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Pyrethroid Insecticides & the Gastrointestinal Environment

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M.S., University of Bordeaux, 2019

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Abstract

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Pyrethroid insecticides are one of the most used classes of insecticides in the world. Derived from a type of chrysanthemum flower, they now represent a family of chemicals that is found in products throughout homes, medical offices, and agricultural settings. Many pyrethroids resist photodegradation, making them invaluable to farmers for large-scale use on vegetables and crops. Their primary mechanism of action is to induce neurotoxicity via actions on voltage-gated sodium channels. While pyrethroids are not nearly as toxic to humans as they are to insects, it is important to understand their potential impact on human health as we expose ourselves to them on a regular basis. In fact, the primary route of exposure to pyrethroids among the general population is through ingestion. However, most studies investigating the role of pyrethroids in human health have looked at their effects in the central nervous system, so knowledge of pyrethroid toxicity in the gastrointestinal (GI) tract is limited. The gut is comprised of several layers, the innermost of which is the gut epithelium. Scattered along the epithelial layer are enteroendocrine cells (EECs), a rare and unique cell type that have many neuronal properties. Coupled with their physical proximity to ingested contents, the gut epithelial environment is highly susceptible to pyrethroid-induced neurotoxicity. Therefore, we investigated the effects of the common pyrethroid deltamethrin on EECs *in vitro* and intestinal function and status *in vivo*. Given the numerous indicators that pyrethroids like deltamethrin are capable of disrupting monoamine signaling pathways, we went on to determine the extent to which longer-term oral exposure to deltamethrin may contribute to neurological dysfunction in a Parkinsonian manner. We found that while chronic exposure to deltamethrin did not induce Parkinsonism, acute exposure paradigms induced intestinal dysmotility and suppressed levels of several GI-related hormones including serotonin, insulin, and leptin. Moreover, we found that deltamethrin interferes with stimulus-induced hormone release by EECs *in vitro*. Thus, it is plausible that deltamethrin could be acting through similar mechanisms to induce intestinal dysmotility. Our findings provide novel insight into the potential for low-dose, oral, pyrethroid exposure to affect GI function and raise important questions about the extent to which such exposures contribute to intestinal dysmotility in humans.

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Chapter 1: Introduction

1.1. General anatomy and functions of the gastrointestinal tract

The gastrointestinal (GI) tract is one of the most underappreciated organ systems in the human body. It is critical for our health and survival because of its promotion of nutrient uptake and regulation of metabolism and immunity, all while providing status updates to every other organ system in the body. The major components of the digestive system include the mouth, esophagus, stomach, small intestine (the proximal duodenum, medial jejunum, and distal ileum), large intestine (the proximal and distal colon in rodents, or the ascending, transverse, and descending colon in humans), and the rectum and anus, with contributions from neighboring organs such as the liver, pancreas, and gall bladder. Each region of the gut differs in its components and properties according to its primary function. The stomach contains an abundance of digestive enzymes, the small intestine has longer villi for better nutrient absorption, and the large intestine contains specialized mechanosensitive cells to help initiate powerful contractions for propelling luminal contents toward excretion (1-3). There are five main layers of the intestines. The outermost longitudinal muscle layer drives peristalsis (the sensory-motor reflex that propels food toward excretion). Embedded between it and the circular muscular layer, which ensures the unidirectionality of digestion, is the myenteric plexus, which forms the outermost layer of the enteric nervous system (ENS). Below the circular muscular layer lies the submucosal plexus of the ENS, followed by the innermost mucosal layer which contains epithelial cells that form both a physical and chemical barrier between the gut lumen and the ENS (Figure 1.1). The majority of all cells within the GI tract are epithelial cells, comprising more than half of all cells in the gut during adulthood (4). Importantly, the epithelial layer is uniquely positioned to serve as one of our first lines of defense against ingested contaminants.

The epithelial layer is arranged into thin, oblong projections called villi, which are most prominent in the small intestine to maximize surface area for absorption and sensation (3). The apical surface of villi is where mature epithelial cells gradually migrate and eventually die after their journey from the depths of the crypt, where they originate as intestinal stem cells (3). Cellular renewal in the intestinal epithelium occurs over a span of days (5), ensuring minimal damage due to rapid epithelial turnover. The epithelial layer consists of many different cellular subtypes. These include (6-8): enterocytes, the most abundant epithelial cells whose primary role is to absorb nutrients; enteroendocrine cells, rare and specialized sensory-transducing cells that produce a range of gastrointestinal hormones, including 5-HT (serotonin), in response to luminal stimuli; mucus-secreting Goblet cells that support intestinal immunity; M-cells that serve as mediators between the lumen and intestinal immune cells; anti-parasitic and chemosensory Tuft cells; and crypt-resident Paneth cells, responsible for secretion of antimicrobial peptides to help prevent infection. These cellular subtypes all work together to create a biological barrier, selectively permitting only essential nutrients and ions to pass through in an effort to protect the host and maintain intestinal homeostasis (9).

1.2. Enteroendocrine cells and the intestinal-luminal interface

Enteroendocrine cells (EECs) are rare and highly specialized cell types in the GI tract. Their scarcity is an anomaly given their critical status in maintaining metabolic homeostasis. EECs produce various gut hormones such as cholecystokinin (CCK), peptide YY (PYY), and glucagon-like peptide 1 (GLP-1) in response to various microbial and dietary stimuli detected within the gut lumen. These hormones in turn send nutritional

signals to the rest of the body to support countless biological processes, such as digestion, blood flow, visceral sensation, and even behavior.

EECs were named according to the main hormone they produce (Table 1.1). For instance (10): A-cells produce ghrelin; D-cells produce somatostatin; EC-cells produce 5-HT; G-cells produce gastrin; K-cells produce glucose-dependent insulinotropic polypeptide (GIP); I-cells produce CCK; S-cells produce secretin; M-cells produce motilin; N-cells produce neurotensin; and L-cells produce PYY and GLP-1. The letter-based naming convention was established at a time when it was thought that EECs produced a single hormone. However, we now understand that this is not the case, as several types of EECs were found to produce more than one hormone (11-13) and in fact have the plastic ability to switch hormonal profiles as they migrate and mature (14). EECs are further characterized according to their location and distribution along the GI tract. A-cells exist most proximally in the stomach, while L-cells are scattered throughout the more distal regions of the small intestine and proximal colon (10). They also tend to cluster near other EECs with similar hormonal profiles (13).

Nearby neurons within the “intrinsic” enteric and “extrinsic” peripheral nervous systems express an array of hormonal receptors, enabling them to respond readily to EEC-derived signals (15, 16). EECs are capable of both local paracrine and long-distance endocrine communication, as is the nature of hormonal transmission. Interestingly, a handful of publications have also highlighted their neuronal properties (10, 17, 18). EECs indeed respond to electrical stimulation (19) and produce action potentials (20), and genetic sequencing has revealed that EECs express several VGSC subtypes (4, 21-23), including Na_v 1.3, Na_v 1.6, Na_v 1.7, and Na_v 1.9. In fact, EECs form synapses with vagal terminals (24), which innervate even the apical regions of mucosal layer villi (25). It is

therefore unsurprising that EECs possess the ability to transmit information on a millisecond timescale via glutamatergic neurotransmission (20). Therefore, EECs are capable of transmitting signals that span the entire temporal spectrum of cell-cell communication.

1.3. Modeling enteroendocrine cells *in vitro*

The rarity of EECs makes them incredibly difficult to study in their native environment. For a long time, we lacked the methodologies to fully elucidate their properties *in vivo*. The development of multi-omics approaches led to an explosion of research that has provided new insights into enteroendocrine cell communication, regulatory pathways, and developmental lineages. Despite these advancements, robust *in vitro* models remain essential for understanding EEC biology. Culture models enable precise manipulation of EECs in a controlled environment and allow functional assessment of EEC responses to various treatments.

STC-1 cells are a murine-derived neuroendocrine cell line that essentially recapitulate native enteroendocrine cells of both the small and large intestine (26). They were first isolated from small intestinal tumors of transgenic mice in the late 20th century (27) and have since been widely used as a screening tool in gastrointestinal biology to better understand the mechanisms and properties of gut hormonal secretion in response to various nutritional stimuli. While they are incredibly useful given their general representation of EECs as a heterogenous cell line, they remain limited by this very feature since it can render difficulty in experimental reproducibility. For this reason, STC-1 cells are often passaged a greater number of times to achieve less heterogeneity between

cell types. These proliferative cells are also of cancerous origin and therefore cannot fully recapitulate enteroendocrine physiology.

Another frequently used EEC cell line is known as the GLUTag cell line, which is derived from a colonic tumor in mice (28). These cells most closely represent intestinal L-cells, native EECs of the distal small intestine and proximal colon, which primarily produce the gut hormone GLP-1. For this reason, GLUTag cells are used most commonly in studies that seek to understand colonic L-cell activity and function. However, they lack the broader EEC representation offered by STC-1 cells and require a matrix to grow efficiently (29).

The only human-derived EEC cell line, NCI-H716, offers a highly relevant model system given its origins. It was first isolated in 1987 from a young adult male with colorectal cancer (30). Like GLUTag cells, this line resembles colonic L-cells and therefore most readily produces GLP-1. The NCI-H716 line is a robust model for understanding human GLP-1 signaling, though it notably does not produce GLP-1 in response to identical stimuli used to induce GLP-1 secretion in other EEC cell lines (31), implying potential cross-species differences.

While cell lines provide incredible utility given their rapid proliferation, ease of manipulation, and general resilience, they cannot replicate native EECs without the natural cellular diversity that exists in the gut epithelium *in vivo*. Primary culture of EECs is therefore ideal, but given their rarity within the intestinal epithelium, rapid turnover rates, and the need for a rich intestinal microenvironment to function normally, primary culture is a challenge. Successful EEC primary culture was first established in 2008, enabling the use of calcium imaging and electrophysiological techniques to better assess EEC function (32), though the cells were only able to survive for 2 days (small intestinal)

or 10 days (colonic) in culture. Moreover, the EECs did not regenerate, possibly due to the absence of epithelial stem cells. The same group has successfully replicated this approach multiple times since then (33), using various regions of the gut and slightly different preparations to improve cell viability. This approach offers a challenging but nonetheless accurate way to study the effects of various compounds on native EEC biology.

These considerations help compare the benefits and disadvantages of using various culture methods to study enteroendocrine cells and reinforce the necessity of using complementary approaches to model native EEC function. STC-1 cells offer the broadest representation of EECs among existing immortalized cell lines. However, STC-1 cells are different enough from native EECs to warrant caution when making any direct comparisons. Additionally, progress with organoid models and co-culture systems has enabled longer term assessment of EEC status and function *in vitro*. Our ability to create EEC-rich environments in culture conditions has enabled us to study native EECs outside of their natural environment and ultimately helps us to better understand their role in gut health and disease.

1.4. Gastrointestinal hormones

Gastrointestinal hormones play a crucial role in regulating digestion, appetite, and visceral sensation (Table 1.1). The GI tract produces more than 20 different hormones to regulate metabolic homeostasis (34). Most of these hormones are made by enteroendocrine cells. Beyond relaying information about the intestinal environment to surrounding cells, gut hormones are responsible for making sure food is broken down efficiently. Gastrin stimulates acid production in the stomach (35), motilin promotes intestinal motility (36), GLP-1 delays gastric emptying and suppresses small intestinal

motility following meals (34), and CCK triggers bile acid release from the gall bladder to facilitate the breakdown of protein and fat (37). Several gut hormones also play a role in appetite regulation, signaling hunger (e.g., ghrelin) or satiety (e.g., CCK, GLP-1) based on feedback from gastric accommodation, which occurs following nutrient absorption in the stomach and duodenum and the detection of accompanying sensations of stretch or pressure (15, 38). Visceral sensation also encompasses the chemical interpretations of nausea and pain. For example, intestinal 5-HT promotes nausea through activation of $5HT_3$ receptors on vagal afferents (39, 40) and modulates the nociceptive response (41, 42). These examples highlight the breadth of actions that gut hormones have locally in the GI tract.

Gastrointestinal hormones also have the critical task of keeping our central nervous system apprised of everything that happens in the gut, representing the most abundant form of gut-brain communication. The proximity of vagal and sympathetic afferents to the intestinal epithelial layer supports this communication. Sensory afferents of the vagus nerve are covered in hormonal receptors (43), enabling EECs to communicate through vagal projections to the nucleus tractus solitarius (NTS) in the brainstem. This is one pathway through which gut hormones moderate feeding behavior. CCK was the first gut-derived hormone to be connected with behavioral modification through its effects on satiety (44). Shortly thereafter, it was reported that vagal afferents are necessary for CCK to centrally regulate appetite (45, 46), though CCK is capable of direct actions on the brain independent of vagal pathways (47) and has been implicated in behaviors such as anxiety and mood regulation (48). These discoveries inevitably led to deeper investigation of the mechanisms and pathways involved in gut-derived hormonal control of central behaviors. It was soon established that gut-derived GLP-1 also has a suppressive effect on food intake

(49, 50). Studies manipulating the vagus nerve investigated its necessity for GLP-1 to reduce appetite (51, 52), but later work found that peripheral GLP-1 administration is sufficient to do so even in the absence of vagal signaling (53, 54). GLP-1 is also tightly coupled to insulin and glucagon production, helping to regulate blood glucose levels (55, 56). As an incretin hormone, it is therefore the case that GLP-1 can indirectly affect food intake through its actions on these hormonal pathways in the pancreas and liver (55), which have both been shown to independently modulate eating behaviors (57, 58). For these reasons, GLP-1 now represents a class of drugs that have revolutionized not only diabetes but also weight loss therapy. Of course, circulating gut hormones released into the bloodstream are capable of direct communication with the brain through central receptor activation as well. The hypothalamus in particular abundantly expresses gut hormonal receptors (59), which is unsurprising given its role in energy homeostasis. Additional EEC-derived hormones that modulate feeding behaviors in the brain include PYY (60), another satiety signal, and ghrelin (61) and insulin-like peptide 5 (62), which both have orexigenic actions. These findings altogether help illustrate a major function of gut hormones in governing central feeding behavior, acting through diverse pathways to regulate metabolism and energy balance in the brain.

Several types of stimuli can trigger the release of gut hormones from EECs. Digested food particles stimulate the release of anorectic intestinal hormones within minutes following a meal (63-65). During the fasted state, orexigenic hormones such as ghrelin (66), the acid-reducing hormone somatostatin (10), as well as motilin, a major driver of the migrating motor complex in the stomach and small intestine that propels undigested food, bacteria, and other luminal contents toward the colon (67) are released. Non-nutritional stimuli can also cause EECs to release hormones. These include bile acids (68-

70), bacterial byproducts (e.g., indole, short-chain fatty acids) (18, 71-73), pathogens and pathogen-associated molecular patterns such as lipopolysaccharides (LPS) (74, 75), chemical irritants (18), catecholamines (18), and even other gut hormones (76, 77). Accordingly, EECs possess a variety of chemosensory transporters, ion channels, and receptors that enable them to identify and convey information about the contents and status of the luminal environment.

1.5. Intestinal monoamines

Gastrointestinal function is tightly controlled by monoaminergic signaling, involving serotonin (5-HT), dopamine (DA), norepinephrine (NE) & epinephrine, and histamine (HA). Each of these monoamines play important roles in the regulation of gut motility, secretion and absorption, immune responses, and gut-brain communication. Of these, 5-HT, dopamine, and histamine are produced locally by cells of the GI tract, while NE and epinephrine alter gut function via autonomic control and circulation, respectively. Although the complex network of signaling molecules involved in normal GI activity is still not fully understood, experimental studies suggest that monoamines are essential for its function.

The family of catecholamines (DA, NE, and epinephrine) are all synthesized from L-tyrosine through the activity of tyrosine hydroxylase (TH). Almost half of all the DA in the human body is produced in the GI tract (78) and mainly originates from dopaminergic neurons in the enteric nervous system (ENS) (79). The study of enteric DA neurons is complicated by the fact that DA is the precursor to NE, the major neurotransmitter of the sympathetic nervous system that densely innervates the GI tract. The existence of DA neurons in the gut was long suspected but not confirmed until observing enteric TH+

immunoreactivity even after extrinsic sympathetic denervation (80). Dopamine appears to have an overall inhibitory effect on gut motility (81) through actions on the D_2 receptor (81) and by activating D_1 receptors on inhibitory nitrergic enteric neurons (82). However, MPTP-treated mice eventually develop a constipation-like phenotype despite an initial increase in gut motility (83), suggesting a potential compensatory role for non-dopaminergic signaling mechanisms.

The sympathetic neurotransmitter NE is synthesized locally in nerve terminals that innervate the intestines, while its counterpart epinephrine is manufactured in the adrenal medulla and released into the bloodstream, where it can then travel to exert its modulatory actions on the GI tract (84). NE and epinephrine have subtle yet distinct differences in their adrenergic receptor subtype binding affinities (85), resulting in differing effects on gut function. For example, they have opposing effects on intestinal blood flow (86), comparably strong promotion of intestinal glucose absorption (87), and epinephrine primarily induces muscular relaxation in the gut (88) while NE can have contrasting effects on gut motility depending on the predominant adrenergic receptor subtype being activated (89). These nuanced differences in receptor interactions allow precise control of GI function in response to physiological demands and highlight the complexity of adrenergic signaling in the gut.

Histamine is synthesized locally in the gut primarily by mast cells, enterochromaffin-like cells (ECLs), histaminergic neurons, and gut microbes, though it also comes from ingested food (90). Like most neurotransmitters, histamine binds several receptor subtypes that each have differing effects upon activation, most of which are expressed throughout the entire GI tract (91, 92). Histamine has a wide array of functions in the gut that appear to be species-dependent (92), including modulation of synaptic

neurotransmission in the ENS (93), stimulation of gastric acid secretion (94), and alteration of intestinal motility (92). However, its canonical role involves regulating the immune response. Clinically, histamine or its biomarker metabolite N-methylhistamine is elevated in patients with irritable bowel syndrome and inflammatory bowel disease, respectively (95, 96). Indeed, histamine is a critical regulator of immune cell activation and recruitment in response to possible threats detected in the luminal environment (97). Evidence from a stroke model suggests that oral delivery of a mast cell suppressant improves gut barrier integrity, reduces circulating histamine and cytokine levels, and increases production of goblet cell-derived intestinal mucosa (98), offering a potential therapeutic avenue for inflammatory pathologies in the GI tract.

