REVISITING BEHAVIORAL VARIABILITY: WHAT IT REVEALS ABOUT NEURAL CIRCUIT STRUCTURE AND FUNCTION

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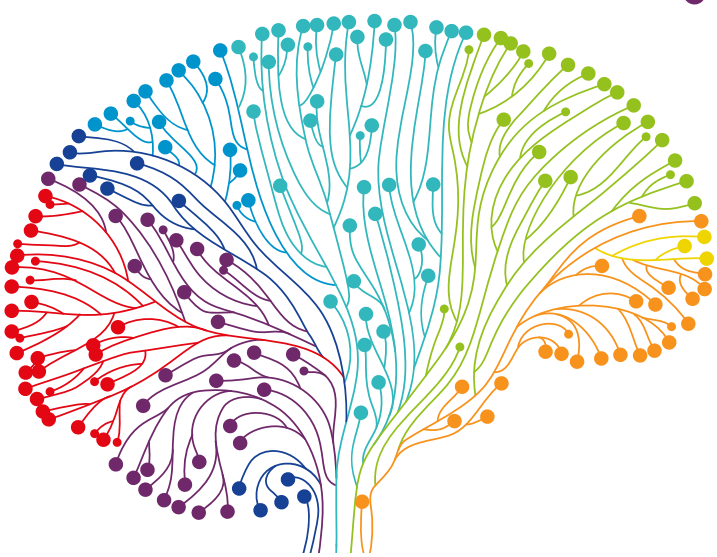

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REVISITING BEHAVIORAL VARIABILITY: WHAT IT REVEALS ABOUT NEURAL CIRCUIT STRUCTURE AND FUNCTION

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EDITORIAL
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Editorial: Revisiting Behavioral Variability: What It Reveals About Neural Circuit Structure and Function

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Keywords: individuality, behavior, genetics, neuromodulation, behavioral variability, behavioral adaptation, neural circuit, internal state

Editorial on the Research Topic

Revisiting Behavioral Variability: What It Reveals About Neural Circuit Structure and Function

Why is animal behavior variable? The main goal of this Research Topic is to showcase the latest research and perspectives that address this fundamental yet often overlooked question in behavioral neuroscience. Five original research articles and seven reviews by leading neuroscientists provide diverse insights on this question through various behavioral models.

Ethologists have long noted that animals and humans often respond differently to the same sensory stimuli. Although variability is common in nature, its study as an essential biological feature has faced friction in lingering ideas, such as small organisms being simple stimulus-response automata. Connectomes, complete maps of neural connectivity, are miraculous accomplishments, but their singular, structural nature can reinforce the feeling that nervous systems are non-varying. On the other hand, these data also revealed many previously unknown synaptic connections, suggesting more alternative routes between neurons and brain regions than necessary for simple stimulus-response routines. Indeed, numerous studies have demonstrated that even a well-defined neural circuit can produce a variety of behavioral outputs. Traditionally, different origins of behavioral variability have been studied in discrete frameworks (such as neural development, learning and memory, reproductive state, and so on). These distinctions do not necessarily reflect the differences in underlying mechanisms, which likely act in superposition in real organisms. To visualize the richness of mechanisms discussed in this Research Topic, we placed the areas covered by each article on a 2-dimensional map. One axis represents the timescale of behavioral variability, and the other axis represents its mechanistic levels (**Figure 1**).

The term "variability" often refers to a within-group difference in observable behavioral outputs that cannot be explained by the factor of interest, e.g., a stimulus or genetic variations within the group. For instance, Darwin was unaware that finches in the Galapagos Islands consisted of multiple species until his colleague ornithologist John Gould pointed that out. In this case, what was initially perceived as anatomical variability within a group turned out to be species-specific characteristics. Knowledge on taxonomy coupled with rigorous quantification of behavior helps distinguish intra- and inter-specific variabilities, as shown by Mueller et al. While it is clear that inter-specific variability is caused by heritable genetic differences between species (though any co-varying environmental effects may also contribute), within-species variabilities might also arise from non-heritable causes such as noise in gene expression due to environmental

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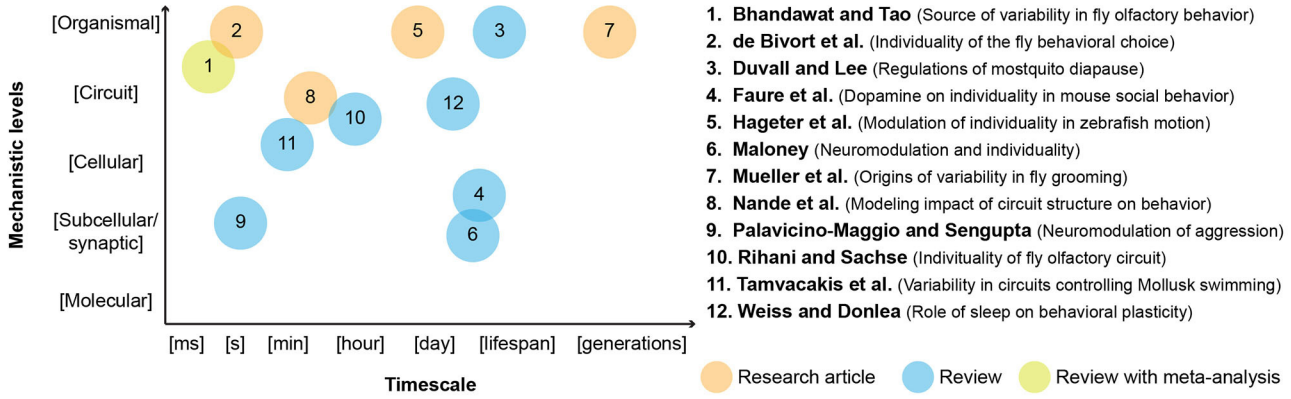

FIGURE 1 | Mapping the scope of articles in this Research Topic. Numbers are given according to the alphabetical order of the first authors.

factors. Both heritable and non-heritable variations affect behavior through multiple cellular and physiological mechanisms, including varying circuit connectivity. Using olfactory-guided behavior of the common fruit fly *Drosophila melanogaster*, Tao and Bhandawat discuss potential genetic contributions for behavioral variability, while Rihani and Sachse illustrate variabilities in neuroanatomy and physiological properties of neural circuits that can be the source of individual behavioral differences. In parallel, Tamvacakis et al. discuss the impact of variability in circuit wiring and gene expression patterns in key neurons driving flexibility in mollusk swimming behavior.

Besides wiring variation, an alternative source of variability is multiple, discrete developmental programs within a species. Lee and Duvall consider egg diapause, an alternative state of arrested development under harsh environmental conditions, in the mosquito *Aedes albopictus*. This is an intriguing example of how external factors drive alternative reproductive strategies within a genetically homogeneous population. Similarly, Hageter et al. demonstrate that temperature fluctuations during Zebrafish development affect specific aspects of turning behavior. Another example is the effect of social experiences, which can profoundly impact animal behavior. Faure et al. discuss how complex social interactions in rodents can reinforce individual differences with significant fitness consequences. As discussed in the above three papers, specific genes likely play an essential role in converting experience during different development timescales into behavioral adaptations. Recent advances in sequencing technology can illuminate key genetic networks that are important for generating behavioral variability in response to changes in environmental conditions.

Genetic, environmental, and stochastic factors underlie stable behavioral idiosyncrasies, but that is not the only source of variability. The same animal often behaves differently when tested at different times, suggesting that parallel factors cause intra-individual fluctuations in behavior. The so-called "internal state" is often used without a clear scientific definition, but several types of "internal states" have been well-studied across species; among them is the general arousal state. Weiss and Donlea discuss how sleep (or the lack of it) can impact the neural functions of developing and mature brains, along with the behavioral consequences of sleep disruption. Arousal levels can be controlled in a behavior-specific manner as well. Palavicino-Maggio and Sengupta describe neurogenetic factors—namely neuromodulators—affecting aggression in *Drosophila melanogaster*. Across animal species, neuromodulation is a key to generating behavioral variability within and among individuals. Underscoring its importance, many articles in this Research Topic touch upon neuromodulation: Faure et al., Tamvacakis et al., Tao and Bhandawat, de Bivort et al., and Rihani and Sachse all discuss the significance of neuromodulation in the context of their behavioral paradigms. A review by Maloney argues that neuromodulation can drive behavioral variability by diversifying the dynamics of a circuit that controls a given behavior. Since many neuromodulators have similar behavioral effects across species, the cellular mechanisms of neuromodulation are critical to understanding how the nervous systems with (largely) identical connectivity can generate variable behavioral outcomes within and across individuals.

While distinguishing inter- and intra-individual variability seems straightforward in concept, a large amount of data and repeated measures from the same individual are often necessary to distinguish these two variabilities (see also Tamvacakis et al.). "Big data" of behavior have become amenable for analysis relatively recently thanks to newly developed computational and experimental toolkits. de Bivort et al., Mueller et al., and Hageter et al. showcase the power of behavioral data collected from a large number of animals when isolating biases characteristic of each animal—or individuality. In all three articles, it is noteworthy that individual behavioral biases are represented as probabilities of exhibiting particular choices rather than the simple presence or absence of a given behavior. Thus, individuality may be expressed as differences in the sequencing or abundance of behaviors rather than their kinematics. Through the meta-analysis of published data, Tao and Bhandawat found that stochastic choice likely generates

larger inter-individual variability in olfactory-guided behavior than genetic or neuromodulatory differences. How stochasticity arises in the nervous system remains an important question in neuroscience. Nande et al. demonstrated through modeling that behavior-specific modular organization of the nervous system makes the behavioral output more robust against perturbation while imparting long-term internal-state-like dynamics. In other words, the difference between what is regarded as a "stereotypical behavior" and a "variable behavior" may reflect differences in the way the neural circuits that control the given behaviors are structured.

The diverse aspects of behavioral variability covered in this Research Topic compel us to ask whether these phenomena can be explained under a single framework. Even a "simple" nervous system turns out to be complex enough to generate behavioral variability. Despite large-scale neural recordings and flourishing "omics" data from molecules to behavior, the level of our current understanding of gene expression regulation, synaptic plasticity, neuromodulation, and circuit development and reorganization still seems insufficient to create cell and circuit models that provide quantitative hypotheses to account for behavioral variability. Rigorous behavioral analysis will also be critical but almost certainly insufficient. Scientists and editors alike love "clean" behavioral data with small error bars that fit together into tidy neurobiological narratives. But the exclusive pursuit of such results limits progress in identifying the origins of behavioral variability, which is so salient to every scientist who performs a behavioral experiment. We hope this Research Topic advances variability discourse in the behavioral neuroscience community and brings us a few steps closer to a mechanistic understanding of the neural functions that generate behavioral variability.

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REVIEW
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Neuromodulation and Individuality

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Within populations, individuals show a variety of behavioral preferences, even in the absence of genetic or environmental variability. Neuromodulators affect these idiosyncratic preferences in a wide range of systems, however, the mechanism(s) by which they do so is unclear. I review the evidence supporting three broad mechanisms by which neuromodulators might affect variability in idiosyncratic behavioral preference: by being a source of variability directly upstream of behavior, by affecting the behavioral output of a circuit in a way that masks or accentuates underlying variability in that circuit, and by driving plasticity in circuits leading to either homeostatic convergence toward a given behavior or divergence from a developmental setpoint. I find evidence for each of these mechanisms and propose future directions to further understand the complex interplay between individual variability and neuromodulators.

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INTRODUCTION

Across a wide range of species, from *C. elegans* (Stern et al., 2017) to humans (Sanchez-Roige et al., 2018), individuals exhibit idiosyncratic behavioral preferences, even when they are genetically identical and raised in similar environments. These differences seem to arise due to underlying stochastic processes during development, causing the same genetic profile to lead to a range of neural phenotypes.

These stochastic processes play important roles in development, however, how they relate to variation in behavior is not always clear. Stochastic processes in development may resolve to highly stereotyped results as well as variable ones, depending on mechanisms to induce robustness (Johnston and Desplan, 2010). Similarly circuits with differing numbers of neurons, connections, and ion conductance can converge on seemingly identical behaviors (Prinz et al., 2004; Daur et al., 2012; Goaillard and Marder, 2021). Understanding when and how variations in the underlying circuit lead to divergence in behavior is crucial to understanding the developmental and ecological context of individuality, defined here as biases or preferences in an individual that differentiate it from other animals in a population.

Individuality plays an important role in the survival of a species (Cohen, 1966; Hopper, 1999). Divergent preferences among a species allow the species to hedge against unpredictable environments by having a range of phenotypes adapted to different possible environments, ensuring some proportion of the population survives regardless of environmental fluctuations (Kain et al., 2015; Xue et al., 2019). The degree to which individuals within an isogenic population show divergent preferences is strongly influenced by genetics, as shown by studies showing differing amounts of individuality between isogenic populations with different genetic backgrounds (Ayroles et al., 2015; Bruijning et al., 2020), demonstrating that intra-genotypic variability is under evolutionary control. This is supported by observed differences in population variability that match theoretical predictions of environments where variability provides a fitness advantage

(Akhund-Zade et al., 2020; Krams et al., 2021). One key proposed mechanism for the regulation of individuality is neuromodulation.

Neuromodulators play a key role in regulating behavior at multiple scales. Neuromodulators are a diverse set of chemicals with a wide range of receptors, kinetics, targets, and roles, however, they have several broadly shared characteristics. Compared to conventional neurotransmitters, neuromodulators are characterized by volume release, broad connectivity, and slower and longer kinetics (Bargmann and Marder, 2013). Because of their ability to trigger widespread shifts in network function across the nervous system, changes in neuromodulation can trigger large shifts in behavior (Lee and Dan, 2012; Taghert and Nitabach, 2012). Within an individual, these shifts allow organisms to adjust their behavior based on context, such as in response to satiety (Marella et al., 2012), social conflict (Rittschof et al., 2014), arousal (Arnsten et al., 2012), experiences (Likhtik and Johansen, 2019) circadian rhythm (Witkovsky, 2004) or stress (Rodrigues et al., 2009). Within eusocial insects, neuromodulators can drive differences in behavior between sub-castes (Kamhi et al., 2015), and help regulate group behavior in response to environmental cues (Kamhi et al., 2017). Neuromodulators and hormones have also been proposed to serve as loci for evolutionary shifts in behavior based on their broad targets affecting a variety of disparate traits, making it easier to coordinate shifts in multiple traits to linked to advantageous behavioral shifts (Cox et al., 2016; Garland et al., 2016). Key to neuromodulators' role in the evolution of behavior is the ability for small shifts in expression levels and localization of elements of the neuromodulatory systems to shift behavior (Katz and Lillvis, 2014), avoiding the need to create *de novo* behaviors and circuits to change behavior in response to evolutionary pressure. Artificial selection experiments have shown that selection pressure can act via changes in neuromodulator levels to drive rapid changes in behavior (Pantoja et al., 2020). Similarly, neuromodulatory systems may serve as loci for individuality—sites where idiosyncratic circuit differences cause idiosyncratic behavior differences (Skutt-Kakaria et al., 2019). Neuromodulatory systems are prime targets to be loci for individuality based on their ability to provide coordinated shifts in function over multiple circuits in the nervous system, and therefore enable coordinated changes in behavior with comparatively few points of variation. A wide range of studies across different behaviors and species have shown that changes in neuromodulators can affect the manifestation of individuality (**Table 1**), suggesting that neuromodulators may play a key (though not exclusive) role in driving individuality among populations.

Despite this clear evidence that neuromodulators play an important role in regulating variation in behavioral preferences in many systems, the mechanisms by which they do so are unclear due to a combination of limited study and the complexity and heterogeneity of neuromodulators. Below, I describe three broad categories by which neuromodulators might affect individuality: variability in neuromodulation, altering circuit function to mask or accentuate circuit variability, and driving plasticity in the underlying circuit. Each of these categories of mechanisms provides different experimental predictions about how neuromodulation affects behavioral individuality, providing an opportunity to deepen our understandings of the myriad of ways neuromodulators might influence individuality in different systems and behaviors.

VARIATION IN NEUROMODULATORS AS A DRIVER OF INDIVIDUALITY

One potential mechanism through which neuromodulators may drive individuality is by being themselves variable between individuals (**Figure 1**). Neuromodulators have strong effects on behaviors, and within an animal shifts in neuromodulators are a driver of trial to trial variability (McCormick et al., 2020). Variation in the amount of neuromodulation, via differences in receptor expression, production of neuromodulators, or activity in neuromodulatory neurons, could drive differences in behavioral preference between individuals. Among genetically diverse populations, variations in the activity of neuromodulatory neurons or mutations in receptors can manifest changes in personality (Sanchez-Roige et al., 2018). Outbred zebrafish populations show significant variation in acoustic startle response that correlate with the physiology of neuromodulatory dorsal raphe neurons (Pantoja et al., 2016), with individuals showing a higher fraction of serotonergic dorsal raphe nucleus neurons active during escape attempts also showing a decreased habituation to startle. Epigenetic changes in expression of neuromodulatory components have also been tied to differences in personality (Cardoso et al., 2015; Puglia et al., 2018; Park et al., 2020). In addition to changes in the global levels of neuromodulation, behavioral variation could also be due to variation in the targets of neuromodulatory neurons, such as has been observed in *C. elegans*, where electron microscopy reveals that neuromodulatory neurons show higher synapse count variation than conventional neurons (Witvliet et al., 2021).

Variation in neuromodulation is limited in its ability to explain all individuality, however. In cases where silencing a neuromodulator leads to an increase in variability, it suggests that the root cause of the behavioral variability is a source other than variability in the direct effect of the neuromodulator in question.

Furthermore, it is difficult to reconcile this explanation with cases where individuality appears to be driven by the asymmetric innervation of known non-modulatory cell types, for example variation in object orientation in *Drosophila melanogaster* is driven by asymmetries in DCN neurons (Linneweber et al., 2020). In these cases, direct variation in neuromodulators cannot account for the observed variability.

NEUROMODULATORS AS SHAPERS OF THE RELATIONSHIP BETWEEN CIRCUITS AND BEHAVIOR

In contrast to conventional neurotransmitters, neuromodulators are frequently insufficient to directly drive activity in neurons, instead altering intrinsic properties of the neuron and filtering the

TABLE 1 | Examples of ties between neuromodulators and individuality.

Study	Species	Population type	Neuromodulators studied	Output studied	Effect on variability in output	Correlation
Stern et al., 2017	*C. elegans*	Isogenic, backcrossed mutants	Serotonin	Roaming Fraction	Decreased Serotonin leads to decreased persistence in preference	Positive
			Tyramine, octopamine, npr-1, daf-7	Roaming speed	Decreased Neuromodulator increases bias toward high or low speeds	Negative
Omura et al., 2012		Isogenic	Dopamine	Roaming Speed	Decreased dopamine decreases variability	Negative
Pantoja et al., 2016	*D. rerio*	Outbred	Serotonin	Acoustic Startle Response Habituation	Decreased serotonin increase habituation	Negative
Kain et al., 2012	*D. melanogaster*	Isogenic	Serotonin	Phototactic Preference	Decreased Serotonin increases population variability	Negative
Honegger et al., 2019	*D. melanogaster*	Isogenic	Serotonin	Olfactory Preference	Decreased Serotonin decreases population variability	Positive
			Dopamine	Olfactory Preference	Increased Dopamine increases population variability	Positive
Krams et al., 2021	*D. melanogaster*	Wild Caught sibling populations from multiple locations	Serotonin	Phototactic Preference	Decreased Serotonin increases population variability	Negative

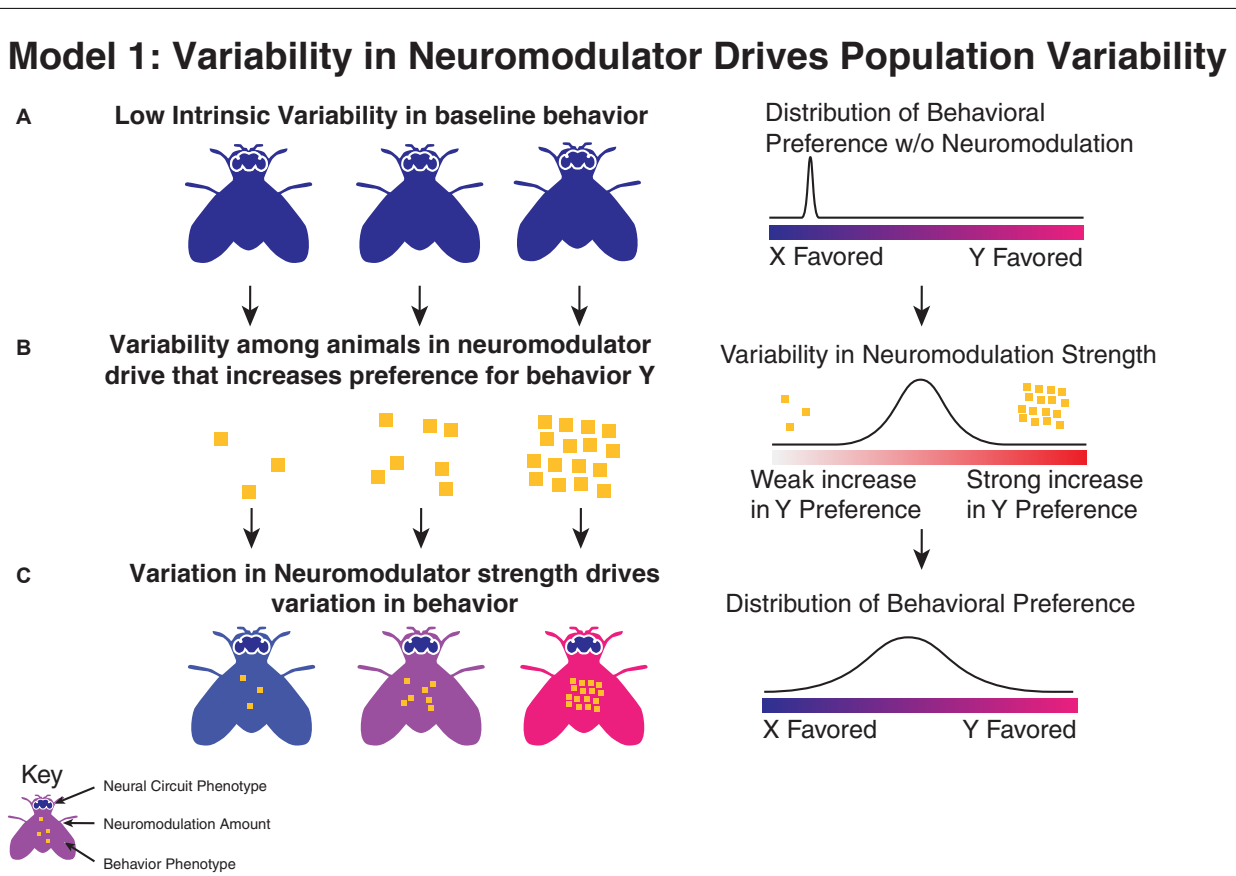

FIGURE 1 | Variation in neuromodulation as driver of behavioral output. **(A)** In this model, individuals show limited variability in their behavior (indicated by individual body color) in the absence of neuromodulation, reflecting low variability in underlying parameters in neural circuits (indicated by brain coloring). **(B)** Individuals instead show significant variability in the strength of their neuromodulatory drive, which drives changes in their behavior. **(C)** This leads to increased variation in the observed behavioral preference.

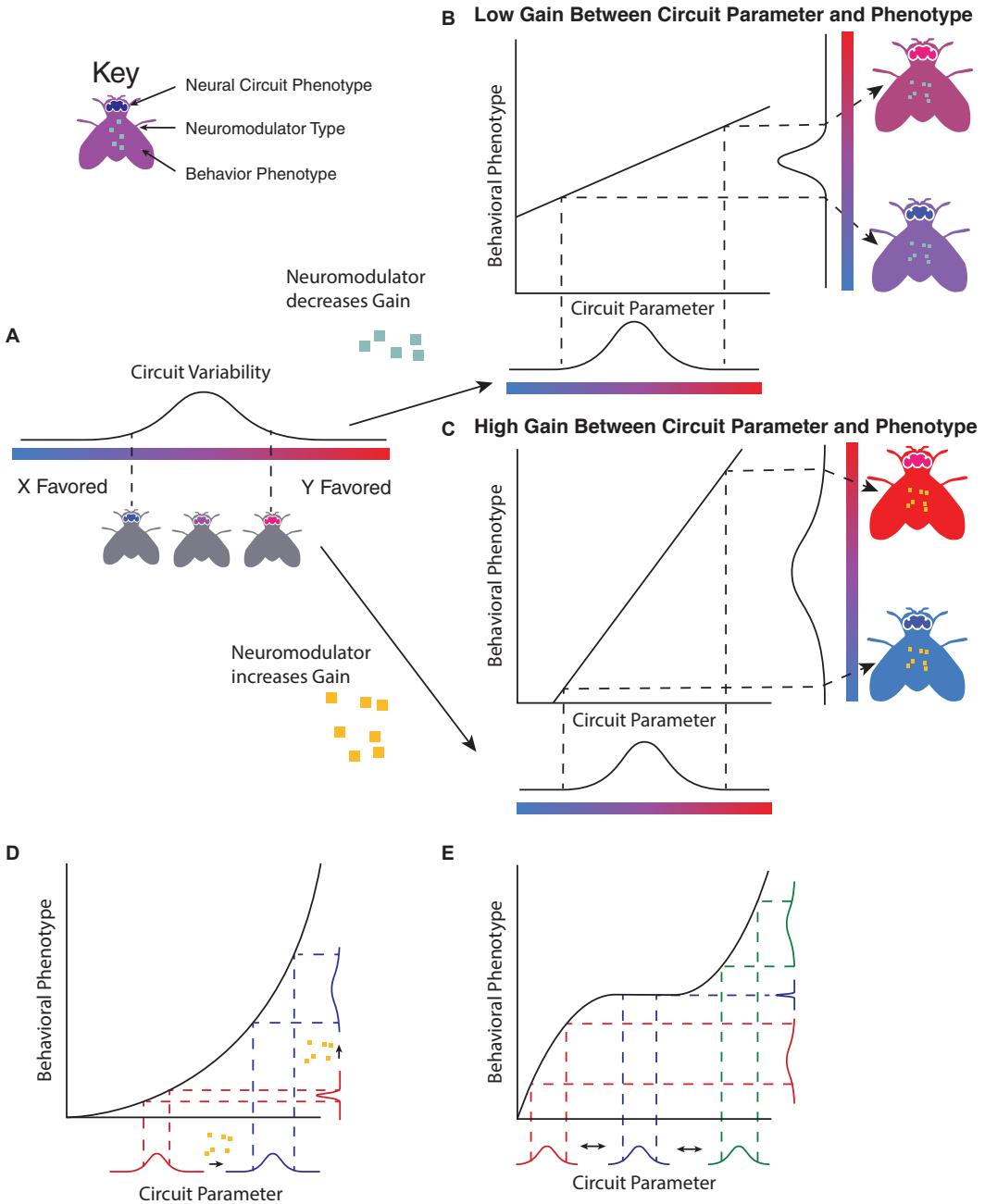

FIGURE 2 | Neuromodulators adjust the relationship between underlying circuit variability and observed variation in behavior. **(A)** In this model, the non-modulatory parameters of the circuit lead to a distribution of behavioral phenotypes in the absence of modulatory input. The addition of neuromodulators either decrease the slope of the relationship between the circuit parameter and behavioral phenotype **(B)**, leading to a decrease in the observed variability, or increase the slope of the relationship between the underlying circuit and the behavioral phenotype **(C)**, increasing the variability in behavior. The change in gain can occur either through by directly altering the relationship, or by altering the mean of a parameter upstream of a non-linear relationship **(D)**. In this example a neuromodulator shifts the mean value of a circuit parameter without altering its variance, however, due to the non-linear relationship between the circuit parameter and the observed behavioral phenotype, the variance of the behavioral phenotype is changed. **(E)** Shifts in parameters by neuromodulators may have inconsistent effects on the variability of observed behavior based on the starting value of the parameter and the relationship between circuit parameter and behavioral phenotype. In this example, variation in a circuit parameter (blue) under some conditions leads to no variation in the observed behavior, however, shifting the mean in either direction increases the variability in the observed behavioral phenotype as fluctuations in the parameter lead to larger changes in phenotype.

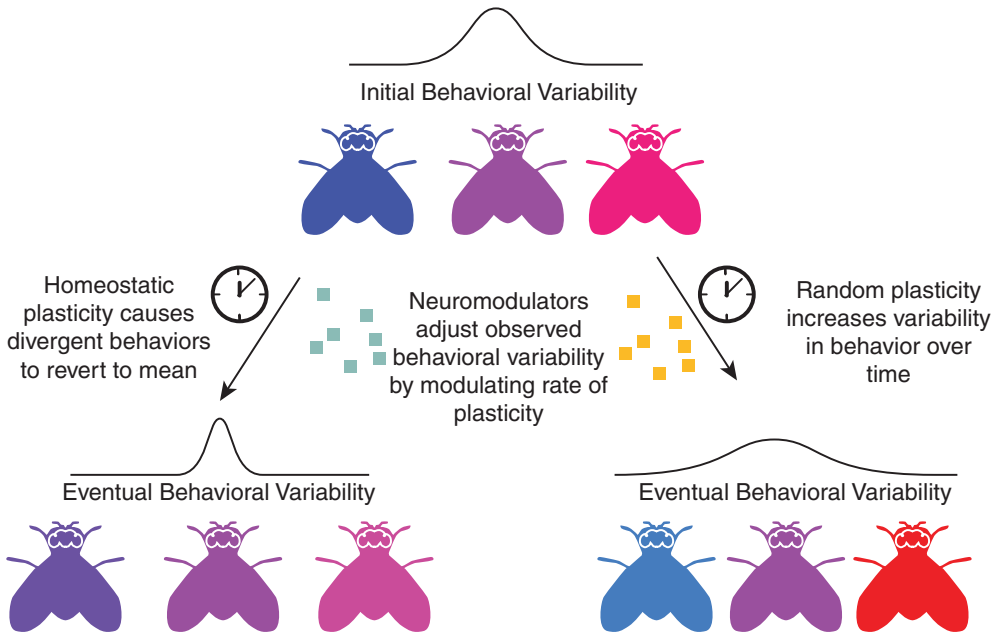

FIGURE 3 | Neuromodulation as a regulator of variability through plasticity. In this model, variability is driven by changes in the variability of underlying circuit components over time, either in a homeostatic mechanism (left) moving behavior closer to a predetermined set point, or through a divergent method (right), leading to further deviation from parameters determined during development.

response to conventional neurotransmitters. Because the activity of neurons and circuits is non-linear and based on a wide range of factors, changes in intrinsic properties due to neuromodulation can either lead to a regime where a large variance in a parameter has little or no effect on the output of a circuit or a regime where small changes lead to large changes in behavior (Goldman et al., 2001; Grashow et al., 2009; Hamood and Marder, 2014; Marder et al., 2014). This observation mirrors similar observations and theory in evolutionary genetics, where certain mutations lead to canalization, suppressing phenotypic variations despite underlying variability in the genes (Félix and Barkoulas, 2015).

Contrary to the previous model, in this case variation is not driven by differences in the neuromodulatory circuit, but rather the amplitude of neuromodulation alters the degree to which variability in other components of the nervous system manifests as idiosyncratic behavioral preferences (**Figure 2**). By changing the relationship between underlying variability in the circuit (**Figure 2A**) and either making the behavioral phenotype less sensitive to changes in the circuit parameter (**Figure 2B**) or more sensitive (**Figure 2C**), the variability in the population can be modulated despite no change in the underlying variability in the circuit parameter.

Clear experimental evidence of the ability of neuromodulators to modulate the manifestation of underlying circuit variability comes from the crustacean stomatogastric nervous system. The crustacean stomatogastric nervous system is divergent among individuals in terms of the constituent neurons (Bucher et al., 2007) and ion channels in individual neurons (Schulz et al., 2007). Variability in connectivity can be ameliorated through neuromodulation, as evidenced by a systematic search of synapse strengths leading to stereotyped rhythmic activity using dynamic clamp between pacemaker neurons (Grashow et al., 2009). Adding two neuromodulators, serotonin and oxotremorine, increased the underlying set of parameters that led to rhythmic bursting—in this way neuromodulators enable a larger distribution of underlying circuit parameters to produce similar behavioral output, increasing the robustness of the circuit.

Compelling experimental evidence for neuromodulation that increases population individuality by accentuating underlying network parameters is more difficult to find, though whether this is due to any evolutionary bias toward neuromodulators promoting robustness or researcher's bias in studying robustness is unclear. Nonetheless, theoretical evidence in simplified models of neuronal circuits highlights that small shifts in conductances consistent with the method of action of neuromodulators change the sensitivity of the circuit to perturbations in other parameters (Goldman et al., 2001; Gutierrez et al., 2013).

A key insight from this work, as well as analogous classic work in evolutionary genetics (Rendel, 1962), is that any change in the mean of a phenotype will also change the variance in a phenotype if the shift in the mean is due to a shift in a parameter with a non-linear relationship to the phenotype, even if the variance in the parameter doesn't

change (**Figure 2D**). In this way, even neuromodulators with straightforward linear effects on one parameter may change the sensitivity of behavioral phenotype to other underlying circuit components, and hence affect the degree of individuality in a population. Similarly, the same neuromodulator may have differing effects based on the underlying state of the neural circuit (**Figure 2E**), leading to inconsistent or does dependent effects of neuromodulators on variability.

NEUROMODULATION OF INDIVIDUALITY THROUGH PLASTICITY

Both previous categories assume that the manifestation of individuality is due to the acute influence of neuromodulators on the observed manifestation of individuality, however, some evidence suggests that even transient changes in neuromodulation in the animals past might drive changes in individuality. Application of the serotonin agonist ANW increases population variability even 5 days after washout (Kain et al., 2012), suggesting that neuromodulators may affect the development of individuality and have long lasting effects on the behavioral preferences of an animal. This is consistent with a large body of literature showing neuromodulators playing a critical role in gating plasticity and learning in a wide range of species (Damme et al., 2021), including *Aplysia* (Barbas et al., 2003), *Drosophila* (Kadow, 2019), mice (Diering et al., 2017), and humans (Likhtik and Johansen, 2019; Damme et al., 2021).

Neuromodulatory changes in learning could manifest in multiple directions (**Figure 3**). Neuromodulation could lead to the refinement of circuits, taking initially noisy developmental connections and applying a learning rule that drives them toward a more functional outcome. This is seen in *C. elegans*, where asymmetry in connections early in development is reduced as animals grow older (Witvliet et al., 2021). This sort of activity-dependent refinement of function, particularly during developmental critical periods has been demonstrated in a wide variety of systems and is influenced by neuromodulators (Shepard et al., 2015).

Alternatively, if development is more tightly controlled than plasticity, plasticity may drive further divergence of circuits over time. Estimations of the genetic heritability of personality traits decrease over time (Briley and Tucker-Drob, 2014), and even among animals raised under similar environments, cumulative changes in the circuit over time could lead to a greater array of idiosyncratic preferences. Even in cases with near perfect homeostatic learning rules, most changes in synaptic plasticity will be driven by spontaneous fluctuations (Raman and O'Leary, 2021), and misalignment between the homeostatic rules and the output behavior could lead to fluctuations in observed behavioral preference over time. This shift is supported by observations of idiosyncratic preferences—animals change their individual preferences over time, even in the absence of stimuli to induce learning (Buchanan et al., 2015; Werkhoven et al., 2021). In these cases, however, the overall distribution of preferences in the population remained constant over time—suggesting that either the divergent and convergent effects of plasticity in the circuit are balanced, or that changes in the range of preferences measured in a population operate via different mechanisms than those determining where in that range each individual occurs.

An additional possibility for neuromodulators to affect individuality through plasticity is by regulating other neuromodulators. Experimental manipulations of one neuromodulator can affect the strength of other neuromodulators (Niederkofler et al., 2015; Niens et al., 2017). Evidence suggests that these processes occur over long time scales, allowing shifts in one neuromodulator to rewire other neuromodulatory systems. Therefore manipulations of one neuromodulator could lead to changes in individuality via another neuromodulator using any of the mechanisms discussed in this paper. This possibility highlights the ways in which these different models can interact, and a given system might involve mechanisms that integrate elements from each of the three abstract models discussed in this paper.

FUTURE DIRECTIONS

How then, does neuromodulation affect individuality? Despite suggestions from various studies, this remains an open question requiring more study. Nonetheless, a number of observations can help determine the answer to this question and categorize the role of neuromodulators in regulating individuality in particular behaviors (**Figure 4**):

- Does past neuromodulation and manipulation of neuromodulation affect individuality, or does neuromodulation alter the rate of change of individuality, suggesting an effect on plasticity?
- Do acute effects of neuromodulators on individuality wash out, suggesting they are reversible?
- Does neuromodulation alter the rate by which individuals change idiosyncratic preferences, suggesting that neuromodulators increase or decrease the rate of plasticity?
- Do individuals maintain idiosyncratic preferences when neuromodulatory systems are silenced, suggesting that individuality is not solely driven by neuromodulatory systems?
- Do neuromodulators have independent effects on the mean of a trait and its variability, suggesting that neuromodulators target specific processes regulating variability?
- Does increased neuromodulation increase or decrease the relationship between underlying circuit variability and behavioral phenotypes, suggesting a role in adjusting the gain of a trait?
- Do neuromodulators alter the effects of other perturbations of behavior, suggesting they are altering the role of other determinants of behavior?

These questions, based on hints from the current literature, will almost certainly lead to conflicting answers. The correlation in serotonin levels and dorsal raphe neuron physiology with behavioral variability suggests shifts in variability in neuromodulation is a part of the answer in the zebrafish

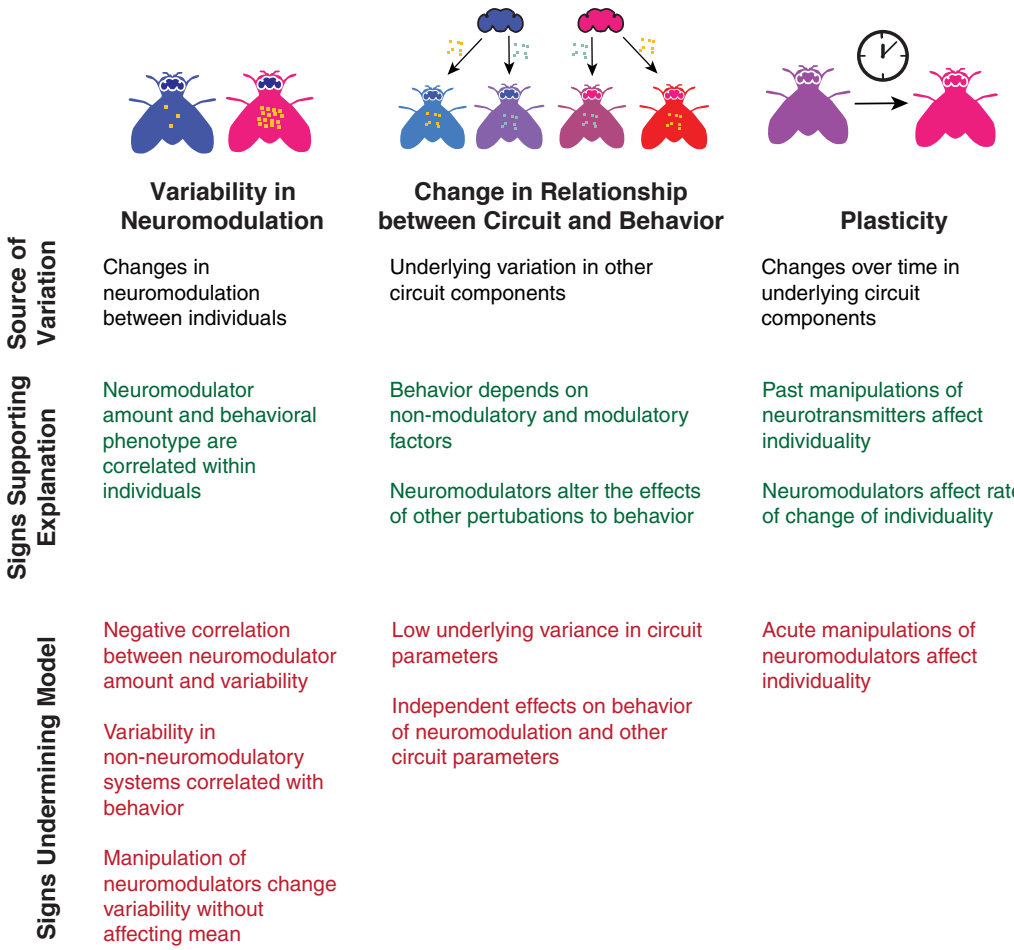

FIGURE 4 | Tests for differentiating between models of how neuromodulators affect individuality. Observations that provide evidence for each model are listed in green, and observations that are contraindicative for each model are shown in red.

acoustic startle response (Pantoja et al., 2016) but that same mechanism struggles to explain how serotonin deficient *C. elegans* demonstrate more variability in roaming (Stern et al., 2017). Neuromodulation increases robustness and decreases variance in behavior in crabs through acute changes in conductances (Grashow et al., 2009), but that doesn't explain how a serotonin agonist can affect individuality 5 days after washout (Kain et al., 2012). These results suggest that none of these models are a universal solution, but instead, that neuromodulators may affect individuality via different mechanisms in different species, circuits and behaviors—and that each case may be a mix of multiple mechanisms.

Apart from the mechanism of action of neuromodulation on individuality, a number of other questions pertaining to neuromodulation and individuality remain unstudied. Does the amount of individuality in individuals change at different points in the lifecycle of an organism, and do these changes correlate to neuromodulator strength? Do changes in the environment or experience of an animal change the amount of individuality manifested in a population, and if so, is this controlled by neuromodulators? Understanding the mechanisms by which neuromodulations influence individuality and how they influence these questions will provide a more detailed understanding of the role and control of individuality in species.

CONCLUSION

The study of variability among populations underlies a foundational question in biology: what principles are generalizable across all individuals and what features are idiosyncratic and optional. Understanding variability is key to understanding developmental and learning rules as well as cognitive and behavioral processes.

Neuromodulators appear to play a key role in regulating individuality in many behaviors. As outlined in this review, there are multiple methods by which they could do so—understanding how and why provides us an opportunity to understand the underlying process by which these behaviors develop. Are neuromodulatory systems inherently less stereotyped than

other neural circuits, and is this difference a major driver in individuality? Or do neuromodulators reveal or conceal widespread variation amongst other components of the nervous system? To what degree are organisms born different, and to what degree do they grow to become different, even continuing into adulthood? Understanding how neuromodulators influence individuality will offer insights into broader questions about the mechanisms that create individuals.

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RM conceived and wrote this review.

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Environmental and Molecular Modulation of Motor Individuality in Larval Zebrafish

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Innate behavioral biases such as human handedness are a ubiquitous form of inter-individual variation that are not strictly hardwired into the genome and are influenced by diverse internal and external cues. Yet, genetic and environmental factors modulating behavioral variation remain poorly understood, especially in vertebrates. To identify genetic and environmental factors that influence behavioral variation, we take advantage of larval zebrafish light-search behavior. During light-search, individuals preferentially turn in leftward or rightward loops, in which directional bias is sustained and non-heritable. Our previous work has shown that bias is maintained by a habenula-rostral PT circuit and genes associated with Notch signaling. Here we use a medium-throughput recording strategy and unbiased analysis to show that significant individual to individual variation exists in wildtype larval zebrafish turning preference. We classify stable left, right, and unbiased turning types, with most individuals exhibiting a directional preference. We show unbiased behavior is not due to a loss of photo-responsiveness but reduced persistence in same-direction turning. Raising larvae at elevated temperature selectively reduces the leftward turning type and impacts rostral PT neurons, specifically. Exposure to conspecifics, variable salinity, environmental enrichment, and physical disturbance does not significantly impact inter-individual turning bias. Pharmacological manipulation of Notch signaling disrupts habenula development and turn bias individuality in a dose dependent manner, establishing a direct role of Notch signaling. Last, a mutant allele of a known Notch pathway affecter gene, *gsx2*, disrupts turn bias individuality, implicating that brain regions independent of the previously established habenula-rostral PT likely contribute to inter-individual variation. These results establish that larval zebrafish is a powerful vertebrate model for inter-individual variation with established neural targets showing sensitivity to specific environmental and gene signaling disruptions. Our results provide new insight into how variation is generated in the vertebrate nervous system.

Keywords: zebrafish, inter-individual variation, individuality, environment, Notch, Gsx, modulation, thermoregulation

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INTRODUCTION

Inter-individual variation, or individuality, is a hallmark of nearly all animal species and contributes to the population's fitness and ability to adapt when confronted with environmental change (Dingemanse et al., 2004; Klein et al., 2017; Horváth et al., 2020). One form of inter-individual variation is sensory and motor biases. Handedness in humans is a familiar example, expressed as a sustained preference for left- or right-hand use, which the expression of a specific hand preference is only partially explained by genetics, suggesting complex interactions contribute to imposing handed phenotypes (Cuellar-Partida et al., 2020). Indeed, significant complexity underlies human handedness. The preferred hand usage is context-dependent, largely independent of other behavioral biases, and shows variable consistency – observed as consistent or inconsistent hand dominance in a task dependent manner (Watson and Kimura, 1989; Souman et al., 2009; Chu et al., 2012). Handed biases are also a conserved form of individual behavioral variation with species as diverse as hagfish (Miyashita and Palmer, 2014), *Drosophila* (Kain et al., 2012; Buchanan et al., 2015), chicken (Rogers, 1982; Casey and Karpinski, 1999), and various vertebrate paw/foot biases (Bulman-Fleming et al., 1997; Brown and Magat, 2011; Giljov et al., 2013; Schiffner and Srinivasan, 2013; Manns et al., 2021) showing sustained individual motor preferences. Despite the prevalence of handed behaviors, mechanisms instructing these behaviors and the variation observed across individuals are still poorly understood.

Research to date shows that binary handed-like behavioral variation is observed in isogenic *Drosophila* (Kain et al., 2012; Buchanan et al., 2015; Linneweber et al., 2020) and clonal fish (Izvekov et al., 2012; Bierbach et al., 2017). Even more complex behavioral modalities in isogenic mouse strains (Freund et al., 2013, 2015; Hager et al., 2014), *Caenorhabditis elegans* (Stern et al., 2017), *Drosophila* (Linneweber et al., 2020), and clonal crayfish (Vogt et al., 2008) species display stable individual phenotypes with significant inter-individual variation at the population level, suggesting external events contribute to behavioral diversity across individuals. Even in humans, external or stochastic factors are likely important as discordant handedness is frequently observed in monozygotic twins (Jäncke and Steinmetz, 1995). These examples suggest that environmental, chemical, or physical events during development, even at early developmental stages, could produce inter-individual differences. In *Drosophila*, the availability of numerous isogenic strains and the ability to assay large numbers of individuals have been instrumental in elucidating key components generating inter-individual variation (Buchanan et al., 2015). When navigating in their environment, *Drosophila* display a turn bias, where individuals preferentially use same-direction turns, and the magnitude of this bias is modulated by genetic background, activity in the central complex, and exposure to environmental enrichment as well as social experiences (Ayroles et al., 2015; Buchanan et al., 2015; Akhund-Zade et al., 2019; Versace et al., 2020). These findings demonstrate that functional variation in the invertebrate nervous system is maintained by specific neural substrates and further modified by gene and environment interaction. In murine models, exploratory behavior is a thoroughly investigated example of inter-individual variation, where phenotype variation is enhanced by environmental enrichment and correlated changes in hippocampal neurogenesis (Freund et al., 2013; Körholz et al., 2018; Zocher et al., 2020). Despite this well-studied mammalian model and other known handed behaviors that suggest changes in neuron number or activity patterns may regulate inter-individual variation, the mechanisms instructing inter-individual differences remain poorly understood. Therefore, two prevailing questions are what neural substrates generate biases and what mechanisms instruct specific bias types, i.e., left versus right-handed or consistent versus inconsistent handedness.

Zebrafish have emerged as a powerful model for elucidating mechanisms that instruct visceral and neural differences between individuals (Gamse et al., 2003, 2005; Dreosti et al., 2014). Moreover, similar to other teleost species, zebrafish have a visual bias, preferentially using the left eye to assess novelty (Bisazza et al., 1997; De Santi et al., 2001; Sovrano, 2004; Andrew et al., 2009). However, this behavioral bias is primarily fixed in the population and offers little insight into inter-individual variation. Larval zebrafish also perform a light-search behavior that is onset by the loss of visual navigating cues, which drives a period of stereotypic leftward or rightward circling (Horstick et al., 2017), consistent with search patterns observed in other species (Bell et al., 1985; Hills et al., 2004, 2013; Gray et al., 2005). An individual's leftward or rightward circling direction is persistent over at least multiple days, observed at equal proportions in the population, and is not heritable (Horstick et al., 2020). The features of light-search share many of the hallmark traits observed in well-established invertebrate models of turn bias that have been instrumental for characterizing mechanisms that regulate inter-individual variation (Ayroles et al., 2015; Buchanan et al., 2015; Akhund-Zade et al., 2019). Moreover, our work has shown that neurons in the habenula and rostral posterior tuberculum (PT) are essential for maintaining zebrafish turn bias (Horstick et al., 2020). Therefore, larval zebrafish is a potentially powerful vertebrate model to determine how inter-individual variation is imposed in the vertebrate brain. What remains lacking is a rigorous analysis of turn bias variation in the population and the identification of external and internal factors modulating inter-individual turn bias differences.

Here, we capitalize on the larval zebrafish turning bias to characterize environmental factors and signaling pathways that modulate inter-individual variation. Previous work identified a persistent left/right turn bias maintained by a habenula-rostral PT circuit and Notch associated signaling pathways (Horstick et al., 2020). However, locomotor features or factors instructing turn direction phenotypes was unexplored. Here we develop a multiplex recording pipeline and a new metric, bias ratio, which permit turn bias recording in a medium-throughput manner and rigorous unbiased analysis of inter-individual variation. Previous work used metrics that weighted behavior on a single trial to categorize turning type (Horstick et al., 2020), which these metrics are potent indicators of bias, yet can easily compound error over serial testing that is typically required to study probabilistic behavior like turning bias. Using

our new testing pipeline, we first characterized turning types, finding previously described left and right turning types and a previously undescribed unbiased turning type in a wildtype strain. We further establish that turning types are distinguishable by unique path trajectory features. Second, we determined that temperature selectively impacts inter-individual variation and rostral PT neurons, establishing a tentative mechanism for temperature dependent regulation of inter-individual variation. Last, we investigate molecular pathways, demonstrating a direct role for Notch signaling using pharmacological inhibition. We establish levels of Notch inhibition that disrupts habenula development and bias, yet well-established Notch mechanisms such as neuronal proliferation or morphological development are unaffected. By testing a mutant associated with Notch signaling, gsx2, we implicate that brain regions beyond that previously described circuit could be important for developing variation in a vertebrate. This work develops zebrafish search behavior as a model for inter-individual variation and reveals how environmental and molecular cues impact specific neural substrates to generate distinct behavior types in a vertebrate.

MATERIALS AND METHODS

Animal Husbandry

All experiments were approved by the West Virginia University Institutional Animal Care and Use Committee. Zebrafish (*Danio rerio*) Tübingen long-fin (TL) wildtype strain was used in all experiments and used as the genetic background to maintain transgenic and mutant lines. Experiments were conducted during the first 7 days post fertilization (dpf), which is before sex determination. Larval rearing conditions were 28°C, 14/10 h light-dark cycle, in E3h media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, and 1 mM HEPES, pH 7.3), and at a stocking density of 40 embryos per 30 mL E3h, unless stated otherwise. *Social environment*: To test the effect of social interaction, we raised larvae under two different social conditions: 20 larvae in a 6 cm petri dish or a single larva per 6cm dish. Social or isolation rearing started at 5–8 h post fertilization (hpf) and continued until testing at 6 dpf. *Temperature*: To test the impact of temperature on the development of turn bias, larvae were raised 1–4 dpf at either 24, 28, or 32°C. At 4 dpf, all groups were moved to 28°C until testing at 6 dpf. To determine if a specific development period was sensitive to elevated temperature, separate groups of larvae were raised at 32°C from either 31–55 hpf or 55–79 hpf, after which they were returned to standard rearing temperature and tested at 6 dpf. *Salinity*: The impact of increased salinity was tested over 4 salt concentrations (1, 2, and 5 ppt – parts per thousand) and standard E3h (∼0.5 ppt) as a control. Larvae were reared in variable salinities from 1 to 4 dpf, and behavior tested at 6 dpf. An elevated salinity stock of E3h was made by adding 9.5g NaCl (Sigma) to standard E3h, creating a 10 ppt stock, which was diluted for working concentrations with standard E3h media. *Environmental enrichment:* Enriched environments were created by adhering mixed size and color (predominately red, blue, gray, and white colors) LEGO® blocks onto the bottom of a 10 cm petri dish. Previously, LEGO® blocks have been used to stimulate novel object recognition and interaction in larval zebrafish (Bruzzone et al., 2020). In addition, 5–8 plastic aquarium leaves were included to float on the surface. Last, dishes were positioned on platforms with mixed white and black shape substrates. A total of four enriched environments were created with variable LEGO® colors and sized blocks, and larvae were rotated daily between enriched environments. As controls, larvae were raised in plain 10 cm dishes placed on either a solid white substrate. For experiments, larvae were maintained in enriched or control dishes from 1 dpf until behavior testing. *Shaking*: We tested the impact of environmental instability on motor bias by continuously shaking larvae from 1 to 4 dpf. At 1 dpf, embryos were placed in a 75 cm^2 cell culture flask (Sigma) with approximately 80 mL E3h. Flasks were propped at 30 degrees on a Stovall Belly Dancer orbital rotator, set to 70 rpm. At 4 dpf, larvae were removed from culture dishes and raised under standard conditions prior to testing at 6–7 dpf.

Transgenic lines used were enhancer trap *Tg(y279-Gal4)* (Marquart et al., 2015) and *Tg(UAS:Kaede)s1999t* (Davison et al., 2007). Mutant line used was $gsx2^{\Delta 13a}$ (Coltogirone et al., 2021).

Behavior Tracking and Analysis

Behavioral experiments were performed on 6–7 dpf larvae, except as noted. All experiments were recorded using infrared illumination (940 nm, CMVision Supplies), a μEye IDS1545LM-M CMOS camera (1st Vision) fitted with a 12 mm lens, and a long-pass 780 nm filter (Thorlabs, MVL12WA and FGL780, respectively). Visible illumination was provided by a white light LED (Thorlabs) positioned above the larvae, adjusted to 40–50 μW/cm^2 (International Light Technologies, ILT2400 Radiometer with SED033 detector). Testing conditions were maintained between 26 and 28°C for all behavioral recording, and all larvae adapted to the recording room conditions for 20 min before recording under matched illumination to recording rigs. Custom DAQtimer software was used to control lighting, camera recording parameters, and real-time tracking as previously described (Yokogawa et al., 2012; Horstick et al., 2017). The camera field of view was set to record four 10 cm dishes simultaneously with one larva per dish for multiplex recordings. A total of four recording rigs were used. Path trajectories of individual larvae are recorded over 30-s recording intervals at 10 fps and analyzed using five measures: net turn angle (NTA), total turning angle (TTA), match index (MI), bias ratio (BR), and performance index (PI) (see **Table 1** for metric reference). A minimum of 100 points were required to be included in the analysis. NTA is the summation of leftward and rightward angular displacement (−leftward, +rightward) over the recording interval, whereas TTA is the sum of absolute values of all angular displacement. MI measures the proportion of events in a series going in the same direction. Leftward and rightward trials are scored as 0 or 1, and MI is the percent of events matching the direction of the first trial in a testing series. For example, a MI = 1 is all events are in the same direction as the first trial, whereas 0.33 is a third of the events matching the first trial. For MI analysis, individuals missing the first dark trial were excluded from analysis. BR is a proportion of directional

TABLE 1 | Reference for metrics and assays.

Behavior metrics	Name	Measure
NTA	Net turn angle	Net sum of leftward and rightward angular movement
TTA	Total turn angle	Absolute sum of all angular movement
BR	Bias ratio	NTA/TTA ratio. Proportion of directional movement
MI	Match index	Proportion of trials matching direction of first trial
PI	Performance index	Average of binarized turn directions (0 = left; 1 = right)
Behavior assays	**Name**	**Measure**
4×	NA	Paired 30 s Light ON and OFF recordings, repeated 4×
8×	NA	Paired 30 s Light ON and OFF recordings, repeated 8×
q4×	Quad 4×	Four repeated '4×' recordings separated by 10 min

turning compared to total turning, calculated by dividing NTA by TTA, e.g., −1 represents that all directional movement in a single trial occurred in a leftward direction, while −0.5 indicates that 50% of all turning was in a net leftward direction (e.g., −200 degrees NTA out of 400 TTA). PI was calculated by averaging binary bias ratios, with leftward trials scored as 0 and rightward 1. Where noted on figures, bias ratios were weighted by the proportion of larvae within a PI group in order to demonstrate changes in the number of larvae within a performance group. In all analyses that required a PI for categorizing larvae, all individuals that had missing trials were excluded. This criteria was necessary to ensure rigorous categorization. For *gsx2* experiments, larvae were housed individually following behavior testing for *post hoc* genotyping. Genotyping was performed as previously described (Coltogirone et al., 2021). In brief, genotypes were confirmed using PCR spanning the deletion: *gsx2* (primers: 5′TGCGTATCCTCACACATCCA, 5′TGTCCAGGGTGCGCTAAC; 134 bp wildtype, 121 bp mutant, and 134/121 bp heterozygous). Previous reports describe that gsx2 mutants have reduced swim bladder inflation (Coltogirone et al., 2021), which was minimized by raising larvae in shallow water dishes. Only larvae with normal swim bladder inflation and balance were used for experiments.

The 4× recording assay was performed by recording larval path trajectories over four recording intervals, each composed of 30 s baseline recordings, immediately followed by 30 s recording following the loss of visible illumination. Each recording interval was separated by 3 min of baseline illumination. The 8× recording was performed in a similar format, including four additional light ON/OFF recording intervals performed in series. The quad 4× (q4×) assay is identical to the 4×, except that the 4× recording interval is repeated four times, separated by 10 min baseline illumination (see **Table 1** for assay reference). A 4× recording strategy was used to test the developmental onset of turn bias. Individual larvae were first tested at 3 dpf, and were separately raised in 6-well plates and retested daily through 6 dpf. For analysis, larvae were grouped as left or right biased based on BR (average BR+, right bias; −, left bias) at 6 dpf when turn bias is well-established (Horstick et al., 2020). To ensure rigorous categorization, larvae with ambiguous responses at 6 dpf (BR between −0.1 and 0.1) were removed.

Pharmacology

Notch signaling was inhibited using the ϒ-secretase inhibitor LY411575 (Sigma, SML050). A 10 mM stock of LY411575 was prepared in DMSO and diluted to working concentrations with a final volume of 0.08–0.1% DMSO for all trials. To test Notch inhibition on turn bias, mid-gastrulation (6–8 hpf) groups of embryos were treated with 0.05, 0.1, 0.15, 0.2, 0.25, 1 or 10 μM LY411575 until 4 dpf; the drug was replaced daily. At 4 dpf, LY411575 was removed and larvae placed in fresh E3h until behavior testing at 6 dpf. Phenotypic categorization was performed at 3 dpf. Individuals were scored as normal (visually no abnormal tail curvature, edema, reduced/decreased swim bladder size, necrosis, or overt abnormal swimming), mild (abnormal touch responsiveness), moderate (tail curvature), or severe (gross developmental defects, necrosis). Only normal larvae were used for behavioral testing. Vehicle controls were 0.08–0.1% DMSO treated.

Labeling and Imaging

Immunohistochemistry

To assay neuronal proliferation, we labeled with anti-HuC/D (Elav protein) (Invitrogen A21271). Control (0.08% DMSO) and LY411575 groups (100 nM and 8 μM) were prepared as described above. At 24 hpf, embryos were fixed overnight using 4% paraformaldehyde in 1× PBS at 4°C. Washes were performed with 1× PBS containing 0.1% TritonX-100. We used primary antibody mouse anti-HuC/D (1:500, Invitrogen, 16A11). Secondary detection was performed with goat anti-mouse IgG1 Alexa 488 (1:500, Invitrogen, A32723). To analyze images, signal intensity of a 56 μm × 6 μm (W × H) region spanning a lateral to midline hemi-section of the anterior spinal cord was recorded using ImageJ. Three sections were measured per larva, averaged and standardized for comparison between groups.

Fluorescent *in situ* Hybridization

To determine the levels of Notch signaling we examined transcript levels of her12 (Jacobs and Huang, 2019). Hybridization chain reaction (Molecular Instruments) probes and labeling technology was used to detect her12 transcripts. Her12 mRNA sequence (NM_205619) was provided to Molecular Instruments to design a custom gene-specific her12 probe detection set. LY411575 and control larvae were treated as described above. At 30 hpf, larvae were fixed overnight using 4% paraformaldehyde in 1× PBS at 4°C. Fixed larvae were washed in 1× PBS containing 0.1% Tween20 and labeled following Molecular Instruments HCR RNA-Fish protocol for whole-mount zebrafish embryos (Schwarzkopf et al., 2021). All images were collected using the same parameters. For analysis,

the percent area of her12 expression was quantified within the spinal cord using ImageJ.

Neuron Temperature Sensitivity

Rostral PT and habenula image stacks were captured and neurons counted in max projections using ImageJ. All imaging was performed using larvae from *Tg(y279-Gal4)/Tg(UAS:kaede)* carrier in-crosses. At 1 dpf, larvae were screened for Kaede and reared at elevated temperatures as described above. Larvae were moved to standard raising conditions at 4 dpf, and live-imaged at 6 dpf. Larvae were anesthetized using MS-222 (Sigma) and mounted in 2% low melting temp agar. To determine if a specific developmental time period was crucial, larvae were similarly prepared and analyzed, yet only raised at elevated temperature during either 31–55 hpf or 55–79 hpf intervals. Controls were raised at standard rearing temperatures.

Neuron Sensitivity to Notch Inhibition

Using *Tg(y279-Gal4)/Tg(UAS:kaede)* carrier in-crosses we performed LY411575 as described above, except treatments ended at 3 dpf when both the habenula and PT could be observed, while attempting to minimize severe morphological phenotypes and death at higher concentrations. We treated embryos at concentrations of 0.1 and 1 μM with a vehicle control. All treatments had a final DMSO concentration of 0.01%. Imaging the habenula and PT was performed as above and neuron counts performed using max projections in ImageJ. Counts were only performed on groups were habenula and PT neurons could be reliable identified. For counting neuron numbers, the larger habenula was classified as the 'left' habenula regardless of hemisphere. Habenula were classified as symmetric if the left to right neuron ratio was less than 2.

Imaging

All imaging was performed on an Olympus Fluoview FV1000. For live imaging, larvae were anesthetized in a low dose of MS222 (Sigma) and embedded in 2% low melting temp agar. Fixed samples were transferred into 70% glycerol/30% 1× PBS and slide-mounted for imaging.

Statistical Analysis

Analysis was performed in R R Core Team (2020). R: A language and environment for statistical computing, 2020), R ggplot2 package (Wickham, 2016) (R Core Team) and Prism (GraphPad). All statistical comparisons were two-sided, unless noted otherwise. Standard error of the mean (±SEM) was used for all experiments, except MAD analysis which display 95% confidence intervals. Cohen D was calculated in R using package effsize. For all experiments, data was collected from a minimum of three independent clutches. Normality was tested using the Shapiro–Wilks test. Normally distributed data was compared using either 1 or 2-way *t*-tests. Non-normal data was analyzed using a Mann–Whitney *U* test or Wilcoxon signed-rank test for 2 or 1-way tests, respectively. To perform multiple comparisons, ANOVAs were performed in GraphPad and multiple comparisons adjusted using a Bonferroni correction. Boxplots show median and quartiles with outliers identified beyond 2.7 standard deviations from the mean.

Permutation and bootstrapping was performed using "sample" R function without and with replacement, respectively. For permutation experiments, bias ratios values were randomized across all individuals in a dataset. Randomization was performed only within the same trial, e.g., reshuffling of bias ratios within the first light off trial. Permutations were simulated 1,000 times and average bias ratios and MAD values calculated using custom R code, and used to plot permutated probability density curves and MAD values. Probability density plots and area under the curve measurements were performed using custom R code. For area under the curve analysis, ±0.3 tails were chosen for comparison, which are approximately two standard deviations from the population average. To generate error bars for MAD analysis, average bias ratios were bootstrapped (1,000 bootstrap replicates) with resampling. For each resampled dataset a MAD was calculated and MAD values across all resampled datasets used to calculate a 95% confidence interval applied as an error bar. A 1-way comparison was used to calculate significance for all simulated dataset comparisons. To generate a *p*-value, the number of resampled dataset MAD values were totaled that fall within or exceed the 95% confidence interval of the comparison group, and this total was divided by 1,000 to produce a final *p*-value. This represents the fraction of simulated experimental groups that fall within a range that supports a null hypothesis of no difference between groups. For example, 600 bootstrapped datasets from a simulated control that fall within or exceed the confidence interval of an experimental group yields $p = 0.60$, implicating that 60% of simulated datasets do not support the statistical difference between compared groups. Direction of comparison is noted in the legend for each dataset.

RESULTS

Turning Behavior During Light Search Shows High Inter-Individual Variation

We developed a multiplexed strategy to record path trajectories to assess inter-individual variation during larval zebrafish light search behavior (**Figure 1A**). Previously, the stereotypic turning was described using a large recording arena (14,400 mm^2) to record single larva (Horstick et al., 2017). Larvae are recorded in 100 mm diameter dishes (7,854 mm^2) for our multiplexed strategy, and robust circling is observed following light extinction (**Figure 1B**). To characterize individual motor biases, we initially recorded larval path trajectories over a series of four intervals of paired 30-s baseline illumination and 30 s following the loss of illumination, with each of these recording pairs separated by 3 min of illumination to restore baseline behavior (Horstick et al., 2020), which we refer to as 4× recording (**Figure 1C**). This recording yields four paired light on and off events per individual. We recorded responses from 374 individuals, representing 1,496 paired baseline and dark responses. The presence of motor bias was previously described using a match index (MI) – the percent of turning trials in which turning direction was the same as the first dark trial (Horstick et al., 2020). Here we

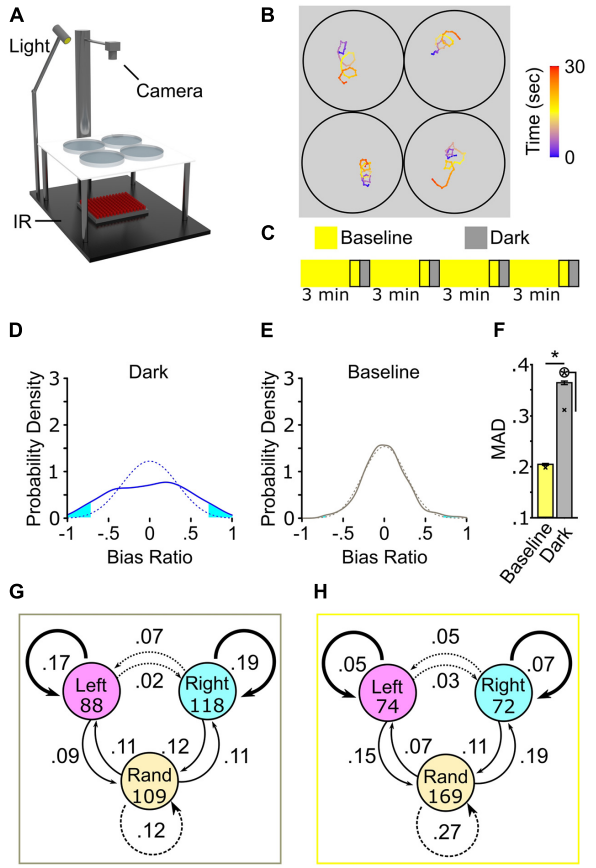

FIGURE 1 | Turning behavior following the loss of light exhibits high inter-individual variation. **(A)** Schematic of multiplex recording rig. **(B)** Illustrative path trajectories following loss of light from multiplex recording. Color scale is time (seconds). **(C)** Diagram of 4× recording. Outlined regions denote recording intervals. **(D–F)** 4× recordings strategy to show high inter-individual variation in turn bias. **(D)** Average bias ratio probability density curve for dark responses (solid blue line, $N = 374$) and paired curve following reshuffling (dotted blue line, average of 1,000 resampled datasets). **(E)** Same as **(D)** except the paired baseline responses (solid gray line, $N = 374$) and random permutation density curve (dotted gray line). Cyan fill shows the area in each tail corresponding to the probability of observing a result more extreme or equal to ±0.3 average bias ratio. **(F)** MAD for baseline (yellow), and dark (gray) responses. $N = 374$. Error bars are 95% confidence intervals generated by bootstrap resampling. Asterisk in circle, $p < 0.05$ to MAD permutation value shown with an X. **(G)** Dark responses (gray outline) and baseline **(H)** (yellow outline) from 4× recording showing frequency of direction change between first (circles) and second (arrows) pairs of responses. Initial N shown in circles. Bold, solid, and dotted arrows delineate responses that produce perfectly matched bias, partial bias, and unbiased responses, respectively.

confirm previous findings showing a significant MI increase following the loss of illumination (Wilcoxon matched-pairs test, $p < 0.001$), showing the number of individuals recorded can be upscaled via multiplexing (**Supplementary Figure 1A**). Overall, our current approach for multiplexed recording recapitulates previous findings. These data show that our multiplexed strategy provides medium-throughput recording, allowing a rigorous analysis of larval zebrafish inter-individual variation.

We calculated a bias ratio by dividing net turning angle (NTA) by total turning angle (TTA – absolute sum of all angular displacement) for each baseline, and dark trial recorded to examine the spectrum of wildtype larvae inter-individual variation during search behavior (**Supplementary Figure 1B**). This metric provides the proportion of same-direction turning within a continuous numerical range bounded by −1 and 1, representing all directional movement in a leftward or rightward direction, respectively. The average bias ratio across the entire population during baseline illumination and light-search did not significantly vary from zero showing no population bias [one-sample t-test against 0, baseline: $t(373) = 0.007842$, $p = 0.9937$; dark: $t(373) = 0.1696$, $p = 0.89$] (**Supplementary Figure 1C**). Despite similar population-level bias ratios between baseline and dark, significant variation is observed in the dark that is not observed during baseline (**Figures 1D–F**). Using a probability density curve, where the area under the curve represents the proportion of individuals in the population, we find that during dark turning, 12.38% of the population displayed a robust sustained turning bias over 4 trials (bias ratio <-0.7 = 6.41%, left bias; >0.7 = 5.97%, right bias) (**Figure 1D**). Conversely, 1.72% of baseline events displayed sustained directional turning (**Figure 1E**). The distribution of bias ratios shows that, following light extinction, a significantly greater number of individuals utilize sustained same-direction turning [$\chi^2(1) = 51.02$, $p < 0.0001$]. To determine whether these distributions were the product of chance, we simulated 'randomized' baseline and dark datasets by resampling bias ratios (1,000 resamples) within each trial (Figures D,E, dotted line). Following randomizing, 2.35% of the simulated dark responses maintained strong directional turning, similar to that observed during baseline. A previous study used mean absolute deviation (MAD) as a metric to quantify variation in a population; a higher MAD represents increased variation across individuals in the population (Buchanan et al., 2015). Here, MAD was calculated for baseline, dark, and simulated datasets. As MAD was generated from the whole population, average bias ratios were bootstrapped (1,000 boots) to generate 95% confidence intervals for statistical comparison. MAD is 44.10% ($p < 0.001$) and 15.79% ($p < 0.001$) reduced in baseline or in randomized dark groups compared to light-search dark responses, respectively (**Figure 1F**), whereas no difference was observed between baseline MAD and randomized baseline responses (**Figure 1F**, yellow bar). These findings show that turn bias during light search behavior shows significant variation beyond what is expected by chance or while larvae navigate in an illuminated environment.

Our analysis, along with findings from previous reports, illustrates robust left and right turners, or turning types, within the population. However, the distribution of bias ratios from 4× recordings shows that over 14% of the population exhibits an average bias ratio consistent with no sustained turn direction ($-0.1 < BR < 0.1$) (see **Figure 1D**). These individuals could represent either a stable unbiased population or endogenous behavioral fluctuation. To evaluate whether unbiased individuals are a sustained turning type in the population, in addition to left/right biased turners, we created a performance index (PI) by transforming all individual trials to either 0 or 1 for

overall leftward or rightward preference per trial, respectively. From these binary values, we created a transition index for the first and second set of responses from the 4× dataset, i.e., left (LL = 0), right (RR = 1), or random (LR; RL = 0.5) responses that can be compared between the first and last response pairs. Using the transition pair PI, we assessed the frequency of turn direction change or conservation (**Figures 1G,H**). During dark trials, 36% of all transitions showed sustained turn direction (left = 17%, right = 19%; average PI = 0 or 1), whereas during baseline illumination 12% of larvae sustained turn direction [$\chi^2(1) = 54.545$, $p < 0.0001$]. Conversely, 21 and 35% of transitions yielded sustained random behavior between dark and baseline recording conditions, respectively (for example, LR to RL or RR to LL; average PI = 0.5) [$\chi^2(1) = 8.615$, $p = 0.0033$] (**Figures 1G,H**). Interestingly, during light-search initially random response pairs transitioned to directional (RR or LL) responses 22% of the time, yielding partial turn bias (average PI 0.75 or 0.25).

To confirm our observations persisted over longer timescales, we ran an additional 8× experiment, testing 189 larvae as before, with four additional light ON/OFF intervals in series. From this extended testing condition, we observed conserved trends demonstrating significant inter-individual variation in turning bias during light-search, yet not during baseline illumination (**Supplementary Figures 1D–F**). Neither 4× or 8× recording showed a change in TTA over time, establishing overall behavior is not disrupted by our assays (**Supplementary Figures 1G,H**). As 4× and 8× experiments were broadly consistent, we focused on the 4× recording strategy for ongoing investigations. Our data show that wildtype larvae exhibit significant inter-individual variation in turn bias during light-search, greater than that expected by chance, with a subset of individuals potentially exhibiting a previously unexplored unbiased turning type.

