TRANSCRIPTIONAL REGULATION OF NEURONAL MORPHOLOGY AND SYNAPSE LOCALIZATION IN DROSOPHILA

by

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A THESIS

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Damage to neural circuits can result in irreversible degenerative disorders. To understand how such circuits form and are maintained, we investigate how transcription factors (TFs) shape the morphology of the Moonwalker Descending Neuron (MDN), a neuron responsible for backward locomotion in *Drosophila melanogaster*. During adulthood, MDN consists of a cluster of four cell bodies and descending neurites. We hypothesize that cross-regulation among TFs directs the MDN's adult morphology and synapse localization. Using the UAS-GAL4 system and RNAi knockdown, we found that the TFs Hunchback (Hb), Engrailed (En), and Pdm2 regulate one another: En activates Hb and Pdm2, Pdm2 activates Hb, and Hb activates Pdm2. Here, I focus on Hb. Using Multicolor Flip-Out, Hb regulates MDN morphology, specifically dendritic branching, axonal bifurcation, and cell body location. By labeling synapses, we also found that Hb regulates synapse localization. We want to know what genes may function downstream of Hb, and started by assaying midline cues. Our midline screen revealed that Robo3 and Lar are expressed in MDN, significantly altering cell clustering and neurite formation. Our data support the model that TF cross-regulation and activation of specific midline guidance cues shape adult MDN morphology and synapses.

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Introduction

You should have seen the road signs that advise people not to text while driving. This is distracting and takes away the focus from the most important task at that moment: driving. But have you wondered why it is distracting to do this multitasking? While you are performing a task, the brain functions in a certain way using a specific pathway, called a neuronal circuit, to allow the execution of that task. However, multitasking can imply that the neurons being utilized to visualize the road while driving are also used to text, or the pathway that allows you to drive is momentarily interrupted while you text[1] .This is how the brain works - to correctly implement the execution of one behavior, all other related behaviors must be excluded. The Moonwalker Descending Neuron (MDN) circuit of the brain activates backward locomotion in *Drosophila melanogaster*, the fruit fly, while also inhibiting the execution of forward locomotion at the same time. This function persists from the embryonic stage all the way through adulthood in the fly[1], allowing researchers to study the function of the MDN at different timepoints (Figure 1.F). Additionally, the MDN circuit undergoes hormone-induced remodeling, similar to humans during puberty, suggesting that findings using the MDN circuit as a model will be translatable to humans[2].

Drosophila melanogaster is the model organism used in this study, primarily because of its short maturation time and ease of culture. The fruit fly has been used for centuries to study multiple biological sciences, including neuroscience. The fly can be used as a model for aging research because, contrary to other species, the fly does not have programmed death, such as Arabidopsis, where programmed death occurs after maturation of seeds. *Drosophila* is more similar to humans in the sense that aging happens through survival and mortality curves, with a peak of

reproduction reached in early life periods[3]. The fly goes through a life cycle that begins at the embryonic stage, proceeds through three larval instar stages, one pupal stage, and finally reaches adulthood, all of this in around ten days[4]. In the pupae stage, flies experience a complete remodeling that humans do not, called metamorphosis. Despite this, some developmental stages still can be used as comparison to the development of humans. In neuroscience, *Drosophila* is a good model organism because of its genetical traceability, its complex behaviors, its well-characterized nervous system, simple neuroanatomy, and its many orthologues. Orthologous genes are genes expressed in distinct species that come from the same common ancestor. For example, the regulatory protein from plant Fu is present in both Arabidopsis, a multicellular plant, and Chlamydomonas, a single cell plant. All of this makes *Drosophila* a good model to start understanding the complexities of the nervous system, despite its simplicity compared to the human brain.

The type of orthologue genes that we study are transcription factors (TFs), which are commonly found across species. TFs are expressed throughout an animal's life and their main function is to turn on other genes. Specifically, TFs regulate gene expression in the nervous system (NS) by binding to a DNA sequence. Gene expression is important for the development of neuronal identity, which is the specific functionality and type of an individual neuron[5,6]. The expression of neuronal identity provides the purpose and identity of a neuron[7]. The generation of neuronal identity happens through two important developmental mechanisms. First, spatial patterning cues that produces different neural progenitors, which in *Drosophila* are called neuroblasts (NBs) and second, NBs sequentially expresses TFs to generate neuronal diversity[8]. Neurons have dendrites and axons that are destinated to different areas of the organism; this is for them to establish

specific synaptic connections, send and receive electrophysiological signals from neurotransmitters, and to respond to different external stimulation[9]. TFs control the expression of different genes, acting as regulators, to aid in the establishment of neuronal identity; each neuron class has a unique combination of TFs. How do they do that, however, is still unknown.

In this paper we are specifically studying how TFs affect gene expression during the development of neuronal identity, and how TFs affect the morphology and connectivity of the MDN. There are two important types of TFs expressed during the development of neuronal identity to which we are concentrating. These TFs are the temporal TFs and the homeodomain TFs. Temporal TFs are sequentially expressed in molecularly distinct neuronal stem cells, generating neuronal diversity[10]. Homeodomain TFs are expressed in mature neurons and are conserved across evolution, they regulate the expression of other genes that determine neuronal identity[11]. The MDN circuit, in which we focus throughout this paper, expresses the temporal TF Hunchback (Hb), and homeodomain TFs Engrailed (En), and Pdm2. These TFs have their own individual expression at different times of the life cycle of the fly. The activation or repression of these transcription factors affect the development of the MDN circuit, which interestingly, has its morphology continuously changing throughout the lifetime of the fly. TFs have also one fascinating function - they can cross-regulate each other; this means that the regulation of one TF can affect the expression of other TFs in a sequential chain of expression. Given this, I specifically asked how does the temporal TF Hunchback (Hb), regulate homeodomain TFs Engrailed (En), and Pdm2? And how do the homeodomain TFs regulate the temporal TF Hb and each other? More importantly, how is this TF cross-regulation important for the morphology of MDN?[10,11]

Morphology is also an important part in the establishment of neuronal identity. We know MDN morphology changes between larval and adult stages. Specifically, in the larvae the MDN neurons are present in a right bundle of two neurons and a left bundle of two neurons. After hormone-induced remodeling during pupae metamorphosis, these neurons are found as a single bundle of four neurons in the middle of the brain during adulthood (Figure 1G). These bundles of neurons, whether in larvae or adults, also do not pass a non-physical line in the middle of the circuit. The establishment of this midline depends on growth cones, which guides axons by providing motile force to their extension and acts as sensors that detect and responds to a variety of environmental guidance cues[12]. These cues are interpreted as positive/attractive forces, "chemoattractant," and negative/repellent forces, "chemorepellents," which shapes the trajectory of a given axon pathway in the central nervous system (CNS)[12]. This midline is present across a variety of species, including humans and *Drosophila*, and they are important in the establishment of morphology during embryogenesis.