The most abundant monoamine in the GI tract is serotonin (Figure 1.2). Although serotonergic neurons are present within the ENS, ~ 90-95% of our body's total serotonin supply is produced by a subtype of EEC called enterochromaffin cells (ECs) (99). These separate pools of serotonin are differentiated based on which tryptophan hydroxylase enzyme they use for serotonin production (TPH1 in ECs, and TPH2 in serotonergic neurons). ECs increase in density towards the distal regions of the gut, primarily localized to the colon (100) where they make up about 70% of all EECs localized there (101). This suggests that mucosal serotonin plays a significant role in colonic functions. Colonic motility is mainly derived from rhythmic, propulsive contractions known as the colonic migrating motor complex (CMMC), also referred to as high amplitude propagating contractions (HAPC) in humans (102). The necessity of gut serotonin in mediating these movements is still a subject of debate. Some research has suggested that EC-derived serotonin is unnecessary for colonic motility (103, 104), while others found that it is in fact required for regular CMMC activity and normal colonic transit (105-107). Further

work is needed to delineate the reasons for these discrepant observations, which could be the result of different experimental approaches, compensatory adaptations, or non-serotonergic mechanisms.

1.6. Intestinal dysmotility

Abnormal movement of the GI tract is known as intestinal dysmotility. The global prevalence of intestinal dysmotility is not well documented, but it was recently estimated that around 40% of people worldwide have some form of functional gastrointestinal disorder (108), an umbrella term that encompasses intestinal dysmotility. Intestinal dysmotility disorders include achalasia, gastroesophageal reflux disease (GERD), gastroparesis, irritable bowel syndrome (IBS), colonic inertia, Hirschsprung's disease, chronic intestinal pseudo-obstruction (CIPO), and dyssynergic defecation (109, 110). Many of these disorders arise independently, but they can also result from underlying conditions such as neurological dysfunction (e.g., neurodegenerative disease, spinal cord injury), muscular impairment (e.g., Ehlers-Danlos Syndrome, visceral myopathy), autoimmune disease (e.g., inflammatory bowel disease (IBD), lupus, scleroderma), or metabolic disorders (e.g., diabetes, thyroid disease, uremia) (110, 111). People with intestinal dysmotility often experience abdominal pain, nausea and vomiting, constipation, diarrhea, bloating, and/or malnutrition (112-115). Most research investigating disorders of intestinal dysmotility is aimed at better understanding the mechanisms in an effort to reduce symptom burden and eventually provide therapeutic relief to affected individuals.

Intestinal dysmotility is a complex phenomenon that is further complicated by our incomplete understanding of healthy intestinal function. The peristaltic reflex is mediated

in large part by the ENS and requires coordinated activity of sensory and motor enteric neurons to trigger muscular contraction above the food bolus and relaxation below the food bolus (116). While true, this is an oversimplification of a complicated system that also involves selective input from interneurons, interstitial cells of Cajal (local pacemakers of the GI tract), intrinsic primary afferent neurons (IPANs), extrinsic vagal and sympathetic input, gut hormones, and microbial signals that all work together to support gut motility. Multiple systems in place to promote intestinal function, even superfluously, ensures host survival. In animal models with constipation (a common symptom among the aforementioned disorders), for instance, it is therefore often the case that multiple systems are indeed affected. Mice with deletion of the metabolic hormone leptin produce fewer stools with less water content and have slower gut transit which is associated with abnormalities in the ENS (including a reduction in markers for interstitial cells of Cajal) and deficits in local gut hormone production (117). Mice with low serotonin availability due to genetic modification of the serotonin transporter (SERT) have slow GI and colonic transit, fewer enteric neurons, and fewer enterochromaffin cells (118). Germ-free mice have slower GI transit (119, 120), structural deficits in the ENS (121), and less peripheral serotonin (122), whereas colonization of the gut microbiome rescues these deficits in a serotonin-dependent manner (123). These models demonstrate a clear role for metabolic hormones, serotonin, and the gut microbiome in intestinal dysmotility and highlight its multifaceted nature that stems from the synchronized efforts of numerous biological components.

The diagnosis of intestinal dysmotility disorders relies on a comprehensive assessment of patient history, blood work, and various exams to identify the problem (124). For example, imaging and radiological tests (e.g., x-ray, CT, MRI, gamma

scintigraphy) to identify structural abnormalities, gastric emptying, stool burden, and whole-gut transit. Endoscopy and breath tests are used to check for mucosal abnormalities and signs of bacterial overgrowth. Manometry exams measure intraluminal pressure, which helps identify propulsive abnormalities, neuromuscular coordination, and degree of strain. Finally, histology assessments can be used to look for signs of pathology such as intestinal inflammation. The complexity of intestinal dysmotility often requires several of these tests to accurately diagnose the condition. The same is true for rodent models, though different techniques are applied in this case. Common assessments include fecal output to measure the amount of stool produced in a given time frame, gut motility assays using a dye to measure transit time, a dye-based gastric emptying test, and bead propulsion assays to measure function of the distal colon (125). These assays enable researchers to model human intestinal dysfunction, identify the potential underlying mechanisms, and test various therapeutic approaches.

1.7. A brief introduction to pyrethroid insecticides

In 1962, a book called Silent Spring by Rachel Carson was published that paved the way for environmental reform around the world. As a result, the infamous insecticide DDT was banned, and countless efforts were made to reinvent pest control products that are far less harmful to humans and the natural environment. Born among these are a class of insecticides called pyrethroids, which today represent the one of the most common insecticides in the world (126).

Pyrethroids are derived from a species of chrysanthemum flower called *Tanacetum cinerariaefolium*. They produce the natural insecticide pyrethrum, whose active ingredient is called pyrethrin. The insecticidal uses of pyrethrums were first described

scientifically in 1909 (127) and were soon studied by scientists around the world in an effort to understand and improve their insecticidal properties. Allethrin, the first synthetic pyrethroid, was discovered in 1949 after successfully deriving it from pyrethrin and was soon commercially produced for residential usage by 1953 (128). Allethrin's potency and stability compared to natural pyrethrins led to the synthesis of numerous other pyrethroids, including the development of those that were able to withstand photodegradation. These more stable pyrethroids, such as permethrin, cypermethrin, and deltamethrin, gained popularity because they could now be applied in large quantities in agricultural settings without breaking down as easily. Today pyrethroids are used not only in agriculture but residentially as pest control for lice, flea, and tick prevention in homes, animal shelters, and veterinary clinics.

Pyrethroids are divided in two categories according to their actions: Type I and Type II. Both types primarily act on voltage-gated sodium channels (VGSCs), extending channel open-time to induce neuronal hyperactivity, while Type II simultaneously inhibit $GABA_A$ receptors, leading to further and more prolonged depolarization (129). Pyrethroids have effects on other ion channels as well, including those responsible for trafficking calcium and potassium (129). Altogether, these mechanisms ensure the total disruption of neuronal function and offer a quick death for most insects. However, they can also lead to a variety of negative outcomes beyond acute nervous system failure and cause harm to countless other species, including humans. In mammalian animal models, the highest levels of pyrethroid exposure led to seizures, tremors, imbalance and incoordination, memory impairment, reproductive toxicity, and even death (130-135).

When recommended usage guidelines are followed and appropriate PPE is worn, pyrethroids pose significantly less risk to human health (136). However, ease of access,

improper usage leading to greater exposures, and mounting evidence linking pyrethroids to adverse health effects are serious concerns. In 2013, the Poison Control Center reported that pyrethroids accounted for over 25% of all cases of poisoning in the category of single substance, non-pharmaceutical exposures (137). Public awareness is therefore insufficient for pyrethroids to be considered safe. Historically, investigations into the safety of pyrethroid usage relied on physical symptoms of overt toxicity, but the disconcerting reality is that most signs of everyday pyrethroid exposures in humans will go unnoticed because they occur at the molecular level.

1.8. Pyrethroid-monoamine interactions

Although it's well-established that pyrethroids act on VGSCs and $GABA_A$Rs, considerably less research has been conducted to determine what else occurs in the nervous system of mammals after low dose pyrethroid exposures. What work has been done has led to the conclusion that pyrethroids disrupt monoamine pathways in animal models. For instance, pyrethroid exposure is associated with hyperactivity (138), a behavioral phenotype thought to be caused by nigrostriatal dopaminergic pathway dysfunction. Indeed, the pyrethroids permethrin and deltamethrin increase striatal dopamine transporter (DAT) expression and dopamine (DA) uptake both *in vitro* and *in vivo* (139-141). Dopamine-producing neurons appear to be especially vulnerable to pyrethroids compared with other monoamines (142, 143). Even so, several pyrethroids were found to alter striatal serotonin (5-HT) levels in rats (144, 145), and this effect was abolished with VGSC and Ca^{2+} channel antagonists (144). Another group reported decreased hippocampal norepinephrine (NE) levels in awake rats after oral exposure to

deltamethrin administered during development (131). These studies represent the increasingly robust evidence that pyrethroids consistently disrupt monoamine pathways in the central nervous system (CNS). However, they fail to address whether this happens in the periphery, especially after the most epidemiologically relevant route of pyrethroid exposure — ingestion.

1.9. Susceptibility of the GI tract to pyrethroids

According to the Agency for Toxic Substances and Disease Registry (ASTDR), ingestion is the most common route of pyrethroid exposure among the general population (134). Indeed, pyrethroids are sprayed annually on numerous crops, especially corn, legumes, grains, and soy (146), all of which make up a considerable portion of the human diet. Information about how pyrethroids affect the mammalian gut is limited, mainly encompassing early experiments conducted by the Environmental Protection Agency (EPA) aimed at testing the limits of pyrethroid toxicity. For instance, gastritis, mucosal erosion, and ulceration were detected in the gut of mice administered oral esfenvalerate (106 mg/kg/day) for 90 days (147). Case reports in humans have also been documented wherein individuals who had accidentally consumed large amounts of pyrethroids while working experienced severe nausea, vomiting, and diarrhea (148, 149). These studies demonstrate the ability of pyrethroids to influence gastrointestinal function.

Enteroendocrine cells are likely first targets of pyrethroids given their physical location in the gut epithelium, and their neuronal properties, including the expression of VGSCs, makes them doubly vulnerable (Figure 1.3). Notably, all VGSC subtypes known to be expressed in the gut except Na_v 1.7 are considered pyrethroid-sensitive, while 1.7—as well as 1.2—is classified as insensitive (150). In other words, higher pyrethroid

concentrations are required to affect these isomers compared with the rest. Thus, the local gut environment is susceptible to oral pyrethroid exposure not only because of physical proximity and the abundance of monoaminergic signaling, but also because the very cells that help form the protective gut barrier (EECs) express VGSCs, the major target of all pyrethroids. This vulnerability invites the possibility that exposures to pyrethroids could induce GI dysfunction, which could eventually lead to longer term GI abnormalities.

1.10. Intestinal dysmotility as an early indicator of neurological disease: an example of Parkinson's disease

The gut-brain axis plays a critical role in maintaining homeostasis across organ systems, enabled through bidirectional communication between the GI tract and brain. It is therefore plausible that disturbances in the periphery might coincide with or even contribute to neurological dysfunction. Several neurological conditions are accompanied by or lead to GI dysfunction. These include stroke, multiple system atrophy, dysautonomia, amyloid lateral sclerosis, multiple sclerosis, Alzheimer's disease, and Parkinson's disease (151). We are learning, however, that it is also sometimes the case that GI dysfunction precedes neurological dysfunction. The best-studied example of this is Parkinson's disease.

Parkinson's disease (PD) is a neurodegenerative disease clinically defined by the progressive loss of voluntary movement, attributable to the death of dopamine (DA) neurons in the substantia nigra pars compacta (SNc) and aggregation of the protein alpha-synuclein (α-syn) throughout the nervous system (152). Notably, PD patients commonly experience gastrointestinal (GI) dysfunction prior to motor symptoms (153, 154). Constipation specifically is one of the earliest and most frequent complaints in

individuals later diagnosed with PD (155), and GI symptoms are predictive of cognitive impairment in PD (156). Furthermore, individuals with PD display aggregates of α-syn throughout the enteric nervous system (ENS) and GI tract along with a range of other enteric abnormalities (157-159). These defects are reproduced by numerous environmental and genetic animal models of PD that report impaired GI function and enteric α-syn pathology prior to motor dysfunction (160-163). These observations came after Braak and colleagues proposed that idiopathic PD begins outside of the central nervous system (CNS). Their post-mortem analyses revealed that in early stage PD, α-syn pathology was limited to the medulla (which contains vagal nerve nuclei) but occurred throughout the rest of the brainstem and mesocortex in late-stage PD (164), suggesting that the vagus nerve is a conduit for PD pathophysiology originating in the periphery. The vagus nerve, together with the ENS and sympathetic innervation from the spinal cord, is a large component of the autonomic nervous system that helps regulate intestinal motility (165). In addition to motor control, the vagus relays sensory information from the gut to the CNS. Vagal sensory afferents are pseudounipolar with cell bodies in the nodose ganglia that project bidirectionally to the gut and the nucleus tractus solitarius (NTS) in the medulla. Interestingly, some individuals with PD exhibit vagal nerve atrophy (166). In fact, vagotomies slightly decrease the risk of PD in humans (167, 168) and even prevent loss of nigrostriatal DA neurons in a peripheral pesticide-induced PD mouse model (161). Altogether, these data suggest that Parkinson's disease involves gastrointestinal pathology and raise the question of whether it could begin in the periphery. While neither genetic nor environmental influences are solely responsible for all PD incidences, genetic factors make up only 10% of all cases (169), which suggests a potentially large role for environmental factors.

1.11. Pesticides in Parkinson's disease

Many pesticides including organochlorines, organophosphates, neonicotinoids, rotenoids, and pyrethroids are linked to neurotoxicity through their ability to induce oxidative stress, neuroinflammation, and cell death (170). Pesticide exposure is a consistent predictor of PD (171, 172). Across epidemiological analyses, the risk of developing PD is doubled after pesticide exposure and tripled with occupational pesticide exposure (173-175). In countries with industrialized agriculture, residential proximity to farms with heavy pesticide use is correlated with increased PD incidences (172), including a PD-risk association for the pyrethroid permethrin (176). These associations are corroborated by experimental studies in rodents demonstrating the capacity of pesticides to alter DA circuits. For example, the organochlorine insecticide dieldrin increased striatal dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2) expression and exacerbated MPTP-induced striatal dopamine depletion (177). Similarly, another organochlorine insecticide, methoxychlor, reduced striatal dopamine and DAT protein levels in mice (178). Paraquat, an herbicide with a similar chemical structure to that of MPTP, was shown to induce neurotoxicity via DAT as evidenced by the fact that DAT-null mice were relatively resistant to paraquat-induced nigral DA neuron loss (179). While most of these studies focus on the central mechanisms of PD, more recent efforts have been made to assess the effects of peripheral pesticide exposure on PD phenotypes in the gut. Exposure to the pesticide rotenone, a mitochondrial complex I inhibitor, alters enteric glial function and induces enteric neuron loss, as determined by the reduction of the pan-neuronal marker PGP9.5 in the submucosal plexus of rats after peripheral rotenone treatment (180). In a more realistic model, oral exposure to rotenone not only

resulted in synuclein pathology in the ENS but also induced synuclein accumulation in the brain and eventually led to the death of nigrostriatal DA neurons in the SNc (181), supporting the notion of gut-to-brain toxicity and highlighting ingestion as a relevant exposure route for pesticide-induced PD pathology. While these are clear indicators that pesticides contribute to PD phenotypes, the precise mechanisms that govern their contribution remain unclear.

1.12. Pyrethroids, intestinal dysfunction, & neurological outcomes

The GI tract is a highly complex organ responsible for the coordinated digestion, absorption, and excretion of luminal contents. Beyond these, it also plays important roles in intestinal immunity, hormonal regulation, and crosstalk with the CNS. Given its constant exposure ingested substances, the GI tract is a target for many ingested neurotoxicants including pyrethroids. Pyrethroids are well-documented insecticides that alter activity and function of neurons in the central nervous system, but their role in disrupting gastrointestinal status and function remains largely unexplored. The presence of EECs within the gut epithelium increases gut vulnerability to pyrethroids because they express VGSCs, the major target of pyrethroids. The critical role of EECs in nutrient absorption, hormonal and metabolic regulation, and intestinal motility implies that any damage to EECs could have serious effects on gut health. Additionally, EECs are the major supplier of intestinal serotonin, a neurotransmitter known to be affected by pyrethroids in the CNS. This dissertation explores this knowledge gap by investigating the effects of pyrethroid exposure on gut function using both *in vitro* and *in vivo* model systems. We aimed to recapitulate realistic exposure scenarios by evaluating the effects of low-dose, oral, deltamethrin exposure on intestinal motility and related signs of intestinal and

metabolic dysfunction. We provide new insights into the potential for oral pyrethroid exposure to disrupt intestinal physiology through actions on EECs. Our work has broad implications for not only intestinal and metabolic disease, but also neurodegenerative conditions such as Parkinson's disease that commonly present with early intestinal dysfunction.

1.13. Figures.

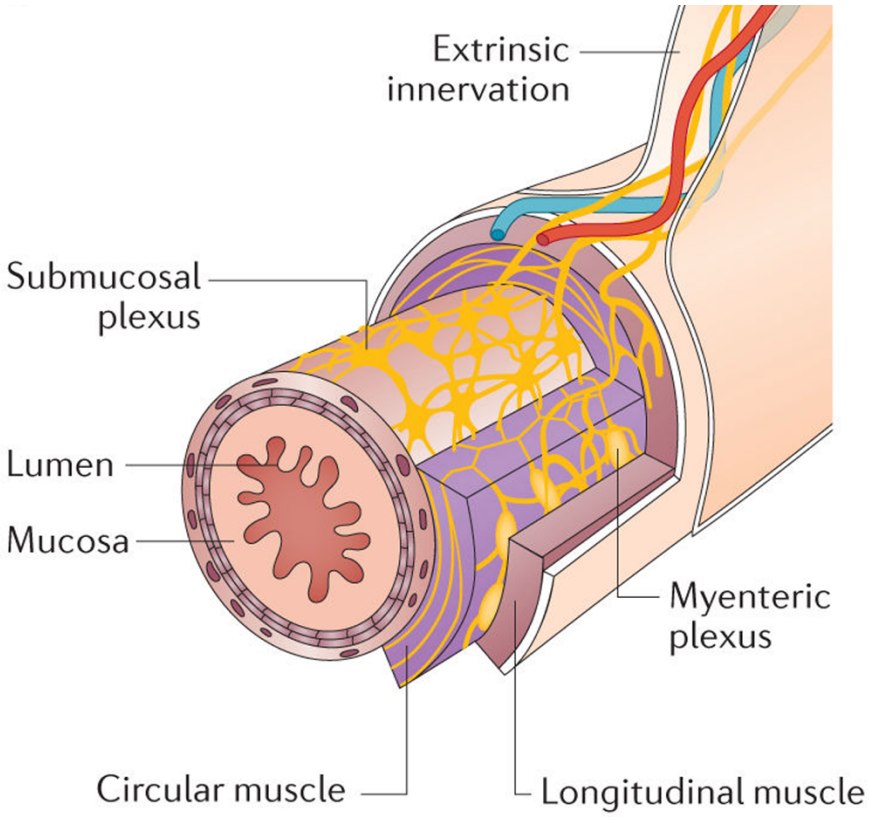

Figure 1.1. The anatomical layers of the gastrointestinal tract.

