Analyzing the Developmental Effect of AKH Suppression on Drosophila Memory

Grant Proposal

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Abstract (RQ)

Gonadotropin-releasing hormone agonists (GnRH-a) are a widely prescribed drug, yet concerns remain regarding their potential long-term cognitive effects when administer during critical periods of brain development. Although short-term cognitive impairments associated with GnRH-a exposure have been reported, the extent to which hormonal suppression during development produces persistent changes in learning and memory circuits remains unknown. This knowledge gap limits the understanding of how hormonal interventions may influence brain maturation.

To investigate the effects of developmental hormone related effects on memory, this study employed Drosophila melanogaster as a model organism, using suppression of adipokinetic hormone (AKH), a functional endocrine analog of GnRH that modulates dopaminergic learning pathways. To mimic the effects of GnRH treatment, AKH suppression was induced solely during the larval developmental window, after which adult flies were assessed using an olfactory habituation choice assay. Habituation was selected as an assay that reflects the plasticity dependence memory mediated by NMDA receptors and GABAergic inhibition, as seen in humans. Memory performance was quantified by comparing behavioral response indices between naive and pre-exposed groups.

Flies that experienced developmental AKH suppression displayed higher odor avoidance responses in adulthood compared to controls, indicating lasting disruptions in memory related neural plasticity. These findings suggest that temporary suppression of hormones during development can produce persistent effects on learning circuits. By modeling developmental hormone suppression in a genetically comparable system, this study provides as a mechanistic insight into hormone regulated brain development and provide as a foundation into discussion regarding the cognitive safety of GnRH based treatments.

Keywords:

Analyzing the Developmental Effect of AKH Suppression on Drosophila Memory

The need for this project stems from the controversy regarding the widespread medical use of GnRH agonists. Concerns are specifically for its use in children and teens because as adolescents, they are still going through essential brain development. Although this drug is extremely effective for endometriosis, gender affirming care, and precocious puberty (the delay of puberty in children who are developing too early) (Casati et al., 2023), this treatment may have side effects on cognition. One side effect of GnRH-a is short-term memory loss, which has been shown through a study where perceived memory decreased throughout subjects during treatment (Newton et al., 1996). Despite widespread clinical use of GnRH agonists in pediatric populations and researched short-term cognitive effects, there remains limited mechanistic understanding of how hormonal suppression during critical developmental periods could have long term effects.

GnRH Gene, GnRH-a Function, and Neurogenesis

Gonadotropin-releasing hormone (GnRH) is mainly associated with regulating the release of sex hormones through its action on the hypothalamic pituitary axis. GnRH is produced in the hypothalamus, where it to GnRH receptors and stimulates the release of sex hormones such as estrogen and testosterone (Casati et al., 2023). However, GnRH receptors are also found in the hippocampus and the preoptic cortex parts of the brain which are essential for cognition, suggesting that it has functions and effects that go beyond reproduction.

In certain illness, cancer and illnesses that rely on sex hormones, GnRH is a widespread treatment. The treatment works by first overstimulating the pituitary gland and gradually desensitizing the receptors and stopping the release of gonadotropin hormones and sex hormones. This ultimately plays a crucial role in our reproductive systems (Casati et al., 2023). While this treatment is extremely

affective, sex hormones that are regulated by GnRH promote neurogenesis, regulates synaptic plasticity, and memory formation (Kim & Casadesus, 2011; Celec et al., 2015). As a result, GnRH is a factor in cognition and suppressing GnRH indirectly alters the hormonal environment that influences cognition.

If the suppression of GnRH occurs during a developmental window when new neurons are forming and synapses are being strengthened; it could have the ability to change how the learning circuits originally form. Thus, the concern about GNRH-a is both about how it affects memory during treatment as well as whether temporary developmental disruption can lead to long lasting changes even after hormone levels return to normal.