Multiple Stable Turning Types Exist With Distinct Locomotor Features

Characterizing changes in locomotor parameters in zebrafish has been a powerful strategy to develop etiological and mechanistic models (Burgess and Granato, 2007; Horstick et al., 2013; Chen and Engert, 2014; Dunn et al., 2016). Therefore, we next aimed to establish what underlying locomotor changes account for unbiased and biased motor types. We hypothesized that three possible modes could generate unbiased behavior: (1) normal turning with high rates of direction switching across trials, (2) reduced same-direction turning within single trials, or (3) weak photo-responsiveness and, therefore, low total turning. To differentiate between these hypotheses, we categorized all larvae based on average PI across all four trials, generating five categories. Across PI groups, we compared the absolute average bias ratio to determine if the magnitude of directional turning changed based on PI. During light search the average bias ratio magnitude significantly changed based on PI [1-way ANOVA $F(4,352) = 10.43$, $p < 0.0001$], where partial and unbiased PI groups showed less overall directional turning (**Figure 2A**). No difference was observed between strong left and right biased turners [PI = 0, 0.603 ± 0.022; PI = 1, 0.58 ± 0.021: $t(352) = 0.7811$ adjusted $p > 0.9999$]. Consistent with earlier observations, no differences were observed across PI groups during baseline [1-way ANOVA $F(4,352) = 2.087$, $p = 0.082$], consistent with an absence of turn individuality (**Supplementary Figure 2A**). Moreover, there was no significant change in TTA during dark turning [1-way ANOVA $F(4,352) = 1.263$, $p = 0.28$] across all PI groups (**Supplementary Figure 2B**). As all PI groups exhibited normal levels of total turning, this ruled out variable photo responsiveness as the basis of different turning types. Unexpectedly, partially biased populations (0.25, 0.75 PI) showed a similar average bias ratio as unbiased larvae (**Figure 2A**). To explain this observation, we reasoned that bias ratio magnitude might vary depending on whether an individual trial matches or opposes the overall larva turning type. For example, for 0.25 PI larvae, leftward matched direction bias ratios compared to rightward opposed direction trials. We analyzed all individual trials between all performance groups to explore this idea, sorting trials into matched or opposing based on the average PI for each individual. Perfect performance trials (0,1) were categorized as all matched, whereas unbiased trials (0.5) as all unmatched. Left and right direction bias ratios did not vary in these groups; therefore, we combined these groups to simplify comparison (**Supplementary Figure 2C**). A significant effect was observed across groups [1-way ANOVA $F(3,1408) = 27.93$, $p < 0.0001$], with trials opposed to overall PI direction showing lower overall bias ratio strength (**Figure 2B**, magenta lines). These data suggest that the basis of unbiased motor types is due to a lower bias ratio or less persistent same-direction turning, yet not a loss of overall turning. Interestingly, we noted that matched bias ratios were reduced in partially matched trials compared to events in the fully matched group [match 0.594 ± 0.013: partial match 0.514 ± 0.015: $t(1408) = 4.046$ adjusted $p = 0.003$] (black line), implicating that the underlying differences between biased and unbiased larvae may be graded.

In order to confirm rigorously the three motor types, we performed a quad 4× assay (q4×), using the standard 4× assay repeated four times, with each recording sequence separated by 10 min of baseline illumination (**Supplementary Figure 2D**). We recorded 114 larvae, and consistent with our previous measures, individuals showed significant inter-individual turn bias variation during light-search (±0.3 probability density tails: 7.34% dark; 0.00037%, randomized dark), and sustained left, right, or unbiased locomotor preferences (**Supplementary Figure 2E**). The cumulative summation of bias ratios provided a qualitative measure of turn performance over time (**Figure 2C**). From this analysis, we noted that some larvae initially categorized as strong or unbiased turners, seemingly switched over time. Therefore, we next aimed to utilize the q4× analysis to quantify bias determination accuracy by comparing the first 4× PI to overall q4× performance. We equally divided the 0 to 1 PI scale for classifying left, right, or unbiased behavior (left ≤ 0.33; unbiased $0.33 < 0.66$; right ≥ 0.66) (**Figure 2D**). Of the larvae that show an initial strong or partial bias during the first 4× interval, 2/96 (2.08%) reverse bias direction during the q4× assay, and 27/96 (28.13%) of these individuals ultimately switch to an unbiased response after serial q4× testing. However, switching is

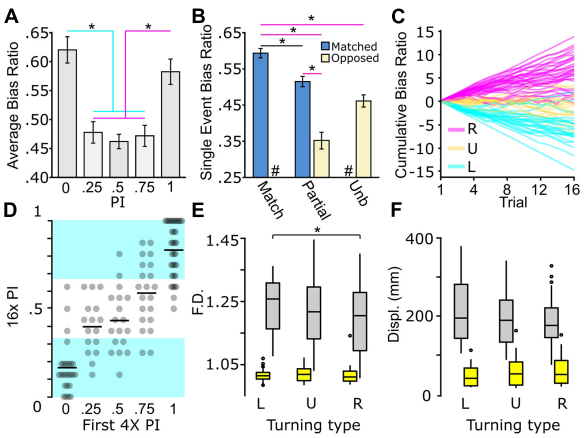

FIGURE 2 | Biased and unbiased motor types present during light-search. **(A)** Absolute bias ratio from 4× recording per PI (PI 0, $N = 66$; PI 0.25, $N = 74$; PI 0.5, $N = 75$; PI 0.75, $N = 75$; PI 1, $N = 67$). **(B)** Absolute bias ratio magnitude for single 30 s recordings occurring in a direction matching (blue) overall average PI direction or occurring in the opposed (beige) direction shown for perfect bias (0,1 PI, $N = 517$), partially biased (0.25, 0.75 PI, $N = 596$), and unbiased (0.5 PI, $N = 301$) populations. **(C)** Cumulative summation of bias ratio over the q4× from strong right (magenta, $N = 34$), strong left (cyan, $N = 34$), and unbiased (yellow, N = 18) individuals. Individuals were selected based on the first 4× average PI (strong right PI = 1, strong left PI = 0, unbiased PI = 0.5). **(D)** Average q4× PI based on initial 4× PI (PI = 0, $N = 66$; PI = 0.25, $N = 74$; PI = 0.5, $N = 75$; PI = 0.75, $N = 75$; PI = 1, $N = 67$). Black bars represent sample mean. Left, right, and unbiased performance categorized within equal thirds of the PI scale. Cyan background highlights average PI with left or right turning type behavior. **(E,F)** Characterization of path trajectory features during baseline (yellow boxes) and dark (gray boxes) responses from individuals tested in the q4× assay. Turning type determined by 16× average PI distribution in **(D)**. **(E)** Fractal dimension and **(F)** Displacement (L, left, $N = 36$; U, unbiased, $N = 40$; R, right, $N = 38$). Asterisk $p < 0.05$.

primarily observed in larvae showing an initially partial bias, as the larvae that displayed an initially strong bias (0, 1 PI) in the q4× assay, 50/59 (84.75%) maintained a left or rightward turning type. Interestingly, at the population level, the 9/114 (7.89%) of unbiased individuals initially categorized with a strong bias was comparable to that expected by random chance, i.e., the same 6.25% likelihood of flipping 4 heads with a coin [$\chi^2(1) = 0.609$, $p = 0.435$]. As expected, classifying unbiased larvae was less accurate, yet a single 4× trial accurately represented 10/18 (55.56%) of individuals. Altogether, the q4× testing strategy confirms our earlier findings and demonstrates the veracity of our recording strategies to detect specific turning types.

As the q4× assay allowed for a rigorous confirmation of turning type, we next wanted to determine whether left, right, or unbiased turning types exhibited unique path trajectory characteristics. A PI was calculated from all 16 trials in the q4× assay for each individual and categorized as left, unbiased, or right type. For each turning type, we examined fractal dimension (F.D.) and displacement (displ) as measures of local search behavior (Tremblay et al., 2007; Horstick et al., 2017). Comparison across all three motor types yielded no differences in the tested motor parameters [main effect due to turn type 2-way ANOVA displ: $F(2,222) = 0.42$, $p = 0.66$; F.D: $F(2,222) = 2.12$, $p = 0.12$], yet the expected changes in behavior following light extinction were observed [main effect due to illumination 2-way ANOVA displ: $F(1,222) = 604$, $p < 0.0001$; F.D: $F(1,222) = 643$, $p < 0.0001$] (**Figures 2E,F**). Interestingly, upon closer inspection, we did notice a small yet significant change in F.D. between left and right turning groups during dark trials [left 1.240 ± 0.012; right 1.200 ± 0.014: $t(222) = 2.974$, adjusted $p = 0.0489$, effect size $d = 0.63$]. This effect was specific, and not observed during baseline [left F.D. 1.021 ± 0.003; right F.D. 1.021 ± 0.004: $t(222) = 0.059$, adjusted $p > 0.9999$] or for displacement. These results show that the difference of left and right turning type also generate mild changes to search pattern behavior, yet not motor trajectories during baseline movement.

Development of Inter-Individual Variation Is Sensitive to Specific Environmental Factors

Many instances of motor and behavioral biases show limited heritability (Collins, 1969; Buchanan et al., 2015; Linneweber et al., 2020). This observation suggests that inter-individual variation is, at least in part, modulated through individual experience with environmental factors. Indeed, previous studies show that social experience and environmental enrichment modify inter-individual variation of specific behaviors (Freund et al., 2015; Akhund-Zade et al., 2019; Versace et al., 2020; Zocher et al., 2020). As larval zebrafish turning bias is not heritable (Horstick et al., 2020), we reasoned that the environment might contribute to overall inter-individual variation or the generation of specific turning types. To test this hypothesis, we first established that turn bias appears at 4 dpf (**Supplementary Figures 3A,B**). Therefore, larvae were exposed to changes in the environment from 1 through either 4 or 7 dpf, dependent on the tested factor. The four parameters we screened were social experience, environmental enrichment, temperature, and salinity (**Figure 3A**). Social interaction and environmental enrichment were selected because each has been shown to modulate inter-individual variation (Akhund-Zade et al., 2019; Versace et al., 2020). For social interaction, larvae are raised in isolation or groups. For enrichment, we generated two environments: (1) an enriched environment where a petri dish was fitted with internal surfaces, diverse color, hiding spots, water surface cover, and dynamic substrate pattern, and (2) an empty petri dish with a uniform white bottom as a control. In addition, we also tested the impact of etiologically relevant temperature (24 or 32°C) and salinity [0.5–5 parts per thousand (ppt)] variations during early development compared to standard rearing parameters (Engeszer et al., 2007; Sundin et al., 2019). Thus, our parameters test factors that generated inter-individual variation in other models and abiotic environmental fluctuations that larvae could encounter in a native habitat.

To determine if any of the tested parameters altered turning type development or magnitude of inter-individual variation, we looked at the average population bias ratio and MAD, respectively (**Figures 3B,C**) (**Supplementary Figure 3C**). Interestingly, the elevated temperature during early development

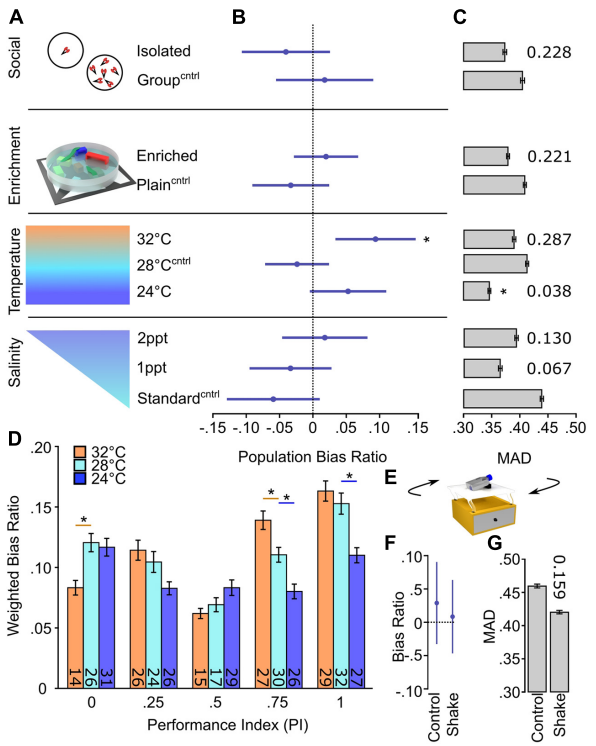

FIGURE 3 | Temperature selectively changes inter-individual variation in turn bias. **(A)** Schematic of environmental manipulations (Social Isolated $N = 87$, Social Group $N = 87$; Enriched $N = 172$, Plain $N = 134$; Temp. 32°C $N = 112$, Temp. 28°C $N = 136$, Temp. 24°C $N = 144$; Standard salinity $N = 102$, 1 ppt $N = 107$, 2 ppt $N = 107$). Each condition has an independent control denoted by superscript Cntrl. **(B)** Average bias ratios across the entire tested population per condition. Statistical comparison performed to 0, denoting no population level bias. **(C)** MAD for dark responses, error bars are 95% confidence levels generated from 1,000 bootstraps. Individual *p*-values shown above bars, calculated by comparing experimental groups to controls. **(D)** Average bias ratio per PI weighted by the percent of individuals. *N*'s indicated within bars. **(E)** Representative diagram of the setup used to shake larvae during early development. Average population bias ratio **(F)** and MAD **(G)** between shake experiments. Number above bar represents *p*-value compared to the control group. Asterisk $p < 0.05$.

caused a significant population shift from random [high temp 0.094 ± 0.044: one-sample *t*-test against 0, $t(112) = 2.157$, $p = 0.033$], implicating a population-level rightward bias, whereas no significant changes were observed in other temperature conditions or any other tested environmental parameter (**Figure 3B**). Conversely, the magnitude of turn bias variation during light-search was only reduced by low-temperature rearing, yet unaffected by other testing conditions (**Figure 3C**). To confirm the observed temperature-dependent changes, we examined the bias ratio per PI, weighted for the number of individuals per PI group. We observed that temperature imposed a significant effect on turn bias persistence [main effect of temperature 2-way ANOVA $F(2,364) = 9.275$, $p = 0.0001$] (**Figure 3D**). Indeed, the tested high temperature resulted in a significant depression of leftward turning [within PI group comparison $t(364) = 3.031$, adjusted $p = 0.0078$; red line] and increase in rightward turning [0.75 PI $t(364) = 2.904$, adjusted $p = 0.012$; red line]. Conversely, low temperature depressed turn bias performance in the population (**Figure 3D**, blue lines). These results suggest a specific temperature-mediated change. However, in larval zebrafish, fluctuating temperature is a stressor, and elevation of stress signaling has been shown to attenuate visual bias in chickens (Rogers and Deng, 2005; Long et al., 2012; Haesemeyer et al., 2018). Therefore, we tested the effect of shaking on turn bias which is a potent stressor for larval zebrafish (Eto et al., 2014; Castillo-Ramírez et al., 2019; Apaydin et al., 2020). Sustained shaking during early development resulted in no population or turn bias magnitude changes (**Figures 3E–G**). Moreover, external temperature impacts the rate of zebrafish development, and based on previous studies, our conditions would lead to an estimated ±13 h shift in development (Kimmel et al., 1995). We show that our temperature assay results in a change in hatching, a developmental marker, yet no gross changes in morphology or survival (**Supplementary Figures 3D–F**). These data illustrate that etiologically relevant temperature fluctuations differentially and specifically affect inter-individual turn bias variation.

Elevated Temperature Impacts Rostral Posterior Tuberculum Specification

A basic circuit involving the rostral posterior tuberculum (PT) and dorsal habenula (dHb) neurons has previously been described for zebrafish turn bias (Horstick et al., 2020). However, in wildtype larvae, no hemispheric differences in these neurons were found to account for left or right turning preference (Horstick et al., 2020). Because we found that elevated temperature disrupted left and right turning balance, we next wanted to determine if elevated temperature caused changes in neurons necessary for turn bias. We reasoned our environmental variables could alter neuronal development, as bias maintaining PT neurons are present as early as 2 dpf (Horstick et al., 2020), and dHb differentiation begins on 1 dpf (Gamse et al., 2003; Amo et al., 2010). First, we wanted to identify if a specific period during early development was sensitive to increased temperature. We found that elevated temperature during either 31–55 hpf or 55–79 hpf intervals did not recapitulate the population shift observed during the 1–4 dpf exposure (**Supplementary Figure 4**); therefore, we selected the full testing duration for further investigation. To visualize key dHb and PT neurons, we used the enhancer trap line *y279:Gal4*, which labels both populations of neurons (Horstick et al., 2020) (**Figure 4A**). In zebrafish, the left dHb is considerably larger than the right dHb (Gamse et al., 2003; Roussigné et al., 2009). We found that elevated temperature did not alter the habenula, and typical left/right asymmetry was observed [2-way ANOVA: interaction between temperature and hemisphere $F(1,56) = 0.070$, $p = 0.79$; effect of hemisphere $F(1,56) = 101.2$, $p < 0.0001$) (**Figures 4B,E**]. No hemispheric differences [main effect of hemisphere 2-way ANOVA $F(1,56) = 0.493$, $p = 0.49$] were observed in the number of y279 positive PT neurons (**Figure 4C**). Therefore, we combined PT measures from both hemispheres. Interestingly, from these combined pools, y279

positive neurons in the PT were reduced after exposure to elevated etiological temperature during early development [high temperature 17.64 ± 0.885; normal temperature 27.59 ± 1.172: 2-tail *t*-test $t(58) = 6.625$, $p < 0.0001$] (**Figures 4D,F** and **Supplementary Figures 4C–F**), establishing a potential neuronal basis for how high temperature during development modifies turn bias inter-individual variation.

Motor Individuality Is Sensitive to Gene Signaling Associated With Neuronal Proliferation

Studies from *C. elegans* (Bertrand et al., 2011) and *Drosophila* (Linneweber et al., 2020) demonstrate that Notch signaling can generate functional asymmetries in the brain that drive unique individual behavioral responses. Established zebrafish mutant lines *mindbomb (mib)* and *mosaic eyes (moe),* E3 ubiquitin ligase and Epb41l5 adapter, respectively, do not directly disrupt the Notch cascade, yet impair Notch signaling (Itoh et al., 2003; Ohata et al., 2011; Matsuda et al., 2016). Indeed, haploinsufficiency in these lines abrogates zebrafish turn bias, suggesting sensitivity to the levels of Notch signaling (Horstick et al., 2020). One of the canonical roles of Notch during early development is the regulation of neuronal proliferation (Appel et al., 2001; Mizutani et al., 2007; Yoon et al., 2008). Therefore, we next aimed to elucidate if turn bias is (1) sensitive to direct Notch antagonism in a dose-dependent manner and (2) if partial Notch inhibition impairs neuronal proliferation.

To disrupt Notch signaling, we used the specific ϒ-secretase inhibitor LY411575, which blocks the activation of the Notch signaling cascade (Geling et al., 2002; Fauq et al., 2007). Previous reports show that treatment with micromolar concentrations of LY411575 starting at mid-gastrulation results in a near-total loss of Notch signaling, which largely recapitulates the *mindbomb* mutant (Jacobs and Huang, 2019; Sharma et al., 2019). Therefore, we used 10 μM as a maximum dose and positive control for inhibitor efficacy across trials. To identify a level of Notch inhibition that could impair turning bias, we LY411575-treated larvae from mid-gastrulation to 4 dpf over 7 concentrations ranging from 50 nM to 10 μM and scored phenotypes at 3 dpf (**Figure 5A**). Developmental exposure of LY411575 up to 100 nM left most larvae morphologically normal, which we used as a maximum dose to test the impact on turn bias. Notch inhibition resulted in a significant change in TTA following the loss of illumination [1-way ANOVA $F(2,152) = 4.614$, $p = 0.011$], causing an increase in overall turning at 100 nM inhibitor treatment compared to controls [vehicle 1175.95 ± 53.34, 100 nM 1411.39 ± 66.50: $t(152) = 2.786$, adjusted $p = 0.018$] (**Supplementary Figure 5**). Whereas turn bias performance was reduced by Notch inhibition [main effect due to treatment 2-way ANOVA $F(2,144) = 8.995$, $p = 0.0002$], with 100 nM inhibitor nullifying bias ratio strength differences due to PI, which was not observed at lower inhibitor concentrations (**Figures 5B,C**). In addition, 100 nM but not 50 nM treatment reduced overall inter-individual turn bias variation in the population (**Figure 5D**). This data suggests that a critical threshold of Notch signaling is required for generating variation in turn bias and overall performance, which is lower than levels necessary for normal gross morphological development. To identify a potential neuronal basis for the loss of bias following Notch inhibition, we LY411575-treated *y279:Gal4* embryos to quantify transgene positive dHb and rostral PT neurons, focusing on the inhibitor concentration that specifically impairs behavior yet not morphological development. Interestingly, we found that the levels of inhibition that abrogate bias also disrupts typical dHb hemispheric asymmetry, producing an increase in reversed and symmetric habenular phenotypes (**Figure 5E**). Similarly, we observed an increase in the smaller 'right' dHb neuron number [vehicle 4.25 ± 1.21, 100 nM 10.22 ± 1.64: $t(15) = 2.870$, $p = 0.012$], consistent with increased habenular symmetry (**Figures 5F,H,I**). Conversely, the rostral PT was unaffected (**Figures 5G–I**).

To confirm that LY411575 exposure impaired Notch signaling, we examined *her12* expression, a downstream target of the Notch signaling cascade that is robustly expressed in the spinal cord, providing an unambiguous region to quantify expression changes (Jacobs and Huang, 2019). Exposure to micromolar inhibitor concentrations resulted in a near-total absence of *her12* expression, consistent with previous reports (Jacobs and Huang, 2019). The *her12* expression was, however, observed in the spinal cord of the 100 nM group at an intensity indistinguishable from controls (**Figures 5E,L**).

A canonical and conserved role for Notch during early development is regulating neuronal proliferation and maintaining progenitor pools, and the loss of Notch leads to increased proliferation (Appel et al., 2001; Cheng et al., 2004; Sharma et al., 2019). Therefore, we next wanted to determine whether the level of Notch inhibition that impairs turn bias individuality also disrupts proliferation. During zebrafish embryonic development, proliferative neurons are readily visualized in the anterior hindbrain using Elav (HuC/D) protein expression as a marker (Kim et al., 1996; Sharma et al., 2019). These proliferative neuron pools expand following high levels of Notch inhibition or in the *mindbomb* mutant background (Itoh et al., 2003; Sharma et al., 2019). Consistent to our observation with *her12* and PT neuron counts, partial pharmacological Notch inhibition (100 nM drug) induced no change in actively proliferating neurons, yet positive controls (8 μM) displayed robust expansion of proliferating neurons (**Figures 5K,M**).

Notch signaling is ubiquitous in the larval zebrafish nervous system (Tallafuss et al., 2009; Banote et al., 2016; Kumar et al., 2017), and pharmacological inhibition is not specific. Consequentially, we next aimed to determine whether proliferative pathways in restricted areas of the brain may also contribute to turn bias. Genomic screen homeobox transcription factors (Gsx1 and 2, formerly Gsh1 and 2) are affecters of the Notch signaling pathway in mouse, and Gsx2 maintains neural progenitor pools in the developing telencephalon (Wang et al., 2009; Pei et al., 2011; Roychoudhury et al., 2020). In larval zebrafish, *gsx2* is predominantly expressed in the pallium, preoptic area, hypothalamus, and hindbrain, with an established putative null TALEN deletion mutant line (Coltogirone et al., 2021). As *gsx2* mutants show no gross

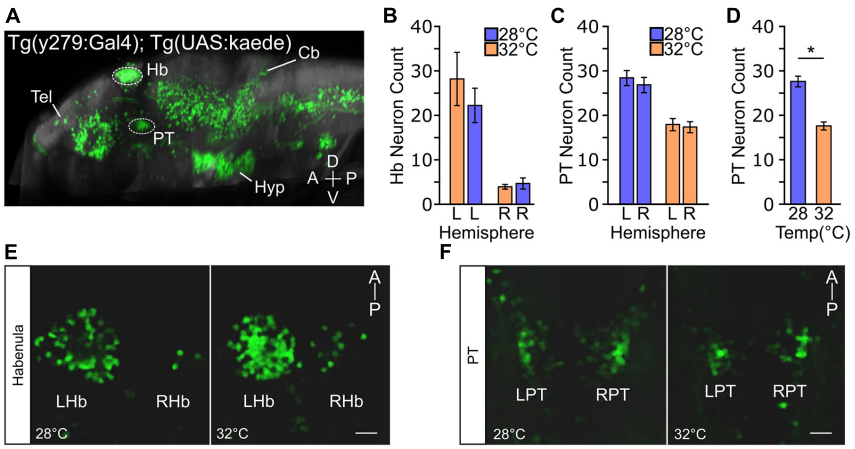

FIGURE 4 | Temperature impacts y279 specified expression in the PT. **(A)** Single sagittal slice of larval zebrafish brain showing expression of enhancer trap *Tg(y279:Gal4)* obtained from the 'Zebrafish Brain Browser' atlas. Circled regions highlight the habenula (Hb) and rostral posterior tuberculum (PT) and white lines show the telencephalon (Tel), hypothalamus (Hyp), and cerebellum (Cb). **(B–F)** Effect of elevated temperature during early development on the expression of y279 in the habenula and PT. **(B)** Expression of y279 in the left and right hemisphere Hb nuclei (28°C purple, $N = 16$; 32°C orange, $N = 14$). **(C)** y279 positive PT neurons (28°C purple, $N = 16$; 32°C orange, $N = 14$). **(D)** Combined left and right hemisphere PT neuron counts (28°C purple, $N = 16$; 32°C orange, $N = 14$). **(E,F)** Representative images showing maximum intensity projections for y279 positive Hb (left habenula, LHb; right habenula, RHb) **(E)** and PT (left PT, LPT; right PT, RPT) **(F)** neurons for larvae raised at 28 or 32°C. Scale bar 20 μm. Asterisk $p < 0.05$.

morphological abnormalities during larval stages, we used these lines to test turn bias. Heterozygous and mutant *gsx2* larvae displayed reduced inter-individual variation and a shift toward less persistent turn bias (**Figure 6A**). The loss of persistent same-direction turning was similarly observed using match index (MI), an analogous metric (**Figure 6B**). Yet, TTA during light-search was not significantly changed across genotypes [1-way ANOVA $F(2,187) = 2.730$, $p = 0.068$], suggesting the loss of same-direction turning is not due to reduced light-driven behavior (**Figure 6C**). Thus, our analysis implies that broad and local haploinsufficient changes in Notch signaling and Gsx2 contribute to inter-individual variation in turn bias behavior, independent of canonical roles in proliferation.

DISCUSSION

Here we reveal that during light-search initiated by the loss of illumination, larval zebrafish exhibit significant inter-individual variation in turn bias, a handed-like behavior. Based on our newly developed assays, we were further able to show mild changes in search behavior correlated with left and right turning types. However, the impact of turning on search motor patterns was specific, as we found no evidence of individual motor changes during baseline illumination, consistent with previous studies (Horstick et al., 2020). We demonstrated a turn bias spectrum across the population which shows the previously described left/right turning types (Horstick et al., 2020). In addition, our analysis revealed a consistently unbiased turning type, supported by multiple independent recording strategies (4×, 8×, and q4×). Furthermore, we show that temperature changes during early development result in sustained changes in inter-individual variation. Finally, we tested how signaling pathways associated with neuronal proliferation affected turn bias development, using either pharmacological inhibition of Notch signaling or a presumable null Gsx2 mutant. Notch and Gsx2 represent canonical broad and regional regulators of proliferation, respectively. Interestingly, turn bias attenuation is observed with partial Notch inhibition and in *gsx2* heterozygotes, suggesting dose-dependent sensitivity. Despite a well-established role for Notch in cell proliferation, the inhibitor concentrations that selectively impairs turn bias did not result in observable changes in proliferation, at least early in development (see **Figure 5**). Our findings confirm that three turning types can be categorically defined, are modulated by specific etiological relevant environmental cues, and are sensitive to internal proliferative associated signaling pathways. One potential caveat is that zebrafish strains are not isogenic and maintain some genetic heterogeneity (Butler et al., 2015), potentially contributing to inter-individual differences. Nevertheless, our work develops larval zebrafish as a powerful model to identify mechanisms generating inter-individual variation in vertebrates.

Determination of Bias

Our findings suggest a 'hemispheric noise' model where turn bias and inter-individual variation is modulated by conflicting brain hemisphere signals in turn bias driving neurons (**Figure 7**). We elucidated that change in bias ratios strength distinguishes unbiased versus biased larvae. Moreover, we establish that this change is not a result of a loss of photo-responsiveness in unbiased individuals (total turning, see **Supplementary Figure 1**); rather a failure to navigate in a single direction during light-search consistently. This observation supports the conclusion that unbiased individuals are not a subset with impaired photo-responsiveness, but a distinct behavioral motor profile during search behavior. Supporting this model,

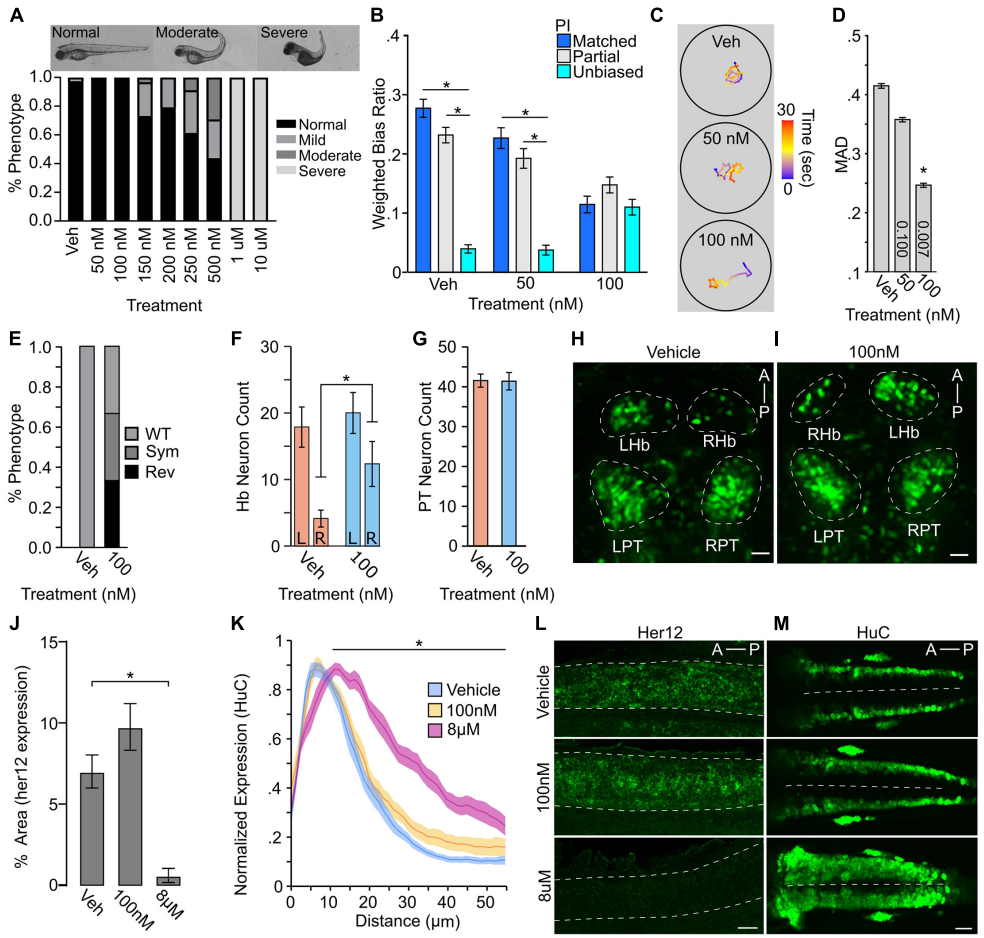

FIGURE 5 | Turn bias is sensitive to levels of Notch signaling. **(A)** Phenotypic counts following Notch inhibitor treatment (Vehicle, *N* = 125; 50 nM, *N* = 88; 100 nM, *N* = 114; 150 nM, *N* = 123; 200 nM, *N* = 120; 250 nM, *N* = 101; 500 nM, *N* = 48; 1 μM, *N* = 43; 10 μM, *N* = 100). **(B)** Weighted absolute bias ratio averages (matched PI = 0,1 blue bar; Partial match PI = 0.25, 0.75 gray bar; unbiased PI = 0.50 cyan bar. **(C)** Illustrative traces for treatment groups. Scale bar color represents time in seconds. **(D)** Effect of Notch inhibition on MAD. *p*-values shown in bar, 1-way comparison of treatment groups to control. **(E)** Habenula symmetry for vehicle controls (*N* = 8) and 100 nM Notch inhibitor treated (*N* = 9) larvae showing proportion with WT, symmetric (Sym), or reversed (Rev) phenotypes. WT larvae have the larger habenula in the left hemisphere. y279 neuron counts in the habenula (**F:** vehicle *N* = 8; 100 nM *N* = 9) and PT (**G:** vehicle *N* = 8; 100 nM *N* = 9). For neuron counts, regardless of hemisphere the larger habenula was classified as the 'left' habenula. **(H,I)** Representative maximum projection images showing y279 labeled neurons in vehicle and control. Scale bar 20 μm. **(J)** Area of her12 expression in the spinal cord following LY411575 treatment (Vehicle, *N* = 12; 100 nM, *N* = 13; 8 μM, *N* = 11). **(K)** Normalized distribution of HuC/D positive neurons following notch inhibition (Vehicle: Blue, *N* = 18; 100 nM: Yellow, *N* = 13; 8 μM: Magenta, *N* = 13). *X*-axis distance spans half the spinal cord (0 micron = lateral spinal cord; 55 micron = spinal cord midline). Comparison shown is between vehicle and 8 μM along the whole length of black bar between matched positions. Ribbons ± SEM. **(L)** Representative images of her12 expression in 27 hpf embryos. Lateral view of spinal cord (dotted outline). Scale bar 20 μm. **(M)** Representative HuC labeling in 24 hpf embryos showing dorsal view. Dotted line denotes spinal cord midline. Scale Bar 40 μm. Asterisk $p < 0.05$.

when we quantify the strength of individual trials, the bias ratios exhibit a step-wise decrease, i.e., PI 1 < 0.75 < 0.5, suggesting accumulating inter-hemispheric noise that degrades overall individual bias persistence. Corroborating this model, previous studies showing that unilateral ablation of rostral PT neurons, which are required for turn bias in larval zebrafish, increases turning strength in the direction ipsilateral to the intact neurons, indicating ablation removes conflicting input from the contralateral hemisphere (Horstick et al., 2020). In pigeons, a classic model for hemispheric specialization and individual variation (Güntürkün et al., 1998; Freund et al., 2016), increased conflict between hemispheres exacerbates visual task latency (Manns and Römling, 2012). Therefore, variable balance in hemispheric signaling may be a conserved mechanism in generating inter-individual variation (Chen-Bee and Frostig, 1996; Linneweber et al., 2020). Inter-hemispheric communication is vital for the function of the visual system (Bui Quoc et al., 2012; Chaumillon et al., 2018), including photo-driven behavior in larval zebrafish (Gebhardt et al., 2019). The counter hypothesis is a 'switching model' where unbiased larvae would display vigorous directional turning, yet in randomly selected directions over sequential trials. This model is consistent with a 'winner take all' circuit function (Fernandes et al., 2021). Indeed, within the primary visual processing center in zebrafish,

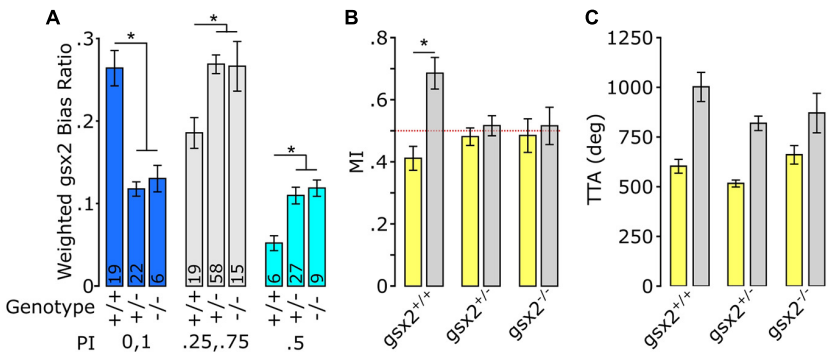

FIGURE 6 | Loss of *gsx2* reduces inter-individual variation. **(A)** Effect of gsx2 genotype on weighted average bias ratio per performance groups (Matched: PI = 0,1; Partial PI = 0.25,0.75; and Unbiased PI = 0.5). Numbers on bars indicate *N*. Weighing was based on the percent of larvae within each PI per genotype. **(B)** MI shows that only gsx2+/+larvae maintain persistent same-direction turning following the loss of illumination. Dotted line at 0.5 indicates random movement. **(C)** TTA between genotypes is not affected (gsx2+/+, $N = 45$; gsx2±, $N = 113$; gsx2–/–, $N = 32$). Baseline (yellow) and dark (gray) responses in **(B,C)**. Asterisk $p < 0.05$.

the optic tectum, neurons operate in a winner take all style during visually guided behavior (Fernandes et al., 2021). However, turn bias is driven by the loss of visual cues that activate rostral PT neurons, which do not project to the tectum (Horstick et al., 2020), implicating that even though turn bias is visually evoked, the mechanism is likely independent of a tectal winner take all mechanism. Despite the neurons maintaining zebrafish turn bias being identified, the underlying mechanism imposing a specific turning type remains unknown (Horstick et al., 2020). Our analysis suggests a model of competitive inter-hemispheric communication modulating the magnitude of inter-individual turn bias variation that is further adjusted by fluctuating and specific variables in the internal and external environment.

Regulation of Individuality

The mechanisms driving unique individual behavioral responses based on sex or sensory context are well described (Asahina et al., 2014; Lewis et al., 2015; Yapici et al., 2016; Marquart et al., 2019; Ishii et al., 2020; Nelson et al., 2020). However, why individuals in a population will show variable response types to a consistent stimulus is poorly understood, especially in vertebrates. One of our goals was to determine what internal and external elements may modulate turn bias variation as a basis to explain how different response types potentially arise. To test external environmental factors, we selected environmental enrichment, social experience, temperature, and salinity. One hypothesis for how factors like enrichment or social experience influence inter-individual variation is through micro-environmental interactions that create unique individual experiences (Kain et al., 2015). However, our data indicate that these interactions do not influence turn bias variation in zebrafish. One possible explanation is that during early development, 1–3 days post-fertilization, larvae are primarily inactive and only begin actively exploring around 4 days post fertilization (Colwill and Creton, 2011; Lambert et al., 2012). Conversely, responsiveness to conspecifics is not observed until 3 weeks (Dreosti et al., 2015; Larsch and Baier, 2018). As bias is established by 4 dpf, the underlying mechanisms may no longer be malleable to environmental experiences beyond this developmental interval.

We also tested temperature and salinity, emphasizing etiological ranges that zebrafish could experience in their native environments (Engeszer et al., 2007; Sundin et al., 2019). Salinity and temperature are critical environmental determinants and have been shown to drive evolutionary changes in stickleback populations (Gibbons et al., 2017). However, we found that only raising larvae at varying temperatures resulted in modifications to inter-individual variation. We show that temperature-dependent

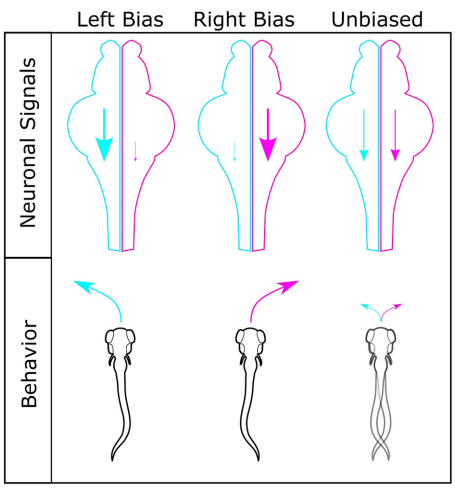

FIGURE 7 | Model for generating different turning types. Interhemispheric differences in turn bias driving motor signals are a potential mechanism for establishing turning types. Left (cyan) and right (magenta) hemispheres shown for left, right, and unbiased motor types, with corresponding motor drive shown by scale of descending arrow. For individuals with robust left or right turning types, a strong ipsilateral turn bias signal persists in a single hemisphere, with limited conflicting input from the contralateral hemisphere. In individuals with similar turn bias drive from both hemispheres, no single turn direction persists due to conflict between hemispheres, resulting in lower bias ratios and unbiased turning types.

effects are not a generic thermal stress response. Etiological increases in temperature have been shown to attenuate turn bias in adult reef fish, implicating a potentially broader thermal sensitivity in bias establishing mechanisms (Domenici et al., 2014). Our analysis establishes that early developmental exposure to etiological temperature fluctuation results in sustained and specific turn bias changes.

Intriguingly, the specification of habenular hemispheric asymmetry is sensitive to the rate of development (Aizawa et al., 2007), and developmental rate is temperature sensitive (Kimmel et al., 1995). This observation could provide a potential mechanism for thermal driven changes in turn bias. However, our testing conditions produced no gross change in the habenula nuclei morphology. This observation, however, does not exclude functional or subcellular changes. Surprisingly, we observe a bilateral reduction in *Tg(y279)* positive PT neurons, which are essential for maintaining turn bias, in the elevated temperature experiments. A primary function of the PT is to integrate diverse sensory inputs (Striedter, 1991; Derjean et al., 2010; Yaeger et al., 2014). However, thermosensitivity of the PT neurons has not been previously described, and we believe this is a novel observation. Future studies identifying the genetic basis of the *Tg(y279)* enhancer trap, which is currently unknown, will be instrumental in elucidating how temperature impacts PT neuron specification and inter-individual variation. The specific abrogation of leftward turning types in increased temperature conditions provides a powerful model to interrogate underlying neural changes in a vertebrate brain associated with individual behavioral patterns.

Last, we wanted to identify molecular signaling pathways regulating turn bias. Biased turning in larvae is largely lost in heterozygotes of mutant lines associated with Notch signaling, yet the impact of direct Notch inhibition was unexplored (Horstick et al., 2020). In *Drosophila* and *C. elegans*, Notch signaling is essential for establishing individual visual navigational strategies and asymmetric chemosensory neuron identities, respectively (Bertrand et al., 2011; Linneweber et al., 2020). Thus, work from several species implicates Notch as a driver of variation at behavioral and neuronal levels. Indeed, we show that partial Notch disruption, using a specific pharmacological inhibitor, disrupts biased turning in larval zebrafish, yet not the ability to respond to illumination changes, establishing a direct role of Notch signaling for turn bias, which is independent of gross morphological development. Despite the established role of Notch in neural proliferation, we found no significant change in proliferative neurons, *her12* expression, or number of PT neurons at the dosages used for behavioral studies. However, we did observe disruption to the typical left/right hemispheric asymmetry of the habenula, observing an increase in reversed or symmetric habenula. Interestingly, similar disruption to habenula symmetry is observed in *mib* zebrafish which have severely reduced Notch signaling (Aizawa et al., 2007). Our results, show a novel Notch dose-sensitivity for habenula asymmetry development, which may be a potential neural basis for the absence of biased behavior following low levels of Notch inhibition. Since Notch signaling is essential for diverse cellular functions, and the precise downstream signaling mechanisms are highly sensitive to the strength of Notch signaling (De Smedt et al., 2005; Shen et al., 2021), the low inhibitor concentrations used may be sub-threshold for disturbing the spatial-temporal patterns of *her12* and HuC/D tested here. In addition, the downstream effects of Notch are dependent on the cellular micro-environments, determined by the local co-expression of Notch receptors, ligands, and auxiliary proteins (Demehri et al., 2009; Bertrand et al., 2011; LaFoya et al., 2016). Therefore, the levels of Notch reduction that impair turn bias, but not morphology, may not be sufficient to alter Notch associated mechanisms impacting proliferation. Nevertheless, subtle changes in Notch could lead to changes in cellular micro-environments, thereby altering downstream signaling cascades, and ultimately impacting turn bias maintaining neurons. Notch haploinsufficiency is known to generate a myriad of defects and disease states, including vasculature defects, seizure, autism, and brain malformations, demonstrating that reduced Notch signaling can disrupt biological functions (Krebs et al., 2004; Connor et al., 2016; Fischer-Zirnsak et al., 2019; Blackwood et al., 2020). However, the pharmacological inhibition used in our current study is not regionally specific. Therefore, we also tested an established zebrafish *gsx2* mutant line, and *gsx2* is predominately expressed in subsets of hypothalamic, preoptic area, pallium, and hindbrain neurons (Coltogirone et al., 2021). The reduction in turn bias in *gsx2* heterozygotes and mutants suggests that turn bias variation is sensitive to local changes in brain regions where *gsx2* is expressed, independent of the previously described rostral PT and habenula (Horstick et al., 2020). As the levels of Notch that reduce turn bias do not impact proliferation, it seems possible that Notch and Gsx2 modulate turn bias by independent mechanisms. Our current analysis identifies two conserved molecular signaling and transcriptional control mechanisms, Notch and Gsx2, and novel neuroanatomical substrates as important for generating variation in turn bias.

Function of Turn Bias and Inter-Individual Variation

Behavioral variation is observed in diverse species and behavioral modalities (Byrne et al., 2004; Elnitsky and Claussen, 2006; Cauchard et al., 2013; Horváth et al., 2020). In zebrafish, even complex neuromodulatory processes such as startle habituation display inter-individual variation with distinguishable 'habituation types' (Pantoja et al., 2016, 2020). Yet, the general question remains, "why do specific behavioral modalities manifest inter-individual differences?" Considering a simple form of inter-individual variation, such as turn bias, may offer insights to these questions. Zebrafish are active hunters during larval stages and predatory success depends on visual input, thus establishing a potent drive to remain in illuminated areas (Gahtan et al., 2005; Filosa et al., 2016; Muto et al., 2017). Following the loss of light and of overt navigation cues, larvae initiate a local light-search, where individual turn bias is triggered, causing looping trajectories (Horstick et al., 2017). Looping search trajectories are observed in various species in the absence of clear navigational cues,

suggesting an efficient systematic strategy (Collins et al., 1994; Conradt et al., 2000; Zadicario et al., 2005). However, even seemingly optimal behaviors may not be advantageous in all contexts (Simons, 2011). Variation in turning types may ensure individuals across the population possess strategies to mitigate erratic environmental challenges, a form of bet-hedging (Simons, 2011; Kain et al., 2015). Similarly, behavioral variation adds unpredictability to a population. Predictable behavioral patterns can be exploited by predators (Catania, 2009, 2010). For aquatic species, this may be advantageous as some heron species, a predator of small fish, use a canopy hunting strategy, covering the water surface with their wings and blocking light (Kushlan, 1976). Prey populations with unpredictable responses would potentially provide a more challenging target (Humphries and Driver, 1970). Even though larval fish may not be the intended target of heron canopy hunting, larval behavioral patterns may persist over their lifespan. Indeed, adult zebrafish display a persistent turn direction preference (Fontana et al., 2019), although the correlation to larval turn bias is currently unexplored. Ultimately, the etiological purpose for turn bias variation is most likely a combination of multiple explanations, including bet-hedging, generating unpredictability, and genetically encoded sources of variation.

DATA AVAILABILITY STATEMENT

Inquiries can be directed to the corresponding author for R scripts and datasets generated in this study.

ETHICS STATEMENT

The animal study was reviewed and approved by West Virginia University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

EH conceived the experiments. EH and JH wrote the manuscript and analyzed data. SL wrote custom R scripts for data analysis. SB provided *gsx2* lines and associated analysis. JH, MW, JS, HC, HP, LB, and CS performed experiments. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.777778/full#supplementary-material

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Variation and Variability in *Drosophila* Grooming Behavior

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Behavioral differences can be observed between species or populations (variation) or between individuals in a genetically similar population (variability). Here, we investigate genetic differences as a possible source of variation and variability in *Drosophila* grooming. Grooming confers survival and social benefits. Grooming features of five *Drosophila* species exposed to a dust irritant were analyzed. Aspects of grooming behavior, such as anterior to posterior progression, were conserved between and within species. However, significant differences in activity levels, proportion of time spent in different cleaning movements, and grooming syntax were identified between species. All species tested showed individual variability in the order and duration of action sequences. Genetic diversity was not found to correlate with grooming variability within a species: *melanogaster* flies bred to increase or decrease genetic heterogeneity exhibited similar variability in grooming syntax. Individual flies observed on consecutive days also showed grooming sequence variability. Standardization of sensory input using optogenetics reduced but did not eliminate this variability. In aggregate, these data suggest that sequence variability may be a conserved feature of grooming behavior itself. These results also demonstrate that large genetic differences result in distinguishable grooming phenotypes (variation), but that genetic heterogeneity within a population does not necessarily correspond to an increase in the range of grooming behavior (variability).

Keywords: *Drosophila*, variability, variation, neural circuits, motor sequence, behavior

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INTRODUCTION

Differences in phenotype arise from differences in genotype. Changes in DNA account for variation in traits among species, and differences between individuals of the same species. Animal behavior contains phenotypes partially under genetic control, and specific genes associated with observable differences in behavior between and within species have been uncovered (Baker et al., 2001; Johanssen, 2014). Different mouse species exhibit variation in monogamy and parental care, and different fly species show variation in courtship song, food preference, and larval digging (Bendesky et al., 2017; Kim et al., 2017; Ding et al., 2019; Markow, 2019; Auer et al., 2020). From endangered species to agricultural crops to virus variants, genetic diversity affects organismal success. Within a species, natural variations in DNA sequences produce individual mice that differ in aggression and or flies that implement different foraging strategies (Anderson, 2016; Allen et al., 2017) and advantageous variants can be selected. Mutant screens have also uncovered gene variants associated with

differences in locomotion, courtship routines, and sleep patterns, among other complex behaviors (Baker et al., 2001; Sokolowski, 2001; Ayroles et al., 2015; Gaertner et al., 2015).

Some behaviors can be performed in different ways even by genetically identical organisms or by the same individual in repeated trials. Behavioral variability can be advantageous as a bet-hedging strategy against unstable environmental conditions (Kain et al., 2015). Phenotypic variability in behaviors ranging from birdsong to escape trajectories can increase individual success, but also fitness in a population, suggesting that variability itself can be a selectable trait. Experiments in *Drosophila melanogaster* demonstrate that the degree of behavioral variability in locomotion is partially controlled by genetic expression of proteins such as teneurin-α, a cell adhesion molecule (Honegger and de Bivort, 2018). Additionally, silencing a subset of neurons in the central complex modifies the degree of variability of locomotor behavior (Honegger and de Bivort, 2018). Differences in neurodevelopment and synaptic connectivity can also result in behavioral variability (Linneweber et al., 2020). Together, these observations suggest that factors at both at the population (genetic) and individual (neuronal) levels contribute to behavioral variability.

Drosophila grooming shows behavioral variability. Fruit flies live in dirty environments, from laboratory vials to rotting fruit, and they perform grooming actions to remove accumulated particulates. Grooming has been observed in several *drosophilid* species and is important for survival (Szebenyi, 1969; Spruijt et al., 1992; Zhukovskaya et al., 2013). Past work demonstrated that the leg movements used in grooming are stereotyped, but the sequences of actions are flexible as opposed to fixed. While the rules underlying grooming do exhibit observable structure in flies (Seeds et al., 2014; Mueller et al., 2019) and in mice (Fentress and Stilwell, 1973; Geuther et al., 2021), different sensory experiences and life histories may influence grooming behavior. These results lead us to ask: how much of variability in fruit fly grooming is under genetic control?

To address this question, we evaluated different features of dust-induced grooming behavior by comparing their values between groups and their range within groups. The raw behavioral data and features we examine here are schematized in **Figure 1**. In particular, we focus on the transition probabilities between actions that compose the grooming sequence, which we refer to as "syntax"; in linguistics, this term refers to the rules that indicate how words and phrases may be combined to form sentences, so it is borrowed here to indicate that action transitions also conform to rules.

First, we evaluated differences in phenotype between genetically-distinct populations, such as *drosophilid* species. Differences in grooming behavior when members of different species are compared can reasonably be attributed to differences in their DNA sequences.

In a wild and genetically diverse population of flies, there may be mutations that change grooming behavior, but lab strains of *Drosophila melanogaster* are largely clonal—all individuals should have the same genotype. We next compared the grooming behavior of different lab strains, and then of individuals within a given lab strain. We hypothesized that genetic heterogeneity might contribute to the magnitude of grooming variability. By interbreeding or isogenizing *melanogaster* lab strains, we generated stocks with high and low genetic diversity, but we find that all groups exhibited similar variability in measured grooming features. Intra-genotypic or phenotypic variability has also been observed in fly locomotor behavior, and some populations exhibit a wide range while others exhibit a narrower one (Ayroles et al., 2015).

Genetic differences among *drosophilid* species and strains may underlie variation in the syntax of their cleaning movements, but all flies show variability in the exact sequence of those movements. Furthermore, even the same fly tested on sequential days revealed sequence variability. The extent of within-fly differences in syntax were similar to between-fly differences: flies were no more similar to themselves over time than they were to other flies on a given day. Finally, flies stimulated using optogenetic manipulation to induce grooming exhibited increased stereotypy, but within-individual grooming variability between stimulation sessions was not fully abolished. These data show that genetic heterogeneity plays a limited role in the variability of grooming behavior, and that differences in sensory experience contribute but do not account for all observed variability. The widespread nature of grooming variability suggests that it may be an important feature, but our experiments indicate the need to search for alternative causes, perhaps including developmental stochasticity, differences in internal state, or noisy neural circuit dynamics.

RESULTS

In this work, N = 390 male flies were covered in dust and their grooming behavior was recorded for approximately 30 min each (**Figure 1A**). We analyzed flies from five *drosophilid* species (*melanogaster*, *santomea*, *sechellia*, *simulans*, and *erecta*), which are genetically distinct—separated by millions of years of evolution—and inhabit different ecological niches. We also examined four common *melanogaster* lab stocks (Canton-S, Oregon-R, Berlin-K, and w1118), and several isogenic lines derived from these parent stocks in our laboratory.

To analyze this large data set, we employed tools from computational ethology (Datta et al., 2019). An automated behavioral recognition system [ABRS, Ravbar et al. (2019)] was used to classify fly behavior into one of five grooming actions (front leg cleaning, head grooming, abdomen grooming, back leg cleaning, wing grooming) and two non-grooming actions (walking and standing). As a note, head grooming consists of actions that use the front legs to clean the antennae, eyes, and face, but sub-movements such as these were not easily detectable using the recording methodology employed here, so analysis was restricted to coarser spatiotemporal scales. After generating ethograms (behavioral time series records) for each fly, several grooming features were extracted (**Figure 1B**). We measure the average amount of time flies spend performing each of the grooming actions (plus standing and walking), the syntax of transition probabilities among these actions, the anterior-to-posterior progression, and the durations of bouts of grooming

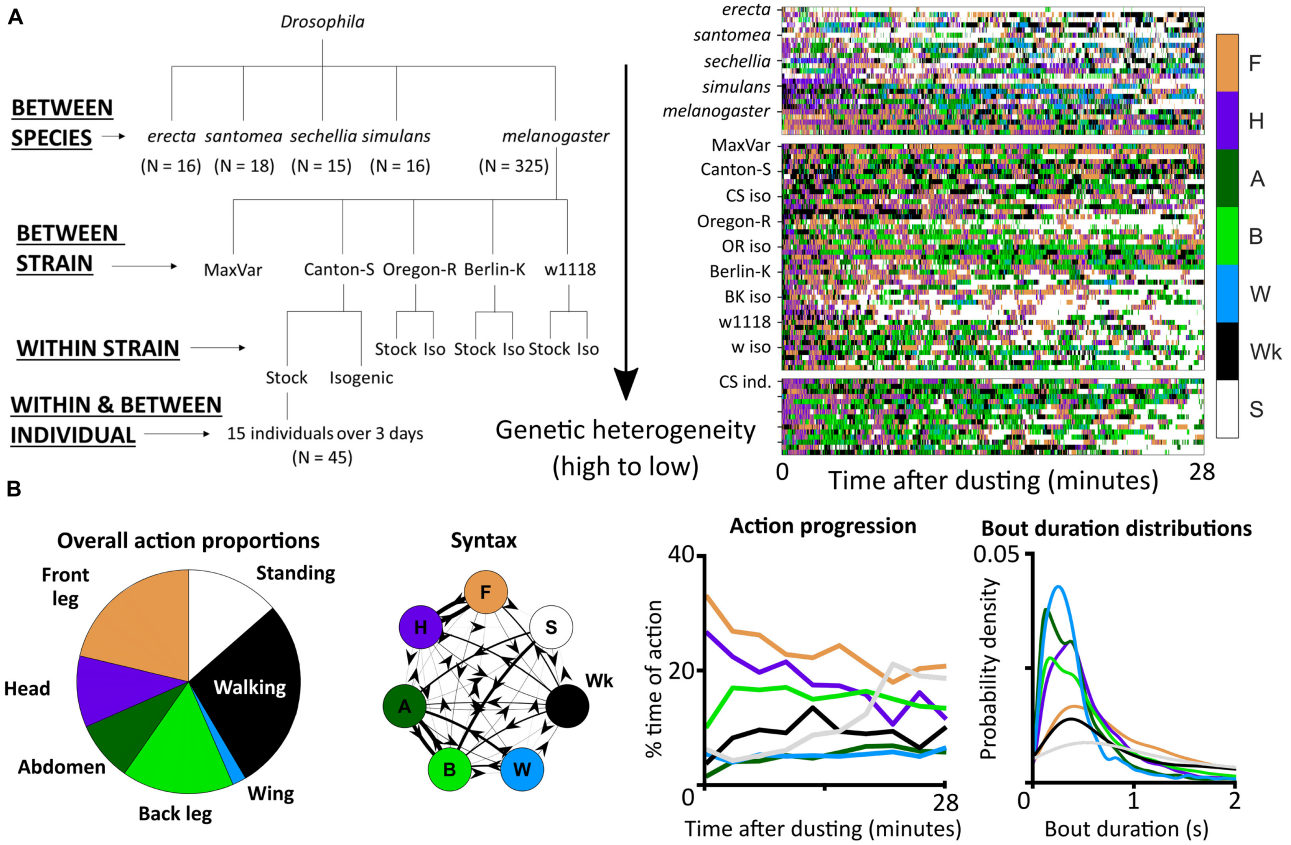

FIGURE 1 | Grooming variability dataset and analysis overview. **(A)** In total, *N* = 390 male flies were dusted and their activity was recorded for approximately half an hour each. Five *drosophilid* species, four *melanogaster* stock lines, one interbred *melanogaster* line, and six isogenic *melanogaster* lines were analyzed for similarity, differences, and stereotypy in grooming and non-grooming behaviors. On the left is a schematic of the different *drosophilid* groups included in this analysis. Higher levels of the tree indicate higher levels of genetic diversity (scale is relative, not absolute). On the right is a sample of ethograms generated by automated annotation of video. Color indicates the occurrence of the five grooming actions (F, front leg cleaning; H, head grooming; A, abdomen grooming; B, back leg cleaning; W, wing grooming) and two non-grooming actions (Wk, walking; S, standing). **(B)** Features scored from ethograms provide summary representations of behavior. Shown here are sample visualizations of behavioral metrics analyzed in this work. On the left, the proportion of time spent in different actions provides the coarsest description of the behavioral response to a dust stimulus. Regardless of genotype, all flies exhibit variable (not fixed) action sequences consisting of the same set of five grooming actions, walking, and standing after exposure to irritant. Next, action transition probabilities (syntax) describe the likelihood of performing consecutive actions. Arrow directions and thicknesses represent the probability of performing an action, given the identity of the previous action. Shown next is an example behavioral progression, which depicts the proportion of time spent in each action over a sliding window. Most flies follow a typical behavioral progression pattern: initial anterior grooming followed by increased posterior grooming. The amount and timing of walking and standing, however, can vary significantly between flies. Finally, action (bout) duration distributions describe the range of action lengths. All example features shown here are scored from Canton-S flies.

actions. We used classification analysis and various measures of stereotypy to quantify the variation (inter-species or inter-strain differences) and variability (intra-strain or intra-individual differences) of these characteristics.

Drosophilids Exhibit a Robust Grooming Response but Different Syntax After Irritant Exposure

Across the *Drosophila* species tested here, five grooming actions were observed consistently, indicating a conserved behavioral response (**Supplementary Figure 1**). Previous work showed that these actions are sufficiently stereotyped to be reliably classified by manual and automated annotation in *melanogaster* (ABRS, see section "Materials and Methods") (Mueller et al., 2019; Ravbar et al., 2019). Here, the ABRS classifier was validated on training data for each species, showing comparable accuracy (see section "Materials and Methods" and **Supplementary Figure 22**), indicating that the movement primitives that make up grooming are stereotyped. No novel species-specific grooming actions were detected. Although some fine-scale movement differences may occur among species, they are beyond the spatial and temporal resolution of the current video and unlikely to affect the analysis of transition probabilities presented here. Analysis of mouse behavior indicates that grooming subroutines are largely stereotyped at high temporal resolution, increasing our confidence in this approach (Wiltschko et al., 2015).

To quantify the behavioral response to dusting, the proportion of time spent grooming (as opposed to walking and standing) was calculated for each fly (**Figure 2A**). The proportions

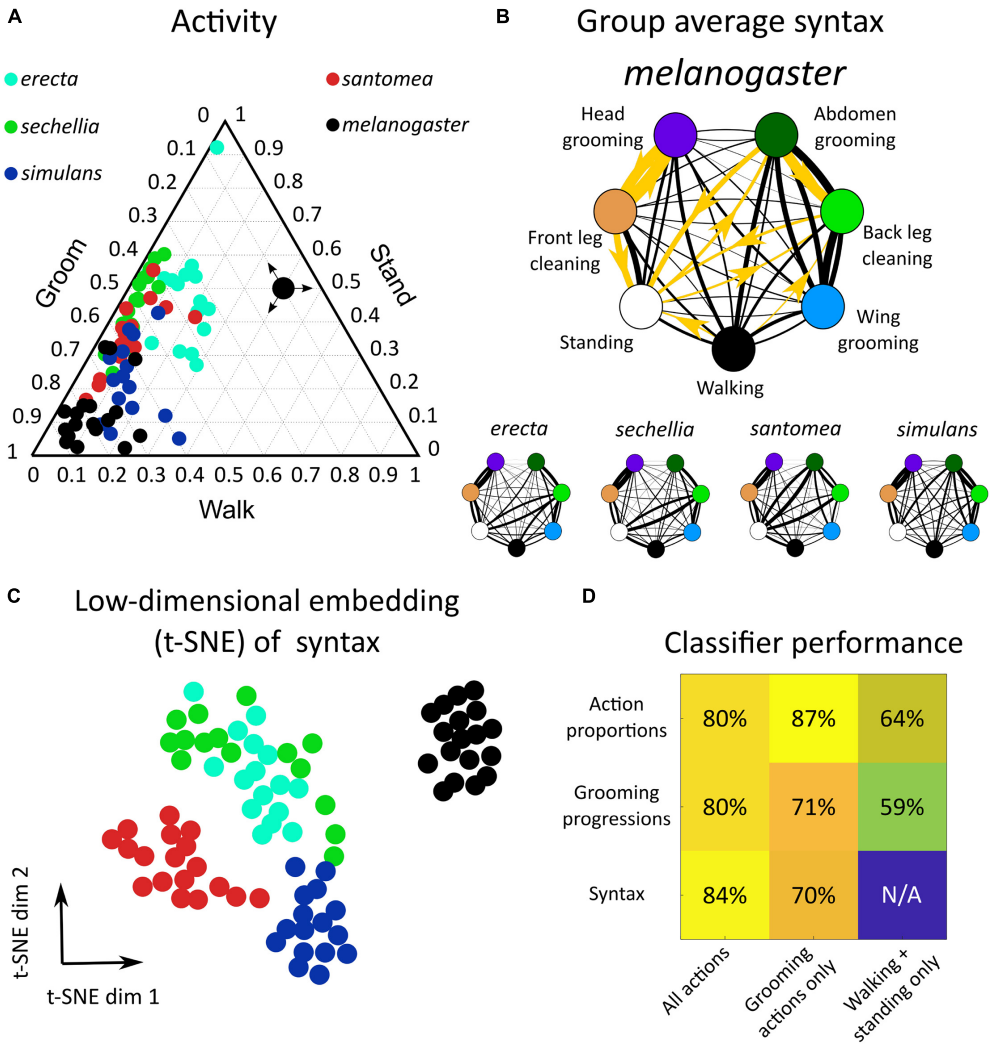

FIGURE 2 | *Drosophila* species share behavioral features but exhibit between-species variation in action proportions and syntax in response to dust stimulus. **(A)** Dusting elicits a conserved behavioral response across *drosophilids*. Shown is a ternary plot of activity proportions for each species examined here (N = 65 flies total). Colored points represent a single fly, with color indicating species. The large black point with arrows indicates how to read activity proportions; the example point corresponds to 10% grooming, 40% walking, and 50% standing. **(B)** *Drosophilid* species produce a probabilistic behavioral sequence (as shown in **Figure 1A**), which can be characterized by the transition probabilities (syntax) between actions [as represented in **Figure 1B**, calculated as in Mueller et al. (2019)]. The mean syntax for each species is depicted as a graph, with nodes representing actions and edges indicating transition probability. Thicker edges indicate higher probabilities. On the *melanogaster* syntax graph, the 10 action transitions exhibiting the largest magnitude differences between *melanogaster* and non-*melanogaster* species are highlighted in gold. These differences are identifiable in anterior motif transitions, which use the front legs to perform grooming actions. Species also differ in their transitions between posterior grooming actions and non-grooming actions (walking and standing) **(C)** Each fly's 42-dimensional syntax vector was plotted in two dimensions after dimensionality reduction using t-SNE. t-SNE preserves local distance structure, indicating that tightly grouped clusters of points are similar to one another. In this case, dimensionality reduction reveals that *drosophilid* species exhibit significant differences in syntax, as syntax vectors congregate by color. **(D)** Classification analysis confirms the qualitative clustering observed in C. Shown is a heat map of accuracy rates of 5-possibility multinomial logistic regression classifiers trained on behavioral features. For these samples, classification at chance would be 20%. Consistent classification accuracy values >20% indicate that species are highly separable by behavioral features. Simple features, such as behavioral proportions and progressions, classify individuals by species with high accuracy when grooming actions are included. Classification using only non-grooming actions (walking and standing) still yields classification above chance, indicating that species differ significantly in their overall activity levels. Syntax also allows for accurate classification, particularly when all action transitions are considered.

of total grooming time between species were all statistically different (Wilcoxon rank-sum test, $p < 0.05$, multiple comparison correction *via* Holm's method; **Supplementary Figure 23**), but all species spent at least 35% of the time grooming, on average. In this analysis, a single stock line (Canton-S, N = 18) was used as the representative *melanogaster* group. Full action proportion distributions are shown in **Supplementary Figure 2**.

Behavior was then examined in more detail by considering all seven actions (five grooming movements plus walking and standing) and the progression of those actions over

time, as in **Figure 1B**. These species exhibited a qualitatively similar behavioral progression, characterized by initial anterior grooming followed by increased posterior grooming, walking, and standing, but the relative proportions and timing of these behaviors over time differed (**Supplementary Figure 3**).

Syntax (the transition probabilities between discrete behaviors) was calculated from the ethogram of each dusted fly ($N = 390$). With seven behavioral states, 42 transitions were possible, excluding self-transitions. Thus, syntax was represented as a 42-dimensional vector for subsequent classification analysis and visualization. Syntax across all flies exhibited high transition probabilities within the anterior grooming motif (front leg cleaning, head grooming) and posterior grooming motif (abdomen grooming, back leg cleaning, wing grooming). The average syntax for each species is illustrated as a weighted, directed graph in **Figure 2B**.

Finally, continuous grooming action duration distributions (e.g., the distribution of how long each head grooming action was) were calculated from ethograms. Distributions of action durations were qualitatively similar across species and had probability peaks between 500 and 750 ms (**Supplementary Figure 4**). When considering the same action, no action duration distributions differed significantly in any pairwise comparison (e.g., comparing head grooming between *erecta* and *santomea*) between any species (two-way Kolmogorov-Smirnov test, no p values < 0.05 after correcting for multiple comparisons using Holm's method).

Several significant differences in behavioral features between *melanogaster* and non-*melanogaster* species were identified. **Supplementary Figure 5** illustrates differences in overall action proportions, around 36% of which differed between species. To compare syntax, transition probability distributions for each action transition (e.g., head cleaning to front leg rubbing) were compared between species in a pairwise manner. 38 of 42 unique syntax elements (90.5%) were significantly different between at least two species (Wilcoxon rank-sum test, $p < 0.05$, multiple comparison correction *via* Holm's method). Overall, 125 of 420 (30%) of pairwise syntax comparisons revealed differences between species (see **Supplementary Figure 24** for all p values).

Of these syntactic differences, 71 (60%) occurred between *melanogaster* and non-*melanogaster* species. In particular, posterior motif grooming transitions (transitions between abdomen grooming, back leg rubbing, and wing grooming) were consistently significantly different, on average, as were transitions between back leg rubbing, standing, and walking. **Figure 2B** illustrates these syntactic differences.

Figure 2C depicts a low-dimensional embedding of species syntax using t-SNE. This visualization suggests that different species possess distinguishable syntax, as points are aggregated by species. Low-dimensional visualizations of all behavioral features are illustrated in **Supplementary Figures 5**, **6**.

Classification analysis was applied to behavioral features to verify this interpretation and quantify the degree of variation between species. Multinomial logistic regression classified flies by species according to behavioral proportions, progressions, and syntax with >80% accuracy (**Figure 2D**). Notably, classification was also possible with accuracy significantly above chance when only considering the proportions and progressions of non-grooming actions, walking and standing, indicating that species also vary in their overall activity levels.

Finally, entropy rates were calculated from syntax transition probabilities to quantify the degree of stereotypy in behavior. An entropy rate of zero would indicate complete stereotypy and perfectly predictable, repeated action sequences, while in this calculation, an entropy rate of one indicates an approximate 37% probability of correctly predicting the next action in a sequence (see section "Materials and Methods"). **Supplementary Figure 7** shows that all species possess average entropy rates between zero and one, demonstrating that grooming sequences are neither fixed nor truly random. *melanogaster* flies possessed the lowest entropy (highest degree of stereotypy) due to high transition probabilities between head cleaning and front leg rubbing (**Figure 2B**). In summary, *drosophilid* species exhibit variation in grooming behavior—visible in proportion of time spent on different actions and the transition probabilities among them—but they all share common cleaning movements and variability in their exact action sequences.

Drosophila melanogaster Strains Exhibit Variation in Grooming Behavior

Next, standard *Drosophila melanogaster* lab strains (Canton-S, Berlin-K, Oregon-R, w1118) were analyzed for differences in grooming features (full ethograms shown in **Supplementary Figure 8**). Behavioral proportions, progressions, and syntax differed between stocks, allowing for classification moderately above chance levels. Comparisons of grooming features can be found in **Supplementary Figures 9–12**.

Overall the proportion of time grooming could account for most of the differences observed between stocks. **Figure 3A** shows a ternary plot of activity, showing that Canton-S flies spend more time walking than other stocks. A t-SNE embedding of the syntax of *melanogaster* stocks is depicted in **Figure 3A**. Similar to the species analysis, all action transition probability distributions were compared in a pairwise manner to look for variation in syntax.

Only 19 of 42 unique syntax element comparisons (45%) differed significantly between any two stocks and, of these, only two within-motif transition (both posterior) differed significantly. Within-motif syntax elements are of particular interest because they represent the most common, most highly stereotyped action transitions observed across flies of all genotypes (see **Figure 2B** for visualizations of these transitions). The syntax element exhibiting the greatest statistically significant difference was the wing grooming to walking transition, shown in **Figure 3B**. The other significantly different transitions also mostly involved transitions to and from walking and standing, perhaps reflecting differences in overall activity levels (**Supplementary Figure 12**).