The basic anatomy of the gastrointestinal (GI) tract, which is divided into six layers: the intestinal serosa (unlabeled outermost layer of the GI tract); longitudinal muscle layer; myenteric plexus; circular muscle layer; submucosal plexus; and mucosal layer. The gut epithelium exists within the mucosal layer, where it makes contact with ingested contents inside the intestinal lumen. The myenteric and submucosal plexuses are what make up the enteric nervous system (ENS), whose innervation of the GI tract is referred to as 'intrinsic.' Extrinsic innervation refers to other components of the peripheral nervous system whose cell bodies are 'extrinsic' to the GI tract but innervate it nonetheless, such as the vagus nerve of the parasympathetic branch and the splanchnic nerve of the sympathetic branch. Figure from (182).

Cell Type	Hormone	Location	Function	Reference
X or A/ P or D1	Ghrelin	Stomach and proximal intestine	Stimulation of food intake	[18,19]
G	Gastrin	Stomach	Gut motility and pancreatic enzyme release	
D	STT	Stomach and intestine	Inhibition of GI hormone	
EC	Serotonin	Stomach and intestine	Inhibition or stimulation of food intake Stimulation of gut motility	
I	CCK	Proximal intestine	Inhibition of food intake and stimulation of the gallbladder	
K	GIP		Stimulation of insulin and gastric acid secretion, nutrient sensing	
S	Secretin		Inhibition of gastric acid secretion and motility	
N	NTS	Distal intestine	Stimulation of gastric acid and bile secretion	
L	GLP-1, GLP-2, PYY, OXM, NTS		Inhibition of food intake, gastric acid secretion, and response in glucose absorption	

Abbreviation: EC, enterochromaffin cells; STT, somatostatin; CCK, cholecystokinin; GIP, glucose-dependent insulinotropic peptide; NTS, neurotensin; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; PYY, peptide YY; OXM, oxyntomodulin.

Table 1.1. Enteroendocrine cell subtypes and their major hormone(s), location in the intestine, and general function. From (183).

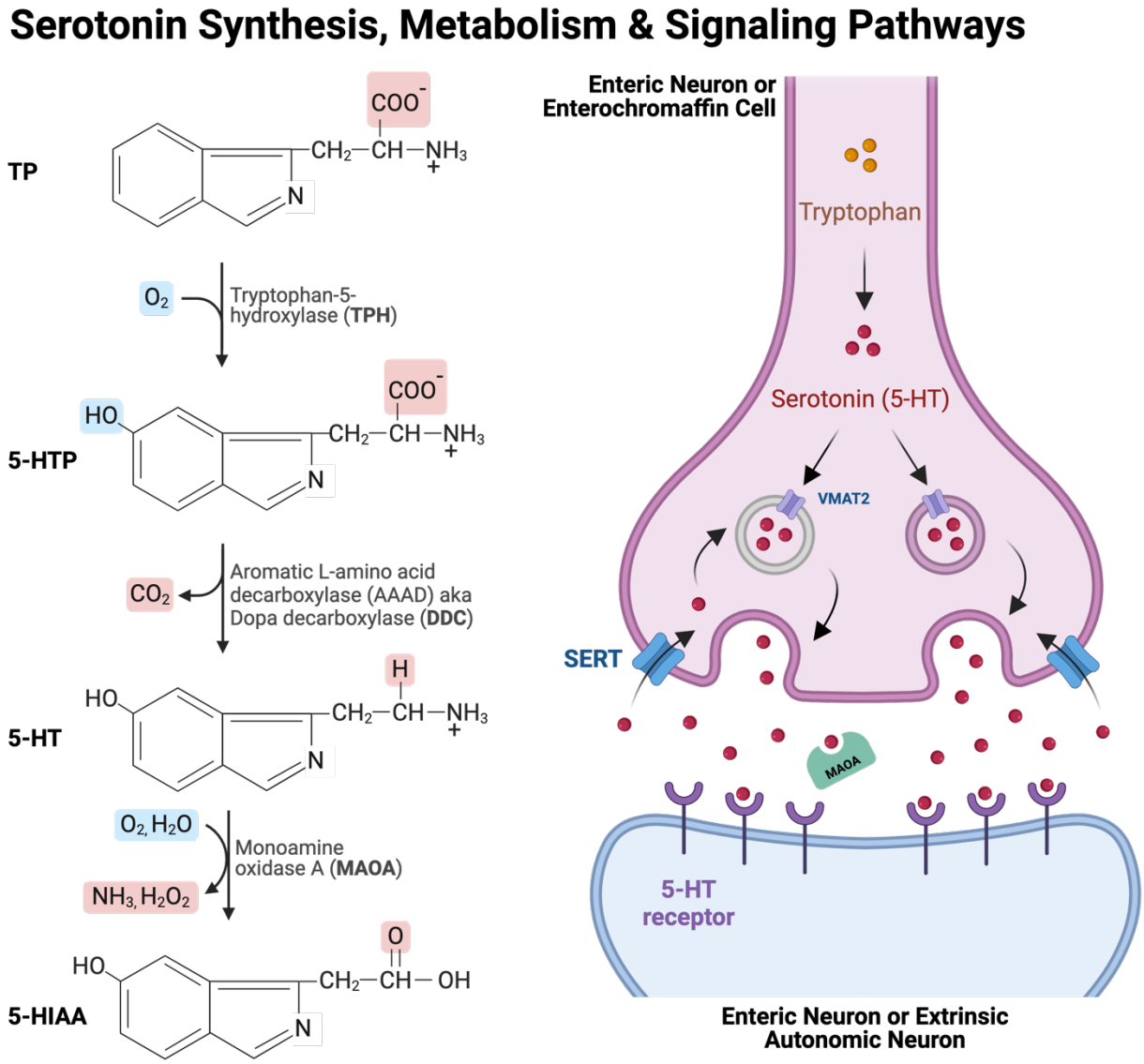

Figure 1.2. Serotonin synthesis, metabolism, and signaling pathways. Most of our body's serotonin supply is produced in the gut, primarily but enterochromaffin cells (ECs) in the gut epithelium. TPH-1 is the rate-limiting enzyme involved in serotonin synthesis in ECs, while TPH-2 is used in the synthesis of neuronal serotonin. Serotonin is released via endocrine, paracrine, and synaptic neurotransmission signaling mechanisms. Modified from a Biorender template (Biorender.com).

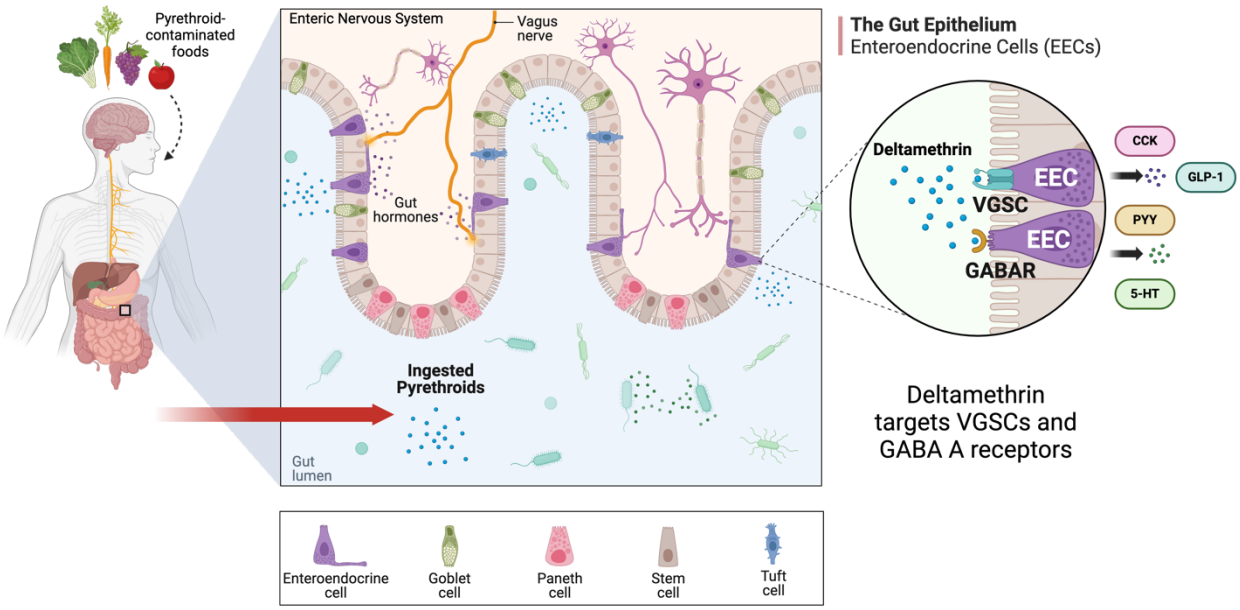

Figure 1.3. The gastrointestinal environment is vulnerable to ingested pyrethroids. Pyrethroids are commonly used insecticides used around the world. Ingestion is the most common route of pyrethroid exposure, and the presence of neuron-like enteroendocrine cells throughout the gut epithelium makes it especially vulnerable to pyrethroid-induced neurotoxicity. Created in Biorender (biorender.com).

Chapter 2: The pyrethroid insecticide deltamethrin disrupts neuropeptide and monoamine signaling pathways in the gastrointestinal tract

This chapter has been submitted for publication as the following:

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2.1 Abstract

Enteroendocrine cells (EECs) are a rare cell type of the intestinal epithelium. Various subtypes of EECs produce distinct repertoires of monoamines and neuropeptides which modulate intestinal motility and other physiologies. EECs also possess neuron-like properties, suggesting a potential vulnerability to ingested environmental neurotoxicants. One such group of toxicants are pyrethroids, a class of prevalent insecticides used residentially and agriculturally. Pyrethroids agonize voltage-gated sodium channels (VGSCs), inducing neuronal excitotoxicity, and affect the function of monoamine-producing neurons. Given their anatomical location at the interface with the environment and their expression of VGSCs, EECs likely represent a vulnerable cell-type to oral pyrethroid exposure. In this study, we used the EEC cell line, STC-1 cells, to evaluate the effects of the common pyrethroid deltamethrin on the functional status of EECs. We find that deltamethrin impacts both expression of serotonergic pathways and inhibits the adrenergic-evoked release of an EEC hormone, GLP-1, *in vitro*. In a mouse model of oral exposure, we found that deltamethrin induced an acute, yet transient, loss of intestinal motility, in both fed and fasted conditions. This constipation phenotype was accompanied by a significant decrease in peripheral serotonin production and an inhibition of nutrient-evoked intestinal hormone release. Together, these data demonstrate that deltamethrin alters monoaminergic signaling pathways in EECs and regulates intestinal motility. This work demonstrates a mechanistic link between pyrethroid exposure and intestinal impacts relevant to pyrethroid-associated diseases, including inflammatory bowel disease, neurodegenerative disease, and metabolic disorders.

2.2 Introduction

Enteroendocrine cells (EECs) are sensory transducing cells of the gastrointestinal (GI) epithelium. Physically located at the interface of the intestinal-luminal environment, EECs are readily exposed to numerous factors, including intrinsic microbiome components and exogenous environmental and dietary molecules. In response to such stimuli, subsets of EECs produce various monoamines and neuropeptides including serotonin (5-hydroxytryptamine; 5-HT), CCK (cholecystokinin), PYY (peptide YY), and GLP-1 (glucagon-like peptide 1). These signaling molecules act to modulate intestinal motility, regulate blood glucose, and transmit satiety signals to the brain (184, 185). Although the role of EECs in microbial and nutrient sensing is well-described (71, 73, 74, 186, 187), whether and how EECs respond to common environmental toxicants is largely unknown.

Pyrethroids are a common insecticide used residentially (e.g., home pest control, gardening, and lice treatment), in veterinary medicine (e.g., flea and tick treatment), and in agricultural industries. Their prevalent and large-scale use in food production and agriculture results in widespread pyrethroid detection within foodstuffs and water, ultimately leading to chronic, low-dose oral exposures to the human population (134). In rare cases of acute toxicity, high-level exposure leads to neurological defects including seizures, discoordination and imbalance, and cognitive impairment (149). These central nervous system (CNS) impacts are likely due to pyrethroid actions on their main molecular targets, voltage-gated sodium channels (VGSCs). Pyrethroids are VGSC agonists, leading to their prolonged hyperactivation (126). While Type I pyrethroids act primarily on VGSCs, Type II pyrethroids simultaneously antagonize $GABA_A$ receptors (GABARs), furthering neuronal excitation by preventing chloride-mediated inhibition.

Through these mechanisms, experimental models have demonstrated that pyrethroid exposure leads to significant disruption of monoamine signaling in neurons, including serotonin and dopamine (138, 139, 141, 144, 145). However, the actions of pyrethroids on non-neuronal cells of the periphery are less described.

Given their location within the intestinal epithelium, EECs represent a likely first cellular target of pyrethroids and other ingested toxicants. Their potential vulnerability to pyrethroid toxicity is highlighted by their expression of pyrethroid-sensitive VGSCs. Using deltamethrin, a prevalent Type II pyrethroid insecticide, we assessed impacts to monoamine pathways and hormonal release in both an EEC culture model and in a murine oral exposure model. We find that deltamethrin dose-dependently interferes with expression of monoamine pathways in EECs *in vitro* and induces acute, but transient, intestinal dysmotility *in vivo*. Correspondingly, we observe that intestinal serotonin concentrations are significantly and transiently reduced after acute deltamethrin exposure. Lastly, we demonstrate that stimulated release of EEC neuropeptides is suppressed by acute deltamethrin exposure both *in vitro* and *in vivo* and is chronically associated with increased food intake. Overall, these data demonstrate a significant impact of a prevalent pyrethroid on EEC status and GI function, suggesting a mechanistic link for pyrethroid-associated diseases that impact the GI tract, including inflammatory bowel disease (188), metabolic syndrome (189-192), and Parkinson's disease (193-195).

2.3 Materials and methods

Chemicals and treatments

For cell culture experiments, deltamethrin (Cat#: N-11579-250MG, purity 99%, Chem Service, Inc, Westchester, Pennsylvania) and bioallethrin (Cat#: P-664N, purity 98.7%,

AccuStandard, Inc, New Haven, Connecticut) were dissolved in sterile dimethyl sulfoxide (DMSO, cell culture grade) to form a stock concentration of 100 mM that was aliquoted and stored at -20C until use. For mouse experiments, deltamethrin was dissolved in filter-sterilized corn oil (Mazola) and prepared fresh one day prior to each use for acute experiments or every 3 uses for chronic experiments, wherein it was stored in the dark at room temperature (RT). Laboratory-grade acetone was used to facilitate deltamethrin's dissolution and was allowed to evaporate overnight. For cell culture, glucose and epinephrine were both purchased from Sigma (Glucose Cat#: G8270-1KG, purity 99.5%; Epinephrine Cat#: E4250-1G, purity 99%), dissolved in PBS, and aliquoted for storage in -20C. Calcium-free media (Gibco Cat#: 21068028) was supplemented with 0.1mM EDTA, filter-sterilized, and stored at 4C. Ensure® (Abbott Pharmaceuticals) mixed-meal nutrient mixtures were prepared fresh on the day of use and mixed with 6% carmine red dye with 0.5% methylcellulose (Sigma Cat#: C1022 and M7027, respectively) prior to gavage. Unless stated otherwise, all stock solutions were filter-sterilized prior to use and were of pharmaceutical or food grade.

Animal husbandry

10-12 week old C57BL/6J male and female mice (The Jackson Laboratory, RRID: IMSR_JAX:000664), as indicated, were co-housed according to treatment group in Emory University's Whitehead Biomedical Research Building rodent facility. Mice were provided standard food and water *ad lib*, except when noted for mixed-meal nutrient stimulation experiments, under a 12h light/dark cycle with lighting at 7AM. At the experimental endpoints, 4-24h or 12 weeks following final treatments or exposures, mice were humanely euthanized via cardiac perfusion with sterile PBS after deep isoflurane

anesthesia. All animal husbandry and experimental procedures were performed in accordance with Emory's Institutional Animal Care and Use Committee (IACUC) protocol #201900030.

Oral exposures

For acute exposures, mice were orally gavaged once with 50 μL of 3 mg/kg deltamethrin (Chem Service, Cat#: N-11579-250MG) dissolved in filter-sterilized corn oil or 50 μL of filter-sterilized corn oil alone as vehicle control using a sterilized metal feeding needle (22G) as described previously (196). This dose aligns with prior work (133, 139, 197, 198) and falls within previously reported no observable effect and no observable adverse effect limits defined by the U.S. EPA (199). Mice were monitored closely for overt signs of acute toxicity, none of which were observed throughout our studies. Corn oil was selected as a vehicle for its longstanding use in the field of pyrethroid toxicology (198, 200-204) and relevance to modeling oral exposures given its use as food (205). For chronic exposure experiments, mice were gavaged once weekly for 12 weeks. Subsequent intestinal assays were conducted within consistent timeframes to account for circadian rhythm. For acute experiments, gavages occurred every 7 mins beginning at 10AM for 4h exposures or 3PM for 24h exposures, while carmine/mixed-meal nutrient gavages (100 μL) occurred every 7 mins starting at 12:30 PM, and fecal output and any motor behaviors occurred in the afternoons at 2PM. For chronic experiments, gavages occurred at 1PM once per week, while fecal output occurred the following morning at 10AM, and tests of total GI transit occurred at 0-, 6-, and 12-weeks post-exposure and lasted from 10AM until 6PM. Food intake was measured only under chronic treatment conditions each week on the day prior

to deltamethrin or vehicle gavage by weighing the amount of food consumed each week per cage, where each cage contained 4 mice. Therefore, food intake per cage = amount of food added the prior week – amount of food remaining at the end of the week.

Intestinal behaviors

Fecal output: Performed as previously described (206). At the indicated timepoints (Acute: 4h or 24h after single oral gavage; Chronic: 24h after each oral gavage), mice were placed in a sterile 1L plastic container to count the number of fecal pellets produced every 5 mins for 30 mins.

Intestinal motility assay: Performed as described previously (207). At the indicated timepoints, mice were orally gavaged with 100 μL 6% carmine red dye 1.5h prior to sacrifice to visually track motility. During tissue collection, the distance traveled (cm) by the carmine dye was recorded as a percentage of the total length of the gastrointestinal tract (small intestine length + large intestine length, excluding cecum). If the dye was in the cecum, it was recorded as traveling 100% of the small intestine length. In a second set of experiments, Ensure (Abbott Pharmaceuticals) was pre-mixed with 6% carmine red dye prior to each mouse receiving 100 μL of the mixture 1.5h prior to sacrifice.