As established before, TFs regulation affects gene expression. We are of the opinion that the cross regulation of TFs affects the expression of the genes that establish specific protein molecules which aim to recognize the midline environmental cues. Because of this, we hypothesized that molecules guiding the cell bodies to the midline may be functioning downstream of the TFs to establish the characteristic morphology of MDN identity. All these elements, together, affect the development of neuronal identity.

This paper also studies how the connectivity of the MDN is affected by TFs. One important aspect of connectivity is the synapses. In the MDN, synapses are located at the axon in the central nervous system (CNS) (Figure 5C) and they travel down along the axon through the ventral nerve cord (VNC) (Figure 5B, 5D, and 5F), which is the equivalent of the human spinal cord. As stated above, the MDN is located at the CNS and the VNC. To study more in detail how the TFs affect MDN morphology and connectivity, we examined the area, depth, and length of the bushy dendrites and the axon. For this, we divided the CNS dendrites into the contralateral (the side with the axon) and the ipsilateral dendrites (Figure 5A). For easier quantification, the VNC was divided into three segments, the T1, T2, and T3 segments (Figure 5D).

Results

Cross-regulation of TFs in the MDN

Previous research found that TFs are expressed at different time points in the MDN. The TFs and En are constitutively expressed throughout the life of the fly, while TF Pdm2 only starts being expressed by the start of the pupae stage, through adulthood (Figure 1F). We hypothesized that these TFs affect each other's expression in the adult MDN. To determine exactly how this happens, we knocked down the expression of one of the TFs by targeting the TFs gene and quantified the pixel intensity of the TFs fluorescence before and after the knockdown at the adult stage of the fly. When we knockdown Hb, we can see that compared to the control, the expression of En shows no significant difference, while the expression of Pdm2 decreases (Figure 1A and 1B). When we knockdown En, the expression of both Hb and Pdm2 decreases (Figure 1A and 1C). Finally, when we knockdown Pdm2, the expression of Hb decreases, while the expression of En shows no significant difference (Figure 1A, and 1D). Based on these results, we conclude that the TF En regulates the expression of the TFs Hb and Pdm2, the TF Hb regulates the expression of the TF Pdm2, and the TF Pdm2 regulates the expression of Hb (Figure 1E).

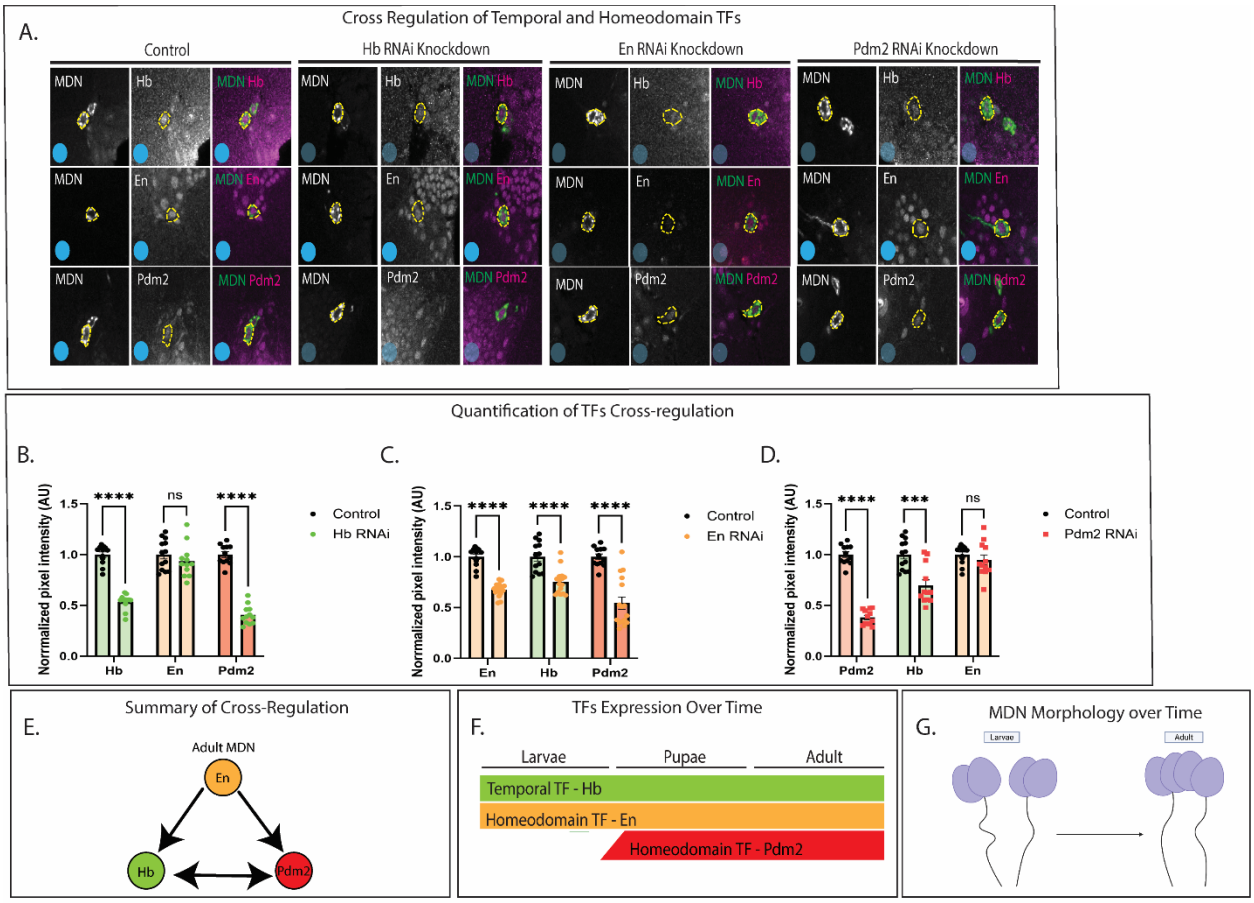

Figure 1. Cross-Regulation of Temporal and Homeodomain Transcription Factors

A) Raw images of the TFs' cross-regulation. Bright blue indicates full expression, and faded blue indicates decreased expression. Ordered by genotype (column) B-D) Quantification of the normalized pixel intensities (AU) of the TFs knockdown. Hb (Green), En (Yellow), Pdm2 (Red). E) Schematic of cross-regulation. F) Timeline of TFs expression through the fly life cycle. G) Morphology of the MDN during larval and adult stages.

TFs affect the morphology of the MDN

To determine how the morphology of the MDN is being affected by TFs, again, we knocked down the expression of the TFs and looked at the morphology images in Imaris after they were imaged using a confocal microscope. The typical morphology of the MDN consists of one cluster of four cell bodies and two descending neurites (Figure 2A). The number of cell

bodies is not affected by TFs (Figure 2E). When we knockdown the expression of the TF Hb, we can see that the number of cell body clusters increases from one to two, but the number of neurites stays the same as control (Figure 2B, 2F and 2G). When we knockdown Pdm2 we see that the number of cell body clusters increases from one to three and the number of neurites increases from two to four (Figure 2C, 2F and 2G). Finally, when we knockdown En we see that the number of cell body clusters increases from one to two and the number of neurites increases from two to four (Figure 2D, 2F and 2G). These results show that TFs affect the morphology of the MDN, specifically affecting the spatial organization of neurons and causing aberrant outgrowth of neurites. This implies that TFs aid in regulating guidance cues and they act as a negative regulator of neurite elaboration.

