UNIVERSITY OF CALIFORNIA SAN DIEGO

Understanding Neurotransmitter Identity and Plasticity in the Circadian Clock Network of Drosophila.

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Biology

by

Koushik Tuppil

Committee in charge:

Professor William Joiner, Chair
Professor Nicholas Spitzer, Co-chair
Professor Chih-Ying Su

2021

The thesis of Koushik Tuppil is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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I would also like to thank Dr. Meilin Wu for all her guidance and help. She is an amazing mentor and friend who helped me with everything from troubleshooting the protocol to teaching me how to use the confocal microscope. She also performed the RNAscope assay for some of the experiments that I will talk about in this thesis.

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ABSTRACT OF THE THESIS

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by

Koushik Tuppil

Master of Science in Biology

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Professor William Joiner, Chair

Professor Nicholas Spitzer, Co-Chair

The clock network in Drosophila is one of the best characterized behavioral circuits and is responsible for circadian control of various bodily functions including sleep, activity, feeding and more. As such, it is important to identify the neurotransmitters used by neurons in this circuit to communicate. In this thesis, I use RNAscope in combination with IHC to quantify expression levels of VAChT, Gad1, and VGlut in LNv and DN1p neurons of the clock network. I also use this approach to quantify expression levels of these markers in 53D10 neurons of the AMMC which may be a sleep-promoting, output pathway for the clock network. I found all three markers well represented in LNv and 53D10 neurons and VAChT and VGlut in DN1ps. Additionally, for all three of these populations, I found a good fraction of cells that co-express markers for multiple classical neurotransmitters. This is the first time clock neurons have been shown to have this property. Since the neurons in the clock network are thought to change their activity with the time of day, they are a good model for investigating which conditions, if any, enable neurotransmitter plasticity in adult Drosophila. Preliminary data from my thesis suggest that changes in light/dark cycles of entrainment may cause changes in VGlut expression in LNv neurons of the clock network.

Introduction:

Identifying the neurotransmitters that neurons in a given circuit use to communicate with each other is often essential to understanding how the circuit contributes to complex behaviors. One of the best characterized behavioral circuits is the circadian clock network in Drosophila. This neural circuit consists of ~75 neurons on each side of the brain that utilize the neuropeptide pigment dispersing factor (PDF) to communicate with each other (Figure 1). Using several interlocking transcriptional and translational feedback loops, these neurons control circadian timing of various bodily functions including sleep, locomotor activity, feeding, eclosion and reproduction. (Dubowy et al., 2017; Beshel et al., 2017). Surprisingly, however, a detailed understanding of neurotransmitter identity within this network of cells remains limited. Acetylcholine (ACh), GABA, and Glutamate have all been previously implicated in circadian control in flies. Small ventrolateral neurons (sLNvs) were found to express glutamate receptors which are believed to be the targets of the glutamatergic posterior dorsolateral neurons (DN1ps) (He et al., 2017; Hamasaka et al., 2007). Similarly, it is believed that sLNv neurons use glycine for reciprocal inhibitory modulation of DN1ps (Frenkel et al., 2017; Franco et al., 2017). sLNv neurons have also been shown to respond to ACh and GABA (Lelito et al., 2012) suggesting the presence of cholinergic and GABAergic neurons which innervate these cells. It is still unclear which cells are responsible for the GABAergic input to the clock network and while there are studies implicating dorsal lateral neurons (LNd) in cholinergic input (He et al., 2017), there could be other unidentified cholinergic cells. In my thesis, I focused on improving our understanding of neurotransmitter identity within the clock network by directly measuring transcriptional markers of classical neurotransmitters in two populations of cells: LNvs and DN1ps.

One of the functions of the circadian clock is to control the timing of the sleep/wake cycle. Neural circuits that integrate this information with other internal cues and externally-derived sensory signals to determine arousal state are unknown. However, in unpublished work

my lab has identified neurons in a subset of the antennal mechanosensory and motor center (AMMC) that may serve such functions. Briefly, these neurons are required for circadian locomotor output, appear to be acutely inhibited by wake-promoting sensory ppk neurons, and promote sleep when thermogenetically stimulated. In my thesis I address the classical neurotransmitter identity of these neurons too. I will refer to these neurons as 53D10 neurons. They are also depicted in figure 1.

For all of my studies, I quantify expression of transcripts that function as proxies for usage of classical small-molecule neurotransmitters. The transcripts I used for this purpose were vesicular acetylcholine transporter (VAChT) for cholinergic neurons, glutamic acid decarboxylase 1 (Gad1), for GABAergic neurons, and vesicular glutamate transporter (VGlut), for glutamatergic neurons. The transcripts I selected for my studies have previously been used as markers for neurotransmitter identity in flies, but not specifically in the circadian clock network (Lacin et al., 2019).

Thus, there exists a possibility that clock neurons co-express these neurotransmitters. Neurotransmitter co-expression in Drosophila is still being actively investigated with varying results. For example, studies have shown that the vast majority of neurons in the adult fly ventral nerve cord use only ACh, GABA, or glutamate (Lacin et al., 2019). Other studies that looked specifically in the lamina of the Drosophila visual system found GABA and ACh co-expression in C2 neurons as well as ACh and glutamate co-expression in L1 and L2 neurons (Kolodziejczyk et al., 2008). However, no study has tried to determine whether individual neurons in the clock network produce more than one classical small-molecule neurotransmitter. In this thesis, I will address that question and extend it to 53D10 putative clock output neurons as well.

