a Qiagen Allprep DNA/RNA Mini Kit. DNA was saved and RNA was extracted per manufacturer recommendations (Qiagen, Germantown, MD, USA). 10 ng total RNA was converted to cDNA using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) and the PCR cycle conditions recommended by the manufacturer: 94ºC – 2:00; 35 cycles: 94ºC – 0:15, 55ºC – 0:30, 68ºC – 1:00; 4ºC – hold. Taqman gene expression assays were utilized for the

genes of interest and are listed in Supplemental Table 2.1. Each assay plate contained a non-template control, positive control derived from pooled adult mouse brain tissue, and a beta actin internal standard. $^{\Delta\Delta}$Ct values were calculated for each animal at every gene tested, and results are expressed relative to gene expression of the vehicle/vehicle control group.

Immunoblotting

Striatal and cortical samples were dissected upon rapid decapitation of animals at 8-10 weeks of age and immunoblotting was performed as previously described [32]. Striata were homogenized, subjected to PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in 7.5% nonfat dry milk in Tris-buffered saline solution to reduce nonspecific binding and incubated overnight in primary antibody. We utilized horseradish peroxidase secondary antibodies (Jackson ImmunoResearch) and enhanced chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). Luminescence and subsequent densitometric analysis were performed using the ImageLab software (Bio-Rad), and all expression values were normalized to β-actin. Samples across multiple gels were randomized on exposure group and sex to avoid batch effects. Primary antibodies were utilized at the following dilutions; all are monoclonal unless otherwise noted: rat anti-DAT (1:1000, Millipore), polyclonal rabbit anti-VMAT2 serum (1:5,000, Covance Custom Immunology Services), rabbit anti-TH (1:1,000, Millipore), mouse anti-COMT (1:1,000, Novus Biologicals) and mouse anti-β-actin (1:5,000, Sigma). The corresponding secondary HRP-linked antibodies were used (1:7,500, Jackson ImmunoResearch). The rabbit polyclonal VMAT2 antibody was raised against the C-terminal region of mouse VMAT2 as previously described by our group [187].

Fast-scan cyclic voltammetry

Voltammetry was performed as previously described [30, 188]. Briefly, mice underwent rapid decapitation at 8-10 weeks of age and the right hemisphere was isolated. The right hemisphere was immersed in oxygenated sucrose aCSF solution (193 mM sucrose, 11 mM D-glucose, 1.2 mM $CaCl_2 2H_2O$, 4.5 mM KCl, 25 mM $NaHCO_3$, 20.5 mM NaCl, 1.2 mM NaH_2PO_4, 2.6 mM $MgCl_2$) for 30 seconds prior to sectioning at 300 μm using a Leica vibratome. Slices were incubated in oxygenated HEPES aCSF solution (19.7 mM HEPES, 11 mM D-glucose, 2.4 mM $CaCl_2*2H_2O$, 25 mM $NaHCO_3$, 126.4 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4 monobasic, 2.6 mM $MgCl_2$) for at least 30 minutes prior to testing. Five recordings were taken from five different sites along a single unilateral dorsal striatal slice for each animal. There was a 5-minute interval between each 2.31 V stimulation. TarHeel CV (University of North Carolina) software and a custom potentiostat (UEI, UNC Electronics Shop) were utilized for application of waveform, stimulus, and current monitoring. To detect dopamine, a waveform of a -0.4 V holding potential versus an Ag/Ag Cl (In Vivo Metric) reference electrode was used, with an applied voltage ramp from -0.4V to 1.0V and back at a rate of 600V/s at 60 Hz. The maximal release was averaged for each striatal slice and carbon-fiber recording microelectrodes were calibrated with dopamine standards. Dopamine uptake was parameterized via tau, a time constant representing the amount of time necessary to return to 2/3 of baseline current after stimulation. Tau is derived from an exponential curve fitted to the dopamine current trace via a least squares constrained exponential fit algorithm that has previously been described and determined to be most accurate for measuring dopamine uptake compared to other kinetic parameters measured via fast-scan cyclic voltammetry [189]. Demon voltammetry software

[189] was used to calculate kinetic constants describing release and uptake of dopamine, via nonlinear logistic regression.

Statistical analyses

Statistical analyses were conducted in GraphPad Prism 8 unless otherwise indicated. Differences between exposure groups were evaluated via one-way ANOVA, unless otherwise indicated, and a significance level of $\alpha = 0.05$ was used throughout.

RESULTS

CORT exposure decreases serum CORT independent of deltamethrin exposure.

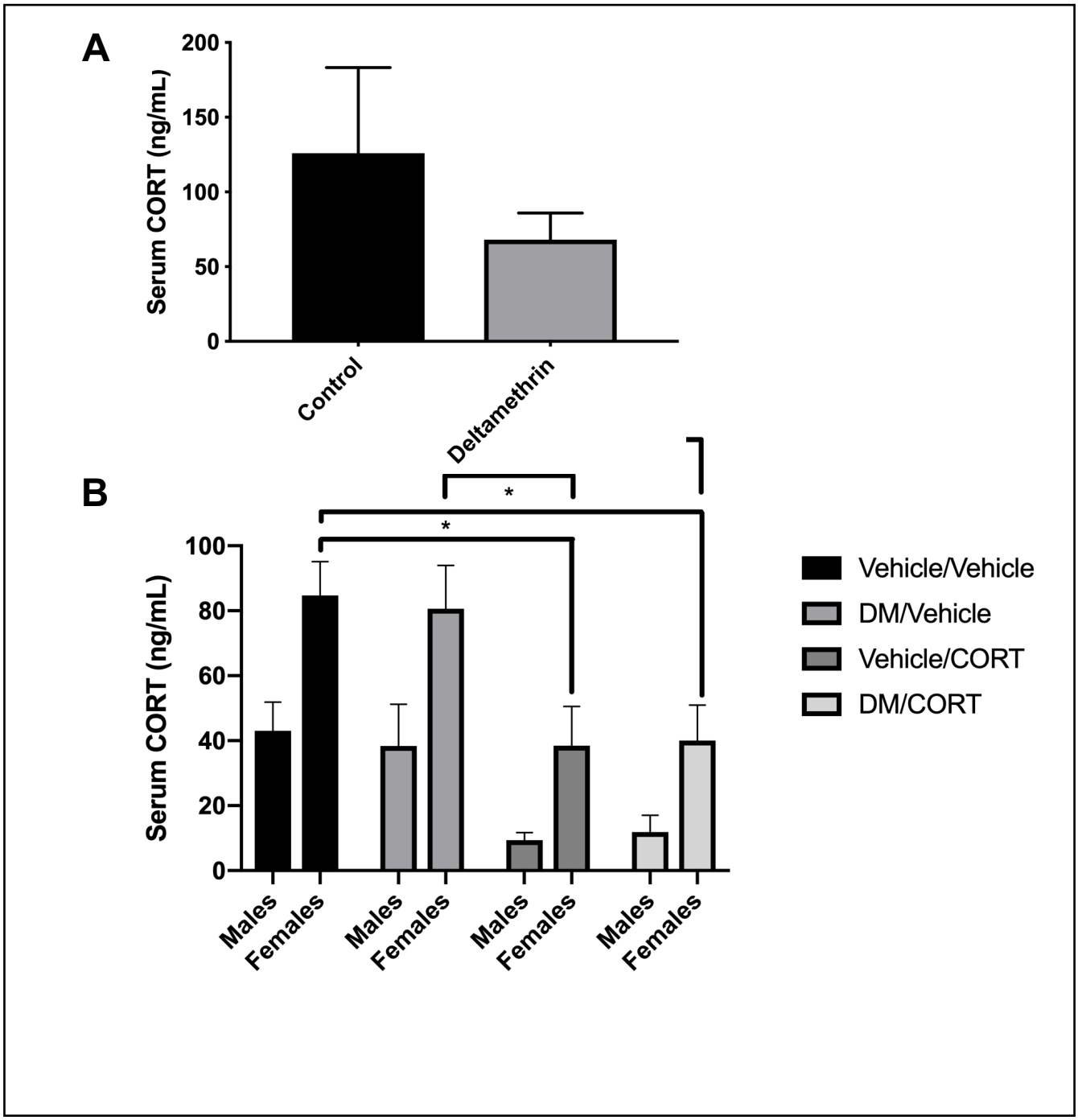
To test whether deltamethrin exposure itself would affect circulating CORT levels in pregnant dams and potentially affect results observed in deltamethrin and CORT-exposed offspring, we assessed serum CORT levels via ELISA. We did not observe statistically significant differences in serum CORT of control and deltamethrin-exposed dams (125.90 ng/mL $\pm$ 57.38 vs. 68.02 ng/mL $\pm$ 17.87, $p = 0.33$) (Figure 2.2). We also did not observe significant alterations in serum CORT of deltamethrin-only exposed male and female offspring compared to the vehicle/vehicle group. However, we did observe significantly decreased serum CORT levels in the CORT-only and deltamethrin/CORT groups in females offspring compared to the vehicle/vehicle group ($p = 0.01$ and $p = 0.01$, respectively), indicating that the CORT exposure itself likely influences circulating CORT levels independent of deltamethrin exposure in females. In addition, there was a significant overall effect of sex on serum CORT levels ($p < 0.0001$), illustrating sex-specific differences in circulating CORT levels.

Figure 2.2: Effect of deltamethrin and CORT exposure on circulating CORT in pregnant dams and their offspring.

A) Serum CORT in dams. Deltamethrin exposure does not affect circulating CORT in dams.

B) Serum CORT in offspring. Deltamethrin exposure does not affect circulating CORT in exposed offspring, but CORT exposure does affect circulating corticosterone independent of deltamethrin exposure in both male and female offspring.

Exposure group differences were assessed two-tailed t test (A) and two-way ANOVA with Tukey's post-hoc test (B). N=7-8 in (A), N=5-7 in (B), and error bars represent SEM. *= $p < 0.05$


A
Serum CORT (ng/mL)
200
150
100
50
0
Control
Deltamethrin
B
Serum CORT (ng/mL)
100
80
60
40
20
0
*
*
Males
Females
Males
Females
Males
Females
Males
Females
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT

Sex-specific alterations in expression of key dopaminergic genes

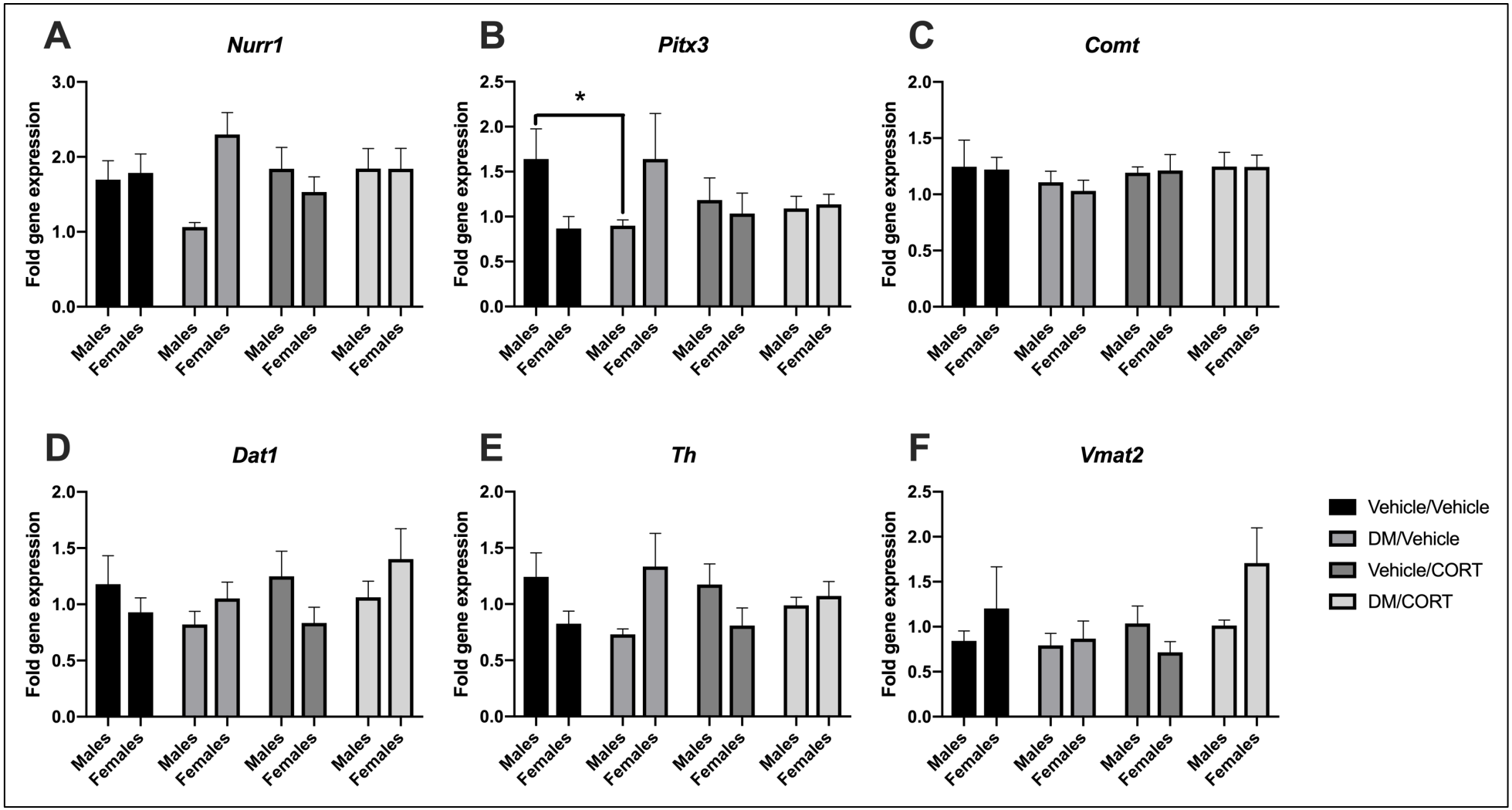
While we have previously studied alterations in the expression of key dopaminergic genes in the midbrain in response to dieldrin and heptachlor exposure, we had not yet investigated changes that occur in response to pyrethroid insecticide exposure [190, 191]. Here, we sought to assess whether expression of *Pitx3* and *Nurr1* transcription factors involved in development of the dopaminergic phenotype, as well as downstream dopaminergic targets *Th*, *Comt*, *Dat1*, and *Vmat2*, were altered in midbrains of offspring exposed to deltamethrin, CORT, or both during neurodevelopment. We also assessed whether expression of *Nr3c1*, the glucocorticoid receptor, would change in response to the aforementioned exposures.

Pitx3 gene expression was significantly decreased in deltamethrin-only exposed males when compared to vehicle/vehicle exposed males (1.64 ± 0.34 vs. 0.90 ± 0.07, $p = 0.04$), but not deltamethrin-only exposed females (0.87 ± 0.13 vs. 1.6 ± 0.51, $p = 0.29$) (Figure 2.3B). There was also no significant difference in *Pitx3* expression in CORT-only and deltamethrin/CORT exposed males, suggesting that CORT exposure may mediate *Pitx3* expression when combined with deltamethrin exposure. Similar expression effects were observed for *Nurr1* transcription factor expression (Figure 2.3A), as well as for *Dat1* and *Th* (Figure 2.3D, 2.3E), though none of these effects reached statistical significance at $\alpha = 0.05$. Interestingly, gene expression of *Pitx3*, *Nurr1*, *Dat1*, and *Th* trended upwards for females exposed to deltamethrin, opposite the trends observed in males, with an intermediate effect in the deltamethrin/CORT-exposed females (Figure 2.3). We did not see significant effects of deltamethrin and CORT exposure on mRNA expression of *Vmat2*, *Nr3c1*, or *Comt* in the midbrain (Figure 2.3).

Figure 2.3*:* Gene expression in adult midbrain.

Males exposed to deltamethrin exhibit decreased *Pitx3* expression in the midbrain. Data is expressed as $2^{\Delta\Delta Ct}$ analysis of qPCR data and compared to expression in the vehicle/vehicle group.

Exposure group differences were assessed via one-way ANOVA. *= $p<0.05$, n=5-8, and error bars represent SEM.


A
Nurr1
B
Pitx3
C
Comt
D
Dat1
E
Th
F
Vmat2
Fold gene expression
Males
Females
*
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT

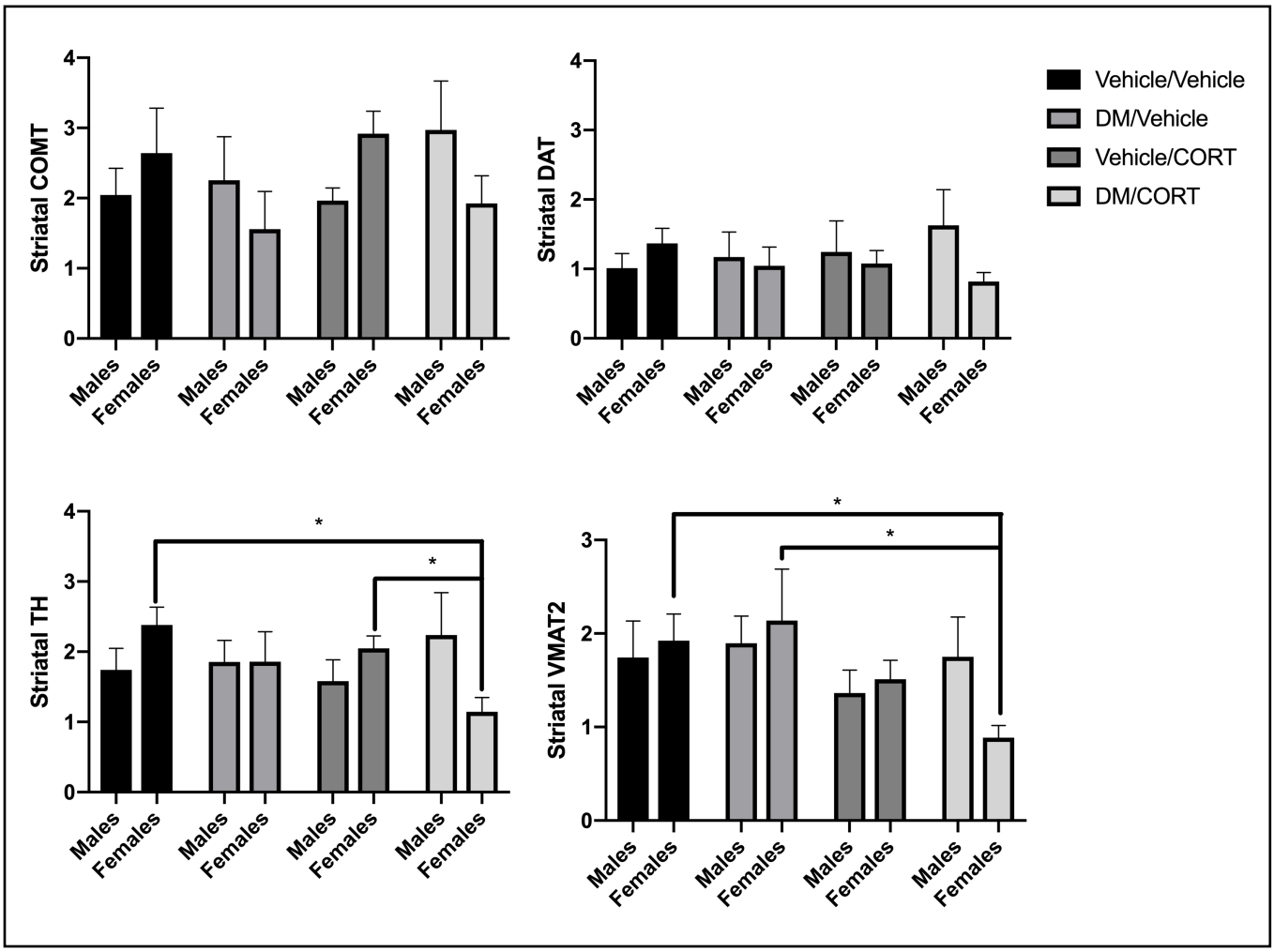
TH and VMAT2 expression are significantly decreased in striatum of females exposed to deltamethrin/CORT, and TH expression is significantly decreased in frontal cortex of males exposed to deltamethrin.