Drosophila as a Model

To investigate whether developmental suppression of hormone signaling can result in lasting changes to memory circuit, a genetically reliable and comparable model organism is required. Drosophila melanogaster, a fruity fly, is widely used to study how the brain learns because of its memory mechanisms and processes that are similar than to those in humans. Although the brain of Drosophila is smaller and less complex than the human brain, it uses the same types of neurons and molecules including dopamine for reinforcement learning, NMDA receptors for synaptic plasticity, and GABAergic neurotransmitter for inhibition, all of which are imperative to building and modifying memory. Although drosophila does not possess the GnRH gene, it still provides as a comparable model for this project because it has a structural equivalent, the adipokine tic hormone (AKH) (Beh-Manahem, 2021). While AKH is not genetically homologous to GnRH, it serves as a functional endocrine analog that modulates dopaminergic learning circuits in a comparable manner.

Flies show a similar process to how GnRH affects cognition through AKH. AKH is released into the blood by endocrine cells in the corpora cardiaca (neuroendocrine glands that function similarly to the vertebrate pituitary gland) and travels to neurons in the suboesophageal zone that expresses AKH receptors. As the AKH binds to these neurons, it alters how they activate dopaminergic neurons and how much dopamine is released during learning (Meschi et al., 2024). Recent studies showed that AKH can change how dopamine can neurons respond during aversive learning which allowed how hunger altered the memories of drosophila. Because dopamine driven learning in flies depends on the synaptic plasticity in the mushroom body, drosophila's primary learning center, AKH's influence on dopamine affects the synaptic plasticity that is required for memory.

Habituation, Synaptic Plasticity, and NMDA

NMDA (N-methyl-G-aspartic acid) receptors that are activated by the NMDA molecule and is an essential factor of synaptic plasticity. Synaptic plasticity is the process where the connections of the neurons change as they get more experienced. Because synaptic plasticity is how the neurons adapt to new experiences or respond with repetition, it can be drawn into memory formation and the process of habituation, a non-harmful, repeated stimuli which results in decreased behavioral responses (Paoletti et al., 2013). Habituation uses non stressful stimulus and directly reflects synaptic plasticity. Essentially, habituation reflects the maturation and flexibility of the neural circuits. As such, habituation provides as a sensitive behavior for detecting subtle disruptions in synaptic plasticity. In Drosophila, habituation depends on synaptic plasticity which is influenced by NMDA receptors and GABAergic interneurons that lessen neural responses over time (Larkin et al., 2010; Das et al., 2024). Human mechanisms also use the NMDA plasticity and GABAergic inhibition.

Habituation is also a better choice than aversive learning tests for this project due to the fact that aversive conditioning requires electric shock, which activates reactions like stress, pain, etc. Because there are so many factors that go into aversive learning it is more difficult to observe if any differences are due to memory circuits or other systems. Therefore, examining habituation learning in Drosophila following developmental AKH suppression provides an approach to isolating long-term effects on memory related plasticity.

Section II: Specific Aims

This proposal's objective is to determine whether altering AKH signaling during the period where Drosophila is still developing can change the maturation of memory and produce long term changes in adult Drosophila cognition.

Our long-term goal is to expand scientific understanding of how GNRH-a treatments could affect the developing brain and its long-term effects on cognition. As the uses of GnRH-a treatment increases in adolescents for conditions such as gender affirming care, it is important to understand whether altering hormone levels during development can produce permanent changes in memory. The hypothesis of this proposal is that altering AKH signaling in Drosophila during development, their larval stage, will lead to quantifiable, significant changes in adult habituation-based memory because if AKH influences how dopaminergic learning neurons form, then flies with AKH suppression during development could show lasting memory differences. The rationale for using Drosophila is that AKH is structurally and functionally comparable to the GnRH gene, where AKH is in the same neuropeptide superfamily and it also impacts memory through synaptic plasticity (Ben-Manahem, 2021). The rationale for using habituation is that it is a simpler, non-stressful form of learning that directly reflects synaptic plasticity without added variables of fear or reward. The work I propose will serve as a model and

foundation of whether hormone changes in larvae can affect in cognition as adults, with the implication on building knowledge into the potential developmental consequences of GnRH-a exposure in children.