Total intestinal transit assay: Performed as previously described in detail (208). After one hour of habituation in an isolated behavioral testing room, mice were orally gavaged with 100 μL carmine red dye solution [6% carmine red dye mixed with 0.5% methylcellulose]. One hour later, mice were split into separate cages where they were housed individually with access to food and water but no bedding. Mice were observed every 15 mins for up to 8 hours or until a red fecal pellet was produced, at which point

mice were returned to their home cage. The difference in time at which the mouse is gavaged and a red pellet is produced equals total intestinal transit time and was recorded in hours.

Cell culture

The mouse (*Mus musculus*) secretin tumor cell line (STC-1, RRID:CVCL_J405) at passage 29 was purchased from the American Type Culture Collection (ATCC, Cat#: CRL-3254) and cultured as described in detail (209). Cells were seeded in 24 or 96-well plates at a density of 80,000 or 20,000 cells/well, respectively. Cells were cultured in DMEM/F12 media with GlutaMAX (GIBCO, Cat#: 10565018) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Cat#: 16140071) and 2% penicillin/streptomycin (5,000 U/mL, Gibco, Cat#: 15070063), modified from work described previously (26). Cells were incubated in a 5% CO_2 humidified chamber held at 37C, and passage numbers were maintained between 30 and 35. Cells were passaged via 5-min incubation in 5mL 0.25% Trypsin-EDTA (Gibco, Cat#: 25200072). Media was changed every other day until treatments, which occurred 3-4 days after seeding once cells reached ~80% confluency.

Cytotoxicity & cell viability assays

LDH assay: Chemical-mediated cell death was assessed using the colorimetric CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Cat#: G1780), which measures lactate dehydrogenase (LDH) released into the supernatant, according to manufacturer's instructions and described in detail previously (210). LDH was quantified 24h after

treatments. Cells were grown to confluency (approximately 48-72h after seeding at 10k cells/well in a 96 well plate) and maintained at 37C until treatments. Maximum LDH release was measured by adding 10 μL lysis buffer per 100 μL media to a subset of untreated control wells and allowing them to incubate for 45 mins. Afterward, 50 μL of supernatant from each well was collected and placed in a new 96 well plate for LDH quantification along with 50 μL of CytoTox 96 Reagent and incubated for 30 mins at room temperature (RT) in the dark. Stop solution was added immediately prior to absorbance reading, which was measured at 490nm using a spectrophotometer.

MTT assay: Cell viability was assessed as described previously (211) using the CyQUANT™ MTT Cell Viability Assay (Invitrogen, Cat#: V13154), which measures cellular redox potential through their ability to reduce the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into an insoluble formazan product. Cells were treated for 24h prior to assessment of cell viability. Cells were resuspended in 100 μL of fresh culture medium (phenol-free) and 10 μL of the 12 mM MTT stock solution was added to each well, including negative control wells without cells. Cells were then incubated at 37C for 3h. All but 25 μL of culture media was removed prior to the addition of 50 μL DMSO with repeated pipetting to ensure the solubilization of the formazan products. After another 37C incubation for 10 mins, the absorbance was read at 540 nm using a spectrophotometer.

Gene expression analysis

RNA extraction: At confluency, STC-1 cells were treated with 0.01% dimethyl sulfoxide (DMSO) vehicle control or 12.5, 25, 50, or 100 μM deltamethrin. Approximately 24h later, cells were collected for RNA extraction as described in detail previously (212). Briefly,

cells were washed gently once with PBS and 250 μL Trizol (Zymo Research, Cat#: R2050-1-200) was added to each well for RNA extraction using the Qiagen RNeasy Mini Kit (Cat#: 74106). In brief, cell lysates were homogenized by sonication (QSonica, ~35-45 setting) for 3-5s before 1/5 volume of chloroform was added to each sample and shaken for 15s. After sitting for 2 mins at RT, samples were centrifuged at 12,000 rpm for 15 mins at 4C. The upper aqueous clear layer was transferred to a QIAshredder (Qiagen, Cat#: 79656) and centrifuged for 2 mins at full speed at RT. The flow-through was combined with a half volume of 70% ethanol and transferred to an RNeasy spin column. Samples were centrifuged briefly for 30s at 10,000 rpm and flow-through was discarded. 700 μL Buffer RW1 was added to the spin column prior to centrifuging again for 30s at 10,000 rpm. Flow-through was discarded and 500 μL Buffer RPE was added to the spin column followed by centrifuge for 30s at 10,000 rpm. This step was repeated but instead centrifuged for 2 mins before discarding flow-through. The RNeasy spin column was then placed in a new collection tube and centrifuged for 1 min at full speed to collect any excess liquid. Finally, the spin column was placed in a new 1.5 mL Eppendorf tube, 30 μL RNase-free water was added, and samples were centrifuged at 10,000 rpm for 1 min to elute RNA. RNA concentrations for each sample were measured using a NanoDrop.

cDNA conversion: RNA was converted to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Cat#: 1708891) following the manufacturer's instructions and described in detail previously (213). Briefly, samples were diluted to 50 ng/μL up to a total of 1 μg RNA. To achieve a 20 μL reaction volume, 4 μL of 5x iScript Reaction Mix and 1 μL iScript Reverse Transcriptase was added for every 15 μL of sample. The complete reaction mix was then incubated in a thermal cycler as follows: priming for 5 mins at 25C, reverse

transcription for 20 mins at 46C, inactivation for 1 min at 95C, and an optional hold step at 4C.

Real-time quantitative PCR (RT-qPCR): was performed for gene expression analysis according to a previously described protocol (214). cDNA was diluted 1:10 to reach a final amount of 10 ng cDNA per reaction. Primer pairs were diluted from 100 μM stocks to 10 μM combined working stocks. Per sample and gene of interest, a 12 μL reaction mix was created: 6 μL SYBR Green PCR Master Mix (Thermo Fisher Scientific, Cat#: 4309155), 2 μL diluted primer pairs, 2 μL DI H_2O, and 2 μL diluted cDNA. Each sample was tested in technical duplicate and appropriate controls and blanks were used (no-template control, cDNA only, DI H_2O only). All primer pair sequences are reported in Supplementary Table 1 and the available key resource table in the Zenodo repository under accession 14803680 (doi.org/10.5281/zenodo.14803680).

Bulk RNA sequencing

Full service, bulk RNA sequencing was performed through Novogene Corporation, Inc. (Sacramento, California). Detailed RNA preparation, sequencing, analysis methods, and source data are found in the Zenodo repository under accession numbers 14775919 and 14803680 (doi.org/10.5281/zenodo.14775919 and doi.org/10.5281/zenodo.14803680).

Library preparation: A total input of 1 μg RNA was to generate sequencing libraries using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA), per manufacturer's instructions. Following mRNA enrichment using poly-T oligo-attached magnetic beads, RNA was fragmented using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). cDNA was synthesized first with

random hexamer primers and M-MuLV Reverse Transcriptase (RNase H-) and amplified by DNA Polymerase I and RNase H. NEBNext Adaptors were ligated and ~150-200bp fragments purified with AMPure XP system (Beckman Coulter, Beverly, USA). Fragments were amplified with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer and amplified products purified (AMPure XP system) assessed for quality on the Agilent Bioanalyzer 2100 system.

Clustering and sequencing: cBot Cluster Generation System using PE Cluster Kit cBot-HS (Illumina), according to the manufacturer's instructions, was used to cluster samples prior to paired-end sequencing on the Illumina platform NovaSeq X Plus Series (PE150) to a depth of 20M reads (6G). Raw sequencing reads are available in the NIH SRA database at accession number PRJNA1218260.

Quality control: Raw data in FASTQ format were firstly processed through *fastp* (RRID:SCR_016962; v0.23.1) to remove adapter and poly-N sequences and reads with low quality from raw data, as described in detail along with quality control files in (doi.org/10.5281/zenodo.14775919).

Genome alignment and analysis: Reference genome (*Mus musculus*, GRCm39/mm39) and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly. Paired-end clean reads were aligned to the reference genome using Hisat2 (RRID:SCR_015530; v2.0.5). Mapped reads were assembled using StringTie (RRID:SCR_016323; v1.3.3b) (215). featureCounts (RRID:SCR_012919; v1.5.0-p3) (216) was used to quantify mapped reads and calculate RPKM for differential analysis._Differential expression analysis was performed using DESeq2 R package (RRID:SCR_015687; v1.20.0) (217). The resulting *p* values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR).

Genes with an adjusted *p* value < 0.05 and $|\log_2(\text{foldchange})| \geq 1$ found by DESeq2 were assigned as differentially expressed. Pathway enrichment was performed using Metascape (RRID:SCR_016620; v3.5.20240901; https://metascape.org) (218). Enriched terms defined by Metascape are selected using Fisher's exact test with a p-value cutoff threshold of 0.02.

All analysis files, including aligned sequences, differential gene expression and Metascape source inputs and outputs are available in the Zenodo repository under accession numbers 14775919 and 14803680 (doi.org/10.5281/zenodo.14775919 and doi.org/10.5281/zenodo.14803680).

Protein quantifications

ELISAs: ELISAs were performed according to manufacturer's instructions and described in detail previously (219). Active GLP-1 release in STC-1 cell supernatant was detected with a GLP-1 (Active) ELISA kit (Millipore, Cat#: EGLP-35K). Samples were diluted 10-fold into kit-provided Assay Buffer prior to adding 200 µl sample/well. The plate was incubated overnight at 4C followed by five washes prior to the addition of 200 µl Detection Conjugate which incubated on a slow shaker for 2 hours at RT. The plate was washed 3 times and Substrate was added to each well for a final 20-min incubation in the dark at RT. Once sufficient fluorochrome was generated, 50 µl Stop Solution was added before reading the plate at an excitation/emission wavelength of 355 nm/460 nm.

Multiplexed ELISAs: Used to detect gut hormones in serum, blood derived from cardiac puncture was phase separated into serum via Vacuette tubes (Greiner-Bio One, Cat#: 454243P) after centrifugation at RT for 10 mins at 1800 rcf and stored at −80C until sample analysis. Multiplexed ELISAs were performed on indicated serum samples

through the Emory University Multiplexed Immunoassay Core. Experiments were performed in duplicate using the U-PLEX Metabolic Hormones Combo 1 for mouse (Meso Scale Discovery, Rockville, MD, USA, Cat#: K15306K-2) with 'blank' replicates that served as negative controls, according to the manufacturer's instructions (220). All samples were diluted two-fold for analyses. Heatmapper (RRID:SCR_016974) was used to visualize gut hormone levels in each treatment condition using the average linkage clustering method and the Euclidean distance measurement method (221).

High Performance Liquid Chromatography (HPLC)

HPLC was performed on indicated samples (ileum, colon, serum) through the Emory University HPLC Bioanalytical Core, and as described previously (222). Samples were resuspended in 300 µL ice cold 0.1M PCA, 0.1 mM EDTA and sonicated on dry ice using probe sonication. Pulses were 1s on, 10s off, and used an amplitude of 25% for 20-30s (QSONIC Q500A, Newtown CT). The homogenated samples were then centrifuged at 13,000 × g for 15 mins at 4C. Sample supernatants were transferred into new 0.22 µM PVDF microcentrifuge filter tubes and filtered through a spin filter at 5000 × rpm for 5 mins at 4C. Reverse-phase HPLC with electrochemical detection was used to measure monoamine concentrations. Protein pellets were dissolved in 500 µL 2% Sodium dodecyl sulfate (SDS). Protein quantification was performed in triplicate in 96-well microplates with SpectraMax M5e spectrophotometer (Molecular Devices, Sunnyvale, CA) using the Pierce BCA Protein Assay Kit (Thermo Scientific, Cat #A55864). Monoamines were quantified using the ACQUITY ARC system equipped with a 3465 electrochemical detector (Waters). Separations were performed using an Xbridge BEH C18, 2.5 µm, 3 x 150 mm column (Waters) at 37C. The mobile phase contained 100 mM citric acid, 100

mM phosphoric acid, pH 3.3, 0.1 mM EDTA, 525 mg/L 1-octanesulfonic acid, and 7% acetonitrile. The detection flow cell was SenCell with 2 mm GC WE and the cell potential was set at 800 mV with a salt bridge reference electrode. The AST position was set at 1 while the ADF was 0.5 Hz. The needle was washed with water and the pump piston was washed with 15% isopropanol. 20 µL of each sample was injected before being eluted isocratically at 0.6 mL/min. The analytes were identified by matching criteria from retention time measures to known standards (Sigma Chemical Co., St. Louis MO). Compounds were quantified by comparing peak areas to those of standards.

Statistical Analyses

All datasets were analyzed using GraphPad Prism 10 statistical software (RRID:SCR_002798; v10.4.1). Comparisons between two groups were generated using two-tailed t-tests for normally distributed data or by Mann Whitney nonparametric tests for non-normally distributed data. Comparisons between more than two groups were analyzed with one-way (one independent variable) or two-way (two independent variables) ANOVAs and follow-up post-hoc tests. Dunnett's multiple comparisons test was used to compare means to a control mean, Šídák's multiple comparisons test was used to compare pre-selected means to one another, and Tukey's multiple comparisons test was used to compare all means to one another. Where indicated, multiple t-test comparisons were performed. Outliers were removed according to the ROUT outlier identification test.

All source data and statistical outputs are included with this manuscript as supplemental material and are also available at the Zenodo repository under accession number 14803680 (doi.org/10.5281/zenodo.14803680). Raw sequencing files are deposited in

the NIH SRA, project number PRJNA1218260, and aligned reads and all analysis outputs in Zenodo under accession 1477919 (doi.org/10.5281/zenodo.14775919).

2.4 Results

Deltamethrin dysregulates monoamine pathways in EECs

To address the effects of deltamethrin on EECs, we first used the STC-1 cell line, a murine transformed cell line that is broadly representative of EEC subtypes (26). We observe that this line expresses a set of VGSCs, namely *Scn2a-Scn11a*, excluding *Scn5a* (Supplementary Fig. S1A). Other than *Scn2a* and *Scn9a*, these VGSCs are sensitive to deltamethrin and other pyrethroids (223). Over a range of relevant doses (0-100μM), we observed little cytotoxicity compared to vehicle controls following a 24hr deltamethrin exposure (Supplementary Fig. S1B, C). VGSC expression, specifically *Scn2a, Scn4a,* and *Scn9a* (but not *Scn3a, Scn5a, Scn8a, Scn10a,* or *Scn11a*), is greatly increased at higher doses at this timepoint (Supplementary Fig. S1D), suggesting that the expression of these channels is indeed affected following deltamethrin exposure.

Since deltamethrin disrupts monoaminergic signaling in CNS neurons (138, 139, 141, 224), we next sought to determine whether these pathways were similarly affected within these specific intestinal endocrine cells. Targeted gene expression analysis following 24hrs of deltamethrin exposure revealed that deltamethrin significantly dysregulates components of monoamine function, in particular serotonin synthesis and release pathways that are central to EEC functions. This includes significant increases in *Tph1*, *Tph2*, *Vmat2*, *Slc6a4*, and *Comt* expression but no effects on *Vmat1, Maoa*, or *Ddc* (Fig. 1A). Exposure to a Type I pyrethroid, bioallethrin, did not induce cytotoxicity

(Supplementary Fig. S2A, B) but was similarly disruptive to monoaminergic gene expression levels (Supplementary Fig. S2C), suggesting shared impacts to these pathways by representatives of both pyrethroid subtypes. Thus, pyrethroids transcriptionally dysregulate monoamine pathways in EECs.

VGSC activation, by pyrethroids and other agonists, leads to an influx of calcium to promote downstream signaling cascades (126). To determine whether the impacts we observed on gene expression in the monoamine pathways were dependent on calcium signaling, we performed identical deltamethrin exposures in the absence of extracellular calcium. Unlike deltamethrin exposures in calcium-replete media, in the absence of calcium, deltamethrin exposures did not result in the same widespread dysregulation of monoamine pathway genes (Fig. 1B). Excluding *Tph1*, expression of other genes, such as *Tph2, Vmat2*, *Slc6a4*, and *Comt* were unaffected. Therefore, the ability of deltamethrin to dysregulate these serotonergic and monoaminergic pathways in EECs is dependent on calcium signaling, and likely their canonical activation of VGSCs.

Deltamethrin inhibits epinephrine-evoked GLP-1 release in STC-1 cells

Other than monoamines, EECs produce a variety of neuropeptide hormones (26), which may also be affected by pyrethroid exposure. To investigate the impact of deltamethrin on STC-1 transcriptional status, particularly on expression of hormonal pathways, we performed bulk RNA sequencing on STC-1 cells treated with vehicle or 100 μM deltamethrin for 24h. We observed 159 differentially expressed genes (DEGs), 142 of which were significantly downregulated in response to deltamethrin treatment (Fig. 2A and our supplemental datasets [Zenodo #14803680 and #1477919]). Pathway analysis revealed that ‘Response to peptide hormone,’ was one of the most significantly enriched

pathways among the downregulated DEGs, along with 'Response to nutrient levels' (Fig. 2B, and Zenodo #14803680), suggesting that deltamethrin indeed transcriptionally dysregulates genes involved in hormonal activities. Notably, the gene encoding preproglucagon (*Gcg*), the precursor to the EEC-derived gut hormone GLP-1, is significantly downregulated with deltamethrin treatment (Fig. 2C). Given that STC-1 cells robustly produce GLP-1 in response to various stimuli (225), we then assessed how deltamethrin functionally impacts EEC neuropeptide signaling by measuring GLP-1 release. STC-1 cells were treated with epinephrine or glucose, two compounds known to stimulate GLP-1 release in these cells (26, 226), resulting in a dose-dependent increase in GLP-1 release, as anticipated (Fig. S3A, B). To determine how deltamethrin may affect the ability of EECs to respond to these stimuli, cells were pre-treated with deltamethrin prior to an epinephrine stimulus. While deltamethrin alone did not affect GLP-1 release (Fig. 2D), pre-treatment with deltamethrin significantly inhibited epinephrine-mediated GLP-1 release (Fig. 2D). These data indicate that deltamethrin interferes with the ability of EECs to respond to key modulatory signals, resulting in significant functional dysregulation across monoamine and GLP-1 signaling pathways.