Because the neurons I chose to study are thought to change their activity with time of day or in response to specific internal or external cues, they were ideal for addressing another question as well. That is, is invertebrate neurotransmitter identity fixed, or can it be modified? In mammals, a number of studies have shown that neurotransmitter identity is not fixed and that neurotransmitter switching occurs in both developing and mature neurons as a way to maintain homeostasis in neural circuits (Spitzer, 2012). For example, physical exercise was shown to induce switching from ACh to GABA in neurons of the caudal pedunculopontine nucleus and thus promote motor skill (Li et al., 2020). Additionally, changes in short and long day photoperiods correlating to the winter and summer days induce changes between dopamine and somatostatin in the hypothalamus of adult rats (Dulcis et al., 2013). This last example is especially relevant to my thesis because light is arguably the most important environmental signal for flies.

In the examples cited above, the underlying mechanisms responsible for neurotransmitter plasticity are still being explored, though there is evidence that they are transcriptionally driven (Guemez-Gamboa et al., 2014). Discovering such mechanisms might be more tractable if neurotransmitter plasticity were found to be conserved in invertebrates. Therefore, I addressed this question in my thesis, again in the context of the circadian clock network because these neurons change their activity with time of day and in response to different light/dark conditions. Specifically, I measured whether neurotransmitter identity of circadian neurons changes in response to twelve hours of light vs dark during the day.

In all my studies, I needed a robust and reliable assay to identify markers of neurotransmitter identity. To address this need, I used a combination of RNAscope and immunohistochemistry (IHC). RNAscope provided good spatial resolution of targeted probes with high selectivity and minimal background noise. IHC allowed me to identify specific classes of clock neurons in which to quantify transcripts by RNAscope. Together, these two techniques

allowed me to investigate neurotransmitter identity, co-expression, and plasticity in the circadian clock network of Drosophila. My hope is that the results of this thesis will lay the groundwork for future studies that address the conditions that influence neurotransmitter identity in the context of the sleep/wake cycle and perhaps other behaviors.

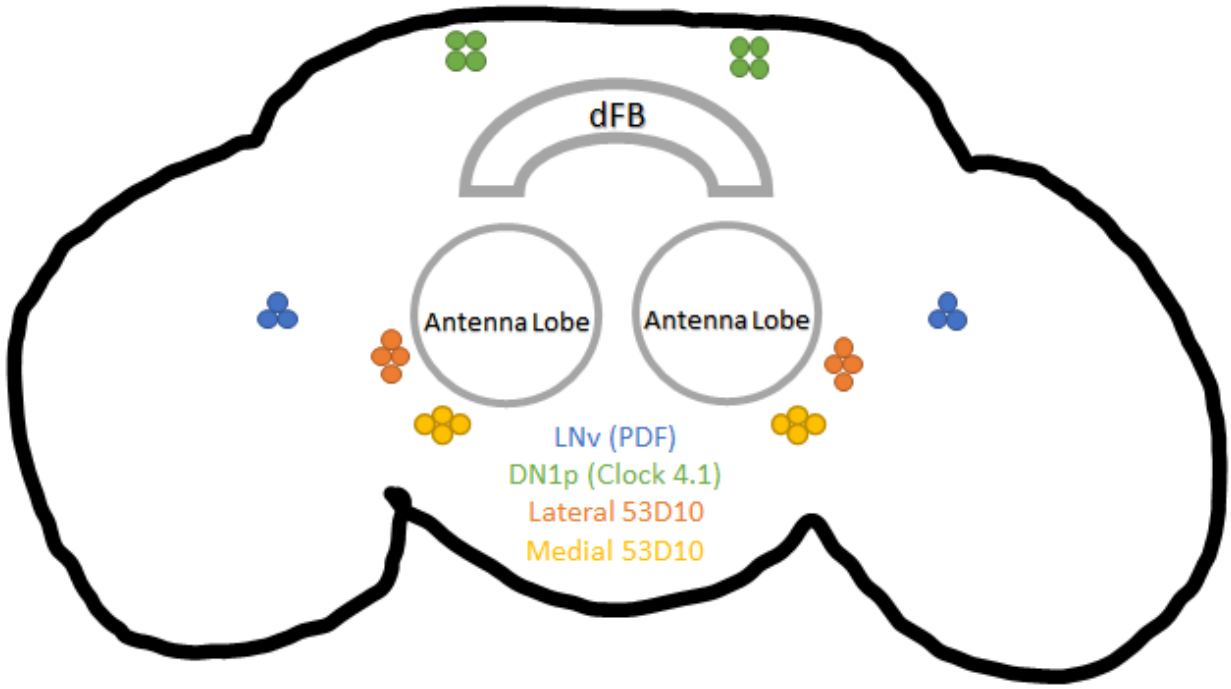


Figure 1: Cartoon image of a fly brain with relevant populations of neurons drawn in different colors to represent their approximate location. Landmarks such as the Antenna Lobes and Dorsal Fan Shaped Body are also drawn.

Results:

Advantages of a Combined RNAscope and IHC Assay

As previously mentioned, a combination of RNAscope and IHC was used for my thesis. This was because RNAscope could provide good spatial resolution of the targeted probes with high selectivity and minimal background noise. When visualized under a fluorescent microscope, targeted mRNA strands can be seen as individual puncta. RNAscope's high selectivity is due to two high affinity probes that need to bind side by side in order to produce a signal. These probe pairs are then bound to a tree-like scaffold which increases the surface area for fluorescent dye conjugation (Wang et al., 2012; Gross-Thebing et al., 2014). Because of these advantages, RNAscope is a good tool to quantify markers of neurotransmitter identity in specific cells that can be genetically targeted and therefore identified by IHC. This combined RNAscope and IHC assay was used for all the following experiments.

RNAscope reveals Neurotransmitter Identity in LNv Neurons of the Clock Network.