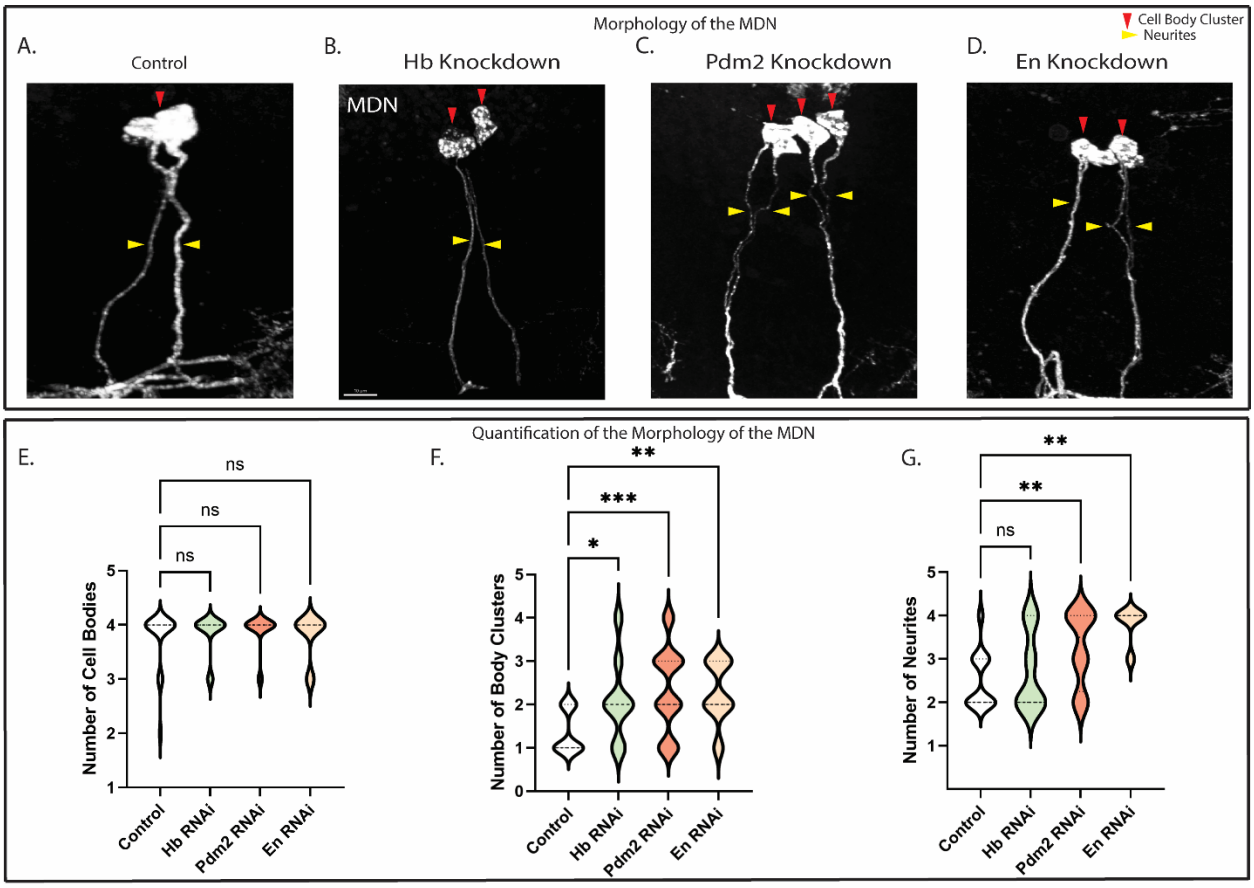

Figure 2. TF effects in the morphology of the MDN

A) Control, normal morphology of the MDN. Four cell bodies, one cell body cluster and two neurites. B) Image of the morphology after the decrease in expression of Hb. Four cell bodies, two cell body cluster and two neurites. C) Image of the morphology after the decrease in expression of Pdm2. Four cell bodies, three cell body cluster and four neurites. D) Image of the morphology after the decrease in expression of En. Four cell bodies, two cell body cluster and four neurites. E) Quantification of the number of cell bodies in control vs when Hb (Green), Pdm2 (Red), and En (Yellow) expression is decreased. E) Quantification of the number of cell body clusters in control vs when Hb (Green), Pdm2 (Red), and En (Yellow) expression is decreased. E) Quantification of the number of neurites in control vs when Hb (Green), Pdm2 (Red), and En (Yellow) expression is decreased.

The TF Hb affects the connectivity of the MDN

As stated before, connectivity is also an essential part of neuron identity. To determine how TFs affect the connectivity in the MDN, we knocked down the expression of Hb and looked at the number of synapses before and after the knockdown of the TF in the axon localized in the VNC. We then quantify the number of synapses in each segment of the VNC. When we knockdown Hb in the T1 segment, there is no significant change in the number of synapses (Figure 3A). When we knockdown Hb in the T2 segment, there is a significant decrease in the number of synapses compared to control (Figure 3B). When we knockdown Hb in the T3 segment, we see an even greater significant decrease in the number of synapses (Figure 3C). These results show that TFs affect synaptic connectivity by regulating the neuron's identity and morphogenesis. Due to a lack of time, the effects of the TFs En and Pdm2 couldn’t be measured.