Oral deltamethrin exposure induces acute constipation in mice

Given the significant impact on motility-regulating monoamine and GLP-1 pathways we observed following deltamethrin treatment of EEC-like cells in culture, we sought to determine potential intestinal functional impacts *in vivo*. We performed a battery of GI assessments in *ad-lib* fed mice following an oral exposure to low-dose deltamethrin (3 mg/kg body weight). After an acute exposure, 4hrs post oral gavage (serum C_{max} time), we observed that deltamethrin-treated male mice displayed

significantly reduced fecal pellet output, compared to those treated with corn oil vehicle (Fig. 3A). This acute effect of oral deltamethrin exposure was not observed in female mice (Supplementary Fig. S4), so male mice were used for the remainder of the study. A terminal dye transit assay further confirmed that total intestinal motility was significantly delayed after deltamethrin exposure in male mice (Fig. 3B). By 24hrs post-exposure, fecal output in treated animals was restored and intestinal transit was improved albeit still impaired compared to vehicle controls, suggesting that deltamethrin's effects, while drastic, are transient (Fig. 3C, D). These data clearly demonstrate that a single, low-dose, orally-administered deltamethrin exposure is sufficient to induce a constipation-like phenotype in mice.

Since we observed a striking *in vivo* intestinal motility phenotype that correlated with dysregulation of monoamine pathways in EECs in culture, we next measured circulating and tissue-resident serotonin levels. In deltamethrin-treated male mice, we detected significantly lower concentrations of serotonin (5-HT) in the ileum (~65% decrease post-treatment) and lower 5-hydroxyindoleacetic acid (5-HIAA, the major serotonin metabolite) levels in serum (~31% decrease), with no difference in colonic concentrations at acute (4hrs) timepoints post-exposure (Fig. 3E). At 24h post deltamethrin exposure, we observed only a significant reduction in serum 5-HIAA (~28% reduction), with no differences between treatment groups in the ileum or colon (Fig. 3F). These data together demonstrate that oral deltamethrin exposure significantly and acutely disrupts intestinal motility and accompanying peripheral monoamine signaling pathways.

Deltamethrin interferes with nutrient-stimulated intestinal hormone signaling *in vivo*

GLP-1 is released *in vivo* in response to glucose and other nutritional stimuli (225). Given our observations that deltamethrin inhibits GLP-1 release in EECs *in vitro*, we assessed how deltamethrin modulates intestinal hormones, including GLP-1, in response to a high nutrient, mixed-meal stimulus *in vivo*. Male mice treated with either deltamethrin or a corn oil vehicle were fasted for 4-6h immediately prior to stimulation with a mixed-meal nutrient oral bolus (Fig. 4A). Similar to our observations in fed mice (Fig. 3B, D), oral deltamethrin exposure resulted in an acute, but transient intestinal dysmotility compared to vehicle controls, even in the presence of a mixed-meal (Fig. 4B, D). To determine the extent to which deltamethrin may interfere with gastrointestinal hormone signaling *in vivo,* we measured an array of circulating intestinal neuropeptide hormones. While GLP-1 concentrations were not affected *in vivo*, we observed significantly less circulating insulin and leptin following acute deltamethrin exposure (Fig. 4C). This effect was transient, with concentrations similar to vehicle controls 24h post-exposure (Fig. 4E). Since both insulin and leptin contribute to satiety, and deltamethrin inhibited their release following a mixed meal, we tested whether deltamethrin modified food intake. Using a chronic treatment paradigm that allows the capture of weekly food intake, we observe an increase in food intake in deltamethrin-exposed mice (Fig. 4F, G), as well as qualitatively impaired GI transit (Fig. 4H). As a whole, these data emphasize a role for deltamethrin in perturbing broad gastrointestinal functions through neuropeptide modulation.

2.5 Discussion

Enteroendocrine cells are among the first cell types to interact with ingested environmental toxicants. Their anatomical location at the interface between the intestinal luminal environment and the nervous system, in conjunction with their neuron-like properties, underscore their vulnerability to neurotoxic compounds. Because pyrethroids are highly prevalent and a common route of their exposure is ingestion (134), they are a relevant exposure to impact EEC function and intestinal physiology. Here, we show that the pyrethroid deltamethrin affects the expression of monoaminergic pathways in EECs *in vitro* and induces *in vivo* perturbations to the gastrointestinal system that are associated with dysregulation of EEC functions, including serotonergic signaling and neuropeptide hormone release. These findings not only establish EECs as vulnerable to pyrethroid toxicity, but also provide insight into the mechanisms by which deltamethrin alters intestinal physiology, which may underlie known gastrointestinal dysfunctions of exposure-associated diseases.

Several studies report various GI disturbances following high-dose or chronic pyrethroid exposures in both animals and humans (134, 146, 149, 188, 227). These include nausea, intestinal pain, dysmotility, and gastrointestinal inflammation—pathologies that overlap with clinical features of irritable bowel syndrome (IBS), which is associated with pyrethroid exposure (228), and inflammatory bowel disease (IBD) (188). As dysregulation of both serotonin and neuropeptide signaling contribute to these diseases and pathologies (229-233), our data supports a direct pathological contribution of deltamethrin exposure to the EEC dysfunctions that characterize these intestinal diseases.

We observe pyrethroid-induced suppression of physiologically stimulated GI hormones GLP-1, insulin, and leptin, which each have broad actions in GI physiology and

metabolism (234-236). Pyrethroid exposure is linked to impairments in glucose metabolism, including diabetes (190, 191, 237, 238), with mixed findings to obesity (239, 240). Interestingly, serotonin has also been implicated as a contributor to obesity and diabetes (241). Given our observations that deltamethrin directly impacts both neuropeptide hormones and serotonin pathways in EECs, it suggests that EEC dysfunctions may be a link between pyrethroid toxicity and further metabolic disruption in the GI tract and systemically.

More broadly, pyrethroid exposure is linked with varying degrees of strength to neurodevelopmental disorders such as autism and attention-deficit hyperactivity disorder (133, 138, 242) and neurodegenerative outcomes relevant to both Parkinson's disease (PD) (139, 141, 143, 194) and amyotrophic lateral sclerosis (ALS) (243), many of which involve established disruptions to monoaminergic pathways, including serotonin (244, 245). PD in particular presents with significant GI dysfunctions (246), though the specific intestinal pathologies which underlie these are not well described. Constipation itself is a prevalent prodrome of PD (153, 155, 247, 248), and pyrethroids are epidemiologically linked to PD risk (136, 176, 249). It is therefore tempting to speculate that environmental exposures, such as to pyrethroids, trigger early pathologies at the site of exposure within the GI tract. In addition, emerging experimental and clinical data implicate GLP-1 signaling in limiting PD pathologies (250-254). Clinical studies have recently demonstrated benefit of systemic GLP-1 agonists for PD symptoms (250, 251). While the protective mechanisms are currently unclear (255), there are known roles for GLP-1 and insulin signaling in PD and other neurological disease risk (256-258).

Our data demonstrate that deltamethrin interferes with GLP-1 release and dampens a nutrient-evoked insulin response, which may contribute to the association

between pyrethroid exposure and these diseases. Serotonergic signaling in the CNS is also impacted post-pyrethroid exposure (144, 145, 259) further validating our observations that these insecticides may contribute to GI dysfunctions via similar mechanisms. Our data herein support the notion that pyrethroid exposures are sufficient to induce dysfunction within vulnerable intestinal cells that lead to relevant prodromal features of these and other diseases.

2.6 Limitations of this study

Both human and murine EECs display significant similarity in their transcriptional repertoires, including the expression of pyrethroid-sensitive VGSCs (260, 261). However, we appreciate that studies in murine models may not fully replicate human intestinal responses to these exposures. In addition, we focused our studies in male mice, based on our initial assessment that female mice did not display an acute constipation-like phenotype post-deltamethrin exposure (Supplementary Fig. S4). Our data provide a foundation for future studies to directly compare physiological outcomes to these exposures based on sex. Lastly, we chose to orally administer deltamethrin in a corn oil vehicle, as a relevant food-grade matrix. Alternative vehicles (*e.g.,* DMSO, ethanol, methylcellulose, glycerol formal), or routes of exposure (*e.g.,* inhalational, systemic) may differentially impact toxicokinetic properties and the intestinal outcomes we observe (202, 262). Nonetheless, our data herein provide a foundational dataset on EEC functional and transcriptional responses, as well as acute intestinal physiologies in response to a low-dose, oral pesticide exposure.

2.7 Conclusion

This study directly addresses pyrethroid-monoamine interactions within the GI tract, a critical step toward understanding the broad health impacts of pyrethroid exposure on intestinal physiology that is linked to many pyrethroid-associated diseases. We provide compelling evidence that the pyrethroid deltamethrin alters intestinal pathways important for serotonin trafficking in EECs, acutely impairs intestinal motility, and diminishes intestinal hormone production in response to physiological stimuli. Our data highlight the continued need to study toxicological impacts in the GI tract that may be associated with long-term exposure to these prevalent chemicals and underlie those diseases epidemiologically linked to exposure.

2.8 Acknowledgments

We thank Isabel Fraccaroli, Tiffany Chen, and Shreya Bandlamudi for scientific support and members of the Emory Division of Animal Resources for technical support. We acknowledge support from the Emory Multiplexed Immunoassay Core (EMIC) and the Emory HPLC Core which are subsidized by the Emory University School of Medicine as Integrated Core Facilities and are supported by the Georgia Clinical and Translational Science Alliance of the NIH (UL1TR002378).

2.9 Figures

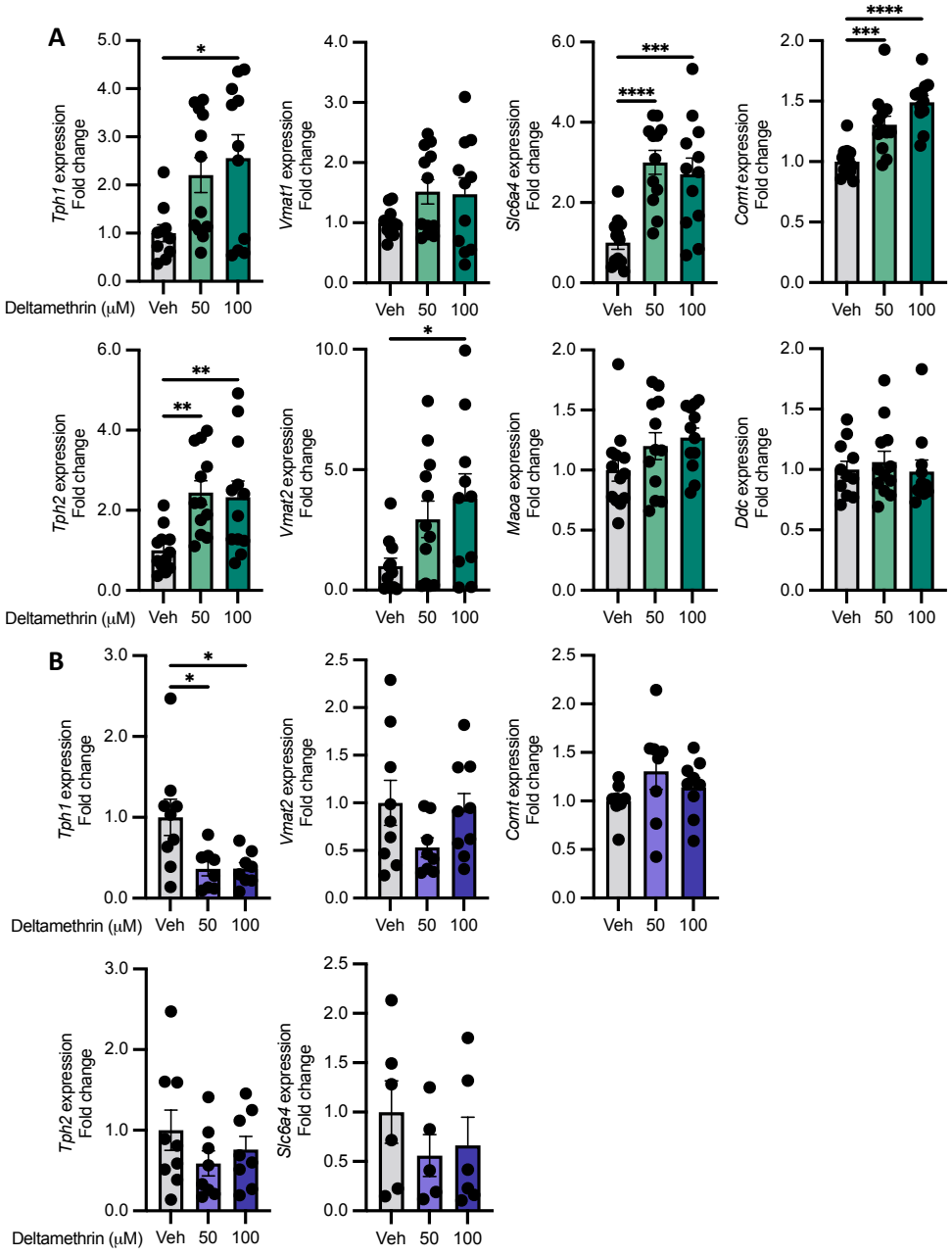


Fig. 2.1. Deltamethrin dysregulates monoaminergic gene expression pathways in STC-1 cells in a calcium-dependent manner. A Relative expression values expressed as fold change from STC-1 cells treated with DMSO vehicle alone (Veh), 50, or 100 μM deltamethrin for 24h**. B** Relative expression values expressed as fold

change from STC-1 cells treated with DMSO vehicle alone (Veh), 50, or 100 μM deltamethrin for 24h in calcium-free media. n = 9-12 (**A**) or n = 6-9 (**B**) independent culture wells per condition. Points represent independent wells (means of technical duplicates) and bars the mean ± SEM. Data compared by ordinary one-way ANOVA with Dunnett's multiple comparisons test comparing each group to the control mean. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.001$, ****$p < 0.0001$.

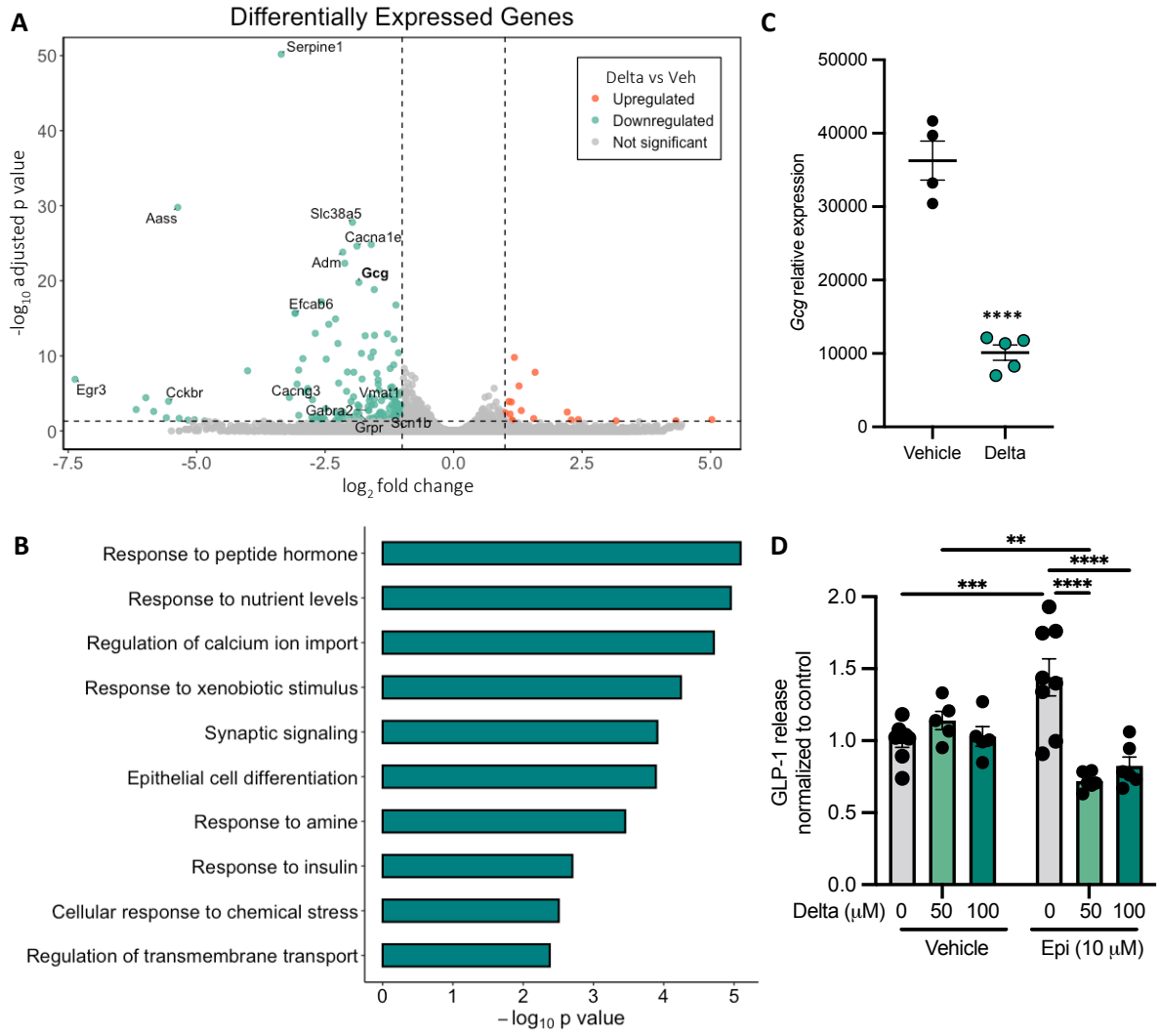


Fig. 2.2. Deltamethrin downregulates gut hormonal expression pathways and inhibits epinephrine-induced GLP-1 release in STC-1 cells. A Volcano plot of differentially expressed genes in STC-1 cells after 24h of 100 μM deltamethrin exposure. Green dots represent significantly downregulated genes following deltamethrin exposure and red dots represent significantly upregulated genes following deltamethrin exposure. **B** Significantly enriched pathways among downregulated DEGs according to Metascape. **C** *Gcg* expression by bulk RNA sequencing in STC-1 cells after 24h treatment with deltamethrin or vehicle control (adjusted p-value = 1.65e-20). **D** GLP-1 release quantified by ELISA and normalized to control after 0, 50 or 100 μM deltamethrin treatment in the

presence and absence of 10 μM epinephrine. **A** Individual data points represent individual genes. **B** Bars represent a set of differentially expressed genes associated with the corresponding enriched pathway. **C, D** Data points represent averages of technical replicates from individual samples where n = 4-5 **(C**) and n = 5-8 (**D**) per group. **A** Differentially expressed genes were determined using a corrected p-value < 0.05 and an absolute $\log_2$ fold change threshold of 1. **B** Enriched terms defined by Metascape are selected using Fisher's exact test with a stringent p-value threshold of 0.02. **C** No further statistical comparisons were made beyond the bioinformatics pipeline used for the identification of *Gcg* as a differentially expressed gene. **D** Data are depicted as mean ± SEM and compared by ordinary two-way ANOVA with Tukey's multiple comparisons test comparing means all means to one another. $^{**}p<0.005$, $^{***}p<0.001$, $^{****}p<0.0001$.