Specific Aim 1: Determine the habituation baseline for normal wild type adult drosophila

Specific Aim 2: Determine whether altering AKH signaling during larval development affects adult habituation

Specific Aim 3: Identify the developmental window in which AKH disruption has the greatest effect on learning as adults

The expected outcome of this work is an understanding of whether or not AKH signals influence the development of memory in Drosophila. If AKH suppression during the larval stage significantly affects adult habituation, it will support the hypothesis that hormone suppression during development can have long-term consequences. Even if the hypothesis is not supported, it will give insight and foundation for other potential side effects of GnRH-a treatment.

Section III: Project Goals and Methodology

Relevance/Significance

This project is significant because it addresses a big gap in our understanding of how hormone manipulation affects memory and cognition, specifically through the use of treatments on adolescents. Because hormone blocking treatments like GnRH-a are becoming more widespread, there is a need to understand the long-term consequences of them, especially when used during critical brain development periods.

Innovation

Currently, there are little no studies that test the effects of hormone manipulation in terms of development and synaptic plasticity (cognition and memory). This project also proposes Drosophila as a model for GnRH studies.

Methodology

I will do is create, measure, and assess a baseline reference of olfactory habituation through the testing of healthy, wild-type, adult Drosophila.

To prepare the odorant for habituation, I will create a 1:100 working dilution of isoamyl acetate. Because isoamyl acetate is only slightly soluble in water, all dilutions will be made in ethanol. A full 1 mL to 99 mL dilution would create more odorant than the experiment requires, so I will first prepare a 1:10 stock solution and then dilute that stock to the final 1:100 concentration. To make the 1:10 stock, I will mix 1 mL of isoamyl acetate with 9 mL of mineral oil. From this solution, I will take 100 µL and dilute it with 900 µL of mineral oil. This produces the final 1:100 isoamyl acetate solution that will be used as the habituation odorant.

Next, I will isolate and measure behavior through a standard T-maze assay that measures odor preference on naïve flies and flies after pre-exposure. I will count the flies in each arm of the T-maze, calculate response indexes, and repeat this trial 2-3 times. After averaging the separate response Index means for naïve and pre-exposed flies, a 2 sample T-Test will be performed to address whether or not there is a statistically significant difference between them, to understand if there are any flaws with the odorant or if the flies are learning. This will be performed in 2 groups, where there will be a 30-minute pre-exposed group to invoke short-term memory, and a 4 day pre-exposed group to invoke long-term memory.

From there, I will manipulate adipokinetic hormone (AKH) signaling in *Drosophila* using the GAL4/UAS system. Specifically, AKH signaling will be suppressed by driving neuronal expression of the UAS transgene. To allow temporal control of this suppression, these flies will be crossed with a temperature-

sensitive GAL80 line (GAL80^ts), which inhibits GAL4 activity at low temperatures and permits GAL4-mediated suppression at higher temperatures.

AKH suppression will be activated during larval development, after which flies will be returned to permissive temperatures in adulthood to restore normal AKH levels. Adult flies will then be tested using the same olfactory habituation T-maze assay, comparing naïve, short-term pre-exposed, and long-term pre-exposed groups. Response indices will be analyzed to identify differences between control flies and AKH-manipulated flies. If these differences are statistically significant, this will indicate that AKH signaling during development influences long-term memory formation in *Drosophila*.

Specific Aim #1: Establish the baseline habituation performance of wild-type Drosophila as adults

Determine the normal olfactory habituation in healthy adult Drosophila. The objective is to assess a baseline habituation curve that shows how the adults should normally respond to repeated odor. It is essentially a reference for comparing the treated flies. Our approach is that we will test on naïve wild-type adult flies and a pre-exposed wild-type group to the non-harmful odor. Right after, the flies will be tested in a standard y-maze olfactory choice assay to measure the attraction to the same odor. This process will be repeated for different times of pre-exposure, specifically 30 minutes and 4 days. Habituation is quantified as the reduction in the difference in the number of flies in the odorant and normal air as a fraction of the total fly's response index. Our rationale for this approach is driven by the research where olfactory habituation was influenced by the GABAergic neurons that affect synaptic plasticity (Larkin et al., 2010).