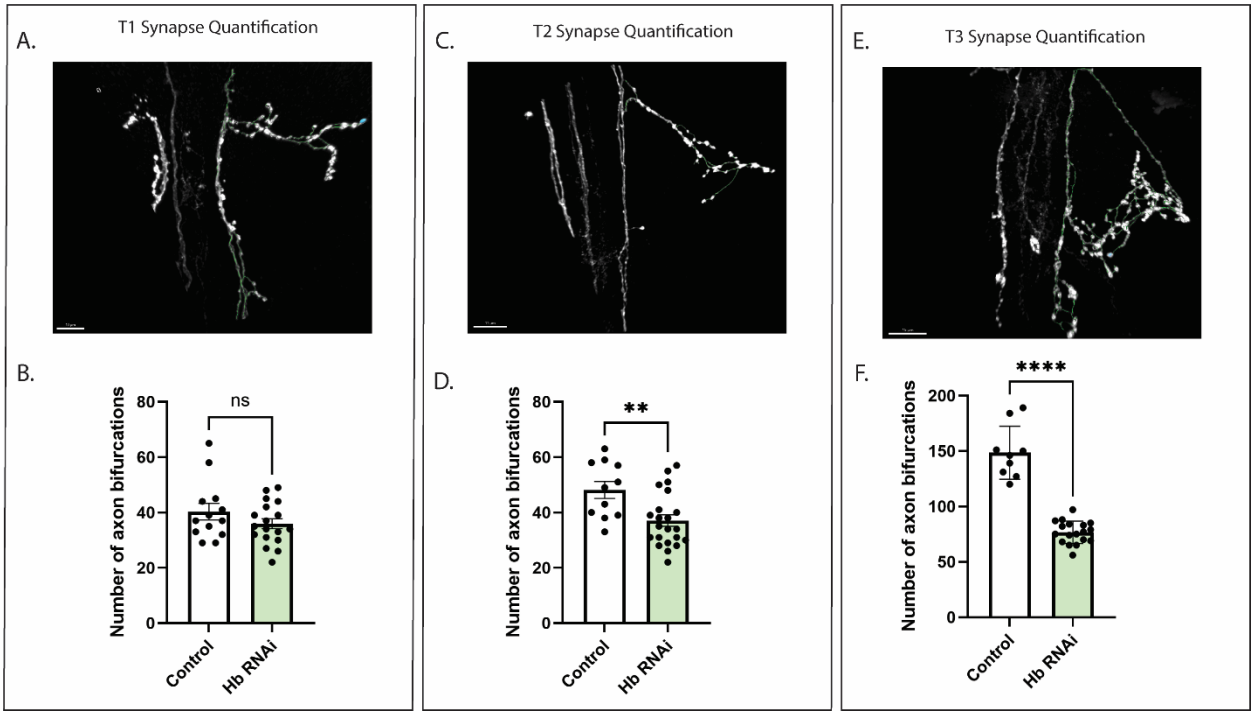

Figure 3. Synapse Number Quantification per Segment

A-B) Image and quantification of T1 segment of the VNC. No significant change in synapse number after Hb expression decreases. C-D) Image and quantification of T2 segment of the VNC. Significant change in synapse number after Hb expression decreases. E-F) Image and quantification of T3 segment of the VNC. Significant change in synapse number after Hb expression decreases.

TFs affect the midline guidance cues in the MDN

Midline guidance cues are environmental cues that guide the trajectory of an axon. We hypothesized that midline guidance cues expressions were regulated by TFs, thus affecting the morphology of the MDN. After looking at literature for known guidance cues[12,13], we assayed their presence in the MDN. From all of them, we were able to confirm the presence of the cues Robo and Lar in the MDN (Figure 4C). We then examined and quantified the effects of Robo and Lar in the MDN morphology. As stated before, the typical morphology of the MDN consists of one cell body cluster with four cell bodies and two descending neurites. The number of cell bodies did not change when we knockdown the expression of Lar and Robo (Figure 4E). When

we knock down the expression of Lar, the number of cell body clusters increases from one to two, while the number of neurites remains two (Figure 4A and 4E) . When we knockdown the expression of Robo, the number of cell body clusters increases from one to two, and the number of neurites increases from two to three (Figure 4B and 4E). To determine how TFs affect the expression of Robo and Lar, we knockdown the expression of the TFs and looked at the expression of Robo and Lar. We did not quantify this part, but we can visually see the decrease in expression of Robo and Lar when we knockdown En, Pdm2, and Hb (Figure 4D). This result supports our hypothesis that TFs are upstream regulators of the midline guidance cues Robo and Lar, affecting morphogenesis.

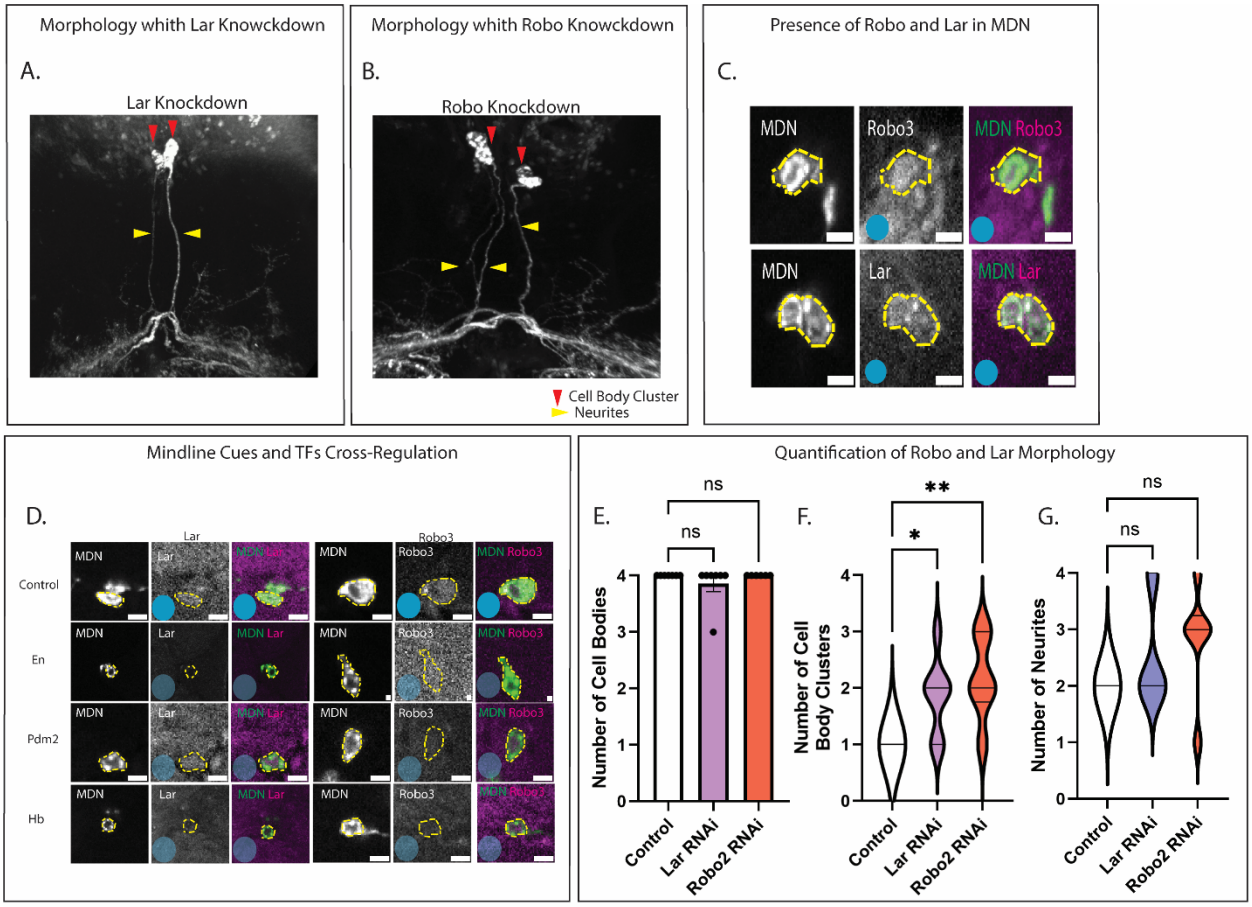

Figure 4. Midline morphology manipulation and TF cross-regulation.