Classification accuracy was moderate but above chance for all features examined; as expected, variation within *melanogaster* was less pronounced than variation between species (compare **Figures 2D**, **3A**). Variation within *melanogaster* stocks appears to be due to differences in overall activity levels, as classification

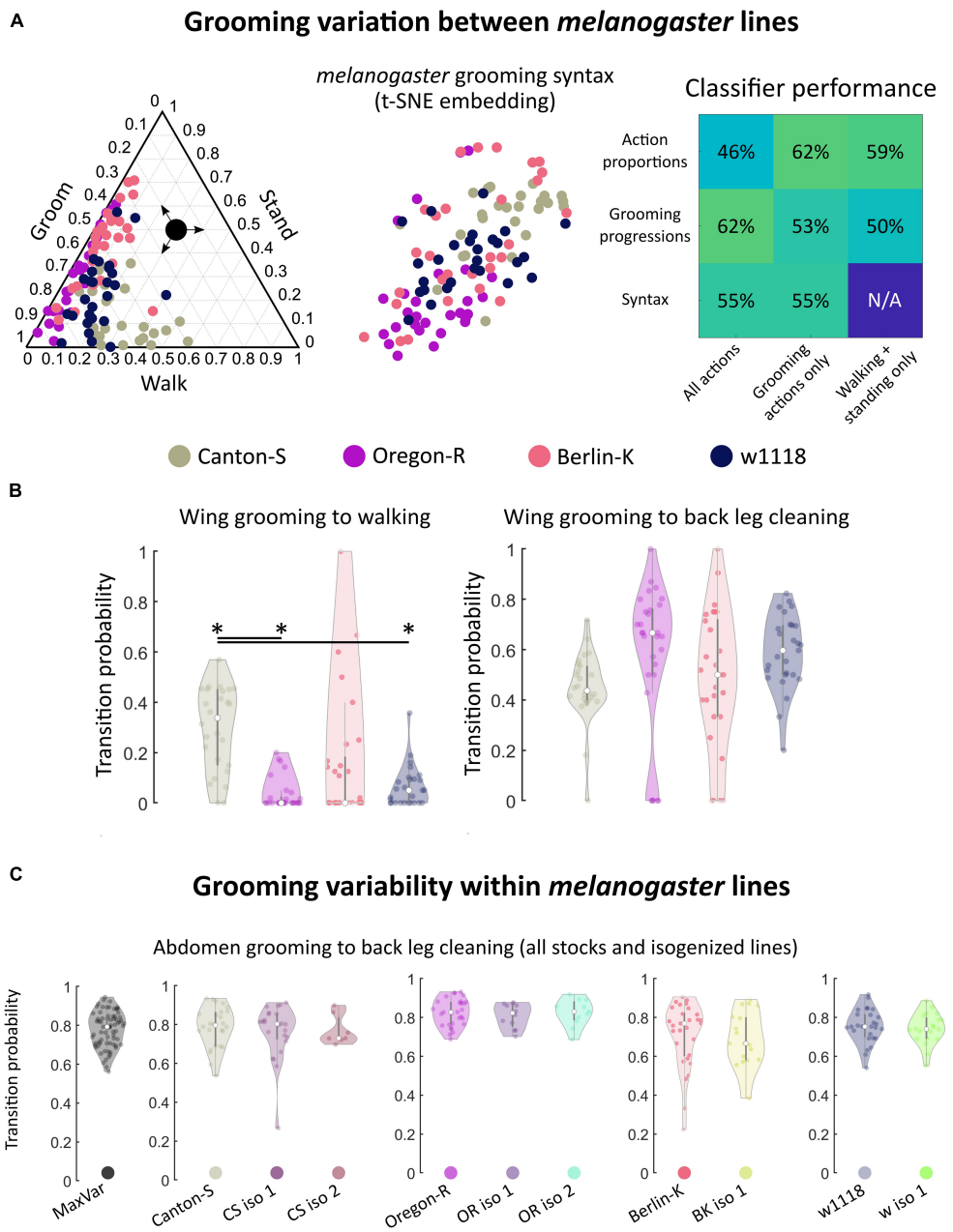

FIGURE 3 | Within *melanogaster*, different stocks differences in syntax activity levels. Genetic homogeneity does not correspond to behavioral stereotypy. **(A)** *melanogaster* stocks (N = 111 flies total) exhibited variation in grooming syntax, though many features were shared. On the left is a ternary plot of grooming, walking, and standing proportions for each stock, similar to **Figure 2A**. Colored points represent individual flies. Shown in the middle is a t-SNE plot of syntax vectors, as in **Figure 2C**. The high degree of overlap in both of these plots illustrates that behavioral responses are qualitatively similar between some individuals of different stock lines. Classifier performance (similar to that shown in **Figure 2D**) is shown on the right. For these data, classification at chance is 25%. Performance above chance is still possible for stock lines. Classification performs similarly well for behavioral features regardless of their complexity; using just walking and standing behavioral proportions provides similar discriminability as using the full syntax. **(B)** Most syntax elements were similar between *melanogaster* stocks, but Canton-S flies walked more than other stocks. Due to differences in activity levels, some walking-related syntax elements differed between Canton-S flies and other stocks. Of the significantly different transitions, only two were within-motif transitions while the rest consisted mostly of transitions to and from walking and standing (**Supplementary Figure 12**). Shown on the left are the wing grooming to walking transition probability distributions for each *melanogaster* stock line. Significant differences in these distributions were observed between lines. On the right, distributions for a posterior grooming transition are shown; the vast majority of action transition distributions did not differ due to their large variances. **(C)** Variances of action transition distributions for stock lines, lines bred for maximum genetic heterogeneity (MaxVar), and lines bred to minimize genetic heterogeneity (iso) were compared (N = 252 total). Genetic homogeneity did not correspond to behavioral variability. Shown as an example are the distributions of abdomen grooming to back leg cleaning transitions. MaxVar flies did not exhibit a higher degree of variability (as measured by the variance of transition distributions) than stock lines. Isogenized lines did not exhibit a lower degree of variability than their parent stocks. *significantly different at $p < 0.05$.

using only non-grooming features (walking and standing proportions and progressions) yielded results similar to classification using full grooming behavior syntax. This is illustrated by the fact that Canton-S flies' higher propensity to walk after grooming their wings is reflected both in their syntax and grooming proportions in **Figure 3B**.

Within Canton-S, activity levels separated male and female flies, as male flies tended to walk more than females (**Supplementary Figure 13**). Male and female flies also possessed somewhat different syntax; classification by syntax was 71%, where chance levels would be 50% for this comparison. This level of accuracy is higher than what was achievable when classifying *melanogaster* stock lines using syntax, but lower than the same comparison for interspecies data.

Since all flies examined showed variability in syntax, we wondered whether the extent of this variability differed among species or strains. **Figure 3B** also illustrates the high degree of variability in *melanogaster* syntax. The wing grooming to back leg cleaning transition exhibited the largest difference between median values of any syntax element (comparison of Canton-S and Oregon-R yielded this difference), but none of these distributions possessed detectable statistical differences due to their concomitantly large variances.

Grooming Behavioral Variability Is Similar Across *melanogaster* Genotypes

To examine the potential relationship between genetic heterogeneity and behavioral variability, each *melanogaster* lab strain was compared to lines bred to maximize genetic heterogeneity (MaxVar) or minimize genetic heterogeneity (isogenic lines). These can be considered outbred and inbred strains. If variability in grooming syntax within a population is strongly related to genetic heterogeneity, we would expect populations with larger genetic heterogeneity to also contain flies with more variable syntax.

All lines, regardless of genetic heterogeneity, exhibit variable grooming (**Supplementary Figure 14**). To quantify variability, the variances of action transition probability distributions were calculated and compared. Only 6/252 (2.4%) transition probability distributions possessed statistically significantly different variances between MaxVar, Canton-S, and the isogenic lines out of all possible pairwise comparisons (Levene's test, $p < 05$ after correction for multiple comparisons *via* Holm's method). Moreover, none of these differences corresponded to within-motif transitions, indicating that variability of common transitions is similar regardless of genetic heterogeneity in a population. These findings also held for Oregon-R (8/252), Berlin-K (19/108), and w1118 (2/108) stock and isogenic comparisons. See **Supplementary Figure 25** for all p values of pairwise action transition distribution variance comparisons.

Figure 3C provides the transition probability distributions for the most common posterior motif transition (abdomen grooming to back leg cleaning) for all stocks and stock-derived isogenic lines. This transition exhibits wide variability in many populations and even populations with smaller variability (CS iso 2) are not different enough to achieve statistical significance after accounting for multiple hypothesis testing.

We also examined stock lines derived from selected wild isolates (Mackay et al., 2012) to determine if these showed more or less grooming variability, as measured by syntax element variance values and Markov entropy. Their variability is comparable to that within lab stocks (**Supplementary Figure 15**).

Finally, we analyzed dust-induced grooming in 15 Canton-S flies that were assayed on three consecutive days. Since a given individual's genome remains constant through the three trials, we could isolate the magnitude of grooming variability that is due to differences in sensory experience (since the dusting protocol does not allow for perfect replication of sensory experience) and life history (since flies will have been exposed to the same irritant several times by the end of the experiment). Ethograms from three example flies are provided in **Figure 4A** (full ethograms are shown in **Supplementary Figure 16**).

Flies exhibited some longitudinal grooming trends, as the total amount of grooming decreased between the first and third days of the experiment. However, the time to completion of 50% of their total grooming did not decrease, suggesting that flies are not simply grooming quicker, but rather are grooming less consistently over time (i.e., punctuating grooming bouts with more walking and standing) (**Supplementary Figure 17**). Importantly, intra-individual variability in syntax across three sessions was of the same magnitude as inter-individual variation in syntax (**Supplementary Figures 18**, **19**); that is, flies were no more similar to themselves over time than they were to other flies on a given day. This suggests that non-genetic factors account for a significant proportion of grooming variability.

Standardizing Sensory Experience Does Not Abolish Grooming Behavioral Variability

To probe the sensory contribution to within-individual variability, we used optogenetic stimulation to induce anterior grooming. 20 Bristle-spGAL4-1 > UAS-CsChrimson flies were tested (Zhang et al., 2020). **Figure 4B** provides ethograms from this experiment, with red bars indicating the three stimulation windows. Even when sensory experience was controlled in this way, flies exhibited variability in their grooming response.

Grooming stereotypy was again quantified using the entropy rate of the grooming syntax. The entropy rate for optogenetically-stimulated flies was lower than for dusted flies ($p < 0.05$, Wilcoxon rank-sum test), indicating a higher degree of stereotypy in grooming (**Figure 4C**). We nonetheless observed within-individual variability between stimulation windows, indicating that standardization of sensory input does not fully abolish grooming variability. **Supplementary Figure 20** quantifies differences in entropy between sessions for three example flies. In addition, optogenetic stimulation resulted in strong anterior motif grooming behavior, rendering all flies' transition probabilities very similar (**Supplementary Figure 21**).

Finally, grooming stereotypy was characterized using edit distance between anterior motif repeats (**Figure 4D**). This metric, used commonly in bioinformatics, describes the difference

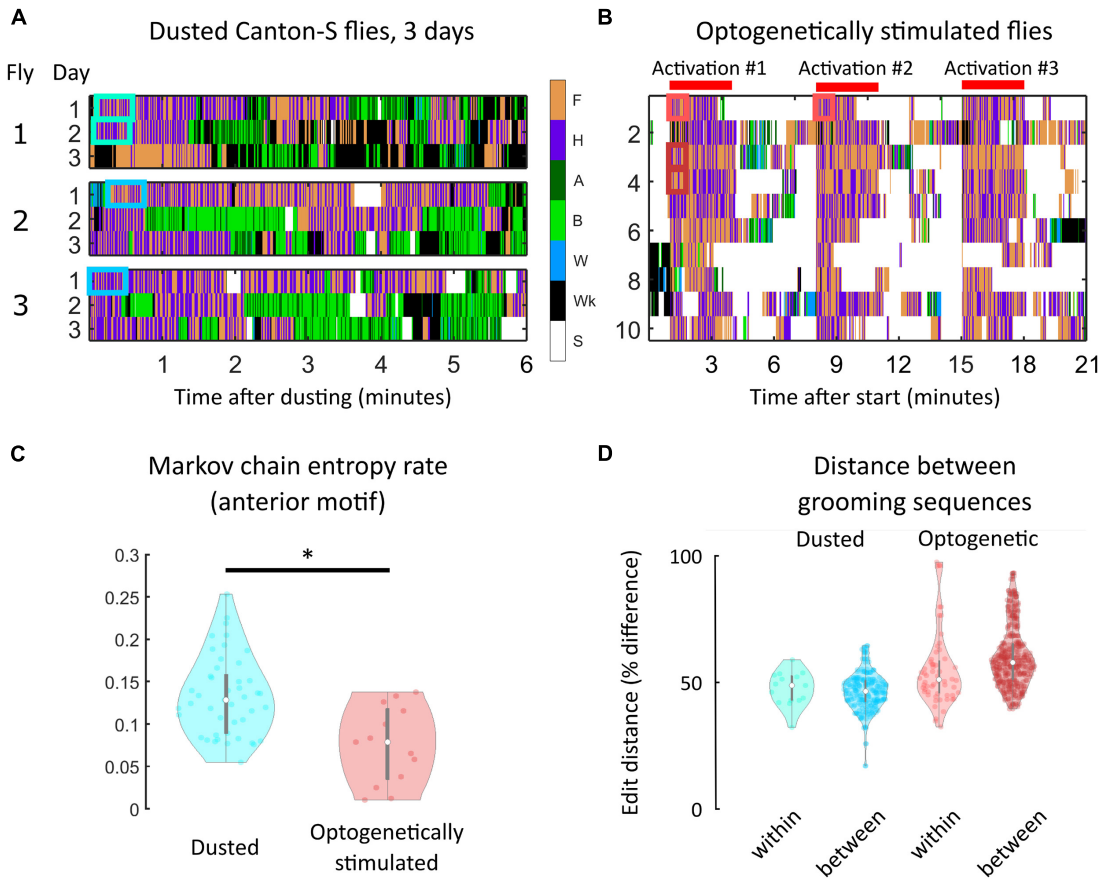

FIGURE 4 | Within-individual grooming differences suggest that non-genetic factors account for a significant portion of variability in behavior. **(A)** Portions of ethograms from three Canton-S flies observed on consecutive days after dust irritant exposure. The differences in ethograms on consecutive days indicate that non-genetic factors must account for some amount of grooming variability. **(B)** Shown are ethograms of 10 Bristle-spGAL4-1 >CsChrimson flies (Zhang et al., 2020). Flies were optogenetically stimulated to induce anterior grooming in three separate 3-min windows, indicated by the red bars. Between these windows, flies still exhibit within-individual grooming variability even though the sensory experience is more uniform than repeated dust exposure. **(C)** Markov chain entropy, a measure of grooming stereotypy, was calculated from anterior grooming syntax. Optogenetically stimulated flies (right) exhibited lower entropies, corresponding to a higher degree of stereotypy, than dusted flies (left). However, optogenetically stimulated flies still exhibited differences in stereotypy between stimulation windows, implicating sources of grooming variability beyond genetic and sensory influences (**Supplementary Figure 19**). **(D)** To assess grooming stereotypy, edit distance between anterior motif repeats was computed. For dusted within-fly comparisons, we computed the edit distance between the first continuous anterior motif sequence lasting 30 s on consecutive days (light blue). For between-fly comparisons, we computed the edit distance between the first continuous anterior motif sequence lasting 30 s on the first day of experiments (dark blue). For all optogentically-stimulated flies, we computed two similar comparisons: within-session [i.e., comparing the sequences labeled "Activation #1" and "Activation #2" in panel **(B)**; light red] and between-fly (i.e., "Activation #1" for each fly; dark red). For each comparison listed, the median edit distance computed corresponded to around 50% of the sequence length, demonstrating the low degree of stereotypy present in grooming sequences. *significantly different at $p < 0.05$.

between two DNA sequences by calculating the minimum number of base pair substitutions, additions, or deletions that would be necessary for the sequences to be identical. Identical sequences would have an edit distance of zero between them, while maximally different sequences would have an edit distance equivalent to the total sequence length (see **Supplementary Methods** for details).

Since edit distance measures the similarity between two sequences (rather than the underlying rules that may generate the sequences), it provides a much stricter definition of stereotypy than Markov entropy, which we use as a measure of stereotypy earlier in our analysis. In addition, it is most useful as a stereotypy measure when it is possible to identify a synchronizing "start" signal between sequences of interest, which is not present in the previously described experiments, but is present for optogenetically-stimulated flies. Therefore, when comparing flies across recording sessions or sequences from different flies, the use of edit distance helps answer the specific question, "Do flies perform repeated, similar sequences or subsequences?"

For all dusted flies, we calculated the edit distance within flies across consecutive days to assess whether flies possess stereotyped repeats. For these comparisons, we compared the first continuous anterior motif sequence lasting at least 30 s on consecutive days, shown by the blue boxes in **Figure 4A**. This particular comparison was chosen to standardize the amount of dust present on the fly to the greatest extent possible and the

short-term grooming history for each sequence, and to ensure that each sequence was long enough to exhibit stereotypy if it exists. Anterior motifs were chosen because they consist of only two actions with high transition probabilities between them, making these sequences the most likely candidates for exhibiting stereotypy. These comparisons yielded a minimum edit distance corresponding to a 39.6% difference between sequences. A similar calculation was made between flies, using the first continuous anterior motif sequence lasting at least 30 s on the first day of experiments. These comparisons yielded a minimum edit distance corresponding to a 41.6% difference between sequences.

Edit distance calculations were also performed for all optogenetically-stimulated flies. Within-fly comparisons (i.e., comparing the sequences labeled "Activation #1" and "Activation #2," red boxes in **Figure 4B**) yielded a minimum edit distance corresponding to a 31.5% difference in sequences. Between-fly comparisons (i.e., "Activation #1" for each fly) yielded a minimum edit distance corresponding to a 42.8% difference in sequences (**Figure 4D**). Together, the low degree of stereotypy present in grooming sequences within and between both dusted and optogenetically-stimulated flies shows that grooming sequence variability is present even when genetics, sensory input, and behavioral history are controlled to the greatest extent possible within this experimental paradigm.

DISCUSSION

Here, we analyzed fly grooming behavior in five different *drosophilid* species and four common *melanogaster* stocks to investigate the relationship between genetic heterogeneity and behavioral variability. Large genetic differences (species-level) correspond to identifiable differences in several grooming features, including the rules governing action transitions known as syntax. Within *melanogaster*, stock lines exhibited smaller variation in grooming syntax, as well as differences in overall activity levels. All flies showed variability in the details of the grooming movement sequence, but increased genetic heterogeneity did not correspond to increased behavioral variability. Analysis of 15 Canton-S flies recorded over consecutive days showed that intra-individual and inter-individual comparisons had similar—high—levels of variability. Optogenetically-stimulated flies also exhibited intra-individual variability in grooming behavior, but less. Taken together, these results demonstrate that large genetic differences result in distinguishable grooming phenotypes, but that genetic heterogeneity within a population does not necessarily correspond to an increase in the range of grooming behavior variability.

Genetic Influences on Behavioral Variation and Variability

Advantageous behavioral phenotypes that are under genetic control can be selected over evolution to produce populations with differing behaviors. Here, we identified significant inter-species variation in grooming syntax, suggesting a genetic basis for group differences in grooming behavior.

Some species differ from *melanogaster* in their propensity to perform anterior grooming actions; the anterior motif actions are significantly less strongly coupled in non-*melanogaster drosophilids*, suggesting that anterior neuronal circuitry or sensory physiology may differ. We also identified differences in grooming behavior between commonly used *melanogaster* stock lines and between male and female Canton-S flies; most of these differences relate to overall activity levels.

Variability itself is a trait that can also be selected for, but is often overlooked (Geiler-Samerotte et al., 2013). At the individual level, randomizing escape trajectories can be beneficial for escaping predators (Wang et al., 2020), and diversity in search paths can be useful when a group is foraging for food. The fate of the passenger pigeons, hunted to death while flocking together, illustrates the dangers of behavioral homogeneity (Murray et al., 2017). The degree of variability in behavior can be selected for as a bet-hedging strategy against unstable environmental conditions (Kain et al., 2015; Krams et al., 2021). Genetic factors contribute to variability in fly visual, olfactory, and locomotor behaviors (Ayroles et al., 2015; Honegger et al., 2019; Linneweber et al., 2020).

The prevalence of variability in *Drosophila* grooming action sequences suggests that non-stereotyped grooming may be advantageous, perhaps for removing diverse distributions or kinds of debris. We examined whether greater genetic heterogeneity within a population corresponded to greater behavioral variability but did not detect any significant impact.

A recent investigation of unstimulated behaviors in different *Drosophila* species detected differences in spontaneous grooming between species and among individuals within a species (Hernández et al., 2020). Using similar methods, they accurately assigned individuals into species categories and assessed variability among individuals. Our findings are complementary: *drosophilid* species show differences in stimulated grooming behaviors as well, suggesting genetic control, but individuals within a species show variability in grooming, indicating that factors other than genes can influence aspects of the behavioral sequence. Hernández et al. (2020) propose that over the long timescales measured in their assay, internal states may explain the observed fluctuation in action transition probabilities. In the shorter timescales we assayed, where flies are responding acutely to dust, we attribute the variability to inherent flexibility in the behavior itself, produced by differences in sensory input and/or intrinsic stochasticity in the neurons or circuits that coordinate the action sequences. These views are not in conflict and together establish that variability in grooming is widespread—potentially even advantageous—with both genetic and non-genetic factors influencing its expression.

Variability also encompasses individuality in animal behavior, typically defined as a trait-like feature that persists stably over several observations. Individuality has been identified in fruit fly turning (Buchanan et al., 2015), mouse roaming behavior (Freund et al., 2013), and bumblebee foraging (Klein et al., 2017), among others (Linneweber et al., 2020; Takagi and Benton, 2020). In both dust-induced and optogenetically-initiated grooming, we did not find evidence for individuality in action sequence patterns at the resolution we analyzed, but this may be because small

contributions from individual tendencies are outweighed by the large amount of variability in the behavior as a whole arising from other causes.

Environmental and Stochastic Influences of Behavioral Variation and Variability

Our analysis of genetic contributions to behavioral variation and variability in the grooming suggests that at the species level, flies show significant differences in the grooming sequence, especially in the syntax of transition probabilities, that allow accurate classification. Differences in behavior between common lab wild-type stocks also support classification, but the accuracy is lower and the effect size of the differences is smaller.

Genetic factors have been implicated in spontaneous (i.e., unstimulated) grooming behavior in *Drosophila melanogaster* (Yanagawa et al., 2020) and in other *drosophilid* species (Hernández et al., 2020). Our results demonstrate that this is true for dust-induced grooming as well. Both spontaneous grooming (Hernández et al., 2020) and dust induced grooming show individual-to-individual variability within a species. The prevalence of sequence flexibility in all species and in controlled experimental conditions suggests that variability itself is a feature of grooming behavior, not a bug. Individuals with overly rigid grooming sequences might not respond as effectively to changing environmental conditions, such as different kinds of debris or the presence of a potential mate or predator.

The causes of grooming variability are still under investigation. Differences in developmental processes such as neural wiring or synaptic connectivity may contribute to behavioral differences between flies, but our experiments show that even individual flies exhibit variability in grooming over repeated trials with dust or optogenetic stimulation. This suggests that non-genetic factors such as sensory stimuli, internal state, previous experience, and circuit noise contribute to the variability we observe in grooming action sequences. The reduction of variability when sensory inputs are optogenetically controlled supports diversity of sensory stimulation as a contributor. The persistence of variability within individuals suggests that intrinsic stochasticity or noise within the neurons or circuits themselves may also play a role, which are possibilities which should be explored further.

MATERIALS AND METHODS

Genetic Stocks

Canton-S, Oregon-R, Berlin-K, w1118, Bristle- spGAL4- 1 (R38B08- AD; R81E10- DBD), and 20XUAS- CsChrimson. mVenus (attp18) stocks were obtained from the Bloomington Stock Center. Isogenic (more accurately, reduced genetic variability) stocks were made by crossing single males to double-balanced stocks and then back-crossing males to the double balancer stock to isolate single second and third chromosomes. Single pairs were mated to reduce variability of X and IV. $\sim$ 2 independent isogenic lines from each *melanogaster* stock were generated; note that many attempts to isogenize result in lethality, as anecdotally reported by colleagues. Maximum Variability stocks were obtained by crossing each *melanogaster* strain to double balancers and then crossing the progeny together and selecting against the balancers. This allowed combination of chromosomes for all four strains. The progeny were allowed to interbreed for several generations to enable recombination in the females.

Drosophilid species stocks were obtained from Tom Turner, UCSB, and the National *Drosophila* Species Stock Center[1].

Data Collection and Processing

Grooming was induced and analyzed as described in Seeds et al. (2014) and Zhang et al. (2020). Three chambers were used in fly dusting assay: dusting chamber (24 well Corning tissue culture plate #3524), transfer chamber and recording chamber. Recording chambers were coated with Insect-a-slip (BioQuip Products Cat #2871A) to discourage wall-climbing and cleaned daily. To control potential circadian effects during assays, trials containing flies of different genotypes were interleaved (allowing for near simultaneity of experiments), and assays were run at the same time each day. Dust-induced grooming assays were performed in 21–23°C. 4–7 day old male flies were anesthetized on ice and transferred to the middle four wells of the transfer chamber. Flies were left in the transfer chamber for 15 min to recover. Approximately 5 mg Reactive Yellow 86 dust (Organic Dyestuffs Corporation CAS 61951-86-8) was added into each of the 4 middle wells of dusting chamber. For fly dusting, the transfer chamber was aligned with the dusting chamber. Flies were tapped into the dusting chamber and shaken 10 times. After dusting, flies and dust were transferred back into the transfer chamber.

Transfer chamber was tapped against an empty pipette tip box to remove extra dust. Dusted flies were then immediately tapped into recording chamber for video recording. The entire dusting process was performed in a WS-6 downflow hood. Approximately 10 individuals were recorded for each genotype. 30 Hz videos were recorded for 50,000 frames (27.78 min) with a DALSA Falcon2 color 4 M camera. A white LED ring right was used for illumination.

Optogenetic stimulation protocol is replicated from Zhang et al. (2020). Further details can be found in the **Supplementary Methods**.

For each set of experimental comparisons (between species, within species, within individual), a single experimenter performed all dusting assays to eliminate experimenter-related differences that may arise. In total, 390 ethograms were recorded. This number includes species data (N = 83), *melanogaster* stocks and isogenic lines = 252), additional male/female Canton-S flies (N = 31), individual Canton-S flies followed for three sessions (N = 45), and optogenetically stimulated flies (N = 10).

Videos were processed through the Automated Behavior Recognition System [ABRS, Ravbar et al. (2019)], trained on a classifier using *melanogaster* flies to generate ethograms. Grooming actions were described previously (Seeds et al., 2014; Hampel et al., 2015). Sub-movements of the grooming actions used in this analysis have not yet been rigorously described and

[1]https://www.drosophilaspecies.com/

may occur on time scales faster than the 30 Hz recording setup can reliably capture, so they were not considered in this work.

Automated behavioral recognition system was used to generate ethograms. Briefly, the raw video frames were pre-processed to generate 3-channel spatiotemporal images (ST images). Features were extracted in three timescales and saved into different channels of ST images: 1. raw frame; 2. difference between two frames; 3. Spectral features extracted from a 0.5 s window. A convolutional network trained by ST images under different light conditions was then used to label the behavior identified in each frame. A different network was trained for classification of each species due to differences in body size and light conditions. All networks achieved >70% validation accuracy within the training protocol, which reserved 20% of frames as test data after training (see **Supplementary Figure 22** for table of classifier performances).

Finally, ethograms were denoised to only include grooming actions that persisted for longer than the approximate duration of one complete leg sweep. Here, we used a cutoff of 150 ms, and eliminated any actions shorter than this duration (fewer than 1% of bouts were removed under this criterion).

Data Analysis

All ethogram features were extracted using custom-written code in MATLAB 2019a. Grooming progression vectors were generated for each fly by calculating the proportion of each action in 10 non-overlapping windows (2.78 min each), yielding a 70-dimensional vector for each fly (10 windows with 7 behavioral proportions). Grooming syntax was defined as the first-order transition probabilities between actions. Syntax for each fly was calculated as described in Mueller et al. (2019).

Bout duration distributions were generated as described in Mueller et al. (2019), using a normalized histogram with 20 bins of equal width for each behavior. Bin width was determined independently for grooming and non-grooming actions, as standing and walking exhibit longer tailed distributions than grooming actions. Thus, duration distribution vectors were 140-dimensional for each fly. Examples of progression, syntax, and duration distribution vectors can be found in the **Supplementary Information**.

Statistics for comparisons between grooming features were calculated using built-in MATLAB functions. t-SNE, and multinomial logistic regression classification analysis were performed using built-in MATLAB functions (**Supplementary Information**).

DATA AVAILABILITY STATEMENT

The original data analyzed in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JS: experimental design. JS and NZ: data collection. JM: data analysis. JM and JS: manuscript writing. JM, JS, and JC: manuscript editing. JS and JC: funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.769372/full#supplementary-material

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REVIEW
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Roles for Sleep in Neural and Behavioral Plasticity: Reviewing Variation in the Consequences of Sleep Loss

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Sleep is a vital physiological state that has been broadly conserved across the evolution of animal species. While the precise functions of sleep remain poorly understood, a large body of research has examined the negative consequences of sleep loss on neural and behavioral plasticity. While sleep disruption generally results in degraded neural plasticity and cognitive function, the impact of sleep loss can vary widely with age, between individuals, and across physiological contexts. Additionally, several recent studies indicate that sleep loss differentially impacts distinct neuronal populations within memory-encoding circuitry. These findings indicate that the negative consequences of sleep loss are not universally shared, and that identifying conditions that influence the resilience of an organism (or neuron type) to sleep loss might open future opportunities to examine sleep's core functions in the brain. Here, we discuss the functional roles for sleep in adaptive plasticity and review factors that can contribute to individual variations in sleep behavior and responses to sleep loss.

Keywords: sleep, plasticity, review, memory, *Drosophila*

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INTRODUCTION

Sleep is a physiological state that has been conserved across evolution, even noted in invertebrates lacking a centralized brain (Hendricks et al., 2000; Shaw et al., 2000; Zhdanova et al., 2001; Raizen et al., 2008; Singh et al., 2014; Nath et al., 2017). Although sleep's physiological functions remain poorly understood, sleep loss has been associated with deleterious effects on health and cognition (Rechtschaffen and Bergmann, 1995; Dinges et al., 1997; Durmer and Dinges, 2005; Spiegel et al., 2005; Banks and Dinges, 2007; Knutson et al., 2007; Grandner et al., 2010). Sleep varies based on previous waking experience (Ganguly-Fitzgerald et al., 2006; Huber et al., 2007; Hanlon et al., 2009; Keene et al., 2010; Beckwith et al., 2017; Kirszenblat et al., 2019; Milinski et al., 2021) throughout the lifespan (Roffwarg et al., 1966; Kales et al., 1967; Feinberg and Carlson, 1968; Cauter et al., 2000; Backhaus et al., 2007; Dijk et al., 2010; Feinberg and Campbell, 2010; Carrier et al., 2011; Vienne et al., 2016; Mander et al., 2017), and between species (Lyamin et al., 2008, 2017, 2018; Siegel, 2008; Lesku et al., 2012), suggesting that sleep has multiple functions. However, because sleep coincides with broad changes in neurophysiology and necessitates a loss of consciousness with reduced responsiveness to external threats, it is likely that sleep evolved, at least in part, to support brain function (Rasch and Born, 2013; Tononi and Cirelli, 2014). Notably, sleep is often

elevated during periods of synaptic reorganization, including early development (Roffwarg et al., 1966; Shaw et al., 2000; Kayser et al., 2014), recovery from neural injury (Singh and Donlea, 2020; Stanhope et al., 2020), and memory consolidation (Walker et al., 2002; Ganguly-Fitzgerald et al., 2006). These findings each suggest that sleep supports plastic remodeling in the brain. Synaptic plasticity allows behavioral flexibility in response to external stimuli, and enables the processing and storage of information (Hughes, 1958; Zucker and Regehr, 2002; Cooke and Bliss, 2006). However, the underlying cellular and molecular mechanisms that support plasticity during sleep remain an area of intense investigation.

The impacts of sleep loss, interestingly, vary widely depending on age, environmental conditions, and genotype. While organisms typically recover from acute sleep disruptions relatively quickly, early-life sleep disruptions can prevent developmental plasticity during critical periods and result in long-lasting changes in circuit connectivity and behavior (Frank et al., 2001; Seugnet et al., 2011; Kayser et al., 2014). Conversely, some individuals withstand sleep loss with few consequences depending on the physiological conditions or genetic factors (Viola et al., 2007, 2012; Lyamin et al., 2008; Keene et al., 2010; Thimgan et al., 2010; Donlea et al., 2012; Lesku et al., 2012). In some cases, sleep disruption even provides an opportunity to weaken maladaptive memories (Poe, 2017). Examining the variables that can influence an individual's sensitivity to sleep loss could provide new insights into the core mechanisms of sleep-dependent plasticity. In this review, we will discuss roles for sleep in the maintenance of neural and behavioral plasticity during development, and learning/memory. Finally, we outline ethologically relevant conditions in which organisms can maintain neural and behavioral plasticity in the face of sleep loss.

DEVELOPMENT

Synaptic plasticity plays a crucial role in brain development, especially in the refining of neural connectivity through the process of pruning (Paolicelli et al., 2011). Defects in synaptic pruning during development are thought to contribute to atypical circuit function seen in neurodevelopmental disorders (Paolicelli et al., 2011; Konopaske et al., 2014; Tang et al., 2014; Cossío et al., 2017; Kim et al., 2017; Neniskyte and Gross, 2017). Daily sleep amounts peak in many species early in development, when the brain is undergoing significant plastic changes (Roffwarg et al., 1966; Jouvet-Mounier et al., 1970; Shaw et al., 2000; Kayser et al., 2014). Studies in humans have found that sleep disruption during development is associated with severe and lasting consequences for behavior and cognition (O'Brien et al., 2004; Halbower et al., 2006; Ednick et al., 2009). While these human studies provide a correlational link between impaired sleep and later cognition, several lines of animal studies described below indicate conserved roles for sleep in neurodevelopment of several species and begin to identify possible mechanisms by which sleep might influence brain development.

Rapid eye movement (REM) sleep is thought to play a particularly important role in development. Infants spend as much as 50% of their time asleep in REM, compared to 25% in adults (Roffwarg et al., 1966; Jouvet-Mounier et al., 1970). This period of increased REM sleep coincides with heightened formation and elimination of synapses in the developing mouse brain (Marks et al., 1995). Previous work found that REM deprivation, but not non-REM (NREM) deprivation, prevents the elimination of newly-formed dendritic spines in layer V pyramidal neurons in the developing mouse motor cortex (Li et al., 2017). Further, elimination of recent spines during REM facilitates the development of new spines at nearby sites. While most newly formed spines are eliminated, persistent spines are strengthened by REM sleep. Notably, similar findings were observed in the adult mouse brain following motor learning (Li et al., 2017).

A unique feature of REM sleep is the occurrence of myoclonic twitches, or spontaneous, discrete, spastic movements of the limbs (Tiriac et al., 2012; Blumberg et al., 2013; Sokoloff et al., 2020). These twitches occur throughout the mammalian lifespan, but are particularly abundant in infancy (Tiriac et al., 2012; Blumberg et al., 2013; Sokoloff et al., 2020, 2021). The development of myoclonic twitches depends on sensory feedback; the spatiotemporal organization of twitches is disrupted in newborn ErbB2 muscle-specific knockout mice which lack muscle spindles and exhibit impaired proprioception in adulthood (Blumberg et al., 2015). Muscle spindles are sensory receptors that relay changes in the length of muscles to the central nervous system and are necessary for intact proprioception (Kröger and Watkins, 2021). These findings suggest that twitches during sleep provide the developing brain with opportunities to refine immature sensorimotor maps and better coordinate limb movements. Twitching during early-life REM episodes, therefore, could facilitate the transformation of uncoordinated movements during infancy to the fine-tuned sensorimotor maps of an adult. Sensory feedback from twitching limbs are thought to contribute to motor learning and sensorimotor integration (Blumberg et al., 2013, 2020; Sokoloff et al., 2015; Rio-Bermudez and Blumberg, 2018; Glanz et al., 2021), as reafference from myoclonic twitches selectively activates brain regions such as the thalamus, cortex, hippocampus, and cerebellum in infant rats (Khazipov et al., 2004; Mohns and Blumberg, 2010; Tiriac et al., 2012; Sokoloff et al., 2015). Because reafference signals from self-movement are gated during waking, sleep disruptions that interfere with twitching, and their corresponding neuronal activity may disrupt sensorimotor maturation (Tiriac and Blumberg, 2016). While these studies provide an important and promising link between early-life sleep episodes and the development of mature sensorimotor representations, the underlying synaptic mechanisms and long-term consequences of myoclonic twitch disruptions remain to be characterized in detail.

A vital role for sleep in early life plasticity is shared across sensory circuits. The study of ocular dominance plasticity (ODP) induced by monocular deprivation (MD) in cats, for example, is a canonical model of critical period plasticity during development that is reliant upon sleep. During an early critical period for visual development, occluding one eye leads to enhanced visual

cortex responses to inputs from the non-deprived eye (Hubel and Wiesel, 1970). Sleep enhances ODP; NREM sleep deprivation prevents enhancement of cortical plasticity, suggesting that sleep is vital for consolidating experience-dependent changes in ocular dominance following MD (Frank et al., 2001). More recent work has found that REM deprivation disrupts cortical plasticity after MD as well, perhaps by disrupting replay-like patterns of activity in the visual cortex (Bridi et al., 2015). Additionally, REM sleep following MD is sufficient to prevent reversal of ODP following subsequent manipulations such as further SD (Bridi et al., 2015), cortical inactivation (Jha et al., 2005), and inhibition of NMDA receptors (Aton et al., 2009). The dependence of ODP on REM sleep parallels studies of sensorimotor development described above, suggesting a vital role for REM sleep in permitting developmental refinement across sensory systems. The consolidation of ODP is also reminiscent of hippocampal memory consolidation during sleep (Diekelmann and Born, 2010; Rasch and Born, 2013). These studies suggest that sleep during development is necessary for the consolidation of plastic changes induced by waking experience, which likely guide appropriate behavioral adaptations to a changing environment. Since ODP (along with other forms of developmental plasticity) occurs during a tightly restricted critical period of development, sleep disruptions early in life could have long-lasting effects on neurophysiology and behavior.

Ontogenetic changes in sleep are conserved; sleep amount and intensity are increased early in life for invertebrates, such as the fruit fly, just as they are in mammals (Jouvet-Mounier et al., 1970; Shaw et al., 2000). In *Drosophila*, 24 h of sleep deprivation following eclosion leads to long-term learning deficits, whereas adults recover from the same duration of sleep loss after one night of recovery sleep (Seugnet et al., 2011). These chronic learning impairments are likely connected with altered dopamine signaling, and can be dampened either by blocking D1 receptor activity during early life sleep loss or by elevating dopamine signaling during the days after developmental sleep deprivation (Seugnet et al., 2011). Additionally, young sleep-deprived male flies, but not mature flies, show deficits in courtship behavior as adults (Seugnet et al., 2011; Kayser et al., 2014). These courtship deficits are accompanied by decreased size of an olfactory glomerulus associated with perception of social pheromones, caused by impaired developmental growth (Kayser et al., 2014). Similarly, 1 week of early life sleep disruption impairs later social bonding in adult prairie voles (Jones et al., 2019). In this study, sleep disruption occurred during the third and fourth weeks of life, which likely falls during a critical period for maturation of GABAergic circuits that contribute to sensory integration (Gogolla et al., 2014). Notably, early life sleep deprivation in prairie voles leads to an increase in parvalbumin immunoreactivity in the primary sensory cortex, a brain region relevant to social bonding (Jones et al., 2019). Chronic changes in parvalbumin signaling could disrupt sensory processing and social behavior by altering excitatory/inhibitory balance (Yizhar et al., 2011). Together, these studies demonstrate that early life sleep is vital for developmental growth of rapidly growing brain regions across many species, and that disrupted sleep during development can result in lasting effects on adult circuitry and behavior.

While human studies have not yet revealed a mechanistic understanding of how sleep promotes neural and cognitive development, animal models indicate that sleep's role in neurodevelopment is evolutionarily ancient. Model system studies, such as those in flies and mice discussed above, have begun to examine how sleep modulates synaptic connectivity in a variety of developing sensory circuits. Further studies in these systems may reveal interventions that facilitate healthy development during insufficient sleep (Seugnet et al., 2011; Kayser et al., 2014; Jones et al., 2019).

LEARNING AND MEMORY

In a variety of species, sleep is required for several stages of memory formation and processing (Walker et al., 2002; Graves et al., 2003; McDermott et al., 2003; Ganguly-Fitzgerald et al., 2006; Seugnet et al., 2008; Krishnan et al., 2016). Indeed, sleep deprivation leads to impaired encoding (Walker et al., 2002; Yoo et al., 2007; Seugnet et al., 2008), consolidation (Graves et al., 2003; Diekelmann and Born, 2010), and retrieval (Gais et al., 2007; Lo et al., 2016; Montes-Rodríguez et al., 2019; Heckman et al., 2020) of recent associations. While even a brief nap restores memory in some assays (Seugnet et al., 2008; Ong et al., 2020), other learning and memory impairments persist after days of recovery sleep (Havekes et al., 2016; Yamazaki et al., 2020; Wu et al., 2021). While it is not clear why recovery from sleep loss varies between these conditions, studies have detected several types of longer-lasting cellular and molecular changes that persist after recovery sleep, including altered gene expression (Gaine et al., 2021), protein synthesis (Tudor et al., 2016; Lamon et al., 2021), and circuit connectivity (Weiss and Donlea, 2021). Interestingly, some types of memories seem to be more vulnerable to sleep loss than others. For example, procedural memories and memories acquired with a conscious motivation or reward benefit from sleep more than declarative or unmotivated memories (Stickgold and Walker, 2007; Diekelmann and Born, 2010). In *Drosophila*, sleep deprivation disrupts consolidation of appetitive sugar reward memories in fed flies, but in not starved flies (Chouhan et al., 2021). Together, these studies indicate that sleep deprivation likely does not have a universal effect on learning and memory, but varies based on physiological, environmental, and behavioral factors.

While the negative impacts of sleep loss on memory formation are typically detrimental, it is possible that targeted sleep disruption could be used to prevent the consolidation of maladaptive memories. Some studies, for instance, suggest that sleep deprivation could be used following trauma to degrade fear memories in patients with post-traumatic stress disorder (PTSD). Studies by Vanderheyden et al. (2015) compared sleep patterns of rats that were susceptible to developing PTSD-like symptoms after trauma to those that were resilient. While susceptible rats exhibited an increase in REM sleep in the hours following the traumatic event, resilient rats slept little during this period (Vanderheyden et al., 2015). Heightened

REM sleep following trauma could lead to consolidation and reactivation of the trauma memory, preventing fear extinction, and resulting in generalization of the fear memory (Poe, 2017). Traumatic events drive activation of the mammalian locus coeruleus (LC) (Passerin et al., 2000; Naegeli et al., 2018), a collection of noradrenergic cells that promote long-term potentiation (LTP) (Izumi et al., 1992; Thomas et al., 1996; Izumi and Zorumski, 1999) and are generally quiescent during REM sleep (Foote et al., 1980). Elevated LC activity during REM sleep following a traumatic event can contribute to enhancement of recently formed emotional memories as seen in PTSD (Wassing et al., 2019). Therefore, behavioral sleep deprivation or pharmacological REM suppression following a traumatic event could lead to interventions to prevent the development of PTSD (Vanderheyden et al., 2014, 2015; Poe, 2017). Conversely, given the importance of sleep in memory consolidation (Rasch and Born, 2013) and emotional processing (Palmer and Alfano, 2017; Tempesta et al., 2018), sleep loss following a traumatic event could prevent consolidation of fear extinction memory in other conditions (Pace-Schott et al., 2015). Recent human studies have produced mixed results (Porcheret et al., 2015; Kleim et al., 2016; Cohen et al., 2017), indicating that the role for sleep in consolidating and/or maintaining traumatic memories varies with context or time elapsed since trauma. Further studies will be required to examine the therapeutic potential of sleep manipulations more clearly.

Synaptic Plasticity and Homeostasis

Although the primary function or functions of sleep are not understood, evidence suggests a strong relationship between sleep and plasticity (Frank et al., 2001; Tononi and Cirelli, 2014). Sleep loss leads to impairments in the plastic processes of learning and memory (Diekelmann and Born, 2010; Rasch and Born, 2013). One prominent hypothesis posits that sleep's function is the renormalization of synaptic strength via downscaling of synapses that are potentiated during wake, thereby constraining excitability and restoring signal-to-noise ratios for neuronal firing (Tononi and Cirelli, 2014). Learning about the environment during waking experience requires strengthening of synapses (Clem and Barth, 2006; Gruart et al., 2006; Tye et al., 2008). According to this synaptic homeostasis hypothesis, sleep deprivation leads to cognitive deficits due to saturation of synaptic connections (Tononi and Cirelli, 2014). Evidence supporting the role of synaptic downscaling during sleep exists in a variety of species (Gilestro et al., 2009; Vyazovskiy et al., 2009; Bushey et al., 2011). At the molecular level, synaptoneurosomes from the cortex and hippocampus of adult rats display increased protein levels of GluA1-containing AMPA receptors after spontaneous and forced wake than after sleep (Vyazovskiy et al., 2008). Sleep has been found to promote synaptic downscaling in the mouse forebrain by internalizing AMPA receptors via the immediate early gene Homer1 (Diering et al., 2017). In addition, the size of the axon-spine-interface, an ultrastructural measure of synaptic strength, increases after several hours of wake compared to sleep in several mouse brain regions (Vivo et al., 2017, 2019; Spano et al., 2019). At the electrophysiological level, amplitude and/or frequency of miniature excitatory postsynaptic currents in several regions of the rodent brain increase during wake and after sleep loss, and decline following spontaneous sleep and recovery sleep (Liu et al., 2010; Bjorness et al., 2020; Khlghatyan et al., 2020). Additionally, firing rates of hippocampal and cortical neurons have been shown to increase with wake and decrease with sleep (Lubenov and Siapas, 2008; Vyazovskiy et al., 2008, 2009; Huber et al., 2013; Norimoto et al., 2018). Studies in *Drosophila* have also found increases in abundance of presynaptic and postsynaptic markers following sleep loss, consistent with the hypothesis of net potentiation during wake (Gilestro et al., 2009; Bushey et al., 2011; Huang et al., 2020; Weiss and Donlea, 2021). Additional work in the fruit fly has found that acute sleep induction is sufficient to reduce abundance of transcripts (Dissel et al., 2015) or protein (Weiss and Donlea, 2021) of synaptic components.

While evidence clearly suggests a role for sleep in synaptic downscaling in some circumstances, other studies have reported synaptic potentiation during sleep (Frank et al., 2001; Aton et al., 2013, 2014). Short periods of sleep loss decrease the number of dendritic spines in the CA1 region of the hippocampus due to increased activity of the actin-binding protein cofilin (Havekes et al., 2016). Suppressing cofilin activity in hippocampal neurons prevents spine loss and cognitive deficits following sleep deprivation, suggesting that disruption of synaptic potentiation during sleep deprivation can lead to defects in memory consolidation (Havekes et al., 2016). Similarly, sleep deprivation leads to decreased spine density in the dentate gyrus (Raven et al., 2019), and disrupts the formation of new spines following learning (Yang et al., 2014). These data indicate that, although evidence supports a general trend for synaptic downscaling during sleep, it is likely that different classes of synapses undergo different forms of plasticity during sleep or that sleep alters synaptic organization differently depending on the organism's developmental state and recent experience.

Several recent studies have sought to understand whether sleep loss differentially affects distinct classes of neurons within a single circuit or brain region. The *Drosophila* mushroom body (MB), which encodes olfactory associative memories, provides an ideal opportunity to examine the local effects of sleep loss on synapse organization. Heroic efforts have untangled the organization of the fly MB with the development of genetic drivers to label each cell type, often with single-cell resolution (Aso et al., 2014a,b) and serial reconstruction of electron micrographs have led to a detailed connectome of the MB circuitry (Li et al., 2020; Scheffer et al., 2020). These studies show that the *Drosophila* MB is an associative learning center that is divided into 15 zones defined by non-overlapping arborization of several cell types, including cholinergic Kenyon Cells (KCs), reinforcing dopaminergic neurons (DANs), and mushroom body output neurons (MBONs) which mediate behavioral valence output (Aso et al., 2014a). Associative engrams can be localized to individual zones of the MB lobes, where plasticity in the connections between odor-encoding KCs and valence-driving MBONs determines the fly's behavioral response to odorant stimuli (Aso et al., 2014b; Hige et al., 2015; Owald et al., 2015). Since sleep loss prior to training can impair acquisition/short-term memory and disrupting sleep after training prevents

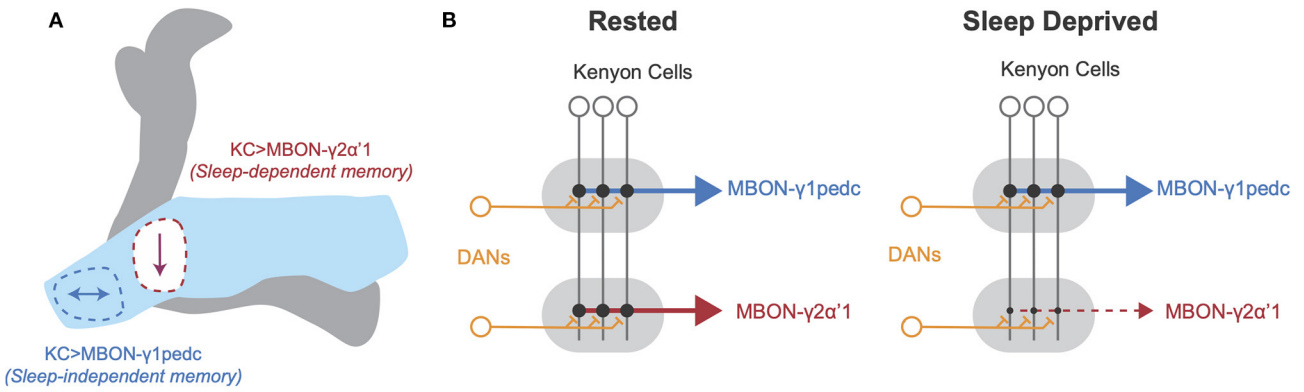

FIGURE 1 | Schematic of local plasticity in *Drosophila* mushroom body after sleep loss. **(A)** Schematic illustration of *Drosophila* mushroom body. The γ lobe (light blue) contains the γ1 compartment, outlined in blue, and the γ2 compartment, outlined in red. Arrows represent changes in connectivity from Kenyon cells to MBON-γ1pedc (left, blue) and MBON-γ2α'1 (right, red). Appetitive memory encoded at KC>MBON-γ1pedc synapses is resilient to sleep loss, but appetitive memory encoded at KC>MBON-γ2α'1 synapses is impaired by sleep loss. **(B)** Schematic of connectivity between neuronal cell types in MB in rested (left) and sleep deprived brains (right). KC axons innervate tiled zones that each receive input from distinct DANs and provide input to unique MBONs. After SD, KC>MBON-γ1pedc connectivity is unchanged, but KC>MBON-γ2α'1 connectivity decreases. Based on findings from Weiss and Donlea (2021) and Chouhan et al. (2021).

memory consolidation (Ganguly-Fitzgerald et al., 2006; Seugnet et al., 2008), it is likely that sleep deprivation alters either synaptic connectivity or plasticity in MB circuits. Overnight sleep deprivation selectively upscales synapses of cholinergic memory-encoding KCs, but not other cell types in the MB, including DANs or large, inhibitory interneurons (Weiss and Donlea, 2021). Further, not all types of KC output synapses are equally impacted by sleep loss; output connections from KCs to different classes of post-synaptic target neurons show wide variations in abundance following sleep loss.

Interestingly, studies by Chouhan et al. (2021), found that flies housed without food did not require sleep after appetitive conditioning to form new memories, unlike fed flies. While appetitive memory is encoded in the KC>MBON-γ2α'1 circuit in fed flies and is sensitive to sleep loss, appetitive memory is encoded in KC>MBON-γ1pedc circuitry in starved flies, and remains intact with sleep loss (Chouhan et al., 2021). Additionally, Weiss and Donlea (2021) found that sleep loss led to decreased connectivity between KCs and MBON-γ2α'1, necessary for sleep-dependent memory consolidation, while KC>MBON-γ1pedc connections, dispensable for sleep-dependent memory consolidation, were unaffected. Sleep loss could therefore disrupt consolidation of recent appetitive memories in fed flies by reducing overall connectivity between KCs and MBON-γ2α'1 (see **Figure 1**). Because plasticity rules can differ widely between MB sub-circuits (Hige et al., 2015), environmental conditions during learning likely influence the strength, retention, and/or decay time of a particular association. These results suggest that different zones of the MB exhibit distinct plasticity rules during sleep, likely based on learning paradigm, internal state, and other previous experience.

Supporting the idea of region and circuit specific changes in plasticity with SD, Puentes-Mestril et al. (2021) examined the effects of sleep loss on ribosome-bound transcripts for activity-dependent regulators of plasticity in excitatory pyramidal neurons and inhibitory parvalbumin-expressing interneurons. While both classes of neurons show increases in plasticity-mediating transcripts in the cortex following sleep loss, SD has little effect on abundance of these transcripts in both cell types in the hippocampus (Puentes-Mestril et al., 2021). Additional work suggests that certain cell types in the mouse hippocampus likely have privileged roles in memory consolidation during sleep (Delorme et al., 2021). Sleep deprivation leads to activation of inhibitory somatostatin-expressing (Sst+) interneurons in the hippocampus, likely due to inputs from increasingly active cholinergic neurons (Delorme et al., 2021). Both pharmacological activation of cholinergic neurons and chemogenetic activation of Sst+ cells in the dorsal hippocampus in the absence of SD leads to deficits in sleep-dependent memory consolidation (Delorme et al., 2021). Notably, both Delorme et al. (2021) and Weiss and Donlea (2021) found that sleep deprivation enhances cholinergic signaling onto GABAergic interneurons in learning/memory-related circuits, which likely increases inhibition onto memory-encoding neurons (see **Figure 2**). Enhanced hippocampal inhibition due to increased Sst+ activity during SD correlates with impairment of memory consolidation by disrupting LTP (Vecsey et al., 2009; Havekes et al., 2016), the reactivation of memory-encoding cells (Stefanelli et al., 2016; Clawson et al., 2021), or hippocampal oscillations (Puentes-Mestril et al., 2019). Similarly, while some inhibition from the *Drosophila* APL interneurons onto KCs is necessary to maintain spatial and temporal sparseness of odor encoding (Lei et al., 2013; Lin et al., 2014), excess inhibition would likely prevent encoding of new odor associations and reactivation of existing memory traces. Interestingly, GABAergic signaling from dorsal paired medial (DPM) and anterior paired lateral (APL) promotes sleep at night, suggesting that these interneurons may be recruited by increased KC activity during SD to promote sleep and sparsen KC representations (Haynes et al., 2015). These studies in both mice and *Drosophila* suggest that increased

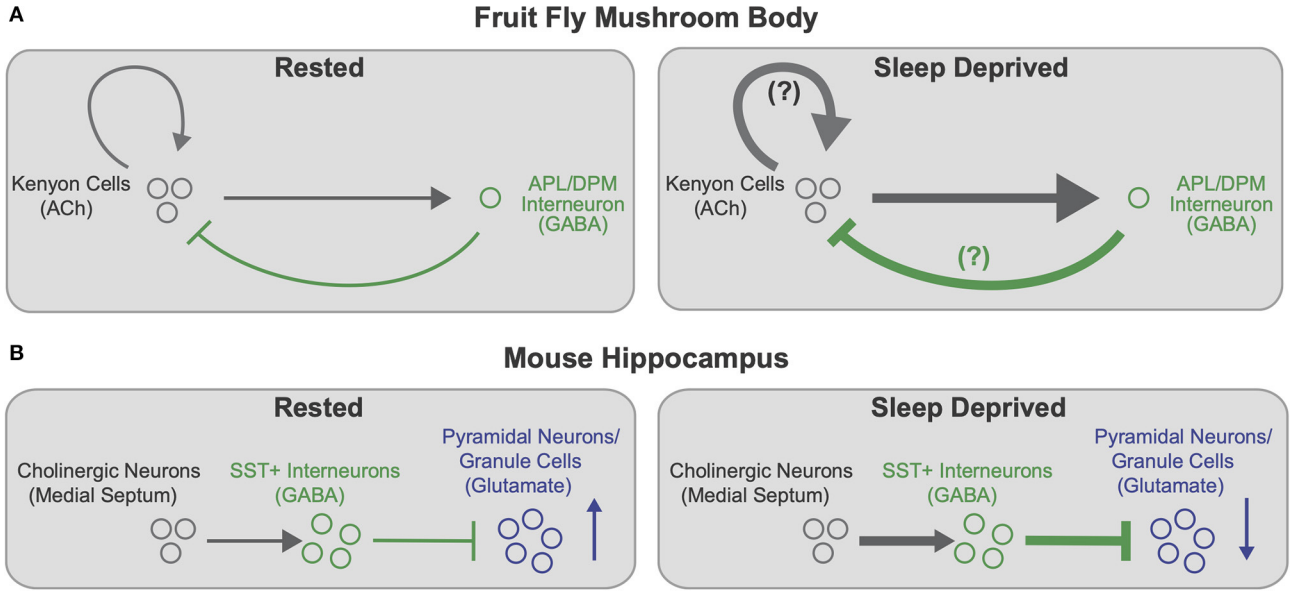

FIGURE 2 | Cell type specific effects of sleep loss on memory-encoding circuits. **(A)** Schematic of connectivity between memory-encoding KCs and APL/DPM interneurons in the *Drosophila* MB in rested (left) and sleep deprived (right) flies. Cholinergic KCs activate GABAergic interneurons, which provide feedback inhibition onto KCs. KCs also synapse back onto other KCs. After SD (right), KC>APL connectivity strengthens, presumably increasing inhibition back onto KCs. KC>KC synapses may also strengthen, further contributing to increased KC>APL connectivity. Increased inhibition from APL/DPM after SD could dampen KC>KC excitation and promote recovery sleep. Based on findings from Weiss and Donlea (2021). **(B)** Schematic of hippocampal circuitry including cholinergic neurons in the medial septum to the mouse hippocampus in rested (left) and sleep deprived (right) mice. Cholinergic neurons activate GABAergic SST+ interneurons in the hippocampus, which inhibit memory-encoding pyramidal neurons/granule cells (principal neurons). After SD (right), enhanced cholinergic signaling increasingly activates SST+ interneurons, thereby heightening inhibition and reducing activity of hippocampal pyramidal neurons and granule cells. Based on findings from Delorme et al. (2021).

cholinergic signaling disrupts learning and memory after sleep deprivation, and that inhibitory drive onto memory-encoding neurons could be recruited to compensate. While these studies find complementary effects of sleep loss in the fly and mouse, these results use different approaches; Weiss and Donlea (2021) measure synaptic active zone reporters in the fly MB while Delorme et al. (2021) and Puentes-Mestril et al. (2021) quantify hippocampal transcript levels of activity-dependent immediate early genes. Additional studies will be required to directly test the relationship between connectivity changes and cell-type specific changes in activity. Ultimately, characterizing the subsets of synapses, cell types, and circuits that are most sensitive to sleep loss will help elucidate the mechanisms by which SD impairs behaviors such as learning and memory.

Sleep not only balances synaptic connectivity, but also influences neuronal firing patterns. In the rodent frontal cortex, fast spiking pyramidal cells show decreased activity during NREM sleep, while slow firing neurons increase their firing rate (Watson et al., 2016). Similar findings were observed in the mouse primary visual cortex, and these changes in firing rates were disrupted by a period of brief sleep deprivation (Clawson et al., 2018). Pyramidal neurons that are active during sleep spindles, oscillatory activity that promotes plasticity underlying memory formation (Schabus et al., 2006; Rasch and Born, 2013; Cairney et al., 2018), are increasingly active over the course of slow-wave sleep (SWS), whereas spindle-inactive pyramidal neurons show decreased activity during SWS (Niethard et al., 2021). These results indicate that sleep can increase the signal-to-noise ratio of neuronal responses by increasing the activity of sparsely firing neurons with the highest selectivity while reducing noise by decreasing activity of faster spiking, less selective neurons (Clawson et al., 2018). Interestingly, sleep during early-life ODP in mice is vital for firing rate homeostasis, indicating a potential life-long role for sleep in normalizing neuronal activity (Hengen et al., 2016; Pacheco et al., 2021).

RESILIENCE TO SLEEP LOSS

Ethological Context

While sleep contributes to many forms of experience-dependent plasticity as described above, individuals can show a wide variation in their responses to sleep loss. Sleep is homeostatically regulated across many species, but both extrinsic and intrinsic factors can influence the responses of an organism to specific sleep challenges. Food-deprived *Drosophila*, for instance, typically reduce their sleep, presumably to maximize foraging opportunities (Keene et al., 2010; Thimgan et al., 2010; Yurgel et al., 2019). While acute sleep-deprivation is typically accompanied by impaired memory and a homeostatic increase in sleep, flies that lose sleep overnight during food deprivation can retain intact memory formation and show little, if any, sleep rebound (Thimgan et al., 2010). Similarly, socially naïve male flies will also forego sleep when paired overnight with a female

TABLE 1 | Summary of experimental or ethologically-relevant conditions that reduce sleep in several species.

Species	Manipulation	Sleep response	Behavioral response	References
Drosophila melanogaster	Sleep deprivation	Decreased sleep, homeostatic rebound	Impaired learning, STM and LTM	Ganguly-Fitzgerald et al., 2006; Seugnet et al., 2008; Li et al., 2009
	Starvation	Decreased sleep, no rebound	Intact memory	Keene et al., 2010; Thimgan et al., 2010; Yurgel et al., 2019
	Stimulants	Decreased sleep	Not measured	Hendricks et al., 2000; Shaw et al., 2000; Andretic et al., 2005
	Courtship	Decreased sleep, no rebound	Not measured	Beckwith et al., 2017; Machado et al., 2017
Frigatebirds	Migration	Decreased sleep in flight, rebound on land	Not measured	Rattenborg et al., 2016
Sandpipers	Mating season	Decreased sleep	Mating success positively correlated with amount of sleep loss	Lesku et al., 2012
Cetaceans	Postpartum	Little to no sleep	Not measured	Lyamin et al., 2005, 2007
Fur seals	In seawater	Greatly reduced REM, no REM rebound	Not measured	Lyamin et al., 2018

fly (Beckwith et al., 2017; Machado et al., 2017). This effect can be replicated by activating pheromone sensing neurons or courtship control circuits and, like starvation-induced arousal, is not followed by a sleep rebound. Similarly, the ability to temporarily offset the need for sleep has also been found in vertebrate species. Fur seals suppress REM sleep for days or weeks when foraging in seawater, accompanied by little to no REM rebound (Lyamin et al., 2018). Migratory frigate birds can reduce the time that they spend asleep by over 90% for ∼10 days while continuously in flight over the Pacific Ocean compared to their sleeping patterns on land (Rattenborg et al., 2016). Similarly, Arctic male sandpipers suppress sleep for a roughly 3 week period annually while they compete for mating partners (Lesku et al., 2012). During mating season, the sun never sets in the high Arctic, allowing males to engage in unlimited visual courtship displays. Because mating success is correlated with the amount of time that male sandpipers spend awake, there is likely selective pressure for genetic factors that can allow male sandpipers to withstand prolonged sleep loss without accruing cognitive deficits or sleep drive. Constant sunlight during this period likely interacts with social and reproductive cues, enabling males to forego sleep for an extended period. Social behaviors can also drive contexts in which mammals can delay the need for sleep. Whales and dolphins, for example, can nearly fully suppress sleep for up to a month after giving birth with no recorded physiological consequences (Lyamin et al., 2005). Importantly, vertebrate sleep stages are characterized by electrophysiological signatures measured with electroencephalography (EEG) (two process model), whereas *Drosophila* sleep is defined by behavioral criteria such as quiescence and increased arousal threshold (Hendricks et al., 2000; Shaw et al., 2000). Recent work has begun to investigate whether sleep in *Drosophila* is composed of distinct stages (Yap et al., 2017; Raccuglia et al., 2019; Tainton-Heap et al., 2021), which may account for variations in plasticity and responses to sleep loss discussed above. While mechanistic studies are not feasible in many of the species mentioned here, the range of contexts in which sleep need can be temporarily offset provides exciting opportunities to understand when sleep is required for plasticity (see **Table 1**).

Intrinsic Factors

Resilience to sleep loss can also be influenced by intrinsic factors that vary between individuals. Human subjects exhibit reliable, stable responses to repeated episodes of sleep loss, suggesting that sensitivity to sleep loss can be a durable trait over time (Dennis et al., 2017; Yamazaki and Goel, 2019). Naturally occurring genetic polymorphisms coincide with an individual's response to sleep loss in flies and humans (Viola et al., 2007, 2012; Donlea et al., 2012; Satterfield et al., 2015). In two of these studies, the same genetic alleles correlated with reduced cognitive impairments and dampened homeostatic sleep pressure after prolonged waking, indicating that the identified loci could contribute to protecting neural functions during sleep loss (Viola et al., 2007; Donlea et al., 2012). Interestingly, the identified human alleles in *per3* and *tnfα* that protected individuals from the consequences of sleep loss did not predominate in the subject populations, consistent with the possibility that these alleles are accompanied with susceptibility to other physiological challenges. Brain structure can also influence sensitivity to sleep loss; variation in functional connectivity between brain regions and hippocampal structure can predict the cognitive impact of sleep loss in human subjects (Yeo et al., 2015; Saletin et al., 2016). While the neural and molecular mechanisms that connect these variations with susceptibility to sleep are not yet known, studies of model systems provide some insights into pathways that might provide protection from insufficient sleep. *Drosophila* and mouse

studies have identified genetic pathways, including circadian rhythm (Mang et al., 2016; Ehlen et al., 2017), and metabolic factors (Thimgan et al., 2010, 2015), that can be manipulated to prevent rebound sleep following extended waking. It is important to note that each of these interventions can temporarily delay the accumulation of sleep debt, but it is unclear how long their protection persists and whether other consequences build as a result. Nonetheless, further examination of the external contexts and internal factors that can confer resilience to sleep loss may provide new insight into the neural functions of sleep and identify controllable interventions to facilitate rapid recovery from sleep loss.

CONCLUSION

In many contexts, sleep is vital for individuals to learn and adapt their behavior to best fit their environmental conditions. Sleep facilitates brain development and circuit refinement, and early life disruptions in sleep can result in long-lasting behavioral changes. Throughout the lifespan, sleep also impacts whether new memories can be effectively acquired and consolidated. While understanding the mechanisms that contribute to sleep-dependent plasticity remain an area of intense interest, many studies have already identified molecular and synaptic connectivity changes that occur during sleep to facilitate memory formation. More clearly identifying these mechanisms and developing strategies to manipulate them could open opportunities to support cognitive processing during sleep loss. Finally, individuals exhibit varying responses to sleep loss due to intrinsic and environmental factors. Understanding the benefits and detriments of variations in sleep, as well as the biological basis for inter-individual differences, will help resolve the function(s) of sleep and elucidate how sleep patterns affect future behavior.

AUTHOR CONTRIBUTIONS

JW and JD wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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The Consistency of Gastropod Identified Neurons Distinguishes Intra-Individual Plasticity From Inter-Individual Variability in Neural Circuits

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Gastropod mollusks are known for their large, individually identifiable neurons, which are amenable to long-term intracellular recordings that can be repeated from animal to animal. The constancy of individual neurons can help distinguish state-dependent or temporal variation within an individual from actual variability between individual animals. Investigations into the circuitry underlying rhythmic swimming movements of the gastropod species, *Tritonia exsulans* and *Pleurobranchaea californica* have uncovered intra- and inter-individual variability in synaptic connectivity and serotonergic neuromodulation. *Tritonia* has a reliably evoked escape swim behavior that is produced by a central pattern generator (CPG) composed of a small number of identifiable neurons. There is apparent individual variability in some of the connections between neurons that is inconsequential for the production of the swim behavior under normal conditions, but determines whether that individual can swim following a neural lesion. Serotonergic neuromodulation of synaptic strength intrinsic to the CPG creates neural circuit plasticity within an individual and contributes to reorganization of the network during recovery from injury and during learning. In *Pleurobranchaea*, variability over time in the modulatory actions of serotonin and in expression of serotonin receptor genes in an identified neuron directly reflects variation in swimming behavior. Tracking behavior and electrophysiology over hours to days was necessary to identify the functional consequences of these intra-individual, time-dependent variations. This work demonstrates the importance of unambiguous neuron identification, properly assessing the animal and network states, and tracking behavior and physiology over time to distinguish plasticity within the same animal at different times from variability across individual animals.

Keywords: injury, neuromodulation, RNA sequencing (RNA-seq), nudibranch behavior, electrophysiology, species differences, individual variability

INTRODUCTION

> "*Unexplained variation in behavior is weak evidence for noisy indeterminacy but strong evidence for unknown modulating factors.*"
>
> – Theodore Bullock (2000)

A goal of studying behavioral variability is to find the source of that variability in the neural circuits that control the behavior. Just as there can be individual differences in behavior, neural circuits can also exhibit individual differences. Even the simplest circuit contains a myriad of physiological and molecular components that are each subject to variability (Goaillard et al., 2009; Marder, 2011). Finding the "unknown modulating factors" in Bullock's words, can lead to a deeper mechanistic understanding of the function of the circuits. However, determining whether there are individual differences in neural circuitry is more challenging than noting differences in behavior because it requires making repeated measurements from the same circuit elements in multiple individuals. Having reliably identifiable neurons and synapses is required to distinguish whether those circuit components vary between individuals or if they are variable over time within an individual.

The nervous systems of gastropods, arthropods, annelids, and nematodes are well-suited for such repeated measurements because they contain individually identifiable neurons (Hoyle, 1975; Leonard, 2000; Brodfuehrer and Thorogood, 2001; Comer and Robertson, 2001; Katz and Quinlan, 2018). The size, number, location, anatomy, and neurochemistry of individual neurons are stereotyped among members of the same species, allowing the neural mechanisms underlying behaviors in some of these animals to be determined using multiple intracellular microelectrode recordings. The neurons are large and resist damage from multiple microelectrode penetrations, facilitating hours-long recordings and even multiple recordings of the same neuron over a course of days. The clear-cut identification of neurons also allows hundreds of recordings to be made from the same neuron in different animals.

Ironically, it is the consistency of the neurons that allows individual differences in neural circuits to be revealed; the identities of the individual neurons are so unambiguous that variations in their properties or synapses do not cause them to be mistaken for a different neuron. Furthermore, the presence of neurons is so highly conserved that the characteristics that are used for identification of a neuron from animal to animal in one species can be used to recognize the same neuron in other species (Croll, 1987; Newcomb et al., 2012). This allows the properties and connectivity of individual neurons to be compared across species, providing the opportunity for natural experiments regarding the functional significance of individual variation.

In this review, we highlight examples of intra- and inter-individual variabilities from the central pattern generator (CPG) circuits underlying swimming in two sea slugs, the nudibranch, *Tritonia exsulans* (formerly *Tritonia diomedea*), and the pleurobranchomorph, *Pleurobranchaea californica*. The work shows that differences that could be mistaken for variation between individuals can be attributed to differences in state of the neurons and synapses over time within an individual. There are also individual differences in the circuits that have no consequence for behavior under normal circumstances but affect the susceptibility of the circuit to a lesion. Without the consistency of neural identification and the ability to monitor neurons over several days, individual differences may appear as "noisy indeterminacy," rather than having causal factors that vary within an individual over time.

A BRIEF HISTORY OF RESEARCH ON VARIABILITY IN INVERTEBRATE NEURAL CIRCUITS

An early strategy employed to study the neural basis of behavior was to focus on behaviors that showed little or no variability, including rhythmic motor patterns produced by CPG circuits (Getting, 1986; Marder and Calabrese, 1996; Marder and Bucher, 2001). However, one of the principles that arose from this work is that even a simple, anatomically defined network can produce a variety of different motor patterns as a result of the neuromodulatory actions of amines and peptides. Amines, such as serotonin (5-HT) can alter membrane conductances and synaptic properties to change the dynamics of the network on a moment-to-moment basis (Harris-Warrick and Marder, 1991). Thus, it became important to identify not only the neurons in the network, but the state of the network to determine the mechanisms underlying various forms of the rhythmic output.

Similarly, research on identified neurons in invertebrates showed that properties of neurons and synapses could be modified by the history of activity though the circuit, leading to various forms of learning and memory (Carew and Sahley, 1986; Menzel and Benjamin, 2013). Thus, any study of the neural basis of individual variability must also take into account the history of neural firing and the history of previous experience of the animal.

Finally, another realization from electrophysiological research on invertebrate neural circuits was that even though circuits are composed of a small number of identified neurons, there are still multiple mechanisms that could produce the same output (Prinz et al., 2004; Rodriguez et al., 2013; Marder et al., 2016). Moreover, individual identified neurons display variations in membrane properties that are not well described by the mean of the population, which makes it difficult to model the circuit (Golowasch et al., 2002). Furthermore, individual differences found in neural circuits do not necessarily translate to individual differences in behavior (Marder, 2011; Marder et al., 2015). Although individual differences in neural circuits may have no consequences for behavior under standard conditions, they might differentiate the behaviors of two individuals when challenged with extreme conditions or injury (Marder and Rue, 2021). Thus, it is important to consider a range of conditions when

assessing the behavioral consequences of individual differences in circuit properties.

INDIVIDUALLY IDENTIFIABLE NEURONS COMPRISE THE CIRCUIT UNDERLYING *Tritonia* SWIMMING

The nudibranch, *Tritonia* provided one of the earliest examples of the roles of identified neurons in the production of behavior (Willows, 1967). The animal produces a stereotyped escape swimming behavior when attacked by a predatory sea star or when encountering a noxious stimulus (Willows and Hoyle, 1969). The escape swim response consists of a series of alternating dorsal and ventral whole-body flexions that lasts about 1 min (**Figure 1A**). The performance of the swim is robust: *Tritonia* reliably swims whenever it is stimulated.

A fictive swim motor pattern is reliably produced *ex vivo* by electrically stimulating a body wall nerve in an isolated brain preparation, allowing the neural basis for the stereotyped behavior to be studied (**Figure 1B**) (Katz, 2009; Katz and Sakurai, 2017). There are three bilaterally represented neuron types that form a CPG circuit (**Figures 1C,D**), which produces the bursting activity underlying the production of the rhythmic dorsal-ventral swim behavior. The three CPG neurons are: ventral swim interneuron-B (VSI), cerebral neuron 2 (C2), and three serotonergic dorsal swim interneurons (DSIs) (Getting, 1989a; Katz, 2009). Each neuron type is uniquely identifiable by its soma position, neuroanatomy, neurotransmitter phenotype, and activity pattern during the swim motor pattern (**Figures 1C,D**). The monosynaptic connections between these neurons have been determined using pair-wise intracellular microelectrode recordings (Getting, 1981). Modeling the properties of the neurons and their synaptic connectivity showed them to be sufficient to cause the rhythmic bursting pattern (Getting, 1989b). Thus, the *Tritonia* swim motor pattern and its neurons are consistent, allowing investigations into the presence and functional significance of variations.