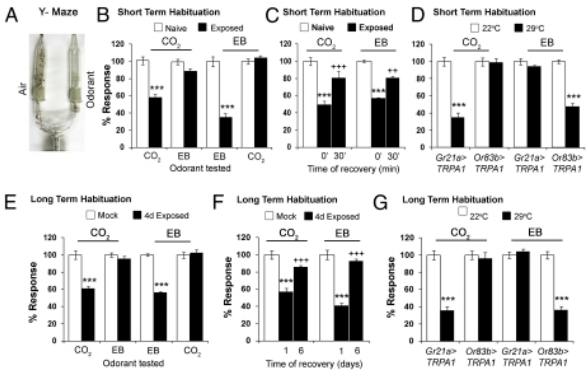

Figure 1: Analysis of the behavioral mechanisms of olfactory habituation in adult Drosophila. The proceeding graphs of B and E show the reduction of avoidance as the flies are exposed, through the comparison between tested naïve and exposed odorant flies (Das et al., 2011).

Justification and Feasibility. The adult olfactory habituation assay is relevant to this aim because it is a thoroughly tested behavioral measure of non-associative learning and drosophila, and it has been shown to rely on synaptic plasticity. Learning and memory have been researched and proven to rely on inhibitory synapses, where they are the specific points of communication where 1 neuron suppresses the activity of another. The neuron delivering the suppression is the GABAergic Neuron—a neuron where synaptic vesicles are filled with GABA, which is the main inhibitory messenger. It works so that the GABAergic neuron releases GABA to bind on to the GABA receptors on the post synaptic neuron. What this does is that it controls how the brain is able to respond to an experience in terms of neural excitability. In many forms of learning the brain must suppress responses to unimportant stimuli or enhance responses to important ones, as shown through aversive learning (Meschi et al., 2024). Strengthening inhibitory synapse onto a neuron can encode idea that a stimulus is harmless like in habituation where repeated stimulus to a non-harmful odor least to gradual reduction in avoidance. This project requires an adult memory assay that both reflects underlying neural learning mechanisms and is meaningfully comparable to human memory systems. To capture the variability of human memory processes, the study distinguishes between short-term and long-term memory formation. These memory states are assessed using separate experimental groups defined by their odor pre-exposure duration, as prolonged exposure is known to induce long-term neural plasticity through changes in protein-dependent pathways.

Figure 1 is relevant to establishing my baseline habituation in adult Drosophila because it shows the standard behavioral pattern that normal flies show during short-term and long-term olfactory habituation. The subsections of B, and D show that the wild-type flies reduce avoidance after pre-exposure, which provides me with data for what my habituation curves should be similar to.

Summary of Preliminary Data. The graph shows the mean avoidance response percentages for naïve wild-type adult Drosophila and a 30-minute pre-exposed group. There is a clear and substantial behavioral shift between the 2 performance indices where the naïve flies exhibited a mean of approximately 85%, while the pre-exposed group showed a mean avoidance of approximately 30%. This difference indicates a successful olfactory habituation, further supported by a statistically significant p-value of 0.01125 calculated using a two-sample t-test. This establishes my baseline habituation curves as well as my experimental design of olfactory habituation as a reliable test for memory in Drosophila.

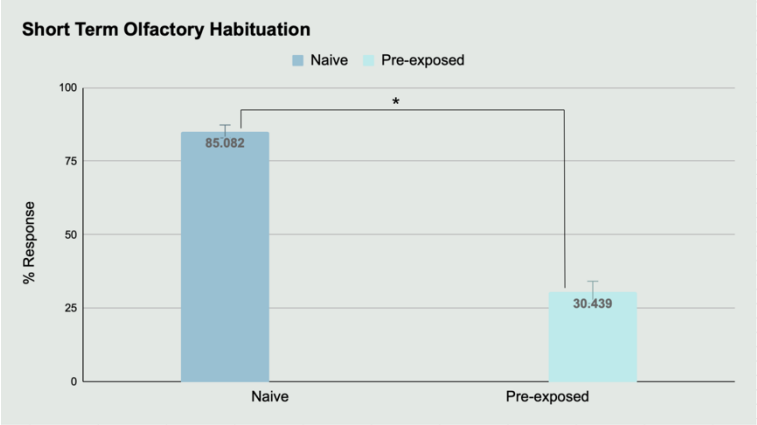

*Figure 2: Naïve flies show significantly higher avoidance responses with a mean around 85% avoidance, than odor pre-exposed flies with around a 30% avoidance. Bars represent mean ± SEM. The bracket represents a statistically significant two-sample t-test (t = 12.513, p = 0.01125) *--(p-value<0.05).*

Expected Outcomes. The overall outcome of this aim is to establish a baseline reference as to how the differences between naïve and pre-exposed habituation Response indexes should look like. This knowledge will be used for both a baseline and a justification that pre-exposure does in fact induce memory.