Previous studies have observed alterations in key dopaminergic proteins in response to various insecticide and chronic stress exposures in the striatum, a highly dopaminergic brain region [67, 101, 136, 190-195]. We evaluated whether combined deltamethrin/CORT exposure would lead to additive effects on dopaminergic protein expression in the striatum via immunoblotting. In striata of females exposed to both deltamethrin and CORT, TH expression was significantly decreased compared to vehicle/vehicle control females (2.23 ± 0.24 vs. 1.15 ± 0.20, $p = 0.01$) (Figure 2.4C). There was a significant difference in TH expression between CORT-only exposed females and deltamethrin/CORT-exposed females, suggesting that dual exposure with deltamethrin may mediate the effect in females (2.47 ± 0.47 vs. 1.15 ± 0.45, $p = 0.02$) (Figure 2.4C). In addition, there was also a significant decrease in VMAT2 expression in the striata of deltamethrin/CORT-exposed females (1.92 ± 0.28 vs. 0.89 ± 0.13, $p = 0.045$) (Figure 2.4D). Furthermore, there was also a significant difference in VMAT2 expression between deltamethrin-only exposed females and deltamethrin-CORT-exposed females, suggesting that dual exposure with CORT mediates the effect on VMAT2 expression (2.14 ± 0.55 vs. 0.89 ± 0.13, $p = 0.02$) (Figure 2.4D). The effects on TH and VMAT2 expression were not observed in striata of males exposed to deltamethrin/CORT. There were no significant alterations in COMT and DAT expression in the striatum of males and females exposed to deltamethrin-only, CORT-only, and deltamethrin/CORT (Figure 2.4A-B). Additionally, we did not observe any changes in expression of NR3C1, which encodes the glucocorticoid receptor, in any of the exposure groups in males or females (data not shown).

Figure 2.4: Protein expression in the striatum

Females exposed to deltamethrin and CORT have significantly decreased TH and VMAT2 expression in the striatum, compared to females exposed to vehicle/vehicle and females exposed to CORT-only. Protein expression in the striatum is expressed as adjusted units (AU) assessed via densitometry analysis and normalized to actin.

Exposure group differences were evaluated via one-way ANOVA. *= $p<0.05$, n=5-7, and error bars represent SEM.

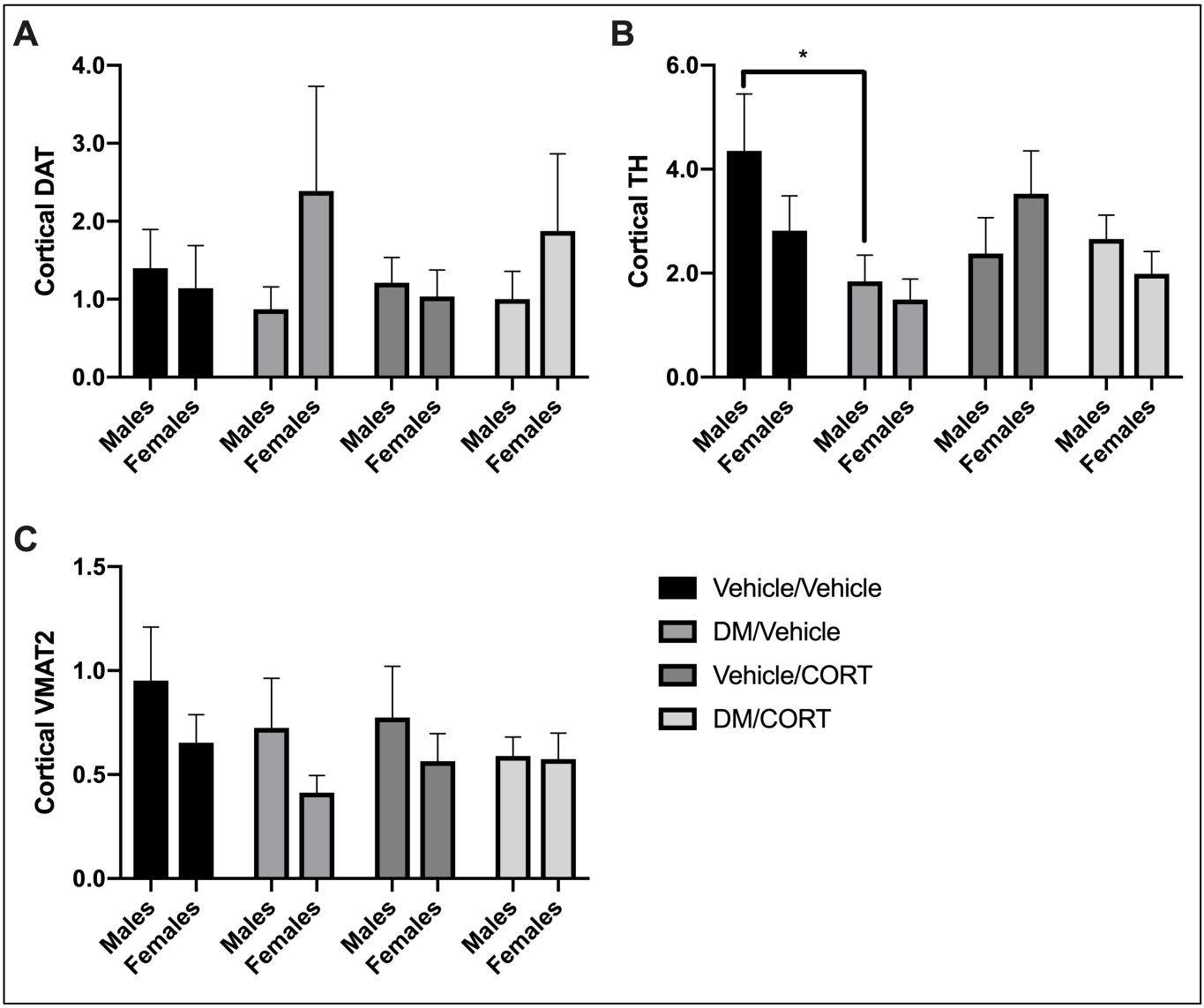

Striatal COMT
Striatal DAT
Striatal TH
Striatal VMAT2
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
Males
Females
*

Next, we measured whether expression of key dopaminergic proteins changes in response to deltamethrin and CORT exposure in the frontal cortex, another brain region that receives dopaminergic projections from the midbrain and has been associated with ADHD pathogenesis. There is a significant decrease in TH expression in the frontal cortex of deltamethrin-only exposed males compared to vehicle/vehicle controls (4.350 ± 1.098 vs. 2.380 ± 0.6870, $p = 0.0340$) (Figure 2.5C), but no significant difference in TH expression in dually-exposed males, suggesting that CORT may mediate the effect of deltamethrin on TH-expression. There is no significant change in expression of VMAT2, DAT, and TH of males and females exposed to deltamethrin-only, CORT-only, and deltamethrin/CORT when compared to the vehicle/vehicle control groups (Figure 2.5A, B).

Figure 2.5: Protein expression in the frontal cortex

Males exposed to deltamethrin have significantly decreased TH expression in the frontal cortex compared to males exposed to vehicle/vehicle. Protein expression in the frontal cortex is expressed as adjusted units (AU) assessed via densitometry analysis and normalized to actin.

Exposure group differences were evaluated via one-way ANOVA. *= $p<0.05$, n=5-7, and error bars represent SEM.


A
Cortical DAT
4.0
3.0
2.0
1.0
0.0
Males
Females
B
Cortical TH
6.0
4.0
2.0
0.0
*
C
Cortical VMAT2
1.5
1.0
0.5
0.0
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT

Deltamethrin exposure significantly slows striatal dopamine uptake rate in males.

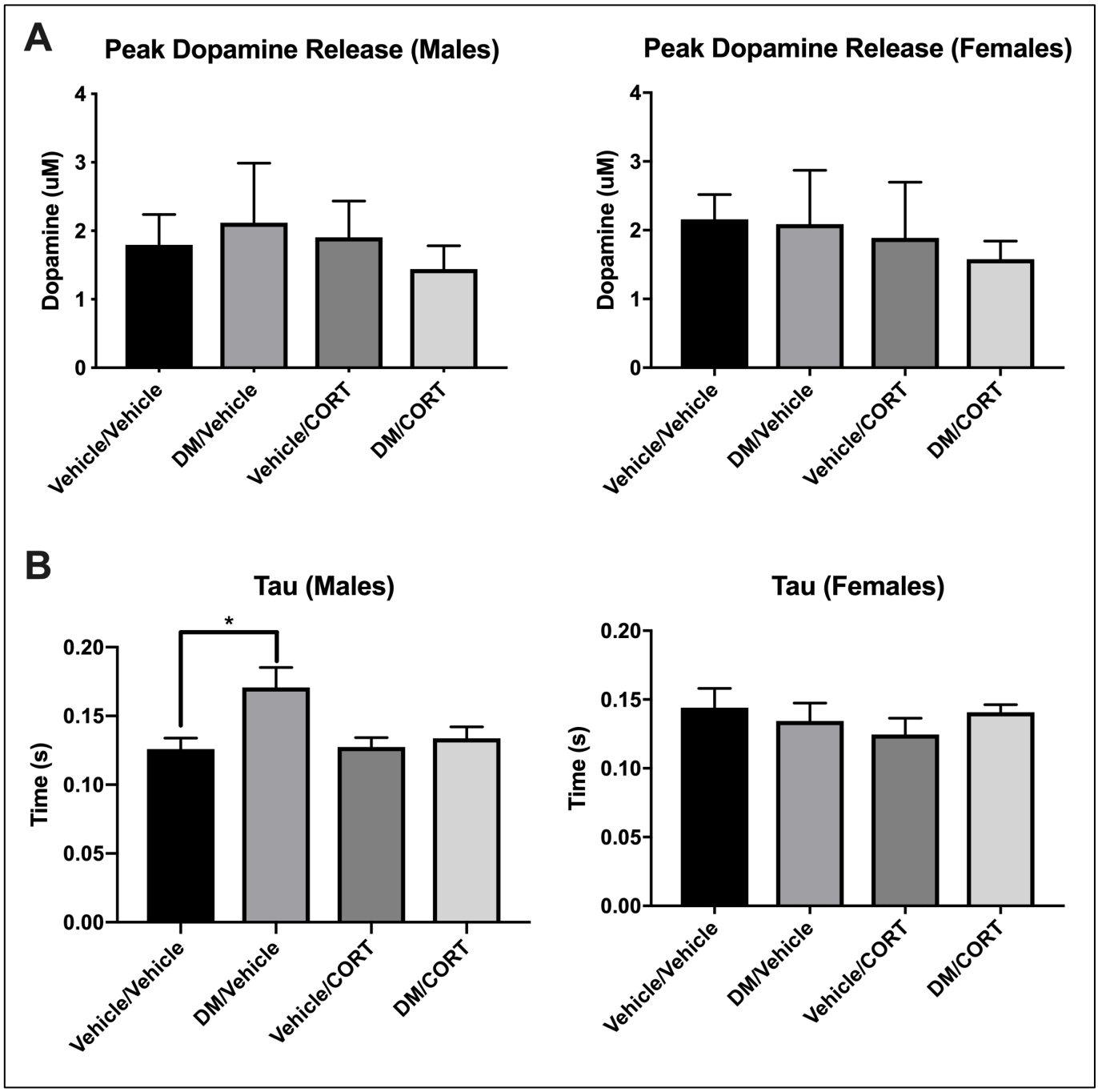
To investigate dopamine release and uptake dynamics *ex vivo* we employed fast-scan cyclic voltammetry as previously described [30, 188, 196]. This technique harnesses dopamine's electrochemical properties and provides data on peak dopamine release in the striatum as well as dopamine uptake by DAT. Functionally, we also do not observe significant changes in peak dopamine release in any of the four exposure groups via voltammetry, in neither males nor females (Figure 2.6A). In contrast, we do observe a significant increase in length of time necessary for dopamine uptake in striata of deltamethrin-only exposed males (Figure 2.6B). Dopamine uptake rate and, subsequently tau, are affected by changes to dopamine transporter function [189], suggesting that there may be more subtle changes in functionality but not overall DAT expression levels. There are no alterations in tau in dually-exposed deltamethrin/CORT males, suggesting that CORT may mediate the effect of deltamethrin on dopamine uptake dynamics (Figure 2.6B). These effects also appear to be sex-specific as there is neither a significant difference in dopamine release nor in tau in females in any of the exposure groups (Figure 2.6).

Figure 2.6: *Ex vivo* electrochemical measurement of dopamine release and uptake via fast-scan cyclic voltammetry.

A) Peak dopamine release. DM, CORT, or DM/CORT exposure does not significantly change peak dopamine release as shown via averaged over all stimulation sites per animal

B) Dopamine uptake measured via tau. Deltamethrin exposure in males does significantly increase tau, a kinetic time constant used to characterize dopamine uptake. Tau values were averaged over all stimulation sites per animal.

Exposure group differences were evaluated via one-way ANOVA. *= $p<0.05$, N=5-7, and error bars represent SEM.


A
Peak Dopamine Release (Males)
Dopamine (uM)
0
1
2
3
4
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
Peak Dopamine Release (Females)
Dopamine (uM)
0
1
2
3
4
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
B
Tau (Males)
*
Time (s)
0.00
0.05
0.10
0.15
0.20
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
Tau (Females)
Time (s)
0.00
0.05
0.10
0.15
0.20
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT

Males exposed to deltamethrin display increased activity in Y maze but not open field.

Given the alterations in mRNA expression, protein expression, and dopamine function, we next investigated whether these changes would result in ADHD-like behaviors. Hyperactivity is a cardinal feature of ADHD symptomology [197]. Genetic models of ADHD as well as animal exposure models of environmental ADHD susceptibility have tested locomotor activity through various methods [67, 71, 198-201]. We assessed locomotor activity over a one-hour period after mice were allowed to habituate to an open field box. We did not observe significant differences in the number of beam breaks in males or females in any of the exposure groups ($p = 0.6199$ for males, $p = 0.5471$ for females) (Figure 2.7A). There was also no significant difference in locomotor behavior during the habituation period (data not shown).

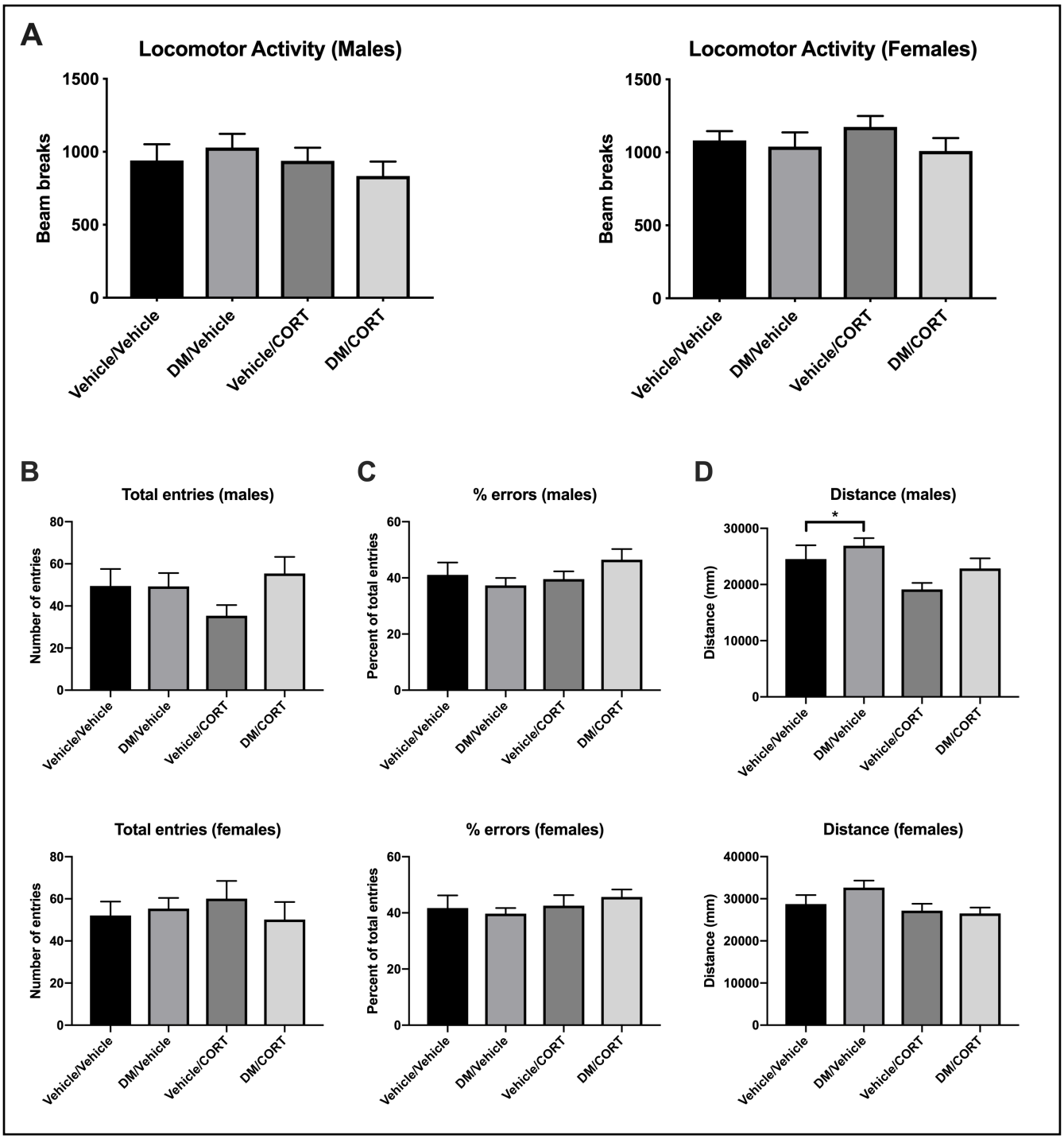
There was no significant difference between exposure groups or sexes in alternation behavior or in the percentage of errors, suggesting that we did not detect alterations in working memory or attention in this assay (Figure 2.7B, 2.7C). However, males exposed to deltamethrin traveled a significantly longer distance in the Y-maze in 8 minutes compared to vehicle/vehicle control males (2452.9 cm $\pm$ 245.4 vs. 2690.8 cm $\pm$ 134.7, $p = 0.0368$), as well as when compared to males exposed to CORT (2452.9 cm $\pm$ 245.4 vs. 1915.0 cm $\pm$ 113.2, $p = 0.0050$) (Figure 2.7D). Thus, while mice did not display hyperactivity in the locomotor activity assay, they do display increased activity in the Y maze assay. This effect appears to be sex-specific, as there is no significant difference in distance traveled among the female exposure groups. Males dually exposed to deltamethrin and CORT do not travel an increased distance compared to the vehicle/vehicle control group (1915.0 cm $\pm$ 113.2 vs. 2284.3 cm $\pm$ 179.9 cm vs., $p = 0.5109$), suggesting that CORT exposure may mediate the hyperactivity effects of deltamethrin in the Y maze test.

Figure 2.7: Behavioral analysis

A) Locomotor activity. Exposure to deltamethrin and CORT does not significantly alter locomotor activity measured via beam breaks. Data is expressed as average number of beam breaks in 60 minutes after a 30-minute habituation period.

B-D) Y maze. Males exposed to deltamethrin display increased activity in the Y maze, but there are no changes in working memory or attention. Mice were allowed to freely explore the Y maze for 8 minutes and arm entries were recorded to assess working memory and attention.

Exposure group differences were assessed via one-way ANOVA. * = $p < 0.05$, n = 7-9, and error bars represent SEM.