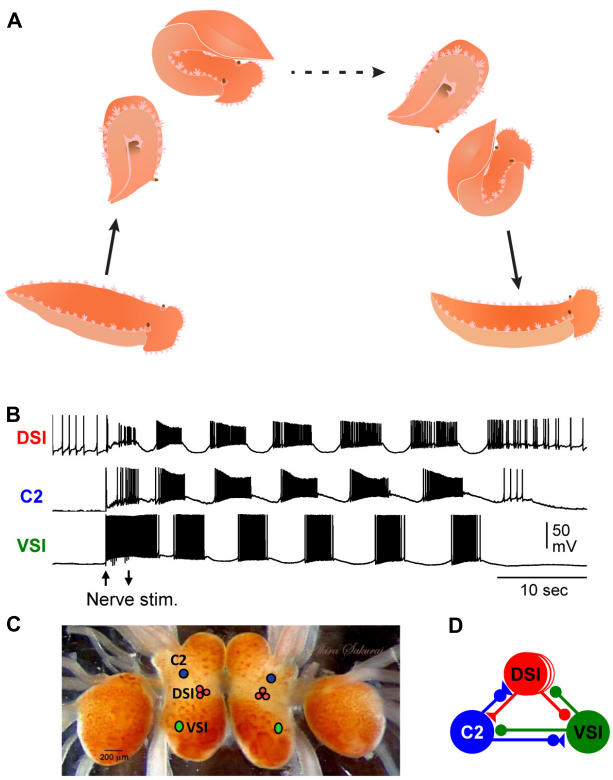

FIGURE 1 | *Tritonia* swimming behavior and swim motor pattern mechanism. **(A)** Illustration of *Tritonia* dorsal-ventral flexion swimming behavior. **(B)** Simultaneous intracellular recordings from identified neurons, DSI, C2, and VSI during a swim motor pattern elicited by stimulation of a body wall nerve (arrows). **(C)** A photograph of the dorsal side of the *Tritonia* brain, showing the locations of the swim interneurons (C2, DSI, and VSI). **(D)** A schematic diagram of the swim CPG. Triangles represent excitatory synapses, circles represent inhibitory synapses, and combined represent multicomponent synapses.

FUNCTIONAL CONSEQUENCES OF INDIVIDUAL DIFFERENCES IN SYNAPTIC CONNECTIONS ARE REVEALED BY NEURAL INJURY

As has been noted in other systems, the strengths of synapses between any particular pair of neurons can be highly variant with little or no effect on the behavioral output of the circuit (Goaillard et al., 2009; Roffman et al., 2012). Theoretically, it is understood that a fixed network topology may still have many solutions to produce the same output (Prinz et al., 2004; Onasch and Gjorgjieva, 2020). Although circuit variation across individuals may have no effect under "normal conditions," behavioral differences might emerge when the system is challenged by environmental changes (Marder and Rue, 2021).

Synapses in the *Tritonia* swim CPG show variation that does not have an effect on the motor pattern in a normal intact system, but causes individual animals to differ in their susceptibility to a midline lesion of the nervous system (Sakurai et al., 2014). Cutting the pedal commissure, which contains the axons of all three CPG neurons, disables swimming behavior in approximately half of the animals tested (Sakurai and Katz, 2009a). Similarly, about half of the isolated brain preparations fail to produce a swim motor pattern after the commissure is cut or action potential propagation is blocked (**Figures 2A,B**). Individual differences in the strength of the inhibitory synapse from C2 to VSI at the time of the lesion cause the differences in susceptibility (Sakurai et al., 2014). Under normal conditions, variation in the strength of this synapse has no effect on the swim motor pattern, but animals with a larger inhibitory component are susceptible to having the motor pattern fail after lesion.

There are also individual differences in recovery from this lesion (Sakurai and Katz, 2009a). The mechanism of recovery involves a reorganization of the CPG through recruitment of additional neurons and involves the activity of the serotonergic

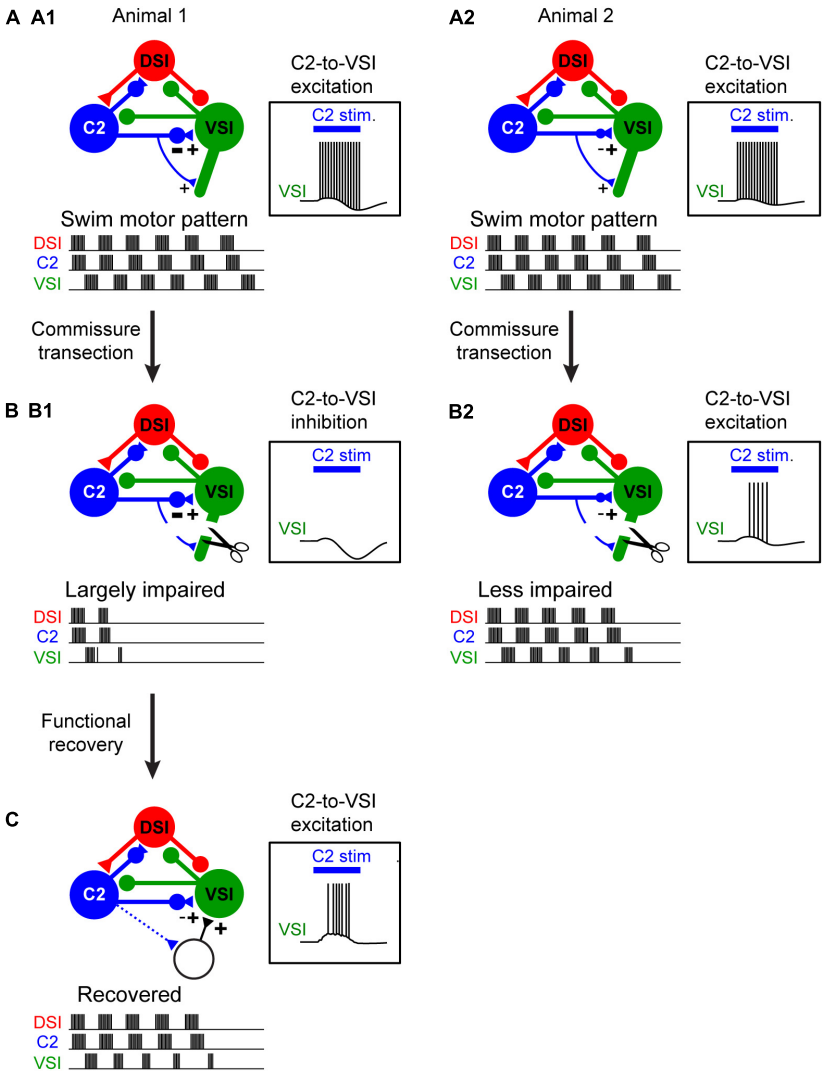

FIGURE 2 | The extent of injury-induced impairment in the swim motor pattern depends on the strength of a particular synapse. **(A1,A2)** Schematic illustration of differences in synaptic strength and motor output under normal conditions. Animal 1 (A1) and Animal 2 (A2) exhibit no apparent difference in the swim motor pattern. C2 synapses on VSI proximally and distally. The two animals differ in the strength of the proximal inhibition. But this has no effect on the ability of C2 stimulation (blue bar) to elicit a spike train in VSI. **(B1,B2)** When the distal synapse from C2 to DSI is cut, the motor pattern in Animal 1 is impaired because the proximal synapse had a strong inhibitory component and C2 fails to excite VSI (B1). However, the motor pattern in Animal 2 is less impaired because the proximal synapse was less inhibitory so C2 continues to excite VSI (B2). **(C)** Injury-induced loss of swim motor pattern is restored within a few hours by the recruitment of unidentified neurons with excitatory synapses to VSI (dotted lines). This recovery also involves the serotonergic DSIs (not illustrated).

DSIs (**Figure 2C**). The extent of recovery was correlated with the change in the depolarization in VSI caused by stimulating DSI and C2 together (Sakurai et al., 2016), implying that serotonergic modulation is involved in the recovery through an unknown mechanism. Neuromodulatory mechanisms have been implicated in recovery from injury in several invertebrate CPG networks across phyla (Puhl et al., 2018; Golowasch, 2019). A connection between injury responses and serotonergic neuromodulation has been proposed based on research in *Aplysia* (Walters and Ambron, 1995). Serotonin also has been implicated in recovery from spinal cord injury (Ghosh and Pearse, 2014; Huang et al., 2021). Although these types of lesions are not likely to occur under natural conditions, the plasticity itself is present and may play a role in maintaining circuit function over the lifetime of an animal.

The injury studies reveal that there can be variation in the system that normally is of no consequence to the behavior. Such hidden variation and its consequences would not have been revealed without the ability to monitor activity from the same neurons over days. The question arises as to whether the hidden differences that were identified are "noisy indeterminacy" or whether the apparent individual differences in synapses reflect the history of the

animal and thus may be an intra-animal difference that emerged over time.

VARIABILITY IN NEUROMODULATORY ACTIONS CAUSED BY SYNAPTIC STATE-DEPENDENCE

Neuromodulation is a means to achieve behavioral flexibility in neural circuits within an individual. It allows a structurally stable circuit to produce different patterns of activity by altering membrane and synaptic conductances (Katz and Calin-Jageman, 2008; Marder et al., 2014). Serotonergic neuromodulation alters motor patterns (Katz, 1995), modifies sensory responses (Sizemore et al., 2020), changes responses to social interactions (Cattaert et al., 2010), mediates learned responses (Brunelli et al., 1976; Jacobs and Gelperin, 1981), and plays a role in recovery from injury (Husch et al., 2012; Ghosh and Pearse, 2014). Neuromodulation has also been noted to be a source of variability between animals (Maloney, 2021). This occurs both in invertebrate circuits with identified neurons such as the stomatogastric ganglion of crabs (Hamood and Marder, 2014), but also in vertebrates, which are not constrained in the same way by the small number of neurons (Parker and Bevan, 2006; Sharples and Whelan, 2017). For example, in zebrafish, variations in serotonergic Raphe neurons cause individual differences in habituation of the acoustic startle response (Pantoja et al., 2016).

In the *Tritonia* swim circuit, serotonin plays an intrinsic modulatory role; it is released from the DSI and enhances the strength of synapses made by the other CPG neurons C2 and VSI (Katz et al., 1994; Katz and Frost, 1996; Sakurai and Katz, 2003). Computer simulations suggest that this neuromodulatory action is necessary for the network of neurons to produce its rhythmic pattern of activity (Calin-Jageman et al., 2007).

The effect of exogenous serotonin on VSI-evoked synaptic potentials was found to vary from individual to individual, sometimes potentiating the synapses and sometimes depressing them (Sakurai and Katz, 2003). The cause of this variability remained a mystery for almost 6 years until it was found that the action of serotonin and indeed the serotonergic DSI was dependent upon the firing history of the neurons that it was modulating (Sakurai and Katz, 2009b). VSI-evoked synaptic currents recorded in a ventral flexion neuron (VFN) exhibit their own homosynaptic potentiation (**Figure 3**); if VSI fires with a spike frequency of just 5 Hz for a few seconds, its synaptic output can increase up to twofold (**Figure 3B**). If a DSI is stimulated to release serotonin when VSI synapses are already potentiated, the serotonin causes the synapses to depotentiate (**Figure 3B**). Additional DSI stimulation has no further effect once the homosynaptic potentiation has been reversed. In addition, DSI heterosynaptically enhances VSI-evoked synaptic currents when stimulated shortly before VSI spikes, thereby increasing the VSI-evoked synaptic currents regardless of their potentiation state (**Figures 3C,D**; Sakurai and Katz, 2009b). In this case, the variability was not

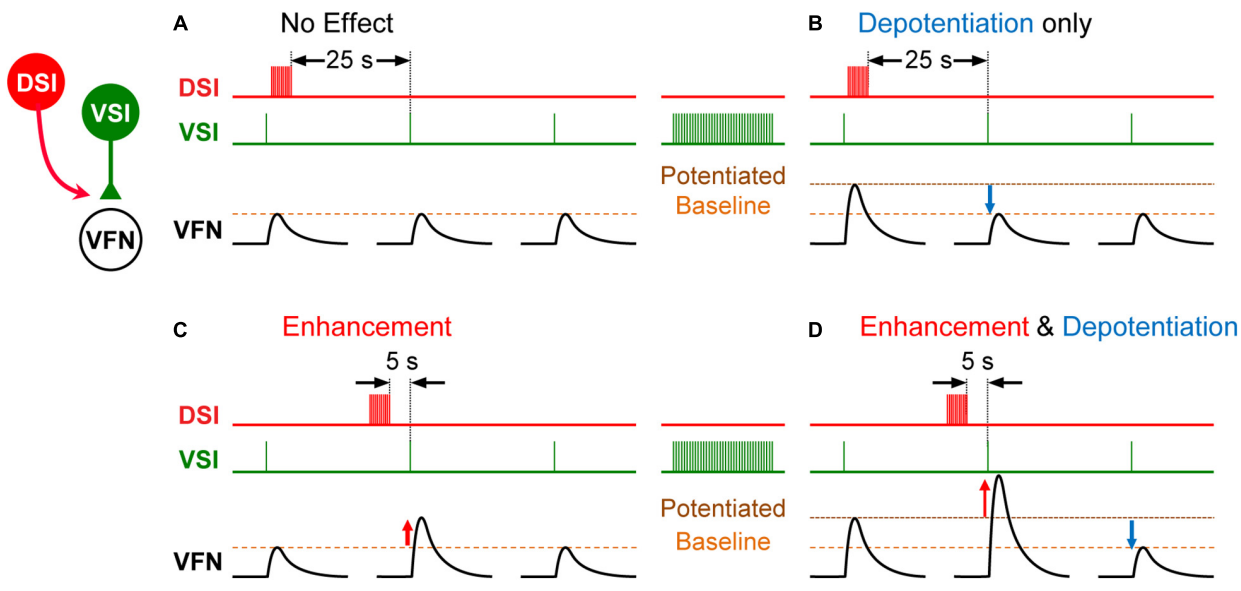

FIGURE 3 | Schematic representation showing state- and timing-dependent neuromodulation of synaptic strength by a serotonergic DSI neuron. **(A)** When the strength of the VSI-to-VFN synapse is at baseline, a DSI spike train delivered 25 s before the next VSI spike has no effect on the synaptic strength. **(B)** After a VSI spike train, the VSI synapse shows post-tetanic potentiation. When potentiated, a DSI spike train delivered 25 s before the next VSI spike depotentiates the synapse. **(C)** When the strength of the VSI-to-VFN synapse was at baseline, a DSI spike train delivered 5 s prior to the next VSI spike produces transient heterosynaptic enhancement (upward red arrow), which lasts for about 15 s. **(D)** When potentiated, a DSI spike train delivered 5 s prior to the next VSI spike produces transient enhancement and a subsequent depotentiation of the synapse. DSI spike train (red trace), VSI spikes (green trace), and the synaptic potentials in VFN (black traces) are shown in each. In each trace, single VSI spikes each produce EPSPs in the postsynaptic VFN neuron. Changes in the synaptic strength are indicated by dashed lines (baseline, potentiated).

inter-individual, it was an intra-individual state- and timing-dependent effect.

VARIABILITY IN BEHAVIOR AND NETWORK SIZE CAUSED BY BEHAVIORAL HISTORY

As it was necessary to know the state of individual neurons in order to assess the effects of neuromodulation, it also may be necessary to know the behavioral history of an individual animal to assess potential variability in how the network will respond to subsequent stimuli. In *Tritonia*, the strength of the swim response and size of the network underlying it vary depending on recent swim history. Although the *Tritonia* swim CPG may be consistent in its composition of neurons, the downstream elements that translate the rhythm into motor output vary. There are over 50 flexion neurons (FNs) that exhibit coordinated bursting that is driven by the CPG (Hume et al., 1982; Hume and Getting, 1982). A subset of FNs exhibit within-animal variability in their participation in the motor program from cycle-to-cycle and across swim episodes (Hill et al., 2012). This network variability may be reflective of some level of behavioral flexibility in this so-called fixed action pattern.

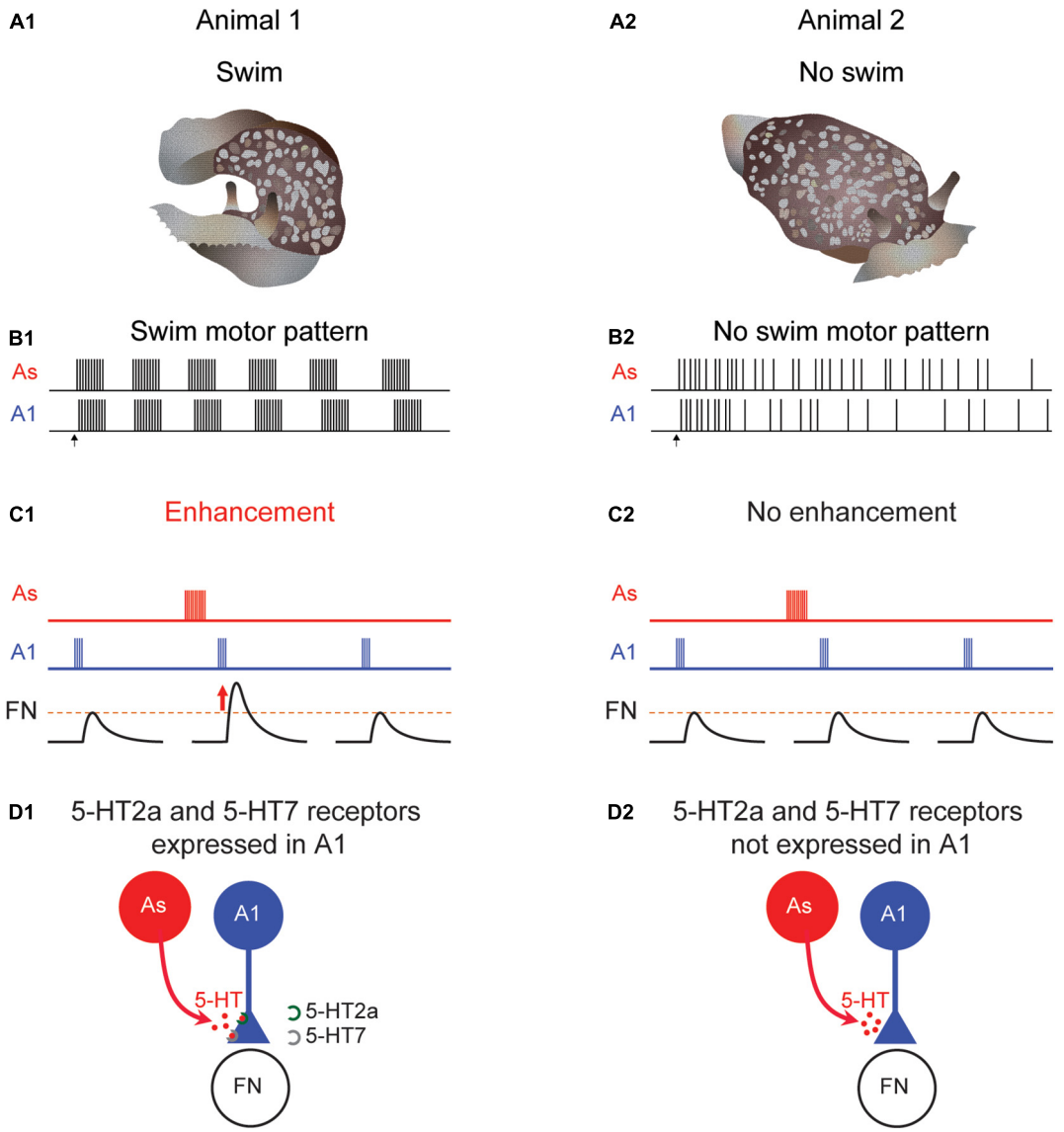

FIGURE 4 | Differences in *Pleurobranchaea* swimming, neuromodulation, and serotonin receptor expression. When tested, some animals swim **(A1)** produce a rhythmic swim motor pattern **(B1)**. At other times, an animal might not swim **(A2)** and would not produce a swim motor pattern **(B2)**. When the animal swims the A1 to follower neuron (FN) synapse is strengthened following serotonergic As stimulation in a swimming animal **(C1)**. But at times when the animal does not swim, there is no enhancement **(C2)**. Serotonin receptor subtypes 5-HT2a and 5-HT7 are present in preparations that produce a swim motor pattern **(D1)** but not in ones that do not **(D2)**.

The *Tritonia* escape swim is a rare event in the animal's life (Wyeth and Willows, 2006). An individual exhibits a consistent response when tested with a strong stimulus at long intervals. However, if stimulated a second time within 5 min, the swim response starts sooner and is more vigorous than after the first stimulus, indicating a form of sensitization (Frost et al., 1998; Hill et al., 2015). This sensitization is correlated with an increased participation of FNs. Stimulating the serotonergic DSIs also increases network size (Hill et al., 2015), plausibly by enhancing the synaptic strength of connections within the network. The participation of a subset of follower neurons is therefore not invariant, but a consequence of the history and activity of the CPG neurons.

VARIATION IN BEHAVIOR, NEUROMODULATION, AND GENE EXPRESSION IN *Pleurobranchaea*

In contrast to *Tritonia*, there is a great deal of individual variability in the generation of a swim response in *Pleurobranchaea* (**Figures 4A1,A2**). On any given day, fewer than 30% of the individual animals respond to strong noxious stimulus with a rhythmic swimming response (Jing and Gillette, 1995; Lillvis and Katz, 2013). However, when tested on different days, the same animal shows different propensities to swim. Furthermore, even when dissected from the animal, the isolated brain is similarly variable in the production of a fictive swim motor pattern (**Figures 4B1,B2**), indicating that the cause of that variability is in the brain and not the periphery. Thus, the apparent individual variability in behavior is not caused by inherent differences between individuals but is most likely temporal variability of each individual.

The swim CPG in *Pleurobranchaea* contains identified neurons homologous to DSI and C2, known as the As and A1 neurons, respectively (Jing and Gillette, 1995, 1999; Lillvis et al., 2012; Newcomb et al., 2012). As with *Tritonia*, the DSI homolog (As) enhances the strength of synaptic potentials evoked by the C2 homolog (A1) (**Figure 4C1**). However, unlike in *Tritonia*, the neuromodulatory effect is sometimes absent; neither As stimulation nor serotonin application causes a change in the amplitude of A1-evoked synaptic potentials (**Figure 4C2**). This variation correlates with the swim motor pattern; preparations that do not produce a swim motor pattern, also do not exhibit serotonergic enhancement of A1-evoked synapses (Lillvis and Katz, 2013). Thus, in this case, variation in the response to serotonin may be the cause of variation in behavior.

The variation in serotonergic neuromodulation is mirrored by differences in the expression of particular serotonin receptors (5-HTRs) in A1. Plucking out the somata of individual A1 neurons from preparations that either did or did-not exhibit the swim motor pattern allowed for single-cell gene expression comparisons. Using single-cell RNA sequencing and single neuron quantitative PCR, Tamvacakis et al. (2018) found that A1 neurons from individual *Pleurobranchaea* that swam expressed 5-HT2a and 5-HT7 receptor subtypes (**Figure 4D1**), whereas, A1 isolated from individuals that did not swim on the day of testing did not express any detectable 5-HT receptor subtype genes (**Figure 4D2**). This stands in contrast to C2 somata isolated from *Tritonia*, which consistently expresses both subtypes and which were consistently modulated by serotonin. It was the ability to unambiguously identity C2 and its homologs in different species (Lillvis et al., 2012) that allowed the mystery of neuromodulatory variability to be solved.

The cause of the fluctuations in gene expression in the *Pleurobranchaea* A1 neuron is still an open question. Although the factors that regulate gene expression have not been examined in this system, work from other systems suggests that regulation of gene expression is likely to be a common cause of neural circuit variation (Benowitz et al., 2018; Friedman et al., 2020). Temporal fluctuations in receptor gene expression may be representative of fluctuations of unknown regulatory factors, which may underlie the variability in genes, modulation, and behavior observed in *Pleurobranchaea*. This is consistent with a model that serotonin neuromodulation is responsible for creating the conditions that lead to the functional swim circuit. In the evolution of behavior and neural circuits, changes to the regulation of cellular expression of neuromodulatory receptors may be a more flexible point for natural selection to act on than other features of neurons (Katz, 2011, 2016).

CONCLUSION

Neural circuits, like behaviors, exhibit individual variability. There are several challenges for neuroscience with regard to such variability. One is to distinguish between consequential and inconsequential individual differences in neuronal and synaptic properties. Some differences might underlie behavioral variability under normal conditions, whereas others might not have any effect on behavior unless the system is stressed (Onasch and Gjorgjieva, 2020). Understanding the effects of individual differences in neural circuit function might help in predicting and possibly ameliorating differential outcomes in injuries and diseases (Prabhakaran et al., 2008; Burke Quinlan et al., 2015; Dopfel et al., 2019). Heritable differences between individuals in neural circuits are the fodder for natural selection. Such differences may accumulate in a population if they have no effect on circuit function under normal conditions, but might be adaptive if conditions change.

A second challenge is to determine whether observed differences in neural circuits are caused by individual idiosyncrasies or whether they represent variations in the histories or states of the individuals. The ability to record from identified neurons for extended periods of time in gastropods has shown the extent to which the properties of individual neurons and synapses can vary within just a week; it is likely that over the course of a lifetime these properties could vary even further. Whether such intra-individual variability is commonly mistaken for inter-individual variability is an open question. Where possible, longitudinal studies of behavior and circuit properties will be necessary to determine whether this is the case.

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Social Determinants of Inter-Individual Variability and Vulnerability: The Role of Dopamine

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Individuals differ in their traits and preferences, which shape their interactions, their prospects for survival and their susceptibility to diseases. These correlations are well documented, yet the neurophysiological mechanisms underlying the emergence of distinct personalities and their relation to vulnerability to diseases are poorly understood. Social ties, in particular, are thought to be major modulators of personality traits and psychiatric vulnerability, yet the majority of neuroscience studies are performed on rodents in socially impoverished conditions. Rodent micro-society paradigms are therefore key experimental paradigms to understand how social life generates diversity by shaping individual traits. Dopamine circuitry is implicated at the interface between social life experiences, the expression of essential traits, and the emergence of pathologies, thus proving a possible mechanism to link these three concepts at a neuromodulatory level. Evaluating inter-individual variability in automated social testing environments shows great promise for improving our understanding of the link between social life, personality, and precision psychiatry – as well as elucidating the underlying neurophysiological mechanisms.

Keywords: dopamine, variability – individual, vulnerability, social behavior, micro-society, ethological analysis

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INTRODUCTION

Inter-individual variability refers to differences in the expression of one or more behaviors between members of a population. For instance, some people express a shyer attitude than others, take more risks, or are more attracted to immediate gains. This variability is also evident in the way one responds to environmental and social challenges, resulting in a heterogeneous expression of emotional, cognitive, and task-related behaviors; and underlies, in particular, the emergence of distinct strategic approaches (i.e., how agents find different solutions to the same problem). In human studies, such behavioral variations have long been associated with the notion of personality (McAdams and Pals, 2006). In animal research, however, behavioral variability has largely been considered as unwanted noise, or as an experimental confound, and thus disregarded. But the consistency of these inter-individual differences across time and contexts has become harder to overlook, and it is now generally acknowledged that animal personalities are ubiquitous, quantifiable, and biologically meaningful (Sih et al., 2004; Bach, 2009; Bergmüller and Taborsky, 2010; Duckworth, 2010; Pennisi, 2016).

While the concept of personality in animals is now increasingly accepted, the mechanisms underlying the generation of inter-individual variability are still poorly understood and a major

current topic in adaptive personality research (Sih et al., 2015). Ecologists have framed the significance of this process from a genetic point of view, proposing that the mechanisms driving individual variability may play a role in evolution by helping segregate species into subpopulations (Pennisi, 2016). However, many teams have observed that even under controlled laboratory conditions, behavioral expression varies much more than expected between virtually genetically identical individuals (Buchanan et al., 2015; Stern et al., 2017; Tuttle et al., 2018), suggesting a key role of the environment in driving individuation processes (Lathe, 2004; Stamps and Groothuis, 2010). Behavioral differences between individuals have been linked with variance in their physiology [e.g., body size, metabolism, neurophysiological properties (Dingemanse and Wolf, 2010)], in local environmental factors (particularly the distribution of resources, such as food, shelter, and breeding opportunities), and in their life history. The latter critically relies on brain plasticity properties, which encode an individual's experiences to shape their response to upcoming environmental challenges in a cumulative manner, thus supporting the behavioral divergence of initially genetically identical mice (Freund et al., 2013). Another point of view is that individuality is an unpredictable outcome of developmental processes (Stern et al., 2017; Wolf et al., 2017; Honegger and de Bivort, 2018). In this stochastic developmental variability hypothesis, individuation results from the accumulation of differences during development that, in turn, generate structural variations in neural connectivity patterns and capacity for plasticity, which then remain stable through adult life (Buchanan et al., 2015; Stern et al., 2017; Wolf et al., 2017; Honegger and de Bivort, 2018). This view is consistent with the definition of individuality as characteristic behavioral traits that persist over a lifetime (Honegger and de Bivort, 2018).

These alternative perspectives on inter-individual variability do not necessarily contradict each other, instead they highlight that distinct forms of individual adaptation or plasticity may operate over different time scales. The influence of the environment on the development of inter-individual variability and personality is most often discussed in terms of a developmental process. In this review we focus instead on the highly dynamic individuation processes that occur across the lifetime as an adaptation to proximal environments, and in response to social interactions in particular. We define adaptation as an animal's flexible adjustment of their behavior over time, in response to situations and by using the cumulative knowledge of their previous experiences. The role of underlying neural components in individuation has been framed in terms of continued developmental processes [e.g., adult hippocampal neurogenesis (Kempermann, 2019)]. Here, we examine the growing evidence that changes in the activity of neuromodulatory networks link social influences with adaptations to egocentric (i.e., non-social) behaviors in adult animals. While multiple neuromodulators are likely involved, we focus here on modifications in dopaminergic circuits, which have been strongly linked to the individualistic expression of exploration behavior. Finally, we discuss how these views, in which circuits are changed through adaptation, can improve our understanding of the link between behavioral trait expression and vulnerability or resilience to psychiatric illness. Each of these aspects will be explored from the perspective of rodent micro-society behavioral paradigms, which are generally large, controlled environments where rodents live in groups (their "micro-society") with automated capture of behavioral information over long periods of time. These testing environments are increasingly developed in neuroscience research laboratories and provide exceptional insight into both naturalistic social interactions and inter-individual behavioral variability.

DEFINING AND MEASURING INTER-INDIVIDUAL VARIABILITY: FROM EXPERIMENTAL CONFOUND TO EXPERIMENTAL OUTCOME

It is easy to recognize qualitatively and anecdotally that each individual is unique in the way it behaves, an idea that incorporates two seemingly contradictory quantitative aspects of behavioral variation. On one hand, intra-individual variation encompasses the diversity in behaviors and actions that occur within the same individual over time and context (for example, an individual facing a binary choice may once choose the first option and another time the second one, or *vice versa*). On the other hand, inter-individual variability can be conceptualized in some ways as the invariability of behaviors. For example, when repeatedly faced with a binary choice, some individuals choose the first option 80% of the time on average, while others will choose the first option on average only 40% of the time. In this sense, inter-individual variation produces a stable behavioral repertoire that characterizes an individual and distinguishes it from its conspecifics (**Figure 1A**). This idea has consequences regarding how behavior is analyzed, as the bulk of behavioral experiments have been designed in ways that ignore or minimize inter-individual variability, stemming both from conceptual limitations and from technical constraints. We argue that, instead, acknowledging and assessing inter-individual variability can clarify the relationships between brain and behavior, as well as between behavioral adaptation and variation. Incorporating measurements of inter-individual variability in behavioral outcomes can be simplified by using large, automated testing environments, such as those that support the study of rodent micro-societies, thus we also discuss some of the advantages and challenges that these environments provide.

The distinction between inter- and intra-individual variability goes against traditional behavioral analysis framework that uses the behavior of the group to derive an average "individual", and to establish the standard deviation from this norm (Bennet, 1987). In this approach, one considers that the information accumulated about populations applies uniformly to their constituent individuals: in other words, sampling a behavior across multiple subjects at the same timepoint would be conceptually the same as using repeated measurements on a single subject, thus there is no need to distinguish between inter- and intra-individual variability. This approach has been heavily exploited to allow

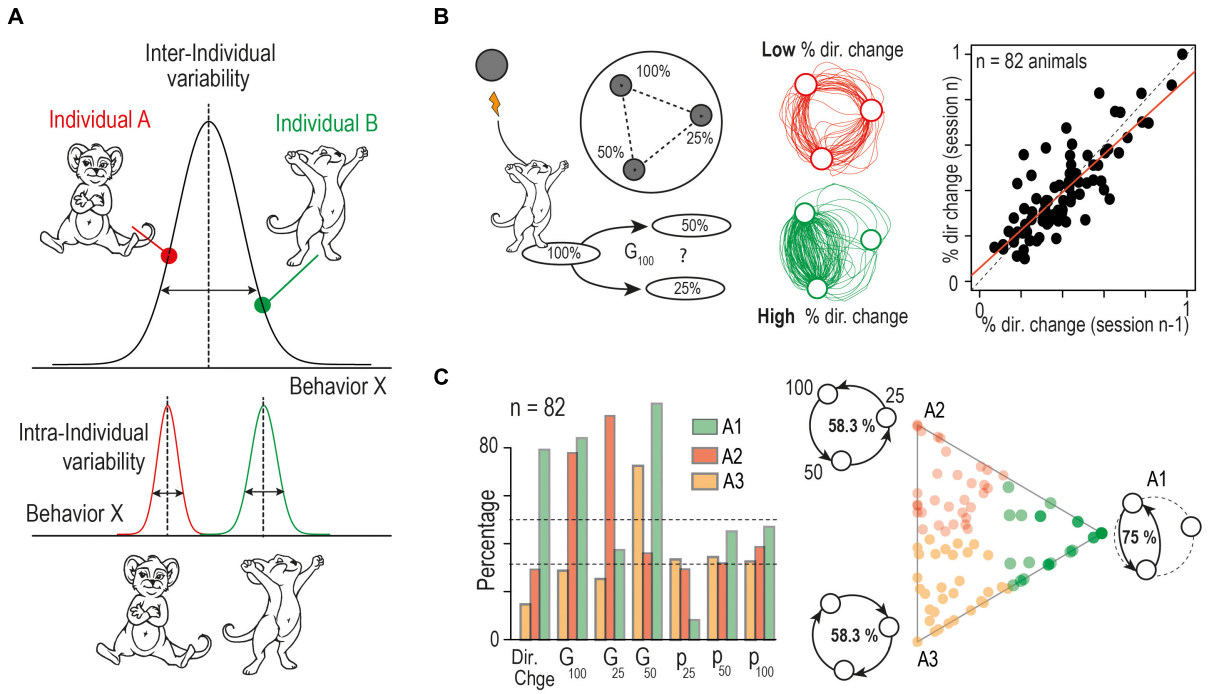

FIGURE 1 | Theoretical framing and experimental distinction between inter- and intra- individual variability. **(A)** Defining intra and inter-individual variability: the frequency and intensity with which an individual exhibits a specific behavior defines a trait. The estimate of the trait expression at the population level defines inter-individual variations. Repeated measurements on individual subjects allows the estimation of intra-individual variations in trait expression. **(B)** (*Left*) Schematic of a behavioral decision-making paradigm where inter- and intra-individual variability is apparent. Mice are placed in a circular open-field with three equidistant targets that are associated with a given probability (100, 50 or 25%) of intracranial stimulation reward delivery when the animal is detected around the target. The animal cannot receive two consecutive stimulations from the same target; thus they must make a sequence of binary choices between the targets. The trajectories and choice or each mouse are quantified using, (i) the percentage of directional change (i.e., returning to the previous target, % dir. change), (ii) the probability to visit each point (P_{100}, P_{50}, P_{25}) and (iii) the probability to choose the option with the highest probability of reward for the three possible "gambles": G_{100} = choice of 50% over 25%, G_{25} = choice of 100% over 50 and G_{50} = choice of 100% over 25%. (*Center*) Example of two trajectories obtained from two different mice across a 5 min session showing individualistic strategies where the mouse in the top trace shows a low propensity for changing direction, resulting in a more circular trajectory, while the mouse in the bottom trace shows a high percentage of directional change, focusing on the most rewarded options. (*Right*) Analysis over concurrent sessions indicates consistent differences in behaviors between mice indicative of low intra-individual variability and high inter-individual variability. Correlation between the percentage of directional changes for two consecutive sessions shows a strong stability between strategy across sessions within individual mice, while behavior between subjects remains variable. **(C)** Archetypal analysis: (*left*) Plot of the three archetypal solutions, and their seven basic variables (see **B**). *Left*: Visualization of the α coefficients using a ternary plot. Each point represents the projection of an individual onto the plane defined by a triangle where the three apices represent the three archetypes (A1, A2, A3). Points are color-coded according to their proximity to the archetypes. Schematic at the three apices illustrate the main behavior of the three archetypes. A1 corresponds to a mouse that alternates only between p_{100} and p_{50}. Such a mouse would reach a 75% success rate. In contrast, mice using a purely circular strategy would have a 58.3% success rate. They either turn in a descending manner (A2: sequence $p_{100} - p_{50} - p_{25}$) or an ascending manner (A3: sequence $p_{25} - p_{50} - p_{100}$). Experimental mice fall somewhere within the boundaries of these extreme behaviors. (Modified from Dongelmans et al., 2021).

meaningful between-group comparisons, particularly in animal research where individuals can indeed largely be considered as identical except when specific conditions are manipulated (e.g., environmental or genetic modifications). However, by assuming that each subject can be described by the behavior of the group, this approach masks the different contributions of intra- and inter-individual variability to overall phenotypic variability. To reveal their balance, one needs to explicitly compare intra- and inter-individual variances by performing repeated measurements on each subject within a testing group (**Figure 1B**). If the multiple expressions of a behavior in the same individual follow their own distribution, the estimates of the inter-(V_{inter}) and intra-individual (V_{intra}) variances will differ, with V_{inter} being greater than V_{intra}. Efforts to standardize these methods to improve study design and interpretation have yielded several measures. For example, the repeatability index (R) has been proposed as a standardized measurement of phenotype consistency across time or contexts. It corresponds to the proportion of the total phenotypic variance [defined as the sum of the inter-individual (V_{inter}) and intra-individual (V_{intra}) variances] that can be attributed to inter-individual variance: $R = V_{inter}/(V_{inter} + V_{intra})$ (Nakagawa and Schielzeth, 2010; Roche et al., 2016). Similarly, analyzing the cumulative value of an estimator over a long-term experiment [e.g., an estimator of exploration level, Freund et al. (2013), Torquet et al. (2018)] highlights the coherence in behavioral differences between individuals across time. For example, a mouse with a low exploration level may increase it over time, but it will typically remain at a lower level than

its congeners. Overall, these approaches demonstrate that intra-individual variation is generally smaller than inter-individual variation, indicating consistency over time in the behavior of individuals, and arguing against "population" assumption in behavioral experiments. Indeed, by defining stable behavioral traits in an individual, relationships can be revealed between the expression of this trait and of other behaviors, physiological characteristics, or brain activity; which may otherwise have remained hidden in a purely group-based analysis.

Time scale is another important consideration for distinguishing between inter- and intra-individual variability in behavioral testing and analysis. Classic tests used to measure individual characteristics or traits often focus on specific behaviors only observed over a short time scale, generally on the order of minutes. Appropriately distinguishing between inter- and intra-individual variability requires instead the generation of repeated measurements over long time frames, on the order of weeks to months. This allows the accumulation of information regarding how an individual behaves in response to the same stimuli over time, which allows not only the estimation of intra-individual variability, but to also estimate inter-individual variations in behaviors and establish individualistic profiles. These longitudinal experiments necessitate the transition to automatic testing and processing, which is already supported in principle and/or in practice by a number of neurobiologists (Dell'Omo et al., 2000; Gerlai, 2002; Vyssotski et al., 2002; Spruijt and DeVisser, 2006; Sandi, 2008; Schaefer and Claridge-Chang, 2012). This idea has led to the development of complex housing environments for laboratory mice (Freund et al., 2013; Shemesh et al., 2013; Weissbrod et al., 2013; Torquet et al., 2018) that allow the integration of automated behavioral testing. These apparatuses engender several significant advantages over traditional testing methods: rodent behaviors can be evaluated without isolating an individual from its social group, measurements for several behavioral parameters can be simultaneously captured, and *post hoc* analyses of behavioral correlations can be used to construct an individual estimator defining each subject (Freund et al., 2013; Torquet et al., 2018; Forkosh et al., 2019).

Longitudinal video tracking of animals over long periods presents its own challenges, namely definitively identifying each individual and correctly assigning their behavioral variables. A single case of mistaken identity would call into question the validity of all the results acquired from months of work. Different solutions to this problem have been proposed. One of them is to dye the fur of the animals with different colors (Shemesh et al., 2013; Forkosh et al., 2019). Another one is to implant radio-frequency identification (RFID) transponders under the skin of the mice to assign an ID number to each subject, while detectors built into the environment can track the identity of mice and confirm or correct video tracking (de Chaumont et al., 2019). Many systems are now able to evaluate the specific postures of individual (e.g., locomotion, self-grooming) or interactive behaviors between two or more identified individuals [e.g., nose to nose contact, playing, aggression, peer grooming, de Chaumont et al. (2012, 2019), Mathis et al. (2018)]. On-line position tracking or *post hoc* pose estimation overcome traditional challenges that result from relying on observer scoring to establish and analyze behavioral patterns. Advances in these technologies are poised to drive the implementation of automated and standardized analysis of behavioral repertoires, which are holistic compilations of behaviors described by an observer, and can be considered to be built at their most basic level from positional changes of an animal over time.

While these automated testing environments generate large data sets, classification and dimension reduction methods can be used to compact this information in order to isolate behavioral domains and to establish correlations between them (Brown and de Bivort, 2018). Clustering methods such as *k-means* or principal component analysis are the most commonly used to discriminate average behaviors, where an individual's distance to each cluster describes the relationship of its behavior to that of its congeners. While these methods have been classically used to aggregate individual data onto typical observations represented by the center of a cluster, they are not the only approaches to analyze behavioral repertoires: archetypal analysis depicts individual behavior instead as a continuum within an "archetypal landscape" defined by "pure" or "archetypal" behavioral patterns. With this method, the most extreme or specialized behavioral profiles possible from the entire data set are first defined as the archetypes. The number of archetypes and their associated behavioral patterns are derived from the dataset in an unsupervised manner, and each individual's behavior can then be described as a convex mixture of archetypal profiles (Cutler and Breiman, 1994; Shoval et al., 2012; Forkosh et al., 2019; Dongelmans et al., 2021). The individuals can be assigned to the archetype that best describes their behavior for experimental grouping purposes, rather than defining groups by an arbitrary threshold on any one continuous variable. For example, strong and stable individual strategies emerge in a decision-making task where mice are required to move between three sites with different probabilities to receive rewarding electrical stimulation (**Figure 1B**). Archetypal analysis uses the key choice parameters from the task to reveal the three most extreme possible strategies: alternating exclusively between the two options with the highest probability of reward (A1), purely traveling in a circular pattern moving from the highest to lowest probability of reward (A2), or from the lowest to highest probability (A3). Therefore, individual behaviors find themselves somewhere between these extreme strategies, and can be defined as a linear combination of each archetype (**Figure 1C**; Dongelmans et al., 2021). These approaches have important consequences for introducing the notion of "personalized" behavioral assessment: by allowing the dissection of the contribution of inter- and intra-individual variability to phenotypic variability, they challenge classic approaches based on the analysis of average group behavior measured at a given moment.

Finally, the implementation of these semi-natural and social testing environments increases the complexity of the research questions that can be addressed, in particular raising questions about (i) how these environments promote the emergence of individual behavioral variability (Kempermann, 2019), and (ii) how animals living in a micro-society deal with complex and ethologically valid decision-making problems. These problems

are defined by the particular conditions of their habitat, notably the food distribution and the social milieu (Dell'Omo et al., 2000; Mobbs et al., 2018). Foraging for food is, for instance, a very important aspect of animal life, and represents one of the basic mechanisms studied in neuroeconomics (Hayden and Walton, 2014; Hayden, 2018; Mobbs et al., 2018). In such closed-economy paradigms, commodities (food or other rewards) are present at all times, in contrast to standard laboratory tests. Thus, the initiation and termination of consummatory behaviors are defined solely by the animal (Timberlake and Peden, 1987; Rowland et al., 2008; Beeler et al., 2010), which significantly modifies our conceptual framing of reward studies. In these environments the dependent variables are rather defined by the sequence of reward related-behavior and the amount of time budgeted by the animal for each of its activities, than by the amount of reward earned by an individual in a restricted amount of time. Social interactions will also constrain the expression of foraging behaviors. An isolated animal must invest time and resources to explore and search for food, while being part of a group may open new opportunities or responsibilities. On the one hand, an individual within a group, may be able to wait for others to find food, on the other hand the group could instead exploit this individual, redistributing the food it has foraged for itself among group members (Barnard and Sibly, 1981; Giraldeau and Dubois, 2008). Overall, foraging for food drives the development of a large number of social interactions, whether cooperative or exploitative, and promotes the development of individual strategies (discussed in detail below).

Once considered nothing more than noise, inter-individual variability is increasingly considered measurable and meaningful, particularly thanks to conceptual and technical advances in behavioral data collection and analysis. The adoption of large, automated testing environments allows the tracking of individual mouse behavior within a micro-society living in a complex environment over long periods of time. In that context, both the processes operating within individuals as well as those operating between individuals at the population level can be described (Giraldeau and Dubois, 2008). These advances are driving new perspectives in understanding behavior and its relationship to underlying neurocircuitry.

THE ROLE OF THE SOCIAL ENVIRONMENT IN THE DEFINITION OF AN INDIVIDUAL

Standard laboratory housing consists in relatively impoverished environments that significantly restrict social contact, housing rats most often in pairs and mice in small groups of up to four congeners (Würbel, 2001). Rodents are, however, social animals; this is aptly evidenced by their repertoire of various interactive behaviors – such as physical contact, vocal communication, aggression, social recognition, imitation, and empathy – that can be considered as hallmarks of sociability, an important personality trait (Gartland et al., 2021). In the wild, mice live in small breeding subpopulations (demes) of 2 to 12 adult members (Crowcroft, 1966; Berry and Bronson, 1992; Beery and Kaufer, 2015) that share territorial defense, while rats generally live in larger colonies that may be divided into smaller sub-groups as a function of resources (Beery and Kaufer, 2015; Schweinfurth, 2020). The structure of rodent groups is highly malleable, with both the size and membership liable to change with resource availability, social competition, or death from predation or disease (Radchuk et al., 2016; Andreassen et al., 2021). The social environment of a rodent is therefore in constant evolution, requiring continual surveillance in order to behaviorally adapt to its changing demands (Webster and Ward, 2011). Adaptations in an individual's behavior can also impact the social structure of the group; driving, in turn, downstream behavioral adaptations in other group members. Understanding the reciprocal interplay between individual behavior and the social environment is therefore crucial to gain insight into how individuals can be behaviorally defined, how their traits are encoded at a neural level, and how these aspects shape their responses to environmental challenges – whether social or not. However, studying fine-scale behavioral interactions in wild rodent populations is challenging (Hughey et al., 2018), considering their large territorial range and the inability to control for genetic or environmental factors (Berry and Bronson, 1992; Macdonald et al., 1999). On the other hand, containing rodents into standard laboratory housing can mask their behavioral profiles. To solve these issues, environmental enrichment can be used, which has proven to widen the set of behaviors rodents can express (Blanchard and Blanchard, 1988; Zocher et al., 2020), as well as the implementation in the laboratory of large, automated testing environments where mice or rats can live in micro-societies (groups that range in size from one to several dozen individuals) under semi-naturalistic conditions (Alexander et al., 1978; Freund et al., 2013; König et al., 2015; Torquet et al., 2018).

Importantly, when rodents live in micro-societies within a closed and enriched naturalistic environment, strong and stable inter-individual variability in behavior emerges, even among isogenic animals. Early studies, such as Rat Park (Alexander et al., 1978), found that rats living in complex social environments show behavioral differences from those in isolated or standard laboratory conditions, but interpretations of these initial studies are limited due to small numbers of animals and few data points (Gage and Sumnall, 2019). More recent studies using large groups of animals with automated data collection have yielded interesting results concerning inter-individual variations. For instance, when forty isogenic mice were placed in a complex environment over a period of months, significant individual differences in explorative behavior and active coverage of the territory, defined as the distribution of space that each animal occupies, were discovered (Freund et al., 2013). This spatial exploratory behavior was negatively correlated with social exploration and play behaviors estimated using manual assessment (Freund et al., 2015), while it was positively correlated with hippocampal neurogenesis (Freund et al., 2013). Our group has developed a semi-naturalistic environment called Souris-City (**Figure 2A**), where groups of ten mice can undergo an extended behavioral analysis over long periods of time (>1 month). Automatic capture of a

large spectrum of behaviors over these longitudinal experiments demonstrates that individualistic behavioral patterns also emerge in these smaller groups of animals, with differences observed in spatial exploration and social behaviors. The Souris-City environment uses a series of RFID-sensing gates to allow the testing of individual cognitive abilities while subjects are temporarily separated from the group (Torquet et al., 2018). Thus, we can clearly distinguish between personality traits (expressed when the animal is alone) and behaviors that could be the direct consequence of a group interaction. The cognitive testing compartment of Souris-City consists of a T-maze, where each side can deliver different drinks. Mice are asked to choose between two different drinks (e.g., water and sucrose), and the position of the bottles is then inverted every three-to-four days, allowing the evaluation of their choice behavior and preferences for each subject. The position of the bottles is then inverted every three-to-four days, allowing for the evaluation of their choice behavior. Interestingly, several subgroups of stable and distinctive patterns of choice strategy consistently emerge, even though animals have low genetic diversity (Torquet et al., 2018). Some individuals systematically track the sucrose solution, while others are more likely to choose the same side of the T-maze, regardless of the drink presented (**Figure 2B**). These different patterns of choice strategy correlate with differences in social and spatial exploratory behavior in the main environment, and with differences in the spontaneous firing of dopaminergic neurons in reward circuits (Torquet et al., 2018). Strikingly, modifying the social environment by regrouping together individuals with a similar initial phenotype (**Figure 2C**) resulted in a fast re-distribution of individual traits, as well as adaptations to the firing pattern of their dopamine neurons. In other words, stable individual behavioral strategies can rapidly change in response to social challenges. This suggests that the dynamic effects of social interactions between individuals generate social specialization and reveal inter-individual differences in various, not necessarily social, behaviors.

The inter-individual variability that emerges in large environments may arise from different social regulation mechanisms. For example, dominance hierarchy within social groups is a naturally occurring and evolutionarily conserved phenomenon which readily emerges in group-housed male or female rodents (van den Berg et al., 2015; Kondrakiewicz et al., 2019). In mice, hierarchy usually develops within a few days and remains stable over weeks (Wang et al., 2011; Williamson et al., 2016). When unfamiliar mice are grouped in a tetrad, they establish a dominance hierarchy that can be analyzed by different pair contests, such as a warm spot occupancy test, territorial urine marking, or by evaluation in the tube-test where one mouse must yield to the other to exit the tube (Wang et al., 2011, 2014; Larrieu et al., 2017; Zhou et al., 2018). An important consequence of the interactions between rodents, and, in particular, of hierarchical organization, is that individuals seem to display markedly different behaviors depending on their social status. In laboratory conditions, dominant animals are more anxious (Larrieu et al., 2017), have higher social interactions, and better social memory (Battivelli et al., 2019) than subordinates. In larger groups of 10–12 mice, dominant males engage further in aggressive behavior, while subordinates modify their foraging behavior to avoid congeners by which they have previously been aggressed (Lee et al., 2018). Interestingly, in wild rodents, the configuration of colonies and overlap of territories may require an individual to act as dominant in its own territory, but perhaps as subordinate when confronted with challengers from other territories (Koolhaas et al., 1987), suggesting that hierarchical position is flexible and context-dependent. Social conflicts and aggressiveness are crucial to determine social status and access to resources, but indeed come at significant energetic cost. This leads individuals to adjust their behavior to reduce conflict, with some specializing in dominance, while others specialize in exploration and vigilance (Bergmüller and Taborsky, 2010). This process, known as "social niche specialization", applies to all group members. It provides an adaptive explanation for the existence of hierarchy, division of labor, and individuality within a rodent group (Bergmüller and Taborsky, 2010).

The division of labor is a property that emerges in social groups. It can take different forms but mainly consists in a specialization in the execution of tasks: not everyone participates in all aspects or stages of a production process. It is an important feature of complex biological systems, particularly in social groups (Cooper and West, 2018), and it is also an active mechanism of individual differentiation (Loftus et al., 2021). Studying the division of labor at the level of resource acquisition in rodent social groups opens up very interesting perspectives for understanding the mechanisms of individuation, as it is indeed a process that allows the emergence of distinct strategies. Division of labor is well-illustrated by the observation of the coexistence within social groups of "producers" that work to search for and acquire food, and "scroungers" that subsist off of what other group members provide (Barnard and Sibly, 1981). For example, when a group of rats is placed in an apparatus where food is delivered by pressing a lever accessible to all, the "producers" press the lever while the "scroungers" simply eat the food delivered while others are pressing the lever (Oldfield-Box, 1967; Ahn et al., 2021). A similar division of labor also appears in experiments in which rats organize themselves to respond collectively to the increasing difficulty of reaching food by diving in a water-submerged corridor (Grasmuck and Desor, 2002). When in a group, some rats readily dive to fetch food, while some animals do not dive, despite successfully diving for food when alone in the apparatus, and instead they obtain their food from the others. This behavior raises the question of whether the "scroungers" are stealing food from their diving counterparts, or are these diving "producers" driven to provide for all of the members of the group, i.e., could this behavior be altruistic, or simply a process of domination? Interestingly, when the divers have the opportunity to stay in a separate place to consume the food alone, some still decide to return to the group location where the food they bring back will be eaten also by non-diving rats (Grasmuck and Desor, 2002). The proportion between "producer" and "scrounger" rats depends on the size of the group (Alfaro and Cabrera, 2021), but, overall, the repartition in each group reflects a collective behavioral balance based on contingencies between animals' individualities and social context. Similar profiles emerge in mice when they must carry food across

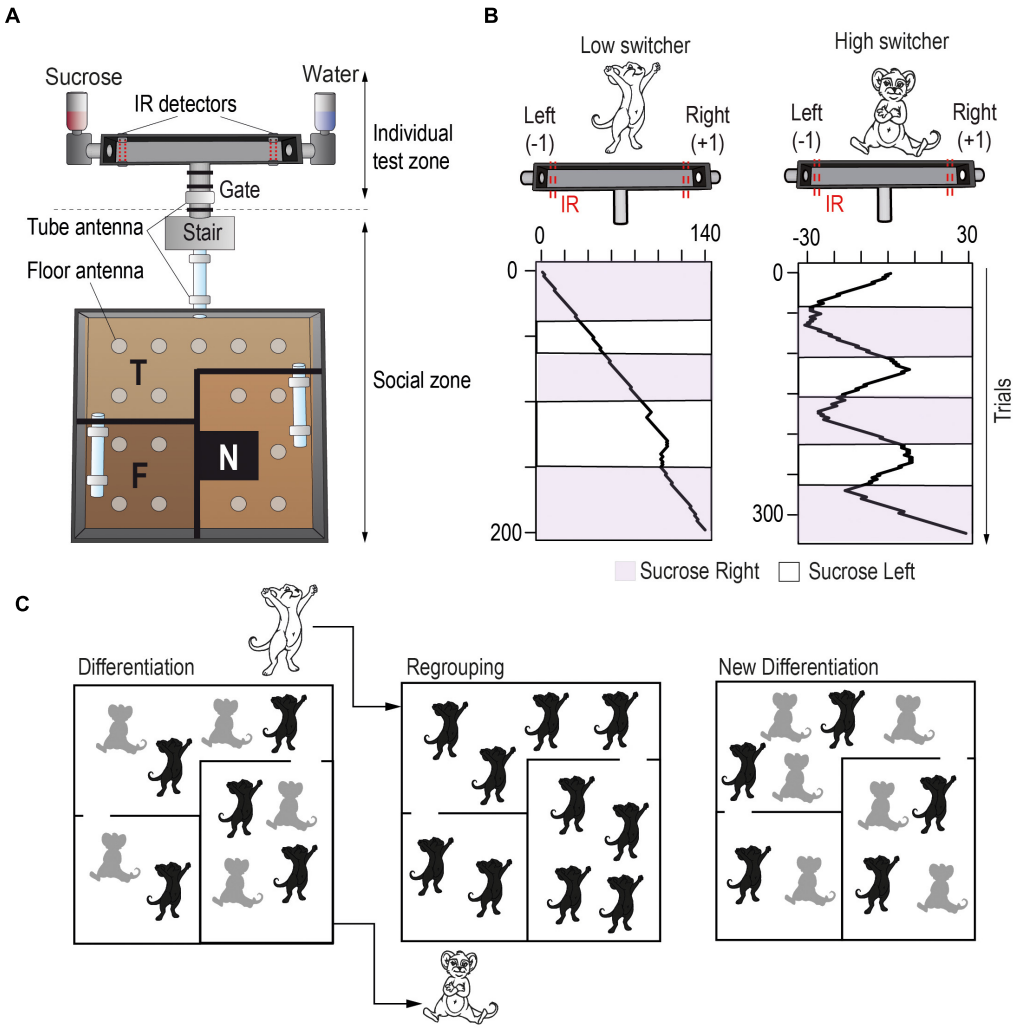

FIGURE 2 | Semi-naturalistic environments allow the study of inter-individual variability with a social context. **(A)** Souris-City environment includes a large and complex living space, in which mice live together and can express sophisticated social and non-social behaviors, and an individual test zone: a T-maze delivering different drinks on each side (e.g., water or sucrose). In the T-maze, mice (inbred male C57BL/6J strain) can perform a cognitive decision-making task spontaneously and isolated from their conspecifics. The various detectors present in the environment allow to follow each individual's behavior and estimate spontaneous individual traits. These are derived from both the general behavior expressed within the social group in the main environment, and the behavior and cognitive performance in the individual test zone. **(B)** Stable and distinctive patterns of choice strategy in the T-maze consistently emerged in independent experiments with, in particular, individuals tracking sucrose (right panel) and individuals constantly choosing the same side independently from the sucrose position (left panel). **(C)** Strikingly, when modifying the social environment by mixing mice from different Souris-City experiments but with similar behaviors, we observed a fast re-adaptation of individual traits, suggesting a social component to this individuation process (Torquet et al., 2018).

a pool of water; some carry the bulk of the food while others do not carry anything (Nejdi et al., 1996). These "producer" mice showed less anxiety in an elevated plus maze compared to the non-carrying "scrounger" mice, an effect interestingly seen both before the food retrieval challenge and maintained afterward – suggesting that underlying behavioral traits influence how labor is divided in social groups. Overall, four principles seem to govern these experiments: (i) taken individually, all animals are capable of solving the task, (ii) the proportion of individuals that share the same trait is related to the size of the group, (iii) individual strategy to solve the task depends on individual traits that pre-exist, and, finally, (iv) the grouping of individuals with the same profile leads to new differentiations. These elements demonstrate that variation in task performance and division of labor are social phenomena, and can be understood in terms of the equilibrium between group demand, information diffusion within the group, and individual motivations.

Large environments that embed complex tasks bring together several social processes that will cause the emergence of strong inter-individual variabilities. Dominance hierarchies and division of labor are specific examples of social niche regulation mechanisms that could help to understand the emergence of individuation, and illustrate that individual behavior is not only the result of developmental process but also of active adaptation

to social challenges. Rather than the idea of a sequence of events over the course of a lifetime that drive an individual toward a phenotype, the concept of social niche specialization instead considers individual variation to be an adaptative process. Animals in large and social environments all encounter slightly different sets of life events, which gives them the opportunity to specialize in a social niche, and, in turn, results in downstream differences between individuals (Bergmüller and Taborsky, 2010; Kempermann, 2019). The development of new analytical tools, such as the continuous analysis of the animals' poses and postures, should make it possible to now better quantify the impact of the environment and of social processes on the mechanisms of individuation and on the neurophysiological consequences of these niche specialization.

DOPAMINE, A NEUROMODULATOR AT THE INTERFACE BETWEEN SOCIAL EXPERIENCE AND BEHAVIORAL TRAIT EXPRESSION

The systematic individual biases that make two individuals different imply strong constraints on neural systems. In particular, it suggests that they are in a way limited in their operating range. The study of the neural bases underlying inter-individual differences mainly focuses on two aspects: (i) pre-existing neurophysiological differences that may explain why individuals respond differently to the same experiences, and (ii) the general plasticity mechanisms that explain how neurophysiological systems adapt as a consequence of individual experience, for example in response to learning or to stress. We propose that these two aspects are coupled: adaptations as a consequence of individual experience lead to differences in individual responses. In turn, these behavioral differences feed forward into changes in an individual's interaction with the environment. Since dopamine circuitry has been implicated in both the stability and flexibility of behaviors (Cools, 2019; Korn et al., 2021), and in various other behaviors, it is thus not only poised to play a central role in the neurophysiological mechanisms of individuation processes, but may be further conceptualized as a central mechanism of a control loop between social influences and behavioral trait expression (**Figure 3**).

Dopamine has long been implicated in reward, aversion, learning and motivation, as well as in various aspects of cognition (Kakade and Dayan, 2002; Schultz, 2007; Bromberg-Martin et al., 2010; Berke, 2018). But it has also been more specifically linked to a cluster of traits that appear to be strong determinants of individual personalities in rodents, including reward seeking (Ikemoto and Panksepp, 1999), novelty seeking, and exploration (Bardo et al., 1996; Kakade and Dayan, 2002; Bunzeck and Düzel, 2006). However, despite the substantial attention paid to dopamine in personality neuroscience (DeYoung, 2013), and despite the evidence pointing toward a link between modulations of dopaminergic function and variations in individual behavior, no comprehensive theory currently explains the role of dopamine in mediating individuation. Beyond the difficulty in precisely defining and measuring a trait, some elements of the physiology of dopaminergic neurons make this problem difficult to assess. Dopamine neurons show a diversity in their projection sites, receptor distribution, and patterns of firing and release; leading to a wide variety of intertwined functional and cognitive roles of dopamine signaling (Cools, 2019). They exhibit a patterned spontaneous firing activity, described as a continuum between two distinguishable rhythms: a tonic slow and regular single spike firing and a phasic bursting mode (Grace et al., 2007; Faure et al., 2014). Regular spiking emerges from intrinsic membrane potential oscillations while the burst-firing pattern critically depends on afferent networks of the dopamine neurons (Grace et al., 2007; Faure et al., 2014). Fluctuation in tonic release is associated with modulation in the firing activity of the spontaneously active population of dopamine neurons. Tonic DA release acts through the gating and modulation of the activity and input sensitivity of downstream neurons and circuits (Dayan, 2012). In contrast, phasic release is specifically associated with the synchronization of burst firing in dopamine neuron populations, and induces a substantially larger dopamine release in terminal regions (Tsai et al., 2009). Phasic dopamine provides a learning signal by encoding the difference between the expected and the actual reward, the so-called reward prediction error (RPE). Dopamine neurons increase their phasic activity first at the presentation of an unexpected reward, and then during the anticipatory phase of this reward after learning (Schultz et al., 1997). Finally, dopamine signaling also depends on clearance mechanisms, relying on the dopamine transporter in the striatum or on catechol-O-methyltransferase action in the cortex (Korn et al., 2021), which adds another layer of control and complexity. This heterogeneity in dopamine release dynamics and sites of action indeed complicates our understanding of how dopamine signaling could influence inter-individual variation and personality. Despite these difficulties, several lines of evidence suggest that variations in the basal activity of dopamine neurons and the tonic level of dopamine impact the expression of individual behavior, particularly those related to reward seeking. Further evidence indicates that dopaminergic activity is actively modulated by social behavior.

Rewards impact the organization of higher-order behaviors: they spur the construction of goals and drive the extraction of information about their presence, predictability, accessibility, and associated costs from the environment. Gathering information about uncertain rewards results in a trade-off between exploration and exploitation (Cohen et al., 2007), which is considered as one of the major axes of trait variation along with locomotor activity, boldness, aggressiveness and sociability (Gartland et al., 2021). Dopamine neuron activity is associated with the level of expression of many behavioral traits related to reward seeking, notably with the level of exploration (Cohen et al., 2007; Frank et al., 2009; Humphries et al., 2012; Schiemann et al., 2012), but also the propensity for risk taking (Onge and Floresco, 2008; Stopper et al., 2014), reaction to uncertainty (Fiorillo et al., 2003; Naudé et al., 2016) and response vigor (Niv et al., 2007). Several studies in rodents have now demonstrated the important role of tonic dopamine neuron activity in setting the balance point for the trade-off between environmental

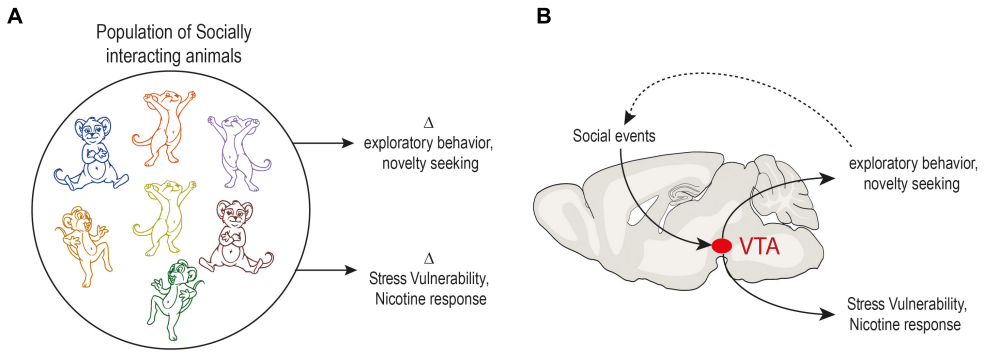

FIGURE 3 | Model of behavioral adaptation in response to social context through dopaminergic system. **(A)** Traits can be viewed as stable individual expressions of a behavioral outputs or preferences – with high inter-individual variability but low intra-individual variability. In a population of animals living in a group, in the same environment, individuals adapt to their social environment. In parallel, traits emerge in a series of non-social behaviors such as exploratory behavior or novelty seeking. Differences in response to stress or drugs such as nicotine are also observed. **(B)** Diagram illustrating a possible mechanism for generating inter-individual variability. Adaptations as a consequence of individual social experience impact dopaminergic system and lead to differences in individual behavior. In turn, these behavioral differences feed forward into changes in an individual's interactions with the environment. This loop allows a social profile to be adjusted to another behavioral profile, for example exploration level. The same type of mechanism could allow the emergence of variability in vulnerability to stress and response to drugs.

exploration and the exploitation of existing knowledge (Beeler et al., 2010; Cinotti et al., 2019; Dongelmans et al., 2021). When mice were presented with two levers in a "closed economy" paradigm where each lever had different relative costs for food, but the two levers frequently switch position, wild-type mice optimally adapted their choices by distributing more effort on the least expensive lever. Hypodopaminergic mice, however, distribute their effort roughly equally between levers expending on average more effort for each pellet earned than wild-type mice, which suggests a role for tonic dopamine in the exploration of options (Beeler et al., 2010). Antagonizing D1-D2 dopamine receptors using systemic injection of flupentixol affects the performance of rats in a 3-armed bandit task with varying levels of uncertainty, resulting in an increase in random choices. A computational analysis reveals that decreasing dopaminergic activity increases exploration, without altering learning rate (Cinotti et al., 2019). Finally, in a recent study, mice were faced with consecutive binary choices in a spatial version of the multi-armed bandit task (**Figure 1B**), having to choose between visiting three sites in an open field delivering an intracranial reward with different probabilities: 100, 50, or 25% (Naudé et al., 2016; Dongelmans et al., 2021). In this task, wild-type mice display individual decision-making strategies, some making more exploitative choices (visiting primarily the 100 and 50% rewarded sites), while others make more exploratory choices (incorporating information gathering about the site rewarded 25% of the time). Chronic exposure to nicotine drives mice toward more exploitative strategies, which was associated with an increase in spontaneous dopamine neuron activity (Dongelmans et al., 2021). Importantly, optogenetically mimicking the increased tonic dopaminergic activity observed under nicotine exposure is sufficient to temporarily and reversibly induce the adoption of an exploitative strategy in mice, suggesting that factors which modulate dopaminergic function can flexibly shift behavioral traits. Together, these findings show the importance of tonic dopamine release in setting the threshold between exploration and exploitation strategies, which is one crucial determinant in adaptive personality in rodents. Modifying ascending dopaminergic activity thus likely modulates arbitration between different strategies, exploiting or exploring certain options, through the gating and modulation of the downstream circuits (Dayan, 2012).

Finally, dopamine is also heavily implicated in establishing and maintaining social relationships. Vertebrate social behaviors are mainly controlled by two evolutionary conserved and interactive neural circuits (O'Connell and Hofmann, 2011): a "social behavior network" composed of midbrain, hypothalamic, and basal forebrain nuclei that is involved in aggressive, reproductive, and communication behaviors (Newman, 2017); and the reward system corresponding to the mesocorticolimbic dopamine network, that allows social behavior to be reinforcing and, thus, adaptive (O'Connell and Hofmann, 2011). Indeed, recent studies demonstrate that dopamine encodes key aspects of social interactions (Gunaydin et al., 2014), that dopaminergic reward prediction errors guide social learning (Solié et al., 2021), and that dopamine has a role in promoting aggressive behavior in mice (Golden et al., 2019; Mahadevia et al., 2021). These findings suggest that the dopaminergic system plays an essential role in social interactions by encoding information about valence (rewarding or aversive social situations), and about social positioning to drive relationship-appropriate behaviors. There is also a growing body of evidence suggesting that social experiences induce long-term modifications in spontaneous dopaminergic activity. Social defeat, an example of a negative social challenge, produces strong and long-lasting changes in spontaneous dopamine neuron activity, dopamine release within the mesolimbic dopamine pathway, and modifies social engagement, notably leading to withdrawal from social interactions (Krishnan et al., 2007; Barik et al., 2013; Chaudhury et al., 2013; Friedman et al., 2014). Social ranking in tetrads of male mice is associated with marked changes in VTA dopamine neuron activity, with higher-rank animals displaying lower

bursting activity (Battivelli et al., 2019). Finally, in Souris City, we have shown that the phenotypic divergence in individual behaviors is mirrored by differences in the firing properties of midbrain dopamine neurons, and that modifying the social environment resulted in a fast re-adaptation of both the animal's traits and the firing pattern of its dopamine neurons (Torquet et al., 2018). Stable decision-making strategies and dopaminergic neurons activity can thus rapidly change upon exposure to social challenges.

Altogether, these diverse – yet intertwined – functions of dopamine signaling suggest that this neuromodulator may link social experience with individualistic behavioral output. We propose in this review that, by triggering rapid modifications in dopaminergic function, the social environment actively alters both social and non-social behaviors, such as the trade-off between exploration and exploitation. This many-to-many relationship, where changes in regulatory influences over dopamine activity induce adaptations in multiple behaviors, has strong implications for the understanding of inter-individual variability and the link between personality, response to environmental risk factors, and mental health outcomes. Social experiences that modify dopamine function, because they would lead to a modification of a certain number of traits (e.g., the level of exploration), would make it possible (**Figure 3B**) to match a social profile to, for example, an exploratory profile (e.g., dominant mice explore less). A subsequent question is then to understand whether vulnerability to psychopathologies could also be extrapolated by social profiles and associated dopaminergic adaptations.

USING INTER-INDIVIDUAL VARIABILITY TO PREDICT MENTAL HEALTH OUTCOMES

Studying the neurobiology of inter-individual variability is essential for understanding how it relates to vulnerability or resilience to psychiatric disease. Mental health disorders are highly heritable, however, their genetic risk factors account for only somewhere between 10–60% of the variance in their distribution (Kreek et al., 2005; Kendler et al., 2012; Fromer et al., 2016; Howard et al., 2019), and they result from complex polygenic interactions that can be common across multiple disorders (Kendler et al., 2003b; Pasman et al., 2018; Wray et al., 2018; Demontis et al., 2019; Linnér et al., 2021). Environmental, social, or cultural factors must therefore also play important roles in determining the incidence of psychiatric disease. Indeed, psychiatric diseases are often, but not always, incited by a precipitating environmental factor: experiencing a stressful life event, for example, has been linked with an increased risk of developing major depressive disorder (Kessler, 1997; Tennant, 2002; Kendler and Gardner, 2010); while exposure to a drug of abuse, and its subsequent availability, is a necessary environmental component for the onset of substance abuse issues (Tsuang et al., 1998; Kendler et al., 2003a; Volkow and Li, 2005). Whether inter-individual variations in behavioral trait expression interact with the social environment to shape vulnerability profiles, and the circuitry on which they may converge, are thus current topics of investigation.

Ample evidence argues that despite equal exposure to a specific psychoactive substance, not all individuals develop an addiction; just as not all individuals will develop depression after a stressful life event. An individual's social milieu may account for this variation in the susceptibility to develop mental illness, as the quality of social relationships in adulthood significantly modulates the development of psychiatric disease, even in the face of strong environmental risk factors. Social support, in the form of healthy romantic relationships, strong familial ties, and community involvement, has been linked with a reduction in the risk of developing mental illness following stressful life events in adults (Syrotuik and D'Arcy, 1984; Kawachi and Berkman, 2001; Coker et al., 2002). Whereas negative social relationships in adulthood, including social isolation, workplace bullying, or intimate partner violence, are linked with a higher incidence of psychiatric illness (Barnett and Gotlib, 1988; Bonomi et al., 2006; Einarsen and Nielsen, 2015; Lacey et al., 2015; Verkuil et al., 2015; Rohde et al., 2016; Leigh-Hunt et al., 2017). Mental health issues, in turn, can perpetuate social isolation and/or maladaptive relationships. The notion of psychiatric vulnerability is thus tightly and bi-directionally linked to an individual's social environment, and as such, one of the overarching consequences of our hypothesis is that a crucial aspect of vulnerability or resilience to psychopathology results from how the active adaptation of neuromodulatory networks in response to social environments constrains the cumulative effect of risk factors.