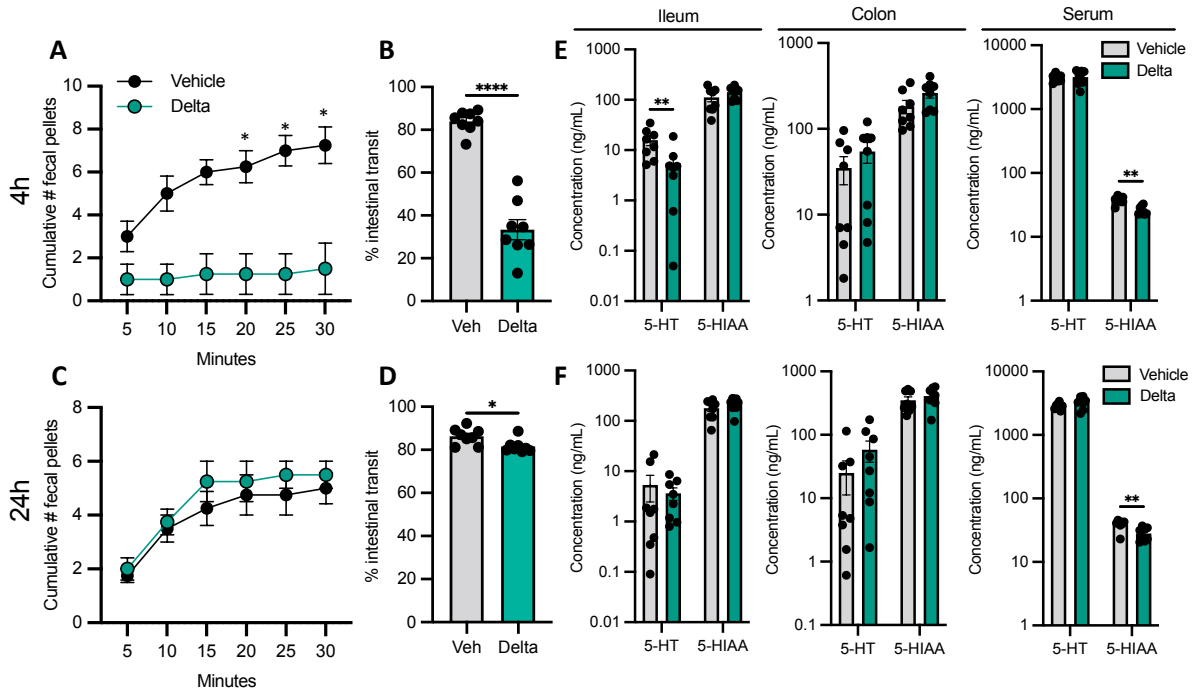


Fig. 2.3. Oral deltamethrin exposure induces acute intestinal dysmotility and limits serotonin production. **A** Fecal output (# pellets produced in 30 mins) after 4h deltamethrin or vehicle exposure. **B** The % intestinal transit of red carmine dye in the GI tract of 4h vehicle or deltamethrin-treated mice immediately after sacrifice. **C** Fecal output after 24h deltamethrin or vehicle exposure. **D** The % intestinal transit of 24h vehicle or deltamethrin-treated mice. **E-F** Peripheral serotonin (5-HT) and 5-HIAA levels at 4h (**E**) or 24h (**F**) post vehicle or deltamethrin exposure in ileum, colon, and serum, as measured by HPLC. All data points represent averages of biological (**A, C**) or technical (**B, D-F**) replicates where n = 4 (**A, C**) or n = 8 (**B, D-F**) per group. Data are depicted as mean ± SEM and compared by Two-way repeated measures ANOVA with Šídák's multiple comparisons test between treatment groups at each time point (**A, C**), Mann Whitney test for non-normally distributed data (**D, E, F**), or two-tailed unpaired student's t-tests (**B, E, F**). *p<0.05, **p<0.005, ****p<0.0001.

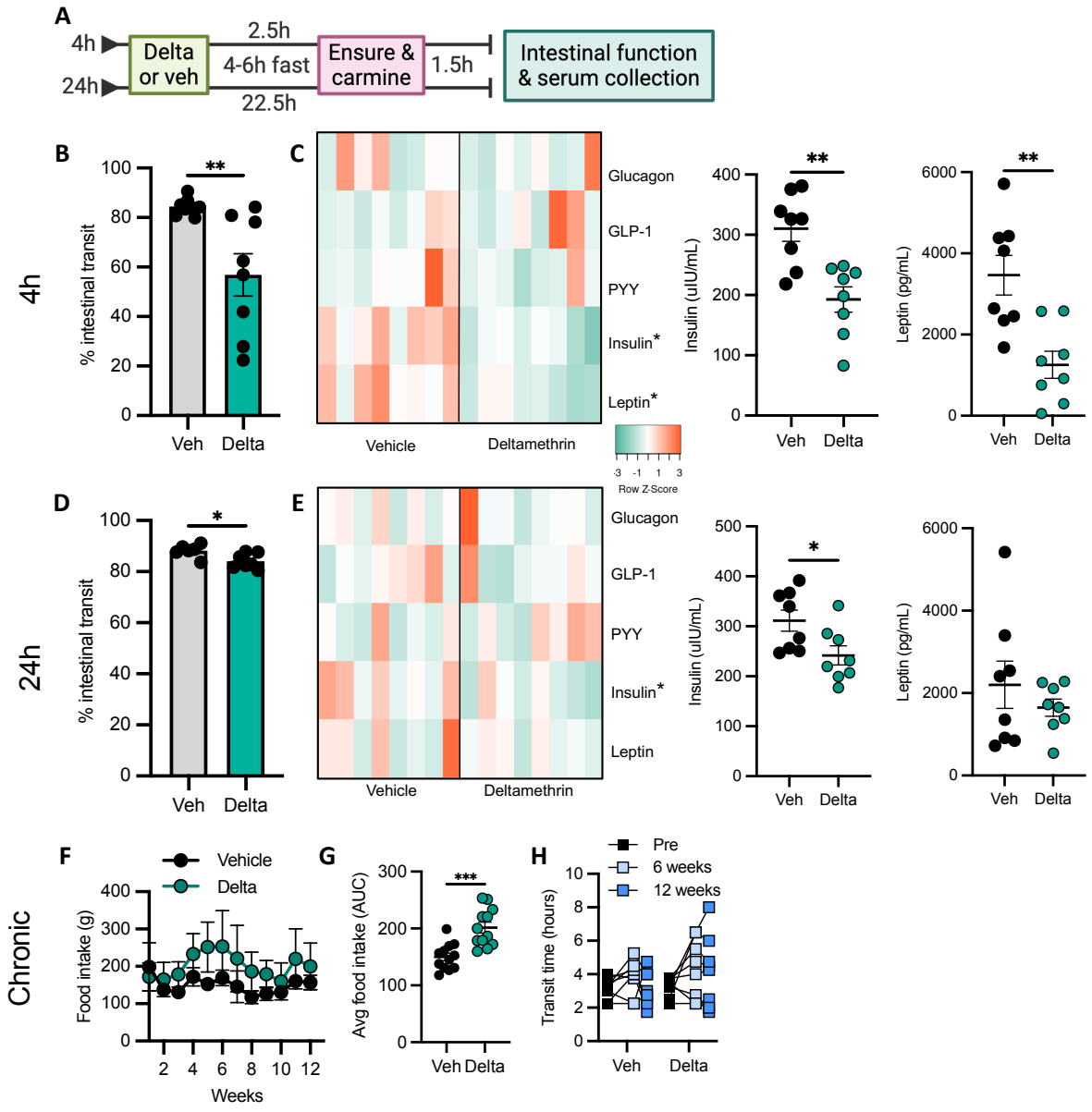
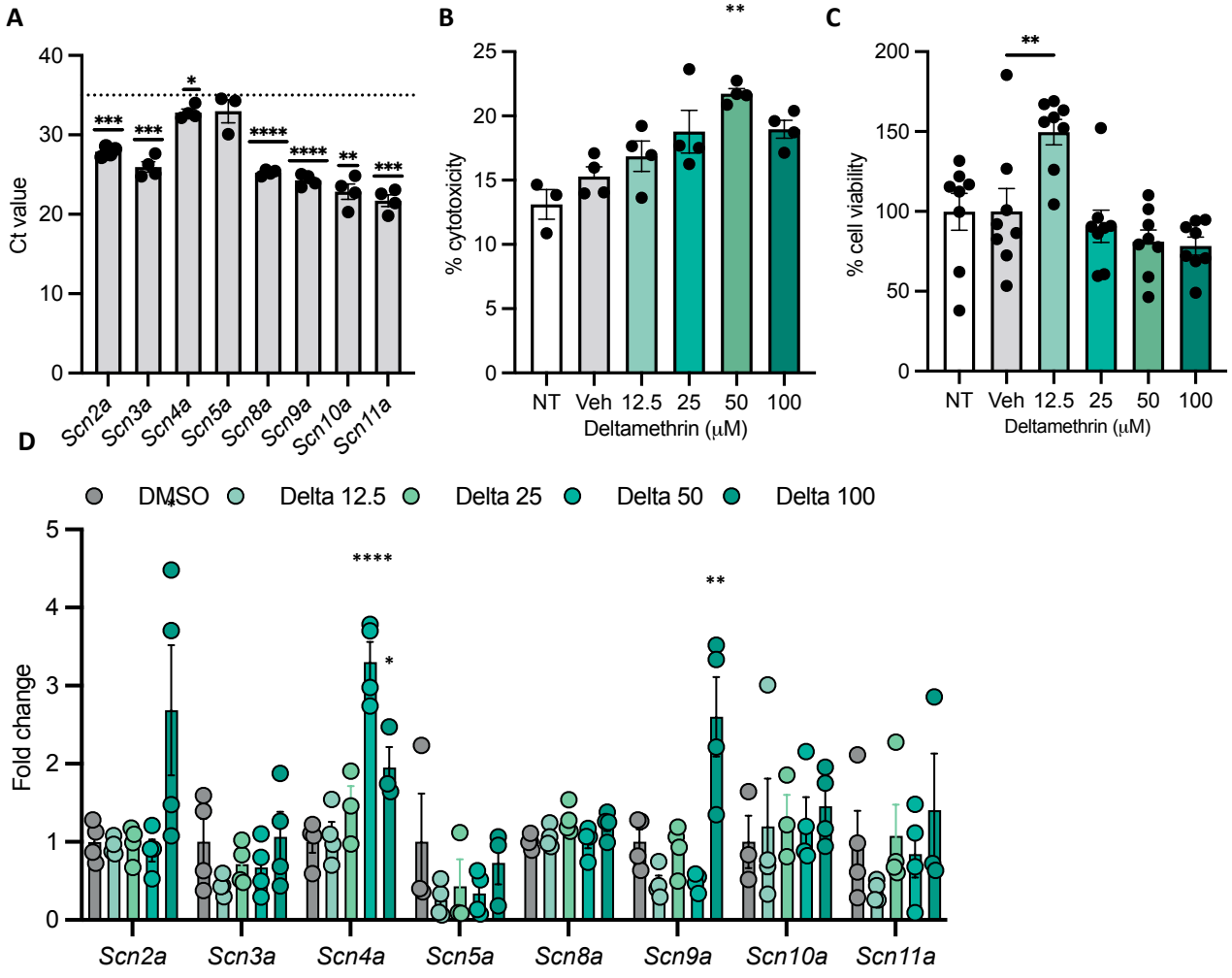


Fig. 2.4. Deltamethrin inhibits release of intestinal hormones following a mixed-meal nutrient-stimulus. **A** Schematic representation of the experimental timeline. **B** % intestinal transit of carmine red dye 4h after exposure to either vehicle or deltamethrin and measured upon sacrifice. **C** Accompanying gut hormone heatmap in serum after 4h exposure to vehicle or deltamethrin, determined by multiplexed ELISA. Insulin and leptin are depicted separately. **D** % intestinal transit of carmine dye after 24h

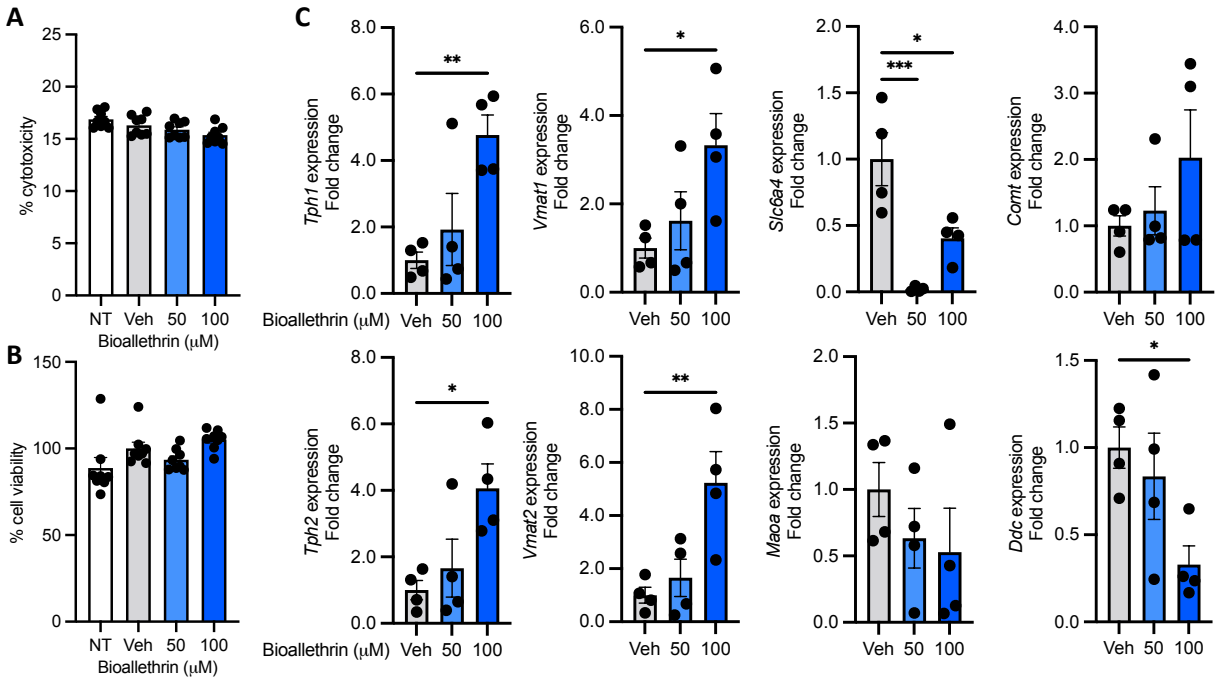
exposure to vehicle or deltamethrin. **E** Corresponding quantification of gut hormone levels in serum by heatmap after 24h exposure to vehicle or deltamethrin, with insulin and leptin depicted separately. **F** Average weekly food intake after chronic exposure to vehicle (black) or deltamethrin (green). **G** Average food intake expressed as area under the curve for vehicle- and deltamethrin-treated mice. **H** Total intestinal transit time in vehicle or deltamethrin-treated mice after 0 (black), 6 (light blue), or 12 weeks (blue) of exposure. **C, E** Concentration units are in pM for GLP-1 (active) and glucagon, uIU/mL for insulin, and pg/mL for leptin and PYY (total). **B-H** All data points represent individual mice (**B, D, H**), their averages after technical duplicates (**C, E**), or the average of two cages of 4 mice per cage (**F, G**), where n = 8 per group. Bars are depicted as mean ± SEM and compared by unpaired student's t-test (**B, C, E, G**), Mann Whitney nonparametric tests for non-normally distributed data (glucagon and PYY in **C, D**), or a Two-way repeated measures ANOVA (**F, H**). *p<0.05, **p<0.005, ***p<0.0005.

2.10. Supplementary Figures

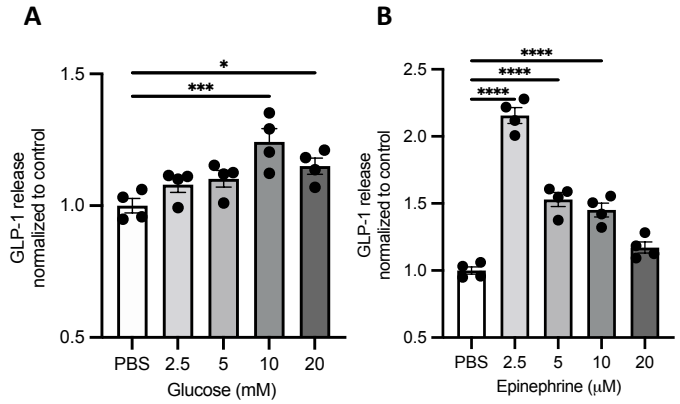


Supplementary Fig. S2.1. STC-1 cells express deltamethrin-sensitive voltage-gated sodium channels. A Ct values of each voltage-gated sodium channel (VGSC) subtype expressed in STC-1 cells. **B** Quantification of % cytotoxicity by lactate dehydrogenase (LDH) detection assay from STC-1 cells treated with 0, 12.5, 25, 50, or 100 μM deltamethrin for 24h. **C** Quantification of % cell viability by MTT assay from STC-1 cells treated with 0, 12.5, 25, 50, or 100 μM deltamethrin for 24h. **D** Fold change of different VGSC subtypes from STC-1 cells treated with 0, 12.5, 25, 50, or 100 μM deltamethrin for 24h, as determined by qPCR. **A-D** All data points represent averages of

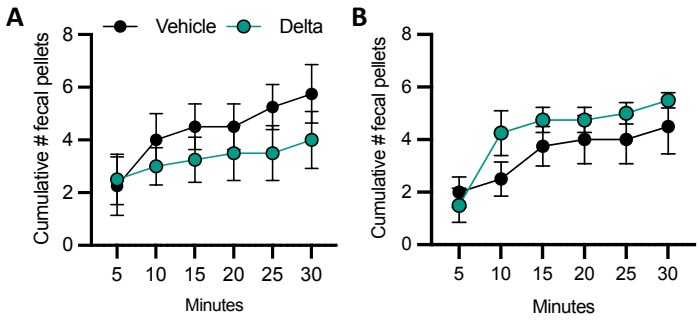
technical duplicates from an individual well where n = 3-4 (**A, B, D**) or n = 8 (**C**). Data are depicted as mean ± SEM and compared by ordinary one-way ANOVA with Dunnett's multiple comparisons tests (**B-D**) or one-sample t-test against a Ct cutoff value of 35 (**A**), represented by a dashed line. *p<0.05, **p< 0.005, ****p<0.0001.