Potential Pitfalls and Alternative Strategies. One potential challenge is that the odorant concentration may be too high or too low, leading to inconclusive and/or nonresponsive results. If this happens, I will adjust the dilution concentration of the odorant to stimulate habituation. Another challenge is selecting an optimal diluting solution. Because isoamyl acetate is not soluble in water, I must pick another solvent. If the solution visible and actively produces side effects to the flies, I will continue to experiment with different solvents. If statistical significance is not reached through these adaptations, I will likely convert to reward-based learning.

Specific Aim #2: **Determine whether AKH suppression during larval development affects adult habituation**

Determine whether altering AKH signaling during larval development affects adult habituation. The objective is to assess whether there is a significant difference between the response indices of AKH manipulated flies and normal wild-type adults. To allow for the suppression of AKH at a specific developmental period, I will have to generate genetically mutated Drosophila. This will be done by crossing 3 different types of Drosophila strains. In order to ensure that the offspring of the flies are ideal, I will first cross virgin male Drosophila that express GAL4 in the AKH secreting cells of the corpora cardiaca, with virgin females containing a UAS strain. Then, I will use the male offspring to cross with female temperature sensitive GAL80 strains. From there, the larval offspring will be cultivated under temperatures around 18 degrees Celsius and cultivate under temperatures around 29 degrees Celsius when they are adults. Our approach stays the same, where I will test on naïve AKH manipulated adult flies and a pre-exposed group to the non-harmful odor. Right after, the flies will be tested in a standard T-maze olfactory choice assay to measure the attraction to the same odor. After multiple trials, I will compute the same performance indices from Specific Aim 1, average them, and compare them to the wild-type group using a 2-sample t-test. Our rationale for this approach is driven by the study conducted to show appetitive long-term memory is induced by GABAergic feedback loops and dopaminergic inhibition and the study conducted to prove that AKH has an influence on dopamine signaling (Paoletti et al., 2013; Pavlowsky et al., 2018).

Justification and Feasibility. AKH suppression is relevant to memory formation because the neuromodulatory systems that regulate metabolism are integrated with neural circuits that influence learning and plasticity. The mushroom body, which is the central area for olfactory learning in Drosophila, relies on the regulation of dopaminergic input that influences change synaptic plasticity. The

dopaminergic neurons do not act alone, where their impact on memory is shaped by the GABAergic feedback loop that affects dopaminergic signaling and stabilizes long-term memory formation (Pavlowsky et al., 2018). The GABAergic interneurons provide inhibitory feedback so synaptic plasticity changes as needed.

This is directly related to AKH signaling because AKH is related to the regulation of dopamine neurons through its function in Drosophila metabolism (Pavlowsky et al., 2018). The dopamine binds to the G-protein coupled receptor on inhibitory neurons, which alters GABA release and synaptic inhibition.

There are existing genetic tools that are required to manipulate AKH signaling at specific developmental stages are well established in Drosophila. The GAL4/UAS system combined with temperature sensitive GAL80 allows for precise suppression of genes (Alphey, 2015). GAL80 binds to the activation domain of GAL4 and suppresses the gene when activated. This will allow me to independently express and activate AKH in the corpora cardiaca and suppress this by cultivating the flies under different temperatures.

Expected Outcomes. The overall outcome of this aim is to determine whether or not early-life AKH suppression has long-term developmental effects on the Drosophila's ability to perform an olfactory habituation assay. I expect to see that the Drosophila's ability to habituate will decrease and weaken, meaning that the AKH suppressed, pre-exposed group will show larger avoidance responses in comparison to the control, pre-exposed group.