A) Image of MDN morphology after decreasing in Lar expression. Increases number of cell body clusters to two. A) Image of MDN morphology after decrease in Robo expression. Increase cell body cluster to two, and neurites to three. C) Raw images of positive Robo and Lar expression in MDN. D) Robo and Lar expression after TFs decrease in expression. Knockdown of all En, Pdm2, and Hb decreases expression of Robo and Lar. E) Quantification of the number of cell bodies in control vs when Lar (Purple) and Robo (Orange) is decreased. F) Quantification of the number of cell body clusters in control vs when Lar (Purple) and Robo (Orange) is decreased. G) Quantification of the number of neurites in control vs when Lar (Purple) and Robo (Orange) is decreased

TFs does not affect the MDN dendrite arborization or axon complexity

Based on the previous results we have gotten, we wanted to dig deeper into how morphology is affected by TFs regulation. As such, we looked at how dendrite arborization (branchiness) and axon complexity were affected when we knockdown the TFs. For this, the

CNS of the MDN was divided into contralateral (axon-side), ipsilateral dendrites and the axon (Figure 5C). And the VNC was divided by the T1-T3 segments (Figure 5D). The individual neuron cell bodies were labeled using Multi Color Flip-Out (MCFO) (Figure 5A and 5E). In the contralateral dendrites of the CNS there was no significant difference in the area, branch depth, branch level, and branch length when we knockdown the TFs compared to control (Figure 6A). In the ipsilateral segment, there was no significant difference between the area, branch level, and branch length of the control and TFs knockdown. Still, there is a significant difference in the depth when we knockdown Hb and Pdm2 but not En (Figure 6B). The axon also has no significant difference between the control and the TFs knockdown in the area, branch depth, branch level, and branch length (Figure 6C). In the T1-T3 segments, there is also no significant difference between control and the TFs knockdown in area, branch depth, branch level, and branch segment (Figure 7A-C). Overall, TFs do not affect contralateral dendrites and axons in the CNS, nor the axon in the VNC. Hb and Pdm2 affect the branch depthness of the ipsilateral dendrites but not En. This shows that TFs have a division of labor regarding the establishment of neuronal identity.

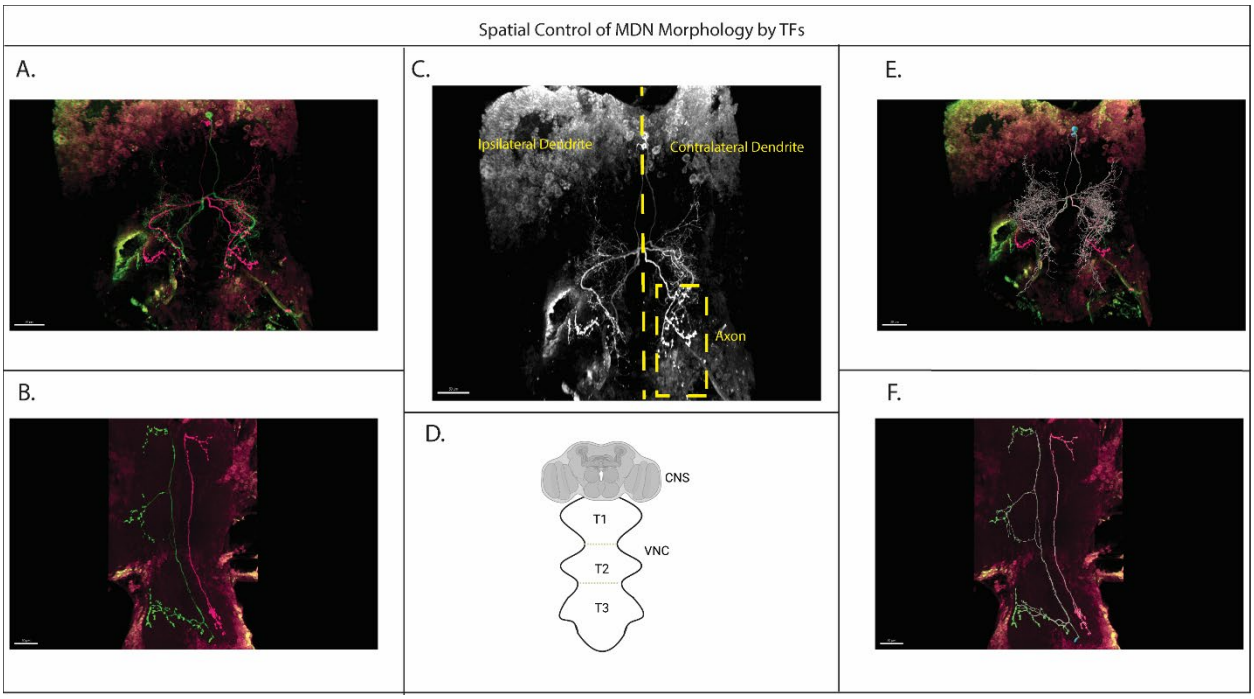

Figure 5. Spatial Control of MDN Morphology by TFs

A) Image of the CNS MCFO MDN. Individual neuron labeled (Green and Pink). B) Image of VNC MCFO MDN. Individual neuron labeled (Green and Pink). C) Demonstration of CNS division. Ipsilateral dendrite (left), contralateral dendrite (right), axon (right, down). D) Demonstration of VNC division in segments, T1, T2, and T3. E) Image of the CNS MCFO MDN with filaments. Individual neuron labeled (Green and Pink). F) Image of VNC MCFO MDN with filaments. Individual neuron labeled (Green and Pink).