Supplementary Fig. S2.2. The pyrethroid bioallethrin dose-dependently disrupts transcriptional serotonergic pathways. **A** Quantification of % cytotoxicity by lactate dehydrogenase (LDH) detection assay from STC-1 cells treated with 0, 50, or 100 μM bioallethrin for 24h. **B** Quantification of % cell viability by MTT assay from STC-1 cells treated with 0, 50, or 100 μM bioallethrin for 24h. **C** Relative expression values expressed as fold change from STC-1 cells treated with 0, 50, or 100 μM bioallethrin for 24h. **A-C** All data points represent averages of technical duplicates from an individual well where n = 8 (**A, B**) or n = 4 (**C**). Data are depicted as mean ± SEM and compared by ordinary one-way ANOVA with Dunnett's multiple comparisons tests (**A-C**). *p<0.05, **p< 0.005, ***p<0.001.



Supplementary Fig. S2.3. Glucose and epinephrine evoke GLP-1 release in STC-1 cells. A Quantification of GLP-1 release by ELISA from STC-1 cells treated with 0, 2.5, 5, 10, or 20 mM glucose for 1h. **B** Quantification of GLP-1 release by ELISA from STC-1 cells after treatment with 0, 2.5, 5, 10, or 20 μM epinephrine for 1h. **A-B** All data points represent averages of technical duplicates from individual samples where n = 3-4 per group. Data are depicted as mean ± SEM and compared by ordinary one-way ANOVA with Dunnett's multiple comparisons test comparing each group to the control (**A-B**). *p<0.05, ***p<0.001, ****p<0.0001.



Supplementary Fig. S2.4. Deltamethrin does not induce intestinal dysmotility in female mice. A Fecal output (cumulative # fecal pellets produced in 30 mins) after 4h of deltamethrin exposure. **B** Fecal output after 24h deltamethrin exposure. **A-B** All data points represent averages of biological replicates from individual samples where n = 4 per group. Data are depicted as mean ± SEM and compared by Two-way repeated measures ANOVA with Šídák's multiple comparisons test between treatment groups at each time point (**A-B**).

Chapter 3: Discussion

3.1 The pyrethroid deltamethrin affects enteroendocrine cell status & function *in vitro*.

We examined the ability of the neurotoxic pyrethroid insecticide, deltamethrin, to alter enteroendocrine cell (EEC) status and function *in vitro*. Our investigation expands upon decades of research demonstrating the effects of pyrethroid exposure in neuronal systems while newly establishing their ability to affect neuron-like cells of the gastrointestinal (GI) tract. Despite their scarcity within the gut epithelium, EECs constitute the largest hormonal reservoir in mammals (263). Loss or dysfunction of EECs has severe consequences to overall development (264), metabolism (265, 266), and intestinal health (264, 267, 268). A fact often shadowed by their reputation as robust producers of GI hormones, EECs possess neuronal properties. In addition to the expression of voltage-dependent ion channels and pre- and post-synaptic markers (24, 269), EECs fire action potentials and release neurotransmitters through synaptic connections formed with neighboring neurons (20). These characteristics, coupled with their accessible location at the interface of the gut lumen and epithelium, render EECs exceptionally vulnerable to ingested neurotoxicants. For these reasons, we looked to model exposures to deltamethrin using an established EEC cell line, STC-1.

Studying native EECs is complicated by their rarity and requisite crosstalk with a diverse community of cells and microbes for normal function. While successful primary EEC culture is technically possible (270), it is much more feasible to screen for EEC functions and response mechanisms using a cell line. STC-1 cells have long been used to study the functions of EECs, primarily through the lens of nutrient sensing and subsequent hormonal activity. Most importantly for our investigation, STC-1 cells possess many of the neuronal qualities observed in native EECs. They express voltage-gated ion

channels (271) and possess markers associated with synaptic neurotransmission (272), which we confirmed with our bulk RNA sequencing analysis (doi.org/10.5281/zenodo.14775919). Notably, we identified their suspected but previously undescribed expression of the major pyrethroid target, voltage-gated sodium channels (VGSCs; Supplementary Figure S2.1A; doi.org/10.5281/zenodo.14775919), though the fact that native EECs and other EEC cell lines express VGSCs has been described (21, 269). Altogether, these features reinforce the suitability of STC-1 cells as an appropriate model for the assessment of pyrethroid exposures in EECs.

We confirmed that deltamethrin transcriptionally disrupts its major targets, VGSCs and $GABA_A$ receptors (Supplementary Figure S2.1D; doi.org/10.5281/zenodo.14775919), as is established in the literature (126). In agreement with the ample evidence supporting a role for pyrethroids in the disruption of monoamine pathways in the central nervous system (139, 143, 194, 224, 259), we reported that deltamethrin significantly affects the majority of genes involved in serotonergic synthesis, reuptake, and metabolism in STC-1 cells (Figure 2.1A). This is an important observation because EECs are the major suppliers of peripheral serotonin, which is essential for normal GI function (106). Pyrethroids are known to alter serotonin pathways in the CNS (141, 144, 145), and our transcriptional findings indicate that deltamethrin may just as easily interfere with EEC-derived serotonin signaling in the gut. A significant limitation to testing this theory *in vitro*, however, is that STC-1 cells do not readily produce serotonin in our hands, nor is there any published evidence of their ability to do so despite their expression of *Tph1/2*. Because of the well-established production of other intestinal hormones by STC-1 cells—especially CCK, PYY, and GLP-1 (26)—we instead turned to these as potential targets for deltamethrin in EEC dysfunction.

Using bulk RNA sequencing, we screened for transcriptional changes in STC-1 cells following 24h deltamethrin exposure and found over 150 differentially expressed genes (DEGs) (Figure 2.2A). Most of these were significantly downregulated with deltamethrin treatment, which could indicate that deltamethrin disproportionately suppresses EEC regulatory activity. Despite the notable impact of deltamethrin on metabolic signaling pathways (Figure 2.2B), *Cck* and *Pyy* were unaffected (doi.org/10.5281/zenodo.14775919), suggesting that not all gut hormones are equally susceptible to deltamethrin toxicity. Interestingly, however, deltamethrin significantly downregulated *Gcg* expression, the precursor to both glucagon and GLP-1 (Figure 2.2C). This selective effect is perhaps due to GLP-1's distinct status as an incretin hormone. Indeed, pyrethroids have been linked to diabetes, insulin resistance, and metabolic dysfunction (189, 273, 274). We therefore investigated whether deltamethrin-induced transcriptional suppression of *Gcg* translated to a functional deficit in GLP-1 secretion.

Somewhat surprisingly, we found that deltamethrin alone did not significantly affect GLP-1 release in STC-1 cells (Figure 2.2D), despite the proposed dependence of GLP-1 release on VGSC activity (19) and voltage-gated calcium channel (VGCC) activation (275, 276). When GLP-1 release was stimulated by epinephrine, however, pretreatment with deltamethrin abolished this effect (Figure 2.2D). This suggests that sequential or co-activation of innate hormone stimulus pathways (in this case, via adrenergic signaling) and voltage-gated cation channels may be required for substantial GLP-1 release in STC-1 cells, and that deltamethrin interferes with this synergy. Indeed, epinephrine-induced GLP-1 release involves the complementary activation of multiple adrenergic receptor subtypes followed by the mobilization of intracellular calcium and cAMP signaling to facilitate exocytosis (226). Deltamethrin's inhibition of this pathway could therefore arise

from its disruption of calcium homeostasis, cAMP activity, or both. In fact, we observed significant transcriptional dysregulation of calcium trafficking pathways with deltamethrin treatment (Figures 2.2A, 2.2B) and of several genes responsible for cAMP regulation (doi.org/10.5281/zenodo.14775919). Transcript-level changes do not necessarily equate to functional impairment, of course, so further manipulations (e.g., the blockade of adrenergic receptors or VGCCs) are required to help elucidate deltamethrin's mechanism of interference, in addition to whether its interference is specific to the presence of monoaminergic stimuli like epinephrine or if it also extends to other GLP-1 secretagogues.

3.2 Deltamethrin-induced gut dysmotility and potential mechanisms.

We found that oral deltamethrin exposure significantly impaired gut motility, inducing a constipation-like phenotype in young adult male mice. This was evident following acute exposure, where we observed a substantial reduction in fecal output (Figure 2.3A) and significantly delayed intestinal transit (Figures 2.3B, D; 2.4B, D). While acute exposure elicited the most robust dysmotility outcomes, longer-term exposures also revealed some features of intestinal dysfunction. For example, after subchronic treatment with deltamethrin (once weekly oral gavages for 3 weeks), mice exhibited decreased fecal output compared to vehicle controls (Figure 3.1), but this effect did not persist with chronic exposure. Chronic deltamethrin treatment (once weekly oral gavages for 12 weeks) did, however, qualitatively impair total gastrointestinal transit (Figure 2.4H). Our findings suggest that oral deltamethrin exposure is transient and does not cause sustained, robust dysmotility in our exposure paradigms. The lack of robust outcomes during our subchronic and chronic paradigms might be due to the timing of our intestinal

assessments. We learned from our acute exposure assessments that dysmotility improves drastically by 24h post-exposure (Figures 2.3C, D; 2.4D), yet our previous subchronic and chronic assessments were performed at 24 and 48h post exposure. Our selected timepoints therefore may have masked transient motility impairments, and perhaps more frequent exposures (e.g., daily) would elicit a stronger outcome. The minimal long-term effects on gut motility also indicate some sort of intestinal recovery or resilience. The GI tract is especially good at withstanding harsh environments due to its constant exposure to xenobiotics through ingestion. Accordingly, the entire gut epithelium undergoes a self-renewal process every few days (277). This has interesting implications for how deltamethrin toxicity is received and "transmitted," and may explain why motility deficits did not persist.

The acute motility deficits we observed could be due to deltamethrin's actions on intestinal serotonin. Given the established effects of deltamethrin on serotonergic pathways in the CNS (144, 145, 259), our evidence that deltamethrin disrupts serotonergic gene expression in EECs *in vitro* (Figure 2.1A), and our associated finding that acute deltamethrin exposure diminishes ileal serotonin and circulating 5-HIAA levels (Figure 2.3E, F), this is a plausible explanation. Indeed, low serotonin levels are associated with constipation-predominant GI motility disorders (106, 278, 279). For example, individuals with constipation-predominant irritable bowel syndrome have significantly lower levels of circulating serotonin compared with healthy controls (280, 281). Moreover, pharmacological intervention using serotonin receptor agonists are a viable treatment option for some people with chronic idiopathic constipation (282-284), as greater serotonin conversely promotes intestinal motility. This clinical evidence strengthens the link between our observations of constipation-like dysmotility and serotonin reduction in

the periphery. However, further experimentation is necessary to determine whether serotonergic disruption is a cause or a consequence of intestinal dysmotility.

Interestingly, colonic serotonin levels were unchanged following acute (4h) exposures (Figure 2.3E, F) despite the fact that most serotonin-producing ECs are localized to the large intestine (100) and that we saw a significant reduction in fecal output at this time point (Figure 2.3A), a measure used as an indicator of colonic function. Given that the average whole gut transit time of a healthy mouse is approximately 3-4h (285, 286), deltamethrin should have reached the distal colon by our experimental endpoint and would have therefore had the opportunity to interact with colonic ECs along the way. The lack of detectable serotonin differences in the colon could indicate a temporal effect, such that serotonin reduction first occurred in the small intestine but a later timepoint would be necessary to capture any colonic serotonin responses. Alternatively, perhaps the detection of deltamethrin in the small intestine triggered a rapid, systemic response that slowed overall transit before the toxicant could reach the colon in meaningful concentrations. This theory conflicts with the classical notion that GI transit speeds up after toxicant or pathogen exposure to quickly expel its contaminated contents, but certain toxicants are known to paradoxically decrease motility instead (287). This may be a protective response by the host to prevent further exposure, but it is likely a reflection of interference with the mechanisms that would normally facilitate rapid expulsion, as is the case with opioid-induced constipation (288) and some anticholinergic drugs (289, 290).

There are a number of other factors that can contribute to intestinal dysmotility independent of (or in conjunction with) serotonin. Beyond serotonin, many gut hormones are also known to regulate intestinal motility, including CCK, PYY, secretin, motilin, and

GLP-1 (291). However, circulating hormone levels in fed mice exhibiting acute constipation were not different between deltamethrin and vehicle-treated groups (data not shown, accompanying dysmotility data in Figure 2.3). This tells us that the acute deficit in gut motility was not due to an effect on gut hormones, although measuring hormone levels without controlling for food intake, as in this case, lacks relevance. Deltamethrin could also be exerting its effects on gut motility more indirectly through the bloodstream, or through actions on the ENS. Indeed, deltamethrin is absorbed rapidly, though incompletely, upon ingestion (292, 293). Despite its low oral bioavailability (262), it can travel to other regions of the body including the central nervous system, as it readily crosses the blood-brain barrier and accumulates in brain tissues within hours following oral administration (293). It's therefore possible that there are central mechanisms involved in deltamethrin-induced gut dysmotility. Deltamethrin could also access the ENS, either through local blood supply or via transport mechanisms across the epithelial layer into the submucosal plexus. While no direct link connecting pyrethroid exposure to ENS dysfunction has been made, pyrethroids including deltamethrin are highly lipophilic and can easily cross or penetrate cell membranes (294). That said, unmetabolized pyrethroid compounds are highly detected in feces after oral administration (295, 296), indicating that a substantial portion of the initial dose does stay in the lumen and pass through the entire GI tract in its whole form. It seems probable that deltamethrin's actions on gut motility are multifaceted and may originate from multiple internal sources given its toxicokinetic profile.

3.3 Deltamethrin dampens the postprandial hormonal response in mice

We demonstrated that acute, oral deltamethrin exposure significantly dampens the postprandial insulin and leptin response in mice (Figure 2.4C, E), while glucagon, GLP-1, and PYY levels were unchanged between vehicle and deltamethrin treatment groups following the mixed-meal nutrient stimulus (Ensure). Unlike in our initial *in vivo* experiment (Figure 2.3), the mice in this experiment were fasted for 4-6h prior to receiving a mixed-meal stimulus, which enables more accurate assessment of nutrient-induced hormonal responses and synchronizes baseline hormone levels across all animals in the cohort. Moreover, all animals received the same nutrient stimulus exactly 1.5h prior to sacrifice, ensuring equal consumption and timing across groups thus ruling out any discrepancies in food intake. Prior exposure to deltamethrin therefore diminishes the normal metabolic response to a mixed meal.

Impaired insulin secretion suggests that deltamethrin either directly suppresses pancreatic insulin production or indirectly acts through other mechanisms that influence insulin signaling. Under normal conditions following a meal in fasted mice, insulin is released within mere minutes and biphasically peaks in circulation first after 10 minutes and gradually rises again between 1h-3h after eating (297). We observed decreased insulin levels in the deltamethrin group at 1.5h post-meal, which suggests that deltamethrin is disrupting normal insulin signaling dynamics either through direct secretion suppression, blunted incretin signaling pathways, or increased clearance of insulin systemically. Pancreatic β-cells possess voltage-gated ion channels (298), especially for calcium, making them susceptible to direct actions by deltamethrin. In addition to its canonical activation of VGSCs, deltamethrin is also an agonist for voltage-gated calcium channels (299, 300). If acting directly on β-cells, one might presume that deltamethrin would actually stimulate insulin release rather than suppress it since calcium influx is necessary

for insulin secretion (301). In this case, perhaps we are observing an effect of insulin depletion during its second rise after initial stores were expelled in excess upon earlier β-cell activation. However, abnormally high intracellular calcium levels can just as well have the opposite effect (301, 302), so deltamethrin may just be impairing insulin release to begin with. Deltamethrin could also be acting through gut-derived incretin hormone pathways, such as via EEC-derived GLP-1, which is normally a strong potentiator of insulin secretion (303). Our prior observation that deltamethrin interferes with stimulus-induced GLP-1 release in EECs *in vitro* (Figure 2.2D) supports this notion, in that a diminished GLP-1 response would translate to an equally diminished insulin response. While we did not observe any effect by deltamethrin on GLP-1 levels themselves *in vivo* (Figure 2.2D), this could simply mean that the β-cell response to GLP-1 is impaired, or it could be a matter of timing, since unlike insulin, circulating GLP-1 levels spike around 5 mins after a nutrient stimulus and begin to decline by ~30 mins (304). Finally, it's also possible that deltamethrin upregulates factors governing insulin clearance and metabolism, most of which occurs in the liver (305). This explanation is supported by the fact that we did not observe any reduction by deltamethrin to circulating C-peptide levels (data not shown), which is produced in the pancreas after proinsulin is cleaved to form both insulin and C-peptide in a 1:1 ratio (306). Deltamethrin was shown to bind hepatocytes (307)and disrupt liver status and structure (308), so deltamethrin-mediated insulin clearance could be altered by its actions on hepatocyte function. Further research examining the effects of deltamethrin on β-cell activation and subsequent insulin secretion, temporal GLP-1 release dynamics, and hepatic insulin uptake is necessary to further elucidate the reasons behind deltamethrin suppression of postprandial insulin.

Similarly, deltamethrin suppressed nutrient-induced leptin levels in fasted mice (Figure 2.4C), which suggests even broader metabolic dysregulation. Leptin is produced by adipocytes and helps regulate satiety after meals (309). In addition to nutrients, leptin release is also partly stimulated by insulin itself (310), so its reduction could be secondary to insulin suppression. Given its role in enhancing insulin sensitivity throughout the body (311), decreased leptin could exacerbate the biological consequences of insulin suppression by diminishing insulin-mediated glucose uptake by cells. Indeed, prior work has found evidence of deltamethrin-induced hyperglycemia coupled with lower plasma insulin in rodents, which was associated with oxidative stress-induced damage to the pancreas and altered glycolysis and gluconeogenesis pathways in the liver (274). Altogether it is clear that deltamethrin impairs hormonal signaling pathways, leading to coordinated dysregulation of metabolic homeostasis.

In humans, there is minimal evidence of pyrethroid exposure leading to metabolic dysfunction. However, recent modeling-based studies using urinary pyrethroid metabolite data collected from the National Health and Nutrition Examination Survey (NHANES) found that recent pyrethroid exposure is associated with obesity, diabetes, and altered glucose homeostasis (240, 273, 312). Given our findings in mice, it's plausible that pyrethroids like deltamethrin could be dysregulating hormonal signaling pathways to increase risk for metabolic disease. These studies reinforce the need for further research on whether pyrethroids induce similar endocrine disruption in humans and highlight an even greater need for determining the underlying mechanism of pyrethroid-induced metabolic dysregulation to better inform public health risk assessments.