Direct causal links between an individual's social environment and the development of mental illness are, however, challenging to establish in human populations. Nevertheless, the proximal social environment has been shown to influence the expression of depression- or addiction-like behaviors in preclinical rodent models; with negative social experience (e.g., isolation from peers, receiving repeated aggression) increasing these types of behaviors, while positive social experience (e.g., housing with peers) has been suggested to buffer the effects of stressors. While social isolation is most commonly used as a developmental stressor, isolation of adult rodents from their cagemates has been shown to promote depressive-like behaviors (Martin and Brown, 2010; Ieraci et al., 2016; Preez et al., 2020) and to increase self-administration of drugs of abuse (Alexander et al., 1978; Bozarth et al., 1989). Mice exposed to repeated aggressions in a chronic social defeat stress (CSDS) paradigm can be divided into differing phenotypes depending on the level of social avoidance exhibited following the CSDS (Kudryavtseva et al., 1991; Berton et al., 2006; Krishnan et al., 2007; Golden et al., 2011), with some mice showing marked depressive-like symptoms while others show stress resilience. Social stress in adult rodents has also been linked to increased vulnerability to develop addiction-like behaviors. Following a repeated social stress in a resident-intruder paradigm, rats showed increased conditioned place-preference to cocaine, sensitized locomotor activation in response to acute amphetamine administration, as well as increased motivation for cocaine self-administration and increased cocaine intake (Covington and Miczek, 2005; Stelly et al., 2016). Remarkably, returning rats to a positive

social environment following resident-intruder stress, rather than leaving them individually housed, can counteract the enduring effects of social stress on cognitive and mood-related outcomes (Ruis et al., 1999; Frijtag et al., 2000), suggesting indeed that the interaction between social environment and stress response is bi-directional in nature and able to be modified continuously in adult rodents. Social stress is not the only factor in group interactions that can reveal individual vulnerability to behaviors linked to psychiatric disease models. The natural social milieu of a rodent, and their place within its hierarchy, can already significantly constrain, or perhaps even amplify, their reactiveness to environmental factors. Dominant mice are more susceptible to negative outcomes following CSDS (Larrieu et al., 2017) or chronic mild stress (Karamihalev et al., 2020), and to experience greater cocaine CPP (Yanovich et al., 2018) than mice lower in social ranking. Likewise, socially dominant rats showed greater cocaine intake in a self-administration experiment (Jupp et al., 2016). A major current limitation to these studies is the use of limited social groupings (using traditional rodent housing and/or single housing animals), as well as the use of acute testing to establish phenotypes. For example, to determine the susceptible vs. resilient mice following CSDS, a social interaction test of less than 5 min is typically used (Cao et al., 2010; Barik et al., 2013; Morel et al., 2017), and the mice are then divided by a median split of their interaction time. By observing mice instead in automated, semi-naturalistic environments over long periods, the characterization of how social stress affects each individual, based on their longitudinal profiles of both social and non-social behaviors, would be quantifiable as continuous variables. This approach would enable the establishment of robust correlations between behavioral trait expression (such as exploration level, Torquet et al., 2018), vulnerability profiles (e.g., by testing drug self-administration), and set the stage for unraveling the underlying circuitry.

As such, the complex neuronal circuits that underpin resilience/susceptibility profiles remain far from understood. We propose that the rapid effects of social experience on VTA dopamine neuron function influence the expression of vulnerable/resilient phenotypes with regard to depressive- or addiction-like behaviors. Individual variations in addiction vulnerability have been linked to spontaneous dopamine neuron activity; rats that show higher basal dopamine neuron firing rates and bursting activity are more likely to exhibit higher novelty or exploratory behaviors and show increased propensity to self-administer psychostimulant drugs (Piazza et al., 1989; Pierre and Vezina, 1996; Marinelli and White, 2000; Suto et al., 2001; Kabbaj, 2006; O'Connor et al., 2021). Spontaneous dopamine neuron firing is elevated following CSDS (Cao et al., 2010; Barik et al., 2013; Morel et al., 2017), an effect which is more prominent in susceptible mice than in resilient mice, as resilient mice instead actively regulate ion channels in response to this social stressor to stabilize dopaminergic cell excitability (Krishnan et al., 2007; Friedman et al., 2014, 2016). Interestingly, one of these studies also indicates that exposure to chronic nicotine, which increases dopamine neuron firing, can increase the potency of a mild social stressor, inducing a vulnerability to the negative effects of a sub-threshold social defeat (Morel et al., 2017). Furthermore, VTA nicotine receptor expression and dopamine neuron response to intravenous nicotine is altered following CSDS (Morel et al., 2017). Together, these results suggest that the modulation of dopamine firing by social defeat stress is instrumental in the development of a susceptible phenotype. Recent studies indicate that postpartum rats show transient changes in dopaminergic activity which are linked with the expression of depressive-like behaviors (Rincón-Cortés and Grace, 2019). The unique social stressor of pup removal further alters dopaminergic activity in postpartum dams, resulting in a decrease of spontaneously active dopamine neurons, which can be rescued by paring housing two pup-separated dams together (Rincón-Cortés and Grace, 2021). These results suggest provide initial evidence that social support may attenuate the effect of stressors by restoring dopamine neuron activity. Recent studies further suggest that the VTA acts as a physiological hub for determining the response to environmental stressors, since other molecular signatures of depression in humans and in rodent models are upstream of the VTA and exert their effects by altering dopamine neuron firing, including modulations in cholinergic (Small et al., 2016; Morel et al., 2017) or noradrenergic input (Isingrini et al., 2016) to the VTA. Understanding how the social environment shapes dopaminergic activity may therefore provide significant insight into individual risk profiles for developing mental health disorders.

Finally, as traits such as novelty seeking and exploration have also been linked to spontaneous dopaminergic activity level, a major open question is whether both the expression of these traits and psychiatric vulnerability share overlapping dopaminergic pathways, and would be therefore vulnerable to the same perturbations by social influence. These ideas have yet to be directly experimentally explored, as they require large experiments with mice living in micro-societies with automated data collection in order to observe correlations and test causative hypotheses. Despite these challenges, studies have recently begun to establish causality between altered dopamine function and psychiatric vulnerability or between dopamine function and level of exploration. Optogenetic experiments have shown that the direct activation or inhibition of midbrain dopamine neurons bidirectionally modulates depression-like behaviors, rescuing or augmenting susceptibility to CSDS in mice (Chaudhury et al., 2013; Tye et al., 2013). Activating or inhibiting dopamine neuron firing using optogenetics also rapidly and reversibly shifts the individualistic level of exploration behavior in a decision-making task (Dongelmans et al., 2021). Given that both dopaminergic activity and decision-making behavioral traits are indeed remodeled when faced with changing social environments (Torquet et al., 2018), whether exploration trait expression and vulnerability to depressive- or addiction-like behaviors correlate, whether they share dopaminergic pathways, and if they can be modulated in parallel by social input remains a topic of current investigation. Emerging relationships between social experience and dopaminergic function thus begin to link inter-individual variability in behavioral trait expression to the idea of an individual's mental health trajectory. Advancing this line of research is poised to shape the future of precision psychiatry.

DISCUSSION

Inter-individual variability consists, in part, of the differential behavioral responses to environmental cues and challenge that define individuals, leading to what we consider to be a personality. How personalities are constructed, maintained, and changed in response to environmental challenges remains an open question. Here, we propose that social environments are major drivers of individuation processes, even in adult rodents, and even after the previously stable expression of behavioral traits. We further contend that adaptations in the activity of neuromodulatory circuits, and dopamine in particular, underlie socially-driven individuation processes. Finally, we suggest that this framework may have useful applications in understanding environmental influence on psychiatric vulnerability. We discuss throughout the important consideration for the field in the context of testing environments, as these complex socially-driven inter-individual differences are best studied in large, semi-naturalistic environments where rodents can live in groups.

Rodent micro-societies must be seen as systems, that is, as an organized set of interacting elements from which specific properties and functions emerge. In insects, mechanisms of social homeostasis (Emerson, 1956) allowing the maintenance of structures, castes, or colony's environment (i.e., an optimal temperature in the nest for example), emerge due to asymmetries in individual environments. What is optimal for one individual is not necessarily optimal for the other. From these asymmetries emerge competition, dominance, division of labor, and marked inter-individual differences. For example, in large environments with a social component, individuals with identical genetic backgrounds are initially exposed to a seemingly identical environment. However, its perception by each individual encompasses a shared component, i.e., the context to which they are all equally exposed, as well as a non-shared component corresponding to the individual's interpretation of environmental cues through the lens of their life history. In this non-shared environment, the social influence differs between individuals (e.g., some exert aggression toward the others, while some are subjected to it) and creates a unique experience. Individual behavioral and physiological adaptations allow the emergence of distinct and stable individual profiles (Bergmüller and Taborsky, 2010; Freund et al., 2013, 2015; Torquet et al., 2018). Thereby, behavioral traits work as a dynamic system where equilibria define stable traits. However, these traits may reorganize rapidly if environmental or physiological conditions change sufficiently. This can be seen in so-called "sociotomy" experiments, where colonies are reorganized by separating or recombining subsets of individuals. In insects, but also in rodents (Torquet et al., 2018), a rapid reconstitution of the task distribution can be demonstrated after such experiments. An interesting consequence of this point of view, still largely unexplored, is that traits associated with vulnerability to psychopathology emerge largely from environmental influence. A question that follows is whether certain environmental conditions (e.g., strong competition. . .) favor the emergence of these traits, how they are distributed in the population and finally whether, like division of labor, variation in trait expression emerges from social life.

A fundamental proposal in the field suggests that personality can be explained by constraints on behavioral adaptation (Sih et al., 2004; Duckworth, 2010; Wolf and Weissing, 2010). Animals can flexibly adjust their behavior over time, in response to situations. However, the fact that two individuals can be more or less aggressive compared to the average population implies a strong coherence in their behavior, and suggests that there is a limit to their respective range of adaptation. This constraint in adaptability defines an individual, gives the feeling of consistency of behavior over time and can be established and/or modified depending on the social context. We can think of these constraints as the individual being caught up in a network of reciprocal interactions between the neuronal circuits shaped by learning and the environment where the individual becomes increasingly specialized. The concepts of brain plasticity and learning thus give singularity to each individual. Brain connectivity and activity can be thought of as a dynamic system, as they control the subjective perception of the environment of an individual, and are themselves modified according to each individual's history. Apart from those very general mechanisms, is it possible to extract the specific role of a given neural circuit in the definition of an individual? Here, we propose that the dopaminergic system, at the interface between adaptation, neuromodulation and decision making, plays a particular role in the control of interindividual differences.

While studies categorizing rodents based on locomotor or social stress-related behaviors provide an entry into the relationship between dopamine neurophysiology, social behavior, and psychiatric vulnerability, they fall prey to some of the same caveats with minimizing interindividual variability in experimental conceptualization. Creating categorical variables from a continuous distribution indeed simplifies data analysis and presentation, however, such an artificial creation of distinct groups can result in a loss of information from the original continuous dataset and a significant limitation of the predictive validity of the variable in question. For example, a categorical value derived from a median split will represent equally those values closest to and those farthest from the median within each group, sacrificing inter-individual variability within the group(s) and thus reducing the power of predictive analyses that can be made using regression (DeCoster et al., 2010). The artificial categorization of continuous distributions indeed significantly facilitates statistical calculations, allowing means comparisons where regression would be more appropriate. Nowadays, with rapidly expanding computational properties, the ability to easily assess nuanced relationships between behavioral distributions and physiological markers is now feasible. The use of large and automated testing environments therefore represents an enormous advantage in predicting psychiatric vulnerability from behavioral or physiological traits, as they are able to measure multiple continuously distributed variables per subject. Thus, while emerging relationships between social experience and dopaminergic function can be linked to the idea of an individual's mental health trajectory, our knowledge to date of the relationships between these factors and psychiatric outcomes remains limited. We propose that the proximal

social environment limits the adaptability of neuromodulatory networks when faced with triggering events for the emergence of psychiatric disease, thus constraining an individual into a more susceptible or more resilient state. This state can be thus predicted from the expression of particular behavioral traits. Notably, this theory, and the results supporting it to date, strongly suggest that positive social connections are a key environmental intervention to support equilibrated mental health. The advancement of trait assessment in large automated testing environments will drive this line of research forward in an unbiased and accurate manner.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Bottlenecks, Modularity, and the Neural Control of Behavior

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In almost all animals, the transfer of information from the brain to the motor circuitry is facilitated by a relatively small number of neurons, leading to a constraint on the amount of information that can be transmitted. Our knowledge of how animals encode information through this pathway, and the consequences of this encoding, however, is limited. In this study, we use a simple feed-forward neural network to investigate the consequences of having such a bottleneck and identify aspects of the network architecture that enable robust information transfer. We are able to explain some recently observed properties of descending neurons—that they exhibit a modular pattern of connectivity and that their excitation leads to consistent alterations in behavior that are often dependent upon the desired behavioral state of the animal. Our model predicts that in the presence of an information bottleneck, such a modular structure is needed to increase the efficiency of the network and to make it more robust to perturbations. However, it does so at the cost of an increase in state-dependent effects. Despite its simplicity, our model is able to provide intuition for the trade-offs faced by the nervous system in the presence of an information processing constraint and makes predictions for future experiments.

Keywords: neural control, modularity, bottlenecks, neural networks, robustness

1. INTRODUCTION

When presented with dynamical external stimuli, an animal selects a behavior to perform—or a lack thereof—according to its internal drives and its model of the world. Its survival depends on its ability to quickly and accurately select an appropriate action, as well as to transmit information from the brain to its motor circuitry in order to physically perform the behavior. In almost all animals, however, there exists a bottleneck between the number of neurons in the brain that make cognitive decisions and the motor units that are responsible for actuating movements, thus constraining the amount of information that can be transmitted from the brain to the body (Smarandache-Wellmann, 2016; Kandel et al., 2021).

In the fruit fly *Drosophila melanogaster*, descending commands from the brain to the ventral nerve cord (VNC) are transmitted through approximately 300 bilaterally symmetric pairs of neurons that have their cell bodies in the brain and have axons project into the VNC (Gronenberg and Strausfeld, 1990; Hsu and Bhandawat, 2016). Recent anatomical studies have shown that these neurons exhibit a modular pattern of connectivity, with the descending neurons clustering into groups that each innervate different parts of the motor system (Namiki et al., 2018; Phelps et al., 2021).

In addition to these anatomical properties, in the fruit fly, manipulating these descending neurons *via* optogenetics has shown that exciting individual neurons or subsets of neurons often result in dramatic and robust behavioral alterations—for example, exciting the DNg07 and DNg08 neurons reliably elicits head grooming, and exciting DNg25 elicits a fast running response (Cande et al., 2018). In many cases, however, it has been shown that exciting the same neuron in different contexts (e.g., walking and flying) often have state-dependent effects (Cande et al., 2018; Zacarias et al., 2018; Ache et al., 2019). In other words, the behavioral effect of stimulating the neuron often depends on the actions that the fly is attempting to perform.

In this study, we use a simplified model of behavioral control to explore how modularity may help increase the efficiency and robustness of behavioral control given an information bottleneck. Specifically, our model predicts that modularity of behavior increases the efficiency of the network and its robustness to perturbations, but also that this modularity increases the amount of state-dependent variability in how behavioral commands are transmitted through the bottleneck. While our feed-forward model is a vast oversimplification of the complicated recurrent circuitry that lives within a fly's ventral nerve cord, we show that it provides intuition into the trade-offs the nervous system is faced with, and makes qualitative predictions as to how the system might respond to inhibition or double-activation experiments.

2. RESULTS AND DISCUSSION

Inspired by the fly ventral nerve cord, we have developed an abstracted model that aims to generate insight into the general problem of behavior control through an information bottleneck. Specifically, we assume that there is a set of N behaviors that are in an animal's behavioral repertoire and that to perform one of these behaviors, the animal must excite a subset of M total binary "motor" neurons (e.g., task 14 requires units 1, 3, and 99 to turn-on, and all the rest to be turned off—see **Figures 1A,B**). However, to model the effect of having limited information transmission from the brain to the motor systems, any commands from the brain must travel through an hidden layer of $R < M, N$ descending neurons (Namiki et al., 2018).

We implemented this model using a feed-forward neural network, with the task being encoded in the top layer, the descending neurons being the hidden layer, and the motor units constituting the bottom layer (see **Figure 1A**). For simplicity, we assume that the brain's intended behavioral output is represented in a one-hot encoded manner, where only one "decision" neuron is turned on at once [i.e., behavior 2 is represented by a first layer of $(0, 1, 0, \cdots) \in \{0, 1\}^N$]. We start with the case where each behavior is randomly assigned a set of k motor neurons that must be activated. **Figure 1B** shows an example of this desired mapping, which we call our behavioral matrix. To perform a behavior, one of the decision neurons has to be activated and pass its signal through the network. The parameters of the network, weights $\{W^{(1)}_{\alpha,\beta}, W^{(2)}_{\alpha,\beta}\}$ and biases $\{B^{(1)}_{\beta}, B^{(2)}_{\beta}\}$, are trained to perform the mapping between the top and bottom layers as accurately as possible (see details in section 4).

Given this model, we would like to study how the network performs as a function of the bottleneck size and the sparsity of the behavioral matrix. The absolute maximum number of sequences that the network could encode is 2^R as each hidden neuron can either be activated or not. However, this simple neural network is incapable of reaching the ideal limit. In **Figure 1C**, the bottleneck size required for accurate encoding is $\sim 20 - 60$ for $N = M = 100$, depending on the sparsity of the behavioral matrix. These values are much larger than the minimal possible bottleneck size, $R = \log_2 100 \approx 7$. While we will explore the potential reasons for this discrepancy shortly, we empirically define the critical bottleneck size, R_c, as the minimal number of neurons in the hidden layer sufficient to reproduce 98% of the behaviors correctly, averaged across multiple random instantiations of the behavioral matrix. See **Supplementary Figure 1** for example learning and loss curves, and **Supplementary Figure 2** for example values of the hidden layer and the weights of the trained network. The values of the hidden layer get more binarized (**Supplementary Figures 2a,b**) as its size decreases, implying that the system is getting pushed out of its dynamic range.

2.1. Characterization of the Model

To explore how the statistics of the behavioral matrix affect the critical bottleneck size, we altered the sparsity of the outputs by manipulating the number of motor neurons activated per behavior (k) while keeping $M = N = 100$ (**Figure 1C**). Note that since our output size is 100 and its encoding is binary, a neural network with k and $100 - k$ activated motor neurons have the same statistical behavior. Thus, sparsity increases as k deviates from 50 in either direction. As evident from **Figure 1C** and the inset therein, as k decreases below 25, the network requires fewer neurons in the hidden layer (a lower R_c) to learn all of the behaviors perfectly, with the decrease starting around $k = 25$. Ultimately, for the sparsest output encoding we tested ($k = 5$), the network requires half the number of neurons compared to the densest ($k = 50$) case ($R_c \approx 24.4 \pm 0.8$ vs. $R_c \approx 57 \pm 2$), indicating that it is more difficult for our model to learn the more complicated patterns that are associated with a denser output. This effect can be more explicitly seen by plotting R_c as a function of the entropy of the behavioral matrix (**Figure 1D**, Equation 4). Furthermore, we note that the shape of the curve, as a function of hidden layer size, R, approaches that of a sigmoid function in the limit of dense output signal (as k approaches 50). Equivalently, sparsity can be varied by fixing k and varying the size of the output layer M (here, keeping $N = 100$ fixed) (**Supplementary Figure 3**). We again find that as the output signal becomes more sparse, that is, as M increases, it is easier to learn the mapping from behavior to motor commands. Moreover, we also notice that the learning curves split into two

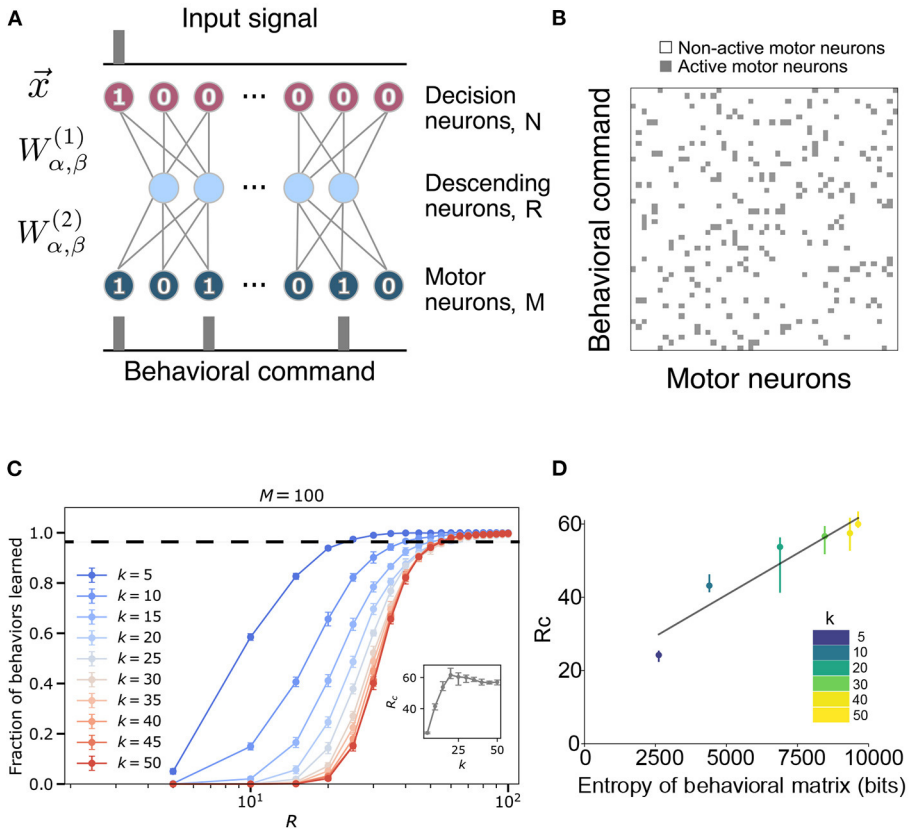

FIGURE 1 | Model construction and parameters. **(A)** The structure of the ventral nerve chord is modeled by a neural network that takes as input a task assignment represented by a binary sequence $\vec{x}$ of length N. The signal travels through a hidden layer (size R) to an output layer (size M), which corresponds to descending neurons and motor neurons, respectively. Each neuron in one layer communicates with all the neurons in the following layer through the weight matrices $W^{(\cdot)}_{\alpha,\beta}$, detailed in section 4. **(B)** An example of a behavioral matrix that indicates the motor units activated for each task. Row i corresponds to the i-th behavioral command (i.e., the i-th neuron activated in the input layer of the network). k is the number of motor neurons needed to execute a given behavior. Columns correspond to different motor neurons [i.e., the jth column indicates whether a particular motor neuron was active (gray) or not (white) in the behaviors]. **(C)** Fraction of behaviors learned as a function of hidden layer size R and fixed input layer size $N = 100$ for varying k and fixed output layer size $M = 100$. The inset shows the critical bottleneck size R_c as a function of k. Each point is averaged over 30 random input-output combinations. Dashed line indicates critical bottleneck threshold. **(D)** Values of the critical bottleneck size R_c for different values of sparsity ($k = 5, 10, 20, 30, 40, 50$) as a function of the behaviorial matrix entropy. Black line is the line of best fit and is provided for visual aid only.

regimes (**Supplementary Figures 3a,b**) corresponding to when M is smaller or larger than N. When $M > N$, the network finds it much easier to learn with the learning ability saturating when the bottleneck size is a certain fraction of the output layer.

2.2. Modularity of Behaviors

While the analyses presented in the previous section involved random mappings between behaviors and motor outputs, we now ask if imposing biologically inspired constraints on this mapping might affect the efficiency of the network. Specifically, we will assume that the behavioral matrix is modular, with similar behaviors (e.g., different locomotion gaits or different types of anterior grooming motions) more likely to require similar motor output patterns. This constraint is motivated from previous anatomical studies in *Drosophila* (Namiki et al., 2018).

To explore the effect of modular structure on our model, we performed a set of simulations with various degrees of behavioral matrix modularity. Specifically, we fixed $k = 10$ and split the behavioral matrix into 5 regions (see inset in **Figure 2A**). If there is no active motor neuron in common between the different clusters, then we have perfect modularity [$\mu = 0.8$, where μ is the fraction of the edges that fall within the modules minus the expected fraction within the modules for an equivalent random network (Newman, 2018), see section 4]. We then allowed for some overlap between regions to generate matrices with a spectrum of modularities (some examples given in **Figure 3C**) between the perfect modular limit and random mixing. We observed that the modular behavioral matrices can be learned more efficiently than random matrices, requiring far smaller critical bottleneck sizes to achieve the correct mapping of behavioral commands (**Figure 2A**). The perfectly modular output matrix (inset **Figure 2A**) was learned

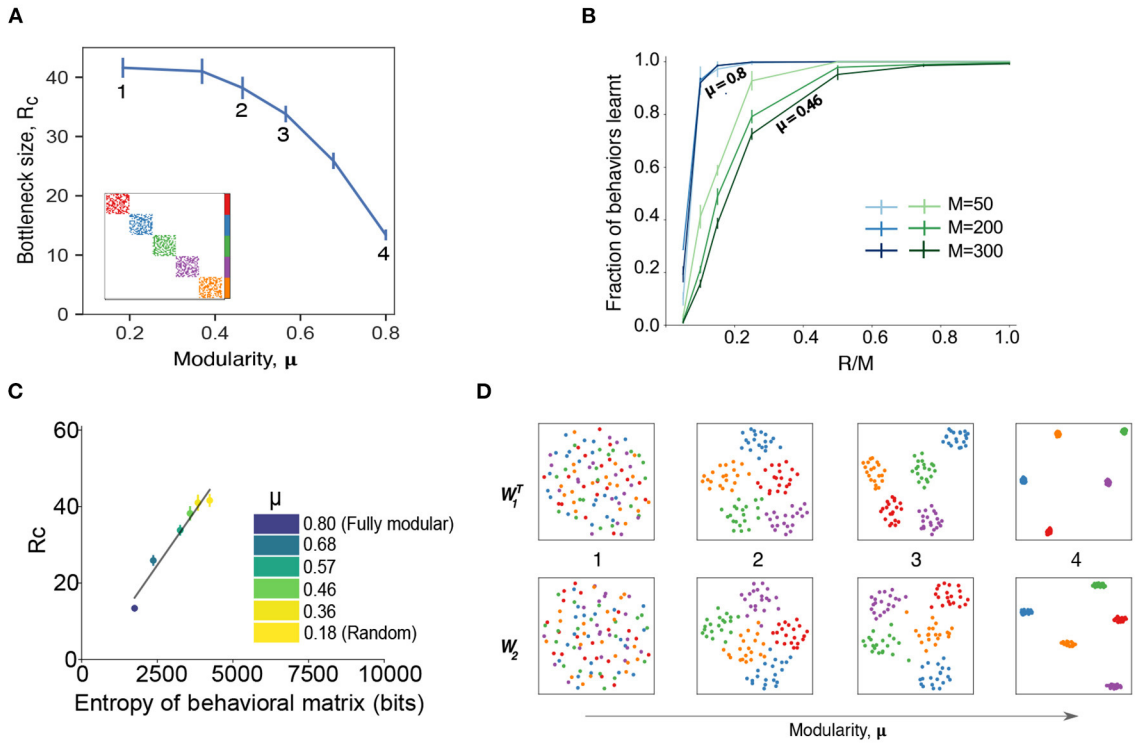

FIGURE 2 | Modularity in the behavioral commands reduces critical bottleneck size and affects other network properties. **(A)** Relationship between the size of the hidden layer R and the modularity of the behavior matrix. Each point corresponds to a set of numerical experiments with 10 different matrices around a given modularity value (see section 4 for details of data generation) for $k = 10$, $M = N = 100$. R_c is defined as the minimal hidden layer size that was able to achieve 98% accuracy in 10^5 epochs of training. Numbers indicate specific cases that are shown in panels **(B–D)** in more detail. Inset shows an example of behavioral command matrix for $\mu = 0.8$ case (point 4). **(B)** Fraction of behaviors learned as a function of the hidden layer size, R for different system sizes with $N = M$ for two levels of modularity ($\mu = 0.8$ and $\mu = 0.46$). Error bars correspond to the standard deviation. Results are averaged over 5 different runs with error bars corresponding to the standard deviation. **(C)** Values of the critical bottleneck size R_c for different values of modularity [$\mu = 0.8$ (fully modular), 0.68, 0.57, 0.46, 0.36, 0.18] as a function of the behavioral matrix entropy. Black line is the line of best fit and is provided for visual aid only. **(D)** Structure of the weight matrices W_1 and W_2 for different modularity values. The dimensionality reduction is performed *via* UMAP (McInnes et al., 2018), a non-linear method that preserves local structure in the data. The point colors correspond to the colors in **(A)** inset: 1 ($\mu = 0.18$, random matrix); 2 ($\mu = 0.46$); 3 ($\mu = 0.56$); 4 ($\mu = 0.8$, perfectly modular matrix with 5 clusters).

with only $R_c = 13$ neurons, which is less than half the number required for the random matrix ($R_c \simeq 35$) with the same amount of sparsity (**Supplementary Table 1**). Note that the dependence of the critical bottleneck layer size on matrix modularity is not linear, just 2 neurons overlapping between clusters makes learning much harder ($R_c = 30$, point #3 in **Figure 2A**).

In addition to making the mapping easier to learn, modularity in the behavioral matrix also helps learning scale with the system size. In **Figure 2B**, we plot the fraction of behaviors learned as a function of the relative size of the bottleneck layer R as compared to the output layer M, for different values of the system size (we assume $N = M$) and for different values of the modularity. Modularity values were chosen to highlight the differences between a perfectly modular matrix ($\mu = 0.8$) and a matrix that has a low amount of modularity ($\mu = 0.46$) while not being completely random. For highly modular behavioral matrices (blue curves in **Figure 2B**), we find that the size of the output doesn't affect the learning ability of the network, as the bottleneck occurs when the size of the hidden layer is a similar fraction of the output sizes. On the other hand, when the behavioral commands aren't very modular, smaller system sizes learn better for a relatively smaller bottleneck size (green curves in **Figure 2B**). This is again a reflection of our model finding it easier to learn the simpler patterns (less entropy) of a more modular behavioral matrix (**Figure 2C**). The similarities between **Figures 1D**, **2C** indicate that the entropy of the behavioral matrix is an important parameter that determines R_c, even while keeping other parameters constant.

Finally, we found that imposing a modular output structure also imposes a modular structure on the weights of the learned network (**Figure 2D**). The modularity in the weights becomes more pronounced as the modularity of the behavioral matrix increases, similar to results found in the study of more generalized artificial neural networks (Zavatone-Veth et al., 2021). Together, these results show that modularity in the behavioral matrix increases the efficiency and scaling properties of the network through creating a concomitantly modular representation within the model.

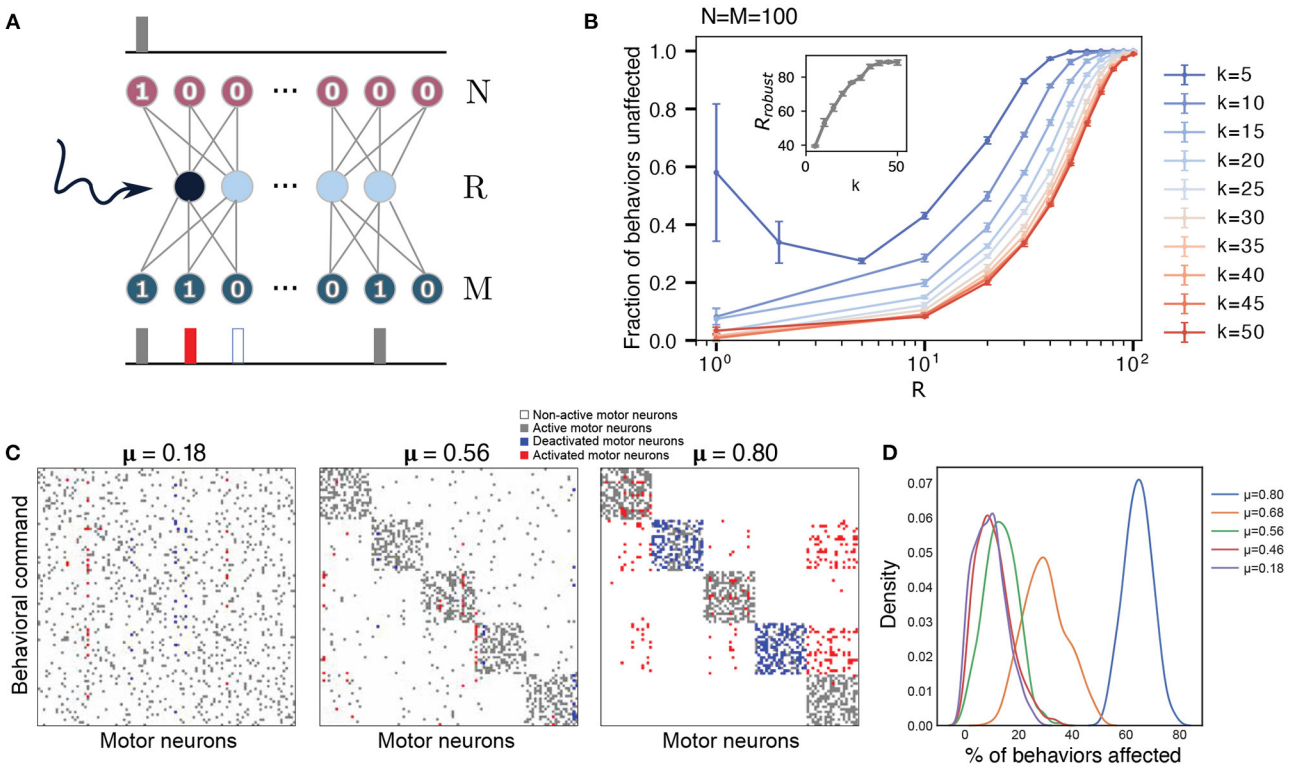

FIGURE 3 | Robustness of the network to perturbations increases with the size of the hidden layer and sparsity. **(A)** Schematic of the perturbation experiment. One of the hidden neurons of the trained network is artificially forced to be on, keeping all other network parameters unchanged. The network is re-run to generate new outputs for each behavioral command. **(B)** Robustness (fraction of outputs that are unaffected by the perturbation) averaged over the effects of activating each hidden neuron as function of the hidden layer size R, with $N = M = 100$, with k varied from $k = 5$ to $k = 50$. The error bars are obtained by considering 10 different behavioral matrices. The inset shows the size of the hidden layer for which such a perturbation leaves 98% of the behaviors unaffected, R_{robust}, as a function of changing sparsity (varying k). **(C)** Example of a hidden layer perturbation on the trained networks' behavior matrices with different modularities (all show with $R = R_c$). In each case, one of the hidden neurons is kept constantly activated, while the rest of the network operates according to the trained weights. White and gray colors correspond to unperturbed motor neurons, non-active and active correspondingly. Blue indicates motor units that have been turned off, and red shows motor units that have been activated. **(D)** Distribution of the number of behavioral commands affected by the hidden layer perturbation. Colors correspond to different degrees of modularity μ. Each distribution was calculated based on 10 different behavioral matrices, all with $R = R_c$.

2.3. Robustness to Perturbations of the Bottleneck Layer

Although the network is capable of reproducing behavioral commands nearly perfectly when it is near the critical bottleneck, it might be prone to errors due to minor perturbations, including noise in the firing of the descending layer. Inspired by previous studies in flies where descending neurons were artificially activated (Cande et al., 2018; Ache et al., 2019), we investigate the robustness of our trained neural networks by manually activating one hidden neuron at a time. We then observe the changes in the output (see **Figure 3A**) to see how these activations affect the mapping between command and behavior. An example of possible outcomes on a set of behaviors under these perturbation is shown in **Figure 3C** (for more examples, see **Supplementary Figure 4**). For each behavioral command, the motor neurons can either remain unaffected—their original "active" or "non-active" state is maintained (gray and white pixels in **Figure 3C**) or their state gets flipped—an "active" neuron gets inactivated or vice-versa (red and blue pixels in **Figure 3C**). The robustness of the network with respect to the activated neuron is calculated as the number of behaviors that are conserved, that is, behavioral commands where all activated motor neurons remain unaffected.

Figure 3B shows the robustness of the network to these perturbations as a function of the hidden layer size R and varying sparsity ($N = M = 100$ is fixed and k is varied), averaged over the effects of activating each hidden neuron and each behavioral command for a randomly generated behavioral matrix (no enforced modularity). For fixed sparsity, the fraction of behaviors that are unaffected increases as the size of the hidden layer increases. At the critical bottleneck size, for example, $R_c = 35$ for $k = 10$, 80% of behaviors were unaffected by the perturbation, indicating that the neural network has some margin of robustness. Robustness increases as we increase the hidden layer size R—the behavioral commands become less sensitive to changes in each individual hidden neuron. As long as the bottleneck layer size is less than the output layer ($R < M$), networks with output signals of high sparsity (lower k) are more

robust on average. The robustness is bounded below by the curve corresponding to maximum output signal density $k = 50 = M/2$. For sufficiently dense output signals $50 \geq k > 5$, the robustness decreases monotonically with decreasing hidden layer size for the entire range of $1 \leq R \leq M$. In contrast, the robustness of high sparsity outputs ($k = 5$) decreases initially with decreasing hidden layer size, but exhibits an increase in both its mean and variance at very small hidden layer sizes ($R < 5$). This behavior is likely caused by an all-or-nothing switching relationship between the hidden neurons and the output neurons.

When applying these perturbations to more modular behavioral matrices (**Figure 3C**), we find that the effects of the activations to the hidden neurons lead to more correlated changes in motor outputs. For these cases at the bottleneck size R_c (which varies depending upon the modularity, see **Figure 2A**), when some of the hidden neurons are activated, they not only affect a certain number of behaviors, but all of these commands tend to belong to the same cluster, which is what we would expect, given the modular structure of the weights in **Figure 2D**. Moreover, activation of a neuron can lead to the complete switch from one type of behavior to the another. An example of this effect is shown in **Figure 3C**. The first matrix in this panel corresponds to a random matrix of behavioral commands (also point #1 in **Figure 2A**). In this case, a particular hidden neuron may be attributed to at most some set of motor neurons as its activation leads to activation of two of them and deactivation of other three. However, in the perfectly modular case, there are some neurons that are responsible for the encoding of the whole cluster (rightmost panel in **Figure 3C**). When a hidden neuron is activated, it causes nearly an entire module of behaviors to be altered. This is in keeping with the previous studies showing that stimulating individual descending neurons in flies can result in dramatic behavioral effects (Bidaye et al., 2014; Cande et al., 2018; Ding et al., 2019; McKellar et al., 2019). Averaging over several behavioral matrices and perturbations (**Figure 3D**), we observe that this pattern holds true in general, with more modular behavioral matrices affected more by perturbations at R_c. This effect is likely due to the different sizes of the hidden layer where the critical bottleneck size R_c (the minimum number of hidden layer neurons needed to ably represent all behavioral commands) occurs, for varying levels of modularity. As the size of the hidden layer controls the susceptibility toward perturbations (**Figure 3B**), highly modular behavioral matrices that have a much smaller R_c (**Figure 2A**), are affected to a larger extent by the perturbations. For example, a fully modular behavioral matrix has $R_c = 13$, but at this size of the hidden layer, it is only approximately 40% robust to such perturbations (**Figure 4A**). This example highlights a trade-off between efficient information compression in the bottleneck layer and robustness in case of failure. In general however, if the constraint is that the size of the hidden layer is fixed, modularity *increases* robustness to perturbations (**Figure 4A**).

Thus, when constrained by a fixed size of the hidden layer, increasing the modularity and sparsity of the behavioral commands helps increase the robustness of the network to artificial perturbations. However, robustness suffers if the goal is to operate the network at the smallest possible critical bottleneck size for a given number of behavioral commands.

2.4. State-Dependency of Behaviors

Previous experimental studies in fruit flies observed that optogenetically activated behaviors in flies often depend on their behavioral state prior to activation (Cande et al., 2018; Ache et al., 2019). This effect can be quantified by calculating the mutual information between the distribution of a fly's behaviors before and after artificial neural activation. We refer to this effect as *state-dependency*. In essence, state-dependency implies that stimulating a neuron in the bottleneck layer will have varying—but predictable based on the input—behavioral results. In order to understand this experimentally observed effect within the framework of our model, we calculated the mutual information between the input and output distributions in the presence of an activated hidden neuron, while varying the size of the hidden layer and modularity (**Figure 4B** and **Supplementary Material 5**, see section 4 for details). This calculation provides a measure of how much information about the input distribution is contained in the output distribution in the presence of artificial activation.

With the input distribution corresponding to the fly's intended behavioral output (the one-hot encoded initial layer from **Figure 1A**) and the modified output corresponding to the set of behaviors that the artificial activation triggers, we see that increasing the bottleneck constraint (reducing R) lowers the overall mutual information—thus, it becomes harder to predict what the triggered behavior will be. On the other hand, a higher amount of modular structure in the output behavioral commands increases the mutual information for a fixed size of the hidden layer, with a maximum increase of around 0.8 corresponding to about a 30% increase between the two extreme values of modularity ($\mu = 0.18$ and $\mu = 0.8$) considered here. Thus, our model predicts that increased modular structure in the behavioral matrix not only increased robustness to perturbations (for a given N, M, and R), but also results in increased state-dependency. These results are consistent with the finding of state-dependency and modularity in the *Drosophila* VNC. In our model, this effect likely results from the fact that the model's weights are segregated at higher modularities (**Figure 2D**), meaning that the effect of stimulating a given bottleneck-layer neuron will be limited to a relatively small number of output behaviors.

It is worth mentioning that we find that the mutual information is proportional to the robustness (**Figure 4A** and **Supplementary Figure 5**) with a proportionality constant $\frac{1}{M}\log_2(N)$ (see section 4). This is a consequence of an absence of stereotypy in our simplified model, that is, multiple inputs don't give the same output on forced activation.

Given these results, we explored what predictions our model makes for two additional types of perturbation experiments that have not, to our knowledge, been systematically performed. First, we asked what the effects would be for deactivating, rather than activating, individual hidden layer neurons (**Figure 5A**). As one might expect for a binary encoded network, the effect of deactivating individual neurons on the robustness of the network is qualitatively similar to that for activation. The network is more robust to the perturbation as the size

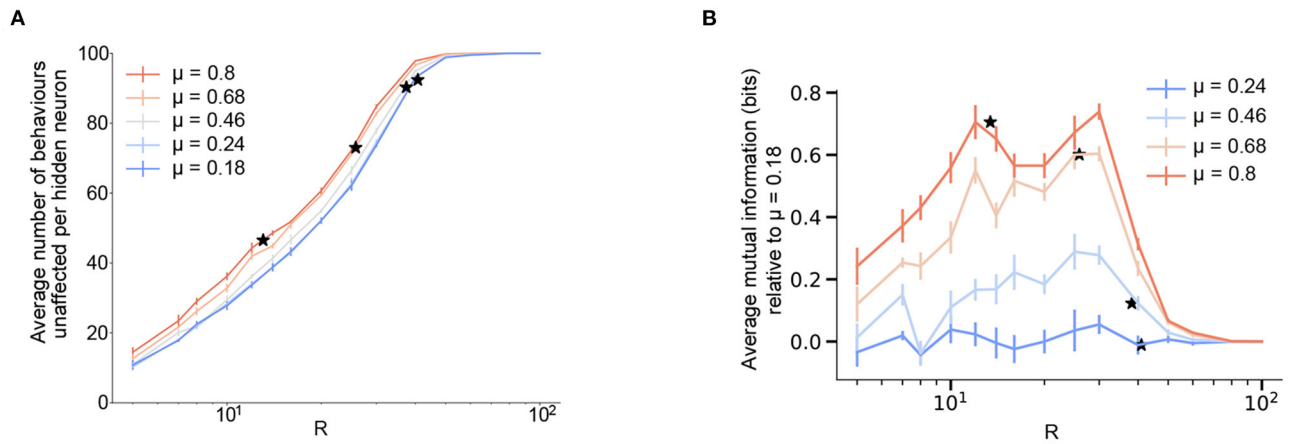

FIGURE 4 | Modularity improves robustness to perturbation and increases state-dependency for a fixed size of the hidden layer. **(A)** Robustness of the network averaged over the effects of activating each hidden neuron as a function of the hidden layer size, R and varying levels of modularity, μ. Here, robustness $\mathcal{A}(\mu)$ is defined as the numbers of behaviors that are not affected upon forcefully activating a neuron in the network. **(B)** Average mutual information (defined as in Equation 9) between the input and output distributions after forced activation of each hidden neuron as a function of the size of the hidden layer R, and varying levels of modularity, μ. To highlight the effects of increasing modularity, we show the results relative to the lowest modularity. The figure for the absolute values is reported in **Supplementary Figure 5**. The mutual information turns out to be $\mathcal{A}(\mu) \times \frac{1}{M}\log_2(N)$ due to the absence of stereotypy. **(A,B)** $N = M = 100$ and results are means over 5 iterations with the error bars corresponding to the standard deviation. Stars correspond to the R_c value for each value of modularity.

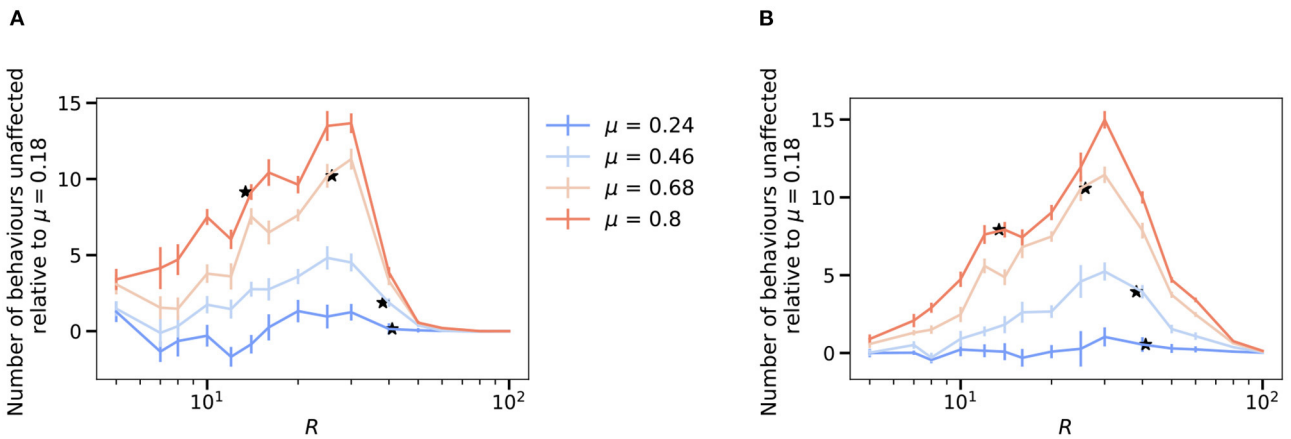

FIGURE 5 | Future excitation and inhibition experiments predict modularity is always associated with improved robustness. To highlight the effects of increasing modularity, we show the results relative to the lowest modularity, $\mu = 0.18$ as $\mathcal{A}(\mu) - \mathcal{A}(\mu = 0.18)$, where $\mathcal{A}$ is the robustness of the network upon de-activating each hidden neuron **(A)** and the robustness upon activating pairs of hidden neurons one at a time **(B)** defined as follows. The figure for the absolute values is reported in **Supplementary Figure 6**. The value of R_c for each modularity value is shown as stars. **(A)** Robustness of the network averaged over the effects of de-activating each hidden neuron as a function of the hidden layer size, R and varying levels of modularity, μ. **(B)** Robustness of the network averaged over the effects of activating a pair of hidden neurons as a function of the hidden layer size, R and varying levels of modularity, μ. **(A,B)** $N = M = 100$ and results are means over 5 iterations with the error bars corresponding to the standard deviation.

of the hidden layer increases. For any given size of the hidden layer, modularity increases the network's robustness to deactivating perturbations.

Similarly, we also explored whether activating pairs of hidden layer neurons (rather than individual neurons) leads to increased state-dependency with modularity as well (**Figure 5B**). We find similar results in this case (averaging over all possible pairs of hidden layer units across many networks).

3. CONCLUSION

Understanding how animals use their nervous system to control behavior is one of the key questions in neuroscience. A key component of most animal's nervous system is an information bottleneck between cognitive decision-making in the brain and the neurons that are responsible for the performance of behaviors. In this work, we use a simple feed-forward neural network, similar to an autoencoder architecture that is commonly

used in deep neural networks (Goodfellow et al., 2016), to understand the consequences of having such a bottleneck and identify different aspects of the network architecture that can still enable robust learning despite having such a constraint. For each set of network parameters, we identify the smallest size of the hidden layer (bottleneck size) that still allows near perfect learning. We find that increasing the sparsity of the output behavioral commands reduces this bottleneck size and increases the robustness of the network.

In addition to sparsity, we find that an increased modularity in the behavioral commands helps to reduce the bottleneck size and increases robustness. This observation could provide an explanation for why such a modular structure has evolved in the behavioral commands in animals, so far observed in flies. Our simple model is also able to predict the experimentally observed state-dependency between behavioral states before and after the forced activation of hidden neurons. We find that lowering the size of the hidden layer reduces state-dependent variability, but state-dependency increases with increasing modularity for a fixed hidden layer size. Overall, the modular nature of the output makes it easier for the network to learn in the presence of a bottleneck, increases its robustness but also leads to a higher amount of state-dependency.

This model described here is obviously simplistic in architecture and dynamics (in that it lacks them) and is highly unlikely to accurately describe the dynamical activity of ventral nerve cord function, where recurrent connections and temporal structure are important features of the system's functioning (Reyn et al., 2014; Phelps et al., 2021). Future work would incorporate the effects of temporal dynamics, as well as using more biophysically realistic neurons. In addition, our model only includes discrete inputs, and understanding how graded controls over more continuous variables (e.g., walking or flight speed) would be interesting for future study. In addition, our interpretation of the results implicitly assumes that the information bottleneck is the fundamental constraint that evolution has to contend with, rather than modularity itself being the constraint and an information bottleneck being the answer that maximizes efficiency. While the ubiquity of information bottlenecks in most nervous systems provides indirect evidence toward our interpretation, future comparative studies will be needed to assess which of the two hypotheses is more likely.

However, despite its simplicity, our model recapitulates several non-trivial features that are observed in experiment, and makes predictions as to the effects of artificially inhibiting neurons or of simultaneously stimulating multiple neurons, allowing for general principles of information-limited motor control to be elucidated, and new hypotheses to be tested.

4. MATERIALS AND METHODS

4.1. Network Architecture and Training

To mimic the structure of the neural chord, we built a feed-forward fully-connected neural network with one hidden layer (see **Figure 1**). The network is constructed with the Python framework PyTorch. The input layer represents decision neurons of number N: they send the signal from the brain down the network leading to a certain behavioral output. The hidden layer of size R represents descending neurons of the neural chord: it transmits the signal down to the motor neurons, which are the output layer of the network of size M. We used the sigmoid as our activation function, serving as an approximation of the transmission of the neural signal. The functioning of the neural network can be understood explicitly from its mathematical definition. The first layer applies a linear transformation on the input sequence $\vec{x}$ *via* the weight matrix, $W^{(1)}_{\alpha,\beta}$ connecting neuron α in the first layer with neuron β in the following equation,

$$a^{(1)}_{\beta} = \sum_{\alpha} W^{(1)}_{\alpha,\beta} x_{\alpha} - B^{(1)}_{\beta}\,, \tag{1}$$

while the second and last layer applies the activation function $\rho(\mathbf{a})$ on $\mathbf{a}^{(1)}$ as,

$$a^{(2)}_{\beta} = \sum_{\alpha} W^{(2)}_{\alpha,\beta} \rho(a^{(1)}_{\alpha}) - B^{(2)}_{\beta}\,, \tag{2}$$

with $\rho(\mathbf{a})$ given by the sigmoid $\rho(x) = 1/(1+e^{-x})$ and $\mathbf{B}^{(1)}$ ($\mathbf{B}^{(2)}$) is the bias, an additive constant. The output of the network is defined as $f(\mathbf{x}, \mathbf{W}) \equiv \mathbf{a}^{(2)}$, where $\mathbf{W}$ contains all the parameters, comprising the biases.

We fixed the size of the input layer ($N = 100$) throughout our experiments, while varying the sizes R, M of the hidden and output layers. We trained the network in the following fashion: we fixed the input and output matrices, i.e., decision and behavior matrices, respectively; we trained the network in a feed-forward manner using stochastic gradient descent with momentum and used the mean-squared error (MSE) loss function to assess learning performance; we stopped training after 10^5 epochs, which corresponds to when the loss curve flattens and the network is no longer learning. The output $\mathbf{y} = f(\mathbf{x}, \mathbf{W})$ of the trained network is then binarized by rounding each entry (using a Heavyside step function centered around 0.5) and the trained weights and biases defining the network are saved for further analysis. Along with these parameters, the number of behaviors learnt, obtained by comparing each entry of the output $\mathbf{y}$ with the imposed behavior, is also stored.

4.2. Modularity

We use the NetworkX 2.5 Python package to calculate modularity using the function 'networkx.algorithms.community.modularity' by treating the output matrix of behavioral commands as an adjacency matrix of a graph. Here modularity is defined as Newman (2018),

$$\mu = \frac{1}{2m} \sum_{ij} \left(A_{ij} - \frac{k_i k_j}{2m} \right) \delta(c_i, c_j) \tag{3}$$

where m is the number of edges, A_{ij} is the adjacency matrix, k_i is the degree (number of connections of a node in a graph) of i and $\delta(c_i, c_j)$ is 1 if i and j are in the same community and 0 otherwise.

4.3. Entropy of the Behavioral Matrix

The entropy of the behavioral matrix depends upon the number of behaviors N, size of the output layer M, sparsity k, number

of modules m, and the noise σ associated with the modules (# of units active outside a module, for e.g., $\sigma = 0$ for perfect modularity). For a random behavioral matrix where for any output k random units are turned "on" the total entropy (in bits) is,

$$S = N\log_2\binom{M}{k} \tag{4}$$

For a modular behavioral matrix with equal sized square modules ($m_{\text{size}} \times m_{\text{size}}$, $m_{\text{size}} = M/m$) the entropy (in bits) is given by,

$$S = N\log_2\left[\binom{m_{\text{size}}}{k-\sigma} \times \binom{M - m_{\text{size}}}{\sigma}\right] \tag{5}$$

4.4. Data Generation

The input data for all of our numerical experiments is always a 100×100 identity matrix. Each row of this matrix corresponds to the signal of performing one behavior from the output matrix. We generated several sets of output behavior matrices. In **Figure 1**, we varied the sparsity of the output matrix by changing the number of randomly activated units in a given row, i.e., the number of 1s. In **Figure 3**, we generated modular behavior matrices by introducing dense and sparse clusters into the output matrix. We start with 5 perfect clusters, i.e., no activated units are in common between 2 different clusters. Then, we generate matrices with different degree of modularity by deactivating some of the units within the cluster and activating the same number of units outside of the cluster so that the sparsity is preserved. In each case we generated 10 different behavior matrices for statistical purposes.

4.5. Checking the Robustness of the Network

We checked the robustness of the network by forcefully activating one of the hidden layer neurons. This is achieved by setting its corresponding weight in the first weight matrix $\mathbf{W}^{(1)}$ to an arbitrarily high value. We propagate the input matrix through the resulting perturbed network to get an output behavior matrix to be compared to the original output. In this way we can monitor how many of the original output behaviors were changed by the forceful activation. These steps are repeated for each individual hidden neuron and the results are averaged over the number of hidden neurons.

4.6. Mutual Information Calculation

Mutual information (MI) between two distributions is the measure of the amount of information one distribution has about the other. For two discrete binary random variables X and Y embedded in $\mathbb{R}^N$ with joint distribution $P(X, Y)$ it is given by Cover and Thomas (2006),

$$I(X;\, Y) = \sum_{x\in\mathcal{X}}\sum_{y\in\mathcal{Y}} P(x,y)\log_2\frac{P(x,y)}{P(x)P(y)} \tag{6}$$

where $P(X)$ and $P(Y)$ are the marginal distributions. In the absence of forced activation, the perfect learning case has a one to one mapping between the input and output distributions and hence the MI is $\log_2 N$. This perfect mapping gets perturbed on forced activation which can lead to one of the three different scenarios: (i) the input-output mapping is still unaffected, (ii) the input gets mapped to another output (stereotypy), and (iii) the input gets mapped to a completely different output that is not part of the original output distribution. This last case suggests that the input possess no information about the output.

Suppose we have N inputs x and M outputs y where we assume that they follow a uniform distribution, that is, $P(x) = 1/N$ and $P(y) = 1/M$. After forced activation, let n_i be the number of inputs associated with each output y_i where $n_i \geq 0$. This gives us $P(x|y_i) = \frac{1}{n_i}$ when $n_i > 0$ and $P(x|y_i) = 0$ when $n_i = 0$. The mutual information then reads

$$I(X, Y) = \sum_{y\in\mathcal{Y}} P(y) \sum_{x\in\mathcal{X}} P(x|y)\log_2\frac{P(x|y)}{P(x)} \tag{7}$$

$$= \sum_{y_i\in\mathcal{Y}'} P(y_i) \sum_{x\in\mathcal{X}'} \frac{1}{n_i}\log_2\left(\frac{N}{n_i}\right) \tag{8}$$

$$= \sum_{y_i\in\mathcal{Y}'} P(y_i)\log_2\left(\frac{N}{n_i}\right) \tag{9}$$

$$= \frac{1}{M}\sum_{y_i\in\mathcal{Y}'} \log_2\left(\frac{N}{n_i}\right) \tag{10}$$

where $\mathcal{X}'$ is the set of n_i inputs associated with each output y_i, $\mathcal{Y}'$ is the set of m outputs with $n_i > 0$. Note that in the absence of stereotypy that is, when n_i is either 1 or 0, the mutual information becomes

$$I(X, Y) = \frac{m}{M}\log_2(N), \tag{11}$$

where m is the number of original outputs that were unaffected by perturbation and hence, the mutual information becomes proportional to our definition of network robustness.

4.7. Statistical Analysis

Error bars in the figures are standard deviations that were calculated by averaging simulation results for 10 different output matrices unless specified otherwise. We used the UMAP (McInnes et al., 2018) method to visualize the structure in weight matrices.

4.8. Code Availability

The code for both our simulations and statistical analysis, can be downloaded from: https://github.com/drahcir7/bottleneck-behaviors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

AN, VD, RR, and GZ performed all the analyses. GB conceived the project and advised on all aspects of the modeling and analysis. All authors wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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The Neuromodulatory Basis of Aggression: Lessons From the Humble Fruit Fly

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Aggression is an intrinsic trait that organisms of almost all species, humans included, use to get access to food, shelter, and mating partners. To maximize fitness in the wild, an organism must vary the intensity of aggression toward the same or different stimuli. How much of this variation is genetic and how much is externally induced, is largely unknown but is likely to be a combination of both. Irrespective of the source, one of the principal physiological mechanisms altering the aggression intensity involves neuromodulation. Any change or variation in aggression intensity is most likely governed by a complex interaction of several neuromodulators acting via a meshwork of neural circuits. Resolving aggression-specific neural circuits in a mammalian model has proven challenging due to the highly complex nature of the mammalian brain. In that regard, the fruit fly model *Drosophila melanogaster* has provided insights into the circuit-driven mechanisms of aggression regulation and its underlying neuromodulatory basis. Despite morphological dissimilarities, the fly brain shares striking similarities with the mammalian brain in genes, neuromodulatory systems, and circuit-organization, making the findings from the fly model extremely valuable for understanding the fundamental circuit logic of human aggression. This review discusses our current understanding of how neuromodulators regulate aggression based on findings from the fruit fly model. We specifically focus on the roles of Serotonin (5-HT), Dopamine (DA), Octopamine (OA), Acetylcholine (ACTH), Sex Peptides (SP), Tachykinin (TK), Neuropeptide F (NPF), and Drosulfakinin (Dsk) in fruit fly male and female aggression.

Keywords: neuromodulator, aggression, serotonin, acetylcholine, dopamine, octopamine, peptides, *Drosophila melanogaster*

INTRODUCTION

Animals display aggression to acquire food, territories, and mating partners (Sturtevant, 1915; Hoffmann, 1987; Lin et al., 2011; Kravitz and Fernandez, 2015; Asahina, 2017; Anderson, 2016; Palavicino-Maggio et al., 2019a). However, the intensity of aggression must be modulated in accordance with changes in the external environment (such as, quality of the resource, size of the competitor, etc.) as well as internal environment (such as internal state, metabolic demands, etc.) (Lim et al., 2014; Li-Byarlay et al., 2014; Anderson, 2016; Asahina, 2017). An innate behavior such as aggression is encoded by genetically hardwired neural circuits. A critical question is, how does a genetically hardwired circuit allow flexible outputs of the same behavior? In other words,

how are different intensities of the same behavior, in this case aggression, computed at the circuit-level? One of the principal mechanisms allowing such behavioral flexibility is neuromodulation (Bargmann, 2012; Bargmann and Marder, 2013; Kim et al., 2017). Neuromodulators are signaling molecules released from neuronal processes, which may alter circuit outputs by modulating the biochemical and electrophysiological properties, metabolic demands, and transcriptional profile of target neurons. Neuromodulators communicate with target neurons via synaptic transmission and/or volume transmission (Civelli, 2012; Marder, 2012; Nadim and Bucher, 2014; Asahina, 2017). Synaptic transmission is a form of point-to-point transmission of neuromodulators between anatomically proximal neurons. Volume transmission, on the other hand, is a form of extra-synaptic mode of transmission in which neuromodulators may be released in a diffuse manner from neuronal endings with the potential to communicate with anatomically distant neurons (Uzelac, 1998; Bucher and Marder, 2013; Nadim and Bucher, 2014; Taber and Hurley, 2014). Compared to volume transmission, synaptic transmission has received and continues to receive more research attention (Taber and Hurley, 2014). Unlike the fast-acting neurotransmitters or gap-junctions, neuromodulation occurs with relatively slower kinetics, over longer time scales, and is well suited to encode persistent behaviors such as aggression (Yurkovic et al., 2006; Kim et al., 2017, 2018; McCormick et al., 2020).

Classical studies in the invertebrate models such as those on the circuit dynamics of chemosensory behaviors in *Caenorhabditis elegans* or stomatogastric nervous system-mediated rhythmic motor pattern generation in crabs and lobsters, have shed light on the myriad ways by which neuromodulators might modify the composition and function of activated neuronal circuits and in effect, modify the outputs of a behavior (Bargmann, 2012; Marder, 2012; Bargmann and Marder, 2013). The fruit fly model of *Drosophila melanogaster* has also been a forerunner in elucidating the neuromodulatory basis of many social behaviors. Findings from the fruit fly model have provided deep, mechanistic understanding of how neuromodulators and their receptors interact within a circuit to modulate aggression (Kravitz and Fernandez, 2015; Asahina, 2017), sleep (Artiushin and Sehgal, 2017; Shafer and Keene, 2021), memory (Margulies et al., 2005), courtship (Greenspan and Ferveur, 2000), locomotion (Clark et al., 2018), etc. In this review, we highlight the current research findings on aggression from the fruit fly model, note findings from the mammalian models for comparison, and speculate on future direction of research. We focus on the aggression-regulatory roles of Serotonin (5-HT), Dopamine (DA), Octopamine (OA), Acetylcholine (ACTH), Sex Peptide (SP), Tachykinin (TK), Neuropeptide F (NPF), and Drosulfakinin (Dsk). It is worth noting that majority of these neuromodulators have primarily been researched in the context of male aggression. Female aggression, on the other hand, is far less known. This review summarizes the current state of knowledge for both male and female aggression in the fruit fly model for each of these neuromodulators.

Systematic analysis of female aggression has had a slow start compared to males. While male aggression has been studied in many species from the 1900's (Sturtevant, 1915), little research was done to understand the neural mechanisms governing female aggression. It is difficult to pinpoint the reasons behind the discrepancy of interest between male and female aggression, but one potential contributing factor could be the general assumption that women, unlike men, do not engage in direct aggression (such as physical assault, threats of harm, etc.) (Denson et al., 2018). However, several exceptions exist to warrant a re-examination of this assumption. Aggression is a common symptom of many psychiatric diseases (Anderson, 2004; Zdanys et al., 2007; Arighi et al., 2012; Fisher et al., 2014; Gotovac et al., 2016; Lukiw and Rogaev, 2017). Diseases such as major depressive disorder (Gulland, 2016), anxiety disorders (McLean et al., 2011), postpartum psychosis (Siegel et al., 1983), post-traumatic stress disorder (PTSD) (Ditlevsen and Elklit, 2012), and dementia, affect women at significantly higher rates than men (Derreberry and Holroyd, 2019). Though PTSD is commonly associated with men, particularly those who have endured trauma because of military combat (Ditlevsen and Elklit, 2012; Crum-Cianflone and Jacobson, 2014), women have a two-fold higher risk of experiencing PTSD after a traumatic experience than their male counterparts (Ditlevsen and Elklit, 2012; Crum-Cianflone and Jacobson, 2014). In addition, instances of hyper-aggression involving direct physical attacks have also been documented in women (Lindberg et al., 2009). These observations strongly suggest that a comprehensive understanding of the neurobiology of aggressive behavior will not be possible by just focusing on male aggression.

In fruit flies, elevated female aggression has been observed under conditions of social isolation (Ueda and Kidokoro, 2002), mating (Bath et al., 2017, 2018, 2021), or nutrient scarcity (Lim et al., 2014). In addition, small populations of neurons have been identified in the fruit fly female brain, whose activation promoted very high levels of female aggression (Palavicino-Maggio et al., 2019b; Schretter et al., 2020). These results suggest that the fruit fly model of *Drosophila melanogaster* is a great model for studying female aggression. Findings from this model may provide fundamental insights about the importance of aggression in female fitness and the circuit logic by which female aggression is governed. With the availability of central brain connectomic data (Scheffer et al., 2020) and automated aggression analysis using machine vision (Schretter et al., 2020) combined with the strengths of fruit fly model, it is only a matter of time before our understanding of female aggression is significantly advanced along with male aggression.

Drosophila melanogaster: A MODEL FOR STUDYING AGGRESSION

Both male and female fruit flies exhibit aggression, and they do so by using a variety of stereotyped motor programs (Chen et al., 2002; Asahina, 2017). These motor programs are well characterized, easily recognizable and highly quantifiable, allowing researchers to perform quantitative aggression assays

and study changes in aggression intensity. Male aggression in *Drosophila melanogaster* can be exhibited by different motor programs such as fencing, wing threat, lunge, boxing, tussling, holding, etc. (Chen et al., 2002). Such an extensive repertoire of motor programs probably helps the fruit fly in adapting its fighting strategy to an ever-changing set of conditions. However, not all the motor programs occur at similar frequencies in a fight suggesting that the context behind each of them is probably different (Chen et al., 2002). For example, the motor program of "boxing" which involves two male flies striking at one another with their front legs, rarely occurs in a fight (Chen et al., 2002; Sengupta et al., 2022). In contrast, the motor program of "lunge," which involves a male fly standing on its hind legs and snapping down on its opponent, is most consistently used in intermale fights (**Figure 1B**; Chen et al., 2002). Similarly, female aggression uses many motor programs such as wing threat, head butt, high-posture fencing, shove, etc. (**Figure 1A**). The motor program of "head butt" is most consistent in female aggression (**Figure 1C**; Sturtevant, 1915; Nilsen et al., 2004; Palavicino-Maggio et al., 2019b), and is executed by a *Drosophila* female extending her torso and striking the conspecific with her head. To analyze changes in aggression intensity, some studies count the frequency of lunges or head butts within a given observation period, some studies count the total number of agnostic motor programs within a given observation period, while some count the percentage of animal pairs exhibiting aggression (Dierick and Greenspan, 2007; Asahina et al., 2014; Koganezawa et al., 2016; Asahina, 2017; Palavicino-Maggio et al., 2019b). In addition, the length of observation period, as well as aggression chamber setups have also varied greatly among studies (Kravitz and Fernandez, 2015). While all the aggression paradigms are correct, the differences among them are likely to influence aggression outputs and therefore, must be kept in mind before comparing results among studies. Other advantages of the fruit fly model include (a) a relatively simple brain ($\sim$ 100,000 neurons compared to $\sim$ 100 billion in humans), (b) advanced genetic and molecular toolkit, (c) a genome with 60% homology to humans, and (d) availability of the hemibrain connectome (Venken et al., 2011; Scheffer et al., 2020; Raji and Potter, 2021). Despite the dissimilarities with the mammalian brain in shape and size, the fruit fly central nervous system shares many similarities with its mammalian counterpart in circuit organization, kinds of neuromodulators used, and mechanisms of neuromodulator storage, release, and recycling (Leyssen and Hassan, 2007; Yamamoto and Seto, 2014). Therefore, findings from the fruit fly model reveal at least some of the general principles of the neuromodulatory basis of aggression in mammals.

SEROTONIN

The monoamine serotonin (5-HT) has been historically linked to aggression in a wide range of species including humans (Coccaro, 1989; Kravitz, 2000; Krakowski, 2003; Manuck et al., 2006). For many years, neurochemical and pharmacological studies upheld a view of a negative association between 5-HT functioning and aggression. This was based on several findings that detected low levels of 5-HT's metabolic product 5-HIAA (5-hydroxyindoleacetic acid) in cerebrospinal fluid of military men with personality disorders, men with violent suicidal tendencies or arsonists (Brown et al., 1979; Linnoila et al., 1983; Virkkunen et al., 1987; O'Keane et al., 1992). However, research in the last decade using sophisticated genetic and pharmacological tools in the invertebrate model of *Drosophila melanogaster* presented a different view. In male fruit flies, increasing and decreasing 5-HT signaling by pharmacological interventions increased and decreased male aggression, respectively (Dierick and Greenspan, 2007). Similar trends were observed upon genetically activating and inactivating 5-HT neurons en masse in brains of male flies (Dierick and Greenspan, 2007; Alekseyenko et al., 2010). First, these observations suggest that the 5-HT system regulates male aggression in both humans and fruit flies. Second, the apparent discrepancy between the valence of the 5-HT signal and aggression outputs in humans and fruit flies suggests that 5-HT's role in aggression regulation is complex and could be potentially influenced by multiple factors such as, magnitude of change in 5-HT signal, brain region involved, downstream 5-HT receptor cascades used, etc. Alternatively, it is also possible that 5-HT's aggression regulatory role is different between humans and fruit flies. Additional research is required to experimentally evaluate these possibilities.

The fruit fly nervous system has $\sim$100 serotonergic neurons (Alekseyenko et al., 2013) and the 5-HT system has been implicated in many behaviors in addition to aggression (such as memory, circadian rhythm, courtship, etc.) (Yuan et al., 2005; Becnel et al., 2011; Sitaraman et al., 2012). To characterize a 5-HT mediated aggression-specific neural circuitry, Alekseyenko et al. (2014) used intersectional genetics (Dionne et al., 2018) and identified a pair of serotonergic neurons with aggression regulatory roles. These neurons were located in the posterior lateral protocerebrum of the central brain (5-HT PLP neurons), a region known to receive visual input from the optic lobe (Pereanu et al., 2010). Activation and inhibition of the 5-HT PLP pair of neurons increased and decreased aggression in male fights, respectively (Alekseyenko et al., 2014). Interestingly, activity manipulation of the 5-HT PLP pair also had mild effects on few other behaviors (Alekseyenko et al., 2014). For example, inactivation of the 5-HT PLP pair of neurons mildly reduced the total amount of sleep per 24 h but did not affect the social behavior of courtship. Locomotion deficits were observed upon activation as well as inactivation of the 5-HT PLP pair of neurons. Since an increased aggression phenotype as well as a decreased aggression phenotype were recorded in fruit fly males with mild locomotion deficits, it is likely that the 5-HT PLP pair's modulatory role on aggression and locomotion are independent of each other. Since a single neuron can receive contacts from many different pre- and post-synaptic neurons (Klaassen et al., 1998), it can potentially influence multiple behaviors by activating non-overlapping circuit partners. Future experiments are required to determine whether this is the case with the 5-HT PLP pair of neurons.

A total of five 5-HT G protein-coupled receptors have been characterized in the fly model (5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_7) (Blenau et al., 2017) and a few of them

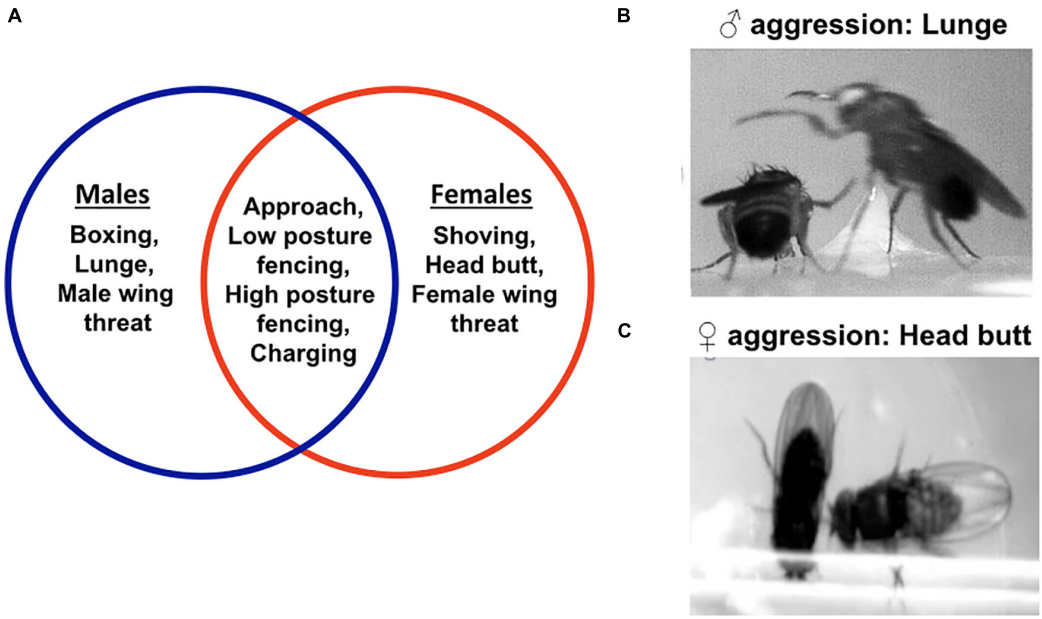

FIGURE 1 | Motor programs used in fruit fly aggression. The Venn diagram shows the different motor programs used in male and female aggression. Some of the motor programs are sexually dimorphic. The motor programs encircled in blue are specific for male aggression. The motor programs encircled in red are specific to female aggression. The intersection enlists the motor programs common to both male and female aggression **(A)**. The most consistent motor program in male aggression is a lunge **(B)**. In a lunge, a male fly stands on its hind legs and snaps down on its opponent. The most consistent motor program in female aggression is a head butt. In a head butt, the female fly extends her torso and strikes the opponent with her head **(C)**.

have already been reported to modulate aggression. For example, the aggression-promoting 5-HT PLP pair of neurons were found to make putative synaptic contacts with 5-HT_{1A} receptor neurons, implicating the latter's involvement in aggression (Alekseyenko et al., 2014). Another study identified two types of 5-HT_{1A} receptor neurons with opposing effects on intermale aggression (Alekseyenko et al., 2019). One of the 5-HT_{1A} receptor neurons was GABAergic, and its activation reduced aggression. The other 5-HT_{1A} receptor neuron was cholinergic, short neuropeptide F receptor$^+$ (sNPFR$^+$) and resistant to dieldrin GABA receptor$^+$ (RDL-GABA$^+$). Activation of this neuron increased aggression. Interestingly, the dendritic fields of both these neurons innervated the LC12 optic glomerulus of the ventrolateral protocerebrum, raising the possibility that visual cues input into the aggression circuit through the GABAergic and cholinergic 5-HT_{1A} receptor neurons.