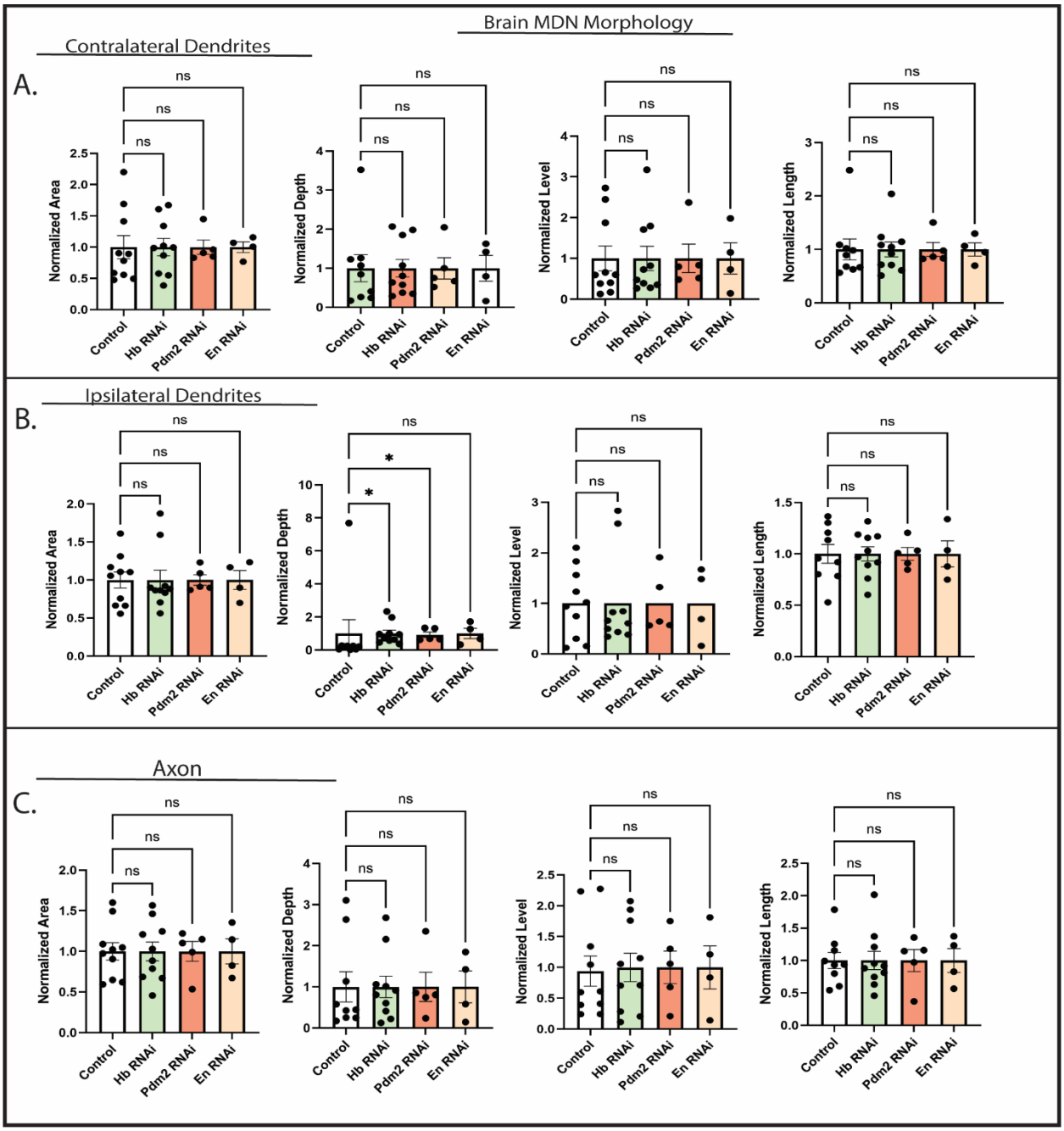

Figure 6. CNS MDN Morphology

Quantification of normalized area, normalized branch depth, normalized branch level, and normalized length. A) Normalized quantification values of the contralateral dendrites. B) Normalized quantification values of the ipsilateral dendrites. A) Normalized quantification values of the CNS axon.

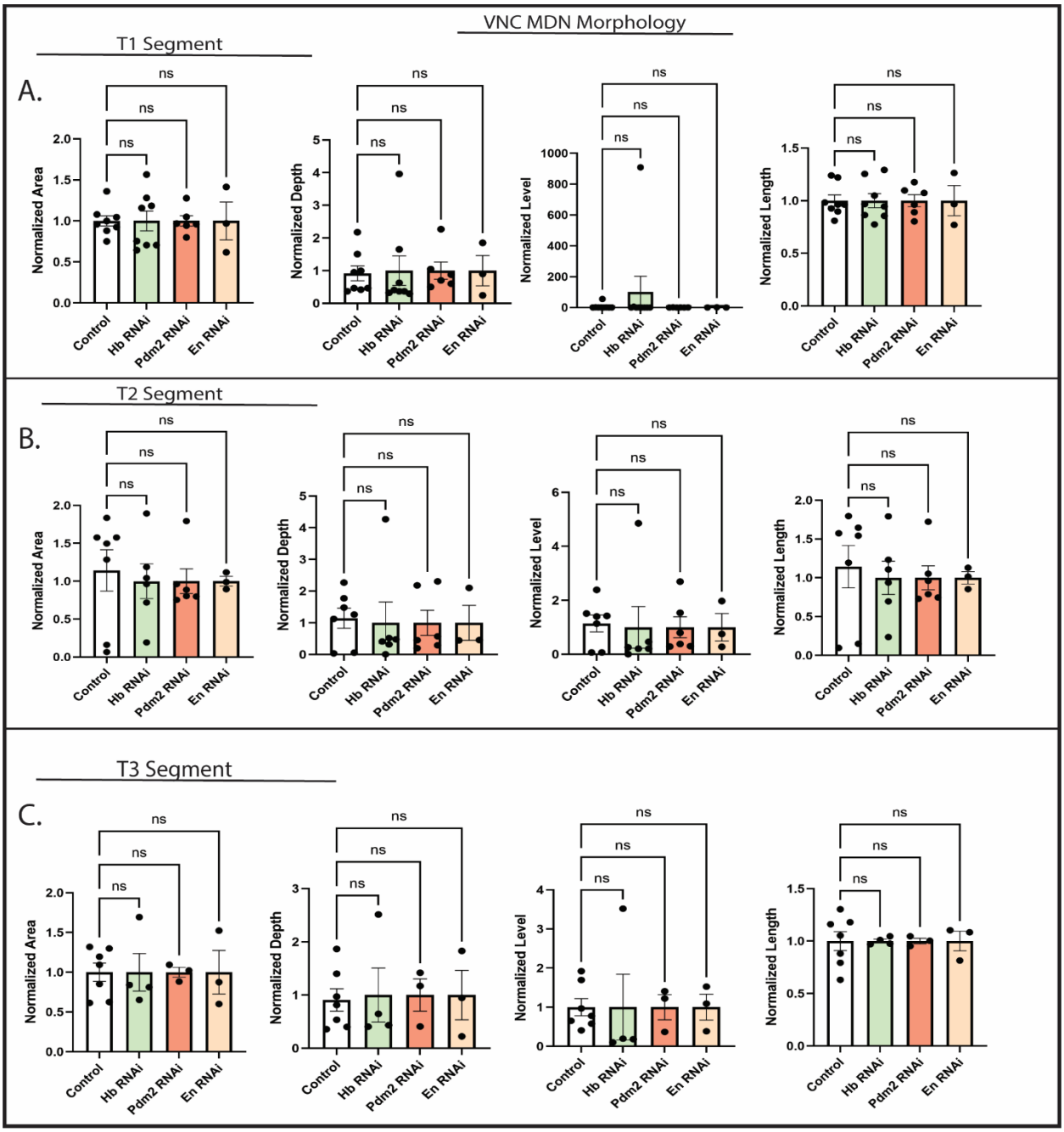

Figure 7. VNC MDN Morphology

Quantification of normalized area, normalized branch depth, normalized branch level, and normalized length. A) Normalized quantification values of the T1 segment. B) Normalized quantification values of the T2 segments. A) Normalized quantification values of the T3 segments.

Discussion

In the Moonwalker Descending Neuron (MDN), the TF En regulates Hb and Pdm2, while HB and Pdm2 regulate each other. This reveals a hierarchical regulation in the TF network of the MDN, with En being an upstream regulator, and establishing the initial conditions for neuronal identity. En, however, is a targeted regulator, as it implies no significance in the contralateral branch depth of the CNS. Hb and Pdm2 are players of a feedback loop, mutually regulating in the MDN. This can explain why they both affect the ipsilateral dendrite branch depth.