A
Locomotor Activity (Males)
Beam breaks
1500
1000
500
0
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
Locomotor Activity (Females)
Beam breaks
1500
1000
500
0
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
B
Total entries (males)
Number of entries
80
60
40
20
0
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
C
% errors (males)
Percent of total entries
60
40
20
0
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
D
Distance (males)
*
Distance (mm)
30000
20000
10000
0
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
Total entries (females)
Number of entries
80
60
40
20
0
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
% errors (females)
Percent of total entries
60
40
20
0
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
Distance (females)
Distance (mm)
40000
30000
20000
10000
0
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT

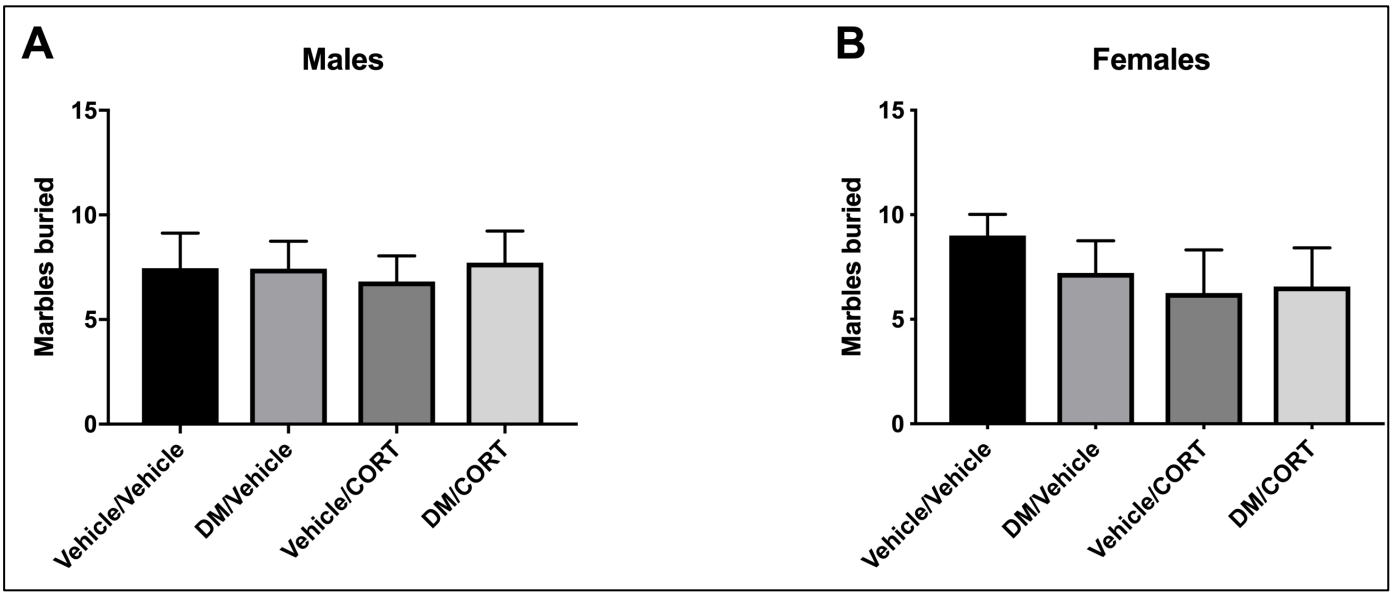
No difference in impulsivity as measured via marble burying in animals exposed to deltamethrin and CORT.

Marble burying behavior has been used previously to test impulsivity in genetic models of ADHD predisposition [80]. While vehicle/vehicle control mice display expected burying behaviors, we do not observe significant differences in the absolute number of marbles buried at least 50% after 30 minutes in male or females in any of the exposure groups ($p = 0.9754$ for males, $p = 0.6903$ for females) (Figure 2.8).

Figure 2.8: Marble burying assay to test impulsivity

We did not observe significant differences in impulsivity in exposed males or females in the marble burying assay. Mice were allowed to explore freely for 30 minutes in a clean cage with 20 marbles placed on top of the bedding. The number of marbles buried at least 50% was scored by two independent scorers and averaged.

Exposure groups differences were assessed via one-way ANOVA, n=7-9, and error bars represent SEM.


A
Males
Marbles buried
15
10
5
0
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT
B
Females
Marbles buried
15
10
5
0
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT

DISCUSSION

The etiology and pathogenesis of ADHD are still unclear, but ADHD continues to present a substantial public health problem and there is a need for additional research probing ADHD-associated neurologic pathways and sources of pathology. The existing data on ADHD suggest that alterations in dopaminergic signaling due to pyrethroid insecticide exposure influence ADHD pathogenesis and are important for studying ADHD risk [67, 86]. Chronic stress exposure also alters the dopamine system and could further potentiate the risk of ADHD [136, 194, 202-204]. Given the independent effects of pyrethroid insecticides and chronic stress on the dopamine system, we hypothesized that combined pyrethroid insecticide and stress hormone exposure during critical periods of neurodevelopment would additively alter protein expression and function of key components of the dopamine system, thereby further increasing the risk of developing ADHD.

Summary of findings

In our study, we exposed pregnant dams to deltamethrin and their offspring to CORT dissolved in drinking water until adulthood and then employed a suite of behavioral and molecular approaches to gain a more comprehensive understanding of alterations to the dopamine system in response to dual exposures. Although deltamethrin did not affect serum CORT levels of male and female offspring, exposure to CORT in drinking water led to a decrease in serum CORT in male and female offspring exposed and unexposed to deltamethrin. Additionally, male offspring exposed to deltamethrin demonstrated a reduction in the expression of the midbrain transcription factor, *Pitx3*. Although changes in mRNA expression for specific dopaminergic phenotypic markers were not observed, a gender- and region-specific effect of TH

and VMAT2 protein expression was detected. The functional consequences of these alterations manifested in a reduction in dopamine uptake in the striatum of male offspring exposed to deltamethrin, which corresponded with increased locomotor activity in the Y maze for these animals.

Glucocorticoid signaling and DM/CORT exposure

No studies have previously examined the effects of developmental pyrethroid insecticide exposure on endogenous CORT production. Thus, our findings provide some novel insight into the effects of developmental exposure to deltamethrin on the HPA axis and CORT levels in offspring. While deltamethrin did not impact CORT levels at the time points we evaluated, we cannot rule out the possibility that assessment at earlier ages would have shown an alteration in CORT that did not persist as they aged and deltamethrin exposure was ceased. In contrast, a few studies have investigated the effects of oral CORT administration on serum CORT production. One study previously reported that adult male mice exposed to 25 μg/mL CORT in drinking water had significantly decreased serum CORT two weeks after exposure [205], but did not have significantly altered endogenous CORT production one month after exposure [186]. Additionally, females exposed to 35 μg/mL CORT for four weeks during adolescence show a significant decrease in plasma CORT levels after forced swim testing, suggesting a dampening effect on the normal stress response following exogenous CORT administration [206].

While these results support the reductions in CORT levels found in our study, these results run counterintuitive to our original hypothesis. Nonetheless, stimulation of the endogenous stress response generated by CORT exposure may provide some insight into the feedback mechanisms that mediate serum CORT levels in our model. In general, the stress

response is mediated via actions of the hypothalamic-pituitary-adrenal (HPA) axis. Ordinarily, an acute stressor causes release of corticotropin-release factor (CRF) from the hypothalamus, subsequent adrenocorticotrophic hormone (ACTH) release from the pituitary, and glucocorticoid release from the adrenal glands. Glucocorticoids then inhibit the hypothalamus and pituitary gland via a negative feedback loop, leading to an overall reduction in CORT release [126, 127]. Under circumstances of early life and chronic stress, HPA axis hyperactivity and impaired negative feedback are typically observed [126, 127], though the type of stressor, sex, genetic predisposition, and environmental factors can all modulate the influence chronic stress has on maturation and function of the HPA axis and downstream CORT levels [128]. Thus, various inputs can impact the function of the HPA axis through alteration to the limbic nuclei as well as adrenal gland. Our results and those of others suggest that chronic oral CORT administration may impact the HPA axis centrally. A previous study showed that oral CORT given during adolescence in rats increases neuronal activity in the paraventricular nucleus of the hypothalamus and caused a blunted CORT response to restraint stress [207, 208]. Additionally, chronic oral CORT was found to result in a reduction in plasma CORT, independent of damage or removal of the adrenal glands [209]. These findings support the central-acting effects of exogenous CORT in mediating serum CORT production and levels. Although deltamethrin did not result in a measurable change in CORT levels at the time points we measured, the impact of chronic CORT exposure suggests a more complex regulation of serum CORT levels, which could significantly affect the development of the dopamine system.

Altered midbrain RNA expression

Via our novel neurodevelopmental combined exposure paradigm, we determined that midbrain expression of *Pitx3* was significantly decreased in adult males exposed to deltamethrin, with a similar trend in *Nurr1* expression. *Pitx3* and *Nurr1* are transcription factors with essential roles in the phenotypic development and maintenance of dopaminergic neurons in the midbrain [54, 210, 211]. *Pitx3* and *Nurr1* expression are normally induced by embryonic day (E) 11.5 in mice and are predominantly localized to the SNpc and VTA in the midbrain, where the densest population of dopamine neurons resides. Highlighting their function in dopamine neuron survival, genetic reduction of *Pitx3* or *Nurr1* in mice results in aberrant differentiation or loss of dopamine neurons in the midbrain and a concomitant loss of dopaminergic projections to the dorsal striatum [212-218]. While both transcription factors are critical in development of the dopamine system, *Nurr1* helps determine and maintain a neuron's dopaminergic phenotype. Conversely, *Pitx3* does so only for a laterally-located subset of midbrain dopaminergic neurons in the SNpc [219]. Lateral subpopulations in the SNpc that express *Pitx3* are also more susceptible to the dopaminergic neurotoxicant, MPTP [220]. Perhaps, we detected *Pitx3* but not *Nurr1* alterations because Pitx3-expressing subpopulations are more sensitive to neurotoxic dopamine perturbations by deltamethrin than other subtypes of dopaminergic neurons found in this region. As far as we are aware, these are the first data evaluating the impact of developmental deltamethrin and CORT exposure on transcription factors associated with development of the mesencephalic dopamine region.

Interestingly, although we observed changes in transcription factors that modulate *Dat1*, *Vmat2*, and *Th* expression, we did not find observable changes in overall expression of these genes in the adult midbrain. While we were able to measure alterations to *Pitx3*, it may be that

these changes were not robust enough to elicit a significant change in mRNA expression of downstream genes. Additionally, alterations in gene expression of these dopaminergic components may be transient and do not persist into adulthood after the exposure to deltamethrin and CORT, making it difficult to capture these changes in our exposure paradigm.

Altered striatal and cortical protein expression

To our knowledge no one has assessed striatal COMT, DAT, TH, and VMAT2, nor has anyone assessed cortical DAT, TH, and VMAT2 after combined exposure to deltamethrin and CORT during neurodevelopment. Utilizing this paradigm, we found no explicit changes in these proteins with CORT exposure alone but did observe changes in striatal TH and VMAT2 following exposure to combined deltamethrin and CORT, while cortical TH was reduced after deltamethrin alone. The striatal expression of TH and VMAT2 in female offspring exposed to a combination of deltamethrin and CORT was significantly decreased, while expression of these proteins remained unchanged in male mice. Previous studies have observed increases in striatal DAT expression at 6 weeks of age, following a neurodevelopmental exposure to 3 mg/kg deltamethrin [67]. We measured striatal DAT expression at 8-10 weeks and did not observe this increase, possibly because the effect of deltamethrin on striatal DAT expression does not persist into adulthood using this exposure paradigm.

Previous studies that have investigated the dopaminergic effects of psychosocial stress provide a few possible explanations for consequences of CORT in dopaminergic neurodevelopment in our study. Rodents exposed to various forms of prolonged behavioral psychosocial stress display decreased density and binding of DAT in the dorsal striatum [194], dorsolateral caudate putamen [136], and nucleus accumbens [136]. However, these results were

observed in male tree shrews [194] and male rats [136] exposed to psychosocial stress in adulthood. Since we employed a neurodevelopmental exposure paradigm and male and female mice received oral CORT from weaning through adolescence, this differential stress exposure paradigm might explain why we did not observe any changes in DAT expression in the striatum and frontal cortex. Potentially, deltamethrin exposure from gestation through weaning increased the susceptibility of dopaminergic neurons in the striatum, and subsequent exposure to oral CORT from adolescence to adulthood then had additional neurotoxic effects that led to a measurable decrease in TH and VMAT2. This could help explain why we did not observe decreased striatal TH and VMAT2 in the deltamethrin-only and CORT-only groups.

In contrast to our findings in the striatum, adult males exposed to deltamethrin-only during neurodevelopment showed significantly decreased TH expression in the cortex. This change in males is not present in the dually-exposed deltamethrin/CORT group. These findings suggest a differential impact of deltamethrin and CORT on the mesolimbic circuitry compared with the nigrostriatal circuitry.

Functional consequences of deltamethrin and CORT exposure

Next, we investigated whether the molecular changes we observed would incur functional consequences via *ex vivo* fast-scan cyclic voltammetry and a battery of behavioral assays. Via electrochemical analysis of dopamine release dynamics, we observed that males exposed to deltamethrin did not exhibit differences in peak dopamine release in the striatum, but males exposed to deltamethrin do show a significant impairment in dopamine uptake. In our electrochemical voltammetry studies, dopamine uptake was parameterized as the time constant tau. Tau describes the amount of time to return to 2/3 of baseline current [189, 221] and is

increased by administration of known pharmacologic dopamine uptake inhibitors such as amantadine [222, 223] as well as cocaine [223]. Our results were surprising, given the lack of effect of deltamethrin exposure on DAT expression in the striatum. These findings suggest that developmental exposure to deltamethrin can elicit a significant impairment in DAT function, that is independent of concomitant changes in DAT expression.

DAT is an integral membrane protein that sequesters cytosolic dopamine into vesicles, thereby controlling dopaminergic signaling pre- and post-synaptically [36]. It acts as a symporter to bind two sodium ions, one chloride ion, and the dopamine substrate, moving between inward and outward facing states relative to the dopamine vesicle [37]. Genetic and pharmacologic manipulation studies of DAT illustrate that DAT function could be affected in many ways [38]. Previous studies show that DAT transport function can be impacted by binding in different conformational states, for example. Cocaine and methylphenidate, a first-line ADHD medication, stabilize DAT's outward-facing conformation [39] whereas other neuropsychiatric medications such as bupropion and modafinil stabilize the inward-facing conformation [40]. Conformation changes in DAT are critical for its proper function as a symporter, as it must move between these conformational states to properly transport dopamine. Perhaps, deltamethrin and CORT exposure abnormally stabilize or impair one of these formations and this leads to a slowed mechanism of dopamine uptake that is independent of changes in DAT expression or dopamine release. There are a few examples of functional *DAT1* mutations that confer changes in dopamine uptake, but not necessarily dopamine release or expression. One group transfected a human *DAT1* mutation associated with Autism Spectrum Disorder, T365M, into cells and observed significantly lower maximal velocity of dopamine influx. However, neither the affinity of dopamine nor overall expression of DAT was affected [224]. In another *in vitro* study of two *DAT1* mutations found in

an adult patient with comorbid Parkinson's disease and ADHD, the DAT1 I312D mutation was associated with significantly lower maximal velocity of dopamine influx but no difference in overall DAT protein expression as well [225]. Thus, in alignment with the aforementioned studies, we did not observe significantly altered DAT expression or release in the striatum of males exposed to deltamethrin/CORT, but dopamine influx velocity was significantly decreased. Dual deltamethrin/CORT exposure seems to attenuate the effect of deltamethrin on dopamine uptake in the striata of adult males, suggesting that CORT exposure could still play a role in determining rate of dopamine uptake.

Given the observed changes in mRNA expression, protein expression, and DAT functionality, we then tested ADHD-like behaviors. Interestingly, males exposed to deltamethrin demonstrate hyperactive behavior in the Y maze, though no differences in attention or impulsivity were measured. It is possible that our behavioral endpoints were not sensitive enough to detect changes at this level, especially given the lack of alterations in DAT expression and peak dopamine release *ex vivo*.

Conclusion

In summary, we established a neurodevelopmental exposure paradigm of joint environmental factors that resulted in novel CORT reduction, RNA expression, striatal and cortical protein expression, and dopamine uptake rate findings. These findings contribute to our understanding of potential mechanisms of action of deltamethrin and oral CORT, both alone and in combination. Due to the dopaminergic effects previously described by our group and others after exposure to either deltamethrin alone or psychosocial stress or CORT alone, we hypothesized there would be synergistic effects in the dopamine system after developmental

exposure to these in combination. Interestingly, we did not observe significant synergistic effects of the combined deltamethrin/CORT exposure on mRNA and protein expression of several key components within the dopamine system. Instead our data suggest that dual exposure to the major stress hormone CORT may actually dampen the effects of deltamethrin on the development of the dopamine system in males. Our study demonstrates a broad effect on the development and function of the dopamine circuit, which may be impacted by temporal aspects of deltamethrin and CORT exposure during neurodevelopment. The rationale for following up on these results is based on our understanding of the "multiple hit" hypothesis, which suggests environmental exposures render a biological system more vulnerable to subsequent environmental insults [226]. In the context of this study, while the initial exposures to deltamethrin or CORT may not manifest in an overt pathological effect on the dopamine circuit, future exposures to stress, deltamethrin, or other environmental factors could unmask more profound neurotoxicological endpoints. Aligned with this, our study highlights the importance of testing multiple environmental exposures in conjunction to better understand how combined exposures present in real-life can affect similar neurodevelopmental systems and could differentially affect particularly vulnerable pediatric populations. First-line treatment with methylphenidate, a stimulant, resolves symptoms of the three domains for the majority of children with ADHD, but treatment response is variable and use is associated with stunted growth [2], neural plasticity effects [3], and substance abuse [4]. Studies also indicate that children with ADHD report deficits in psychosocial well-being and family life [5]. Thus, while significant clinical and research advancements have been realized, ADHD continues to present a major public health burden. Further study of predisposing mechanisms and pathophysiology is therefore merited.

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SUPPLEMENTAL MATERIALS

Supplemental Table 2.1: Taqman Gene Expression Assays

Gene of Interest	Assay ID
β-actin	Mm02619580_g1
Dat1/Slc6a3	Mm00438388_m1
Vmat2/Slc18a2	Mm00553058_m1
Comt	Mm00514377_m1
Th	Mm00447557_m1
Nurr1/Nr4a2	Mm00443060_m1
Pitx3	Mm01194166_g1
Nr3c1	Mm00433832_m1

CHAPTER 3: Combined neurodevelopmental exposure to deltamethrin and corticosterone is associated with *Nr3c1* hypermethylation in the midbrain of male mice

ABSTRACT

Attention-Deficit Hyperactivity Disorder (ADHD) is one of the most common neurodevelopmental disorders and manifests inattention, hyperactivity, and impulsivity symptoms in childhood that can last throughout life. Genetic and environmental studies implicate the dopamine system in ADHD pathogenesis. Work from our group and that of others indicates that deltamethrin insecticide and stress exposure during neurodevelopment leads to alterations in dopamine function, and we hypothesized that exposure to both of these factors together would lead to synergistic effects on DNA methylation of key genes within the midbrain, a highly dopaminergic region, that could contribute to these findings. Through targeted next-generation sequencing of a panel of cortisol and dopamine pathway genes, we observed hypermethylation of the glucorticoid receptor gene, *Nr3c1*, in the midbrain of C57/BL6N males in response to dual deltamethrin and corticosterone exposures during development. This is the first description of DNA methylation studies of *Nr3c1* and key dopaminergic genes within the midbrain in response to a pyrethroid insecticide, corticosterone, and these two exposures together. Our results provide possible connections between environmental exposures that impact the dopamine system and the hypothalamic-pituitary-adrenal axis via changes in DNA methylation and provides new information about the presence of epigenetic effects in adulthood after exposure during neurodevelopment.

INTRODUCTION

Attention-Deficit Hyperactivity Disorder (ADHD) affects 7-10% of children. It is defined by inattention, hyperactivity, and impulsivity symptoms that appear by age 12 and can continue well into adulthood [1, 197]. While the exact pathophysiology is still unclear, studies reveal several potential genetic and environmental contributors and point to neurotransmitter systems that are especially responsive to these perturbations. In particular, multiple genetic studies have implicated key dopaminergic components such as receptors, enzymes, and transporters in ADHD pathogenesis [6]. Gene variants in the dopamine receptor 5 gene (*DRD5*) predict age of ADHD onset, while variants in the dopamine transporter (*DAT1*) gene are associated with increased severity of hyperactivity and impulsivity symptoms [7, 8]. In multiple cohorts of children, changes in DAT expression are also associated with ADHD [10-12]. Furthermore, in mouse models of genetic alterations in the dopamine system, several ADHD-like behaviors have been described. For example, a *Dat1* knockout mouse model exhibits increased activity in a locomotor assay and a *Dat1* overexpressing mouse has increased impulsivity [13-17]. Notably, first-line treatments of ADHD such as methylphenidate target dopamine and norepinephrine reuptake inhibitors and treatment efficacy is associated with *DAT1* genotype [18], further implicating the dopamine system in the etiogenesis of ADHD.