While it has been shown that females with low serotonin levels exhibit higher aggression (Westergaard et al., 1999; Kästner et al., 2019), systematic studies on the role of 5-HT on aggressive behavior in all model systems have primarily focused on males. Continued examination of different components of the serotonergic system is necessary to comprehensively understand its bearing on aggression in both males and females.

DOPAMINE

Like 5-HT, Dopamine (DA) has also been shown to regulate aggression in vertebrate and invertebrate models (Ryding et al., 2008; Seo et al., 2008; Alekseyenko et al., 2013). Release of DA from the nucleus accumbens has been correlated with increased aggression in rats (Van Erp and Miczek, 2000). Activation of the ventral tegmental area (VTA) dopaminergic neurons has been shown to increase isolation-induced aggression in mice (Yu et al., 2014). Another study by Mahadevia et al. (2021) identified a subgroup of the VTA dopaminergic neurons that selectively projected to the lateral septum, and whose activity was necessary for maintaining baseline aggression in mice.

The fruit fly has ∼ 125 DA neurons in each brain hemisphere (Xie et al., 2018). Blocking synaptic transmission from DA neurons en masse generated hyperactive flies that displayed increased locomotion, and rarely engaged in either courtship or aggression (Alekseyenko et al., 2010). Using intersectional genetics, Alekseyenko et al. (2013) identified two pairs of morphologically distinguishable DA neurons in the fly brain with aggression regulatory roles: the tritocerebral neurons (T1) and the protocerebral posterior medial 3 (PPM3) neurons. Activating and inactivating the T1 and PPM3 pairs of neurons enhanced male aggression without any major effect on the behavior of locomotion (Alekseyenko et al., 2013). These observations suggest (i) activity manipulation of the T1 and PPM3 pairs of neurons has selective effects on aggression, (ii) relationship between DA signaling and aggression is not linear, with higher or lower amounts of DA-signaling resulting in an increase in aggression intensity. This kind of relationship is also known as the "U-shaped relationship." A similar U-shaped relationship has been reported for DA and spatial working memory in the rodent model, with increased and decreased DA signaling in the

prefrontal cortex inducing working memory deficits (Zahrt et al., 1997; Cools and D'Esposito, 2011). While these findings indicate a need for maintaining an optimal concentration of basal DA for cognitive functions such as aggression or working memory, the mechanistic basis of U-shaped effect is largely unknown.

Pharmacological studies in the murine models have highlighted the involvement of DA receptors in aggression regulation. Indeed, the current therapeutic interventions for treatment of aberrant aggression include antagonists of different DA receptors (Kudryavtseva, 2005; Khushu and Powney, 2016). For example, Haloperidol, which is primarily a D2-receptor antagonist, has been routinely used to treat violent behavior in aggressive patients, especially those suffering from psychosis (Nelson and Trainor, 2007; Khushu and Powney, 2016). However, the administration of these drugs is often complicated by negative side effects such as sedation, metabolic disorders, and tardive dyskinesia (de Almeida et al., 2005; Nelson and Trainor, 2007; Palavicino-Maggio and Kuzhikandathil, 2016), suggesting modulation of different biological processes through multiple site receptor-action. Therefore, though DA system has been shown to be necessary for aggression, details of the circuit mechanisms through which DA and its receptors specifically modulate aggression, remain largely unknown. In *Drosophila*, four G-protein coupled receptors (Dop1R1, Dop1R2, DD2R, and DopEcR) have been identified (Yamamoto and Seto, 2014). Of them, Dop1R1 has been found to regulate different types of arousal states: it positively regulates sleep-wake transitions (a form of endogenous arousal) and negatively regulates startle-induced arousal (a form of exogenous arousal) (Lebestky et al., 2009). In Alekseyenko et al. (2013), the presynaptic endings of dopaminergic T1 intermingled with the DD2R neurons in the protocerebral bridge and that for PPM3 neurons intermingled with the Dop1R1 neurons in the fan-shaped body and noduli of the central complex. While this raised the possibility that aggression regulatory T1 and PPM3 neurons interacted with DD2R and Dop1R1 receptors as downstream targets, direct experimental evidence demonstrating DD2R's and/or Dop1R1's involvement in aggression is still lacking.

Studies researching the function of DA in female aggression have been predominantly described in the context of courtship. Often when mated, immature, or older females come into encounter with a courting male, they may engage in pre-or post-mating female aggression that contains defensive aggressive behavior such as fleeing, kicking, and shoving, indicating rejection (Speith, 1952; Manning, 1966; Connolly and Cook, 1973; Ueda and Kidokoro, 2002; Sakurai et al., 2013; Bontonou and Wicker-Thomas, 2014; Bussell et al., 2014; Zhou et al., 2014; Li et al., 2016; Bath et al., 2017, 2018). Dopaminergic inputs have been noted to drive this circuit and govern female receptivity behavior (Zhou et al., 2014; Rezával et al., 2016; Ishimoto and Kamikouchi, 2020). This circuit is comprised of (R) neurons found in the ellipsoid body of the central brain (Martín-Peña et al., 2014; Omoto et al., 2018); PPM3 transmits DA specifically to R4d neurons, and activation of these neurons has been demonstrated to prolong the duration of this type of defensive behavior (Ishimoto and Kamikouchi, 2020).

The DA system, just like any other neuromodulator, is complex with regulatory roles in many behaviors. With more than 100 DA neurons sending arbors to different parts of the brain, the same brain region potentially generating paradoxical effects of activation or inactivation of downstream circuit depending on the DA receptors used, and a non-linear relationship between DA and male aggression at least in the fruit fly model, understanding the specifics of DA's aggression regulatory role is not straightforward. Nevertheless, findings from the fruit fly model provide relevant entry points into unraveling DA's regulatory roles in both male and female aggression.

OCTOPAMINE

Noradrenaline (NA) has been implicated in mammalian aggression (Yanowitch and Coccaro, 2011). Research in male mice indicated that perturbation of the NA signaling reduced aggression (Marino et al., 2005). The invertebrate ortholog of noradrenaline is Octopamine (OA). There are about ∼ 100 OA neurons in fruit fly brain (Busch et al., 2009; Farooqui, 2012). In the fruit fly model, OA is necessary for maintaining baseline aggression in both males and females (Zhou et al., 2008). Almost all the studies investigating how OA deficiency affects fly aggression used a deletion-mutant of *Tyramine ß-hydroxylase* gene ($T\beta h^{nM18}$) (Hoyer et al., 2008), that encodes a key biosynthetic enzyme in OA synthesis. $T\beta h^{nM18}$ males performed reduced lunges and increased male-male courtship toward conspecific males (Certel et al., 2007). $T\beta h^{nM18}$ females performed reduced number of head butts in female-female pairings (Zhou et al., 2008). These observations suggest OA signaling regulates aggression in both male and female fruit flies. Subsequent reports investigating the role of OA signaling in aggression focused on male aggression. Four kinds of OA-receptors (OAMB, Octβ1R, Octβ2R, and Octβ3R) have been characterized in *Drosophila* (El-Kholy et al., 2015). Watanabe et al. (2017) found that, OAMB receptor neurons labelled by a *GAL4* driver made from the cis-regulatory element of the *OAMB* gene (*R47A04-GAL4*), resulted in decreased aggression and increased courtship in male-male encounters. Altogether, these reports suggest that OA-signaling regulates appropriate behavioral choices in males.

In *Drosophila*, as in most species, males court females as potential mates and never attack them. Male-male pairings, on the other hand, are characterized predominantly by aggression with little or no courtship (Fernández et al., 2010; Wang et al., 2011; Monyak et al., 2021; Sengupta et al., 2022). In that regard, the increased courtship and decreased aggression phenotypes of the $T\beta h^{nM18}$ intermale fights could likely result from aberrant sex recognition. One of principal sensory modalities guiding sex-recognition and behavioral decisions in fruit flies is its pheromone system (Fernández and Kravitz, 2013). Indeed, elimination of some of the male-enriched pheromones such as (*z*)-7-tricosene (7-T), results in reduced aggression and increased courtship in male-male encounters (Wang et al., 2011). Sensory neurons expressing the chemoreceptor gene

Gr32a (Gr32a neurons) have been identified to mediate the behavioral effects of 7-T. Mutant males lacking *Gr32a* (*Gr32a* −/−) or males with ablated Gr32a neurons have been shown to phenocopy the decreased aggression and increased intermale courtship behaviors of the $T\beta h^{nM18}$ males (Wang et al., 2011; Andrews et al., 2014). These results suggest that sex-dependent pheromonal inputs processed by sensory Gr32a neurons are transduced upstream at least by the OA system for maintaining the appropriate balance of aggression and courtship in male-male pairings. Consistent with these findings, axons of Gr32a neurons have been found to make putative synaptic contacts with OA neurons in the suboesophageal ganglion (Andrews et al., 2014).

The above section suggests that OA signaling regulates aggression by modulating the pheromone-brain axis in fruit flies. A recent study (Jia et al., 2021) showed that OA signaling could also regulate aggression by modulating the gut-brain axis. The microbiome, a collection of microbes such as bacteria, archaea, fungi, and viruses, inhabit almost all the exposed surfaces of the body, with humans having the greatest density in their gastrointestinal tract or gut (Hsu et al., 2019). Using the fruit fly model, Jia et al. (2021) showed that gut microbiome selectively promoted both male and female aggression using OA neuromodulation. Germ-free males exhibited reduced aggression and a concomitant downregulation in OA signaling. Apart from a reduced expression in two major genes of the OA biosynthesis pathway, *Tyrosine Decarboxylase 2* (*Tdc2*) and *Tyramine ß-Hydroxylase* (*Tßh*), the germ-free males also displayed reduced Tdc2 immunoreactivity in subsets of OA neurons in the central brain. An interesting question is, how are signals from the gut transmitted to OA neurons in the central brain?

Does an enhancement of OA signal increase aggression in *Drosophila* males? Enhancing OA signaling in the less aggressive group-housed flies, by either feeding them the OA agonist Chlordimeform (CDM) or genetically overexpressing the *Tßh* gene, increased aggression (Zhou et al., 2008). But the same treatment was unable to raise the intensity of aggression among more aggressive socially naive males (Zhou et al., 2008). One hypothesis is, under normal conditions neural circuits are already saturated with OA signaling in socially naïve males and thus, any further enhancement of OA signaling does not result in an increase in aggression intensity. It would be interesting to overexpress the OA receptors in socially naïve flies and subsequently test the effect of enhancing OA signaling on aggression. It is worth mentioning that OA feeding alone did not increase aggression in group-housed flies in another study but did increase aggression upon *OAMB* overexpression in *R47A04-GAL4* neurons (Watanabe et al., 2017). The discrepancy in the observed outputs of aggression intensity could potentially result from different feeding strategies, chamber set ups and/or aggression scoring protocols.

ACETYLCHOLINE

The neuromodulatory role of Acetylcholine (ACTH) in aggression was initially suggested in the 1970s when ACTH-treated animal models revealed variation in aggression levels (Bandler, 1969; Silverman, 1969, 1971; Igić et al., 1970; Allikmets, 1974). Furthermore, known acetylcholine receptors (AchRs), nicotinic (nAchRs) and muscarinic (mAchRs) receptors also have been implicated in aggression regulation (Bandler, 1969, 1970; Berntson et al., 1976; Picciotto et al., 2015). Male and female cats, for example, exhibited aggressive behaviors in response to cholinergic agonists; however, muscarinic antagonists inhibited aggression. Nicotine and other nAChR-targeting drugs have been shown to reduce aggression in animal models (Bandler, 1969, 1970; Igić et al., 1970; Driscoll and Baettig, 1981; Yoburn and Glusman, 1984).

ACTH is found in many excitatory synapses in the *Drosophila* central nervous system (Buchner, 1991; Shih et al., 2019). Studies suggest that fruit fly has ten nAChRs and three mAChRs (Su and O'Dowd, 2003; Collin et al., 2013; Ren et al., 2015; Silva et al., 2015). While it is believed that nAChRs mediate fast excitatory synapses currents, mAChRs have been discovered to function as both excitatory and inhibitory modulators (Collin et al., 2013; Ren et al., 2015; Bielopolski et al., 2019).

It is possible that cholinergic signaling has opposing behavioral effects in both males and females. Enhanced female chasing, aggression, and territorial behavior, for example, were discovered upon activating *R26E01-GAL4* labeled neurons (McKellar and Wyttenbach, 2017; Palavicino-Maggio et al., 2019b). An intersectional study further revealed that neurons in the female fly brain's pC1 region (pC1α neurons) were cholinergic, expressed female isoform of the sex determination gene *doublesex* (*dsx*), and were responsible for this behavior (Palavicino-Maggio et al., 2019b). Other studies found another subset of neurons, known as the aIPg neurons (Cachero et al., 2010) that were also cholinergic and expressed sNPF, implying that an excitatory neural network regulated female aggression as well (Schretter et al., 2020). Both aIPg and pC1 cholinergic clusters have been found to mediate female aggression, with activation promoting persistent aggressive behavior and inhibition reducing aggression (Palavicino-Maggio et al., 2019b; Deutsch et al., 2020; Schretter et al., 2020; Chiu et al., 2021). Furthermore, additional research discovered that cholinergic neurons in the pC1 circuit also facilitate female receptivity during courtship behavior (Zhou et al., 2014; Rezával et al., 2016). The extent to which acetylcholine in neurons regulates female aggression and how this regulation coincides with that of mating behavior remains unknown.

In contrast, it has been found that blocking a single cholinergic neuron increases aggression in males (Alekseyenko et al., 2019), and feminizing cholinergic neurons in male brains similarly alters aggression (Mundiyanapurath et al., 2009). The brains of fruit flies include many cholinergic neurons, many of which are in areas that provide sensory information to the central brain (Kitamoto et al., 1995; Yasuyama and Salvaterra, 1999; Salvaterra and Kitamoto, 2001; Olsen et al., 2007). The detailed mechanism by which cholinergic neurons regulate aggression in males and females is unknown, and this has raised several questions, such as (i) are female cholinergic neurons distinct from the male cholinergic neurons? (ii) are there morphological distinctions amongst neuronal arbors? (iii) is there any variation in the quantities of acetylcholine or the transmitter release machinery?

SEX PEPTIDE

Seminal proteins have been found to have a sexually dimorphic effect on female and male behavior in both vertebrates and invertebrates (Cooke et al., 1998; Heifetz and Wolfner, 2004; Wigby and Chapman, 2005; Yapici et al., 2008; Yang et al., 2013; Lee et al., 2015; Garbe et al., 2016; Asahina, 2018; Isaac, 2019).

In fruit flies, mating has been shown to modulate female aggression, suggesting a link between neural circuits of mating and aggression (Nilsen et al., 2004; Bath et al., 2017). During copulation, the male's seminal fluid delivers a sex peptide (SP) (Chen et al., 1988; Aigaki et al., 1991; Chapman et al., 2003; Liu and Kubli, 2003; Heifetz and Wolfner, 2004; Feng et al., 2014), which activates SP receptors expressed in sex peptide sensory neurons that connect post-synaptically to the sex peptide abdominal ganglion (SAG) neurons (Yapici et al., 2008; Häsemeyer et al., 2009; Yang et al., 2009; Rezával et al., 2012; Bath et al., 2020). According to one study, female *Drosophila* mated with older males exhibit lower aggression reflecting changes in sex peptide activation (Bath et al., 2020).

SAG neurons have also been shown to be female-specific and implicated in post-mating behavior (Feng et al., 2014; Wang et al., 2020). Anatomical studies have shown that axons of SAG neurons project directly to the central complex of the brain (Feng et al., 2014; Wolff et al., 2015; Wang et al., 2020), ipsilaterally into the flange (periesophageal) area, and bilaterally into the superior medial protocerebrum, which includes the pars intercerebralis (PI) (Wang et al., 2020). Intrinsically, PI is a mammalian hypothalamus homolog that governs many processes, including sleep, alertness, locomotor cycles, aggression, and eating (De Velasco et al., 2007; Erion et al., 2012; Cavanaugh et al., 2014; Davis et al., 2014; Barber et al., 2016). However, the significance of SAG neurons in female aggression, is unknown.

Interestingly, in the pC1α activated female aggression study (Palavicino-Maggio et al., 2019b), *dsx* labeling was identified in the abdominal ganglion area, which also contains SAG neurons. According to electron microscopy (EM) data analysis (**Figure 2**) and other studies, SAG neurons project a vast number of putative synaptic input connections to pC1α neurons (pC1a-pC1e) (Zheng et al., 2018; Schretter et al., 2020; Wang et al., 2020). Neuronal tracings also revealed pC1α neurons have reciprocal connections within the pC1α neuronal cluster (**Figure 2**). Given that SAG neurons provide a significant number of synaptic inputs to pC1α neurons, is it possible that SAG neurons also regulate female aggression? This still remains an open question.

TACHYKININ

Tachykinins (Tk) constitute a group of evolutionary conserved neuropeptides present in both vertebrates and invertebrates wherein they perform a multitude of functions in controlling behavior, physiology and development (Jiang et al., 2013; Nässel et al., 2019). Substance P, a member of the Tk family has been linked to aggression-induction and regulation in many studies (Bhatt et al., 2003; Katsouni et al., 2009). Genetically knocking-out its potent Tk receptor, *Neurokinin-1 Receptor* (*NK1*) in the mice model has been reported to reduce aggression in resident-intruder experiments and alter nociceptive reflexes and analgesia (De Felipe et al., 1998). How tachykinins regulate aggression levels has been systematically studied in the fruit fly model (Asahina et al., 2014). Activation of a subset of male-specific Tk neurons (*Tk-GAL4^{FruM}*) robustly increased male aggression, while their silencing reduced aggression. Immunostaining experiments revealed that *Tk-GAL4^{FruM}* neurons expressed acetylcholine in addition to the neuropeptide Tk, thereby suggesting that acetylcholine may play an additional role in this circuit. A deletion mutation in the *Tk* gene in the homozygous form significantly reduced aggression, a phenotype that was rescued by expressing Tk neuropeptide in the Tk neurons. This suggests that at least part of the aggression modulatory function of the Tk neurons is mediated by the Tk peptides. *Drosophila* tachykinin has two known receptors: Tachykinin-like receptor 86C (TakR86C) and Tachykinin-like receptor 99D (TakR99D) (Birse et al., 2006; Poels et al., 2009; Pavlou et al., 2014). Owing to differential sensitivity to ligand Tk (Asahina et al., 2014, Asahina, 2017), both these receptors have been postulated to have non-overlapping roles in aggression regulation. The TakR99D receptor has a higher sensitivity to Tk and is postulated to mediate baseline aggression. TakR86C is postulated to regulate transient, intense bursts of aggression as seen during thermogenetic activation of *Tk-GAL4^{FruM}* neurons (Asahina et al., 2014; Asahina, 2017). Future investigations delineating how and which TakR99D and TakR86C receptor neurons interact with the Tk-GAL4^{FruM} neurons are necessary to characterize the circuit-mechanisms involved therein.

Tk-GAL4^{FruM} neurons are specified in males by the *fruitless* (*fru*) gene, a central component of the sex determination pathway (Wohl et al., 2020). Transcripts from the P1 promoter of the *fru* locus are spliced differently in males and females (Demir and Dickson, 2005). By gene-targeting, *fru* alleles *fru^{M}* and *fru^{F}* have been generated, which force male-specific and female-specific P1 *fru* splicing in females and males, respectively. The resulting *fruM* females are said to be masculinized, and the resulting *fruF* males feminized (Demir and Dickson, 2005). *Tk-GAL4^{FruM}* neurons are absent in wild type females but are present in the *fruM* females, where they are comparable to their male counterpart in number and morphology (Asahina et al., 2014; Wohl et al., 2020). Strikingly, optogenetic activation of the *Tk-GAL4^{FruM}* neurons in *fruM* females induced the male-specific motor program of "lunge," albeit at a low frequency, against wild type females or feminized males. *Tk-GAL4^{FruM}* activation did not induce female-specific "head butts" in these fights. Since lunge is a male-specific fighting pattern, these observations argue that *Tk-GAL4^{FruM}* neurons are a part of a neural circuit whose activation is sufficient for releasing significant amounts of male-patterns of aggression in females.

An intriguing question in the field of behavioral neuroscience is, how are male and female patterns of aggression encoded in the brain? In the fruit fly model, several features of male and female aggression are sexually dimorphic. Some of the motor programs used in male aggression, such as lunge and boxing, are male-specific (**Figure 1B**; Sturtevant, 1915; Hoffmann, 1987; Chen et al., 2002; Nilsen et al., 2004; Palavicino-Maggio et al., 2019a)

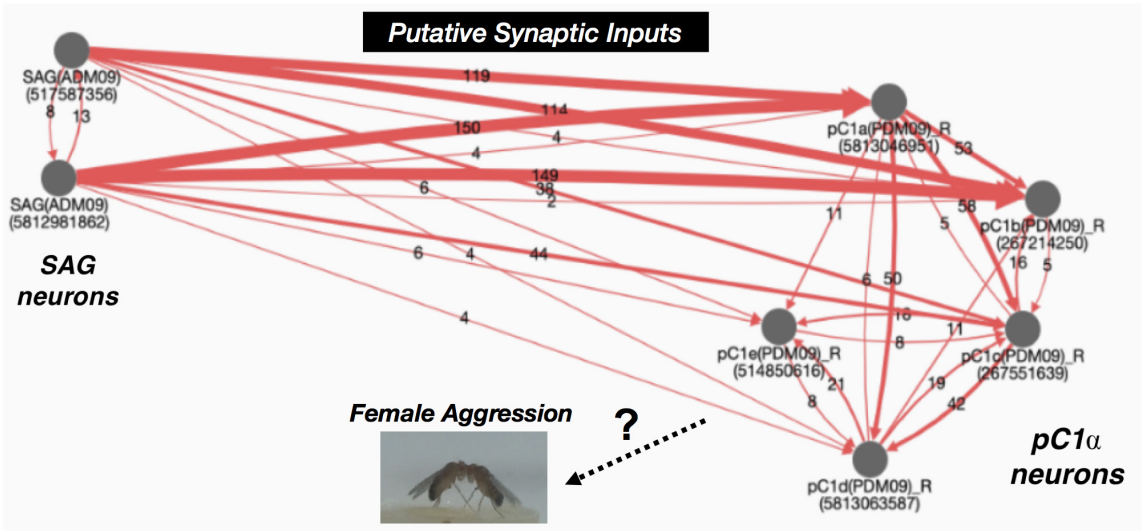

FIGURE 2 | Connectivity graph of SAG neurons and pC1α neurons. SAG neurons project major inputs into the pC1α neurons. pC1α neurons make reciprocal connections within the pC1 neuronal cluster. Red lines indicate synaptic connections and the numbers within the arrows represent number of shared synapses. Arrows indicate putative target. Numbers underneath traced neurons indicate the ID number from the neuPrint server (https://neuprint.janelia.org/) (Zheng et al., 2018; Scheffer et al., 2020; Wang et al., 2020).

and some in female aggression such as head butt, shove, are female-specific (**Figure 1C**; Manning, 1960; Ueda and Kidokoro, 2002; Nilsen et al., 2004; Palavicino-Maggio et al., 2019b). Social hierarchy or "dominance," a condition in which the dominant fly retains possession of the resources (such as food and territory) to the exclusion of the subordinate conspecific, is frequently established in most male fights but not in female fights (Chen et al., 2002; Nilsen et al., 2004; Palavicino-Maggio et al., 2019b). In addition, females often share resources during fights, unlike their male counterparts (Chen et al., 2002; Nilsen et al., 2004). What genes and neurons encode the sex-specific differences in aggression? A previous study found that *fruM* females and *fruF* males fought using significant amounts of male and female patterns of aggression, respectively (Vrontou et al., 2006). These results suggest that the sex-specific differences in fruit fly aggression are genetically encoded by at least the *fru* gene. Findings from Wohl et al. (2020) further refine our understanding by identifying a small group of male-specific neurons *Tk-GAL4*^{FruM} whose activation induced male patterns of aggression in masculinized females. Overall, these results provide an important framework on which to further research the neurobiological determinants of sexual dimorphism of aggression.

NEUROPEPTIDE F

Neuropeptide Y (NPY) is a 36-amino acid peptide that belongs to the NPY family of peptides along with peptide YY (PYY) and pancreatic polypeptide (PP). NPY is expressed widely in the mammalian brain and functions through five known G-protein coupled receptors Y1, Y2, Y4, Y5, and Y6. The NPY system regulates feeding, energy, homeostasis, stress, etc. (Reichmann and Holzer, 2016; Huang et al., 2021). NPY has also received a lot of attention because of its anxiolytic properties that are primarily mediated by Y1 receptor activation (Karlsson et al., 2008; Reichmann and Holzer, 2016). Perhaps not surprisingly, increased territorial aggressive behavior was reported in *Y1* knockout mice (Karl et al., 2004). NPY has been identified in invertebrates including fruit flies where it is called NPF to reflect the change from tyrosine (Y) to phenylalanine (F) in the C-terminal end (Fadda et al., 2019). To probe the role of NPF in aggression using the fruit fly model, Dierick and Greenspan (2007) genetically perturbed NPF signaling by blocking synaptic transmission from NPF neurons labeled by an *NPF-GAL4* driver. Compared to parental controls, a higher percentage of males engaged in aggressive interactions when synaptic transmission from *NPF-GAL4* neurons was blocked (Dierick and Greenspan, 2007). In contrast, another study detected a slight increase in aggression upon thermogenetic activation of the *NPF-GAL4* neurons (Asahina et al., 2014). These results possibly point toward the necessity of an optimal level of NPF signaling for maintaining baseline aggression, an effect also seen with DA (Alekseyenko et al., 2013). In other words, a U-shaped relationship potentially exists between the NPF signal and aggression. However, it is worth mentioning that the chamber set-ups, as well as aggression scoring parameters, were vastly different between Dierick and Greenspan (2007) and Asahina et al. (2014). Additional experiments may have to be performed before directly comparing results from the two studies.

The *NPF-GAL4* labels ∼ 30 neurons that extend their neuronal processes throughout the central brain and VNC, and at least some of these cells are male-specific (Shao et al., 2017). The next question is, are all or a specific subpopulation of the NPF neurons required for increasing aggression? Dierick and Greenspan (2007) expressed the

female-specific *Transformer* gene in the *NPF-GAL4* labeled cells (*NPF-GAL4/UAS-TRA*) to eliminate *NPF* expression in the male-specific NPF cells. Interestingly, *NPF-GAL4/UAS-TRA* males recapitulated the aggression-inducing phenotype of the synaptically blocked *NPF-GAL4* neurons (Dierick and Greenspan, 2007). This result raises the possibility that male specific NPF neurons were regulators of baseline aggression. NPF is known to bind to a single receptor NPF receptor (NPFR) (Chung et al., 2017). Presently, we do not know which NPFR neurons synaptically connect to NPF cells to regulate aggression. Next, we also do not know how NPF system regulates female aggression. Since *NPY* deletion has been found to increase depressive behaviors in female mice (Nahvi and Sabban, 2020; Nahvi et al., 2021), similar to its male counterparts, it would be interesting to investigate how genetically and/or pharmacologically manipulating the NPY/NPF system influence female aggression.

DROSULFAKININ

Neuropeptide Cholecystokinin (CCK) has been studied extensively for its anxiogenic effects. It is synthesized as a 115 amino acid preprohormone which is proteolytically cleaved to generate many biologically active peptides (Netto and Guimarães, 2004; Bowers et al., 2012). Administration of CCK tetrapeptide (CCK-4) induced panic attacks in humans (Bradwejn, 1993). RNA interference (RNAi) mediated knockdown of *CCK* in the VTA of mice resulted in manic-like phenotypes (Arey et al., 2014). CCK is also present in the gastrointestinal tract (GI) where it regulates many important GI functions such as satiety and food ingestion (Moran, 2000; Moran and Kinzig, 2004). The *Drosophila* ortholog of *CCK* is called *Drosulfakinin* (*Dsk*), and its modulatory role has been studied in male sexual arousal (Wu et al., 2019) and satiety (Nässel and Williams, 2014). In *Drosophila*, the *Dsk* gene encodes three mature peptides: Dsk 0, Dsk1, and Dsk2. Of these, Dsk1 and Dsk2 are known to be CCK-like peptides. Two G-protein coupled receptors have been identified for the Dsk peptides: CCKLR17D1 and CCKLR17D3 (Nässel and Williams, 2014; Wu et al., 2019).

One of the first glimpses of Dsk's connection in fruit fly aggression came in 2014 when Williams et al. (2014), reported octopaminergic signaling regulated male aggression by controlling Dsk expression in insulin producing cells. A more detailed analysis of Dsk's role in aggression has come from a study by Wu et al. (2020). Genetically knocking out *Dsk* (*Dsk* −/−) reduced male aggression without interfering with locomotion or courtship (Wu et al., 2020). However, *Dsk* −/− males also exhibited increased feeding behavior (Wu et al., 2020). Genetically silencing and activating a subpopulation of ∼ 8 Dsk neurons in the fruit fly brain labeled by a *Dsk-GAL4* driver reduced and increased male aggression, respectively. These results suggest that both the Dsk molecule as well as the *Dsk-GAL4* labeled neurons are necessary for male aggression. Of the two Dsk receptors, loss-of-function mutants of *CCKLR17D1* (*CCKLR17D1-/y*) and not *CCKLR17D3* (*CCKLR17D3-/y*) recapitulated the reduced aggression phenotype of the *Dsk* −/− males (Wu et al., 2020). Moreover, the aggression-promoting effect of activated *Dsk-GAL4* neurons was lost in the *CCKLR17D1* mutant background suggesting CCKLR17D1 receptor system acts downstream to mediate the aggression promoting role of activated *Dsk-GAL4* neurons (Wu et al., 2020).

Dsk-GAL4 neurons were reported to be synaptically connected to a subset of male-specific P1 neurons, popularly known as $P1^a$, whose activation has been shown to simultaneously increase aggression and courtship in male-male pairings (Hoopfer et al., 2015). When $P1^a$ neurons were activated in *Dsk* −/− males, its aggression-promoting effect was severely suppressed while its courtship-promoting effect was preserved, suggesting that the Dsk system acts downstream of activated $P1^a$ neurons to promote male aggression. Overall, these research findings provide important insights into Dsk's aggression modulatory role. However, many outstanding questions remain. The Dsk system in fruit flies, like that of mammals, is implicated in both aggression and feeding behavior and right now, we do not know whether the same or different subsets of Dsk neurons regulate feeding and aggression (Moran, 2000; Moran and Kinzig, 2004; Nässel and Williams, 2014; Wu et al., 2020). Furthermore, we also do not know whether one or both CCK-like Dsk peptides are necessary for aggression regulation.

In contrast to Wu et al. (2020), another contemporary study by Agrawal et al. (2020) reported that *Dsk* knockdown using RNAi increased social-isolation mediated aggression (Agrawal et al., 2020). Since the two investigations (Agrawal et al., 2020; Wu et al., 2020) differed in multiple aspects, it is hard to speculate on the possible reasons behind the seemingly contradictory findings. Some of these differences are as follows: (i) chamber set-ups and scoring paradigms were different making direct comparison of results difficult (ii) techniques used for reducing *Dsk* expression were different. It is possible that Dsk signaling was perturbed to different degrees in the two studies, thereby resulting in different aggression outputs (iii) use of different *Dsk-GAL4* drivers. Unlike the *Dsk-GAL4* used in Wu et al. (2020), the *Dsk-GAL4* used by Agrawal et al. (2020) targeted a group of Dsk neurons that included the Dsk^+ insulin-like peptide Dilp2-producing neurons in the PI of the fly brain (Nichols, 1992; Nichols and Lim, 1995; Söderberg et al., 2012; Asahina et al., 2014; Agrawal et al., 2020). Interestingly, *Dsk* RNAi in the Dilp2-producing neurons using a *Dilp2-GAL4* driver also increased social-isolation mediated intermale aggression. These results suggest that reduced Dsk signaling in the PI increased intermale aggression upon social isolation. In view of the results from Agrawal et al. (2020) and Wu et al. (2020), one hypothesis is, neurons within the Dsk population exert heterogenous effects on aggression. Future investigations employing genetic mosaic techniques, such as mosaic analysis with repressible cell marker (Wu and Luo, 2006), may be used to stochastically label individual or reduced subsets of Dsk neurons and analyze their roles in aggression.

Finally, in common with most neuromodulators, Dsk's role in female aggression is poorly understood. Wu et al. (2020) reported that *Dsk* knockout (*Dsk* −/−) suppressed female aggression. But a detailed picture of Dsk-mediated female-specific aggression

circuitry is lacking. An interesting question is whether the Dsk-mediated aggression circuit in males and females involves common or sexually dimorphic set of neurons. Just like any neuromodulator, the Dsk system is complex, with links in multiple behaviors, likely by the recruitment of different peptides, receptors, and neurons. Future experiments addressing some of the questions addressed here may help understand important aspects of Dsk's role in aggression.

CONCLUSION AND FUTURE DIRECTIONS

Neuromodulation constitutes a principal mechanism for generating flexible outputs of a stereotypical behavior such as aggression in both vertebrates and invertebrates. Aggression released at different intensities may be considered as flexible outputs of the behavior. Research in the fruit fly model of *Drosophila melanogaster* have made important strides in identifying specific groups of cells in the central brain system as parts of circuits whose activity manipulation changes the intensity of aggression. However, a comprehensive understanding of the circuit dynamics is lacking. In other words, finer details of how neuromodulation is achieved mechanistically are limiting. There are several ways through which neuromodulators may encode different intensities of aggression. For example, neuromodulators can coordinate multiple neuronal circuits encoding the fly's internal and external states to compute and release aggression at a certain intensity (Bargmann, 2012; Marder, 2012). Neuromodulators can also effectively reconfigure new circuits from existing ones by recruiting new neurons or excluding current members and in doing so, alter the output intensity of aggression (Bargmann, 2012; Marder, 2012). In addition, neuromodulators can modify the excitability of an existing circuit to release aggression at an intensity appropriately matched with internal and external environments (Bargmann, 2012; Marder, 2012). At the present moment, we do not know which of these mechanisms are at work during aggression in male-male or female-female encounters. However, with the recent advances in the fly toolkit such as (i) connectomic data showing anatomical connections between different brain regions, (ii) an ever expanding genetic and molecular toolkit making precise manipulation of neuronal activity possible, and (iii) a rapidly growing set of imaging tools allowing researchers to investigate neuronal structure and function across several spatial and temporal scales, it will not be long before a comprehensive picture of the neuromodulatory basis of aggression regulation, from sensory processing to behavior computation, starts emerging.

AUTHOR CONTRIBUTIONS

CP-M and SS conceived and designed the review. Both authors contributed equally to the article and approved the submitted version.

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REVIEW
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Shedding Light on Inter-Individual Variability of Olfactory Circuits in *Drosophila*

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Inter-individual differences in behavioral responses, anatomy or functional properties of neuronal populations of animals having the same genotype were for a long time disregarded. The majority of behavioral studies were conducted at a group level, and usually the mean behavior of all individuals was considered. Similarly, in neurophysiological studies, data were pooled and normalized from several individuals. This approach is mostly suited to map and characterize stereotyped neuronal properties between individuals, but lacks the ability to depict inter-individual variability regarding neuronal wiring or physiological characteristics. Recent studies have shown that behavioral biases and preferences to olfactory stimuli can vary significantly among individuals of the same genotype. The origin and the benefit of these diverse "personalities" is still unclear and needs to be further investigated. A perspective taken into account the inter-individual differences is needed to explore the cellular mechanisms underlying this phenomenon. This review focuses on olfaction in the vinegar fly *Drosophila melanogaster* and summarizes previous and recent studies on odor-guided behavior and the underlying olfactory circuits in the light of inter-individual variability. We address the morphological and physiological variabilities present at each layer of the olfactory circuitry and attempt to link them to individual olfactory behavior. Additionally, we discuss the factors that might influence individuality with regard to olfactory perception.

Keywords: insect, antennal lobe, odor, sensory processing, olfactory behavior, neural circuits

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INTRODUCTION

Researchers studying animal behavior are confronted with the diversity of behavioral outputs among individuals. Even individuals with nearly identical genotypes display different behavioral personalities. It is important to note that variability across individuals does not always reflect idiosyncratic behavior. A specific behavior is considered as a trait of individuality if it designates behavioral features that differ among conspecifics and persist over trials. This phenomenon has been described in humans (Johnson et al., 2009), rodents (Freund et al., 2013), fish (Vogt et al., 2008) and insects (Schuett et al., 2011) comprising various behaviors, such as startle, social, reproductive, locomotor, phototaxis, aggression as well as olfactory behaviors (Vogt et al., 2008; Schuett et al., 2011; Honegger and de Bivort, 2018). The vinegar fly *Drosophila melanogaster* represents a powerful genetic model organism to investigate variability among individuals. In

fact, animals with the same genotype can be studied at a behavioral, physiological, anatomical and molecular level. Several studies that analyzed behavioral variability in *Drosophila* strongly contributed to our present knowledge regarding relevant brain regions and underlying genes that might be involved in idiosyncrasy (Kain et al., 2012; Ayroles et al., 2015; Buchanan et al., 2015; Honegger et al., 2020). Notably, the vinegar fly exhibits individual behaviors that persist over days in phototaxis (Kain et al., 2012), spontaneous locomotor biases (Buchanan et al., 2015), thermal preference (Kain et al., 2015), leg postural dynamics and locomotion (Todd et al., 2017), object-fixated locomotion (Linneweber et al., 2020), olfactory learning (Smith et al., 2021) and innate odor-guided behavior (Honegger et al., 2020). Even though individuality is present in every behavior and might shape the personalities of animals, it is rather disregarded and not taken into consideration in the final data presentation. Additionally, the link of individual differences between brain structures and physiology to the idiosyncratic behavior is still poorly understood. The comprehension of individual behavior and its relationship to brain structure and function will shed light on the strategies used by animals to differentiate themselves from others and allow them to adapt to environmental fluctuations. Individuality is a highly interesting phenomenon which gives important insight into how neural circuits develop and what internal as well as external factors are determining a behavioral output.

In this review we focus on the sense of smell of the vinegar fly, since this offers an ideal model system to study inter-individual variability. Over the last decades, numerous studies have identified the anatomical, molecular and genetic basis of the fly's olfactory behavior (Harris, 1972; Venkatesh and Naresh Singh, 1984; Siddiqi, 1987; Stocker, 2001; Jones et al., 2007; Kwon et al., 2007; Vosshall and Stocker, 2007; Pask and Ray, 2016; Gomez-Diaz et al., 2018; Yan et al., 2020). Moreover, functional imaging as well as EM based connectomic studies have elucidated in great detail the associated brain circuits involved in the processing of olfactory information (Wang et al., 2003; Berck et al., 2016; Grabe et al., 2016, 2020; Horne et al., 2018; Zheng et al., 2018; Frechter et al., 2019; Bates et al., 2020; Marin et al., 2020). Such information will help us to highlight subjects to variability at the olfactory circuit level that will take this field a step further and decipher the observed differences in the behavioral output of different individuals.

Flies detect odors with the help of olfactory sensory neurons (OSNs) present on the third antennal segment and the maxillary palps (Stocker, 1994; **Figure 1A**). These olfactory appendages are covered with sensilla and each sensillum houses between one to four OSNs (Venkatesh and Naresh Singh, 1984; De Bruyne et al., 2001). Each OSN expresses one specific chemosensory receptor from two gene families—odorant receptors (ORs) or ionotropic receptors (IRs)—in combination with not only one, but several co-receptors (i.e., Orco, Ir8a, Ir25a, and/or Ir76b) as recently shown (Task et al., 2020). All OSNs project their axons to the antennal lobe (AL) and converge upon one specific olfactory glomerulus (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999, 2000; Benton et al., 2009). A given odor activates different OSN classes and their respective glomeruli in a combinatorial manner (Grabe and Sachse, 2018). Interglomerular connections are provided by local interneurons (LNs) (Wilson and Laurent, 2005; Seki et al., 2010; Mohamed et al., 2019). Following pre-processing, the olfactory information is transferred to higher brain centers, such as the mushroom bodies (MB) and the lateral horn (LH), through olfactory projection neurons (PNs) (Jefferis et al., 2007; Lin et al., 2007; Fişek and Wilson, 2014). The LH is believed to primarily mediate innate behavior (e.g., De Belle and Heisenberg, 1994; Jefferis et al., 2007; Das Chakraborty and Sachse, 2021), while the MBs form olfactory associative memories (e.g., Heisenberg, 2003; Hige, 2018). The processed odors information is subsequently translated into a behavioral output.

In this review we would like to revisit the anatomical and functional features of the olfactory circuitry at different processing levels in *Drosophila* in the light of inter-individual variability and discuss what that might imply for individualized odor-guided behavior. A first step toward identifying the origins of inter-individual differences in odor-guided behavior in flies is to give an overview of the morphological and physiological variabilities present at each layer of the olfactory circuitry (**Figure 1B**). Furthermore, we will describe the factors that might support the emergence of olfactory personalities (**Figure 1C**). We also explore at what processing level connections and cellular properties become specific to each individual animal. Finally, the link between the differential connectivity in the olfactory circuit and odor-preference individualities is discussed.

FACTORS THAT INFLUENCE OLFACTORY INDIVIDUALITY

Genetic and environmental traits together shape the individuality of animal behavior. Animals with similar genetic background adapt their gene expression to the available resources present in the environment (Honegger and de Bivort, 2018; Koyama et al., 2020). Even among individuals with the same genotype reared under the same environmental condition, differences in the phenotype were noted in genetic studies (Lin et al., 2016). Moreover, during the life course of flies, the expression of genes is plastic leading to changes in the individuality of an animal (Juneja et al., 2016; Lin et al., 2016).

Genes underlie transcriptional variation between individuals that influence different behavioral outputs (Jin et al., 2001). Studies showed variations in genes associated with olfactory perception in *Drosophila melanogaster*. Genetic variation in specific olfactory receptors or genes associated with neural development and the later processing in the central nervous system induces divergent odor guidance behavior among individuals of the same population (Richgels and Rollmann, 2012; Brown et al., 2013). This aspect is further discussed in the section "Variability at the level of olfactory sensory neurons." The genotypic variation is also observed in other traits such as lifespan or morphological and anatomical structures (e.g., brain, wing, thorax, or eye size) (Carreira et al., 2016; Buchberger et al., 2021). Studies using the Drosophila Genetic Reference Panel (DGRP) have found the genetic origin involved in the variation

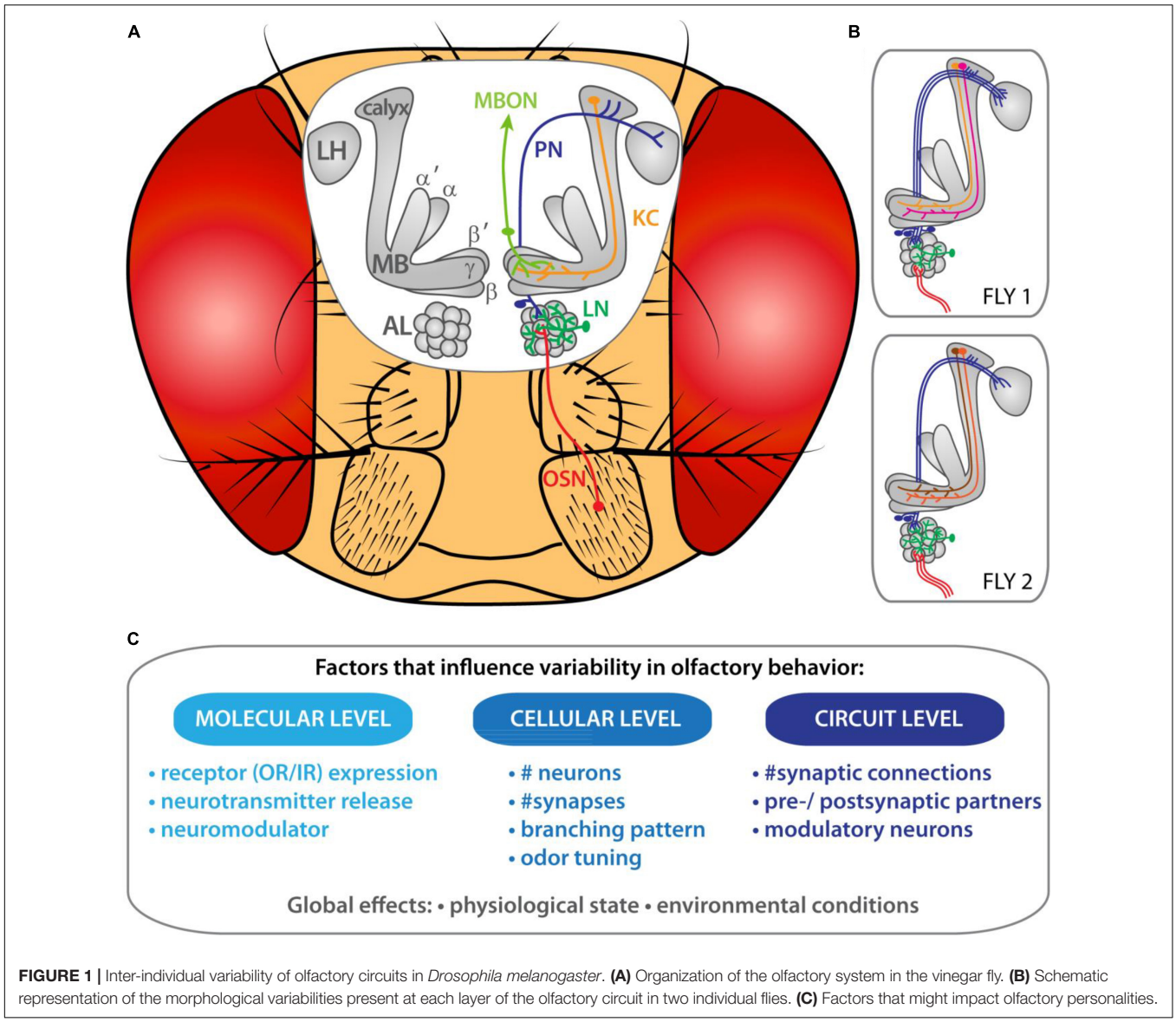

FIGURE 1 | Inter-individual variability of olfactory circuits in *Drosophila melanogaster*. **(A)** Organization of the olfactory system in the vinegar fly. **(B)** Schematic representation of the morphological variabilities present at each layer of the olfactory circuit in two individual flies. **(C)** Factors that might impact olfactory personalities.

between individuals expressing specific behaviors such as flight performance (Spierer et al., 2021), virgin egg retention (Akhund-Zade et al., 2017), aggressive behavior, immune response against pathogens (Guzman et al., 2021) as well as mating behavior (Gaertner et al., 2015).

In addition, researchers studied the implication of neuromodulators, such as serotonin or dopamine, with regard to inter-individual variability (Maloney, 2021). Interestingly, idiosyncrasy in olfactory behavior was reduced in a dose-dependent manner when the flies were fed on food containing the serotonin synthesis inhibitor alpha-methyltryptophan (Honegger et al., 2020). In contrast, activating the contralaterally projecting, serotonin-immunoreactive deutocerebral neurons (CSDn) had no effect on behavioral variability (Honegger et al., 2020). The former result is in line with a previous study showing that the neuromodulator serotonin affects the degree of idiosyncrasy in phototaxis behavior (Kain et al., 2012). The later result concerning the CSDn is consistent with the fact that synaptic connectivity of serotoninergic neurons is heterogeneous across glomeruli but stereotypic across individual flies (Coates et al., 2017). However, some degree of inter-individual variability has also been observed for the OSN-CSDn connectivity in a few glomeruli, as e.g., DA2 and VM2 (Coates et al., 2017, 2020). PNs and LNs express a diversity of serotonergic receptors that might be responsible for the effect of serotonin on the variability (Sizemore and Dacks, 2016). Additionally, also the neurotransmitter dopamine has been shown to have an impact on variability in odor-guided behavior. Dopaminergic neurons innervate the MB lobes in a compartmentalized manner and are crucial for associative learning (Aso et al., 2014). A mutation of the dopamine receptor gene (Dop1R1) induced lower variability in olfactory behavior than control flies, while a higher variability could be observed in flies that have been fed with the dopamine precursor L-DOPA (Honegger et al., 2020). In fact, Dop1R1

facilitates synaptic plasticity in the MBs (Kim et al., 2007; Qin et al., 2012). The effect of dopaminergic neurons in the MBs with regard to odor-tracking behavior was further investigated by Zolin et al. (2021). This study showed that dopamine can contribute to multiple forms of behavioral modulation by conveying motivational as well as instructive signals that shape current behavior and dictate future behavior through learning (Zolin et al., 2021). In general, neuromodulators seem to affect individuality at different levels: (i) variation in the amount of neuromodulation by differences in receptor expression, production of neuromodulators or activity in neuromodulatory neurons; (ii) alteration of circuit function to mask or accentuate circuit variability; (iii) facilitating plasticity of neural circuits. Hence, all these data suggest that serotonin and dopamine may control the degree of variability between individual flies (though not exclusively).

A recent study investigated biological mechanisms that affect variability/individuality with regard to locomotor behavior (de Bivort et al., 2021). A large data set of vinegar flies walking in Y-shaped mazes was evaluated by taking different biological mechanisms into consideration: the neuromodulator serotonin, white genotype, heterogametic sex and temperature. The results revealed that serotonin levels affected the variability of turn number, but had no strong effect that was consistent across behaviors. Notably, white genetic disruption correlated with small reductions in variability in turn bias and turn switchiness. Concerning the effect of sex on behavioral variability, male flies exhibited variability that was less in turn bias and greater in the number of turns as well as turn switchiness. On the other hand, high temperature significantly decreased the variability with regard to number of turns and turn switchiness but had no effect on turn bias variability. Overall, this study provided evidence that the effect on variability of the biological mechanism, as mentioned above, was behavior-dependent (de Bivort et al., 2021).

Developmental and growth conditions represent another important factor that has an influence on behavioral individuality. These variations are non-genetic and derive from stochastic microenvironment effects such as e.g., food sources. Interestingly, Honegger et al. (2020) demonstrated that an acute switch in the food diet from cornmeal/dextrose food to a commercial flake food led to an increase in variability of odor preference in flies. Environmental effects on behavioral variability were also investigated in other insects. For instance, the change of food quality does not impact the variability in risk-taking behavior of clonal pea aphids (Schuett et al., 2011), but influences the variability of risk-taking and activity in the beetle *Phaedon cochleariae* (Tremmel and Müller, 2013). Taking an example from the visual system of flies, Linneweber et al. (2020) showed that personalities in form of object orientation have a developmental origin. They demonstrated that stochastic variation of the axonal projections in the Dorsal Cluster Neurons within the medulla shapes the visual orientation of flies (Linneweber et al., 2020). In addition, the correlation between behavioral variability and the genetic diversity was investigated. Notably, the genetic background has no influence on the phenotypic variability with regard to visual orientation (Linneweber et al., 2020), while the degree of variability in locomotor handedness is itself genetically determined and thus heritable (Ayroles et al., 2015; Buchanan et al., 2015).

In the following section, we are discussing potential mechanisms underlying variability regarding olfactory behavior of flies. Further investigations are of course needed to verify whether these factors indeed influence the personalities observed. Exposure of flies to odors can influence the inter-individual variability in size and responsiveness of olfactory glomeruli in the AL. In fact, a 4 days exposure of flies to carbon dioxide or ethyl butyrate induced a significant increase in the volume of the responsive glomerulus (Sachse et al., 2007) in an odor-specific manner. In addition, the odor responses of second-order neurons (i.e., LNs and PNs) as well as the behavioral output were modulated by long-term odor exposure. Hence, the sensory environment can affect the morphology and physiology of the respective neurons in the first olfactory center, while it has been shown to shape the circuit organization in higher brain centers as well. For instance, the size, number and active zone density of microglomeruli (i.e., PN-KC synaptic contacts) in the MB calyx region is activity-dependent, since it is altered when the synaptic transmission is abolished in PNs (Kremer et al., 2010). These results were confirmed by another study showing that chronic deprivation of synaptic transmission of PNs reduced drastically the amplitudes of postsynaptic calcium transients of the AL as well as presynaptic calcium signaling in the MB calyx evoked by the odors methyl cyclohexanol, 3-octanol, apple and banana (Pech et al., 2015). Pech et al. (2015) also showed that prolonged exposure to apple reduces postsynaptic calcium signaling in the apple-responsive glomerulus DL5. Furthermore, the number of microglomeruli has been shown to be affected by associative olfactory learning (Baltruschat et al., 2021). These parameters (i.e., sensory deprivation, olfactory learning) should be tested in a paradigm that directly links the variabilities observed to olfactory personalities in individual flies.

Thus, epigenetic mechanisms, genetic variation, developmental growth and environmental conditions can shape the personality of an animal's individuality (**Figure 1C**; Mollá-albaladejo and Sánchez-alcañiz, 2021). However, the variability of behavioral traits and their genetic and non-genetic origins need to be further studied to enlighten the evolution of personality traits.

VARIABILITY AT THE LEVEL OF OLFACTORY SENSORY NEURONS

Flies rely on the detection of odor stimuli in the environment to find nutritive food, to avoid toxic compounds and to identify suitable ecological niches and mating partners. The first layer responsible of these tasks represents the OSNs expressing different types of receptors (i.e., ORs, IRs) as introduced above. It is conceivable that individuality in odor preferences derives from inter-individual differences at the peripheral olfactory organs, i.e., at the level of the first-order sensory neurons. Potentially, the expression levels as well as types of chemosensory

receptors and/or neurotransmitter receptors might vary between individuals and impact the odor-evoked responses in OSNs (**Figures 1B,C**). For instance, odor-guided perceptions among *Drosophila* individuals of the same population was linked to genetic variation in ORs (Or22a/Or22b, Or35a, and Or47a) (Richgels and Rollmann, 2012). Another study revealed the effect of genes associated with neural development and the later processing in the central nervous system on variation in odor preference to 2,3-butanedione (Brown et al., 2013). However, the idea that variations in the expression levels of olfactory receptors and neural developmental genes enhance variability should be further investigated to find evidence for morphological or physiological changes in olfactory responses of afferent neurons.

The axons of OSNs converge onto a discrete glomerulus within the AL in the brain (**Figure 1A**). Notably, the number of OSNs innervating a given glomerulus varies across flies (Grabe et al., 2016), and structural variations in synaptic connections between OSNs and PNs have been identified (Tobin et al., 2017). A recent study tackled the dynamic of the cellular processes by which OSNs target axons precisely to a specific glomerulus in the ipsi- and contralateral AL (Li et al., 2021). During that process, OSN axons first form multiple ipsilateral branches, while only those branches that are close to their eventual glomerular target will be stabilized later on. The precise dynamic state of the branches (extending, retracting and stationary) varies between individuals (Li et al., 2021). One possibility is that the number of branches and therefore the strength of the diverse synaptic connections varies between individuals, which would represent an additional factor to facilitate individualization. However, the influence of these developmental differences on the variability of olfactory responses is unknown and needs to be further explored.

A recent study showed that certain neuronal populations of the olfactory circuit are predictive for individual behavioral responses (Churgin et al., 2021). Based on two-photon imaging measurements paired with behavioral assays, Churgin et al. (2021) built a model and found that idiosyncratic calcium dynamics as well as presynaptic densities of OSNs could predict the odor preference of flies. Furthermore, Churgin et al. (2021) investigated the capacity of predicting individual behavioral responses from the calcium dynamics in PNs which will be discussed in the following section.

Second-, third- and higher-order neurons are shown to exhibit morphological variations between individuals with regard to wiring and synaptic connectivity and could therefore provide the neural substrate in the brain to support individualities in odor-guided behavior. We will summarize in the following section the so far described inter-individual variabilities at the different olfactory processing levels.

VARIABILITY AT THE LEVEL OF THE ANTENNAL LOBE

The *Drosophila* AL possesses 58 identifiable glomeruli (Grabe et al., 2015). The glomeruli are organized in a consistent spatial pattern and have genetically determined shapes, positions and sizes across individuals as well as stereotyped OSN inputs and PN outputs (Laissue et al., 1999; Couto et al., 2005; Fishilevich and Vosshall, 2005; Jefferis et al., 2007; Lin et al., 2007; Grabe et al., 2015). LNs innervate the AL and provide intra- and inter-glomerular inhibition (i.e., presynaptic inhibition of OSNs, feedforward inhibition onto PNs) (Wilson and Mainen, 2006; Olsen and Wilson, 2008; Root et al., 2008; Mohamed et al., 2019). Highly comprehensive characterization of LNs in the *Drosophila* AL was established previously and has led to the categorization of LNs based on neurotransmitter profiles, connectivity, as well as morphological and physiological properties (Chou et al., 2010; Seki et al., 2010). Hence, different classes of LNs exhibit morphological and physiological differences. Moreover, a considerable variability in the density of arborizations and thicknesses of their processes is present within each category. This finding raises the following question: Does the variability of the same LNs across different individual flies represent the origin of the LN's morphological and physiological diversity? Indeed, the number of distinct innervation patterns in ipsilaterally projecting LNs exceeds the estimated total number of ipsilaterally projecting LNs within an individual AL. In other words, there are far more anatomical classes of LNs across individuals than there are LNs in an individual fly brain (Chou et al., 2010). This finding indicates that LN arborization patterns are not completely stereotyped across flies and seem to be rather unique in each fly (**Figure 1B**). Furthermore, physiological studies on specific GAL4 lines that label a small population of LNs identified diverse functional properties. Nevertheless, the properties of these LNs are not drawn randomly from the entire distribution of LN properties. In fact, odor response properties, i.e., mean, maximum odor-evoked and spontaneous firing rates were less variable across small populations of LNs than across all LNs. All these data indicate that the coarse properties of these neurons might be genetically pre-programmed, but do also reflect developmental plasticity and sensory experience (Chou et al., 2010). Along that line, a recent study demonstrated that activating or inhibiting different populations of LNs reduced variability in olfactory behavior (Honegger et al., 2020).

The olfactory information formed at the level of the AL is sent to higher brain centers *via* PNs. PNs extend their dendrites into a single glomerulus and project their axons to innervate the LH and MB. The olfactory system of the fly possesses two types of PNs: uniglomerular PNs (uPNs) that innervate a single glomerulus, and multiglomerular PNs (mPNs) that branch within subsets of glomeruli. uPNs have been intensively studied and could be classified due to their specific odor response profiles as well as their steretyped branching patterns in the AL and LH (Marin et al., 2002; Wong et al., 2002; Wang et al., 2003; Wilson et al., 2004; Bates et al., 2020), while mPNs could only be classified into two broad categories based on their innervated glomeruli in the AL (Strutz et al., 2014). However, the number of uPNs innervating a given glomerulus is not stereotypic and varies across animals (Grabe et al., 2016), while we do not have this information about mPNs. Moreover, recordings of odor responses of uPNs innervating specific glomeruli reveal some degree of inter-individual variability (Honegger et al., 2020). However, functional and anatomical clustering among the uPN population is still possible despite their inter-individual

differences, since the targeted glomerulus is strictly conserved among individuals (Jefferis et al., 2007; Murthy et al., 2008). Nevertheless, the differences across odor-evoked PN responses might still reflect and explain the individuality observed in odor preferences: Honegger et al. (2020) characterized odor responses of dozens of animals to a dozen odors, in PNs of the AL. They observed that responses of some glomeruli were very different across individuals, but consistent across multiple presentations of the same odor within an individual. Moreover, this study revealed that the within-fly responses were closer correlated than between-fly responses. All these data demonstrate that PN responses to odors differ significantly across individuals and are idiosyncratic (Honegger et al., 2020). A recent study further investigated the link between neuronal responses and individual odor preferences (Churgin et al., 2021). Churgin et al. (2021) could predict the idiosyncratic odor preference of flies using the calcium imaging responses of PNs. Overall, the results of this study suggest that physiological variations in PN responses might be driven by the developmental variability of OSN populations leading to individuality in odor preference behavior.

Similar to *Drosophila*, also other insects exhibit inter-individual variabilities in their olfactory circuits with regard to neuronal wiring, synaptic connectivity as well as anatomical features. The olfactory glomeruli of the noctuid moth *Spodoptera littoralis* can be clearly identified in different ALs of different individuals. However, several types of biological variability were observed here as well: For instance, some glomeruli were lacking in some individuals which indicates either the absence of the corresponding OR/IR/OSN type or a mistargeting to another glomerulus during development. Contrary to *Drosophila*, the AL of *Spodoptera littoralis* varies in its global shape which leads to changes in the spatial location of the glomeruli. Interestingly, several other moth species also exhibit variations in the number and size of their glomeruli in the AL, such as *Mamestra brassicae* (Rospars, 1983), *Manduca sexta* (Rospars and Hildebrand, 1992, 2000), and *Bombyx mori* (Rospars and Chambille, 1981; Rospars, 1983; Kazawa et al., 2009).

VARIABILITY AT HIGHER BRAIN CENTERS – THE MUSHROOM BODY LEVEL

The MBs are composed of approximately 2,500 intrinsic neurons known as Kenyon cells (KCs). The KC's dendrites form the MB calyx while their axonal fibers form the output lobes of the MB (γ, α′/β′, α/β lobes). The main olfactory inputs received by the MB calyx are provided by PNs from the AL. Anatomical and physiological studies showed that on average 6–8 PNs innervate each KC (Caron et al., 2013; Gruntman and Turner, 2013; Bates et al., 2020). Caron et al. (2013) characterized the glomerular origin of those PNs that converge onto one KC by photolabeling individual KCs. Their study showed that the majority of individual KCs integrates random and not stereotyped combinations of glomerular inputs (**Figure 1B**). Notably, neither the odor tuning nor anatomical features or developmental origins dictate a specific organization of the glomerular inputs to an individual KC. Moreover, electrophysiological responses of KCs to different odors in a fly line labeling 23 α/β neurons revealed distinct odor response profiles of KCs among individuals (Murthy et al., 2008). It is well established that learning and experience-dependent behavior rely on the plasticity and the described random organization of the MBs (Bilz et al., 2020). The inter-individual variability of KC responses and the random PN-KC connectivity facilitates flexibility of the olfactory system to adjust to environmental changes, previous experience and internal state. However, these data raise the question whether the random organization of glomerular inputs to the MBs could also account for the observed individuality in odor-driven behavior. Indeed, a given odor will activate different sets of KCs in different flies and trigger behavioral outputs that are likely to vary across individuals (**Figure 1B**).

One specific feature of the MB circuit is that the output to further brain areas is conveyed by solely 34 MB output neurons (MBONs) that can be categorized into 21 cell types. Dendrites of each MBON type innervate distinct subregions of the MB lobes. These numbers reflect the heavy convergence from the KCs onto MBONs (Tanaka et al., 2008; Mao and Davis, 2009; Aso et al., 2014). Many studies have characterized odor-evoked responses of MBONs, which usually normalize and average the measured odor responses between flies, leading to the loss of information concerning inter-individual variability as mentioned above. In contrast, the study by Hige et al. (2015) clearly emphasizes variability of odor responses of MBONs across flies by demonstrating that some MBONs with uniquely identifiable anatomy have diverse tuning properties in different animals. Interestingly, across all MBONs, the α2sc neurons exhibit the greatest amount of variability (Hige et al., 2015), a MBON type that is required for the retrieval of aversive olfactory memories (Séjourné et al., 2011). However, the odor tuning patterns of MBON-α2sc from the two brain hemispheres of the same animal are strikingly similar indicating that processes coordinated across both hemispheres must dictate the tuning patterns of this MBON type. To assess whether the variable tuning properties derive from fluctuating levels of population activity in KCs or by the functional connectivity between KCs and MBONs, Hige et al. (2015) demonstrated that the KC-MBON-α2sc connection differs among individuals, while the calcium responses in the KC axon bundle were similar from fly to fly. Hence, the individual-specific connectivity of MBON-α2sc enables the neurons to extract different information among individuals, even from presynaptic KCs that exhibit a similar overall population tuning. Moreover, the diversity in wiring across flies might be caused by synaptic plasticity, since mutants of the rutabaga gene encoding a calcium-dependent adenylyl cyclase required for learning, reduced (but did not abolish) the tuning variability of MBON-α2sc across flies (Hige et al., 2015). Adaptive plasticity of calcium activity of MBONs was also reported in a recent study (Hancock et al., 2022). These findings suggest that elements implicated in learning processes and plasticity also influence the variability across flies. Hence, individualized coordination of tuning observed at the KC-MBON level might represent one

of the origins of individuality in olfactory responses. However, additional behavioral experiments are necessary to provide the link between plasticity and individuality.

VARIABILITY AT HIGHER BRAIN CENTERS – THE LATERAL HORN LEVEL

The LH represents a higher-order brain center that processes different sensory modalities including olfactory information (Frechter et al., 2019; Das Chakraborty and Sachse, 2021). Several studies have documented the role of the LH with regard to innate behavioral responses by encoding hedonic valence to odor cues, while the LH is also processing learned responses to previously encountered odors (Strutz et al., 2014; Dolan et al., 2019). The spatial organization of the LH is determined by the position of the PN axonal terminals that either directly project from the AL (most of mPNs) or that relay the olfactory information from the AL *via* the MBs (all uPNs and some mPNs) (Li et al., 2021). Comprehensive maps of higher olfactory centers of *Drosophila* reported in previous studies revealed a clear stereotypy of the branching patterns of PN axons in the LH (Marin et al., 2002; Wong et al., 2002; Jefferis et al., 2007). So far, the LH connectivity is less well understood than the MB circuitry (Das Chakraborty and Sachse, 2021). LH neurons (LHN) could be classified based on morphological, neurotransmitter and polarity information using the EM connectomic dataset as well as their odor response properties (Jeanne et al., 2018; Dolan et al., 2019; Frechter et al., 2019). However, some functional cell types exhibited a high degree of variability in their odor responses and were difficult to classify (Frechter et al., 2019). Moreover, recent analyses of the EM-based connectomics data showed that the PN input to LHNs of the same cell type can vary (Dolan et al., 2018; Jeanne et al., 2018). The origin of this response variability could either result from differences in the number or strength of inputs to that cell type across animals or just experimental factors. The latter suggestion was excluded by the study of Frechter et al. (2019) by providing evidence that no apparent relationship between cell-recording parameters (i.e., cell capacitance, membrane/pipette resistance) and the strength of the response could be found. Additionally, taking together all the recent advances in characterizing the cellular composition of the LH and analyzing the connectivity to PNs of the AL, it is very likely that synaptic partners are variable among individuals. Indeed, some LHNs receive synaptic inputs from glomeruli that differ between flies and even between both brain hemispheres (Cachero et al., 2020). These findings could either result from technical issues or reflect biological variability at the level of the PN-LHN connections (Cachero et al., 2020). However, the impact of these variable connections on inter-individual differences in odor-guided behavior in flies is so far unknown and requires the analysis of circuit elements in large numbers of individuals.

Overall, the morphological and physiological differences at each level of the olfactory circuitry probably contribute to the individuality seen in olfactory behavior. It is most likely the combination of all these differences between individuals that shapes a specific olfactory behavioral output. Hence, the diverse connectivity of the olfactory circuit optimizes its ability to respond appropriately to a rich array of olfactory experiences and a changing environment.

VARIABILITIES BETWEEN BRAIN HEMISPHERES

The majority of OSNs in *Drosophila* projects from the antennae bilaterally to both brain hemispheres by collaterals passing *via* the antennal commissure (Stocker et al., 1990; Couto et al., 2005). Nevertheless, the connectivity of OSNs between the brain hemispheres are diverse (Tobin et al., 2017). Neuronal tracing from serial EM sections showed that the number of PNs in glomerulus DM6 varies between two and four, and PN counts are often different between the right and left side. In fact, the right brain hemisphere possesses larger dendritic path length and a higher number of OSN synapses (Tobin et al., 2017). Multiglomerular neuron synapses of LNs and mPNs and presynaptic contacts of uPNs were also in greater numbers on the right than on the left side (Tobin et al., 2017). Moreover, Bates et al. (2020) explored the numerical stereotypy of 58 uniglomerular PN (uPNs) types across both hemispheres and revealed that the uPN number is twice as numerous on the left side in glomerulus VA1d.

In addition, asymmetric odor stimulation has been shown to evoke distinct activation in the left and right brain hemisphere as a result of contralateral inhibition (Mohamed et al., 2019). It could be shown that odor responses in a specific cluster of third-order LHNs, so-called ventrolateral protocerebrum neurons (VLPn) were suppressed by presynaptic LHNs when an odor was presented to the contralateral side. Thus, a lateralized odor stimulus is distinctively detected by higher-order neurons through contralateral inhibition leading to an enhanced perception of odor concentration gradients between both brain hemispheres (Mohamed et al., 2019). Hence, also variability with regard to odor lateralization between flies should be taken into consideration and quantified, since it is conceivable that this could differ between animals and might be another factor for individuality of odor preference behavior.

CONCLUDING REMARKS

For long, researchers believed that odor responses are highly stereotypic across different individuals of the same species and that the variability in animal behavior is just due to limitations in methodological approaches. Researchers also considered that the majority of the quantitative differences might be the product of noisy developmental processes and thus not relevant. However, this idea should be re-evaluated since various recent studies have shown that flies, similar to other animals, exhibit an individualized perception of odors (Thomas-Danguin et al., 2014; Trimmer et al., 2019; Honegger et al., 2020; Kermen et al., 2020; Ruser et al., 2021). These findings reveal the genetic sources of variations and should change our concept about the insect brain and its reproducibility of putative "hard-wired" properties. Moreover, studies on inter-individual differences in

the neuronal wiring of other modalities (vision, locomotion, etc.) (Vogt et al., 2008; Schuett et al., 2011; Honegger and de Bivort, 2018), enlighten us on the presence of non-genetic variability that has an effect on the individuality in animal behavior. In this review, we describe morphological and physiological variabilities that occur in the olfactory circuit between individual flies. The possible link between genetic or environmental factors and odor-preference individualities is also discussed, but still needs to be proven. Potential factors that would cause variable behavioral responses and support "olfactory personalities" are mentioned and discussed as well. This review raises two questions: First, are the occurring variations at the molecular, cellular and circuit level arbitrary or do they facilitate potential adaptations of the brain to environmental fluctuations? Second, what might be the benefit for the animal's fitness and survival? One could argue that it is more costly for animals to preserve structure and function of their neuronal circuits across individuals, since the biophysics and development processes need to be constrained that build and maintain biological systems. In addition, during learning, brain centers responsible for assigning context- specific values take advantage of random and individualistic connectivity patterns as shown for the MBs (Caron et al., 2013; Hiesinger and Hassan, 2018; Zheng et al., 2018). This variation is also beneficial for innate behavior, as it allows an animal to adapt to unpredictable environmental conditions and fluctuations (Honegger and de Bivort, 2018).

Addressing the origin and significance of variable connectivity throughout the nervous system will increase our understanding of personality variations. This aspect will require analysis of circuit elements in a large number of individuals of a given species. To conclude, it can be stated that the variability throughout the olfactory system supports odor-preference individualities.

AUTHOR CONTRIBUTIONS

KR and SS conceived and wrote the review. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Maternally Instigated Diapause in *Aedes albopictus*: Coordinating Experience and Internal State for Survival in Variable Environments

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The Asian tiger mosquito, *Aedes albopictus,* is one of the most dangerous invasive species in the world. Females bite mammalian hosts, including humans, to obtain blood for egg development. The ancestral range of *Ae. albopictus* likely spanned from India to Japan and this species has since invaded a substantial portion of the globe. *Ae. albopictus* can be broadly categorized into temperate and tropical populations. One key to their ability to invade diverse ecological spaces is the capacity of females to detect seasonal changes and produce stress-resistant eggs that survive harsh winters. Females living in temperate regions respond to cues that predict the onset of unfavorable environmental conditions by producing eggs that enter maternally instigated embryonic diapause, a developmentally arrested state, which allows species survival by protecting the embryos until favorable conditions return. To appropriately produce diapause eggs, the female must integrate environmental cues and internal physiological state (blood feeding and reproductive status) to allocate nutrients and regulate reproduction. There is variation in reproductive responses to environmental cues between interfertile tropical and temperate populations depending on whether females are actively producing diapause vs. non-diapause eggs and whether they originate from populations that are capable of diapause. Although diapause-inducing environmental cues and diapause eggs have been extensively characterized, little is known about how the female detects gradual environmental changes and coordinates her reproductive status with seasonal dynamics to lay diapause eggs in order to maximize offspring survival. Previous studies suggest that the circadian system is involved in detecting daylength as a critical cue. However, it is unknown which clock network components are important, how these connect to reproductive physiology, and how they may differ between behavioral states or across populations with variable diapause competence. In this review, we showcase *Ae. albopictus* as an emerging species for neurogenetics to study how the nervous system combines environmental conditions and internal state to optimize reproductive behavior. We review environmental cues for diapause induction, downstream pathways that control female metabolic changes and reproductive capacity, as well as diapause heterogeneity between populations with different evolutionary histories. We highlight genetic tools that can be implemented in *Ae. albopictus* to identify signaling molecules

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and cellular circuits that control diapause. The tools and discoveries made in this species could translate to a broader understanding of how environmental cues are interpreted to alter reproductive physiology in other species and how populations with similar genetic and circuit organizations diversify behavioral patterns. These approaches may yield new targets to interfere with mosquito reproductive capacity, which could be exploited to reduce mosquito populations and the burden of the pathogens they transmit.