Section III: Resources/Equipment

Drosophila & Drosophila Handling

Material	Price	Justification
Wild-Type Drosophila	$12	It is normal fruit flies without any genetic mutations. Provides as a control group to measure normal

		habituation and compare against the AKH- manipulated flies.
Yeast	$5.95	Used as food to raise larvae into adult flies.

Drosophila Hormone Manipulation

Material	Price	Justification
GAL 4 Drosophila		This will provide as the Drosophila strain that allows for the expression of adipokinetic hormone soley in the corpora cardiaca.
UAS Drosophila		This drosophila strain provides the binding site for the GAL4, allowing the actual activation of adipokinetic hormone in a specific area.
GAL 80 (temperature sensitive) Drosophila		This is the drosophila strain that allows for the suppression of adipokinetic hormone through temperature. It binds to the GAL4 activation domain at lower temperatures, allowing for adipokinetic hormone suppression.

Habituation Test

Material	Price	Justification
T-Maze Assay		This is the choice assay used to test olfactory habituation. It is a widely used contraption for testing habituation.
Isoamyl Acetate	$9	This is the odorant that will be used to test the avoidance of flies. The drosophila is naturally repulsed by the odor and are proven to work as a stimulus for habituation.
Mineral Oil	$2.58	Used as the solvent to dilute the odorant. Because we do not want a high concentration of odorant, a solvent by which isoamyl acetate (odorant) is soluble in is imperative. Mineral oil exposure does not have any effect on Drosophila on its own.
Cotton Balls/Strips or Filter Paper	$8.99	Used to deliver odorants where they absorb exact volumes to provide consistent odor exposure across multiple trials and experiments.
Pipettes	$28.99	Needed to apply precise, consistent odor volumes for both dilution and exposures between habituation trials.

Section V: Ethical Considerations

This study uses Drosophila Melanogaster, an invertebrate model organism that is not subject to vertebrate animal research regulations; however, ethical principles of minimizing harm and distress will still be followed. All experimental procedures are designed to avoid pain and stress by using non-

aversive learning paradigms, specifically olfactory habituation rather than shock-based conditioning. Flies will be handled gently using standard laboratory techniques, and environmental conditions such as temperature, humidity, and nutrition will be carefully controlled to prevent unnecessary physiological stress. Genetic manipulation of AKH signaling will be restricted to established GAL4/UAS and GAL80 systems widely used in Drosophila research and will not induce physical injury. At the conclusion of experiments, flies will be humanely euthanized using carbon dioxide anesthesia followed by freezing, consistent with accepted laboratory practices for invertebrates. All biological waste will be disposed of according to institutional biosafety guidelines. The use of Drosophila allows this research to address important questions about developmental hormone signaling and cognition while avoiding the ethical risks associated with vertebrate or human studies

Section VI: Timeline

NOV 1-14	NOV 14-28	NOV 28-21	DEC 1-7	DEC 7-14	DEC 14-19	BREAKKKKKK	JAN 6-13	JAN 13-20	JAN 20-27	JAN 27-31	FEB 1-7	FEB 7-14
Pick model organism + justification					Dec. Fair							FEB FAIR
		5 articles	Redo 3 articles	Last Articles		Mas articles 3-4	2 articles	2 articles				
			Odorant Dilution					Odorant Dilution	Odorant Dilution			
			Naive Contol T-maze assay					Naive Contol T-ma	Naive Contol T-maze assay			
			Pre Exposed Assay					Pre Exposed Assay	Pre Exposed Assay			
			RI Calc					RI Calc	RI Calc			
			Sample Stat test					Sample Stat test	Sample Stat test			
			POSTER					Comparison	Comparison			
			Redo 3 articles									
			1 patent	#2 patent								
					Breed mutated Fli	Breed mutated Flies						
						Any write ups						
						Grant Work		Grant Work	GRANT due 27			
							AKH induction?	AKH induction?	AKH induction?			
			Prensentation Pra	Prensentation Practice				Prensentation Practice		Prensentation Practice		
										Posterboard	Posterboard	

Section VII: Appendix

$$Performance\ Index\ Formula: \frac{(\#\ of\ flies\ in\ non\ odorant\ arm - \#\ of\ flies\ in\ odorant\ arm)}{Total\ \#\ of\ flies}$$

Section VIII: References

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