Environmental factors also contribute to ADHD risk, and both chemical and psychosocial exposures such as maternal smoking, psychosocial stress, and low socioeconomic status have been described to date [61]. We recently found that neurodevelopmental exposure to deltamethrin, a pyrethroid insecticide, increases DAT and dopamine receptor 1 (DRD1) expression in the striatum and produced ADHD-like hyperactivity, inattention, and impulsivity behaviors [67]. Additionally, we implicated elevated levels of pyrethroid metabolites in urine

with ADHD in children in the United States [67]. Others found that this association was higher in boys and in children with hyperactive-impulsive symptoms [86]. Pyrethroid insecticides are synthetic analogues of pyrethrins and are a derivative of the *Chrysanthemum cinerariaefolium* flower. Their insecticide properties primarily come from its ability to bind the α subunit of voltage-gated Na^+ channels and hold them open to induce neuronal hyperexcitability [72]. Moreover, type II pyrethroids, such as deltamethrin, can also inhibit $GABA_A$ receptors and voltage-gated chloride channels to further potentiate excitability [73]. In addition to these targets, effects on dopamine dynamics and DAT expression have been described by our group and others [99-101, 108], and indicate that pyrethroid exposure may be particularly important to study in understanding mechanisms of ADHD development.

Children of lower socioeconomic status are also more likely to be diagnosed with ADHD [69, 111]. Potentially, this effect could be mediated by higher levels of psychosocial stress that are often associated with factors affecting low socioeconomic status groups [112, 121, 175]. Psychosocial stressors activate the hypothalamic-pituitary-adrenal (HPA) axis and cause release of the major stress hormone, which is cortisol in humans and corticosterone in mice [125]. Animal studies highlight some of the effects of increased early-life stress and implicate both the dopamine system and ADHD-like behaviors. For example, chronic stress in rodent models leads to increased dopamine release in the medial prefrontal cortex via glucocorticoid effects in the ventral tegmental area [135] as well as decreased DAT and DRD2 expression in the striatum [136]. Separation of rodent pups from their mothers induces stress, and these offspring later exhibit hyperactivity and inattention behaviors that respond to methylphenidate [71].

One important mechanism through which environmental exposures, particularly during the developmental period, could impact long term neurobehavioral risk is DNA methylation. DNA

methylation is a critical regulator of protein expression and a mechanism by which environmental exposures affect neurodevelopment in human and animal studies [227-230]. Our work illustrates the role of differential DNA methylation in human offspring neurodevelopment [150-152, 231-236] and others have demonstrated that exposure to persistent organic pollutants alters DNA methylation in humans and animal models [153-158, 162]. We identified changes in transcription factors that regulate expression of key dopaminergic genes after neurodevelopmental exposure to pesticides, suggesting that protein alterations we see may be mediated by changes at the level of the gene [191, 193]. Furthermore, exposure to chronic stress is also associated with altered DNA methylation of genes involved in neurodevelopment and more specifically, development of the dopamine circuit [165, 237], including those encoding dopamine receptors [174] and, as shown in our recent study, those related to cortisol response [238]. Lower DNA methylation of *DRD4* is also associated with an increase in ADHD symptoms in children at age 6 [9], which further indicates that DNA methylation is relevant for our understanding of ADHD pathogenesis.

Neurodevelopment is tightly regulated and highly vulnerable to environmental perturbations that have lasting effects on neuropsychiatric outcomes [62-65]. Given the importance of proper neurodevelopment to outcomes later in life, we sought to improve upon the current animal models in order to recapitulate real-world exposures that could occur in particularly vulnerable groups of children and affect ADHD risk. Low socioeconomic groups are exposed to increased levels of environmental contaminants [139], including pyrethroids [140-142], experience higher levels of stress [112, 121, 175], are at increased risk of developing ADHD [69, 111]. We surmise that DNA methylation is an important molecular mechanism to examine because changes in DNA methylation could potentially occur in response to pyrethroid and stress exposure during

critical periods of neurodevelopment. As these exposures have not been previously tested in an animal model, our approach provides a novel platform to assess the environmental factors involved in ADHD. We evaluated DNA methylation within genes that encode key dopaminergic components: catechol-o-methyltransferase (*Comt*), an enzyme responsible for dopamine metabolism; the dopamine transporter (*Dat1*), which is the primary mode of dopamine removal from the synapse; dopamine receptor 4 (*Drd4*); tyrosine hydroxylase (*Th*), the enzyme essential for dopamine synthesis; and the vesicular monoamine transporter (*Vmat2*), which packages dopamine into synaptic vesicles. We also assessed DNA methylation of two transcription factors necessary for proper dopamine neuron development: *Nurr1* (nuclear-receptor related 1 protein; transcription factor) and *Pitx3* (pituitary homeobox 3; transcription factor). Finally, we also measured DNA methylation of *Nr3c1* (nuclear receptor subfamily 3 group C member 1), which encodes the corticosterone-responsive glucocorticoid receptor. We hypothesized that these exposures could be related to ADHD pathogenesis via their documented effects on the dopamine system, and that combined neurodevelopmental exposure to both deltamethrin and chronic stress would be associated with synergistic changes in DNA methylation of these key components of the dopamine system and glucorticoid response.

METHODS

Animals

Eight-week-old C57BL/6NCrl wild-type mice (Charles River Labs) received food and water *ad libitum* and were maintained on a 12:12 dark/light cycle. Females were dosed with 3 mg/kg deltamethrin every three days for two weeks prior to breeding in triplicate. Deltamethrin dosing continued during breeding and gestation. One male and one female mouse per litter were

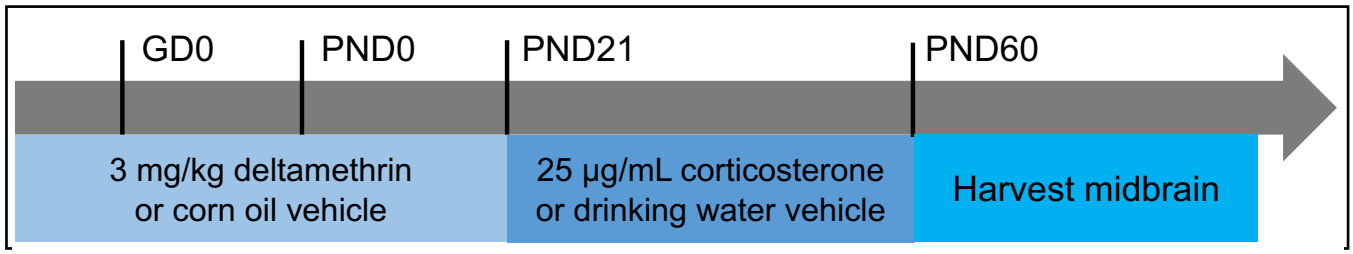
utilized for each experiment to reduce confounding due to litter effects. Throughout, an independent litter represents one sample (n = 1). All experiments were approved by the Institutional Animal Care and Use Committee at Emory University and were conducted in accordance with the National Institutes of Health Guide of Care and Use of Laboratory Animals.

Exposure paradigm

We utilized a deltamethrin dose of 3 mg/kg based on our previous study showing dopaminergic effects upon neurodevelopmental exposure to 3 mg/kg deltamethrin in male offspring. Additionally, this dose models a realistic dose in the human population [76, 176-183] and is lower than the developmental no observable adverse effect limit (NOAEL) determined by the United States Environmental Protection Agency. Adult C57BL/6J females were exposed to deltamethrin (N = 17) or vehicle (N = 20) every 3 days during gestation, lactation, and weaning at postnatal day (PND) 21. Deltamethrin was administered via corn oil dissolved in peanut butter to minimize trauma to pregnant mice. Corticosterone (CORT) was dissolved in the drinking water to minimize handling stress and reduce variation in CORT levels seen in behavioral chronic stress paradigms [184, 185]. CORT doses were prepared as previously described [186]. Offspring were continuously exposed to CORT (N = 15) or drinking water vehicle (N = 22) from adolescence through adulthood (PND21-60) (Figure 3.1). Water bottles were weighed daily to assess intake and mice were weighed at weaning and at least once a week to assess proper weight gain. At PND60 offspring were sacrificed and midbrains dissected for DNA methylation and RNA expression studies.

Figure 3.1: Exposure paradigm timeline.

Adult C57BL/6J females were exposed to deltamethrin or vehicle every 3 days during gestation, lactation, and weaning at postnatal day (PND) 21. Deltamethrin was administered via corn oil dissolved in peanut butter to minimize trauma to pregnant mice. Corticosterone (CORT) was dissolved in the drinking water to minimize handling stress and reduce variation in CORT levels seen in behavioral chronic stress paradigms [184, 185]. Midbrains for DNA methylation and RNA expression assays were harvested at 8-10 weeks.


GD0
PND0
PND21
PND60
3 mg/kg deltamethrin
or corn oil vehicle
25 µg/mL corticosterone
or drinking water vehicle
Harvest midbrain

DNA and RNA Sample Isolation

Mice underwent rapid decapitation at 8-10 weeks of age and the midbrain was isolated and immediately flash frozen. Total RNA and DNA were extracted with a Qiagen Allprep DNA/RNA Mini Kit (Qiagen, Germantown, MD, USA) following the manufacturer's recommended protocol, and each were stored at -80^{o}C until assayed.

DNA isolation, bisulfite conversion, library preparation and sequencing

DNA, isolated as described above, was quantified via a Qubit fluorometer dsDNA high sensitivity kit (Thermo Fisher Scientific, Waltham, MA) and 2 μg DNA was bisulfite-converted using a Zymo EZ DNA Methylation Lightning Kit according to manufacturer guidelines (Zymo Research, Irvine, CA). We evaluated DNA methylation around CpG islands residing within the following key dopaminergic genes: *Comt* (catechol-o-methyltransferase), *Dat1* (dopamine transporter), *Drd4* (dopamine receptor 4), *Nurr1* (nuclear-receptor related 1 protein; transcription factor), *Pitx3* (pituitary homeobox 3; transcription factor), *Th* (tyrosine hydroxylase), and *Vmat2* (vesicular monoamine transporter 2). We also evaluated DNA methylation of *Nr3c1* (nuclear receptor subfamily 3 group C member 1), which encodes the corticosterone-responsive glucocorticoid receptor. For genomic coordinates, see Supplemental Table 3.1.

DNA methylation was assessed utilizing targeted next-generation sequencing BisPCR2 methodology described previously [239]. Briefly, primers were designed to amplify bisulfite-modified regions of interest in intervals of approximately 300 base pairs (for primer sequences, see Supplemental Table 1) and included partial adapter overhangs: PCR#1 Left Primer Overhang: 5′-ACACTCTTTCCCTACACGA CGCTCTTCCGATCT-3′; PCR#1 Right Primer Over- hang: 5′-GTGACTGGAGTTCAGACGTGTGCTCTTCC GATCT-3′. Up to 5 primer pairs

were multiplexed per PCR reaction. Primers were diluted to a final concentration of 5 μM and approximately 30 ng genomic DNA was amplified utilizing the following PCR cycle conditions: 95ºC – 2:00; 35 cycles; 95ºC – 0:15, 56ºC – 1:30, 72ºC – 0:30; 72ºC – 2:00; 4ºC hold. PCR reactions were size-restricted and purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA) at a 1:1 volume ratio. For each sample, 5 μL of each multiplex reaction was pooled, and the subsequent pool was concentrated using AMPure XP beads at a 1:1 volume ratio. A subsequent PCR reaction incorporated Illumina DNA sequencing barcodes unique to each sample (see Supplemental Table 3.2). Barcode primers were diluted to a final concentration of 5 μM and 1 ng of pooled DNA from PCR #1 was amplified using the following PCR cycle conditions: 95°C – 1:00; 8 cycles: 95ºC – 0:30, 56ºC – 0:30, 72ºC – 1:00; 72ºC – 1:00; 4ºC hold. PCR products were again purified using AMPure XP beads at a 1:1 volume ratio and concentrations measured using the Qubit fluorometer dsDNA high sensitivity kit prior to sequencing. Targeted next-generation sequencing was performed on an Illumina MiSeq instrument using the reagent V2 kit according to manufacturer instructions. Sequencing was conducted by the Emory Integrated Genomics Core at Emory University (Atlanta, GA, USA).

mRNA Expression Assays

After isolation, we converted 10 ng total RNA to cDNA using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) and the PCR cycle conditions recommended by the manufacturer: 94ºC – 2:00; 35 cycles: 94ºC – 0:15, 55ºC – 0:30, 68ºC – 1:00; 4ºC – hold. Taqman gene expression assays were utilized for *Nr3c1* and *Actb* (beta actin). Each assay plate contained a non-template control, positive control derived from pooled adult mouse brain tissue, and a beta actin internal standard. $^{\Delta\Delta}$Ct values were calculated for each

animal at every gene tested, and results are expressed relative to gene expression of the vehicle/vehicle control group.

Data analysis pipeline

Sequences were aligned to the bisulfite-converted NCBI GRCm38 reference genome using the BS-Seeker2 pipeline and Bowtie2 alignment tool [240-242]. Briefly, Illumina adapter sequences were trimmed, paired-end reads for each sample were aligned, and then the unaligned sense and antisense sequences were aligned and both sets of files merged via SAMtools [243]. Methylation was called on the merged file via BSseeker2, and total number of reads, number of methylated reads, and percentage methylation were reported. CpG sites with coverage below 250x were removed. To compare methylation at individual loci across exposure groups we used the R/Bioconductor package limma [244]. This package fits a linear model for each CpG site and exposure group, and then develops an empirical Bayes estimation to assess whether there is a significant difference between groups. All analyses were stratified by offspring sex, since previous studies of deltamethrin and stress exposure observed sex-specific effects [67, 136]. The Benjamini-Hochberg correction was used to adjust for multiple comparisons and an adjusted p-value was calculated for methylation at each CpG site.

RESULTS

Dual exposure to deltamethrin and CORT is associated with increased methylation of *Nr3c1* in males, but not females.

After quality control, 24 CpG sites met our criteria and represented 5 genes: *Nr3c1, Nurr1, Pitx3, Dat1, Th,* and *Vmat2*. There was a significant increase in average percent methylation of a CpG site at chr18: 39489427 (*Nr3c1*) in males exposed to deltamethrin and CORT compared to unexposed males (1.57% ± 1.51% vs. 3.00% ± 2.19%, adjusted p-value = 1.53×10^{-10}) (Figure 3.2). There was no significant difference in methylation at this site in any of the other groups or in females (Supplemental Table 3.3). There were also no significant differences in average methylation across any of the other CpG sites studied. The *Nr3c1* CpG site falls within the NR3C1_6 amplicon. It also resides within a CpG island containing 190 CpGs present in the promoter region of *Nr3c1* (Figure 3.2A). A scatterplot representation of individual sample methylation values is shown in Figure 3.2B, and also highlights that, interestingly, a proximal CpG site we also tested did not express any observable methylation.

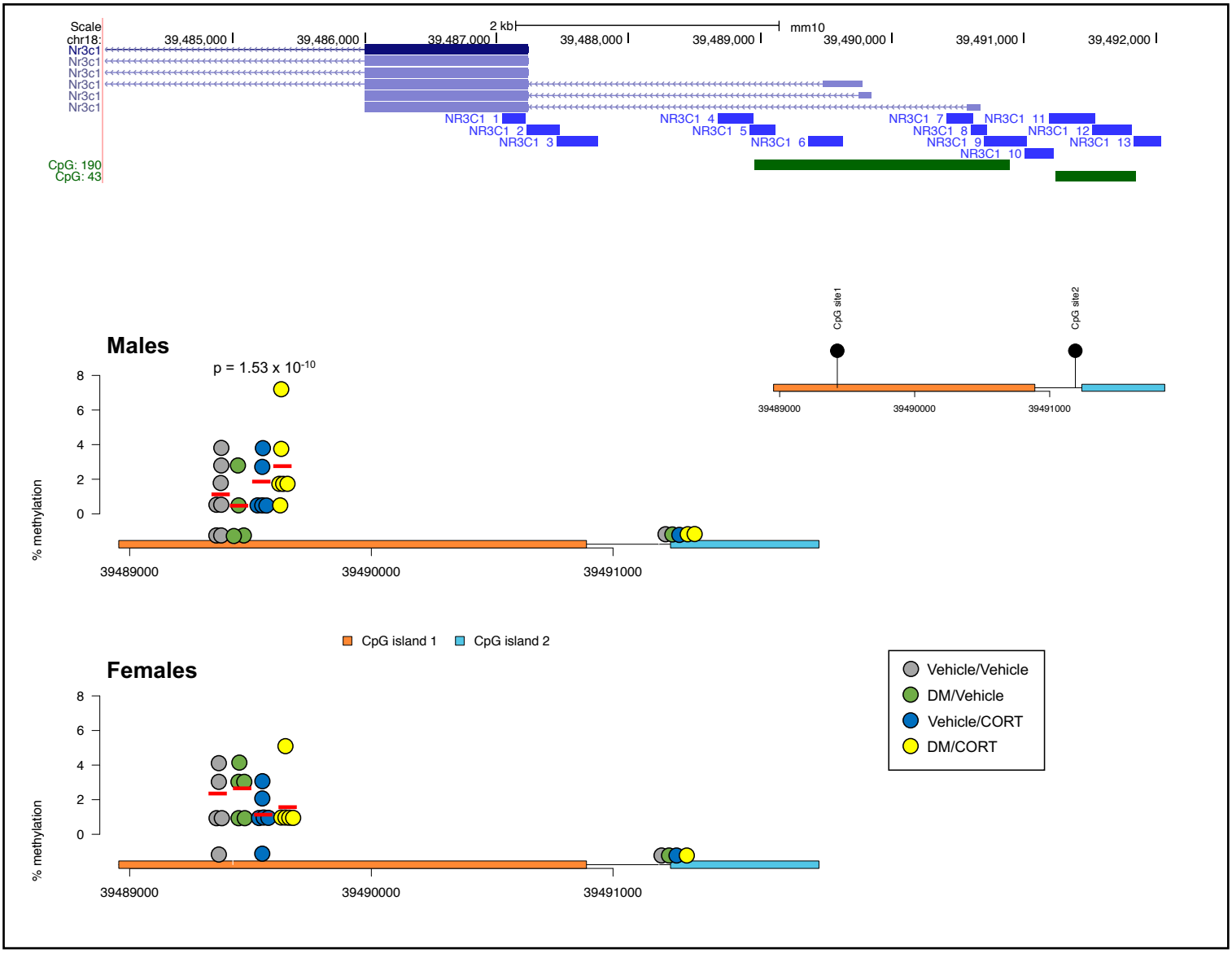
Figure 3.2: Midbrain DNA methylation at *Nr3c1*

A) Location of regions of interest and CpG islands assessed in *Nr3c1*.

Image obtained from http://genome.ucsc.edu, GRCm38/mm10. NR3C1 = Nuclear Receptor Subfamily 3 Group C Member 1 [245]

B) Scatterplot representation of percent methylation of each sample.

Red lines indicate average percent methylation for group. Empirical Bayes testing followed by Benjamini-Hochberg correction were conducted and stratified by sex. For males, n = 5-7. For females, n = 6. DM = deltamethrin. Circles represent individual samples with sequencing coverage of at least 250x.