Keywords: diapause, *Ae. albopictus*, reproduction, mosquito, seasonal change

INTRODUCTION

Insects are successful across huge geographic ranges due to their capacity to physiologically and behaviorally adapt to a wide variety of environments. In tropical climates, the annual range of temperature is usually small; however in temperate climates the environmental conditions optimal for survival prevail during specific seasons. This requires animals to energetically provision themselves or their offspring to successfully endure harsh winters (Tauber and Tauber, 1976). To determine the appropriate reproductive behavior and allocation of nutritional resources, this adaptive response requires the nervous system to integrate environmental cues and internal state (gravid or not). Understanding how animals adapt to survive unfavorable environmental conditions is critical for understanding their behavior, distribution, and population growth as well as speciation and interspecific interactions. This is particularly important for *Ae. albopictus* (Skuse, 1894), a highly invasive mosquito vector of arthropod-borne disease that has expanded its range to include temperate and tropical regions on every continent except for Antarctica (Bonizzoni et al., 2013; **Figure 1A**). Climate change modeling predicts that these mosquitoes will continue to expand and redistribute their geographical range, increasing net and new exposure to *Aedes*-borne pathogens (Ryan et al., 2019).

Entering the state of preprogrammed developmental arrest called diapause is a successful strategy to survive harsh environmental conditions. Diapause is a dynamic process that includes several phases; pre-diapause stages include "induction" and "preparation" and diapause itself can be divided into "initiation," "maintenance," and "termination" stages (Koštál, 2006). During diapause induction, which occurs well in advance of seasonal change, animals are sensitive to cues that predict the arrival of unfavorable environmental stressors. Generally, this only occurs at a genetically predetermined life stage. This sensitive stage may take place within the lifetime of the diapausing individual or, as in the case of *Ae. albopictus*, in the preceding generation. Seasonal shifts are likely anticipated by measuring daylength (photoperiod). Both the shortening of daylength and static short photoperiod induce diapause, causing individuals to undergo physiological and behavioral changes. Next is the preparation phase, when individuals provision themselves or their offspring by accumulating lipids, proteins, and carbohydrates. Once diapause is initiated, development is arrested and diapause is maintained as animals exhibit reduced metabolism, developmental arrest, and increased sensitivity to certain environmental cues (such as light and temperature) in preparation for diapause termination phase. Diapause termination occurs spontaneously in some species, but in others, the resumption of development may be initiated by external stimuli, which signal the eventual return of favorable conditions (Golden and Riddle, 1984; Sommerville and Davey, 2002; Milonas and Savopoulou-Soultani, 2004). This period of dormancy is characterized by changes in insulin signaling, metabolism, cell-cycle arrest, and stress-response genes (Lees, 1955; Hansen et al., 2011; Poelchau et al., 2013a). Diapause has independently evolved multiple times and can occur at distinct developmental stages (embryonic, larval, adult). Typically, a species is only capable of entering diapause at a specific developmental stage, one factor that differentiates diapause from quiescence, which is a dormant state that can occur during any developmental stage and can be quickly terminated upon exposure to favorable conditions (Tauber and Tauber, 1976).

Mosquitoes employ a diverse set of diapause strategies; *Wyeomyia smithii* enter diapause as larvae living inside pitcher plants (Bradshaw and Lounibos, 1977), whereas *Culex* mosquitoes enter diapause as adult females (Eldridge, 1987). In this review, we focus on *Ae. albopictus*, which exhibits maternally instigated embryonic diapause. In temperate populations of *Ae. albopictus* where fall/winter conditions are too harsh for adult survival and reproduction, pupae and adult females detect seasonal changes in daylength and produce diapause eggs that enter developmental arrest as pharate larvae inside the chorion of the egg (Wang, 1966; Mori et al., 1981; Denlinger and Armbruster, 2014). These developmentally arrested pharate larvae remain non-responsive to hatching stimuli for a period of time, thus preventing autumnal hatching and larval mortality during cold winter conditions. Under long day photoperiods similar to those found in spring and summer (e.g., 16 h light:8 h dark), females from temperate populations produce non-diapause eggs that complete embryonic development and are immediately responsive to hatching stimuli (**Figure 1A**). In tropical environments, temperatures are permissive for reproduction year-round and tropical *Ae. albopictus* populations do not undergo diapause (Hawley, 1988; Pumpuni, 1989; **Figure 1A**). This results in seasonal differences in the relative abundance of immature and adults mosquitoes in temperate regions (**Figure 1B**). While ancestrally temperate and tropical strains can interbreed and share genetic and anatomical organization (Boyle et al., 2021), they differ in their reproductive responses to environmental cues. It is unclear if the physiological differences between ancestrally temperate vs. tropical strains or

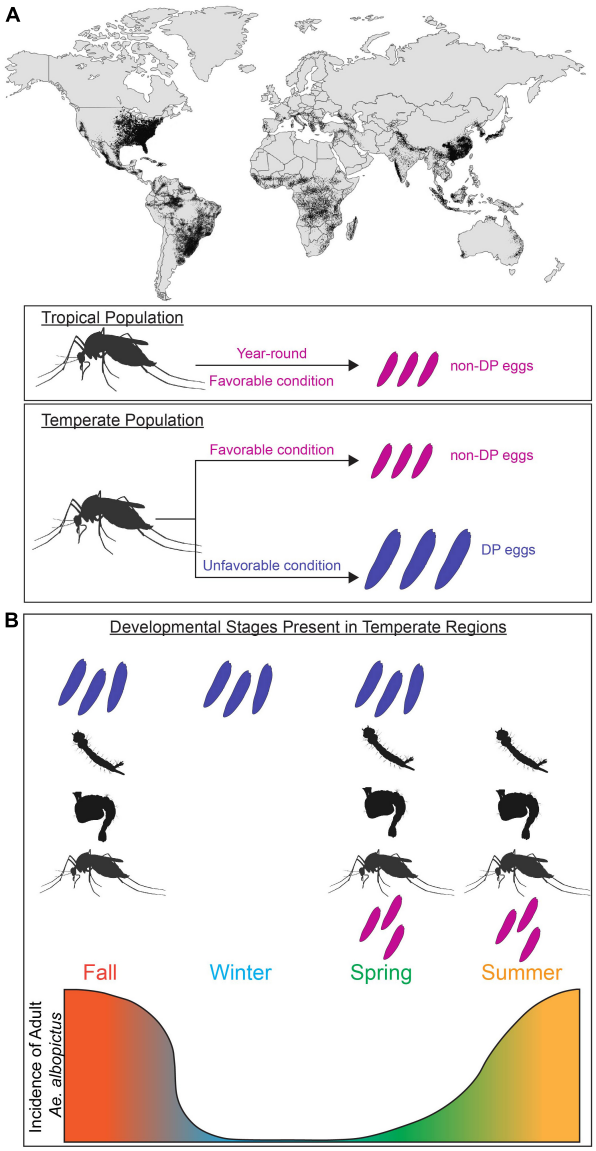

FIGURE 1 | *Aedes albopictus* global invasion and population expansion in temperate and tropical regions. **(A)** Global map of predicted distribution of *Ae. albopictus* population (adapted from Kraemer et al., 2015). Adult females found in tropical regions lay non-diapause eggs (magenta) majority of year-round. In temperate regions, adult females found in favorable conditions (magenta) lay non-diapause eggs. In unfavorable conditions, adult females alter their reproduction (blue) and lay diapause eggs (blue). **(B)** Incidence of *Ae. albopictus* at different life stages in temperate regions. Temperate population have unique subsets of developmental stages present (diapause and non-diapause egg, larva, pupa, adult) in each season. Incidence of adult *Ae. albopictus* in temperate region is depicted by the gradient graph.

between females actively producing diapause vs. non-diapause eggs arise in neural or reproductive circuits, or elsewhere entirely. The evolutionary history of *Ae. albopictus* has resulted in populations with distinct responses to environmental cues, which provides researchers the opportunity to study neural circuits that control these responses in groups with behavioral variation.

EVOLUTIONARY HISTORY OF *Aedes albopictus* AND BEHAVIORAL VARIATION IN DIAPAUSE

Understanding the evolutionary history of *Ae. albopictus* is critical to inform our understanding of how variation in reproductive behaviors arose and has been maintained. In its ancestral range in Asia, *Ae. albopictus* is found in both temperate and tropical environments, spanning from India to Japan (Hawley, 1988), and this invasive species has now become established on every continent except for Antarctica (Lounibos, 2002; Bonizzoni et al., 2013; **Figure 1A**).

The first case of *Ae. albopictus* in the continental United States was reported in 1985, when an established population was discovered in Texas (Sprenger and Wuithiranyagool, 1986). Trade records implicate the use of imported water-containing tires as breeding sites in facilitating the dispersal of *Ae. albopictus* from a Japanese population (Sprenger and Wuithiranyagool, 1986; Hawley et al., 1987). These strains collected in Texas were sensitive to photoperiod and females exposed to short daylengths produce diapause eggs, suggesting that they originate from an ancestrally temperate population (Lounibos et al., 2003). Likely through multiple introductions and geographic spread, *Ae. albopictus* now inhabits temperate regions of the North American east coast and midwest, and subtropical and tropical regions in the Caribbean, Mexico and Central America (Moore, 1999; Lounibos et al., 2003; Bonizzoni et al., 2013). The transition of North American populations from temperate to tropical regions resulted in the gradual loss of an environmental requirement to enter diapause under natural conditions, with an environmental breakpoint found in northern Florida (Lounibos et al., 2003). However, females collected from tropical regions in Florida have been reported to produce diapause eggs in laboratory settings, suggesting that diapause-induction pathways remain intact in these animals.

At almost exactly the same time that the species was detected in Texas, *Ae. albopictus* was also first detected in Brazil, near Rio de Janiero (Forattini, 1986). While the exact origin of this invasion is unknown, Brazilian populations are thought to have originated from an ancestrally tropical population due to the absence of diapause induction in this sample (Forattini, 1986; Hawley et al., 1987). Although *Ae. albopictus* is now broadly distributed across the Amazon basin, they are thought to have remained genetically isolated from North American populations (Birungi and Munstermann, 2002; Bonizzoni et al., 2013). It remains controversial whether diapause competence can emerge from a founding population of tropical origin that does not produce diapause eggs. Although Lounibos et al. (2003) reported that females from temperate regions of Brazil were able to produce diapause eggs, despite the likely tropical origins of their founders, earlier findings suggested that short photoperiods were never capable of inducing diapause if the founding population was of a tropical origin and did not originally undergo diapause (Craig, 1993; Hanson and Craig, 1994). Interestingly, populations from similar latitudes in the northern vs. southern hemispheres showed noticeable differences in their ability to

produce diapause eggs when exposed to short day conditions in the laboratory. Animals collected from the northern hemisphere showed higher rates of diapause egg production in laboratory settings compared to those collected from similar latitudes in the southern hemisphere although both groups showed significant heterogeneity in their responses (Lounibos et al., 2003). These findings provide evidence for the emergence of diapause in populations from an assumed tropical origin (e.g., subtropical/temperate Brazilian populations), and ongoing loss of diapause in now-tropical populations from a temperate origin (e.g., Floridian and Caribbean populations). Thus, diapause competence is thought to be determined by rapid evolution induced by the selective pressures of the local environment as well as whether the origin of the founding population is tropical or temperate. However, it is difficult to exclude the possibility of multiple establishment events. Within the United States, there is evidence for rapid evolution in diapause incidence and seasonal timing in *Ae. albopictus* populations, supporting the conclusion that these adaptive phenotypes are critical for the high invasion potential of this species (Lounibos et al., 2003; Urbanski et al., 2012). Recent work mapping putative diapause-associated SNP clusters throughout the *Ae. albopictus* genome suggests that the evolution of diapause in *Ae. albopictus* is polygenic (Boyle et al., 2021).

The evolutionary history of *Ae. albopictus* has led to widely distributed global populations with varying reproductive responses to environmental cues. These changes likely have a genetic basis and although these populations share genomic and anatomical commonalities, the basis for variation in diapause competence remains an area for exploration.

MATERNAL RESPONSES TO ENVIRONMENTAL CUES: DIAPAUSE INDUCTION

Environmental Cues

Appropriate diapause entry requires accurate anticipation of seasonal changes. As winter approaches, the days become reliably shorter; laboratory studies and observations in the field have implicated photoperiod as the major stimulus for diapause initiation (Denlinger, 1986; Armbruster, 2016). Critical photoperiod (CPP) is the daylength that induces diapause entry in at least 50% of the population. Although generally insufficient to induce diapause alone, lower temperature can interact with photoperiod to increase diapause incidence at a given daylength (Pumpuni and Craig, 1992). Reduced larval nutrition has also been shown to increase the CPP and diapause incidence, meaning that animals that experience early nutritional stress enter diapause at higher rates earlier in the autumn, suggesting crosstalk between nutritional state and diapause entry (Pumpuni and Craig, 1992). Other environmental cues that signal seasonal change are also thought to play a role in diapause initiation and are reviewed in Tauber (1987). The CPP and the temporal dynamics of diapause entry have implicated circadian clock cells as seasonal sensors, as they are poised to detect light/dark cycles and influence physiology (Armbruster, 2016).

Circadian Clock Involvement

The circadian system has been suggested to play a key role in seasonal tracking, but the mechanism by which mosquitoes utilize this circuitry to interpret seasonal dynamics and initiate diapause entry remains unclear. There are three main models in the field. The first model is referred to as an external coincidence model, proposed by Bünning (1936). Bünning hypothesized that since the circadian clock already provides information related to light/dark cycles, organisms likely use their circadian clocks to measure daylength and initiate photoperiodic responses (**Figure 2A**). To detect long days, the light cue entrains the clock to determine a light-sensitive period in the late night and early morning. Diapause is initiated as the light-sensitive period shortens to match the critical photoperiod. In a contrasting internal coincidence model, proposed by Pittendrigh and Minis (1964), light entrains distinct dawn and dusk oscillators, so that a change in the phase of these two oscillators induces diapause entry. In the third model, called the hourglass timer or hourglass interval timer model, internal circadian clocks are not involved in initiating diapause and organisms measure day/night length using an independent system. For example, the accumulation of a chemical substance may initiate diapause after surpassing a critical threshold, or there may be an independent genetic basis for seasonal tracking (Bradshaw et al., 2003; Bradshaw and Holzapfel, 2010). These models may not be mutually exclusive but could occur in combination to mediate diapause initiation (Goto, 2013; Meuti and Denlinger, 2013).

Circadian rhythms are driven by endogenous molecular clocks, which consist of auto-regulatory loops of proteins that rhythmically repress expression of their own genes. Critical circadian genes include *period (per)*, *timeless (tim)*, *clock (clk)*, *cycle (cyc),* and *cryptochrome (cry).* The genes and regulatory mechanisms of circadian rhythms are deeply conserved across the animal kingdom (Wager-Smith and Kay, 2000; Sandrelli et al., 2008; Beer and Helfrich-Förster, 2020). Neuropeptides, notably Pigment Dispersing Factor (PDF), coordinate circadian rhythms between clock cell subgroups in the insect brain. Despite this general conservation, there are some features in which *D. melanogaster*, the most well-characterized organism, is the outlier among insects. Specifically, *D. melanogaster* has a single cryptochrome (CRY), whereas most insects, including mosquitoes, have both light-sensitive and -insensitive cryptochromes (Gentile et al., 2009; Baik et al., 2020; Beer and Helfrich-Förster, 2020). Although the total number of clock cells varies widely between species, ranging from ∼80 to 90 in *Aedes aegypti* and *Anopheles coluzzi,* to 150 in *D. melanogaster,* and ∼400 in *Apis mellifera*, there is a similar pattern of clustering into anatomically distinct subgroups. Interestingly, PDF seems to play a conserved role in communicating across these subgroups and brain regions (**Figure 2B**; Vafopoulou and Steel, 2012; Fuchikawa et al., 2017; Beer et al., 2018; Baik et al., 2020) and PDF-expressing neurons have been implicated in mediating photoperiodism in multiple species, notably *Riptortus pedestris* and *Culex* mosquitoes. However, PDF may perform this role independent of its circadian functions (Ikeno et al., 2014).

Studies performed in multiple species indicate that functional circadian clock components are required for appropriate diapause entry (Denlinger and Armbruster, 2014) by detecting

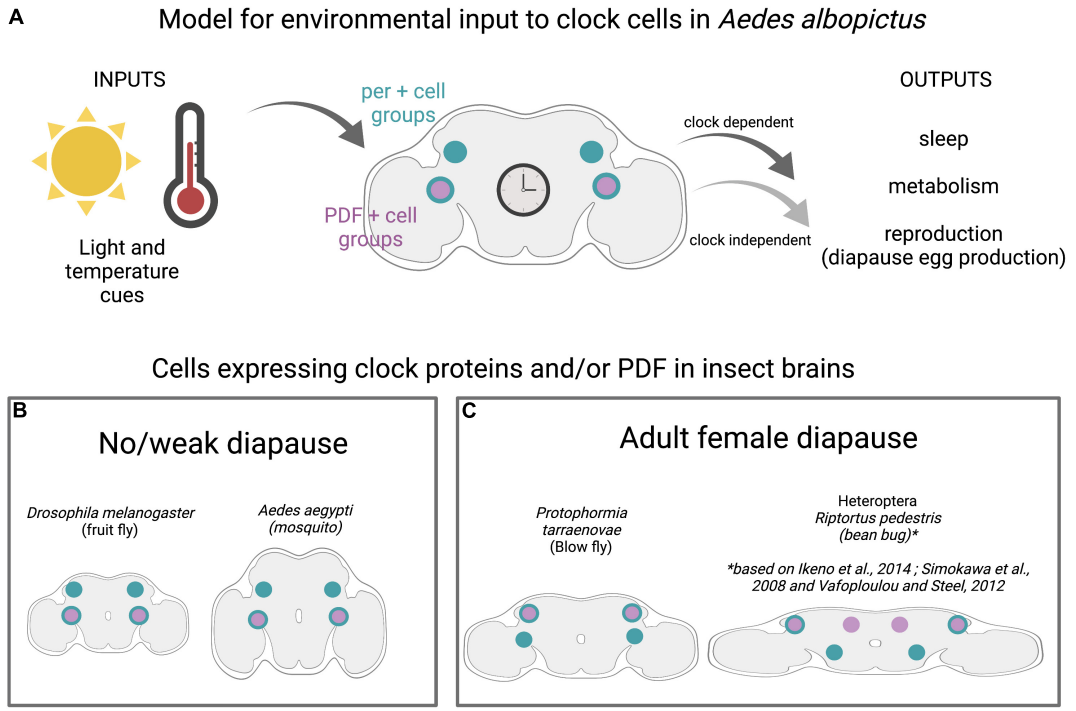

FIGURE 2 | Clock cells in diapausing and non-diapausing insects. **(A)** Model for environmental inputs to predicted clock cell subgroups in *Ae albopictus* that are candidates for regulating diapause entry in adult females. Light and temperature cues are detected by central brain clock cells expressing the neuropeptide Pigment Dispersing Factor (PDF) shown in purple and/or core clock genes (denoted by period (per) in teal). Predicted clock subgroups are based on observations in *D. melanogaster* and *Ae. aegypti* (Baik et al., 2020). **(B)** Insects with no/weak diapause and those with adult female diapause **(C)** show conservation of clock anatomy, including distinct subgroups of central brain cells that express combination of per and PDF.

and regulating photoperiodic entrainment to appropriately sense daylength and signal reproductive system (Tauber, 1987; Armbruster, 2016). The circadian system has been linked to diapause regulation in multiple insects, and a recent study from the Arctic archipelago of Svalbard proposes that seasonal synchronization is dependent on the circadian clock in birds, even under constant light conditions (Appenroth et al., 2021). In *Drosophila triauraria*, allelic differences in SNPs and deletions in *tim* and *cry* between diapausing and non-diapausing strains are associated with diapause incidence although genetic linkage analysis suggests that *tim* and *cry* have independent effects on the occurrence of diapause, unlike their action in the circadian clock (Yamada and Yamamoto, 2011). In the bean bug, *R. pedestris*, *per* and *cyc* genes modulate diapause induction, and neurons in the pars lateralis are involved in photoperiod responses (Shimokawa et al., 2008; Ikeno et al., 2010). Studies in *Cx. Pipiens* have revealed the presence of an oscillating circadian network that is essential for diapause initiation in these mosquitoes (Meuti et al., 2015). The neuropeptide PDF is secreted by specific clock cells, regulates circadian- and light-mediated behaviors in *D. melanogaster*, and initiates diapause entry in the blow fly *Protophormia terraenovae* (Hamanaka et al., 2005; Shiga and Numata, 2009). Clock genes have been identified in *Ae. albopictus,* and transcriptional profiling shows higher levels of *tim* and *cry1* transcripts in whole bodies of non-blood-fed females reared under short day conditions compared to those reared in long day conditions (Huang et al., 2015). This suggests that diapause-inducing photoperiod modulates the expression of *tim* and *cry1* genes. Although diapause-inducing short day conditions alter maternal clock components, their roles in diapause induction may be independent from their role in the circadian function, as has been proposed for *tim* (Bradshaw and Holzapfel, 2010). Although molecular clock components and central brain anatomy are generally conserved between diapausing and non-diapausing insects (**Figures 2B,C**) and recent work has mapped the clock neuron anatomy in *Ae. aegypti* and *An. coluzzi*, the circadian clock circuitry and the molecular mechanisms underlying the translation of seasonal cues to diapause responses in *Ae. albopictus* remain uncharacterized (Summa et al., 2012; Baik et al., 2020). Whether allelic differences in clock genes are present in different *Ae. albopictus* populations and whether these associate with diapause behaviors (as in *D. triauraria*) also remains unstudied.

Clock genes may affect downstream metabolic pathways to appropriately allocate energy reserves. This suggests that short photoperiod is detected by clock cells in the brain of *Ae. albopictus* females and this signal is translated into hormonal cues that determine the diapause fate of her offspring. A connection between the circadian system and insulin signaling pathways is observed in *D. melanogaster,* where PDF and short Neuropeptide F (sNPF) inhibit reproductive

dormancy by modulating insulin producing cells. Furthermore, genetic manipulations of PDF-expressing neurons, including the sNPF-producing small ventral Lateral Neurons (s-LNvs), affect reproductive arrest (Nagy et al., 2019). This suggests that neural connections between the clock and reproductive systems may be critical for coordinating egg provisioning and development, but whether these connections exist in *Ae. albopictus* and whether their modulation is dependent on diapause state are areas for future research.

PATHWAYS INVOLVED IN DIAPAUSE EGG PRODUCTION: DIAPAUSE PREPARATION AND INITIATION

Diapause induction involves the alteration of signaling processes in adult females prior to the developmental arrest of their offspring. Recent studies performing RNA-seq high-throughput sequencing have generated a global transcriptome analysis of blood-fed and non-blood-fed adult female *Ae. albopictus* reared in diapause and non-diapause inducing conditions (Huang et al., 2015). The transcriptional profiles of these mosquitoes undergo drastic changes in pathways involved in blood digestion, hormone synthesis, vitellogenin synthesis, insecticide resistance, and the circadian clock system. In non-blood-fed females reared in diapause-inducing conditions, potential regulatory elements of diapause induction (i.e., transcripts in pathways related to energy production and nutrient provisioning) were upregulated compared to both non-blood-fed females reared under long day conditions (Huang et al., 2015). These findings indicate that, in response to environmental conditions that induce diapause, females undergo changes in their nutrient provisioning pathways to appropriately produce diapause eggs.

Cell Cycle Arrest

During diapause, development is arrested at a fixed stage. Specifically, cells in target organs, including the primordial imaginal structures in larval and pupal stages, halt their differentiation and progression through the cell division cycle (Nakagaki et al., 1991; Tammariello and Denlinger, 1998). Cell cycle arrest is one of the unifying themes of diapause, and positive cell cycle regulators and DNA replication-associated transcripts are downregulated during diapause induction in *Ae. albopictus*. Notably, transcripts for the positive cell cycle regulator, *proliferating cell nuclear antigen (pcna)*, are downregulated and similar patterns of *pcna* expression are reported during diapause induction in flesh flies, drosophilids, cotton bollworms, and apple maggots (Flannagan et al., 1998; Koštál et al., 2009; Bao and Xu, 2011; Ragland et al., 2011). The exact phase of cell cycle arrest varies between species. The flesh fly *Sarcophaga crassipalpis* undergoes larval diapause with most brain cells in G0/G1 phase (Tammariello and Denlinger, 1998), whereas diapausing *Bombyx mori* embryos have cells that are halted in G2 phase of cell division (Nakagaki et al., 1991). In the jewel wasp *Nasonia vitripennis*, 80% of cells halt at G0/G1 and 20% halt in G2 phases, a notably high proportion of subdominant cell cycle phase (Shimizu et al., 2018). Similarly, transcripts associated with cell proliferation are down-regulated in female *Ae. albopictus* under diapause-inducing conditions even prior to blood feeding, presumably to allocate energy for alternative metabolic pathways (Huang et al., 2015). These findings indicate that critical cellular processes are modulated in the adult female well in advance of the actual developmental arrest of her offspring.

Insulin/FOXO Signaling

The insulin signaling/FOXO (*forkhead transcription factor*) pathway has been connected with many features of diapause including: arrested reproduction, extended lifespan, metabolic suppression, fat hypertrophy, and enhanced stress tolerance (Sim and Denlinger, 2013). The insulin pathway is employed in other types of reproductive arrest including dauer formation in *Caenorhabditis elegans* (Gottlieb and Ruvkun, 1994). PI3K is a component of the insulin-signaling pathway with naturally occurring variants that segregate with the ability to enter adult diapause in *D. melanogaster* (Williams et al., 2006). Interestingly, transcriptome analysis of *Ae. albopictus* oocytes showed that targets of FOXO are upregulated in females reared in diapause-inducing conditions, suggesting an important role for FOXO during the early stages of diapause egg production (Poelchau et al., 2011). Further evidence in *Culex* mosquitoes indicates that insulin signaling acts through downstream FOXO pathways to appropriately stockpile lipid reserves (Sim and Denlinger, 2008). Cold and desiccation tolerance are also important components for the survival of diapause eggs that may be linked to insulin signaling. Cold tolerance is achieved through upregulation of protective antioxidant enzymes that are regulated by FOXO in mosquitoes (Sim and Denlinger, 2011). Corazonin and CAPA neuropeptides affect the resistance to metabolic and desiccation stress in *D. melanogaster* and may also play roles in mediating desiccation resistance in diapause eggs (Zandawala et al., 2021).

Although multiple insulin-like peptides (ILPs) are encoded in *Aedes* and *Culex* genomes, ILP1 (but not ILP5) has been implicated in mediating diapause in *Culex* through the insulin receptor, suggesting that ILPs play distinct roles. Together, these studies suggest a model in which short days lead to a shutdown in insulin signaling that subsequently releases the repression of *FOXO*, leading to diapause induction. To fully understand the influence of insulin pathways in diapausing insects, it will be necessary to characterize the spatiotemporal expression and release of ILPs as well as their distinct or combinatorial regulation of lipid storage and cold tolerance (Sim and Denlinger, 2009). The *Ae. albopictus* genome encodes homologs of both ILP1 and ILP5 (Clark et al., 2016), although their roles in diapause entry remain untested. The involvement of ILP pathways in growth and development suggests that future studies to understand the specific role(s) of insulin signaling in diapause will require targeted spatial or temporal manipulations to limit these disruptions to specific timepoints and/or tissues of interest.

Nutrient Sensing and Storage Pathways

Aedes albopictus females generally require protein from a blood meal to develop their eggs, although autogenous strains have been reported to produce eggs without a blood meal (Hawley, 1988; Chambers and Klowden, 1994). *Ae. albopictus*

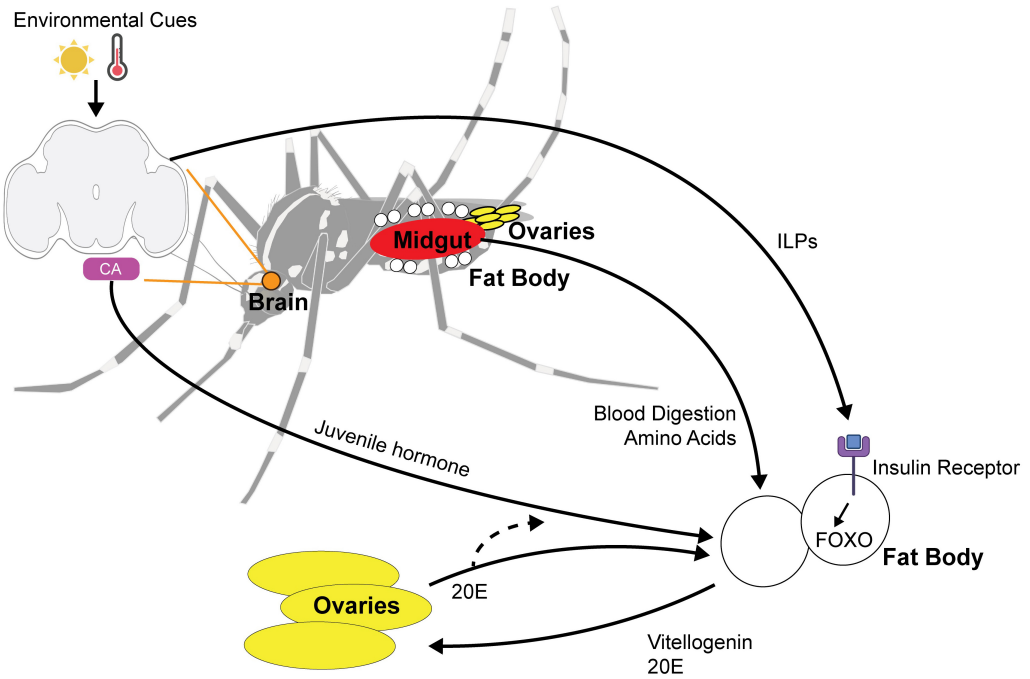

FIGURE 3 | Multi-organ signaling pathways for maternally instigated diapause in female *Ae. Albopictus*. Environmental cues such as photoperiod and temperature are sensed by the central brain and signal the fat body using (1) ILPs (insulin-like peptides) and (2) juvenile hormone released by the corpora allata (CA). ILPs bind to insulin receptor and activate FOXO pathway. Blood nutrients are digested by the midgut and the integration of environmental cue and blood-fed status trigger the release of vitellogenin and 20E signaling to the ovaries to alter reproduction in diapause state. 20E feedback to the fat body and is likely to affect juvenile hormone biosynthesis.

diapause eggs are larger and contain approximately 30% more lipids than non-diapause eggs, presumably due to an upregulation of lipid storage genes and a downregulation of lipid mobilization genes during the diapause initiation period (Reynolds et al., 2012). The identity of lipids also differs, with diacylglycerides and triacylglycerides being particularly abundant in diapause eggs (Batz and Armbruster, 2018). This suggests that blood nutrients must be utilized differently by the female to produce diapause eggs, which are more energetically costly. Nutrient utilization involves coordination between multiple tissues including the gut, ovaries, and fat body (**Figure 3**). The fat body is an organ that plays similar roles to the mammalian liver and is crucial for nutrient sensing, lipid storage, and endocrine signaling to the brain and reproductive organs.

Prior to blood feeding, ovary maturation remains in a previtellogenic state of arrest and the production of vitellogenin, the major yolk protein precursor, is repressed by the fat body. After a blood meal, multiple factors trigger vitellogenin protein synthesis in the fat body. Vitellogenins are then released into the hemolymph and taken up by the developing oocytes (Hansen et al., 2004, 2014; Attardo et al., 2005). Even before blood feeding, diapause-inducing conditions increase the expression of genes involved in amino acid synthesis and metabolism, suggesting that short day exposure primes females to differentially utilize blood protein compared to long day exposure (Huang et al., 2015). Interestingly, alanine-specific transferases are upregulated in females exposed to short day conditions that have not yet blood fed. In *B. mori*, alanine has been proposed to play a unique role in cryo-protection of diapause eggs, potentially revealing a requirement for females to provision their eggs with this specific amino acid (Suzuki et al., 1984). Furthermore, blood-fed females raised in diapause-inducing conditions have increased degradation of valine, leucine, and isoleucine, suggesting that amino acid handling may undergo changes during preparation to lay diapause eggs. Females reared in diapause-inducing conditions also demonstrate an upregulation in global metabolic pathways (phosphoenolpyruvate carboxykinase transcript), fatty acid metabolism (fatty acid synthase, fatty acid desaturase, delta(9)-desaturase 2), transcripts related to blood digestion (*trypsin*), detoxification (*glutathione transferase*, *thioredoxin peroxidase*), stress response, 20-hydroxyecdysone (20E) synthesis (*CYP302A1* and homolog of *Spook*), and vitellogenesis (Vitellogenin-A1 precursor) in response to a blood meal. Notably, upregulation of the vitellogenin synthesis gene *PVG1* was greater in females living in diapause-inducing conditions (Huang et al., 2015). This suggests that, although the general digestive and reproductive functions associated with blood feeding are similar, females show alterations in gene expression levels that allow them to differentially utilize nutrients for the production of diapause eggs. Although *Ae. albopictus* females reared under short day conditions have larger body sizes, no association between egg production per female, lifespan, or blood feeding propensity has

been found (Costanzo et al., 2015). However, diapause status of females was not tested in this work, and systematic study of host-seeking or blood-feeding behavior to determine whether biting frequency or meal size(s) differ in females preparing to lay diapause vs. non-diapause eggs remains uncharacterized.

Juvenile Hormone Signaling

Hormonal control of diapause is a common theme in insects and, in preparation for laying diapause eggs, females must coordinate JH, 20E, and insulin signaling to appropriately provision their offspring. Juvenile hormone (JH) plays critical roles in development and reproduction and has been linked to adult diapause in insects (Spielman, 1974; Saunders et al., 1990). Previous work in *Culex* mosquitoes demonstrated that long nights (short daylength) suppress JH synthesis through intermediate suppression of insulin signaling (Sim and Denlinger, 2013). In *D. melanogaster,* neuropeptides produced by circadian clock cells (PDF and sNPF) signal to insulin-producing cells (Nagy et al., 2019). In *D. melanogaster,* insulin receptors are present on the corpora allata (the tissue that synthesizes JH) and disruption of the insulin receptor also alters key enzymes required for JH synthesis (Belgacem and Martin, 2007).

Before a blood meal, JH induces the primary follicles to enter a resting stage, priming the fat body for vitellogenin synthesis as soon as a blood meal is consumed (Clements, 1999). In non-blood-fed females, genes encoding putative JH-inducible proteins are upregulated under short day conditions (Huang et al., 2015) and elevated JH-induced signaling likely enhances the fat body's competence for vitellogenin synthesis, potentially increasing vitellogenesis for additional nutrient provisioning of diapause eggs once a blood meal is consumed. The overall transcriptional responses to a blood meal are similar under both short and long day conditions including the upregulation of PVG1 and trypsin genes, which reflects the transcriptional upregulation of vitellogenesis, blood digestion, and detoxification genes (e.g., glutathione S-transferase and thioredoxin peroxidases), consistent with previous studies (Ribeiro, 2003; Sanders et al., 2003; Dana et al., 2006).

Juvenile hormone synthesis may also be regulated by steroid hormone levels associated with egg development; recent work in cabbage beetles demonstrated that levels of 20-Hydroxyecdysone (20E), a steroid hormone critical for egg development, are regulated by environmental cues and that 20E affects JH biosynthesis and reproductive diapause in adult females (Guo et al., 2021). Cytochrome P450 monooxygenases are a superfamily of enzymes involved in hormone synthesis and insecticide resistance (Hlavica, 2011). 20E-synthesizing Cytochrome P450 enzyme (CYP302A1) and the homolog of Spook are upregulated in response to a blood meal under both long and short day conditions, consistent with the role of 20E in promoting egg development (Clements, 1999; Huang et al., 2015). However, CYP314A1, which encodes an enzyme that catalyzes the final step in conversion of ecdysone to 20E, is uniquely upregulated in blood-fed females under diapause-inducing short day conditions (Huang et al., 2015). Although the overall transcriptional responses are similar and suggest that nutrients are used to support vitellogenesis under both short and long days conditions, changes in maternal hormone signaling may tailor egg production for the increased nutritional demands of diapause eggs.

After a blood meal JH-inducible proteins are generally downregulated during non-diapause reproduction; however, blood-fed females under short-day conditions showed upregulation of four putative JH-inducible proteins (Shapiro et al., 1986; Huang et al., 2015). This upregulation of JH-induced signaling under diapause-inducing conditions in blood-fed females suggests that reproductive endocrine signaling is altered during diapause induction. Interestingly, diapause eggs themselves show reduced levels of JH (Batz et al., 2019).

These findings suggest potential mechanisms for the integration of environmental cues, sensed by the circadian system and reproductive status, reflected in 20E levels, to affect JH signaling. Further investigation to understand how these hormones regulate diapause will be challenging as they are critical for both embryonic development and molting and will require tools to manipulate JH pathways with spatial and temporal precision.

SIGNATURES OF DIAPAUSE EGGS: DIAPAUSE MAINTENANCE

Diapause eggs undergo drastic morphological, physiological, and metabolic changes to survive the winter. Diapause eggs have reduced metabolism for long-term survival and are larger in size, particularly in width and volume, due to increased egg lipid reserve (Wang, 1966; Lacour et al., 2014). Diapause eggs of *Ae. albopictus* also become desiccant- and cold-resistant and are capable of tolerating temperatures as low as –10°C (Hanson and Craig, 1995). This enhanced desiccation resistance is associated with increased surface hydrocarbons (Urbanski et al., 2010). Scanning and transmission electron microscopy analysis of egg composition showed that the dark endochorion layer shrinks, likely due to the compaction of fatty acids that creates a physical barrier against ice formation, and that the serosal cuticle, which secretes the waxy layer, thickens for stronger sclerotization and chitinization (Kreß et al., 2016). Egg hardiness is likely due to both qualitative and quantitative changes of the egg shell.

Transcriptome data of oocytes destined to become diapause or non-diapause eggs show drastic changes in functional pathways that regulate metabolism, cell maintenance, and endocrine signaling (Poelchau et al., 2013a). Diapause eggs have decreased metabolism, which is reflected by the abundance of phosphoenolpyruvate carboxykinase transcript (*pepck*) in diapause-destined oocytes. PEPCK enhances the gluconeogenesis pathway to switch over to anaerobic metabolism. During metabolic suppression, many animals decrease aerobic metabolism and shift largely to anaerobic metabolism, favoring the activity of glycolysis and gluconeogenesis, the pentose phosphate shunt, and the PEPCK-succinate pathway to generate ATP (Hahn and Denlinger, 2011). PEPCK overexpression could reflect a maternally provisioned regulatory cue or the initiation of the gluconeogenic pathway in preparation for diapause (Hahn and Denlinger, 2011; Poelchau et al., 2013a). The *pepck*

transcript is upregulated in non-blood-fed females reared in diapause-inducing conditions, suggesting that it may also play a role in metabolism prior to egg production (Huang et al., 2015).

Diapause as a state of developmental arrest is reflected by cell cycle arrest and accompanying overexpression of transcripts involved in DNA replication and transcription. In particular, *inhibitor of growth protein* (*ing1*, AALF016435; LOC109399745) and *bhlhzip transcription factor bigmax* (AALF005213; LOC109405346) are abundant in diapause-destined oocytes. In *D. melanogaster*, ING1 likely interacts with the transcription factor *p53*, which regulates cell-cycle arrest in response to stress (Lunardi et al., 2010). The *Bhlhzip bigmax* transcription factor is involved in metabolism and energy sensing pathways and is a likely target of FOXO, an important regulator of diapause in *Cx. pipiens* that functions downstream of insulin signaling (Sans et al., 2006; Sim and Denlinger, 2008; Alic et al., 2011).

Lastly, transcriptomic profiles of endocrine signaling pathways showed increased levels of *rack1* activated protein kinase C receptor and decreased levels of ecdysone inducible protein L2 (*eip*) in diapause oocytes (Poelchau et al., 2011). RACK1 is reported to have a role in ecdysone signaling, which is critical for vitellogenesis, and is differentially expressed in diapausing cricket embryos (*Allonemobius socius*) (Quan et al., 2006; Reynolds and Hand, 2009). Moreover, RACK1 is an integral component of the mammalian circadian rhythm circuitry in mice (Robles et al., 2010), consistent with the hypothesis that photoperiod is the likely environmental cue initiating diapause transition. This suggests RACK1 may play roles in environmental cue detection by the female as well as diapause induction in her eggs. The *eip* homolog *imp-l2* in *D. melanogaster* is important for neural and ectoderm development (Garbe et al., 1993) and is essential for enduring periods of starvation (Honegger et al., 2008). Furthermore, both *rack1* and *eip* are implicated for regulating the size of ovaries in *D. melanogaster* (Kadrmas et al., 2007; Honegger et al., 2008) and may act similarly in regulating the size of diapause eggs in *Ae. albopictus*.

Hormonal signaling pathways are also likely contributing to diapause maintenance in *Ae. albopictus* embryos independently from their roles in adult females. Diapause eggs show lower levels of JH and JH pathway-associated transcripts compared to non-DP eggs when measured using LC-MS, which suggest that JH levels are reduced in diapause (Poelchau et al., 2013b; Batz et al., 2019).

Although diapause maintenance may appear to be an inactive period, early vs. late diapause embryos show distinct gene expression profiles—highlighting the dynamic nature of diapause (Poelchau et al., 2013b).

DIAPAUSE TERMINATION

Although our understanding of the mechanisms of diapause termination are incomplete, *Ae. albopictus* embryos terminate maternally instigated diapause after a period of time to enter the "quiescence" phase, in which they become responsive to cues that signal the return of favorable conditions and development resumes (Batz et al., 2020). If unfavorable conditions persist, development may remain suppressed in post-diapause quiescence but this form of dormancy is distinguishable from diapause because it can be terminated immediately upon exposure to favorable conditions (Tauber and Tauber, 1976). In temperate conditions, embryos often terminate diapause during the winter, thus regaining responsiveness to environmental cues while still suppressing their development in the quiescence phase. This can lead to a stockpile of eggs that are primed to hatch when favorable conditions return, resulting in a relatively synchronous springtime emergence (Lacour et al., 2015). Non-diapause embryos also enter quiescence if hatching stimuli are not present immediately after embryonic development; however, these eggs hatch as soon as they receive an appropriate stimulus (Judson et al., 1965).

Stimuli often used to terminate *Ae. albopictus* embryonic diapause in the laboratory include exposure to long daylengths, direct application of JH and chilling (not freezing) temperature (Spielman, 1974). In the field, *Ae. albopictus* eggs terminate diapause and enter quiescent phase in advance of the return of favorable conditions, usually indicated by the low temperatures and extended photoperiods associated with early spring (Vinogradova, 2007; Lacour et al., 2015). Consistent with the finding that JH abundance is reduced during diapause, the juvenile hormone-analog, pyriproxyfen, can also terminate diapause when directly applied (Suman et al., 2015; Batz et al., 2019). Although hatching is generally measured as the binary output of diapause exit, diapause is a dynamic state and both photoperiod and temperature can be thought of as regulating the *rate* of diapause development under natural conditions. As diapause progresses, gene expression patterns tend to become more similar to those of quiescent embryos, supporting the concept of diapause as a dynamic process and suggesting that diapause duration may be endogenously timed (Poelchau et al., 2013b). Although *Wy. smithii* uses daylength as a cue for termination (Bradshaw and Lounibos, 1977), few species have been shown to require a specific stimulus to end diapause. Laboratory studies often show that long daylengths can terminate both laboratory- and naturally induced autumnal diapause. However, it may be misleading to assume that long daylength serves as a direct termination signal considering that quiescence dormancy phase is maintained during longer daylength. This suggests diapause termination may occur after a predetermined phase, induced by a combination of environmental cues, or initiated by the integration of daylength dynamics, depending on the species.

Female *Ae. albopictus* that produce diapause eggs are unlikely to survive winter conditions and are presumed to die. Whether these adult *Ae. albopictus* females are capable of switching back to non-diapause egg production remains unexplored. The accuracy of behavioral changes as indicators of natural diapause termination is dependent on how closely timed are the behavioral output to the reactivation of the endocrine system (Tauber and Tauber, 1976). Interestingly, species in which adults enter and exit diapause themselves show altered behaviors and genetic expression patterns post-diapause. For example, in some tick and mosquitoes species that enter diapause as adults, the readiness to consume a blood meal is used as a behavioral indicator

to determine the end of diapause (Wilkinson, 1968; Washino, 1970; Spielman and Wong, 1973). In *Cx. pipiens* females, which enter diapause as adults, *cry2* expression profile is changed after diapause termination and may serve as a biomarker for other diapausing insects (Meuti et al., 2015).

FUTURE RESEARCH DIRECTIONS

While extensive work has been carried out to characterize the differences between diapause and non-diapause eggs and the environmental cues sufficient to induce this period of suspended development, there is a major gap in our understanding of how adult females translate seasonal cues to initiate diapause egg production. Specifically, the mechanism by which gradual environmental changes are sensed by the female and translated into a binary reproductive switch remains unknown.

The variation in diapause physiology and behavior of *Ae. albopictus* populations across global habitats provides a unique system to understand the adaptation of seemingly similar nervous system circuitry to different environmental conditions. Furthermore, *Ae. albopictus* serves as a unique biological model to study the maternal effects on the fate of her over-wintering progeny to maximize survival. It is possible that mothers reprogram the developmental timeline of their offspring through epigenetic mechanisms but this remains unstudied.

New discoveries and tools developed in *Ae. albopictus* could be applied to study other invasive species that undergo distinct forms of reproductive diapause arrest. By expanding the field's toolkit, these strategies could be applied to study and control a broad range of disease vectors and crop pest species.

Genome Editing

Whole genome sequencing of *Ae. albopictus* (Palatini et al., 2020) and the emergence of new genome editing tools allow for the unprecedented ability to manipulate genes and cells to examine their role in regulating diapause (Riabinina et al., 2015; Chaverra-Rodriguez et al., 2018). Piggybac- and PhiC31 genetic transformation has been demonstrated in a number of mosquito species, including *Ae. albopictus* (Labbé et al., 2010) and CRISPR-Cas-based genome editing has reliably generated knock-in and knock-out mutants in multiple mosquito species including: *Ae. albopictus, Ae. aegypti*, *An. gambiae*, and *Cx. quinquefasciatus* (Kistler et al., 2015; Anderson et al., 2019; Liu et al., 2019; Macias et al., 2019). Additionally the Q binary system has been successfully applied in *Ae. aegypti* and *An. gambiae* (Riabinina et al., 2016; Matthews et al., 2019), which allows for the application of a genetic toolkit to label and manipulate defined subsets of cells and is now poised to be applied in other species.

Recent work has identified chromosomal regions enriched for SNPs that differ between temperate and tropical strains (Boyle et al., 2021) and candidate genes in these regions could provide a basis for investigating the genetic regulation of diapause using targeted genome-editing. However, previous work suggests that generating disruptive mutations to a single locus will likely be uninformative to identify the genes regulating diapause in *Ae. albopictus*. Evolutionary history suggests diapause is polygenic and would likely require multiple genetic manipulations to alter diapause behavior (Boyle et al., 2021). Additionally, most candidate genes for controlling diapause entry are involved in many essential physiological processes (i.e., insulin signaling) and globally disrupting these genes is expected to cause lethality and pleiotropic effects. Genetic tools that allow spatial (i.e., tissue-specific drivers) or temporal (i.e., inducible drivers) control over transgene expression will likely be required to overcome pleiotropy associated with many of these genes to understand the specific roles that they play in diapause. "Split" binary systems, including the Q system, represent one approach to limit the expression of effectors to a restricted subset of cells by "splitting" the QF2 transcription factor into two parts with the DNA binding domain and activation domain expressed under the control of different drivers. This means that functional QF2 is only produced in cells that co-express both domains (Riabinina et al., 2019; Younger et al., 2022). These tools could be applied for targeted manipulation of developmentally essential genes only in restricted subsets of cells. For example manipulating only insulin receptor-expressing cells within the clock system by combining *InR* and *per* split driver lines. However, this approach relies on the identification and validation of driver lines to allow genetic subpopulation sectioning.

Temporal control of experimental manipulations may be even more critical for understanding the roles of specific signaling pathways or cells in diapause. Optogenetic and chemogenetic tools allow researchers to activate or silence cells of interest with temporal control depending on when the exogenous stimulus is applied. These have recently been deployed in *Ae. aegypti* (Jové et al., 2020; Sorrells et al., 2021), but may not be ideal for the long timescale of activation associated with responses to environmental cues that change over the course of days. The development and application of experimentally inducible transgenes in mosquitoes also provides potential avenues for understanding the roles of fundamental genes in specific behaviors by disrupting or rescuing gene function with temporal specificity (Chen et al., 2021). For example, these tools could allow researchers to manipulate maternal insulin-producing cells specifically during diapause induction and egg provisioning, while leaving the insulin signaling intact during development.

While forward genetic screening is currently too low-throughput and labor intensive to be feasible, candidate and RNAi-base approaches have been successfully applied in *Ae. albopictus* and could allow for temporally specific disruption of targets of interest (Xu et al., 2018).

Mapping and Characterizing Maternal Diapause Induction Circuitry

As noted above, the ability for female *Ae. albopictus* to optimize their behavior and reproduction by integrating internal metabolic and reproductive states with external environmental conditions is crucial for species propagation. The exact circuitry and mechanisms underlying this adaptive process remain unclear. Genetic and technological advances in the field now allow researchers to identify cells that respond to environmental cues to understand the mechanisms underlying diapause development,

physiology, and behavior. A possible circuit may consist of detection of environmental cues by either sensory neurons that relay that information to clock cells or circadian clock cells themselves, which in turn release neuropeptides to control circulating levels of ILPs and signal to the distant tissues, including the fat body and ovaries, to alter nutrient utilization and JH levels. Mapping the anatomical and functional connections between cells that detect seasonal cues and those that control nutrient allocation is a critical first step to connect the female's response to environmental cues with her reproductive physiology.

Using reporters such as GCaMP, researchers can measure the acute activity of target cells in response to environmental cues during the critical period of diapause transition. The functional role of target cells may be directly tested using genetic or pharmacological manipulations to determine if diapause can be effectively induced or if these manipulations are sufficient to block environmentally induced diapause. The application of these cutting-edge genetic tools in *Ae. aegypti* has established their feasibility and the field is now poised to anatomically and temporally map diapause-inducing circuitry in *Ae. albopictus* and to compare these neural circuits in different behavioral states (diapause vs. non-diapause egg production) and between populations with varying diapause competence (Jové et al., 2020; Zhao et al., 2021).

Metabolic Profiling of Diapause Preparation

Although metabolomic methods have been applied to study insecticide resistance and embryonic diapause, the changes in metabolic profiles of female *Ae. albopictus* preparing to lay diapause eggs has not yet been characterized (Huang et al., 2015; Batz and Armbruster, 2018). Untargeted metabolomic technology allows researchers to simultaneously assess amino acids, lipids, polyols, fatty acids, and metabolic intermediates in insect and have demonstrated that diapause eggs show distinct metabolic profiles compared to non-diapause eggs with particular enrichment of diacylglycerides and triacylglycerides (Colinet et al., 2012; Batz and Armbruster, 2018). Untargeted metabolomic approaches provide an unbiased snapshot of organismal physiology and may identify unexpected metabolic changes that can be used as the basis for subsequent targeted experiments, although they lack the specificity and pathway coverage of targeted analysis (Macel et al., 2010). Additionally, the metabolites that are detected vary depending on which extraction and separation methods are used (Cajka and Fiehn, 2016). Targeted metabolic profiling is available to measure triglyceride/lipoprotein levels in adult females from laboratory and field populations preparing to lay diapause and non-diapause eggs using assays such as the colorimetric sulfophosphovanillin (SPV) (Knight et al., 1972; Van Handel, 1985; Men et al., 2016) or the Glycerol-3-phosphate Oxidase (GPO) with *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS) methods (Sawabe and Moribayashi, 2000). Both are attractive methods to quantify the total lipid reserves from single adult females as they are fast- and high-throughput assays and have been used to measure total lipid content in other mosquito species and *D. melanogaster*. Metabolic changes correlated with diapause behaviors will yield new insight into how the brain integrates metabolic and reproductive status to undergo reproductive switch at appropriate seasons.

Maternal Behaviors in Preparation for Diapause Egg Production

Many outstanding questions remain regarding how adult females detect seasonal cues to initiate the drastic switch to diapause state. As obligate blood-feeders, females consume a blood meal to obtain the protein required to complete reproduction and lay eggs. The effects of diapause-inducing conditions on the host-seeking and blood-feeding behaviors of adult females are poorly characterized. Using validated assays, the differences in host-seeking, blood feeding, and engorgement behaviors can be compared between females preparing to lay diapause and non-diapause eggs. Previous studies suggest while *Ae. albopictus* females suppress their drive to find and bite hosts in the days following a blood meal, they are capable of multiple host-feedings without laying eggs, particularly if they have a smaller body size (Klowden and Briegel, 1994; Farjana and Tuno, 2013). Females preparing to lay diapause eggs may require multiple bouts of blood feeding to obtain the nutrient levels necessary to lay lipid-rich diapause eggs and may also show altered host-seeking regulation.

A recent study characterized egg laying behavior of *Ae. albopictus* reared in favorable and unfavorable conditions (Reinbold-Wasson and Reiskind, 2021). They focused on "skip-oviposition" behavior, broadly defined as female mosquitoes distributing eggs among multiple oviposition sites during a single gonotrophic cycle. In both favorable and unfavorable conditions, *Ae. albopictus* females spread their eggs widely, suggesting that this egg-laying behavior is unaltered by diapause state. However, other groups have reported that females distribute their eggs more broadly across oviposition sites when tested in summer conditions compared to fall conditions (Fonseca et al., 2015). Further critical assessment of feeding and reproductive behaviors in females reared in diapause-inducing conditions could identify new potential targets to disrupt these behaviors or more efficient ways to deploy mosquito control strategies throughout the year, for example if oviposition sites are reliably denser in the fall compared to the summer.

Ecology and Population Biology

The differences in reproductive physiology and behavior of females in temperate and tropical conditions are interesting examples of evolutionary divergence. These temperate and tropical populations share similar genetic components, neural circuitry, and anatomy yet produce distinctly different behavioral outputs in response to environmental conditions. These differences raise intriguing research questions about the genetic and neural basis of maternally induced diapause and the plasticity of these behaviors.

As climate change and urbanization open new areas for territorial expansion, tracking and characterizing new invasions

around the globe may provide opportunities to examine the plasticity of diapause and to uncover the mechanisms by which these selective pressures shape behavior. These studies could address the conflicting observations on whether diapausing ability is gained or lost throughout history. Furthermore, it could yield insights into the evolutionary timeline of the emergence and disappearance of adaptive behaviors induced by environmental changes, especially as climate change contributes to the expansion of this species' geographical range (Ryan et al., 2019). Work in *Wy. smithii* has demonstrated genetically based shifts in CPP toward shorter more "southern" CPPs in populations in more northern latitudes – consistent with an adaptive response to prolonged warm season (Bradshaw and Holzapfel, 2001). Altogether, these findings suggest that *Ae. albopictus* populations that are able to adapt to harsh winters are poised to expand their geographic ranges. Furthermore, *Ae. albopictus* diapause affects seasonal species abundance and this can influence inter-species interactions and competition.

Interaction With Artificial Environmental Conditions

Due to global urbanization, the increased presence of artificial light at night (ALAN) has greatly impacted the diapause physiology and behavior of temperate mosquito strains. A recent field study showed that ALAN exposure interferes with daylight as a cue for seasonal dynamics and significantly reduced diapause incidence in *Ae. albopictus* (Westby and Medley, 2020). Similarly, diapausing adult female *Cx. pipiens*, which undergo reproductive arrest, inappropriately averted diapause state by becoming reproductively active when exposed to ALAN in laboratory settings (Fyie et al., 2021). In these females, whole body fat content was significantly reduced, egg follicles were larger, and blood-feeding increased (Fyie et al., 2021). Likewise, *Mamestra brassicae* caterpillars exposed to low intensities of artificial light at night as larvae showed disrupted pupal diapause initiation as moths (Van Geffen et al., 2014). ALAN exposure likely interferes with photoperiod dynamics as an environmental cue for seasonal timing, resulting in improperly initiated diapause. As *Ae. albopictus* continues to expand its range, urbanization and ALAN will be important factors that determine seasonal biting patterns and the risk of disease transmission. From a basic science perspective it will be interesting to understand how these factors interact with the neural circuitry that controls diapause.

APPLICATIONS FOR MOSQUITO CONTROL AND PUBLIC HEALTH

Aedes albopictus poses increasing threats to public health largely due to its ability to live in a broad geographic range and to vector a number of arboviruses that threaten human health (Ibáñez-Bernal et al., 1997; Paupy et al., 2012; Lindh et al., 2019). Diapause can lead to the synchronized seasonal abundance of *Ae. albopictus,* which can contribute to interspecific competition and could directly influence pathogen transmission cycles (Joy and Sullivan, 2005). In temperate regions, predictable seasonal changes in the incidence of larval vs. adult mosquitoes allow vector control strategies to be efficiently targeted to specific life stages at certain times of year. For example, prioritizing larvicidal control in the early- to mid-spring will be particularly effective as eggs exit diapause, enter quiescence and hatch because there are relatively few adult mosquitoes present at this time of year (**Figure 1B**; Lacour et al., 2015).

Understanding diapause pathways in *Ae. albopictus* could facilitate the development of new methods of mosquito control by creating tools to disrupt reproductive and/or vectorial capacity. For example, deploying compounds that block diapause entry or promote inappropriate diapause exit could reduce mosquito populations in temperate conditions. Alternately, finding ways to inappropriately induce diapause in tropical conditions could also suppress the populations.

New control strategies are urgently needed as this species continues to expand its range (**Figure 1A**). The risk of severe outbreaks of emerging vector-borne diseases is an increasing threat for more of the world (Bonizzoni et al., 2013; Ryan et al., 2019). Targeting the pathways that control and optimize reproduction may be a particularly effective approach to reduce mosquito populations, prevent vector-host interactions, and limit the spread of vector-borne disease.

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REVIEW
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Mechanisms of Variability Underlying Odor-Guided Locomotion

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Changes in locomotion mediated by odors (odor-guided locomotion) are an important mechanism by which animals discover resources important to their survival. Odor-guided locomotion, like most other behaviors, is highly variable. Variability in behavior can arise at many nodes along the circuit that performs sensorimotor transformation. We review these sources of variability in the context of the *Drosophila* olfactory system. While these sources of variability are important, using a model for locomotion, we show that another important contributor to behavioral variability is the stochastic nature of decision-making during locomotion as well as the persistence of these decisions: Flies choose the speed and curvature stochastically from a distribution and locomote with the same speed and curvature for extended periods. This stochasticity in locomotion will result in variability in behavior even if there is no noise in sensorimotor transformation. Overall, the noise in sensorimotor transformation is amplified by mechanisms of locomotion making odor-guided locomotion in flies highly variable.

Keywords: *Drosophila*, odor-guided locomotion, variability, stochastic, circuit

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INTRODUCTION

Variability is a hallmark of behavior and is observed across timescales (Tinbergen, 1951). On long timescales, variability has been studied in the migratory behavior of birds; birds display inter-individual variability in migratory patterns, timing, and kinematics such as migratory speed (Potti, 1998; Trierweiler et al., 2014; Fraser et al., 2019; Phipps et al., 2019). On shorter timescales, many studies have looked at variability in movement kinetics, kinematics, and endpoints of reaching movements (Gordon et al., 1994; Messier and Kalaska, 1999; van Beers et al., 2004; Wu et al., 2014). Even when movement kinematics, such as walking speed, is constrained to a constant value, studies in humans have shown that there is variability in properties such as step length and width (Sekiya et al., 1997; Collins and Kuo, 2013).

Given the ubiquity of behavioral variability, it is unsurprising that odor-guided locomotion in fruit flies or *Drosophila melanogaster* also shows variability. One large body of literature has focused on the idea of behavioral valence (attraction vs. repulsion) of flies to odors. Attraction or repulsion of a fly to an odor source is usually measured as the fraction of time a fly spends within an odorized region. These studies often utilize a wide array of odors and a wide range of behavioral assays ranging from a trap assay where a population of flies chooses between two odor traps to assays with a single fly in an arena with a single odorant zone (**Figure 1A**). Yet, regardless of the experimental setup or the odors used, there is a large variability in attraction (**Figure 1A** and methods) (Larsson et al., 2004; Semmelhack and Wang, 2009; Knaden et al., 2012; Jung et al., 2015; Badel et al., 2016; Honegger et al., 2020; Tao et al., 2020). As a simple illustration of the large variability, consider an experiment in which the standard deviation (SD) in attraction is 0.09 (**Figure 1A**), one of the lowest values in our

survey of the literature. A SD of 0.09 with a mean attraction of 0.5 means that 95% (± 2 SD) of attraction would fall between 0.32 and 0.68, a large range.

Recently, research on odor-guided locomotion has moved past simple measures of valence to the moment-by-moment change in locomotion that accompanies attraction or repulsion. This advance parallels advances in ethological techniques to perform pose estimation (Mathis et al., 2018; Graving et al., 2019; Pereira et al., 2019), identification of behaviors (Dankert et al., 2009; Kabra et al., 2013; Berman et al., 2014; Wiltschko et al., 2015; Tao et al., 2019), and high throughput experimentation (Branson et al., 2009; Buchanan et al., 2015; Werkhoven et al., 2019). In the context of fly locomotion and how odors affect it, one insight from studying the detailed mechanism is that fly locomotion is comprised of sequences of discrete movement states, i.e., flies move at a surprisingly constant speed and curvature for extended periods before making sudden changes. This persistence means that instead of deciding on speed and curvature on every step, flies make decisions at the beginning of a "state" which can last several steps (hundreds of milliseconds). As we will discuss at length in this review, this persistence means that each decision will be important and small differences in choices will drive large variability in sensory experience and the spatial spread of a population of flies.

The effect of locomotor persistence on variability is well-described by a recent study that employed a hierarchical hidden Markov Model (HHMM) (Tao et al., 2019). The HHMM is an unsupervised method to infer states based on speed and curvature in an unbiased way. The authors found that flies use about ten states – each state defined by characteristic speed and curvature that does not change much during the state – to walk around a small circular arena. These states are persistent and last about a second, a time during which a fly takes 10 steps on average. Although each fly in the dataset could have its own set of states, a single set of states modeled all the flies. Since flies utilize a single set of state, flies likely utilize the same building blocks during locomotion. These building blocks account for locomotion both before the odor was turned on and during the odor period (Tao et al., 2019). Although flies use the same states, there is large fly-to-fly variability in the time spent performing each state both in the absence and presence of odors. The variability in state usage results in behavioral variability since there is a large difference in speed and curvature between states. In contrast to between states, this model shows a tight distribution of kinematics within a state, implying that flies maintain consistent kinematics (speed and curvature) for about a second – a time during which the fly takes ∼10 steps. Qualitatively, these states represent characterizations of different types of walking, stopping, and turning states.

The HHMM model shows that locomotion consists of persistent states where each state represents different types of walking, stopping, and turning states. The insights from the HHMM model – that persistence of a state can cause variability – can also be captured by a much simpler model with four states – walk, stop, turn, and boundary (Tao et al., 2020; **Figure 1B**). Each transition into a given state is well-described by the average kinematics (e.g., speed), but different transitions can have widely different speeds. The persistence is shown by the fact that states last on average 700 milliseconds within which the variation in speed is much less than the variation observed across states (**Figure 1B**). The result of this variation is that the tracks of the fly and attraction to odors are highly variable even though each fly is executing the same algorithm (**Figure 1B**).

Both the variability in olfactory behavior (**Figure 1A**) and the role of the nature of locomotion itself in creating this variability (**Figure 1B**) has not been systematically explored. Here, we will review potential mechanisms behind variability in odor-guided locomotion. At any moment a given fly has a given locomotor or search algorithm which is determined by its sensory environment and its state acting on its locomotor circuits. Odors affect attraction and repulsion by changing how these different locomotor states are used, and how different locomotor variables such as speed and curvature are chosen in a given state (**Figure 1C**). Thus, variability in olfactory behavior can result from differences in sensorimotor transformations which in turn can result from irreversible genetic differences, from reversible neuromodulatory differences, or from sampling noise. We will draw on work aimed at understanding both variability in odor valence and odor-driven locomotion. We will emphasize that the noise in sensorimotor transformations when coupled with persistence in locomotion can be an important source of variability in genetically identical flies. The review is organized into four main sections. In the first section, we will orient the reader on the structure and function of the fly's olfactory system. In the remaining three sections, we will discuss variability arising from genetic differences, neuromodulation with an emphasis on hunger, and from sampling noise in turn.

SIGNAL PROCESSING IN THE *DROSOPHILA* OLFACTORY CIRCUIT

Olfactory processing in *Drosophila* can be broken down into three layers of processing (**Figure 2A**). First, odors are detected by the receptors of ∼1,400 olfactory receptor neurons (ORNs) located in the antennae and maxillary palps. These olfactory organs have hair-like protrusions that each house the dendrites of one to four ORNs (Vosshall et al., 1999). ORNs can be segregated into distinct classes based on the expression of 51 receptor types (de Bruyne et al., 1999, 2001; Bates et al., 2020). At the signal detection level, odorant-binding proteins (OBPs) facilitate the transport of odorants to bind with olfactory receptors (ORs). Beyond OBPs and ORs within a single sensillum, ORN signal transduction will be influenced by sensillar morphology, lymph fluid biochemistry, and physiological crosstalk between sensillar cells (Schmidt and Benton, 2020).

Olfactory signal transduction will ultimately lead to ORN spiking activity. The rate of spiking increases immediately following odor onset, then adapts to a stable but elevated level. The level of activation for each class of ORN is dependent on the odorant, and also has a non-linear dependence on its concentration within the odor plume (Hallem et al., 2004; Hallem and Carlson, 2006). The relationship between odor concentration and ORN spiking response also depends on stimulus history (Nagel and Wilson, 2011; Martelli and Fiala, 2019). At odor offset, the neural activity of many types of ORNs

A

Study	Odors or Optogenetics	Assay	Lowest Variability	Highest Variability	Valence Range	Starvation (hours)
Larsson et al., 2004	2 odors	Trap	0.08	0.12	-1 to 1	48
Knaden et al., 2012	120 odors	Trap	0.11	0.74	-1 to 1	24
Semmelhack and Wang, 2009	ACV	Quadrant		0.27	-1 to 1	50
Badel et al., 2016	39 odors	Tethered flight	0.15	0.31	-1 to 1	4-6
Jung et al., 2015	2 odors	Ring			0 to 1	18-24
Tao et al., 2020	Chrimson Orco	Ring		0.25	0 to 1	15-21
Honegger et al., 2020	4 odors	Linear	0.09	0.14	0 to 1	0

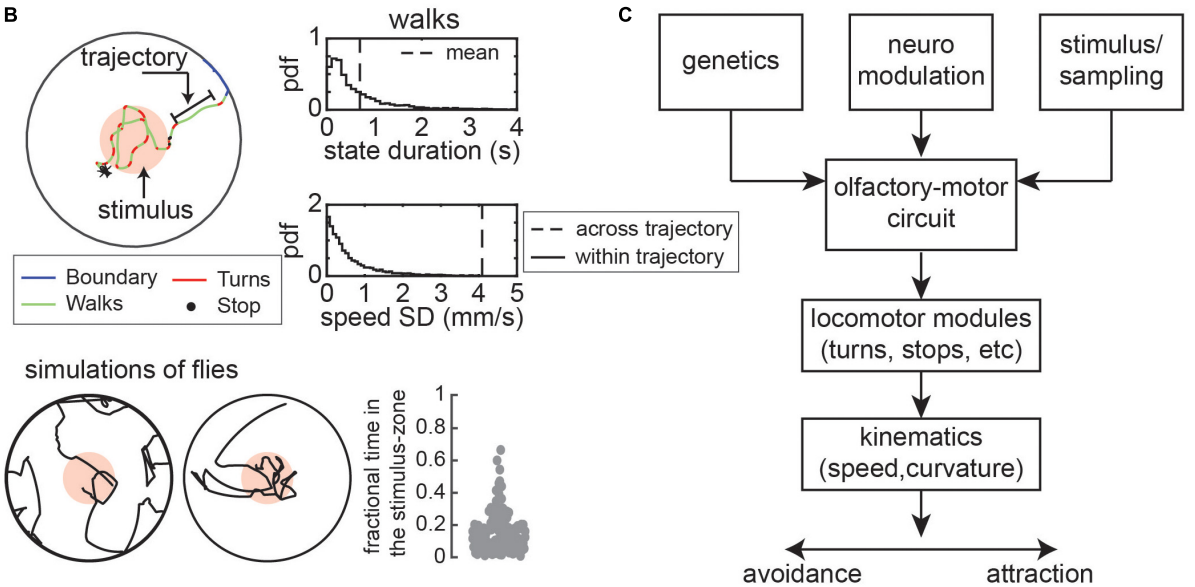
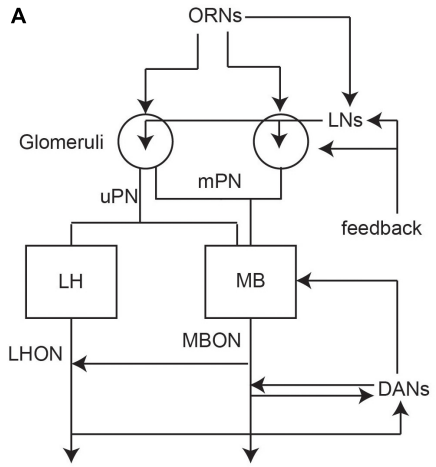


FIGURE 1 | Persistence of locomotor states is an important contributor to variability in olfactory behavior. **(A)** Examples of variability in attraction to odors. Most sources of variability is measured as the standard deviation (SD) in attraction index with the exception of Knaden et al., 2012 and Jung et al., 2015, where it is represented by the interquartile range. **(B)** Top: In a circular arena with a concentric odor zone, fly locomotion can be represented as discrete states such as walks and turns (different colors) which last 700 millisecond on average (dotted line). During each state flies move with relatively stable speed and curvature as compared to across trajectories (characterized by the SD). Probability density distributions for durations and speed SD of walking trajectories are shown on the right. Bottom: This persistence leads to variability in sample trajectories. Over many samples, simulations of flies (n = 116) show a high a high degree of variability in the movement path and time spent in the odor zone (SD = 0.12). **(C)** Genetic factors, neuromodulation, and the dynamics of olfactory stimulus and sensorimotor sampling all can cause variability in the olfactory-motor circuit. This will result in variability in the performance of locomotor modules such as turns which results in variability in the time averaged attraction to odors. Panel **(B)** is adapted from Tao et al. (2020).

is inhibited for an extended period that can last for upwards of a few seconds.

The ORNs project to 51 glomeruli in the antennal lobe where they synapse with second-order projection neurons (PNs) which carry information into higher-order olfactory processing centers (Bates et al., 2020). PNs can be classified into uniglomerular PNs (uPNs) that receive input from a single glomerulus and multiglomerular PNs (mPNs) that receive input from multiple glomeruli (**Figure 2A**; Bates et al., 2020). In addition to the PNs, local neurons (LNs) connect multiple glomeruli within the antennal lobe through lateral connections (**Figure 2A**). The computation in the antennal lobe results in an increase in the separability in odor representations and a decrease in variability in response to a given ORN class (Bhandawat et al., 2007; Olsen et al., 2010; Wilson, 2013).

From the PNs, olfactory information is next transmitted to third-order processing centers called the mushroom body (MB) and the lateral horn (LH) (**Figure 2A**). In the MB, PNs form random synapses with on average 7 Kenyon cells (KC) in the MB calyx (Jefferis et al., 2007; Butcher et al., 2012; Caron et al., 2013). The output of the MB calyx converges into a small set of 34 output neurons called mushroom body output neurons (MBONs) that are separated into 15 different compartments (Tanaka et al., 2008; Aso et al., 2014b; Bates et al., 2020). Functional studies have shown that the MBON activity patterns likely encode the valence of an odor. This valence can be remapped or learned through synaptic plasticity brought about by dopaminergic neurons (DANs) that enervate each compartment of the MBONs (Aso et al., 2014b). DANs in turn can receive inputs from both the MBONs as well as input



B

layer	function	types of variability
ORNs	Odor detection	1. Signal transduction 2. Odor driven neural response
PNs	Odor identification and discrimination	3. Idiosyncrasies in circuit structure 4. Reversible circuit dynamics
MB	Valence encoding	5. Idiosyncrasies in MBON valence tuning 6. Valence learning and adaptation
LH	Innate odor valences and locomotor programs	

FIGURE 2 | Information flow and properties of the *Drosophila* olfactory circuit. **(A)** Information flow of the fly olfactory circuit. First order olfactory receptor neurons (ORNs) detect odors and synapse with uniglomerular and multiglomerular projection neurons (uPNs and mPNs, respectively) in the glomeruli. Local neurons (LNs) provide lateral connections. PNs synpase into the mushroom body MB and lateral horn (LH), which act as third order processing centers. Dopaminergic neurons modulate MB activity. Mushroom body output neurons (MBONs) and lateral horn output neurons (LHONs) carry information into higher order circuits. **(B)** A table of the main function at each layer of the olfactory circuit as well as where variability will arise.

from the lateral horn output neurons (LHONs) (Dolan et al., 2019; Li et al., 2020).

The LH is comprised of local neurons and output neurons. These neurons receive excitatory input from both the uPNs and mPNs as well as inhibitory input from the mPNs (Bates et al., 2020). The LHONs and MBONs project downstream into multiple fourth-order processing centers. The MB and the LH are highly interconnected via both direct connections (Aso et al., 2014b; Dolan et al., 2019) as well as via recurrent connections from MBONs to PN axons in the LH (Bates et al., 2020). The specific function of the LH is currently being actively investigated, but specific classes of neurons have been shown to drive innate odor valence as well as specific locomotor programs such as turning or wingbeat frequency during flight (Dolan et al., 2019; Varela et al., 2019).

The MB and LH represent what is the final stage of the relatively stereotyped olfactory circuit. From here olfactory information form multiple convergent and divergent pathways to higher order circuits as well as recurrent pathways to the aforementioned layers of olfactory processing neurons (Bates et al., 2020; Scheffer et al., 2020; Scaplen et al., 2021). Recent studies have shown that these higher order circuits, especially those in the central brain allow flies to integrate and switch between multisensory information such as wind and visual cues during odor guided locomotion to generate a representation of the direction of the olfactory source (Suver et al., 2019; Okubo et al., 2020; Matheson et al., 2021). Variations in circuit activity at the level of the central complex may ultimately explain variability in movement reorientation when the fly is turning during olfactory guided locomotion. The role of central complex in odor-guided locomotion is discussed in detail in other recent reviews (Hulse et al., 2021; Fisher, 2022).