I first examined neurotransmitter identity of LNv neurons of the clock network. These cells were identified by labeling them with fluorescent antibodies that targeted the cell-type specific marker PDF. The two remaining fluorescent channels available on our microscope were used to visualize pairwise probes of VAChT, Gad1, and VGlut by RNAscope (figures 2a-c). In each experiment, RNAscope signal shows up as puncta that can be quantified. Regions of Interest (ROIs) were drawn around the labeled neurons and the puncta within each ROI were counted to determine gross expression levels of the respective neurotransmitter marker. Only the puncta present in the same stacks as the neurons were included. During the analysis, I did not attempt to distinguish between small and large LNvs (sLNvs and lLNvs). However, the data

is likely more representative of the lLNvs as they were more easily labeled than the sLNvs. It's unclear why this is the case, but it may be because the lLNvs are larger and thus have more PDF for IHC to recognize.

Using this approach, I quantified the percentage of LNv neurons that express each neurotransmitter marker, as depicted in figure 2d. Using a minimal detection threshold of 1 molecule of transcript/cell, VAChT and VGlut were individually represented in ~85-95% of LNvs, whereas Gad1 was represented in only ~35% of examined neurons. Even when using a stricter threshold of 5 molecules of transcript/cell, the same general trend was observed, albeit with slightly lower percentages of neurons considered positive for each marker. Additionally, I also found that individual LNvs co-express pairs of neurotransmitter markers, as depicted in figure 2e. LNvs most commonly co-expressed VAChT and VGlut, however, I also detected Gad1 with either VAChT or VGlut as well.

These results are surprising because all three neurotransmitters were well represented in LNv neurons. This suggests that there may be functional subsets of these neurons that have yet to be discovered. Because animals for these experiments were not entrained to a strict light/dark cycle, it is also possible that heterogeneity of marker expression reflects time-of-day-dependent changes in use of different neurotransmitters over the 24 hour circadian cycle. More experiments will need to be conducted to test this hypothesis.

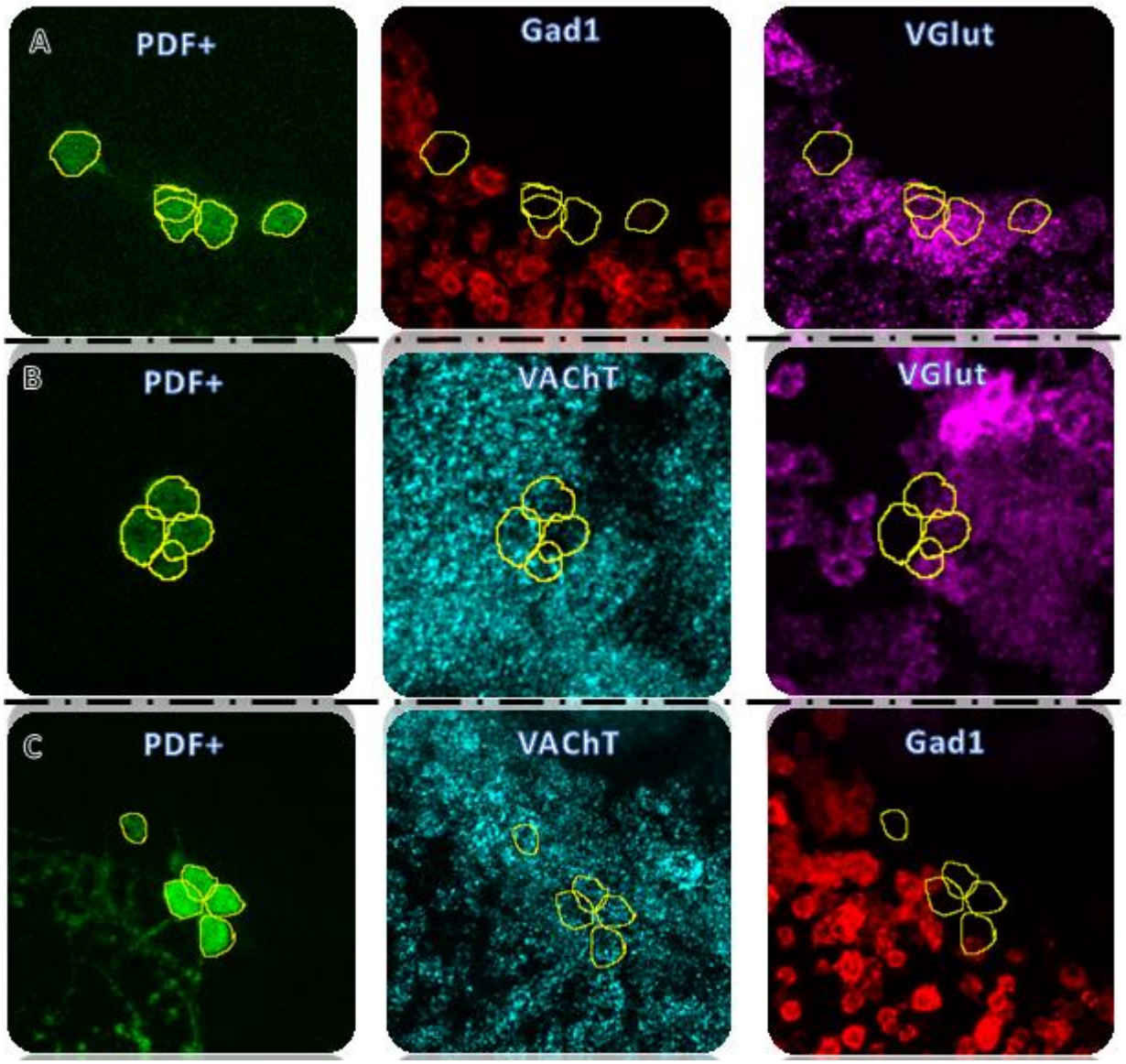
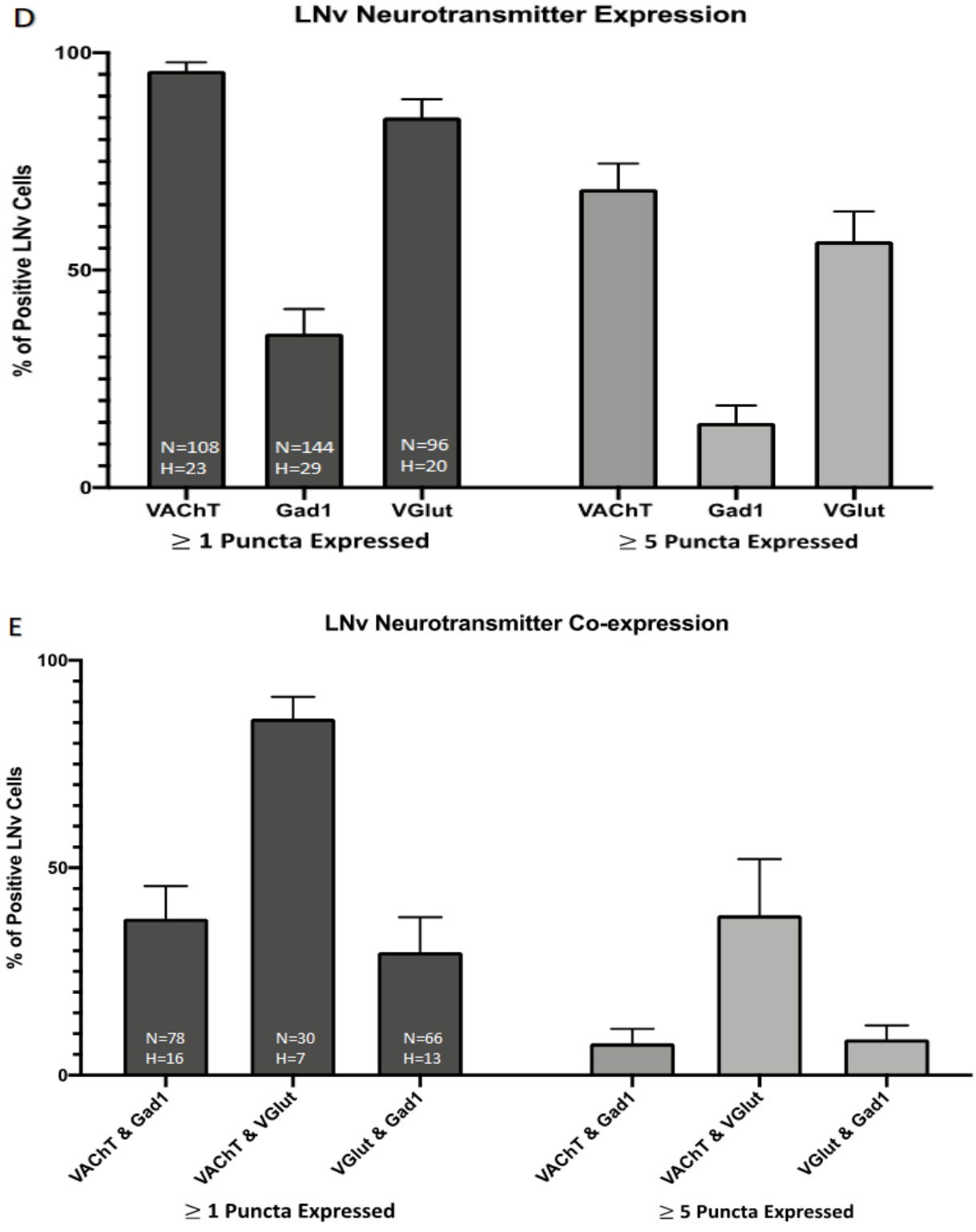