The morphology of the MDN is affected by the molecular identity established by the TFs En, Hb, and Pdm2, which also regulate the midline guidance cues, Robo and Lar. The fact that only the number of cell body clusters and neurites are being affected, but not the number of cell bodies themselves, shows that TFs regulation does not play a role in the generation or survival of neurons, but only in the organization and differentiation after their formation. This is confirmed when midline guidance cues Robo and Lar affect the morphology of the MDN, and it is confirmed that they are regulated by TFs expression. The effect of TFs in morphology and connectivity is also corroborated when the decrease in Hb expression affects the number of synapses in the VNC axon, which implies that there will also be a reduction in the axon targeting with other neurons, affecting the proper connections that allow MDN behavior. Overall, TFs are essential for establishing appropriate neuronal identity in the brain.

This formation of molecular identity is essential for people to live a normal and happy life. Neurological diseases can include neurodevelopmental, neurobehavioral, and neurodegenerative illnesses. These distinct categories of neurological diseases also involve distinct disorders, and they can easily overlap. Neurodevelopmental and neurobehavioral diseases can include famous disorders such as attention deficit/hyperactivity disorder (ADHD),

autism, and learning and intellectual disabilities, which affect brain growth and development and are composed of behavioral impairments originating from the brain[14,15]. Mood disorders can also include neurological connotations, overlapping the disciplines of neuroscience and psychiatry. The cells of the central nervous system in neurodegenerative diseases, such as Alzheimer's Disease and Parkinson's, stop working or die, and the symptoms usually worsen over time[16].

It is known that neurological diseases are one of the illnesses that are less understood and most difficult to study. We still cannot understand the complexities of how the brain functions in everyday tasks, much less why it is malfunctioning. As everyone is unique and genetics vary across populations, studying neurological diseases is very challenging. The goal of studying neurological diseases, using *Drosophila* as a model organism, is to understand the basis of neurology. Because how can we understand what is wrong when we do not know how it is when it is right? How does it look in a correct neuronal pathway in a specific brain region? What are the factors that hint to you that something is wrong in that region?

As said before, neuronal identity establishes the purpose and function of a neuron. Neurons are the cells that receive and send the signals from the brain to the body and reverse. For each part of the body to work correctly, a specific and precise neuron must be present to be able to receive or send those signals. Failure of this process may result in various consequences, ranging from weak to significant effects. Just imagine what could happen if one neuron were wrongly established in the incorrect region. This neuron is incapable of receiving a signal from the body; the absence of response could trigger a chain effect that could have major disastrous consequences, such as the inability to express the correct genes. To state it in simple words, imagine that because of erroneous identity establishment in the amygdala, you are incapable of processing your emotions. This could result in emotional dysregulation, which could end up in

an individual with problems processing happiness. Thus, being diagnosed with depression, or in another scenario, the person is incapable of regulating their anger, resulting in anger issues.

For reasons such as this, the importance of establishing neuronal identity in the brain is clear, not just for an individual to live a normal and happy life but also because neurological problems can affect families, societies, and populations. Researchers from all over the world work every day to contribute their part in understanding a bit more about the essential organ that we call the brain.

Materials and Methods

Fly Culture:

To start the fly culture, we deposit the chosen female and male flies in a food vial or bottle and incubate them at 25°C. Female flies are from the genotype UAS-myr::HA; Adult MDN split-Gal4. The female fly contains a labeled Moonwalker Descending Neuron (MDN) that could be tagged in the larval or adult stage. TF's experiments include the use of both female larvae and adult tagged MDN. We generate different crosses by placing these flies with males expressing distinct transgenes. The type of experiment determines the male fly genotype used in the cross we will do. Examples for these experiments include using gene knockdown or overexpressing a gene. All flies used in these experiments are obtained from sources such as the Bloomington Drosophila Stock Center and are afterwards taken care of and grown in vials with fly food inside the laboratory. The fly food contains yeast, sugar, cornmeal, water, and agar.

UAS-GAL4 Binary Expression System:

The UAS-Gal4 system uses an MDN-specific driver line to express Gal4, which binds to the upstream activation sequence (UAS) of the added transgene. This promotes the expression of the transgenes, which in this case are an HA-epitope tag attached to the membrane and the machinery necessary to knock down a gene of interest via RNAi (Figure 8). This system is used in female and male flies to express the gene of interest.

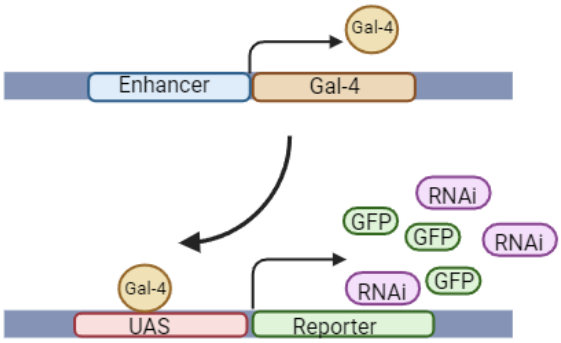

Figure 8. UAS-GAL4 Binary Expression System Visualization

Illustrations of DNA sequences. The top sequence is the driver line, and the bottom sequence is the reporter line.

RNAi Knockdown:

Messenger RNA (mRNA) is expressed by the gene knockdown method. A dicer cuts a double-stranded DNA segment, becoming a short-interference RNA (siRNA). The RNA-Induced Silencing Complex (RISC) recruits the siRNA, which guides the mRNA to bind. Finally, the mRNA is cleaved and degraded, ultimately causing a decrease in the gene expression of interest, which in this study, belongs to the transcription factor chosen (Figure 9). All RNAi used in these experiments originates from male fly genotypes.

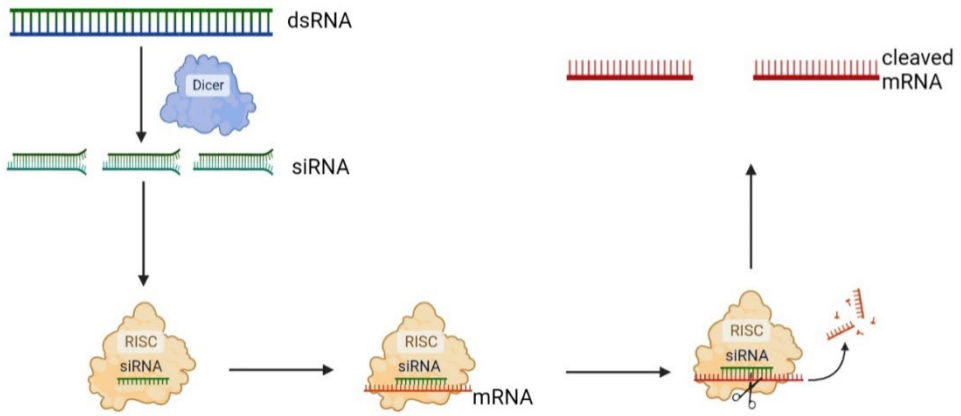

Figure 9. Demonstration of RNAi Knockdown Method

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Multi Color Flip-Out (MCFO)[17]

Used to label individual neurons in different fluorescent color, avoiding overlapping of neurons and allowing quantification of individual neurons. Different fluorescent tags are attached to the backbone of a non-fluorescent GFP, resulting in a protein named spaghetti-monster GFP (smGFP). Expression is controlled using the UAS-GAL4 binary expression system. A second level of control is done by incorporating a cassette into the smGFP. This way, the expression of the smGFP depends on the activation of transcription and the recombination that removes the interruption cassette. This cassette uses the flp-out technique, where Gal-4 is preceded by a stop cassette flanked by two FRT sites. A Flippase (FLP) is a site-specific recombinase that only recognizes FRT (FLP Recognition Target), which enables recombination. The FLP Recombinase is expressed using heat shock (8 min), FLP binds to the two FRT sites and excises (cuts) between the two FRT sites, ligating them together and expressing the multiple reporter fluorescent proteins tagged to target cells (Figure 10).