3.4 Chronic exposure to deltamethrin does not induce Parkinsonism

We decided to investigate how pyrethroid exposure might contribute to Parkinson's disease-like phenotypes for several reasons. These include: 1) the well-established relationship between pesticide exposure and increased PD risk (153, 175, 176, 249, 313); 2) the ability of pyrethroids to disrupt monoamine signaling pathways including dopamine (133, 138-141, 143, 145, 194); and 3) our previously discussed finding that deltamethrin induces intestinal dysmotility in a constipation-like manner, which is a common non-motor symptom and prodrome of PD (247, 248, 314, 315). We tested for signs of pyrethroid-induced locomotor dysfunction, intestinal dysmotility, and nigrostriatal dopamine pathway degeneration in male and female young adult mice after chronic exposure to 3 mg/kg deltamethrin or vehicle control administered orally once weekly for 12 weeks. In short, long-term oral exposure to deltamethrin does not induce Parkinsonism.

We did not observe any deficits in motor behaviors in deltamethrin-treated male and female mice, either over time within-subjects or compared to vehicle controls (Figure 3.2A-E). These included hindlimb scoring (hindlimb rigidity), wire hang (grip strength), open field (locomotion and anxiety-like behaviors), pole test (grip, balance, and coordination), and sticker removal test (sensorimotor coordination). This finding is consistent with one report that long-term occupational pyrethroid exposure in humans is not associated with impaired motor performance (316). However, because disruptions to these abilities are preceded by alterations to dopaminergic signaling pathways (317), we proceeded to test whether long-term deltamethrin exposure alters striatal dopamine-handling pathways. Chronic deltamethrin exposure did not affect striatal dopamine levels and its metabolites (Figure 3.2F), nor did it alter striatal tyrosine hydroxylase (TH) or

dopamine transporter (DAT) levels (Figure 3.2G), the loss of which are considered early indicators of dopaminergic degeneration (317).

In the gut, we did observe mild indicators of long-term effects of deltamethrin on gut motility. For example, while both treatment groups developed significantly slower whole gut transit over time, the deltamethrin group substantially more so than vehicle (Figure 3.3B). Additionally, fecal output was qualitatively less in deltamethrin-treatment animals compared to controls (Figure 3.3A). In alignment with our prior findings, it appears that the major effects of oral deltamethrin exposure predominantly involve intestinal status. Though subtle, these data suggest that deltamethrin exposure can exacerbate intestinal dysmotility that occurs naturally over time.

Finally, we decided to examine motor outcomes in a mouse model of synucleinopathy. There is some evidence that pyrethroid exposure affects alpha synuclein levels in the rodent brain (140), leading us to hypothesize that deltamethrin could exacerbate or accelerate the onset of PD-like symptoms through interactions with synuclein. Even in a synuclein disease model, however, deltamethrin did not induce signs of motor dysfunction (Figure 3.4A-E), though we assessed these animals using a subchronic paradigm (3 mg/kg deltamethrin, once weekly, for 3 weeks) rather than chronic. It's possible that longer-term exposure is necessary to observe any deltamethrin-mediated impact on any PD-like behavioral or intestinal manifestations in this model. Notably, however, we did observe a significant increase by deltamethrin in several metabolic hormones in the male animals (Figure 3.5A), some of which were conversely suppressed with acute instead of subchronic exposure (Figure 3.5B). These data suggest that although deltamethrin does not interact with synuclein to impair motor behaviors, it does appear to affect factors important for metabolism and energy homeostasis. Once

again, these findings align more closely with our work demonstrating that deltamethrin interferes with nutrient-stimulated gut hormone release discussed previously.

These experiments collectively demonstrate that while deltamethrin does not induce Parkinsonism or accelerate synucleinopathy-related motor impairment, it does have measurable effects on intestinal dysmotility and metabolic hormone regulation. Despite prior evidence that pyrethroids induce dopamine dysregulation in the CNS (139, 141), our research shows that chronic deltamethrin exposure is not sufficient to drive dopaminergic signaling disruption or locomotor deficits in mice, even in a mouse model of synucleinopathy. However, the observation that deltamethrin worsened the age-related increase in intestinal transit and disrupted metabolic hormone levels underscores our conclusion that oral deltamethrin exposure appears to predominantly influence GI-related physiologies. That said, it remains possible that deltamethrin could contribute to PD risk through actions on intestinal function given the prodromal GI features associated with PD (246). Assessments using different dosing paradigms, durations of exposure, aged animals, or other disease models are necessary to determine whether it is possible for deltamethrin to have more pronounced effects on neurodegenerative processes.

3.5 Conclusions and future directions

This dissertation sheds light on a new role for pyrethroid toxicity in mediating gastrointestinal dysfunction, possibly through interactions with serotonergic or GLP-1-producing enteroendocrine cells. This work creates a new avenue of research for the field and recognizes the susceptibility of the GI tract to ingested neurotoxicants like pyrethroids. Intestinal dysmotility is a tremendous burden for affected individuals and can drastically impact quality of life. It is therefore critical to study any potential causes

of intestinal dysmotility, including those that stem from environmental sources. There is currently insufficient evidence that pyrethroids mediate intestinal dysmotility epidemiologically, so more work is required to establish how pyrethroids could be interfering with regular gut function. This is especially true for populations who are predisposed to GI disorders or metabolic dysfunction for genetic or other environmental reasons.

Future work should focus on elucidating the underlying mechanisms of deltamethrin-mediated intestinal dysfunction. For instance, if deltamethrin impairs EEC-derived serotonergic signaling leading to acute intestinal dysmotility, then pharmacological intervention using serotonin receptor agonists or else by direct stimulation of enterochromaffin cells should rescue this phenotype. If co-administration of serotonin receptor agonists along with deltamethrin exposure does indeed prevent constipation, then this would be strong evidence that a serotonin deficit is mediating the observed disruption to intestinal motility. Moreover, it will be necessary to determine whether deltamethrin is directly interacting with EECs to induce these effects or whether it is acting via secondary signaling mechanisms. Given our *in vitro* finding that deltamethrin alters monoaminergic gene expression in EECs in a calcium-dependent manner (Figure 2.1B), it's possible that deltamethrin physically interacts with EECs to induce excitotoxicity through mechanisms involving the activation of calcium signaling pathways. Identifying which specific receptors and ion channel subtypes that deltamethrin preferentially targets on EECs would enable pharmacological blockade of those targets to test whether deltamethrin-induced toxicity can be prevented. Finally, future work should address the extent of involvement of the enteric nervous system to

help distinguish the different contributions of EECs vs. the ENS to deltamethrin-induced gut dysmotility.

Deltamethrin's suppression of the metabolic hormones insulin and leptin following a nutrient stimulus also requires further investigation. To determine how deltamethrin is driving these outcomes, future studies should examine deltamethrin's actions on pancreatic β-cells, hepatocytes, and adipocytes to gain insight into how it might be playing a role. Moreover, temporal assessments of deltamethrin's effects on insulin and leptin secretion dynamics over time will help guide our understanding of its initial site of action. In line with this, we did not observe nutrient-induced suppression of incretin hormones *in vivo*, but it will be necessary to check GLP-1 hormone levels at earlier timepoints (e.g., 10, 15, 30 mins post-stimulus) to gain a more accurate idea about whether deltamethrin interferes with the GLP-1 response *in vivo* as it does *in vitro* (Figure 2.2D).

Finally, although our results indicate that deltamethrin does not induce Parkinsonism, its actions on gut physiology and metabolism may still be relevant for neurodegenerative disease risk. Because our goal was to mimic real-life exposure scenarios to the greatest extent possible, we did not pursue more aggressive dosing paradigms. Still, it remains possible that longer or more frequent exposures, higher dosing, or the use of aged animals could produce neurodegenerative outcomes.

This body of work offers a new perspective regarding the potential health effects of pyrethroid insecticides on the gastrointestinal tract. Through our characterization of the short-term outcomes of pyrethroid toxicity on intestinal motility and enteroendocrine cell status and function, we have laid the groundwork for future studies to determine precisely how pyrethroids exert their toxicity within the local gut environment. Our findings

necessitate further investigation into the role of deltamethrin in human gastrointestinal health and provide an interesting and relevant foundation for addressing unanswered questions at the intersection of toxicology, gastrointestinal health, and epithelial biology.

3.6 Figures

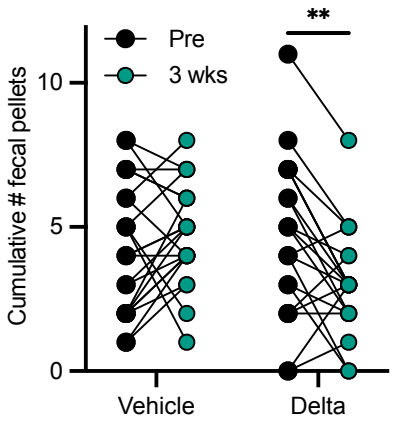


Fig. 3.1. Subchronic exposure to deltamethrin reduces fecal output. Mice were orally gavaged with corn oil vehicle control or 3 mg/kg deltamethrin dissolved in corn oil once weekly for 3 weeks. Fecal output is displayed as pre-treatment (black circles) compared to post-treatment (green circles) in vehicle or deltamethrin-treated mice. n = 21 per treatment group. Statistical comparisons were made using a Two-way repeated measures ANOVA with follow-up Šídák's multiple comparisons test, which compares pre vs. post within each treatment group. **p<0.005.

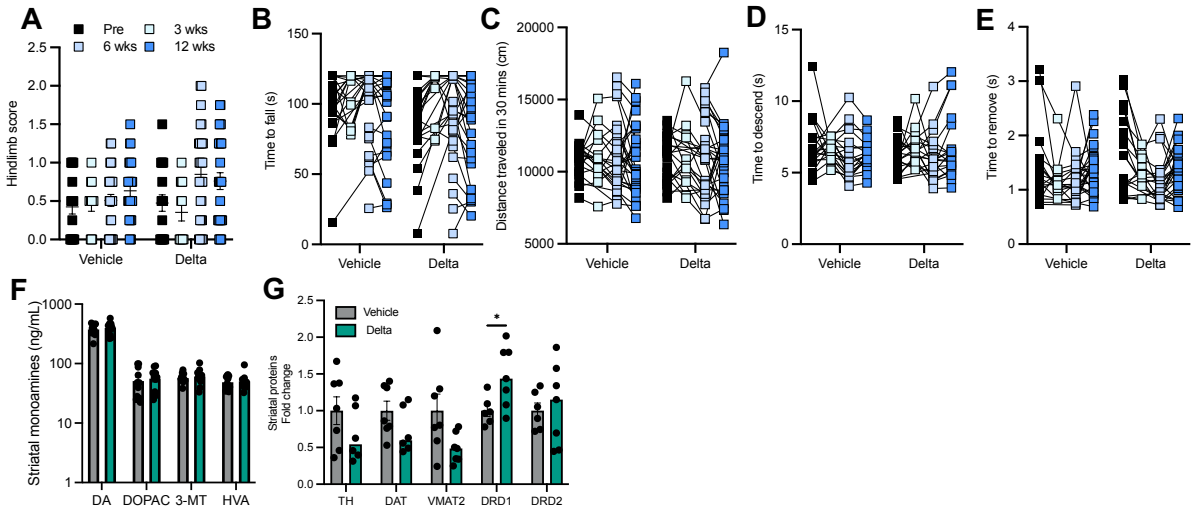


Fig. 3.2. Chronic, low-dose, oral deltamethrin exposure does not induce Parkinsonism or associated disruption to nigrostriatal dopamine pathways. **A.** Hindlimb scoring over time in vehicle or deltamethrin-treated male and female mice. **B.** Wire hang test over time in vehicle or deltamethrin-treated male and female mice. **C.** Distance traveled in the open field test over time in vehicle or deltamethrin-treated male and female mice. **D.** Pole test assay measurements over time in vehicle or deltamethrin-treated male and female mice. **E.** Sticker removal assay results over time in vehicle or deltamethrin-treated male and female mice. **F.** Striatal dopamine and metabolites after chronic vehicle (gray) or deltamethrin (green) exposure in male and female mice. **G.** Dopaminergic proteins in the striatal tissue of vehicle or deltamethrin-treated mice. n = 20-28 mice (**A-E**), n = 12 (**F**), or n = 6-7 (**G**) per treatment group. Statistical comparisons were made using a mixed effects model ANOVA (**A-G**) or multiple individual t-tests (**F**, **G**). *p<0.05.

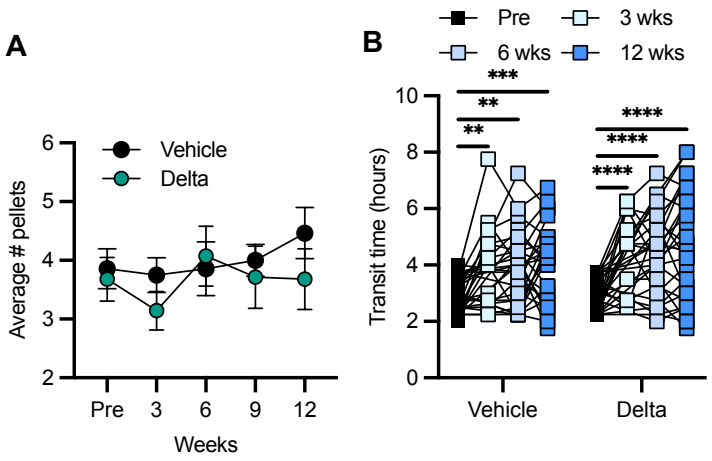


Fig. 3.3. Chronic, low-dose, oral deltamethrin exposure worsens age-related gut dysmotility. A Average fecal output in 30 minutes over time in vehicle vs. deltamethrin-treated male and female mice. **B** Total gastrointestinal transit time over time in vehicle or deltamethrin-treated male and female mice. n = 28 (**A**) or n = 12-28 (**B**) per group. Statistical comparisons were made using an ordinary two-way repeated measures ANOVA (**A**) or a mixed-effects model ANOVA with a Dunnett's multiple comparisons test comparing means over time within each treatment group (**B**). **p<0.005, ***p<0.001, ****p<0.0001

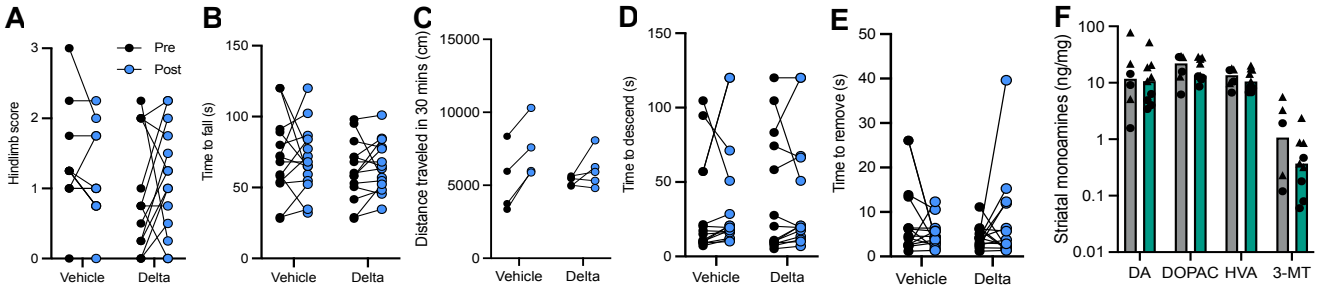


Fig. 3.4. Subchronic, low-dose, oral deltamethrin exposure does not accelerate Parkinsonism in a mouse model of synucleinopathy. A Hindlimb scoring at baseline (black) compared to experimental endpoint (blue) in vehicle or deltamethrin-treated male and female mice. **B** Wire hang values at baseline and endpoint in vehicle and deltamethrin-treated male and female mice. **C** Quantification of distance traveled in open field assay over time in vehicle or deltamethrin-treated mice. **D** Pole test assay results over time in vehicle or deltamethrin-treated mice. **E** Average time to remove sticker from nose over time in vehicle or deltamethrin-treated male and female mice. **F** Measurement of striatal dopamine and metabolites in vehicle (gray) or deltamethrin-treated (green) mice. n = 12-14 (**A, B, D, E**), n = 4-5 (**C**), or n = 6-9 (**F**). Statistical comparisons were made using a mixed effects model ANOVA (**A-E**) or multiple independent t-tests (**F**).

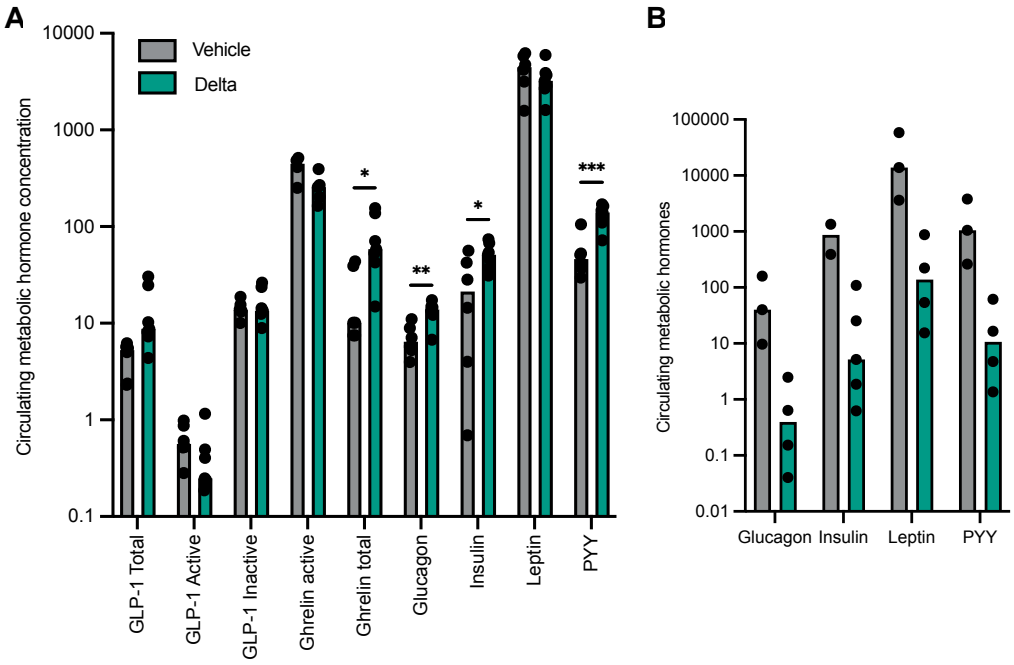


Fig. 3.5. Oral deltamethrin exposure alters metabolic hormone levels in a mouse model of synucleinopathy. A Measurement of metabolic hormones in serum of male mice subchronically (1x week for 3 weeks) treated with vehicle (gray) or deltamethrin (green). **B** Metabolic hormone levels in serum of male mice treated acutely (24h exposure) with vehicle or deltamethrin. Concentration units are in pM for GLP-1 and glucagon, uIU/mL for insulin, and pg/mL for ghrelin, leptin, and PYY (total). N = 6-7 (**A**) or n = 3-4 (**B**). Statistical comparisons were made using multiple individual t-tests. *p<0.05, **p<0.005, ***p<0.001.

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