Figure 2: RNAscope data suggests LNvs express VAChT, Gad1, and VGlut transcripts. For all panels, ROIs are drawn and max projections of only the stacks with PDF+ neurons are shown. Representative example are shown of RNAscope and IHC being used to label (A) LNv neurons, Gad1, and VGlut (B) LNv neurons, VAChT, and VGlut (C) LNv neurons, VAChT, and Gad1. (D) Average percent of LNv neurons that express ≥1 vs. ≥5 detectable transcripts of the indicated marker. (E) Average percent of LNv neurons that co-express ≥1 vs. ≥5 detectable transcripts of the indicated marker. N is equal to the number of neurons. H is equal to the number of hemispheres. RNAscope and imaging done by Dr. Meilin Wu.

Figure 2 Continued



RNAscope reveals Neurotransmitter Identity in DN1p Neurons of the Clock Network.

I then examined the neurotransmitter identity of DN1p neurons of the clock network. To isolate these neurons, GFP was expressed under control of the DN1p-specific driver clock 4.1m-Gal4. I then used IHC to label the GFP in DN1p neurons as shown in figures 3a and 3b. Just as I did for LNv neurons, I performed RNAscope on pairwise combinations of the three neurotransmitter markers. I then drew ROIs around the GFP labelled cells and quantified marker expression in them. Representative images for pairwise comparisons of neurotransmitter markers in GFP-labeled neurons are shown in figures 3c and 3d.

Using a minimal detection threshold of 1 molecule of transcript/cell, I found that VGlut and VAChT were expressed in >80% of DN1p neurons. Using a more conservative detection threshold of 5 molecules of transcript/cell, VAChT representation dropped to ~50% of neurons, whereas VGlut representation remained relatively unchanged. Just as I saw for LNvs, VAChT and VGlut co-expression appeared to be common in DN1p neurons. ~75% of neurons had at least 1 molecule of both VAChT and VGlut, and ~38% of neurons had at least 5 molecules of each transcript. These results are depicted in figures 3e and 3f.