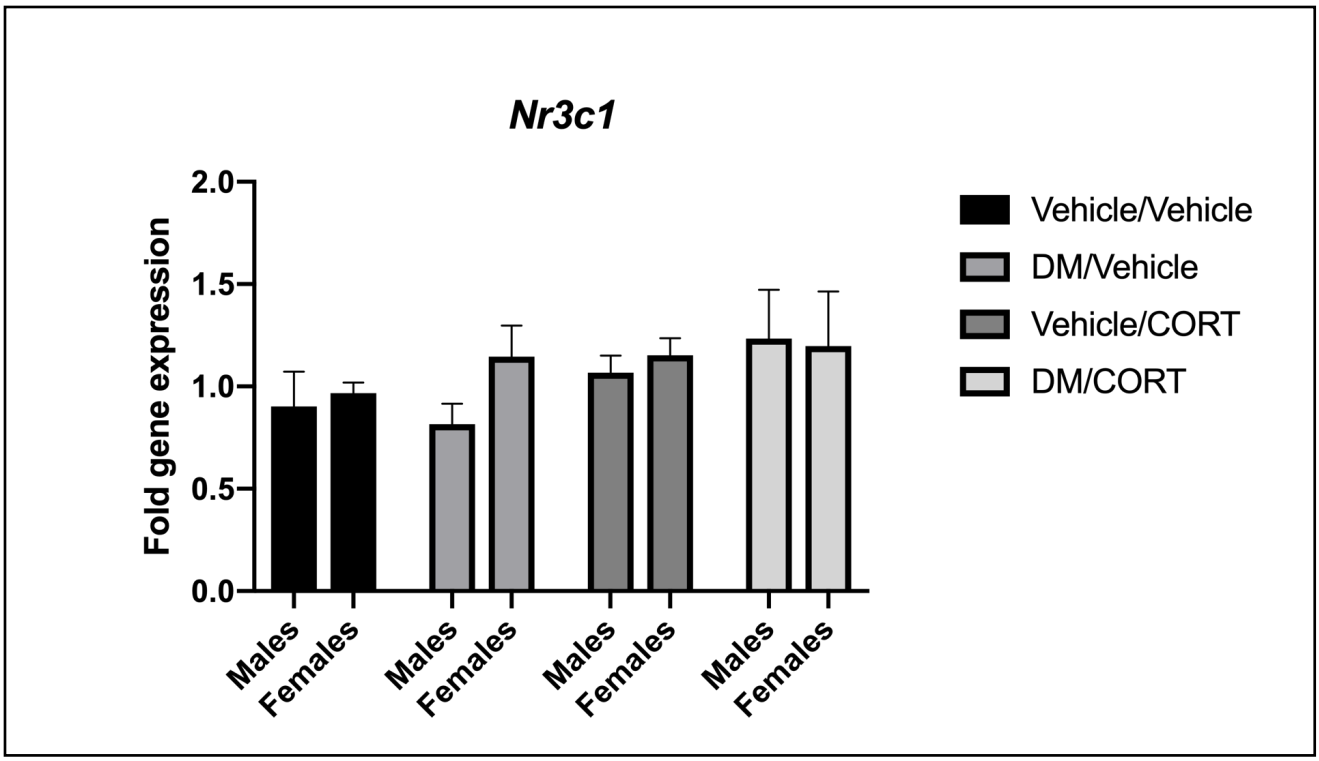

Scale
chr18:
39,485,000
39,486,000
39,487,000
2 kb
39,488,000
39,489,000
mm10
39,490,000
39,491,000
39,492,000
Nr3c1
NR3C1 1
NR3C1 2
NR3C1 3
NR3C1 4
NR3C1 5
NR3C1 6
NR3C1 7
NR3C1 8
NR3C1 9
NR3C1 10
NR3C1 11
NR3C1 12
NR3C1 13
CpG: 190
CpG: 43
CpG site1
CpG site2
39489000
39490000
39491000
Males
p = 1.53 x 10-10
% methylation
CpG island 1
CpG island 2
Females
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT

Developmental exposure to deltamethrin and CORT does not significantly alter midbrain *Nr3c1* RNA expression.

Given the observed changes in DNA methylation in the midbrain we then examined whether mRNA expression would concomitantly be affected. Interestingly, we did not observe any significant differences in midbrain *Nr3c1* RNA expression in males or females in any of the exposure groups via two-way ANOVA (Figure 3.3). There were also no significant differences between expression values in males versus females. A spearman correlation analysis was also conducted to determine whether DNA methylation and RNA expression of *Nr3c1* of each midbrain sample were correlated. We did not observe any significant correlation between the two (data not shown).

Figure 3.3: Gene expression in adult midbrain.

There is no significant difference in midbrain *Nr3c1* expression across all groups in males and females. Data is expressed as $2^{\Delta\Delta Ct}$ analysis of qPCR data and compared to expression in vehicle/vehicle group. Exposure group differences were assessed via two-way ANOVA. *= $p<0.05$, n=5-8, and error bars represent SEM.


Nr3c1
Fold gene expression
2.0
1.5
1.0
0.5
0.0
Males
Females
Males
Females
Males
Females
Males
Females
Vehicle/Vehicle
DM/Vehicle
Vehicle/CORT
DM/CORT

DISCUSSION

Summary of results

We utilized a novel combined neurodevelopmental exposure paradigm to model how environmental exposures known to increase risk of ADHD independently might affect DNA methylation of ADHD-relevant genes, when these exposures are combined. We hypothesized that, due to the known dopaminergic effects of both deltamethrin and CORT, we would observe synergistic effects in genes important for proper dopaminergic development, such as the *Nurr1* and *Pitx3* transcription factors as well as key dopaminergic components such as *Comt*, *Dat1*, *Drd4*, *Th*, and *Vmat2*. We assessed DNA methylation in adult offspring exposed to deltamethrin during gestation and weaning and to oral CORT during adolescence utilizing a recently-developed targeted next-generation sequencing protocol. We measured DNA methylation in the midbrain because this is where the majority of the brain's dopamine cell bodies reside, and we hypothesized we would be most likely to observe whether synergistic effects of deltamethrin and CORT exposure happen in the dopamine system. We did not observe differential DNA methylation in any of our targeted CpG sites in the dopaminergic *Dat1*, *Th*, *Vmat2*, *Drd4* genes, nor in the *Nurr1* and *Pitx3* transcription factors. Additionally, we examined whether DNA methylation of the gene encoding *Nr3c1*, the CORT-responsive glucocorticoid receptor, would be affected in this dopaminergic brain region. There was a significant increase in DNA methylation at a CpG that resides within a CpG island in the 5' region of the *Nr3c1* gene in males exposed to both deltamethrin and CORT, but not either exposure by itself. We did not observe this in females, indicating that this effect is sex-specific. Given the observed change in DNA methylation at *Nr3c1* we then tested whether this increase in DNA methylation would lead to a decrease in *Nr3c1* mRNA expression in the same region. Interestingly, we did not observe a

significant decrease or increase in *Nr3c1* mRNA expression in the midbrain. We also did not observe a significant correlation between DNA methylation and mRNA expression levels from each midbrain sample in any of the exposure groups in males or females.

Possible explanations

Effects of developmental deltamethrin and CORT exposure on dopaminergic protein expression and functional dopamine outputs have been documented independently [67, 136, 246]. However, effects of these exposures, either alone or in combination, on DNA methylation of dopaminergic genes and the glucocorticoid receptor have not been described in male and female mice. We sought to investigate whether there were persistent effects on DNA methylation after these neurodevelopmental exposures, so we assessed methylation at 8-10 weeks of age. It is possible that we did not see significant differences in methylation in any of the studied dopaminergic genes or in *Nr3c1* in females because alterations in methylation did not persist into adulthood. It is also possible that while increased DNA methylation of *Nr3c1* in males exposed to deltamethrin and CORT endured, corresponding *Nr3c1* RNA transcript expression changes were not persistent, and this is why we did not detect them. Furthermore, while most studies of environmental epigenetic mechanisms have focused on DNA methylation, additional epigenetic regulatory mechanisms such as miRNA expression or histone modification exist and could also be altered in our exposure scenario [247].

Cell-to-cell heterogeneity poses a unique challenge in studies of DNA methylation [248], and particularly within solid tissues such as the brain where differences in cell composition could greatly affect detectable epigenetic marks [249]. We extracted both DNA and mRNA from the entire midbrain instead of cutting it in half for each individual isolation, in an attempt to reduce

some of this variability. However, while the midbrain is primarily dopaminergic, additional cell types such as GABAergic populations [250] and various glial populations [251] are present. This cell-to-cell heterogeneity could have contributed to the variability we observed in our DNA methylation results.

Potential mechanisms

Effects of CORT and stress exposure on *Nr3c1* methylation have been studied extensively in humans by our group as well as others [152, 238, 252-258] and early life stress in a mouse model led to hypermethylation of a CpG island shore proximal to the *Nr3c1* promoter in the hypothalamus [259]. However, this is the first description of *Nr3c1* hypermethylation within the dopaminergic midbrain in response to deltamethrin and CORT. Interestingly, there was no hypermethylation in the CORT-only exposed males or females, nor in the deltamethrin-only exposed males or females. Perhaps, combined effects of deltamethrin and CORT on *Nr3c1* in this region were necessary to achieve a detectable methylation difference. Glucocorticoid receptors are present in the midbrain's substantia nigra and ventral tegmental area and glucocorticoid receptors are expressed in dopaminergic neurons in these areas [137]. In vitro studies also show that pyrethroid insecticides antagonize the glucorticoid receptor [260]. It is therefore possible that *Nr3c1* hypermethylation occurs due to direct effects of deltamethrin and CORT on glucocorticoid receptor expression, or through indirect effects of deltamethrin and CORT on dopaminergic neurons that express the glucocorticoid receptor.

We observed *Nr3c1* hypermethylation in males exposed to deltamethrin and CORT but not females. In our previous study of developmental deltamethrin, we did not observe dopaminergic effects of deltamethrin in females either [67]. Moreover, this same sex-specific

effect is seen in an epidemiologic study of children with ADHD who were exposed to pyrethroid insecticides [86]. ADHD also affects males significantly more than females in the general population and this could possibly be due to variability in the dopamine system [261, 262]. Some studies indicate that estrogen may protect dopaminergic neurons from toxicant insults [263-266]. Possibly, the deltamethrin exposure in females did not sufficiently alter glucocorticoid receptor expression due to a lack of dopaminergic effects in the midbrain.

The HPA axis, dopamine, and ADHD

While glucocorticoid function has not been studied as extensively in ADHD development as dopamine function, one study of a rat ADHD model showed that administration of a glucocorticoid receptor agonist (dexamethasone) led to increased dopamine in the striatum and prefrontal cortex. Dexamethasone also ameliorated hyperactivity behavior and inattention measured via a Y-maze assay [267]. Lastly, plasma corticosterone in an ADHD rat model and plasma cortisol in boys with ADHD was significantly lower [268], further implicating the HPA axis and glucocorticoid response in ADHD pathogenesis. In humans, we previously illustrated that increased *NR3C1* methylation is associated with early neurodevelopmental indicators in infants. First, *NR3C1* hypermethylation is associated with altered infant cry acoustics, a measure of stress responsiveness and neurological status [254]. Additionally, we showed that there is a significant positive association between placental *NR3C1* promoter methylation and infant movement and attention measured via the NICU Network Behavioral Scales [269]. Functional polymorphisms in NR3C1 have also been implicated in differential ADHD outcomes. In a cohort of children with ADHD, functional *NR3C1* polymorphisms affected ADHD symptom severity [270]. Another study revealed that functional polymorphisms in NR3C1 mediate

methylphenidate treatment response in children with ADHD [271]. Thus, both rodent and human studies provide evidence that functioning of the glucocorticoid receptor and HPA axis are important in both dopaminergic function and ADHD.

Future directions, impact

To measure DNA methylation we used a recently-developed targeted next-generation sequencing approach first described by Bernstein et al. [239]. While this BisPCR^2 method has been utilized in human tissue samples and cell lines [272-275], zebrafish and sea bass [276-278], and a mouse model of intestinal cancer [279, 280], this is the first described use of the method in a mouse model of brain development. Mouse models of neurodevelopment are particularly tractable for studies of environmental exposure and neuropsychiatric disease. Epigenetic mechanisms are important intermediates for environment x gene interactions and this targeted next-generation sequencing approach provides a cost- and time-efficient method of study. In addition, this exposure paradigm establishes a novel exposure approach that is more relevant to environmental exposure in vulnerable populations than individual exposure models utilized to date. Results of our study provide possible connections between environmental exposures that impact the dopamine system and the HPA axis and contribute to our current understanding of the complex etiopathogenesis of ADHD.

ACKNOWLEDGEMENTS

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SUPPLEMENTAL MATERIALS

Supplemental Table 3.1: Loci of interest and BisPCR2 primers

Target	ROI (UCSC mm10)	Prime	Primer name
COMT	chr16 18425236-18427661	ACACTCTTTCCCTACACGACGCTCTTCCGATCTT AGATTTTTGAGTTTAAGGTTAGTT	Ms.COMT_F3
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAAATTACTCTAAAAATACACCC	Ms.COMT_R3
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTT TGTGGGATTATGGGAATTAGTTTA	Ms.COMT_F4
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATCCTTACTAACCCTCCAAACCTTA	Ms.COMT_R4
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTT TTTAGTGTTTTTGAGTTTTAAGGG	Ms.COMT_F5
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAACACATATAAAAAAAATCTAATCTTAACT	Ms.COMT_R5
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTT AATGGGTTAGGTTTTGGATGTG	Ms.COMT_F6
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAAAACAACAAACCAAAATAATTCATA	Ms.COMT_R6
DAT	chr13 73535639-73537643	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTTTTGAAGTAGGTTGATTGGAAG	Ms.DAT_F3
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCTAACCAAATCACAACAAATCTC	Ms.DAT_R3
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGATTTGTTGTGATTTGGTTAGGA	Ms.DAT_F4
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACATAAATCTCCCAACAACTAATTTC	Ms.DAT_R4
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTAGTTGTTGGGAGATTTATGTAGG	Ms.DAT_F5
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAACCAAAAACCACTAAACCTAAAC	Ms.DAT_R5
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTTGTTTAGGTTTAGTGGTTTTTG	Ms.DAT_F6
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATCTTAATCCTTACCTACCTCCAAC	Ms.DAT_R6
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTGGAGGTAGGTAAGGATTAAGAT	Ms.DAT_F7
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCATATCCCCCTAAAAAACAACAAC	Ms.DAT_R7
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTATGGGTTTTTGGGGTTATTTTTATA	Ms.DAT_F8
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCAACTCTCTCACAAACACAAATAC	Ms.DAT_R8

DRD4	chr7 141290934-141295185	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGGGTTTTGGAGGTGTTAATTATTA	Ms.DRD4_F3
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTACTCCTACACTAAAATCCTTCCCA	Ms.DRD4_R3
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGTGGTTTGTTTTTTGGTTAGGAT	Ms.DRD4_F4
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTCCCCATAATACCACTACTAAACC	Ms.DRD4_R4
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAGTGGTATTATGGGGAATAGTAG	Ms.DRD4_F5
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTCAAAATAACTAAAAATCCAAAC	Ms.DRD4_R5
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTTATTTTGAGTTAGGGTGTTTGTAG	Ms.DRD4_F6
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATATTCCCAAAAAACAATAATCATC	Ms.DRD4_R6
NR3C1	chr18 39487955-39491890	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAATTTTTTGTTGTTTGGAATTTGT	Ms.NR3C1_F1
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAATCCTTAACTCCCCCTAATAAAA	Ms.NR3C1_R1
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGGAGTTTATTGGTAAATATTAATTATAA	Ms.NR3C1_F2
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAAAATAAACAACCAAATTTAAATC	Ms.NR3C1_R2
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAATTTGGTTGTTTATTTTGTTATT	Ms.NR3C1_F3
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATTCTTCAAAATTATACCTTAAAAAATTTA	Ms.NR3C1_R3
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTATGTTTAGGGTTTTAAGTGGTAAGGT	Ms.NR3C1_F4
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCAACAAATCTAAACACATTTCTCC	Ms.NR3C1_R4
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGAAGGGAGAAATGTGTTTAGATT	Ms.NR3C1_F5
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAACTTTTTCCCCCTAAAAAAAA	Ms.NR3C1_R5
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTTGGTAAAAGTTTGTTAAGTTT	Ms.NR3C1_F6
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTAAAAACTCTCCCCTCCCC	Ms.NR3C1_R6
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGTGTTATTTTAGTAGAGGGGTTA	Ms.NR3C1_F7
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAATAAAAAACCTAACAACAC	Ms.NR3C1_R7
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGTTGTTAGGTTTTTTATTTT	Ms.NR3C1_F8
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAATAAATTCTACTTTACAACTTCTCCC	Ms.NR3C1_R8
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTGTAAAGTAGAATTTATTTTTTTT	Ms.NR3C1_F9
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTTACTTCTAAACCTACACACACCC	Ms.NR3C1_R9
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGGTGTGTGTAGGTTTAGAAGTAA	Ms.NR3C1_F10
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAAACAAAAAAATCCCTAAAACTCA	Ms.NR3C1_R10
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTTTGAGTTTTAGGGATTTTTTT	Ms.NR3C1_F11

		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATAAAAAAACAAAAATTACTTCCTC	Ms.NR3C1_R11
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATTTTTGTTTTTTTATAGGTGTTAG	Ms.NR3C1_F12
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAAACAACCTTTACATTTTCCATC	Ms.NR3C1_R12
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGATGGAAAATGTAAAGGTTGTTTA	Ms.NR3C1_F13
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTTCATAACTCCTCTCCTAAAAAAAA	Ms.NR3C1_R13
NURR1	chr2 57110607-57117857	ACACTCTTTCCCTACACGA CGCTCTTCCGATCTTTTAGGGTATTTATTAGAAGAAAATTGATA	Ms.NURR1_F4
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAACTAACCAAATAAAACAAAATTCC	Ms.NURR1_R4
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGAATTTTGTTTTATTTGGTTAGTT	Ms.NURR1_F5
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTAAAACCCAACAATATACCCTCAC	Ms.NURR1_R5
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGAGGGTATATTGTTGGGTTTTAG	Ms.NURR1_F6
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAACTCTCCCTCCAATAAAAATCTATAC	Ms.NURR1_R6
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGATTTTTATTGGAGGGAGAGTTT	Ms.NURR1_F7
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACACTCCTATATCTAACTACCAAATAC	Ms.NURR1_R7
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTGGTAGTTAGATATAGGAGTGTT	Ms.NURR1_F8
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAACTTCCAAATACAACATAACCC	Ms.NURR1_R8
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGTTATGTTGTATTTGGAAGTT	Ms.NURR1_F9
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACAACAATCCTCCATTAAAATAAAAAA	Ms.NURR1_R9
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTATTTTAATGGAGGATTGTTGTT	Ms.NURR1_F10
		GTGACTGGAGTTCAGACGTGTGCTCTTCC GATCTCCAAACTAAATATATATCACCCTATTTC	Ms.NURR1_R10
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTTTGGTTAATTGAATATTTTTTTT	Ms.NURR1_F11
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCAACAACTTTCCAACTCTAAATC	Ms.NURR1_R11
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTTAGAGTTGGAAAGTTGTTGAGG	Ms.NURR1_F12
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCAAAAAAACAAAACAAAACAAAAC	Ms.NURR1_R12
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTGTTTTGTTTTGTTTTTTTGAG	Ms.NURR1_F13
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAATAAATCTACATCTACCCAACCC	Ms.NURR1_R13
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTGAAGATTTTATTTAATAGTATTTTAAA	Ms.NURR1_F14
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATTTAATTATCCAAACCTTACTAAC	Ms.NURR1_R14
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTATAGGTTGTTTATTTGTTGGGATAAG	Ms.NURR1_F15
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCAAAACAAACATATTTTCAAAAAC	Ms.NURR1_R15
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTATATGTTTGTTTTGGATATTAATT	Ms.NURR1_F16