GENETICS AS A SOURCE OF VARIABILITY IN *DROSOPHILA* ODOR-GUIDED LOCOMOTION

At each step described above, variability can arise from genetic differences which can affect different aspects of the sensorimotor transformation as reviewed below. First, subtle changes in genes that are directly involved in various aspects of olfactory processing can affect sensorimotor transformation. There is a growing body of evidence particularly at the level of ORNs that supports contribution due to this mechanism. Even in isogenic flies, accumulations of polymorphisms can lead to behavioral variability (Mollá-Albaladejo and Sánchez-Alcañiz, 2021). For instance, naturally occurring single nucleotide polymorphism (SNP) in OBPs 99a-d has been shown to contribute to the phenotypic variability in the aversion to benzaldehyde (Wang et al., 2007). The authors found in a follow-up study that SNPs in different OBPs in the 99a-d complex can have a varied effect on olfactory behaviors (Wang et al., 2010). Similarly, natural polymorphisms in multiple ORs have been found to have a significant association with variations in odorant-specific valence (Rollmann et al., 2010; Richgels and Rollmann, 2012).

Single nucleotide polymorphisms can also affect olfactory behavior via network pathways involved in olfactory signal

transduction, neurogenesis, and neural connectivity (**Figure 2B**; Swarup et al., 2013; Arya et al., 2015). A recent study provides evidence that genetic variation in the Or22 locus leads to significant differences in the functional neural response properties of its corresponding class of ORN, which in turn correlates with a preference for ethyl hexanoate, an odor that strongly stimulates this ORN (Shaw et al., 2019, 2021).

In addition to single-neuron effects, individuality in the genetic code can lead to wiring and structural variability in neural circuits (**Figure 2B**). A recent study looking at a large population of inbred flies over 9 different behavioral assays showed that individual differences in genes related to development (e.g., Hedgehog signaling, Wnt signaling) and neural function (e.g., vesicle release) may be involved with behavioral variability (Werkhoven et al., 2021). This study also implicated genes involved in cellular respiration and protein translation in behavioral variability.

Despite recent efforts, the mechanistic effect of variability of most genes on animal-by-animal variability in odor guided locomotion is still unknown. These effects may present themselves through careful anatomical and functional studies. In the antennal lobe, electron microscopy studies show that the connectivity from ORN to PN are variable. In one study, the authors found that there is a high degree of synaptic variability, which leads to the contamination of ORN spike count information (Tobin et al., 2017). Some variability in this connectivity will be compensated for. For instance, one hemisphere may have smaller PN dendritic sizes but compensate with more synapses to generate similar postsynaptic membrane potential responses to pre-synaptic ORN input. In addition to the ORN to PN connections, LNs have also been found to exhibit variability in fine-scale connectivity patterns which undergo both developmental and experience-dependent plasticity (Chou et al., 2010). However, the extent to which this variability leads to variability in sensory processing and ultimately behavioral variability is unclear.

Finally, an important mechanism for genetic variability is the plasticity effect of different genes that alter olfactory valence (**Figure 2B**). In the MB, there are many genes shown to be important for olfactory memory (Kahsai and Zars, 2011). It has been shown that while the tuning of individual MBON compartments is the same across hemispheres of an individual fly, the tuning of these compartments is different across animals. This source of individuality is linked to the rutabaga (*rut*) gene (Hige et al., 2015). In the MB, both the *rut* and *dunce* gene are involved in the synthesis and degradation of cAMP, and mutations in these genes have been shown to affect signal transduction (Renger et al., 2000).

While these studies show that genetic variability can lead to individuality through potential changes in signal transduction and circuit wiring, they will not be the only source of this variability. For example, a recent study in the fly visual system showed that left/right wiring asymmetry for a set of neurons called the dorsal cluster neurons is caused by stochastic wiring during development and not genetic differences. The extent of the wiring asymmetry explains the ability of individual flies to orient toward a visual object (Linneweber Gerit et al., 2020).

NEUROMODULATION MAY DRIVE SHIFTS IN VALENCE THROUGH CHANGING EXCITATORY-INHIBITORY BALANCE

A second mechanism for variability is through internal states such as hunger which have been shown to drastically alter the behavioral valence of odors through neuromodulation (**Figure 2B**). In the antennal lobe, such neuromodulators act upon both the LN and uPN to generate variability in attraction to odors. In a recent study, it was found that feeding flies a serotonin synthesis inhibitor (alpha-methyltryptophan) or expressing a mutant allele of the dopamine receptor gene (Dop1R1) resulted in a decrease in the variability of odor preference. Meanwhile, feeding flies a dopamine precursor (L-DOPA) increased odor preference variability (Honegger et al., 2020).

The effect of serotonin on the antennal lobe neurons is likely a result of action of a well-studied group of serotonergic neurons, that modulate both LN and PN activity, called the contralaterally projecting serotonin-immunoreactive deuterocerebral (CSD) neurons (Zhang and Gaudry, 2016). These neurons are conserved among multiple insect taxa (Kent et al., 1987; Python and Stocker, 2002; Dacks et al., 2006). Interestingly, it was found that thermogenetic activation of the CSD neurons did not change the attraction to or variability in the attraction to the odors (Honegger et al., 2020). However, a recent paper in larvae showed that CSD neurons are necessary for hunger-driven changes in olfactory behavior. When satiated, larvae avoid geranyl acetate; when hungry, CSD neurons cause an increase in attraction to geranyl acetate by directly potentiating attraction mediating uPN responses while indirectly inhibiting aversion mediating mPN responses (**Figure 3A**; Vogt et al., 2021). The circuit motif of hunger promoting activity in attraction mediating neurons and reducing activity in aversion mediating neurons appears in both the antennal lobe (Root et al., 2011; Ko et al., 2015) and mushroom body (MB) (Tsao et al., 2018).

Since the level of hunger can play a key role in behavioral variability, most laboratory studies control hunger though controlling starvation time. In the antennal lobe, the duration of starvation leads to a negative exponential change in PN activity (Root et al., 2011). In the same study, it was shown that the mean time spent finding food follows a similar pattern. Such a mechanism suggests that changes in valence caused by variability in hunger levels should be less at large starvation values (**Figure 3B**). However, most studies show that even after long periods (24+ h) of starvation, there is still a high degree of valence variability (**Figure 1A**). In such scenarios, variability can still arise from neuromodulation. One potential explanation is because while the average effect of hunger on neural activity across individuals and trials saturates after long starvation periods, there is still variability in neural activity around the average that can reflect variability in activity in the antennal lobe, the effect of other sensory and higher order circuits that input into the antennal lobe, and variability in the amount of neuromodulation. Furthermore, while we have highlighted one potential mechanism of hunger, this state affects behaviors

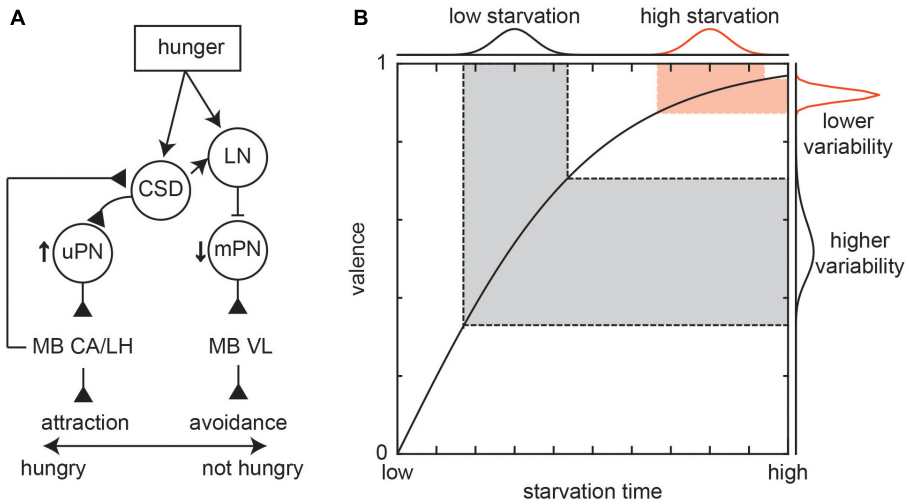


FIGURE 3 | Effect of internal states on behavioral variability. **(A)** Effect of hunger on larvae attraction or avoidance to geranyl acetate. When hungry, the CSD neuron potentiate attraction mediating uPN responses while LNs inhibit aversion mediating mPN responses through glutamatergic mPNs. This leads to a switch from avoidance to attraction through downstream connections to the mushroom body calyx (MB CA), mushroom body vertical lobe (MB VL), and lateral horn (LH). Figure based on Vogt et al., 2021. **(B)** The variability in behaviors such as attraction depends on the relationship between the behavior and internal states like hunger (represented by starvation time). In this cartoon, two groups of flies that have the same variance in starvation times, the flies that are starved more should show less variability in valence. However, experiments typically show a higher level of valence variance than that predicted by theoretical average relationship curves.

through a multitude of parallel mechanisms. For instance, this variability may reflect an increase in exploratory drive in a bid find the food source. This process is driven by a metabolic pathway where starvation drives an increase in the adipokinetic hormone, which in turn drives octopaminergic cells to promote foraging associated hyperactivity (Yang et al., 2015; Yu et al., 2016).

While hunger is the most well studied and one of the most important internal states for odor guided locomotion (especially in the context of food odors), there are many other internal states that can affect odor guided locomotion. For instance, the nutritional and social history of flies can affect both olfactory driven locomotion and attraction to specific odors (Lebreton et al., 2014; Jung et al., 2018; Huang et al., 2020). Finally, beyond internal states, trial-by-trial variability may arise from differences in the behavioral state of the fly. For instance, flies are attracted to CO_2 when in an active foraging state but avoid CO_2 when moving at a slower speed (van Breugel et al., 2018). The internal states and mechanisms described here exemplify a wider range of processes; some of these processes are detailed in other recent review (Grunwald Kadow, 2019; Lin et al., 2019; Maloney, 2021; Devineni and Scaplen, 2022).

VARIABILITY IN SENSORIMOTOR TRANSFORMATION IS AMPLIFIED BY STOCHASTIC AND PERSISTENT BEHAVIORAL CHOICES

In nature, flies will often navigate complex landscapes involving multiple odor sources where rather than a continuous odor gradient, flies experience odors as pulses – odor plumes – resulting from turbulent winds (Crimaldi and Koseff, 2001; Celani et al., 2014). To navigate these environments, the *Drosophila* will either fly or walk as it approaches the odors. There will be variability in sensorimotor transformations underlying the navigational strategies during each phase. Here, we will focus on the walking phase of odor guided locomotion.

Far from the odor source, the frequency of plume encounters is small. A fly will encounter a pulse of odor such as the one shown in **Figure 4A** (from an actual experiment) and respond with the corresponding ORN activity (**Figure 4A**). The behavioral variability comes from two sources. First, odorant history and differences in ORN activity experienced by flies across separate odor encounters will lead to changes in the average locomotor kinematics such as speed and curvature (**Figure 4A**). Studies in wind tunnels show that the temporal dynamics of these sensorimotor transformations is complex and dependent on odor concentration and wind (Álvarez-Salvado et al., 2018; Demir et al., 2020). Recently studies have used open loop optogenetics to dissect the individual effects of ORN activation. A recent study using optogenetics show that even trial-by-trial differences in locomotion when crossing a static stimulus zone can lead to differential ORN activity (Tao et al., 2022).

A second source of noise is the stochasticity in locomotor kinematics across locomotor state transitions and the decision to transition between states. While the average sensorimotor transformation can be predicted from ORN activity in studies where other external factors like wind is controlled, there will be a high level of variability around this average. As such one way to think about this is that given the same olfactory stimulus information, flies will modulate their future locomotion by sampling from a probabilistic distribution (**Figure 4A**). The

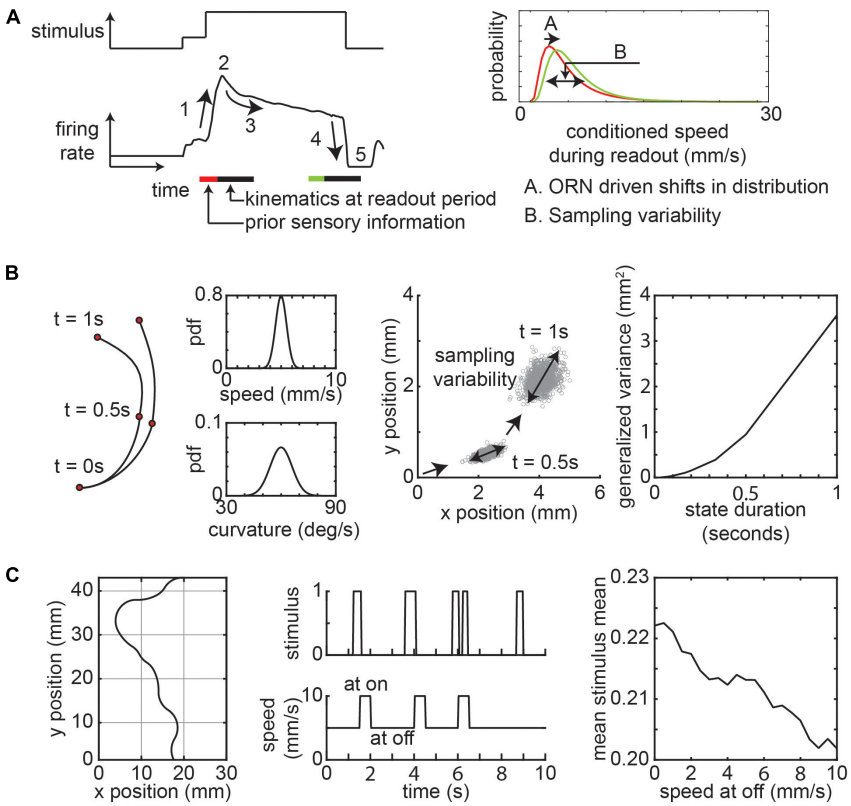


FIGURE 4 | Variability due to sensorimotor transformations and sampling. **(A)** A schematic of odor stimulus and ORN response. The response is characterized by a rising edge (1), peak response (2), adaptation (3), falling edge (4), and inhibition (5). The speed bouts of curved walks (readout period) conditioned on ORN activity follows a lognormal distribution. The distribution changes based on ORN activity. **(B)** Variability in sensorimotor transformation will result in sampling variability. Left: Toy example of two consecutive instances of curved walk with constant speed and curvature sampled from normal distributions. Middle: Positions from 1,000 simulations starting at position (0,0) facing in the positive × position with a trajectory persistence of 0.5 s show variability increases with consecutive samples. Right: The generalized variance in positions after 5 s increase with increasing state persistence. **(C)** Effect of locomotor strategy on sensory experience. Left: Sample 10 s trajectory of a fly moving through an environment with constant average stimulus intensity, but with variable frequencies at each spatial block (bounded by gray). Middle: Stimulus experienced by the fly during the period as it chooses a lower speed when it experiences no odors. Right: The mean of the mean stimulus experienced by simulations of flies as a function of off speed (n = 5,000/speed at off). See methods for further details about simulations in **(B,C)**.

properties of this distribution (such as mean and variance) may be estimated by past ORN experience (Tao et al., 2022). If flies continuously update their speed and curvature on a moment-by-moment basis, then the positional variability due to sampling noise will be small. However, the variability arising from sampling noise is magnified when flies maintain relatively consistent kinematics for long (hundreds of milliseconds to seconds, **Figure 1B**) periods. This can be shown using a simple agent-based simulation where the agent moves at a constant speed and curvature based on samples from a gaussian distribution at fixed time intervals (**Figure 4B** and methods). The resultant spread of the flies in space increases as the interval between samples increases (**Figure 4B**). This means that two flies starting at the same position in space experiencing similar odor stimulus will have divergent positions and paths at the end of an instance of a locomotor state. In a spatially inhomogeneous odorant environment, this spatial dispersion in positions will have knock-on effects as the sensory experience of different flies diverge leading to greater variability in behavior.

In addition to locomotor kinematics, decisions to transition between walking, turning, and stopped states have been shown to be stochastic. How flies implement these decisions is dependent on the type of decision as well as the environment that the fly is locomoting in. For instance, flies implement stochastic sequential integration of odor plume encounters in transitioning from stops to walks and use the timing of odor encounters to modulate the transition from walks to stops (Demir et al., 2020). Furthermore, flies can bias their upwind turning based on the combination of the frequency and the intermittency of odor encounter (Álvarez-Salvado et al., 2018; Demir et al., 2020; Jayaram et al., 2022).

As the fly moves closer to the odor source, the frequency of odor encounters will increase. Effects discussed above will be further exacerbated as frequent odor encounters will drive history dependent ORN firing rate adaptation which creates a potential for greater variety in possible responses. Consider a temporally changing olfactory environment where the mean and variance of the stimulus is spatially conserved, if flies adopt a simple strategy of slowing down when not experiencing an odor plume, the mean in odor experience will increase (**Figure 4C** and methods). This increase in mean odor experience will depend on how much the fly decreases its speed. The gain in the ORN dose-response curve decreases with an increase in stimulus mean

and variance (Gorur-Shandilya et al., 2017). At the population level, the sensitivity to odorant concentrations follows a power-law distribution and this response sensitivity adapts to stimulus intensity (Si et al., 2019). This means that flies can experience vastly different sensory input based on both statistics of the odorant environment and how the fly chooses to locomote within the environment.

In addition to the effect of recent sensory experience in driving behavioral variability, the sensorimotor transformations also exhibit adaptations over the course of tens of seconds to minutes. In a static odor landscape, the timescale of this adaptation coincides with changes in the attraction index (Tao et al., 2022). This adaptation likely reflects a longer timescale change in the perception of the odor based on the motivation of the fly to continue the search for the odor. A recent study showed that there is a large variability in the distance flies traveled on food patches before deciding to give up (van Breugel, 2021). Using an agent-based model of variable decision making, the author showed that this variability may enhance the metabolic efficiency in finding the food source. In the MB, DANs modulate MBON neurons and induce plasticity of KC to MBON connections to cause changes in odor valence (Aso et al., 2014a). The output of MBONs makes many connections with the LH, which is thought to drive innate behaviors and different motor programs (Dolan et al., 2019). This suggests that the longer timescale adaptations in locomotion and valence can be driven by the MB. This process, which depends on each flies' experience and internal states may be a potential way to explain the variability in longer timescale odor valence and locomotion (Grunwald Kadow, 2019).

CONCLUSION

Behavioral variability is a central feature of natural behaviors. Odor-guided locomotion performed by *Drosophila* is a key model system to study principles and sources of behavioral variability. Traditionally, variability is commonly attributed to genetic and neuromodulatory factors. Indeed, even in isogenous populations, small amounts of genetic variability may cause variability in phenotype expression. Such a process may allow a population of animals to limit the risk of going extinct in an expectedly ever-changing environment. Meanwhile, neuromodulation allows animals to flexibly control their behaviors in response to their internal needs or wants. But beyond these factors, another less discussed source of variability arises from stochasticity of behavioral choices and their persistence. Over multiple rounds of decision, this source of variability will drive noticeable variability in attraction and spatial position across a population of flies.

The presence of persistent locomotion is a ubiquitous feature of locomotion ranging from sharks to *Drosophila* to humans (Reynolds and Frye, 2007; Humphries et al., 2010; Rhee et al., 2011). This feature is predicted to provide ethological benefits in many environments by multiple theoretical frameworks for animal search ranging from Lévy flights to infotaxis. For instance, the power-law distribution of trajectory persistence during Lévy walks, although controversial, is predicted to be optimal in environments with random and sparse odor sources (Viswanathan et al., 1999). Meanwhile, infotaxis predicts long persistent path trajectories far from an odor source that shorten in duration in regions with high odor information accumulation (Vergassola et al., 2007). While potentially suboptimal, the infotaxis framework allows animals to reliably locate an odor source (Loisy and Eloy, 2021). But beyond potential ethological benefits of long persistence trajectories, there is a growing source of literature that shows how these frameworks that generate long persistence trajectories can arise naturally from biomechanical mechanisms of locomotion and neural mechanisms of decision making (Calhoun et al., 2014; Reynolds, 2015, 2021; Abe Masato, 2020).

Meanwhile, the presence of noisy sensorimotor transformations can arise from a multitude of factors. First, genetic, biomechanical, metabolic, and history-dependent experiences can influence idiosyncratic differences in sensorimotor transformations. Second, internal and external behavioral states can influence locomotor transformations across sensory experience. Finally, there will be natural, uncontrollable variations in locomotor speed and curvature likely arising from motor noise or various sources of noise in the brain (Faisal et al., 2008). Even in highly practiced tasks such as arm reaching, small variations in neuronal activity in the premotor cortex of monkeys has been shown to drive trial-by-trial movement variability (Churchland et al., 2006). During odor-guided locomotion where the goal of the animal is not to control the kinematics of locomotion explicitly and precisely, these sources of noise in locomotor kinematics will be larger. But beyond the biological origins of movement variability, this variability can be ethologically beneficial as a lack of movement variability can result in rigid locomotor search patterns that limit the ability of an animal to effectively search for resources.

MATERIALS AND METHODS

Data Curation

Standard deviations (SD) reported in **Figure 1A** were obtained from the relevant articles through the raw data when available or through estimation of error bounds using WebPlotDigitizer (Rohatgi, 2021). As most studies report the standard error of the mean (SEM), the SD was calculated by multiplying the SEM by the square root of the reported sample size. For papers with box plots, WebPlotDigitizer was used to obtain the interquartile range. Below is a table of the relevant figures that error bounds were reported from, and the method used.

	Figure number	Method
Larsson et al., 2004	Figure 7	WebPlotDigitizer
Knaden et al., 2012	Figure 1	WebPlotDigitizer
Semmelhack and Wang, 2009	Figure 2	WebPlotDigitizer
Badel et al., 2016	Figure 1	WebPlotDigitizer
Jung et al., 2015	Figure 3	WebPlotDigitizer
Tao et al., 2020	Figure 1	Data
Honegger et al., 2020	Figure 1	Data

Agent Model of Sampling Noise Variability

The speed was sampled from a normal distribution with a mean of 5 mm/s and an SD of 0.5 mm/s. The curvature was sampled from a normal distribution with a mean of 60 degrees/s and an SD of 3 degrees/s. For each simulation the duration of a trajectory is fixed, and the sampling rate was set to 30 Hz. A 1,000 agents were initialized at the origin (x = 0 mm, y = 0 mm, and an orientation θ = 0 degrees). At the start of each trajectory, each agent selects from the speed and curvature distribution. The position of each agent was then updated as follows:

$$\theta(t) = \theta(t-1) + \frac{k(t-1)+k(t)}{2} \quad (1)$$

$$x(t) = x(t-1) + s(t) * \cos(\theta(t)) \quad (2)$$

$$y(t) = y(t-1) + s(t) * \sin(\theta(t)) \quad (3)$$

Where k is the sampled curvature and s is the sampled speed. After the agent has moved for the set duration, the agent initiates another trajectory by resampling from the speed and curvature distribution. This process repeats until a time of 5 s has passed.

The spread of agents at the end of the 5 s period can be approximated by a bivariate Gaussian distribution. These end positions were fit to a bivariate gaussian density function using MATLAB. The spread of this distribution was characterized by the generalized variance:

$$GV = det(\Sigma)$$

Where Σ is the covariance matrix.

Agent Model of Locomotion Induced Changes in Sensory Input

To simulate a dynamically changing environment with conserved stimulus properties, we first segmented the odor space into grids of 10 mm by 10 mm. The temporal pattern of odor stimulus in each grid is modeled as a square wave with a 20% duty cycle and variable frequency sampled from a gaussian distribution centered around 0.5 Hz with a standard deviation of 0.1 Hz.

A 5,000 agents were initialized at the origin (x = 0 mm, y = 0 mm, and an orientation θ = 0 degrees). Each agent is set to move in trajectories lasting 0.5 s. At the end of each trajectory, the agent update its speed based on its latest sensory experience. If the agent is in an odor plume (stimulus = 1) at the time of trajectory transition, the agent will initiate a trajectory with a speed of 10 mm/s (On stimulus speed) and a curvature of 60 degrees/s. If the agent is instead not in an odor plume (stimulus = 0), then the agent will initiate a trajectory with a speed slower than or equal to 10 mm/s (Off stimulus speed) and a curvature of 60 degrees/s. The direction of curvature is random (50/50 left vs. right). The position of each agent is updated as described in equations 1 to 3. For each agent, we calculated the mean in stimulus over 2 min. **Figure 4C2** shows the mean of the stimulus mean over all agents.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Precise Quantification of Behavioral Individuality From 80 Million Decisions Across 183,000 Flies

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Individual animals behave differently from each other. This variability is a component of personality and arises even when genetics and environment are held constant. Discovering the biological mechanisms underlying behavioral variability depends on efficiently measuring individual behavioral bias, a requirement that is facilitated by automated, high-throughput experiments. We compiled a large data set of individual locomotor behavior measures, acquired from over 183,000 fruit flies walking in Y-shaped mazes. With this data set we first conducted a "computational ethology natural history" study to quantify the distribution of individual behavioral biases with unprecedented precision and examine correlations between behavioral measures with high power. We discovered a slight, but highly significant, left-bias in spontaneous locomotor decision-making. We then used the data to evaluate standing hypotheses about biological mechanisms affecting behavioral variability, specifically: the neuromodulator serotonin and its precursor transporter, heterogametic sex, and temperature. We found a variety of significant effects associated with each of these mechanisms that were behavior-dependent. This indicates that the relationship between biological mechanisms and behavioral variability may be highly context dependent. Going forward, automation of behavioral experiments will likely be essential in teasing out the complex causality of individuality.

Keywords: handedness, fluctuating asymmetry, variability, high-throughput behavior, automation, ethology

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INTRODUCTION

Individual animals exhibit idiosyncratic behavior, even when their genetics and rearing environment are held constant. This variability is termed intragenotypic variability (Stamps et al., 2013) and likely arises in part due to stochastic effects during development (Vogt, 2015; Honegger and de Bivort, 2018), which, in a quantitative genetic framework, are classified as microenvironmental plasticity (Morgante et al., 2015). Intragenotypic variability in animal behavior is likely a major component of animal personality, an ecologically and evolutionarily important dimension of variation (Freund et al., 2013; Bierbach et al., 2017). A single genotype giving rise to a broad distribution of random phenotypes may constitute an adaptive evolutionary strategy, termed "bet-hedging," to increase the probability that for any fluctuation in the environment,

some individuals will be fit, increasing the odds that a lineage never goes extinct (Hopper, 1999). While bet-hedging has strong theoretical foundations, in the context of animal behavior it has limited evidence [but see Kain et al. (2015) and Akhund-Zade et al. (2020)]. A challenge in studying bet-hedging is that behavioral variability is difficult to measure; larger sample sizes are needed to precisely estimate the variance of a trait, compared to the mean. This is largely because the former requires sampling phenotypes in the tail of a distribution, which are rare by definition.

Increasing behavioral assay throughput via automation is an effective way to attain the sample sizes needed to study variability. This can be achieved through miniaturization and parallelization of imaging platforms in a lab context (Kain et al., 2012; Churgin et al., 2017; Pantoja et al., 2017; Stern et al., 2017; Barlow et al., 2021). While the up-scaling of experiments is easiest with small, lab-adapted animals, such approaches do work with species beyond the common genetic models (Crall et al., 2016, 2018; Bierbach et al., 2017; Ulrich et al., 2018). Gains in data throughput can be achieved with the help of robots that automate animal handling (Alisch et al., 2018), move cameras between arenas (Alisch et al., 2018; Crall et al., 2018) or track a single animal over long periods of time (Johnson et al., 2020). Automation of analysis is also essential, and innovations in animal centroid tracking (Panadeiro et al., 2021), body-part tracking using neural networks (Hausmann et al., 2021) and behavioral classification (Kabra et al., 2013; Berman et al., 2014; Todd et al., 2017) constitute a rich tool set for rapidly extracting behavioral measures from digital data sets.

High-throughput, automated behavioral assays have been used to investigate the variability of *Drosophila* behavior (Mollá-Albaladejo and Sánchez-Alcañiz, 2021; Mueller et al., 2021; Steymans et al., 2021; Werkhoven et al., 2021). The species' deep genetic toolkit facilitates the study of proximate mechanisms controlling variability such as neurotransmitters (Kain et al., 2012; Honegger et al., 2020), neural circuits (Buchanan et al., 2015; Skutt-Kakaria et al., 2019; Honegger et al., 2020; Linneweber et al., 2020), genes (Kain et al., 2012; Ayroles et al., 2015; Wu et al., 2018), environmental variation (Akhund-Zade et al., 2019), and social effects (Alisch et al., 2018; Versace et al., 2020). Of these studies, the three that have assayed the greatest number of individuals (Ayroles et al., 2015; Buchanan et al., 2015; Skutt-Kakaria et al., 2019) all employed a common behavioral assay: spontaneous locomotion in Y-shaped mazes. As flies walk freely in these arenas, they make a left-vs-right choice every time they cross through the center of the maze. Individual flies make hundreds of such choices per hour. This yields a large data set per individual, which in combination with a high throughput of individuals, makes this assay particularly amenable to the study of variability. Beyond the number of left-right choices made and their average handedness, the Y-maze assay also produces behavioral measures related to the higher-organization of turn sequences and their timing (Ayroles et al., 2015).

Individual left-vs-right turning bias is correlated with counterclockwise-vs-clockwise bias in open arenas (Buchanan et al., 2015) indicating that the behavioral measures in this assay are not entirely geometry-dependent. Humans may exhibit a comparable form of locomotor bias in the curvature of their trajectories when trying to walk straight without visual feedback (Souman et al., 2009). The left-right symmetry of this assay evokes the phenomenon of fluctuating asymmetry, in which individual variation in the extent of morphological asymmetry is used as a measure of developmental stability (Van Valen, 1962; Debat et al., 2011). Indeed, both left-vs-right turn bias in Y-mazes and morphological traits examined for fluctuating asymmetry tend to have average values (typically close to left-right symmetry) that are robust across genotypes and selection (Pélabon et al., 2006; Ayroles et al., 2015).

Here, we took advantage of the high precision and throughput of the Y-maze assay to characterize the distribution of individual behaviors and their variability along different experimental axes. We collected nearly all the data from Y-maze experiments conducted by lab members since this assay was devised in 2010. In descriptive analyses, we characterized the distribution of individual Y-maze behavioral measures, and their correlations, with unprecedented precision. In hypothesis-driven analyses, we examined the effects on variability of manipulations of serotonergic signaling, the gene *white* [previously shown to affect phototactic variability; Kain et al. (2012)], sex, and temperature. On the whole, these analyses reinforce the finding that genotype and the choice of behavioral measure itself have consistently large effects on measures of variability (Akhund-Zade et al., 2019), though some environmental manipulations can have large effects in a behavior-dependent fashion.

RESULTS

We collected experimental records from hundreds of experiments examining the Y-maze behavior of 183,496 individual flies (**Figure 1**). In total, these flies made 79.8 M left-right choices. Four behavioral measures were recorded for each fly (Ayroles et al., 2015): turn bias (percent of turns to the right), number of turns, and turn switchiness. The last is a measure of the degree to which flies alternate between left and right turns, normalized by their turn bias. A fly making exactly as many left (right) followed by right (left) turns as expected in a binomial model has a switchiness value of 1. Lower switchiness indicates fewer LR/RL turn sequences, and, conversely, longer streaks of L or R turns. The fourth measure, turn clumpiness, captures the non-uniformity of turn timing, i.e., the extent to which flies made choices in bursts. We changed the formula for the last measure midway through the data collection period [compare Buchanan et al. (2015) and Werkhoven et al. (2021)], making this measure hard to compare across experiments; therefore we excluded it from further analysis. In addition to behavioral data, the record for each fly also included metadata about the experimental circumstances, including (**Table 1**): the fly's genotype, experimental conditions, temperature during behavior, age of the fly, the experimenter who recorded the

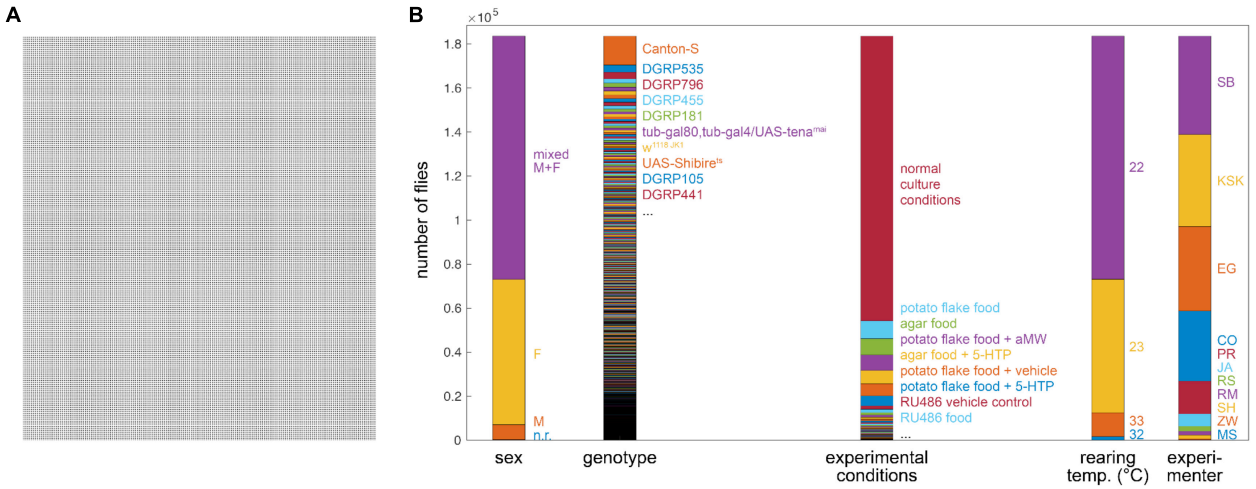

FIGURE 1 | Depiction of grand Y-maze data set (n = 183,496)—**(A)** Visualization of 183,496 flies (each dot is a fly). **(B)** Breakdown of flies into important metadata categories. Height of each color segment indicates the number of flies with that metadata value. Bars align to panel **(A)**.

behavioral data, the ID# of the array of arenas ("tray") in which it behaved, the ID# of the imaging box in which it behaved, the date, the number of arenas in its tray, the software used to record its behavior, the software used to produce its behavior measures, and its sex. The proportions of all flies for five of these metadata categories are shown in **Figure 1B**.

The size of our data set allows some of the most precise estimation of behavioral distributions across individuals to-date. We computed kernel density estimates of the distributions of turn bias, number of turns and turn switchiness (**Figures 2A,D,G**). The distributions of all measures are essentially unimodal, with the distribution of handedness appearing roughly Gaussian (**Figure 2A**). However, it deviates from that distribution in a number of ways: it is denser at its mode and in tails corresponding to strong turning biases around 0.1 and 0.9. This is reflected in a kurtosis greater than three (**Figure 2B**; see below). The empirical distribution of handedness is technically trimodal, with small peaks corresponding to flies with biases very close to 0 and 1. Most flies in these peaks performed fewer than 50 turns, indicating that these peaks may be the consequence of undersampling within these individuals.

To assess the precision of measures quantifying these distributions we looked at the distribution of estimates (under bootstrapping) of the mean, standard deviation, skewness and kurtosis of the behavioral distributions (**Figures 2B,E,H**). These were generally quite narrow, indicating precise estimation, and generally broader for the higher-order statistics. This was expected as the higher-order statistics have exponential terms that render them more sensitive to sampling error. But their precision did not always decrease monotonically (**Figure 2H**). To extend this analysis, we computed the standardized moments of each distribution, up to the 20th moment, for each behavioral measure (**Figures 2C,F,I**). To our surprise, the data provided robust estimates even of the 20th moment of turn bias and turn switchiness. This was true even in 10-fold subsamples of the turn switchiness data, but was not the case for number of turns (**Figure 2F**) or odd moments of the turn bias data (**Figure 2C**). This indicates that the reliability of estimates of high-order distribution statistics depends on the underlying distribution, not just the sample size.

In our studies of turn bias in Y-mazes (Ayroles et al., 2015; Buchanan et al., 2015; Akhund-Zade et al., 2019; Werkhoven et al., 2021), we operated under the assumption that the mean turn bias was 0.5 in all genotypes. For example, this assumption was the basis of a decision to not model the interaction of genetic variation for the mean and variability of turn bias in Ayroles et al. (2015). On close examination of this measure in our new data set, we found evidence that the mean turn bias may not be 0.5 (**Figure 3**). The mean of turn bias in the grand data set was 0.496 (**Figure 3A**), indicating a slight left bias to Y-maze turn choices. This slight left bias was also present in the distribution of genotype, sex and experimenter (**Figures 3B–D**) mean turn biases, suggesting that the apparent left bias in the grand mean is not likely attributable to imbalance among the metadata covariates. Indeed, a linear model with 11 meta variables as predictors (all but date, which renders the model rank deficient) and 636 coefficients has a turn bias intercept of 0.485 (SE 0.0099). The apparent effect of experimenter (**Figure 3D**) was not strongly seen in the above model (lowest p-value = 0.04 across 10 experimenters; nor in a model with only genotype and experimental condition as the other predictors: lowest p-value = 0.11). In contrast, 47/569 genotypes have significant effects ($p < 0.05$) in a linear model where genotype is the sole predictor of turn bias (**Figure 3E**). This is a significant enrichment, and supports the conclusion that the average turn bias is under biological control.

Since our behavioral data was multidimensional (turn bias, number of turns and turn switchiness were measured for each fly), we were also able to investigate the joint distributions and correlations of these measures. We first tested whether there

TABLE 1 | Y-maze data set variables.

Data variable name	Notes
flyID	Number linking this fly's data to other digital records
handedness	Turn bias behavioral measure
numTurns	Number of turns behavioral measure
switchiness	Turn switchiness behavioral measure
lev_handedness	Levene-transformed turn bias, for linear modeling of variability in turn bias
lev_numTurns	Levene-transformed number of turns, for linear modeling of variability in number of turns
lev_switchiness	Levene-transformed turn switchiness, for linear modeling of variability in turn switchiness
genotype	String indicating the genotype of fly
expCond	String indicating the experimental conditions
5htpagar	Flies reared on agar media supplemented with 10 mM 5-HTP
5htpagar25	Flies reared on agar media supplemented with 25 mM 5-HTP
5htpagar50	Flies reared on agar media supplemented with 50 mM 5-HTP
5htpnormal	Flies reared on cornmeal-dextrose media supplemented with 10 mM 5-HTP
5htppotato10	Flies reared on potato media supplemented with 10 mM 5-HTP
5htppotato25	Flies reared on potato media supplemented with 25 mM 5-HTP
5htppotato50	Flies reared on potato media supplemented with 50 mM 5-HTP
aMWnormal	Flies reared on cornmeal-dextrose media supplemented with 10 mM aMW
agar	Flies reared on control agar media
amwagar	Flies reared on control agar media supplemented with 15 mg/mL ascorbic acid
amwpotato10	Flies reared on potato media supplemented with 10 mM aMW
amwpotato20	Flies reared on potato media supplemented with 20 mM aMW
amwpotato25	Flies reared on potato media supplemented with 25 mM aMW
amwpotato50	Flies reared on potato media supplemented with 50 mM aMW
ctrlaanormal	Flies reared on control potato media
ctrlaapotato	Flies reared on control potato media supplemented with 15 mg/mL ascorbic acid
d10gal80heatshock	Flies subjected to heat-shock at day 10 of development (Ayroles et al., 2015)
d14gal80heatshock	Flies subjected to heat-shock at day 14 of development (Ayroles et al., 2015)
d1gal80heatshock	Flies subjected to heat-shock at day 1 of development (Ayroles et al., 2015)
d3gal80heatshock	Flies subjected to heat-shock at day 3 of development (Ayroles et al., 2015)
d4gal80heatshock	Flies subjected to heat-shock at day 4 of development (Ayroles et al., 2015)
d5gal80heatshock	Flies subjected to heat-shock at day 5 of development (Ayroles et al., 2015)
d6gal80heatshock	Flies subjected to heat-shock at day 6 of development (Ayroles et al., 2015)
d7gal80heatshock	Flies subjected to heat-shock at day 7 of development (Ayroles et al., 2015)
d8gal80heatshock	Flies subjected to heat-shock at day 8 of development (Ayroles et al., 2015)
d9gal80heatshock	Flies subjected to heat-shock at day 9 of development (Ayroles et al., 2015)
darkreared	Flies reared in darkness
gal80heatshock	Flies subjected to heat-shock post-eclosion, prior to behavioral assay
grownat18	Flies reared in incubators at 18°C
grownat20	Flies reared in incubators at 20°C
grownat23	Flies reared in incubators at 23°C
grownat25	Flies reared in incubators at 25°C
grownat30	Flies reared in incubators at 30°C
heritability	Flies are the progeny of single parents selected for turn biases (Buchanan et al., 2015)
intenseenrichment	Flies reared in high intensity enrichment population cage (Akhund-Zade et al., 2019)
irtest	Fly behavior was measured using infrared rather than white illumination
mildenrichment	Flies reared in mild intensity enrichment vials (Akhund-Zade et al., 2019)
normal	Standard rearing conditions
potato	Flies reared on potato media
ru486	Flies reared on media supplemented with ru486
ru486control	Flies reared on ru486 control media
single	Flies reared in single housing
expTemp	Temperature during behavior acquisition (°C)
age	Middle of range of ages post-eclosion of fly in that experimental group. E.g., age = 6 typically reflects experimental flies ranging from 4 to 8 days old
experimenterID	Name of experimenter who collected the behavioral data
trayID	Identifying # of the arena array tray in which the fly behaved
boxID	Identifying # of the imaging box in which the fly behaved
date	String encoding the date of the behavioral experiment
arrayFormat	The number of mazes imaged per tray
mazeNum	ID number of the maze the fly occupied within its tray

(Continued)

TABLE 1 | (Continued)

Data variable name	Notes
acquisition	Software used to collect that fly's behavioral data
Ymaze31	Custom LabView software http://lab.debivort.org/neuronal-control-of-locomotor-handedness/y%20maze%20v3.1.vi
roitracker	Custom LabView software similar to above
autotracker	Custom MATLAB software, eventually published as MARGO
autotrackerv2	Custom MATLAB software, eventually published as MARGO
margo	Custom MATLAB software: http://lab.debivort.org/MARGO/
analysis	Software used to compute that fly's behavioral measures
sex	Fly's sex. "Both" indicates that both males and females were used in this experimental group, in unspecified proportion
eyeColor	State of the *white* genetic locus. See **Figure 5**. + indicates wild type, − null, and m mini-white alleles

might be a correlation between turn bias and number of turns, specifically a negative correlation arising from higher sampling error in estimating turn bias for flies making fewer turns. Counter to this prediction, we observed a slight positive correlation ($r = 0.036$; $p = 4^{*}10^{-52}$). Incidentally, we noticed the effects of the discreteness of number of turns as a measure, and the resulting limited values that turn bias can take on, as a fractal-like (Trifonov et al., 2011) structure in the scatter plot of absolute turn bias vs. number of turns (**Figure 4A**).

Next, we examined the joint distribution of turn switchiness and number of turns (**Figure 4B**). This two-dimensional distribution had two conspicuous features: an uncorrelated mode containing the vast majority of the flies, and a smaller mode exhibiting a negative linear relationship between turn switchiness and number of turns. The flies in this second mode were nearly all reared on potato flake media [which was sometimes supplemented with drugs targeting the neurotransmitter serotonin; Dierick and Greenspan (2007), Kain et al. (2012), and Krams et al. (2021)]. Of these flies, approximately 296 flies were reared on media including the serotonin inhibitor aMW, 429 were reared on the serotonin precursor 5-HTP, and 942 were reared on control media. Notably, being reared on potato food was not a guarantee that a fly fell in this part of the distribution; the vast majority of flies in such rearing conditions fell in the predominant uncorrelated mode of the joint distribution along with flies fed on standard cornmeal-dextrose media.

Finally, we used the Y-maze data set to revisit several previously examined hypotheses about the proximate mechanisms regulating behavioral variability. We first asked whether the distribution of measures of turn bias variability across genotypes was consistent between the distribution seen in Ayroles et al. (2015) and the other genotypes present in our data set. The lines examined in that paper come from the Drosophila Genome Reference Panel [DGRP; Mackay et al. (2012)], a collection of inbred lines established from the natural population of flies in Raleigh, NC USA. The remaining 339 genotypes in our data set come from a variety of sources, mostly lab stocks, and include 165 lines expressing the transgenic driver Gal4 (Brand and Perrimon, 1993) in neural circuit elements (Jennett et al., 2012). Thus, these genotypes do not represent a sample from a natural population. The distribution of their genotype-wise variability in turn bias was largely similar to that observed in DGRP lines (**Figure 5A**), with genotypes exhibiting coefficients of variation in handedness ranging from less than 0.2 to more than 0.4.

Neuromodulation may have a special role in the control of behavioral variability (Maloney, 2021), e.g., phototaxis (Kain et al., 2012; Krams et al., 2021) and olfactory preference (Honegger et al., 2020). We conducted experiments to see if serotonin modulation controls variability of locomotor behaviors in the Y-maze. Specifically, we measured the variability of turn bias, number of turns and turn switchiness in DGRP lines which were treated with alpha-MW (a serotonin synthesis inhibitor), 5-HTP (a biosynthetic precursor of serotonin) (Dierick and Greenspan, 2007) or their respective control media. These treatments generally had small effects on behavioral variability across genotypes (ranging from a −10% to a 7% increase), with the exception of the effect of 5-HTP on variability in the number turns, which, in two versions of the experiment increased variability by 16 and 25% (**Figure 5B**). Overall, these results imply that although serotonin levels can affect the variability of turn number, there is not a strong effect that is consistent across behaviors.

We previously determined that the effect of serotonin on phototactic variability was dependent on the gene *white*, which encodes a transmembrane transporter that imports the serotonin precursor tryptophan into neurons. We scored the flies in our Y-maze data set for their white genotype, which could range from wild type to homozygous null, with intermediate conditions of (likely) partial rescue by the expression of the "mini-white" allele at non-endogenous transgenic insertion sites (Klemenz et al., 1987). Lines with homozygous null alleles at the endogenous white locus exhibited higher variability in number of turns, with the exception of lines that were also heterozygous for mini-white at a transgenic locus. The molecular function of White suggests that its disruption should produce a behavioral phenotype like serotonin synthesis inhibition, which had no effect in our pharmacological manipulations (whereas feeding flies serotonin precursor increased variability, like *white* disruption). *White* genetic disruption was associated with small reductions in variability in turn bias and turn switchiness (**Figure 5C**), consistent with the small decreases seen in the aMW

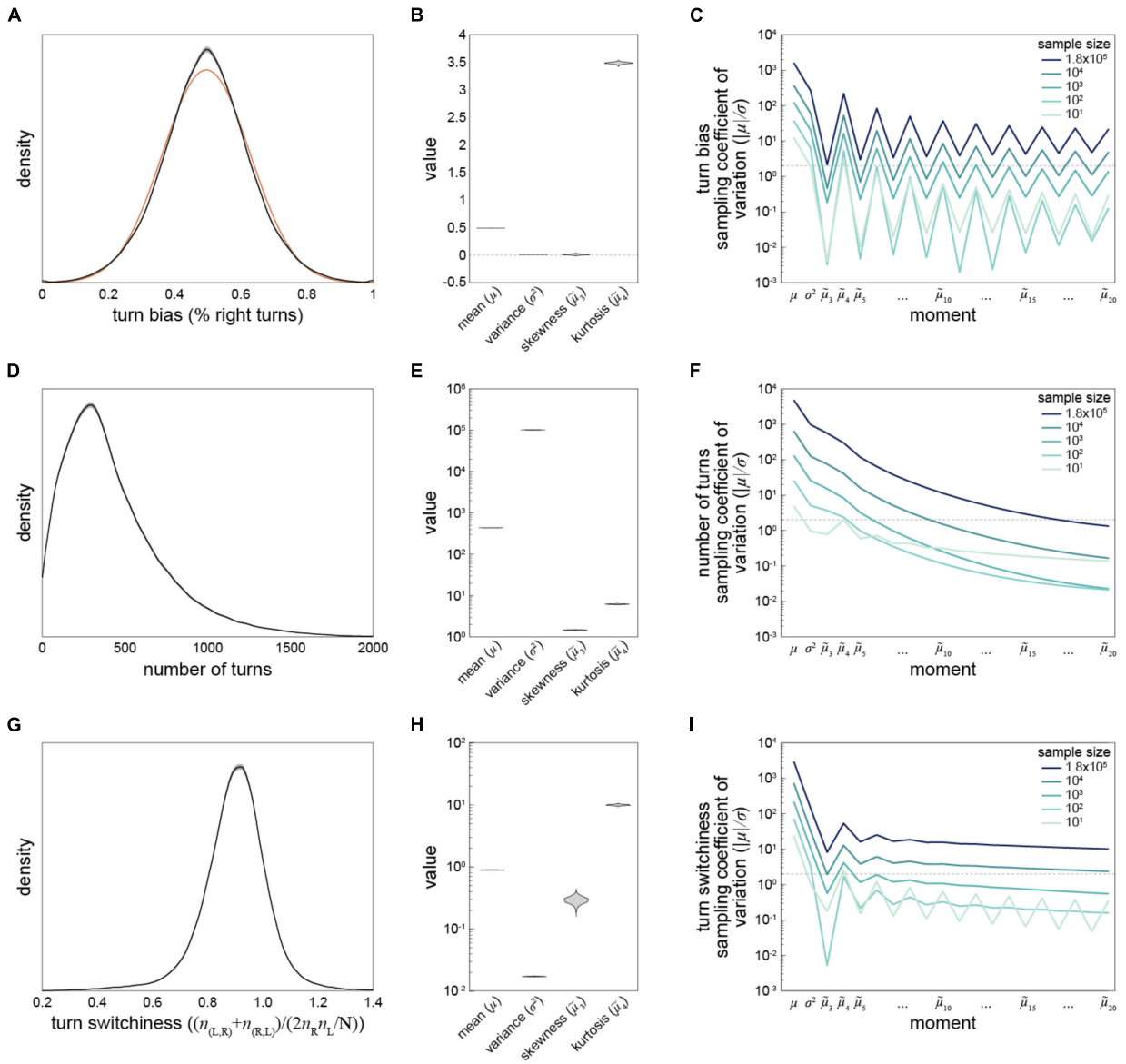

FIGURE 2 | Estimation of statistics describing three Y-maze behavioral measures—**(A)** Kernel density estimate of the distribution of turn bias across all flies in the data set. Gray interval is the 95% CI as estimated by bootstrap resampling. Orange line is the Gaussian distribution that best fits the data. **(B)** Violin plot of estimation distributions of four statistical moments describing the distribution of turn bias. Each violin is a kernel density estimate of the distribution of each statistic's value across bootstrap samples from 1,000 replicates. **(C)** Average bootstrap estimate of the mean, variance, and subsequent 18 standardized moments of the distribution of turn bias, as a function of the size of the data set. Darkest line corresponds to the complete grand Y-maze data set, and lighter lines random subsets. Dotted line at $|\mu|/\sigma = 2$ indicates the threshold for moment estimate significantly different from 0 at $p = 0.05$. **(D–F)** As in panels **(A–C)** for number of turns as the behavioral measure. **(G–I)** As in panels **(A–C)** for turn switchiness as the behavioral measure. Note log y-axes in panels **(C,E,F,H,I)**. Data from all 183,496 flies were used in these analyses.

pharmacological experiments (**Figure 4B**). Overall, we found some agreement in the effects of serotonin pharmacological experiments and *white* disruption, but not perfect agreement, suggestive of behavior-dependent complexity in the relationship between *white*, serotonin, and variability.

It has been hypothesized that individuals of the heterogametic sex will exhibit greater trait variability due to noise in gene compensation (James, 1973), though a recent meta-analysis found no significant sex-bias in the variances of 218 mouse traits (Zajitschek et al., 2020). We fit linear models to Levene-transformed turn bias, number of turns, and turn switchiness data, with genotype and sex as predictors, to test for the effect of sex on behavioral variability. Males had variability that was −6.8% ($p < 0.001$), 7.5% ($p < 0.001$), and 1.8% (n.s.) greater than that of females in turn bias, number of turns, and turn switchiness respectively.

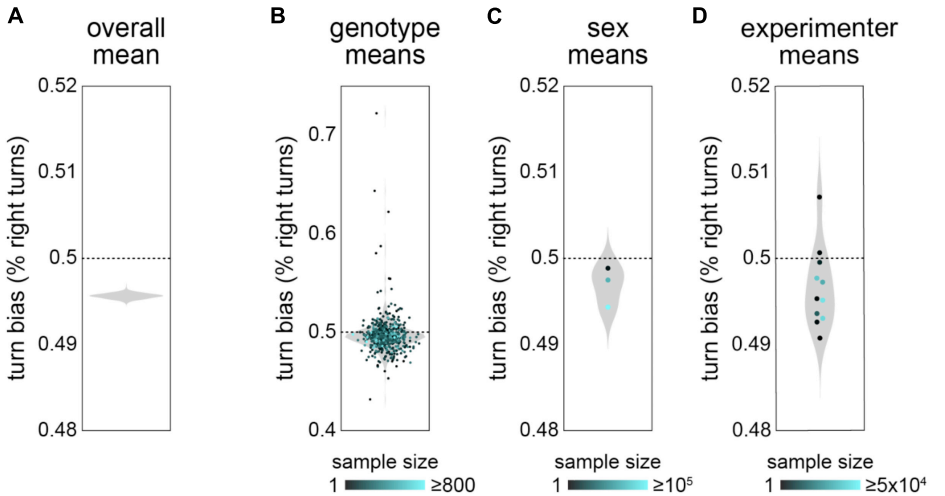

FIGURE 3 | Mean turn bias appears slightly asymmetrical—**(A)** Violin plot of estimate distribution for the mean of turn bias across the grand data set, exhibiting an apparent slight left-bias of 49.6%. Violin is a kernel density estimate (KDE) of this statistic from 1,000 bootstrap replicates. **(B)** Mean turn bias for each genotype (points). Violin is the KDE of genotype means. Point color indicates the number of flies recorded for that genotype. **(C)** As in panel **(B)**, but with flies grouped by sex. The three points correspond, from top to bottom, to males only, females only and mixed sex. **(D)** As in panel **(B)**, but with flies grouped by experimenter. Note: the groups with the highest apparent right-bias have low sample sizes. **(E)** Histogram of p-values from a linear model with each genotype as a predictor. Brown bars represent effects significant at $p < 0.05$. Dotted line indicates the expected distribution under the null model. Data from all 183,496 flies were used in these analyses.

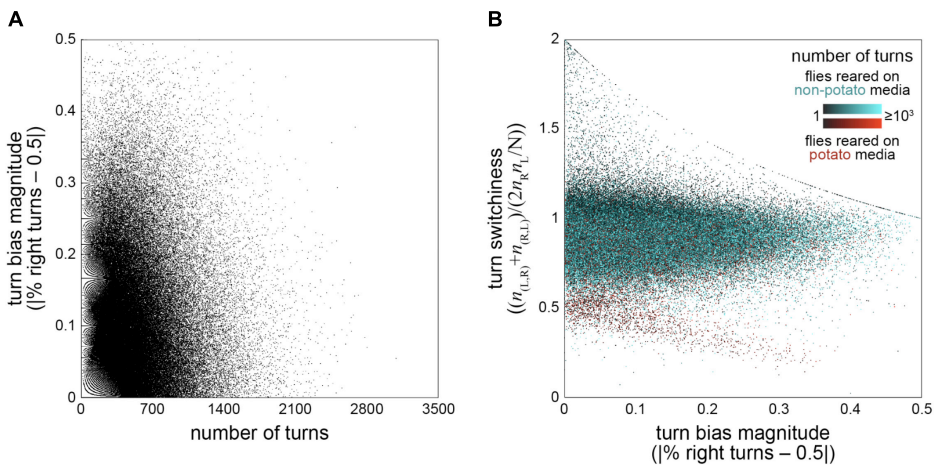

FIGURE 4 | Correlations between behavior measures—**(A)** Turn bias magnitude vs. number of turns. Each point is a fly (n = 183,496). Fractal-like pattern at left is a consequence of the limited turn bias values that are possible for a given discrete number of turns. r = 0.0357, $p < 10^{-50}$. **(B)** Turn switchiness vs. turn bias magnitude. Each point is a fly and colored on a scale depending on whether the flies were reared on cornmeal-dextrose agar media (black-cyan; n = 157,321) or F4-24 potato flake media (black-red; n = 26,175). Point color value indicates sample size, with dark flies making fewer turns. Curvilinear features are a consequence of limited switchiness values possible for a given turn bias magnitude, a constraint that arises most obviously in flies making fewer turns (dark points).

Lastly, we examined the effect of temperature during behavioral testing, with the hypothesis that flies would exhibit higher variability at high temperature (32–33°C) than at room temperature (22–23°C). This would be consistent with a mechanism in which heat pushes neural circuits out of the range in which physiological buffering keeps circuits operating similarly despite latent developmental and genetic variability (Tang et al., 2012; Rinberg et al., 2013). We examined this specifically for genotypes that had paired experiments at low and high temperature, and did not express any temperature-sensitive effectors. We found that high temperature had no effect on turn bias variability, but significantly decreased number of turns variability and turn switchiness variability by 37 and 32% respectively (**Figure 5D**). Temperature does affect the mean number of turns, typically increasing it by making flies more active. Our analysis controlled for this by assessing mean-normalized variability (the coefficient of variation: μ/σ). Overall, our analyses of the effects of potential proximate mechanisms controlling variability revealed a complex picture with (often small) effects of serotonergic regulation, white genotype, sex and

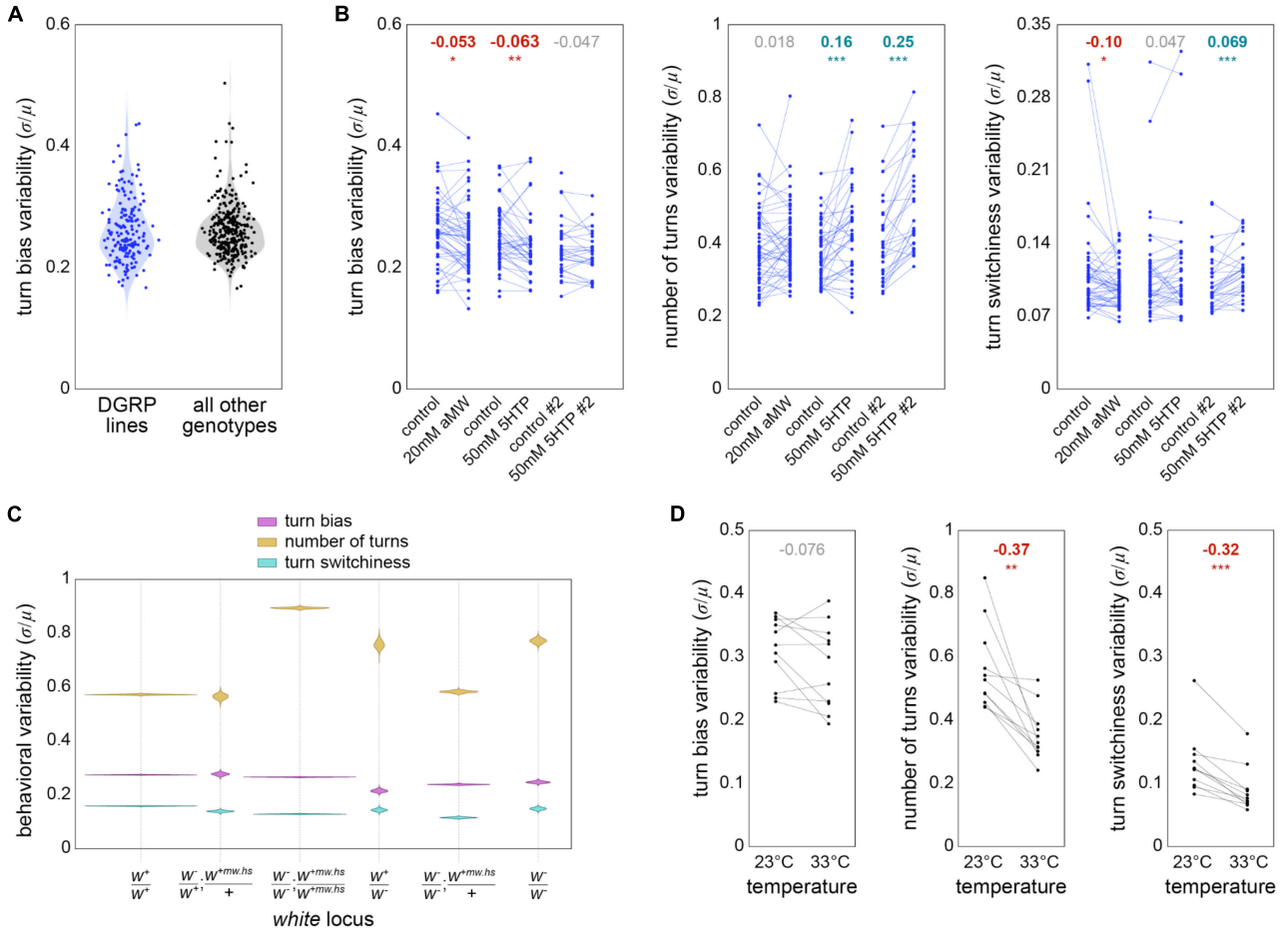

FIGURE 5 | Factors potentially affecting behavioral variability—**(A)** Variability (measured as the coefficient of variation) of turn bias for DGRP genotypes (blue; n = 223 genotypes comprising 64,838 flies) and non-DGRP genotypes (black; n = 3466 genotypes comprising 118,658 flies). Violins are the KDE of genotype variabilities (points). **(B)** Variability of turn bias (left), number of turns (middle), and turn switchiness (right) for DGRP genotypes in six pharmacological experimental conditions targeting serotonin. Each point is a genotype in a particular experimental condition. Lines pair genotypes across a drug medium and its associated control medium. Numbers at top indicate the effect size from control to drug treatment. Bold effect sizes are statistically significant and colored by the direction of their effect (red = lower variability; cyan = higher). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. n = 157 genotypes comprising 38,316 flies. **(C)** Violin plot of estimation distributions for the variability of turn bias (magenta), number of turns (gold) and turn switchiness (turquoise) vs. genotype of the white gene.+ indicates wild type, + *mw.hs* the "mini-white" allele typically used to mark a transgenic insertion, and - a null allele [typically w^{1118}; Hazelrigg et al. (1984)]. *white* genotypes are ranked in estimated order of expression disruption. The site of $w^{+mw.hs}$ insertion varied by line; the semi-colon notation in the panel label indicates that this site might be on a different chromosome than the endogenous *w* locus. n = 85,551, 1,863, 75,866, 1,484, 14,888 and 3,844 flies, respectively. **(D)** Variability of turn bias (left), number of turns (middle) and turn switchiness (right) for genotypes tested at 23 and 33°C. Lines pair genotypes across temperature conditions. n = 11 genotypes comprising 10,060 flies. Effect sizes and significances indicated as in panel **(B)**.

temperature. For all of these manipulations, the direction of effect on variability was behavior-dependent.

DISCUSSION

We gathered Y-maze data collected by lab members back to the origination of this assay 11 years ago. This large data set comprised the behavioral measures of over 180,000 individual flies that made a total of nearly 80 million left-right choices. With it, we were able to estimate the distribution of three measures of individual behavior with unprecedented precision, even out to the 20th standardized statistical moment (**Figure 2**). In exploratory analyses, we noticed two surprising patterns: (1) a discrete change in the relationship between turn bias magnitude and turn switchiness in a subset of animals that had been reared on potato flake media used for pharmacological experiments, and (2) that flies appear to have a slight left bias in their Y-maze choices. Finally, we used our data set to test several hypotheses pertaining to proximate control of variability in behavior, finding significant behavior-dependent effects of drugs targeting serotonin, mutation of the *white* gene (which encodes a channel that imports serotonin precursor), sex and temperature. Compared to the effects of genotype and the choice of behavior measure, the effects of these manipulations were generally small and context-dependent, underscoring the

complexity of relationships between axes of biological regulation and behavioral variability.

Admittedly, a motivation for this study was the desire to explore a very large data set reflecting the work over many years of many lab colleagues. In that spirit, it is fun to think about how throughput might be expanded another order of magnitude in the coming years. One possibility is robotic fly-handling (Alisch et al., 2018), which has yet to be deployed at scale in support of a large screen. Another possibility is tracking flies using capacitive sensors (Itskov et al., 2014) instead of with cameras. This would remove the need for long optical axes that force our behavior boxes to be tall, allowing a dense, vertical packing of arenas within a minimal bench footprint.

While increasing throughput through further automation is an appealing possibility, and perhaps essential for certain classes of experiments (like experimental selection for variability, which would require testing thousands of individual flies per generation for a year or more), it is not without conceptual consequences. One of these is how to assess small effects that are extremely statistically significant due to large sample sizes. Two examples from this study are the apparent slight left turn bias (**Figure 3**) and the significant positive correlation between turn bias magnitude and number of turns (**Figure 4A**). A turn bias of 0.496 compared to an expected value of 0.5 is indeed a small discrepancy, but it might nevertheless be biologically significant given the consistent failure of artificial selection experiments to evolve directional asymmetry in a variety of fly morphological characters (Carter et al., 2009). Another aspect of working with large data sets is that sampling error is likely to be small compared to inadvertent biases in the data [Meng, 2018; see Bradley et al. (2021) for an important example]. I.e., accuracy is unlikely to improve with further observations, but instead with the harder work of addressing systematic miscalibration, misunderstandings of what is being measured, or structure in the data leading to effects like Simpson's paradox. A way forward among these challenges may be to conduct experiments and analyses under a variety of biological conditions, increasing the odds that inferences generalize across contexts (Voelkl et al., 2020), an approach that would also be boosted by throughput and automation.

With caveats of big data in mind, we want to consider possible errors that might explain the apparent slight (but highly significant) left mean turn bias. All experimenters who conducted these experiments are right-handed. It is formally possible that chiral manipulation during the experimental set-up imparted a slight chirality to turning in the Y-maze, though we cannot think of a convincing mechanism by which this would happen. We also cannot think of mechanisms by which small, inevitable asymmetries in our behavioral rigs would impart a consistent left bias to behaviors measured across several generations of rigs and tracking software versions. Arguments in favor of the apparent left turn bias being real are previous reports of small mean asymmetries in wing size and shape (Klingenberg et al., 1998), possible indirect effects of conspicuously asymmetrical anatomical features like the gut, or the contribution of the Asymmetric Body, a small neuropil abutting the premotor Central Complex that is consistently larger in the right hemisphere (Wolff and Rubin, 2018).

While we found that our data set allowed the precise estimation of the distribution of individual behavioral scores, we also saw that the stability of higher-order moment estimates depended strongly on the behavioral distribution in question (**Figure 2**). Thus, there is not necessarily a simple rule for how large a sample is needed to estimate higher order statistics of its distribution. In the joint distribution of turn bias magnitude and turn switchiness, we observed two distinct modes between these measures, and, to our surprise, found that most of the points falling in the rarer mode came from experiments where flies were reared on potato flake food (**Figure 4B**). These flies comprised a relatively small subset of multiple experiments, in both control and drug conditions, from many genotypes. Thus, rearing on potato media is the best explanatory variable we could find for this mode of variation. We previously observed that acutely switching flies from cornmeal-dextrose media to potato media increased their variability in odor preference (Honegger et al., 2020). Perhaps this perturbation also alters the correlation structure (Lea et al., 2019), in a subset of flies, between turn bias and turn switchiness. Since these measures may relate to the paths animals take through natural environments, a food-dependent change in turning might alter foraging statistics, perhaps adaptively.

Finally, we used this large data set to examine hypotheses about proximate mechanisms controlling variability. We found many significant effects, such as 5-HTP or disruption of the *white* locus increasing variability in number of turns, disruption of white decreasing variability of turn bias and turn switchiness, males exhibiting slightly lower variability in turn bias but higher variability in number of turns, and conducting experiments at high temperatures lowering variability in number of turns and turn switchiness (**Figure 5**). We expected temperature to increase variability per results in the crab stomatogastric ganglion (Tang et al., 2012; Rinberg et al., 2013), but our high temperature experiments did not push the flies to their critical thermal limits (Kellermann et al., 2012). Thus, perhaps even higher temperature manipulations might result in consistent increases in variability across behaviors.

Our variability results indicate a complex, behavior-dependent relationship between many biological mechanisms and behavioral variability, which likely parallels the complexity of mechanisms controlling the means of behavioral traits. Experimental automation, and the high throughput it permits, made these and other findings on behavioral individuality feasible. However, individual projects drawing on tens of thousands of flies have already identified genetic (Ayroles et al., 2015) and neural circuit (Buchanan et al., 2015) regulators of variability as well as complex gene x environment x behavior interactions affecting variability (Akhund-Zade et al., 2019; Versace et al., 2020). Inferences that were uniquely possible with data from hundreds of thousands of flies include the slight left-bias in turning and precise estimation of high statistical moments of behavioral distributions. The enduring scientific value of such results remains to be seen. Regardless, further automation of behavioral assays will speed up both large and

small scale projects and, more importantly, liberate researchers from mindless, repetitive behavioral assays.

MATERIALS AND METHODS

Data and Analysis Code

All behavioral measures and metadata values, along with the code underlying analyses are available at http://lab.debivort.org/precise-quantification-of-behavioral-individuality/ and https://zenodo.org/record/5784716.

Assays Over Time

Since the locomotor handedness Y-maze assay was developed, there have been several changes to the experimental protocol. While we are confident that the data collected through these iterations are comparable, these changes potentially represent confounding variables for the grand analysis presented here. The structure of each fly's assay is represented in our raw data table by several entries (see **Table 1** for definitions): expTemp, trayID, boxID, arrayFormat, acquisition, and analysis. We found no significant effects of these variables on mean the means or variabilities of the behavioral measures analyzed in this study.

Typical Fly Handling

Unless otherwise indicated (via the expCond variable), the default culture conditions were cornmeal-dextrose media containing tegosept (Lewis, 1960) and incubation on the bench or in incubators at 21–25°C with 12/12 h light cycles. Our source of media was Scientiis, LLC (Baltimore, MD, United States), product ID: BuzzGro, until 2013, at which point we switched to media produced by the Harvard Fly Food core facility. The recipes are nominally the same between these sources. Flies were generally anesthetized under CO_2 to load them into y-mazes, though a small portion of flies were anesthetized by ice or loaded without anesthetization. Flies were given a period of 15–30 min of acclimation to the Y-mazes after loading before data collection began.

Pharmacological Experiments

Experimental flies receiving drug treatments were reared from egg-laying in drug-supplemented media (or control media). Drug media are indicated in the expCond metadata variable (see **Table 1**). To supplement media, drug was added to distilled, deionized water, which was then added to dry potato flake media, or drug was added directly to agar media liquified momentarily in a microwave oven. To attain the final concentrations of aMW, the following concentrations were used per media vial: 10 mM = 131 mg/60 mL; 25 mM = 327 mg/60 mL; 50 mM = 655 mg/60 mL. For 5-HTP, the following concentrations were used: 10 mM = 10.1 mg/60 mL; 25 mM = 330 mg/60 mL; 50 mM = 661 mg/60 mL. Fifteen milligrams ascorbic acid was added to each 60 mL media vial as an anti-oxidant in 5-HTP treated groups and their controls. The two 5-HTP experiments presented in **Figure 5** were conducted on potato media and cornmeal-dextrose media (#2) but are otherwise identical. To control for the average dose of experimental flies, prior to drug experiments we measured the average number of progeny to eclose following a 24 h parental egg-laying session, on cornmeal-dextrose media, for each of the DGRP lines (Akhund-Zade et al., 2020). The number of parental animals for drug experiments was adjusted proportionally, line-by-line, to target an identical number of progeny on the drug media for each line.

Behavioral Assay

Data was collected in Y-shaped mazes arrayed in trays (Buchanan et al., 2015; Alisch et al., 2018; Werkhoven et al., 2019) and imaged in enclosed behavioral boxes (Werkhoven et al., 2019) under diffuse white LED illumination typically provided by custom LED boards (Knema LLC, Shreveport, LA, United States). The number of Y-mazes per tray varied, as indicated by the arrrayFormat variable. Individual Y-mazes had 3-fold rotational symmetry, and ended in circular "cul-de-sacs" where the fly could turn around before making a subsequent choice. Trays were fabricated from three layers of acrylic, making up the floor (clear), walls (black) and a lid-holding layer (black). The surface of the floor layer was roughened to encourage flies to walk on it, using a random orbital sander and 200 grit sand paper until 2013 and a sand-blaster thereafter. Lids over each maze were cut from clear acrylic. All acrylic parts were cut to shape by a laser engraver. Schematics for trays and imaging boxes are available at https://github.com/de-Bivort-Lab/dblab-schematics/tree/master/Ymaze. Trays were imaged in opaque enclosures constructed from aluminum extrusion and laser-cut acrylic panels (https://github.com/de-Bivort-Lab/dblab-schematics/tree/master/Behavioral%20Box). A variety of USB digital cameras (often made by PointGrey) with resolution exceeding 1 MP were used to capture video of behaving flies for real-time tracking at 6–30 Hz. The default assay length was 2 h. Fly centroids were computed in real time using background subtraction implemented in a variety of custom software environments coded in LabView or MATLAB. The centroid tracking software used in recent experiments was MARGO (Werkhoven et al., 2019).

Statistics and Analysis

Analysis was conducted in MATLAB 2017b (The Mathworks, Natick, MA, United States) using custom functions. 95% confidence intervals estimated by bootstrapping were estimated as $\pm$ twice the standard deviation of values across bootstrap replicates. For the analysis of the effect of temperature on variability (**Figure 5D**), the 23°C groups include experiments conducted at 22°C and the 33°C groups include experiments conducted at 32°C. Genotypes were only included in the temperature analysis if they had data recorded at both temperatures and did not express any thermogenetic constructs. Thus, most genotypes in this analysis were controls for thermogenetic experiments or wild type lines. Significance in the serotonin pharmacological and temperature experiments was assessed by paired t-tests, and all reported p-values are nominal.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

BB oversaw the project and conducted data analysis. All authors collected the data and edited the manuscript.

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