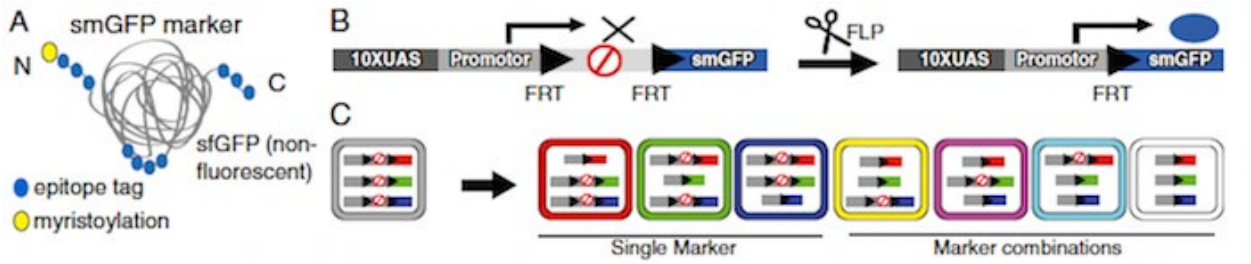

Figure 10. Multi-Color Flip Out Schematic

Dissection Protocol

Cultured flies are put to sleep with CO_2 gas and kept sedated on ice. In a dissection petri dish filled with sillagaurd, the brain tissue is dissected from the rest of the body with forceps in hemolymph-like 3 buffer (HL3B) and placed on a lysine coverslip. The coverslip is then situated on a 24 culture well plate, where is soaked in 4% Paraformaldehyde (PFA) (Adults: 40 min). After soaking time, the brains are then washed fast three times with the buffer Phosphate Buffered Saline and Tween at 10X (PBST).

Immunohistochemistry:

After finishing the dissection protocol, the wash is removed and tossed, and Block (0.5% normal donkey serum) is added. Tissue stays in block for 40 minutes at room temperature or overnight. Block is tossed away, and the primary antibody diluted with PBST is added. Primary antibody then binds to the transcription factor protein of interest, as well as the HA tag expressed in MDN. Primary antibody is removed, and tissue is again washed with PBST three times fast and refrigerated overnight. Primary antibody is removed, and tissues are washed again three times and refrigerated overnight. Wash is removed and the secondary antibody with fluorescent protein (lines 488, RRX or 647) is added and refrigerated overnight. The secondary antibody then binds to an antigen specific to the primary antibody (i.e., rat, mouse, guinea pig or rabbit).

The tissue is rewashed three times and refrigerated overnight. Finally, the tissue goes through the DPX protocol (Figure 11).

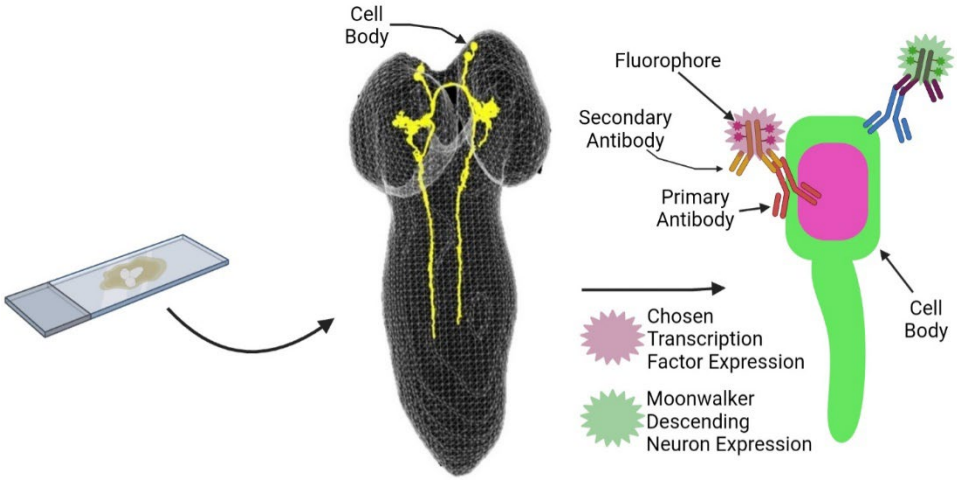

Figure 11. Depiction of the Immunofluorescence.

Labeling is done in the neuron's plasma membrane; it can tag cell bodies, dendrites, and axons.

Mounting Tissue (DPX) Protocol

The brain tissues in the 24 culture well plate is washed with ethanol (EtOH) for various times. Larvae are washed with EtOH at 30%, 50%, and 75% for 10 minutes each time, then washed twice with 100% EtOH for 10 minutes each time. Adults are washed with EtOH at 30%, 50%, and 70% for 12 minutes each time. Another was with EtOH at 90% for 15 minutes, and a final wash was with EtOH at 100% two times for 18 minutes. Afterwards, the coverslip is transferred with forceps to a glass plate filled with xylene in a fume hood. The larvae are washed with xylenes two times for 10 minutes. Adults are washed with xylenes two times for 18 minutes. Coverslips are mounted in a labeled glass slide using Dibutylphthalate Polystyrene Xylene (DPX). The adult brain requires a “bridge” in the glass slide.

Microscope Imaging

Mounted coverslips in labeled glass slides are imagined in a confocal microscope (Zeiss 900) using the ZEN microscopy software. The results are 3D structures of the labeled MDN circuit.

Data Analysis

Using different software, the samples are analyzed statistically (Prism), or by image, shape, or form (ImageJ2 and Imaris). Data and 3D structures are analyzed and quantified (Imaris). Finally, snapshots of 3D structures and images made online are mounted in this paper (Adobe Illustrator, BioRender).

Software

1. Imaris: an interactive microscopy image analysis software. Used for visualization of 3D images and cell bodies, and for quantifying regions.
2. Fiji/ImageJ2: basic image viewer and annotation tool for multidimensional image data.
3. Adobe Illustrator: a design and drawing program.
4. Prism/GraphPad: perform basic statistical tests, data organization, and 2D scientific graphing.
5. BioRender: This is used to create most of the science figures in this paper.

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