NURR1	chr2 57110607-57117857	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTATAACTTAAAAAAACTCCATCTC	Ms.NURR1_R16
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTAAAATTAGTTATTTTAGGTAATTT	Ms.NURR1_F17
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAAAAAAACTCCCACAATTTTAAAA	Ms.NURR1_R17
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTAATGAGAAGTTTGGAAATTTTTGT	Ms.NURR1_F18
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTAACTACCTCCCTCTCCTAC	Ms.NURR1_R18
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTAGGAGAGGGAGGTAGTTAG	Ms.NURR1_F19
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCACCCAAATAAACTACCAAAATAA	Ms.NURR1_R19
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGGTAGTTTATTTGGGTGGATTTT	Ms.NURR1_F20
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTACAAAACACACCTCTACCCTCTC	Ms.NURR1_R20
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTAGTAGAAGTGGAGATAGTTGTTT	Ms.NURR1_F21
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATATCAACTCTATCAAAAATTAACC	Ms.NURR1_R21
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAATTAAGAAGAAAGTATGGAGGGAGA	Ms.NURR1_F22
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCCCTTCCTAATCTTAAAAAAAACC	Ms.NURR1_R22
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTTAAGATTAGGAAGGGATTGAG	Ms.NURR1_F23
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAACTAAAAATAAACAACCCCACAAC	Ms.NURR1_R23
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGTGTGTGTGTGTAATATATATTT	Ms.NURR1_F24
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACAACAACTAAAAAAACAACC	Ms.NURR1_R24
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTATTTTAAGTTTTTTTTAGATGTTG	Ms.NURR1_F25
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTTCTATTTTAAACCCAACCTAACC	Ms.NURR1_R25
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAAATAGAAGTAATTTTAGTTTATAGG	Ms.NURR1_F26
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAAAAAACAACATATCTACTTAACC	Ms.NURR1_R26
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTTTTTTTAGAGTGAGGAAAGAGG	Ms.NURR1_F27
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTACACCTAAAACACATACCAAACTCC	Ms.NURR1_R27
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTTGGAGTTTGGTATGTGTTTTA	Ms.NURR1_F28
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAATCAAACCCCCTACTAACAAAAT	Ms.NURR1_R28
	chr2 57122389-57125335	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGATATTGATATTTTGAGAAGAAAAA	Ms.NURR1_F31
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAAAAAATAAAAAACACAACTTCC	Ms.NURR1_R31
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAATGGTAATTTTTAAGATTAGTTTTGTTA	Ms.NURR1_F32
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACCATATAATTCCTTTAACCTTTCAC	Ms.NURR1_R32
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTATTTGTATTTTATAGAAATTTAGTTG	Ms.NURR1_F33

NURR1	chr2 57122389-57125335	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATACTTTTAAAAACTTATTAACCCC	Ms.NURR1_R33
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGTTTTTAAAAGTATTTGTTAGGG	Ms.NURR1_F34
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAAAAACTTCAATAAAACCAATCC	Ms.NURR1_R34
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGTTTTTAAAAGTATTTGTTAGGG	Ms.NURR1_F35
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAATTTTCACTAACAATTTCCTATCC	Ms.NURR1_R35
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTATAAAGTTTAGTGTATTTTGGGGATAG	Ms.NURR1_F36
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAAAAAACCCCTTTAAATAACAAC	Ms.NURR1_R36
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTATTTAAAGGGGTTTTTTTTGTAGG	Ms.NURR1_F37
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAACACCCAAAAATTATCAAATAACTT	Ms.NURR1_R37
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTGGGGTGTTGATATATAGAAAATT	Ms.NURR1_F38
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAACACAACTACCAACATAAAAAAAA	Ms.NURR1_R38
PITX3	chr19 46134818-46138182	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTTTGGTTTTTTATGTTATATGGG	Ms.PITX3_F3
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCCCCAAAAATAACATTTCTTAATTAT	Ms.PITX3_R3
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTGTGTGGAAGGATAGAGGTATATATA	Ms.PITX3_F4
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTTTCCTCCAAAATAAAAAAACAAA	Ms.PITX3_R4
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTATTTTGGAGGAAAGGAGTGA	Ms.PITX3_F5
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCCCAAAATCTAAAAAAAATACCAA	Ms.PITX3_R5
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTTGTTTTTGGTTTTAGTTTTAGG	Ms.PITX3_F6
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATAAATAACCCAATCCCCAAC	Ms.PITX3_R6
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGGGGATTGGGTTATTTATAGTAG	Ms.PITX3_F7
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCCCATCCCAAATAACTAATAACT	Ms.PITX3_R7
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGTGGTAGGTAAGGTAGTAGGAG	Ms.PITX3_F8
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTACCACATTCTAACCCTCAAACTC	Ms.PITX3_R8
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGGTTAGAATGTGGTAGGTTATAGG	Ms.PITX3_F9
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAAACCTCCCTCCATAAAATTTAA	Ms.PITX3_R9
	chr19 46146389-46148852	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGAAAATTTATTTTTTGGTTGAGTT	Ms.PITX3_F13
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTATTAACCCACTCAAAACTCCTTAAC	Ms.PITX3_R13
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAATTAGATTAGGAATTTGAGGGTATT	Ms.PITX3_F14
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAACAAAATTCCTCTACAAAACAAC	Ms.PITX3_R14
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTTGTAGAGGAATTTTGTTTTAAGGT	Ms.PITX3_F15

		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAAAAATCCAAATCTAAAACCTCC	Ms.PITX3_R15
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGGAGGTTTTAGATTTGGATTTTTT	Ms.PITX3_F16
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAACTCATACTTTCCTTCCCAACTACT	Ms.PITX3_R16
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGGGAAGGAAAGTATGAGTTATTA	Ms.PITX3_F17
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTAAAAACCTTTACCAAAAAACAC	Ms.PITX3_R17
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAGTTTTAGGATTAGTTTTGGTTTTT	Ms.PITX3_F18
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACACTATAAAACTCACTCTCTCCTCC	Ms.PITX3_R18
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAGGAGAGAGTGAGTTTTATAGTGTAGT	Ms.PITX3_F19
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAAAAACCCAAAACCTCAATTAAC	Ms.PITX3_R19
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAATTGTGGTTAATTGAGGTTTTG	Ms.PITX3_F20
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTCTCCAAATTATCTTCTTTAAAAAAAA	Ms.PITX3_R20
TH	chr7 142893550-142896160	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGGGTTGATTTTTGATGGTTTTAT	Ms.TH_F3
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATACCTCTAACCAAACCCCACTATAC	Ms.TH_R3
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAAGGATTATTTAAAATGTTTGGTGT	Ms.TH_F4
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAACATTAAACTTACATCTCTAAAAACTT	Ms.TH_R4
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTAATTAAATAATTTTTGGTTTTTG	Ms.TH_F5
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTATATTTCAATACACACAATACATCC	Ms.TH_R5
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGAAGGTTAGATTGGTTAGAAAATTA	Ms.TH_F6
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAACCCCTATAACCTACAAAAAAAA	Ms.TH_R6
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTAGGTTATAGGGGTTTAGGGAAT	Ms.TH_F7
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAACTTAAAAAAAACCAAAAAAAC	Ms.TH_R7
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGTTTTTTTTAAGTTTAGTTTTTTTTGTTT	Ms.TH_F8
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAAATCAATATACCCAAACCAATC	Ms.TH_R8
VMAT2	chr19 59259852-59262467	ACACTCTTTCCCTACACGACGCTCTTCCGATCT TTAGTAGGTTTTTGTTGATAATAGG	Ms.VMAT2_F3
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT TAAAATATAAATAAATTAATTAAAAAACAA	Ms.VMAT2_R3
		ACACTCTTTCCCTACACGACGCTCTTCCGATCT GTTTTTTTGGGTTATTTTATGAATATG	Ms.VMAT2_F4
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CCACCCAAAACTACCTCTAAAC	Ms.VMAT2_R4
		ACACTCTTTCCCTACACGACGCTCTTCCGATCT GGAGGTTAGTATTTTAGTTTTTTTT	Ms.VMAT2_F5
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CAAAATTATATCTCCACACTACCC	Ms.VMAT2_R5
		ACACTCTTTCCCTACACGACGCTCTTCCGATCT GGGTAGTGTGGAGATATAATTTTGTAG	Ms.VMAT2_F6

		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT CTCCCCTCAAACACTAAAATAAC	Ms.VMAT2_R6
		ACACTCTTTCCCTACACGACGCTCTTCCGATCT GTTATTTTAGTGTTTGAGGGGAG	Ms.VMAT2_F7
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTAAACAACATATTATCTAACAACAAC	Ms.VMAT2_R7
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTATTTAGTATAGGTTTTTAGGAGTT	Ms.VMAT2_F8
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAAAAAAAACAAACCCACAAATTAC	Ms.VMAT2_R8
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTTTGTAATTTGTGGGTTTGTTTTTTT	Ms.VMAT2_F9
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAATTTATACTATCCCTAAATCCCCC	Ms.VMAT2_R9
		ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGTTAGTTGGGGGTTTTAGTTGTTT	Ms.VMAT2_F10
		GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAAAATTTCTCATTATAACATTCCCTCA	Ms.VMAT2_R10

Supplemental Table 3.2: Barcode Primer Sequences

Index	Barcode	Primer Sequence
Index_1	ATCACG	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGT
Index_2	CGATGT	CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGT
Index_3	TTAGGC	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGT
Index_4	TGACCA	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGT
Index_5	ACAGTG	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGT
Index_6	GCCAAT	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGT
Index_7	CAGATC	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGT
Index_8	ACTTGA	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGT
Index_9	GATCAG	CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTCAGACGTGT
Index_10	TAGCTT	CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTCAGACGTGT
Index_11	GGCTAC	CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTCAGACGTGT
Index_12	CTTGTA	CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGT
Index_13	AGTCAA	CAAGCAGAAGACGGCATACGAGATTTGACTGTGACTGGAGTTCAGACGTGT
Index_14	AGTTCC	CAAGCAGAAGACGGCATACGAGATGGAACTGTGACTGGAGTTCAGACGTGT
Index_15	ATGTCA	CAAGCAGAAGACGGCATACGAGATTGACATGTGACTGGAGTTCAGACGTGT
Index_16	CCGTCC	CAAGCAGAAGACGGCATACGAGATGGACGGGTGACTGGAGTTCAGACGTGT
Index_17	GTAGAG	CAAGCAGAAGACGGCATACGAGATCTCTACGTGACTGGAGTTCAGACGTGT
Index_18	GTCCGC	CAAGCAGAAGACGGCATACGAGATGCGGACGTGACTGGAGTTCAGACGTGT
Index_19	GTGAAA	CAAGCAGAAGACGGCATACGAGATTTTCACGTGACTGGAGTTCAGACGTGT
Index_20	GTGGCC	CAAGCAGAAGACGGCATACGAGATGGCCACGTGACTGGAGTTCAGACGTGT
Index_21	GTTTCG	CAAGCAGAAGACGGCATACGAGATCGAAACGTGACTGGAGTTCAGACGTGT
Index_22	CGTACG	CAAGCAGAAGACGGCATACGAGATCGTACGGTGACTGGAGTTCAGACGTGT
Index_23	GAGTGG	CAAGCAGAAGACGGCATACGAGATCCACTCGTGACTGGAGTTCAGACGTGT

Index_24	GGTAGC	CAAGCAGAAGACGGCATACGAGATGCTACCGTGACTGGAGTTCAGACGTGT
Index_25	ACTGAT	CAAGCAGAAGACGGCATACGAGATATCAGTGTGACTGGAGTTCAGACGTGT
Index_26	ATGAGC	CAAGCAGAAGACGGCATACGAGATGCTCATGTGACTGGAGTTCAGACGTGT
Index_27	ATTCCT	CAAGCAGAAGACGGCATACGAGATAGGAATGTGACTGGAGTTCAGACGTGT
Index_28	CAAAAG	CAAGCAGAAGACGGCATACGAGATCTTTTGGTGACTGGAGTTCAGACGTGT
Index_29	CAACTA	CAAGCAGAAGACGGCATACGAGATTAGTTGGTGACTGGAGTTCAGACGTGT
Index_30	CACCGG	CAAGCAGAAGACGGCATACGAGATCCGGTGGTGACTGGAGTTCAGACGTGT
Index_31	CACGAT	CAAGCAGAAGACGGCATACGAGATATCGTGGTGACTGGAGTTCAGACGTGT
Index_32	CACTCA	CAAGCAGAAGACGGCATACGAGATTGAGTGGTGACTGGAGTTCAGACGTGT
Index_33	CAGGCG	CAAGCAGAAGACGGCATACGAGATCGCCTGGTGACTGGAGTTCAGACGTGT
Index_34	CATGGC	CAAGCAGAAGACGGCATACGAGATGCCATGGTGACTGGAGTTCAGACGTGT
Index_35	CATTTT	CAAGCAGAAGACGGCATACGAGATAAAATGGTGACTGGAGTTCAGACGTGT
Index_36	CCAACA	CAAGCAGAAGACGGCATACGAGATTGTTGGGTGACTGGAGTTCAGACGTGT
Index_37	CGGAAT	CAAGCAGAAGACGGCATACGAGATATTCCGGTGACTGGAGTTCAGACGTGT
Index_38	CTAGCT	CAAGCAGAAGACGGCATACGAGATAGCTAGGTGACTGGAGTTCAGACGTGT
Index_39	CTATAC	CAAGCAGAAGACGGCATACGAGATGTATAGGTGACTGGAGTTCAGACGTGT
Index_40	CTCAGA	CAAGCAGAAGACGGCATACGAGATTCTGAGGTGACTGGAGTTCAGACGTGT
Index_41	GACGAC	CAAGCAGAAGACGGCATACGAGATGTCGTCGTGACTGGAGTTCAGACGTGT
Index_42	TAATCG	CAAGCAGAAGACGGCATACGAGATCGATTAGTGACTGGAGTTCAGACGTGT
Index_43	TACAGC	CAAGCAGAAGACGGCATACGAGATGCTGTAGTGACTGGAGTTCAGACGTGT
Index_44	TATAAT	CAAGCAGAAGACGGCATACGAGATATTATAGTGACTGGAGTTCAGACGTGT
Index_45	TCATTC	CAAGCAGAAGACGGCATACGAGATGAATGAGTGACTGGAGTTCAGACGTGT
Index_46	TCCCGA	CAAGCAGAAGACGGCATACGAGATTCGGGAGTGACTGGAGTTCAGACGTGT
Index_47	TCGAAG	CAAGCAGAAGACGGCATACGAGATCTTCGAGTGACTGGAGTTCAGACGTGT
Index_48	TCGGCA	CAAGCAGAAGACGGCATACGAGATTGCCGAGTGACTGGAGTTCAGACGTGT

Supplemental Table 3.3: limma analysis results

Chromosome	Gene	CpG site	Average % methylation	Unadjusted p-value	Adjusted p-value
Females					
18	NR3C1	39489427	0.02	0.36	1.00
19	PITX3	46135545	0.00	1.00	1.00
19	PITX3	46136645	0.00	1.00	1.00
19	PITX3	46137299	0.00	1.00	1.00
19	PITX3	46146677	0.00	1.00	1.00
19	PITX3	46147262	0.00	1.00	1.00
19	PITX3	46147738	0.00	1.00	1.00
2	NURR1	57110993	0.00	1.00	1.00
2	NURR1	57111690	1.00	1.00	1.00
2	NURR1	57112163	0.00	1.00	1.00
2	NURR1	57112879	0.00	1.00	1.00
2	NURR1	57113842	0.00	1.00	1.00
2	NURR1	57124422	0.00	1.00	1.00
2	NURR1	57124777	0.00	1.00	1.00
19	VMAT2	59261587	0.00	1.00	1.00
19	VMAT2	59261588	0.00	1.00	1.00
19	VMAT2	59261759	0.00	1.00	1.00
13	VMAT2	73536419	0.00	1.00	1.00
13	VMAT2	73537270	1.00	1.00	1.00
7	TH	142893979	0.00	1.00	1.00
18	NR3C1	39491190	0.00	NA	NA
2	NURR1	57111654	NA	NA	NA
2	NURR1	57113379	0.00	NA	NA

2	NURR1	57125080	0.00	NA	NA

Chromosome	Gene	CpG site	Average % methylation	Unadjusted p-value	Adjusted p-value
Males					
18	NR3C1	39489427	0.02	0.00	0.00
19	PITX3	46135545	0.00	1.00	1.00
19	PITX3	46137299	0.00	1.00	1.00
19	PITX3	46146677	0.00	1.00	1.00
19	PITX3	46147262	0.00	1.00	1.00
19	PITX3	46147738	0.00	1.00	1.00
2	NURR1	57110993	0.00	1.00	1.00
2	NURR1	57111690	1.00	1.00	1.00
2	NURR1	57112163	0.00	1.00	1.00
2	NURR1	57112879	0.00	1.00	1.00
2	NURR1	57113842	0.00	1.00	1.00
2	NURR1	57124422	0.00	1.00	1.00
2	NURR1	57124777	0.00	1.00	1.00
19	VMAT2	59261588	0.00	1.00	1.00
19	VMAT2	59261759	0.00	1.00	1.00
13	VMAT2	73536419	0.00	1.00	1.00
13	VMAT2	73537270	1.00	1.00	1.00
7	TH	142893979	0.00	1.00	1.00
18	NR3C1	39491190	0.00	NA	NA
19	PITX3	46136645	0.00	NA	NA
2	NURR1	57111654	0.00	NA	NA
2	NURR1	57113379	0.00	NA	NA
2	NURR1	57125080	0.00	NA	NA
19	VMAT2	59261587	0.00	NA	NA

CHAPTER 4: Urinary pyrethroid insecticide metabolites and allostatic load are associated with increased ADHD prevalence in children ages 6-18 years in NHANES

ABSTRACT

Attention-Deficit Hyperactivity Disorder (ADHD) is one of the most common neurodevelopmental disorders in the United States. Children with ADHD exhibit inattention, hyperactivity, and impulsivity that influences their ability to function academically, socially, and occupationally in adulthood. Several epidemiologic studies of ADHD have identified environmental exposures that could increase the susceptibility of certain groups to ADHD. Utilizing the National Health and Nutrition Examination Survey (NHANES), we examined whether pyrethroid insecticide and increased stress exposure would increase prevalence of ADHD in children ages 6-18 years living in the United States. To more comprehensively model stress exposure, we developed a novel Allostatic Load Index that encompasses both biological markers of stress reactivity and sociodemographic factors known to increased psychosocial stress. We found that 3-phenoxybenzoic acid (3-PBA) pyrethroid metabolites in the urine were associated with increased prevalence of ADHD, as well as increased allostatic load. Additionally, the presence of 3-PBA above the LOD multiplicatively interacts with allostatic load score to increase prevalence of ADHD. This is the first description of a pediatric allostatic load score in the NHANES dataset and provides a more inclusive understanding of the role of the exposome on children's health.

INTRODUCTION

Attention-Deficit Hyperactivity Disorder (ADHD) affects approximately 7% of children in the United States [1]. Children with ADHD exhibit inattention, hyperactivity, and impulsivity features that can impact their academic, personal, and occupational lives through adulthood.

Although several pharmacologic treatments are currently available, there are known long-term health risks to prolonged use of methylphenidate and amphetamines such as stunted growth, alterations in cortical plasticity, and risk of substance abuse later in life [2-4]. Additional research is therefore needed to further dissect potential mechanisms of ADHD pathogenesis and identify potential preventative strategies for populations that are at increased risk.

Several studies of ADHD risk have identified environmental toxicant exposures that could make specific populations of children particularly susceptible to ADHD. In the National Health and Nutrition Examination Survey (NHANES), our group and others have shown, for example, that exposure to currently-used pyrethroid insecticides, measured via urinary pyrethroid metabolite 3-phenoxybenzoic acid (3-PBA), increases ADHD prevalence [67, 86]. Pyrethroid insecticides are considered safe alternatives to phased-out insecticides including organophosphates, such as DDT and chlorpyrifos, though a large body of epidemiologic, animal model, and in vitro work has now illustrated that they have neurotoxic effects [97, 281-284]. Pyrethroids are commonly used residentially and agriculturally and their primary mechanism of action is blockade of the α subunit of the voltage-gated sodium channel, though type II pyrethroids can also target GABAergic signaling. Other environmental toxicant exposures associated with ADHD include maternal smoking 12-14], bisphenol A [285], organophosphates [286-290], and lead [287]. Interestingly, pyrethroid insecticide metabolites are found in higher concentrations in low-income children [139].