Previous studies have suggested that DN1p neurons use glutamate to communicate with LNv neurons to modulate rhythmic behavioral patterns in Drosophila (Hamasaka et al., 2007). The hypothesis that DN1p neurons are glutamatergic is further supported by my RNAscope data. However, it was surprising to find VAChT also expressed in many DN1p neurons. This result suggests that some DN1p neurons have dual neurotransmitter identity, though the functional relevance of this finding is unclear. It is possible that producing both ACh and glutamate could be important for tuning cholinergic and glutamatergic feedback to other clock neurons such as LNvs. Alternatively, the balance between these two neurotransmitters may convey time of day information to other areas of the brain. Notably, DN1p neurons have

been proposed to function as just such an interface (Collins et al., 2012), though without invoking release of multiple neurotransmitters. In either case, my data suggest that there may be a functional subset of DN1p neurons that have yet to be discovered. My data also raise the possibility that DN1p neurons have heterogeneity in usage of neurotransmitters over the 24 hour circadian cycle. Follow up experiments will be required to address these possibilities more directly.

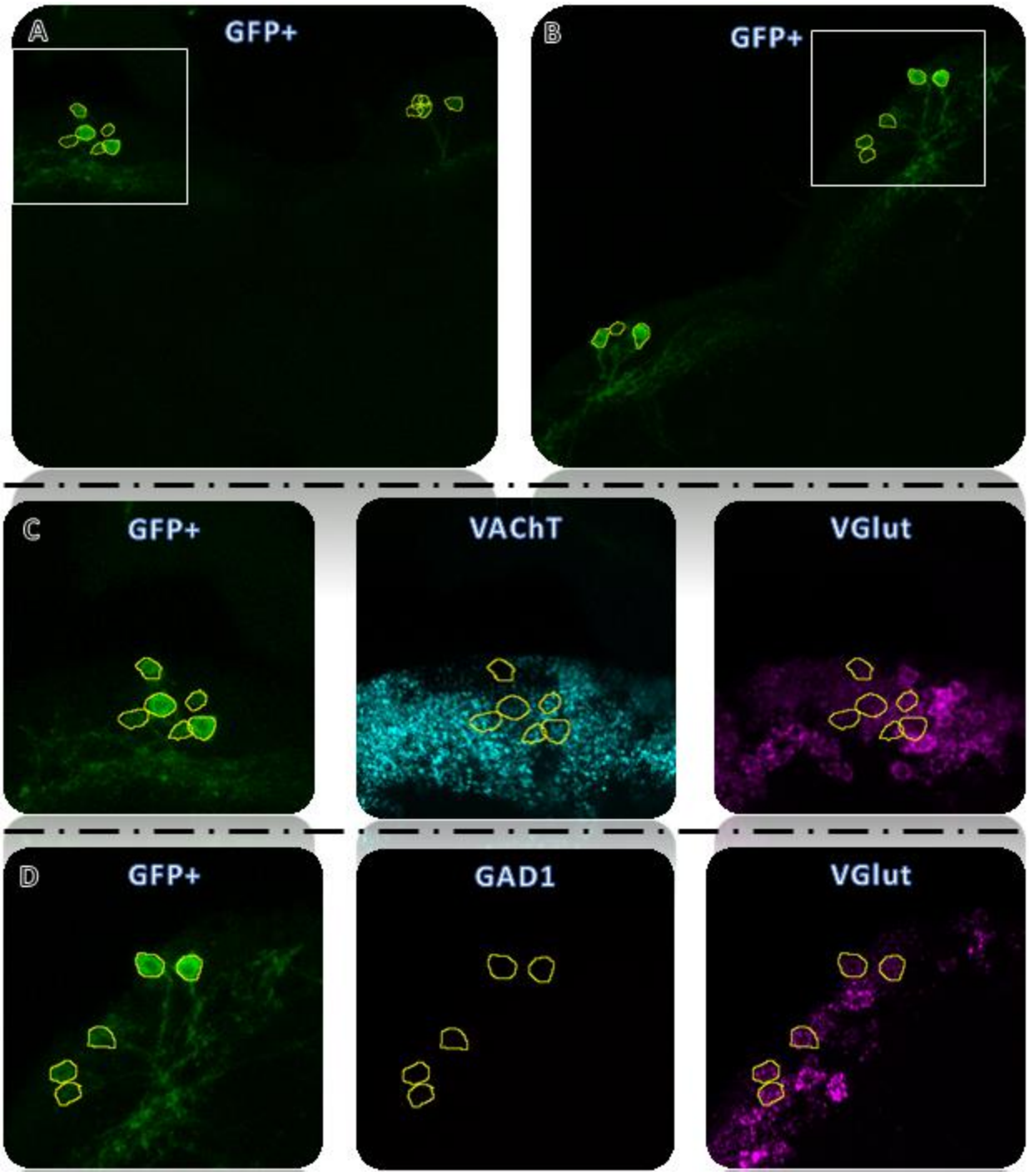
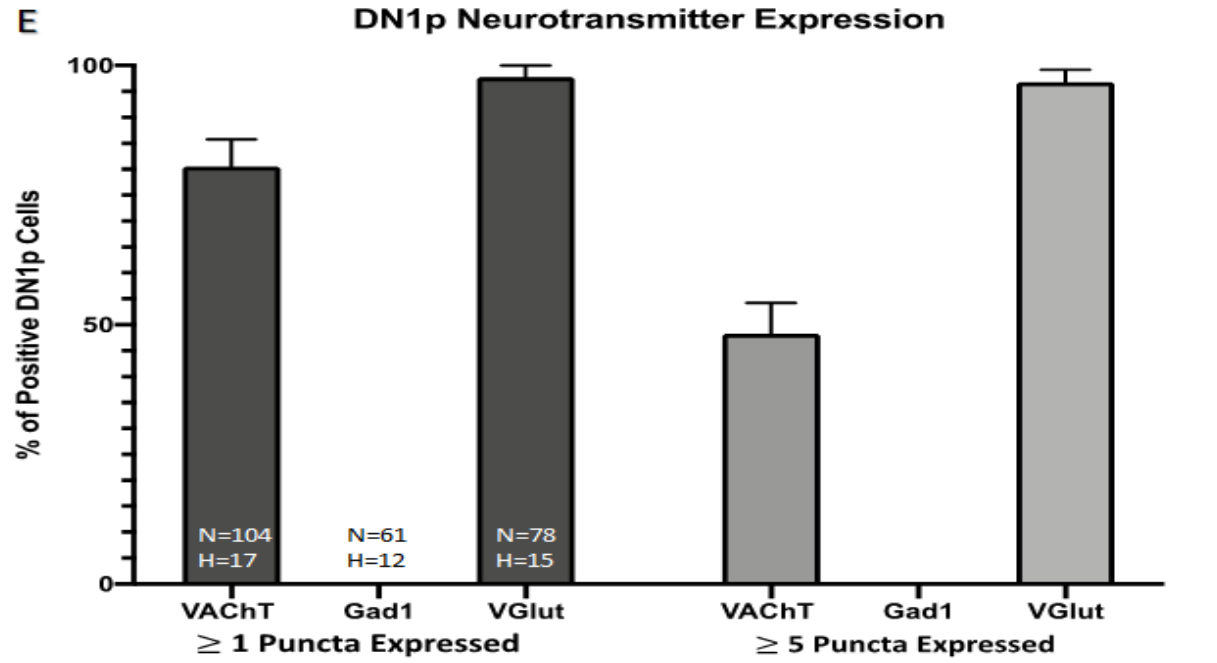
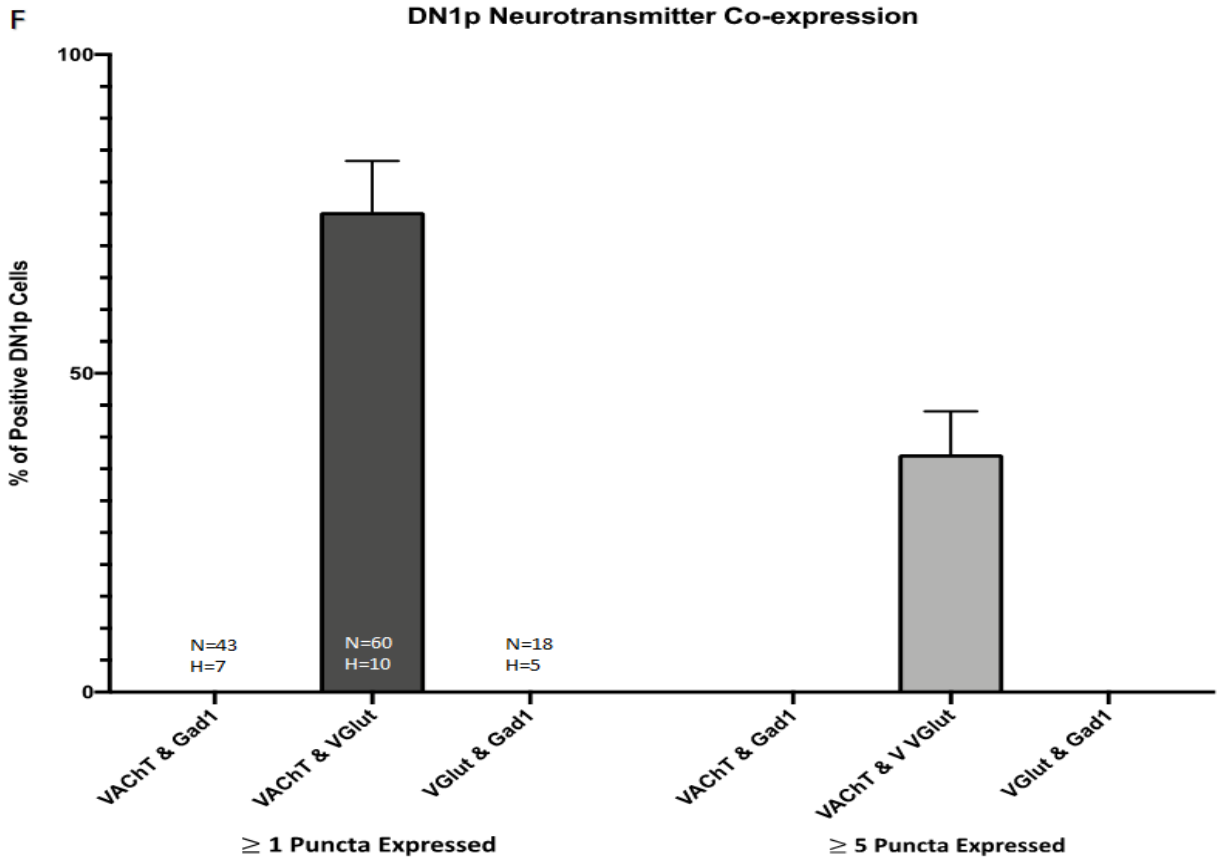


Figure 3: RNAscope data suggests DN1p neurons co-express VAChT and VGlut transcripts. For all panels, ROIs are drawn and max projections of only the stacks with GFP positive neurons are shown. (A)(B) Both hemispheres of a fly brain with DN1p neurons labeled by GFP are shown. A white box is drawn around the hemisphere which is zoomed in to show (C) DN1p neurons, VAChT, and VGlut and (D) DN1p neurons, Gad1, and VGlut. (E) Average percent of DN1p neurons that express ≥1 vs. ≥5 detectable transcripts of the indicated marker. (F) Average percent of DN1p neurons that co-express ≥1 vs. ≥5 detectable transcripts of the indicated marker. N is equal to the number of neurons and H is equal to the number of hemispheres. RNAscope and imaging done by Dr. Meilin Wu.