Children of families with socioeconomic disadvantage are also at increased risk, and this relationship has been identified in multiple epidemiologic studies [69, 70, 291-297]. One quasi-experimental epidemiologic study followed up on these mostly correlational results and observed a dose-dependent negative association between family income and risk of ADHD [70].

Potentially, this effect could be mediated by higher levels of psychosocial stress that are often associated with factors affecting low socioeconomic status groups [112, 121, 175]. Psychosocial stress is also associated with an increased risk of ADHD both in children who experience stressful life events [298-300] and in mothers who experience stressful life events or conditions before, during, and after their pregnancy [301]. Early life stress and allostatic load, a measure of how chronic stress affects the body, are associated with additional neurodevelopmental disorders as well [302-304]. In rodent models, chronic stress leads to diverse neurodevelopmental alterations [305-311], including ADHD-like behaviors such as hyperactivity and inattention [136, 312, 313]. Thus, complex and cumulative chemical and psychosocial factors have been shown to increase the risk of ADHD in children.

Bruce McEwen and others have conceptualized the ideas of allostasis and allostatic load to better study the biological effects of environmental stressors in adults. Broadly, allostasis refers to ways in which the body maintains a stable state through physiologic changes. Allostatic load refers to the eventual consequences and imbalances that occur in the body after prolonged adaptation to environmental challenges. Stressors, then, are environmental exposures that induce physiological and behavioral allostatic responses [123, 314-319]. McEwen and colleagues developed a method of indexing allostatic load in humans based on data from the MacArthur Successful Aging study. Their measure of allostatic load includes physiologic responses that occur in response to stressors in the hypothalamic-pituitary-adrenal (HPA) axis, cardiovascular, and metabolic systems [316]. Examples of measures include systolic and diastolic blood pressure, cholesterol levels, waist-hip ratio, urinary cortisol, and urinary catecholamines [316, 320, 321]. Thus, they are able to measure downstream biological effects of various environmental stressors – both at the level of the individual and their broader lived environment.

While plenty of previous studies have examined the effects of chronic stressors and stressful environments in children, few have attempted to modify the allostatic load model parameters for a pediatric population. This may be because children maintain different physiologic parameters throughout their development, making it difficult to ascertain physiologic differences in a study population with an age range analogous to those in studies of adults. Additionally, for many of the physiologic parameters that contribute to the allostatic load score in adults, there are not similar clinical parameters – examples include serum triglycerides, for example (American Academy of Pediatrics). We therefore chose to create our own model of allostatic load in children, based upon physiologically-relevant measurements available in the NHANES dataset as well as sociodemographic information made available via the parent-reported NHANES questionnaire. Instead of controlling for sociodemographic factors that we know to be associated with low socioeconomic status and neurodevelopmental outcomes in children and could confound our results, we added them to our allostatic load score so that they could be included in our assessment. We also looked to previous studies that calculated an allostatic load score in adults exposed to lead in the NHANES cohort [322] and assessed inflammatory markers in children exposed to social adversity prospectively [323].

We hypothesized that exposure to pyrethroid insecticides and chronic psychosocial stress, represented by allostatic load, together would be associated with increased prevalence of ADHD in children 6-18 years in the United States. To study this, we aimed to complete the following objectives in the publicly-available CDC NHANES cohort: a) determine whether presence of pyrethroid metabolites in urine was associated with ADHD prevalence in the studied cohort, b) characterize allostatic load in children as a measure of stress by utilizing relevant

sociodemographic factors and biological markers, and c) determine whether pyrethroid insecticide exposure and allostatic load interact to increase the risk of ADHD.

METHODS

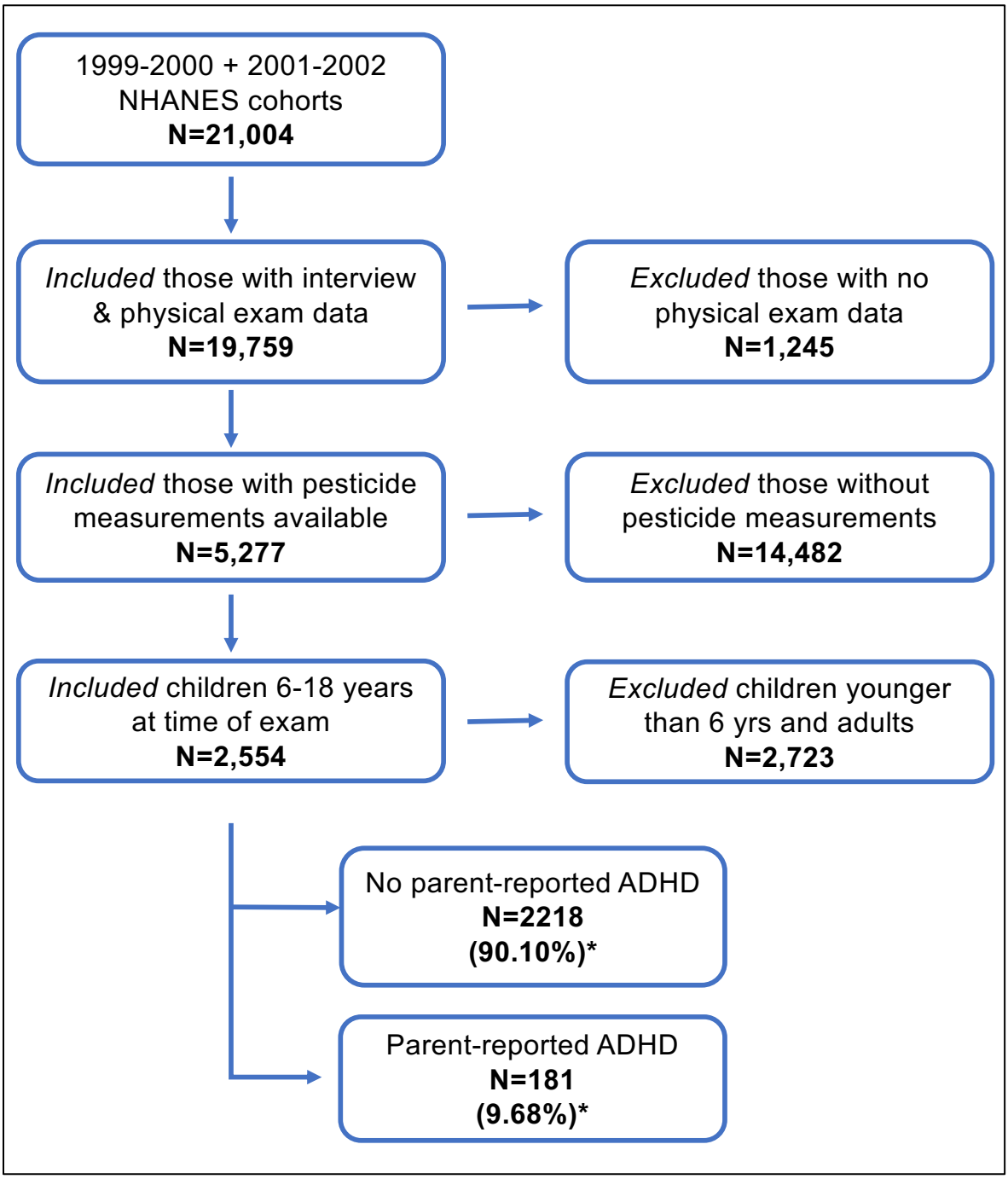
NHANES

The National Health and Nutrition Examination Survey (NHANES) is conducted by the Centers for Disease Control every other year across the United States and utilizes a complex oversampling method to ensure proper sampling of underrepresented groups. Data are publicly available at https://www.cdc.gov/nchs/nhanes/index.htm. While the study is conducted every other year, the same measurements and questionnaire components are not included every cycle. We determined that the only available survey years with usable data on urine pyrethroid metabolite (3-phenoxybenzoic acid) levels and ADHD diagnosis were the 1999-2000 and 2001-2002 cohorts. Only NHANES participants who underwent demographic questionnaire, physical exam, and pesticide measurement assessments were included and children had to be 6-18 years at the time of the physical exam (Figure 4.1). Of the 2,554 eligible children, complete data were available for 2,404 of them. When weighted according to guidelines from the National Center for Health Statistics, the 2,404 subjects represent 53,629,318 children in the United States.

Figure 4.1: Flowchart of study population selection.

The only available survey years with usable data on urine pyrethroid metabolite (3-phenoxybenzoic acid) levels and ADHD diagnosis were the 1999-2000 and 2001-2002 cohorts. Only NHANES participants who underwent demographic questionnaire, physical exam, and pesticide measurement assessments were included and children had to be 6-18 years at the time of the physical exam. Of the 2,554 eligible children, complete data were available for 2,404 of them. When weighted according to guidelines from the National Center for Health Statistics, the 2404 subjects represent 53,629,318 children in the United States.

Reported sample size is unweighted whereas the proportions marked with an * are weighted to account for survey sampling methods.


1999-2000 + 2001-2002
NHANES cohorts
N=21,004
Included those with interview & physical exam data
N=19,759
Excluded those with no physical exam data
N=1,245
Included those with pesticide measurements available
N=5,277
Excluded those without pesticide measurements
N=14,482
Included children 6-18 years at time of exam
N=2,554
Excluded children younger than 6 yrs and adults
N=2,723
No parent-reported ADHD
N=2218
(90.10%)*
Parent-reported ADHD
N=181
(9.68%)*

Development of Allostatic Load Index

We developed a composite measure of allostatic load based on prior studies of early life stress in children and adults (Table 4.1). Most of these studies utilize adult physiologic parameters that do not necessarily have relevant pediatric analogs. We attempted to substitute these physiologic indicators with guidelines from the American Academy of Pediatrics and the American Academy of Family Physicians wherever possible, to better reflect potential stress responses in children. Each component of the allostatic load was coded as an ordinal variable and scored dependent on previous studies and clinical guidelines wherever possible. Family Poverty-Income Ratio was separated into tertiles (Low, Moderate, High), maternal age at child's birth was separated into weighted quartiles, and serum C-reactive protein was separated into weighted tertiles.

Table 4.1: Allostatic Load Parameters

Abbreviations: HS (high school), GED (General Educational Development), HbA1C (glycated hemoglobin)

Percentiles for maternal age and serum C-reactive protein were calculated using sample weights provided by the National Center for Health Statistics. Plasma HbA1c, serum triglyceride, and total serum cholesterol were categorized based on clinical lab values outlined by the AAP (American Academy of Pediatrics) and AAFP (American Academy of Family Physicians).

Table 4.1: Allostatic Load Index Parameters

Parameter		3-phenoxybenzoic acid		Reference
		Below LOD	Above LOD	
	N	% (SE)	% (SE)	
Family Poverty-Income Ratio				Freeman and Corey 1993
0: Low (PIR ≥ 1.5)	1088		72.86 (3.23)	
1: Moderate (PIR 1-1.5)	354		79.03 (3.16)	
2: High (PIR <1)	962		79.37 (2.84)	
Does family own or rent home?				Slopen et al 2015
0: Own	209		87.67 (4.02)	
1: Rent	1414		73.40 (2.83)	
Covered by health insurance?				Wagner-Schuman et al 2015, Slopen et al 2015
0: Yes	1890		74.85 (2.67)	
1: No	482		80.30 (3.81)	
Maternal age at birth (weighted quartiles)				Slopen et al 2015
0: 30-44 years	178		60.18 (10.73)	
1: 25-30 years	210		79.30 (5.23)	
2: 21-25 years	247		74.86 (5.80)	
3: <21 years	1769		77.03 (2.74)	
Reference Parent's Education Level				Slopen et al 2015
0: More than HS	855		70.43 (3.38)	
1: HS graduate or GED	546		77.00 (4.34)	
2: Less than HS	1003		83.60 (1.88)	
Parent marital status				Slopen et al 2015
0: Married	1238		71.79 (3.19)	
1: Single parent	316		79.54 (4.34)	
2: Divorced, separated, widowed	850		81.09 (2.51)	
Mother smoked when pregnant?				Slopen et al 2015, Wagner-Schuman et al 2015, Stroud et al 2014
0: No	1581	25.69 (2.83)	74.31 (2.83)	
1: Yes	265	19.25 (4.44)	80.75 (4.44)	
Serum C-Reactive Protein (weighted tertiles)				McEwen 2015, Zota et al. 2013
0: ≤ 0.01 mg/dL	1709	24.24 (2.80)	75.76 (2.80)	
1: 0.011-0.08 mg/dL	320	26.32 (4.83)	73.68 (4.83)	
2: ≥ 0.08	375	22.96 (4.15)	77.04 (4.15)	
Plasma HbA1C				Zota et al 2013
0: Normal (<5.7%)	2387	24.44 (2.61)	75.66 (2.61)	
1: Pre-diabetes (5.7-6.49%)	13	8.45 (6.32)	91.55 (6.32)	
2: Diabetes (>6.5%)	4	10.29 (11.14)	89.71 (11.14)	
Serum triglycerides				Zota et al 2013, AAP guidelines
0: Acceptable (<150 mg/dL)	2069	25.05 (2.67)	74.95 (2.67)	
1: Borderline (90-129 mg/dL)	200	17.01 (3.93)	82.99 (3.93)	
2: High (>130 mg/dL)	135	23.69 (5.09)	76.31 (5.09)	
Total cholesterol				McEwen 2015, AAFP guidelines
0: Acceptable (<170 mg/dL)	2015	25.38 (2.88)	74.62 (2.88)	
1: Borderline (170-199 mg/dL)	273	17.66 (4.12)	82.34 (4.12)	
2: Elevated (>200 mg/dL)	111	18.76 (3.13)	81.24 (3.13)	

Pyrethroid insecticide metabolite measurement

Urine samples were collected from children six years and above, and were processed by the Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention. The pyrethroid metabolite, 3-phenoxybenzoic acid (3-PBA), was measured via high-performance liquid chromatography/electrospray chemical ionization/tandem mass spectrometry [178, 324]. The limit of detection (LOD) of the urinary 3-PBA metabolite was 0.1 μg/L.

Data analysis

Model 1: Association between urinary pyrethroids and caretaker-reported ADHD

We utilized logistic regression to assess the association between self-reported ADHD and urinary pyrethroid metabolites. Since urinary 3-PBA measurements were right-skewed and not normally distributed, we dichotomized the exposure variable into above-LOD and below-LOD categories, as in previous studies [67, 86]. Adjustment for potential confounders included any factors that were associated with pyrethroid insecticide exposure and neurodevelopmental outcomes in prior studies [67, 86, 292, 325]. Included confounders were: gender, child age at time of exam, self-reported race/ethnicity, family poverty-income ratio quintile, health insurance status at time of exam, maternal smoking, urine creatinine, urine 3-PBA, $\log_{10}$-transformed urine organophosphate pesticide metabolite (DMAP), and $\log_{10}$-transformed blood lead levels.

Model 2: Association between allostatic load and caretaker-reported ADHD

We utilized logistic regression to assess the association between allostatic load score and self-reported ADHD, while adjusting for presence of urine 3-PBA. The allostatic load score was

coded as an ordinal variable. We adjusted for child gender, child age at time of exam, self-reported race/ethnicity, urine creatinine, dichotomized urinary 3-PBA pyrethroid metabolite, $\log_{10}$-transformed urine organophosphate pesticide metabolite (DMAP), and $\log_{10}$-transformed blood lead levels.

Model 3: Association between allostatic load quartiles and caretaker-reported ADHD

We utilized logistic regression to assess the association between allostatic load score and self-reported ADHD, while adjusting for presence of urine 3-PBA. The allostatic load score variable was divided into "Minimal", "Low", "Medium", and "High" based on quartile thresholds. We adjusted for child gender, child age at time of exam, self-reported race/ethnicity, urine creatinine, dichotomized urinary 3-PBA pyrethroid metabolite, $\log_{10}$-transformed urine organophosphate pesticide metabolite (DMAP), and $\log_{10}$-transformed blood lead levels.

Model 4: Multiplicative interaction between allostatic load and urine 3-PBA in caretaker-reported ADHD

To test the multiplicative interaction between allostatic load and presence of urine 3-PBA, we performed a logistic regression with allostatic load score as an ordinal variable and urine 3-PBA dichotomized as previously described in Model 1. We adjusted for child gender, child age at time of exam, urine creatinine, $\log_{10}$-transformed urine organophosphate pesticide metabolite (DMAP), and $\log_{10}$-transformed blood lead levels.

Model 5: Multiplicative interaction between allostatic load quartiles and urine 3-PBA in caretaker-reported ADHD

To test the multiplicative interaction between allostatic load and presence of urine 3-PBA, we performed a logistic regression. The allostatic load score variable was divided into "Minimal", "Low", "Medium", and "High" based on quartile thresholds and urine 3-PBA dichotomized as previously described in Model 1. We adjusted for child gender, child age at time of exam, urine creatinine, $\log_{10}$-transformed urine organophosphate pesticide metabolite (DMAP), and $\log_{10}$-transformed blood lead levels.

All analyses were conducted using SAS 9.4 and SUDAAN 11.0.1 software and National Center for Health Statistics sample weights were used.

RESULTS

Descriptive statistics

Of 2,404 eligible children ages 6-18 years with complete data available, caretakers of 9.68% (n = 181) of children reported a prior diagnosis of ADHD. A slightly higher proportion of children with urinary 3-PBA levels above the LOD identified as "non-Hispanic Black" or "Other Hispanic", did not have health insurance, were covered by Medicaid/CHIP, and had mothers who smoked at some point during pregnancy (Table 4.2). Males were 3 times more likely to be diagnosed with ADHD than females (OR = 3.08 95% CI = 1.97, 4.83), which aligns with national data that indicate there is increased prevalence of ADHD amongst boys compared to girls [291] (Table 4.2).

Table 4.2: Demographics of NHANES subset

Abbreviations: LOD (limit of detection), SE (standard error), ADHD (attention-deficit hyperactivity disorder)

All estimates are adjusted for survey design and sample weight. Maternal smoking was defined as any smoking, at any point during pregnancy. When weighted, the 2404 subjects represent 53,629,318 individuals.

Table 4.2: Demographics of NHANES subset

Characteristic	Total (N = 2404)	3-phenoxybenzoic acid	
		Below LOD (N = 542) % (SE)	Above LOD (N = 1862) % (SE)
Gender			
Female		24.15 (2.57)	75.85 (2.57)
Male		24.50 (3.19)	75.50 (3.19)
Caretaker-reported ADHD			
Yes		21.27 (4.29)	84.23 (4.27)
No		24.59 (2.60)	74.32 (3.02)
Race/ethnicity			
Non-Hispanic White		26.86 (3.65)	73.14 (3.65)
Non-Hispanic Black		14.47 (2.32)	85.53 (2.32)
Mexican American		26.81 (1.87)	73.19 (1.87)
Other Hispanic		17.52 (4.56)	82.48 (4.56)
Other Race – incl. multi-racial		27.97 (5.87)	72.03 (5.87)
Covered by health insurance?			
Yes		25.15 (2.67)	74.85 (2.67)
No		19.70 (3.81)	80.30 (3.81)
Covered by Medicaid/CHIP?			
Yes		14.01 (2.70)	85.99 (2.70)
No		28.08 (3.02)	71.92 (3.02)
Mother smoked when pregnant?			
No		25.69 (2.83)	74.31 (2.83)
Yes		19.25 (4.44)	80.75 (4.44)

Detectable urinary 3'-PBA and maternal smoking are associated with increased prevalence of ADHD

Children with a urinary 3-PBA measurement above the LOD were two times more likely to have ADHD (OR = 2.03, 95% CI = 1.18, 3.50) (Table 4.3). There was a detectable difference in odds of ADHD diagnosis in any of the race/ethnicity subgroups or in children not covered by health insurance. However, children of mothers who smoked at some point during pregnancy were also twice as likely to be diagnosed with ADHD (OR = 2.24, 95% = 1.34, 3.76) (Table 4.3). Covariates that were included in our models were: gender, child age, self-reported race/ethnicity, family poverty-income ratio, health insurance status at time of exam, maternal smoking, maternal age, urine creatinine, urine 3-PBA, $\log_{10}$-transformed urine organophosphate pesticide metabolite (DMAP), and $\log_{10}$-transformed blood lead levels.

Table 4.3: Presence of detectable urinary 3-phenoxybenzoic acid metabolites is associated with increased prevalence of ADHD

Abbreviations: OR (odds ratio), CI (confidence interval), 3-PBA (3-phenoxybenzoic acid), LOD (limit of detection), DMAP (dimethyl alkylphosphate)

All estimates are adjusted for survey design and sample weight. Model was adjusted for gender, child age, self-reported race/ethnicity, urine creatinine, urine 3-PBA, $\log_{10}$-transformed urine organophosphate pesticide metabolite (DMAP), and $\log_{10}$-transformed blood lead levels.

Table 4.3: Presence of detectable urinary 3-phenoxybenzoic acid metabolites is associated with increased prevalence of ADHD

Characteristic	Adjusted Odds Ratio (95% CI)	Sattherwaite p value
Gender		<0.0001*
Female	Referent	
Male	3.08 (1.97, 4.83)	
Race/ethnicity		0.2087
Non-Hispanic White	Referent	
Non-Hispanic Black	0.73 (0.40, 1.31)	
Mexican American	0.35 (0.16, 0.78)	
Other Hispanic	0.58 (0.20, 1.66)	
Other Race – incl. multi racial	0.57 (0.17, 1.90)	
Covered by health insurance?		0.79
Yes	Referent	
No	1.13 (0.46, 2.74)	
Mother smoked when pregnant?		0.02*
No	Referent	
Yes	2.24 (1.34, 3.76)	
Urine 3-phenoxybenzoic acid		0.01*
Below LOD	Referent	
Above LOD	2.03 (1.18, 3.50)	
Urine DMAP	0.96 (0.55, 1.66)	0.88
Blood lead	0.98 (0.23, 4.11)	0.97

Significant positive association between allostatic load and ADHD

Next, we tested whether allostatic load was associated with increased prevalence of ADHD. Children with a higher allostatic load score were significantly more likely to have ADHD (OR = 1.15, 95% CI = 1.01, 1.31) (Table 4.4) when adjusting for gender, child age, self-reported race/ethnicity, urine creatinine, urine 3-PBA, $\log_{10}$-transformed urine organophosphate pesticide metabolite (DMAP), and $\log_{10}$-transformed blood lead levels. There was no significant interaction between gender and allostatic load score in increasing the odds of having ADHD ($p = 0.263$). When allostatic load score was divided into quartiles, higher allostatic load score was not significantly associated with caretaker-reported ADHD (Table 4.4), when adjusting for the same variables listed above.

Table 4.4: Allostatic load is associated with ADHD prevalence

The overall adjusted odds ratio was calculated using a no-interaction model. Allostatic load score * Gender interaction was then modeled, and an interaction p-value was calculated. All estimates are adjusted for survey design and sample weight. Factors adjusted for include gender, child age, self-reported race/ethnicity, urine creatinine, urine 3-PBA, $\log_{10}$-transformed urine organophosphate pesticide metabolite (DMAP), and $\log_{10}$-transformed blood lead levels.

Table 4.4: Allostatic load is associated with ADHD prevalence

	Adjusted OR (95% CI)	**P-value**
Allostatic load score	1.15 (1.01, 1.31)	0.03*
3-PBA	1.56 (0.85, 2.83)	0.18
Allostatic load score*Gender Interaction		
Boys	Referent	0.26
Girls	1.18 (0.88, 1.58)	
Allostatic load Quartiles Analysis		
Allostatic load quartiles		0.40
Minimal	Referent	
Low	1.30 (0.54, 3.12)	
Medium	1.75 (0.61, 5.01)	
High	2.22 (0.86, 5.78)	

Presence of urinary 3-PBA metabolites significantly interacts with allostatic load to increase ADHD prevalence.

We observed significant multiplicative interaction between urinary 3-PBA and allostatic load score ($p = 0.04$) (Table 4.5). In children with urinary 3-PBA above the LOD, there is a significant association between allostatic load score and prevalence of ADHD (OR = 1.16, 95% CI = 1.02, 1.32), when adjusting for gender, child age, self-reported race/ethnicity, health insurance status at time of exam, maternal smoking, maternal age, urine creatinine, urine 3-PBA, allostatic score group, $\log_{10}$-transformed urine organophosphate pesticide metabolite (DMAP), and $\log_{10}$-transformed blood lead levels. There is not a significant association between allostatic load score and prevalence of ADHD in children with no detectable urinary 3-PBA metabolites (OR = 1.13, 95% CI = 0.96, 1.34).

Table 4.5: Multiplicative interaction of urinary 3-phenoxybenzoic acid and Allostatic Load Index

Multiplicative interaction of 3-phenoxybenzoic acid*allostatic load was modeled. All estimates are adjusted for survey design and sample weight. Factors that were adjusted for include gender, child age, self-reported race/ethnicity, urine creatinine, urine 3-PBA, allostatic score group, log_{10}-transformed urine organophosphate pesticide metabolite (DMAP), and log_{10}-transformed blood lead levels.

Table 4.5: Multiplicative interaction of urinary 3-phenoxybenzoic acid and allostatic load

	Adjusted OR (95% CI)	Interaction P-value
Allostatic load*3-PBA		
3-PBA above LOD	1.16 (1.02, 1.32)	0.04*
3-PBA below LOD	1.13 (0.96, 1.34)	
Allostatic load quartiles*3-PBA		0.43
3-PBA below LOD		
*Minimal allostatic load	Referent	
*Low allostatic load	2.04 (0.21, 19.81)	
*Medium allostatic load	4.07 (0.58, 28.83)	
*High allostatic load	10.39 (1.09, 99.05)	
3-PBA above LOD		
*Minimal allostatic load	3.49 (0.55, 22.00)	
*Low allostatic load	4.27 (0.78, 23.52)	
*Medium allostatic load	5.32 (0.95, 29.89)	
*High allostatic load	6.24 (1.16, 33.62)	

DISCUSSION

Summary of conclusions

In concordance with two previous reports assessing the relationship between urinary 3-PBA and ADHD, we found that there is a significant positive association between the presence of 3-PBA in urine and ADHD (OR = 2.03, 95% CI = 1.18, 3.50), as well as a significant positive association between maternal history of smoking during pregnancy and ADHD (OR = 2.24, 95% = 1.34, 3.76). To further study whether pyrethroid insecticide and psychosocial stress exposure interact to potentiate risk of ADHD, we developed an 11-part allostatic load composite score based on adult allostatic load composite scores [316, 319, 322] and modified it to better reflect the pediatric population in NHANES. After adjusting for child gender, age, race, urine 3-PBA, urine creatinine, $\log_{10}$-transformed urine DMAP, and $\log_{10}$-transformed blood lead level, we found a significant positive association between allostatic load score and caretaker-reported ADHD (OR = 1.15, 95% CI = 1.01, 1.31). Given the higher prevalence of ADHD in boys, we assessed whether sex modified the association between allostatic load and ADHD. We did not observe a statistically significant association, but our analysis was limited by a relatively small sample size of girls with ADHD – 4.53% of girls sampled versus 15.12% of sampled boys. Lastly, we studied whether allostatic load and presence of urinary pyrethroid metabolites interact to increase risk of ADHD. We observed significant multiplicative interaction between the two – in children with detectable 3-PBA, the association between allostatic load and prevalence of ADHD is statistically significant (p = 0.0441). In children with urinary 3-PBA above the LOD, there is a significant association between allostatic load and prevalence of ADHD (OR = 1.16, 95% CI = 1.02, 1.32), but there is not a significant association between allostatic load and prevalence of ADHD in children with no detectable urinary 3-PBA metabolites (OR = 1.13, 95%

CI = 0.96, 1.34). This suggests that presence of urinary pyrethroid insecticide metabolites changes the association between allostatic load and prevalence of ADHD.

Limitations

There are several limitations to address in our study and its use of the CDC's NHANES cohort. Firstly, NHANES is a cross-sectional study and thus temporality of the exposures and health outcomes cannot be ascertained. Perhaps, having ADHD leads to behaviors that increase potential for pyrethroid insecticide exposure, or lead to socioeconomic factors and physiologic changes that would increase allostatic and psychosocial stress. Second, NHANES cycles after 2002 unfortunately did not concurrently examine ADHD and urinary 3-PBA levels, making it impossible to use data from more recent NHANES cycles or combine additional cycles to increase sample size. Furthermore, exposure to 3-PBA may occur if pyrethroids in the environment are hydrolyzed and is not necessarily all from ingestion of parent pyrethroid compound. Thus, exposure misclassification may have occurred in some instances. We would not expect this misclassification to be differential and any bias introduced would therefore be expected to skew results towards the null hypothesis. Lastly, certain high-risk populations are left out of NHANES sampling, including homeless and incarcerated populations. Children that experience homelessness or who have caretakers that are incarcerated would be particularly interesting to study because these populations are associated with greater psychosocial stress exposure [326-329].

Allostatic load in pediatric populations

Pediatric populations present unique challenges for comprehensive assessments of allostatic load. This is because biomarkers of increased allostatic load such as blood pressure, cholesterol, or triglycerides could also be elevated due to inherited conditions that would present early in life and potentially confound results. In addition, it is possible that these physiologic metrics are elevated in adults that have experienced increased psychosocial stress over their entire lives, and that these physiologic effects have not yet fully manifested in developing children. Moreover, sociodemographic data on children are often collected via the caretaker. It is likely that children living in circumstances that induce high amounts of psychosocial stress also have caretakers that experience elevated psychosocial stress. The increased allostatic load of caretakers could thus alter their children's allostatic load directly and indirectly, and lead to measurement errors unique to stressed populations. Lastly, the CDC's NHANES study was designed to study exposures in the entire adult and pediatric population in the United States. Most data on adults are more robust and complete than data on children included in the study. One potential reason for this is that it is simpler and less time-consuming to find volunteers for and consent adults for physical exam and biospecimen studies than it is to consent children and their caretakers. This limitation restricted our sample size and subsequently our study's power to discern true associations between pyrethroid insecticide exposure, allostatic load, and ADHD, and could potentially explain why we did not see the hypothesized interaction effects between pyrethroid insecticide exposure and allostatic load.

Overall, we illustrated that pyrethroid insecticide exposure and increased stress, characterized by an allostatic load score, are associated with increased ADHD prevalence in children 6-18 years old, in the US and their interaction is multiplicative. This is the first description of a pediatric allostatic load score in the NHANES dataset and contributes to

relatively sparse studies of children included in NHANES. Further validation and refinement of a pediatric allostatic load composite score would provide a valuable resource for more comprehensively studying the effects of psychosocial stress in children in NHANES, allowing for a broader representation of the experienced environment and a more comprehensive understanding of the role of the exposome on children's health.

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CHAPTER 5: DISCUSSION

SUMMARY OF FINDINGS

Overall, we sought to understand how environmental exposures during neurodevelopment would impact ADHD pathogenesis. We employed a comprehensive approach using an animal model to study molecular, pathologic, and functional changes, as well as a human cohort to take a more population-based approach. Since genetic and environmental studies indicate the dopamine system is especially important in ADHD, we focused on dopaminergic alterations in our first two aims. In these two aims, our goal was to understand how pyrethroid insecticide and stress exposure together would impact the dopamine system in the context of ADHD. Based on previous studies showing independent effects of deltamethrin and chronic stress on the dopamine system and ADHD risk [67, 86, 295], we hypothesized that deltamethrin and stress would cause synergistic effects when studied in combination. To better study how dual environmental exposures could impact the dopamine system in the context of ADHD, we created three new research tools. First, we developed a novel neurodevelopmental exposure paradigm in mice that models deltamethrin insecticide and stress hormone exposure from the gestational period through adulthood. Second, we adapted a novel targeted next-generation sequencing method previously described in human and *in vitro* samples to study combined exposure in a developmental mouse model. This technique allows for higher-throughput evaluation of DNA methylation from multiple samples and multiple targets on a single sequencing run. In our last aim, we took a population-based approach to translate some of our findings to an epidemiologic study. In doing so we generated a third new research tool - a comprehensive index to measure psychosocial stressors specifically for children.

In aim 1 we utilized our neurodevelopmental exposure paradigm, exposing pregnant dams to deltamethrin and their offspring to CORT dissolved in their drinking water until they

reached adulthood. We hypothesized that we would observe synergistic alterations in dopaminergic midbrain RNA expression, striatum and frontal cortex protein expression, dopamine release dynamics, and ADHD-like behaviors. We found that deltamethrin had no effect on serum CORT levels of male and female adult offspring, but oral CORT administration did lead to a significant decrease in serum CORT of females exposed to deltamethrin and vehicle. Secondly, we assessed RNA expression in the midbrain of *Nurr1, Pitx3, Comt, Dat1, Th,* and *Vmat2*. Male offspring exposed to deltamethrin exhibited reduced expression of the transcription factor *Pitx3*. We did not observe altered RNA expression of *Nurr1, Comt, Dat1, Th,* or *Vmat*2 but there was a sex- and region-specific effect of deltamethrin and CORT exposure on TH and VMAT2 protein expression. TH and VMAT2 protein expression were significantly decreased in the striatum of females exposed to deltamethrin and CORT and TH protein expression was significantly decreased in the frontal cortex of males exposed to deltamethrin. Functionally, male offspring exposed to deltamethrin had reduced dopamine uptake in the striatum and increased locomotor activity in the Y maze. We therefore rejected our hypothesis and did not observe synergistic effects of combined deltamethrin and CORT exposure. However, our results still contribute to our understanding of potential mechanisms of action of deltamethrin and oral CORT, both together and separately, within the dopamine system. Our data suggest that dual exposure to CORT may actually dampen the effects of deltamethrin on the development of the dopamine system, and that is a sex-specific effect in males.

In aim 2, we again used our neurodevelopmental exposure paradigm and hypothesized that DNA methylation of key dopaminergic genes as well as the CORT-responsive glucocorticoid receptor would be significantly altered after exposure to deltamethrin and oral CORT. We also hypothesized that there would be synergistic effects of combined deltamethrin

and oral CORT exposure on DNA methylation in these genes. To study this, we utilized a relatively new method of DNA library preparation and targeted next-generation sequencing. A data analysis pipeline was developed to assess sequencing data since most previously-published analyses and available packages utilized either human Illumina arrays or were capable of comparing methylation for only two exposure groups, instead of four exposure groups. We assessed DNA methylation in the midbrain of adult male and female offspring. We did not observe significant differences in DNA methylation of the dopaminergic genes *Dat1*, *Th*, *Vmat2*, or *Drd4*, nor in their transcription factors *Nurr1* and *Pitx3*. However, in males exposed to deltamethrin and CORT there was a significant increase in DNA methylation at a CpG site residing within a CpG island in the 5' region of the *Nr3c1* gene. This change did not occur in females exposed to deltamethrin and CORT, suggesting that this effect is sex-specific. To follow up on this result, we then tested whether *Nr3c1* mRNA expression was decreased in the midbrain. We did not observe a significant change in *Nr3c1* mRNA expression, nor was there a significant correlation between DNA methylation and mRNA expression levels. However, it is possible that there are additional regulatory interactions at play or that DNA methylation perturbations in response to deltamethrin and CORT persisted longer than perturbations in mRNA expression in the midbrain. No other studies have measured DNA methylation changes in our target genes in response to deltamethrin and CORT and our methodology contributes to the field as well.

In aim 3, we studied the effects of pyrethroid insecticides and stress in children in the United States via the NHANES cohort. To more comprehensively study stress exposures, we drew upon previous work that parameterizes stress as a function of allostatic load [320-322, 330] and adapted it to better suit psychosocial stress experiences in children. We then studied whether

there was an interaction between this index of stress and urinary levels of 3-phenoxybenzoic acid (3-PBA), a metabolite of pyrethroid insecticides. Consistent with previous reports, we observed a positive association between urinary 3-PBA and risk of ADHD, as well as between maternal history of smoking during pregnancy and ADHD. Our allostatic load score contained 11 components, with a maximum possible score of 20. In our study, the range of scores was 1-15 points and the median score was 6 points. We found a significant positive association between allostatic load score and ADHD when allostatic load score was coded as an ordinal variable, but not when subdivided into quartiles. We then investigated whether allostatic load and presence of urinary pyrethroid metabolites multiplicatively interacted to increase the risk of ADHD. We illustrated that there was significant multiplicative interaction between these two variables – in children with urinary 3-PBA levels above the LOD, there was a significant association between allostatic load score and ADHD. This association was not significant in children with urinary 3-PBA levels below the LOD, suggesting that pyrethroid exposure modifies the association between allostatic load and prevalence of ADHD. This work contributes a novel metric of stress in children that encompasses biological markers as well as sociodemographic variables. In addition, it is one of few studies examining children's neurodevelopmental outcomes in the NHANES cohort.

FUTURE DIRECTIONS

Our findings contribute to what is currently known about dopaminergic development, risk of ADHD, and the role of pyrethroid and stress exposures in neurodevelopment – independently and in combination. These data also uncover interesting additional research questions to pursue. To begin, we could potentially modify our neurodevelopmental exposure paradigm to study

whether differential dopaminergic alterations occur at earlier timepoints during development. For example, it is possible that mRNA expression differences in the midbrain are present in adolescence but not adulthood. Moreover, we conducted our exposures sequentially – deltamethrin was administered during gestation and through weaning while oral CORT was administered from weaning through adolescence. We hypothesized that there would be sequential synergistic effects in the dopamine system after this combination of exposures. It is possible that we did not observe synergistic effects because these two exposures have to occur at the same time to observe a detectable change in dopaminergic function. Lastly, while both the behavioral chronic stress and oral CORT exposure paradigms have been validated by various groups [136, 186, 194], it would be informative to compare the pathologic and functional changes that occur in the dopamine system after both of these exposure routes. This would be useful for comparing studies of chronic stress exposure in rodents since many of these studies have not utilized consistent stress exposure paradigms and yielded differential results. Potentially, one exposure model provides more robust results than the other.

Secondly, to our knowledge, this is the first investigation of DNA methylation changes that occur in dopaminergic genes of the midbrain after pyrethroid insecticide and stress exposure. We could expand upon our targeted next-generation sequencing approach and conduct genome-wide analyses via whole genome bisulfite sequencing or reduced represented bisulfite sequencing (RRBS) to capture DNA methylation changes that arise throughout the genome. Utilizing more of an epigenome-wide study approach, we could then investigate whether there are DNA methylation differences amongst related neuronal gene networks. Bioinformatics tools such as weighted correlation gene network analysis (WCGNA) would allow us to examine DNA methylation changes that occur in a more comprehensive way. In this initial DNA methylation

study, we observed a lot of variability between midbrain samples. We harvested midbrains at 8-10 weeks, and it is possible that DNA methylation alterations were not long-lasting, and this time frame induced some variability. Future studies that collect all midbrains as close to the same time point as possible could address this potential issue. Cell-to-cell heterogeneity within the midbrain could also play a role in this variability since we were not able to differentiate neuronal and non-neuronal cells via gross dissection. A recent study utilized a fluorescence-activated cell sorter (FACS) to isolate neuronal nuclei from the prefrontal cortex of adult C57BL/6 mice [331] and provides a potential approach to help address this issue in the future.

Third, our epidemiologic findings in the NHANES cohort suggest that there are important interactions between toxicant and psychosocial environmental exposures in children. Since few studies have examined outcomes in children surveyed in NHANES, it would be useful to motivate future NHANES cohorts to include more detailed biological and demographic measurements at additional ages so that researchers may build a more robust measurement tool of allostatic load and other environmental factors. In addition, we were only able to utilize the 1999-2000 and 2001-2002 NHANES cohorts because no other survey years contained both pyrethroid metabolite and caretaker-reported ADHD measurements. Ideally, upcoming NHANES surveys will ensure better overlap in the measured outcomes between survey years so that more NHANES cohorts can be combined. This would allow us to greatly increase our sample sizes and further refine our exposure and outcome variables. For instance, children with ADHD exhibit variation both in disease trajectory and treatment outcomes. Examining whether pyrethroid insecticide exposure predisposes children to more of a hyperactive vs. inattentive ADHD phenotype, or whether children who experience higher allostatic load realize greater

benefit from behavioral therapy than those who increase lower allostatic load, would provide substantial clinical and public health benefit.

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