Figure 3 Continued





RNAscope reveals Neurotransmitter Identity in 53D10 Neurons of the AMMC.

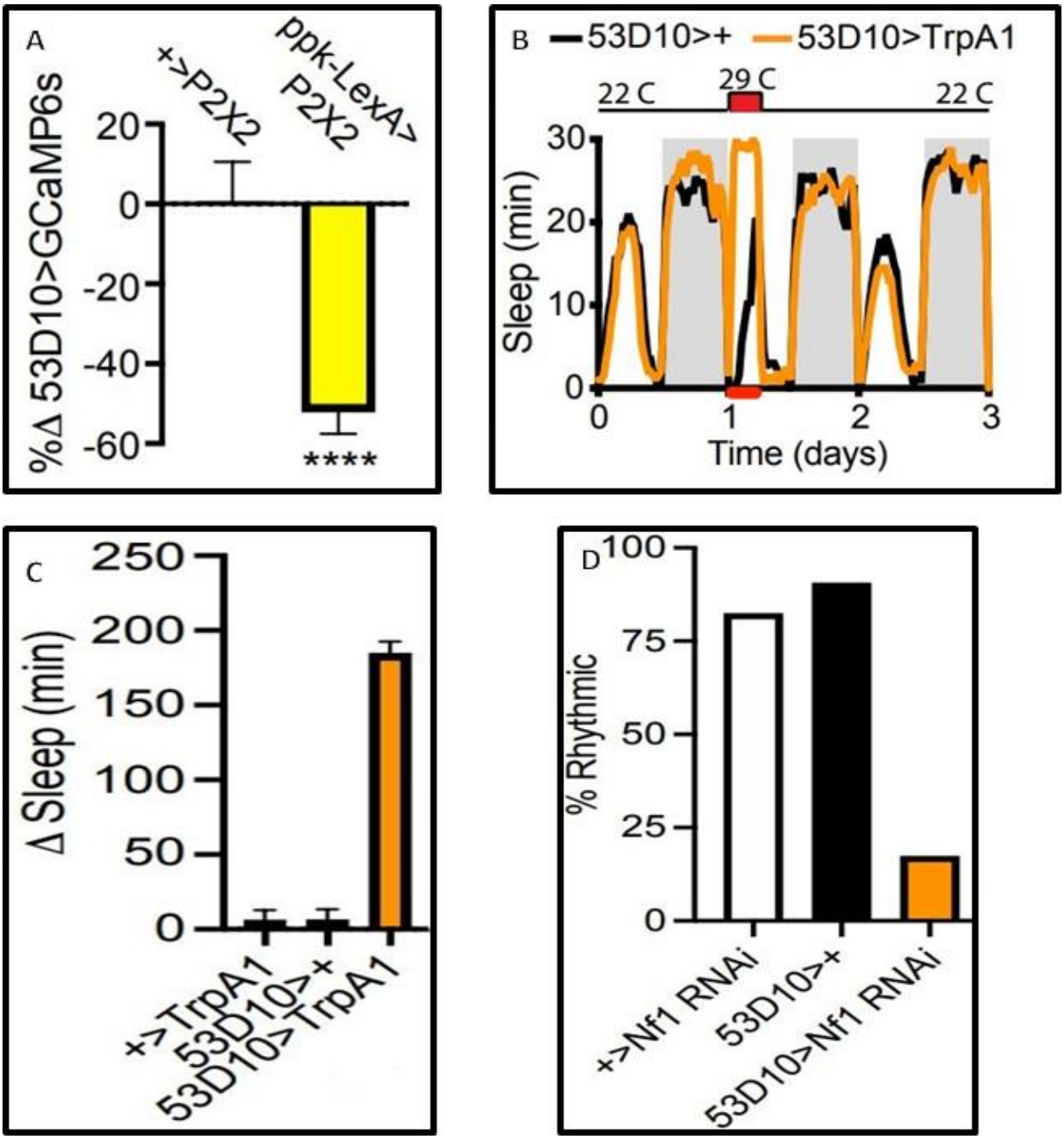
As mentioned in the introduction, unpublished data from my lab suggests that 53D10 neurons in the AMMC may be responsible for integrating externally derived sensory signals with internal cues from the clock network and perhaps other neurons. This hypothesis is supported by several findings. First, chemogenetic activation of wake-promoting sensory ppk neurons acutely inhibits 53D10 neurons (Supplemental Figure 1a), whereas direct thermogenetic activation of 53D10 neurons promotes sleep (Supplemental figures 1b and 1c). These results suggest that 53D10 neurons function as an arousal-regulating gate that is opened by activity of sensory neurons. Second, knockdown of the Nf1 gene in 53D10 neurons causes locomotor arrhythmicity (supplemental figure 1d), suggesting that the circadian clock may also regulate the opening of such a gate. Because of this data, we believe that 53D10 neurons are crucial for regulating sleep/wake cycles and function as an output pathway from the clock network. As such, it is important to understand the neurotransmitter identity of these neurons.

To isolate these neurons, GFP was expressed under control of the 53D10 specific driver 53D10 Gal4. I then used IHC to label the GFP in 53D10 neurons as shown in figures 4a and 4b. Just as I did for LNv and DN1p neurons, I performed RNAscope on pairwise combinations of the three neurotransmitter markers (except VAChT and VGlut). I then drew ROI's around the GFP labelled cells and quantified the fraction of them that expressed each neurotransmitter marker. Representative images for these pairwise comparisons are shown in figures 4c and 4d.

Using this approach, I identified at least one molecule of each neurotransmitter marker in over half of all 53D10 neurons. Even when using a more conservative estimate of neurotransmitter identity (5 molecules/cell), each neurotransmitter could be assigned to ~30-50% of all 53D10 neurons. Additionally, both VAChT and Gad1 as well as VGlut and Gad1 were found to be co-expressed in these neurons. These results are depicted in figure 4e and 4f.

While performing this analysis, I noticed that the 53D10 neurons could be further classified into medial or lateral clusters, and that these clusters expressed different neurotransmitter markers. With further analyses, I found that medial 53D10 neurons express both Gad1 and VGlut whereas the lateral 53D10 neurons expressed Gad1 and VAChT. Low levels of VAChT were found in the medial neurons, and similarly low levels of VGlut were found in the lateral neurons, however, these were rare occurrences. These results are depicted in figures 4g and 4h. The medial and lateral clusters can be visualized in figures 4a and 4b where a dashed line separates the two clusters.

The clear distinction in neurotransmitter markers used by the medial and lateral 53D10 neurons suggests heterogeneity in their functions. However, we don't yet know what these functions are and how they relate to regulation of the sleep/wake cycle and sensory gating. It is also unclear what role, if any, dual neurotransmitter identity of 53D10 neurons plays in these functions.



Supplemental Figure 1: 53D10 neurons in the AMMC may be responsible for integrating external stimuli with internal cues from the clock network. (A) Chemogenetic activation of wake promoting ppk neurons inhibits 53D10 neurons. (B)(C) Thermogenetic activation of 53D10 neurons acutely increases sleep. (D) Knock down of Nf1 in 53D10 drivers causes arrhythmicity. Figures kindly provided by Dr. Joydeep De and Dr. William Joiner.

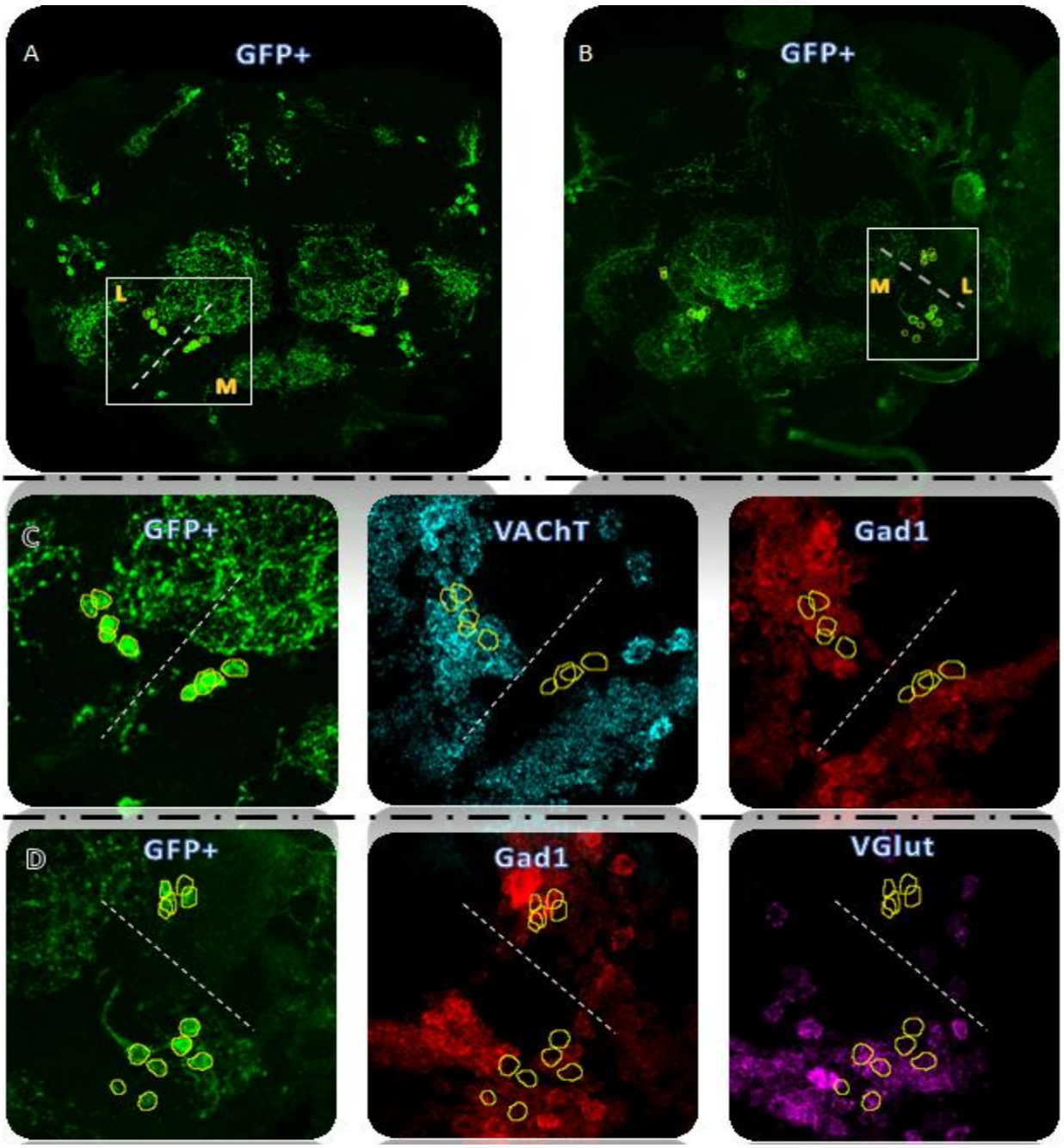
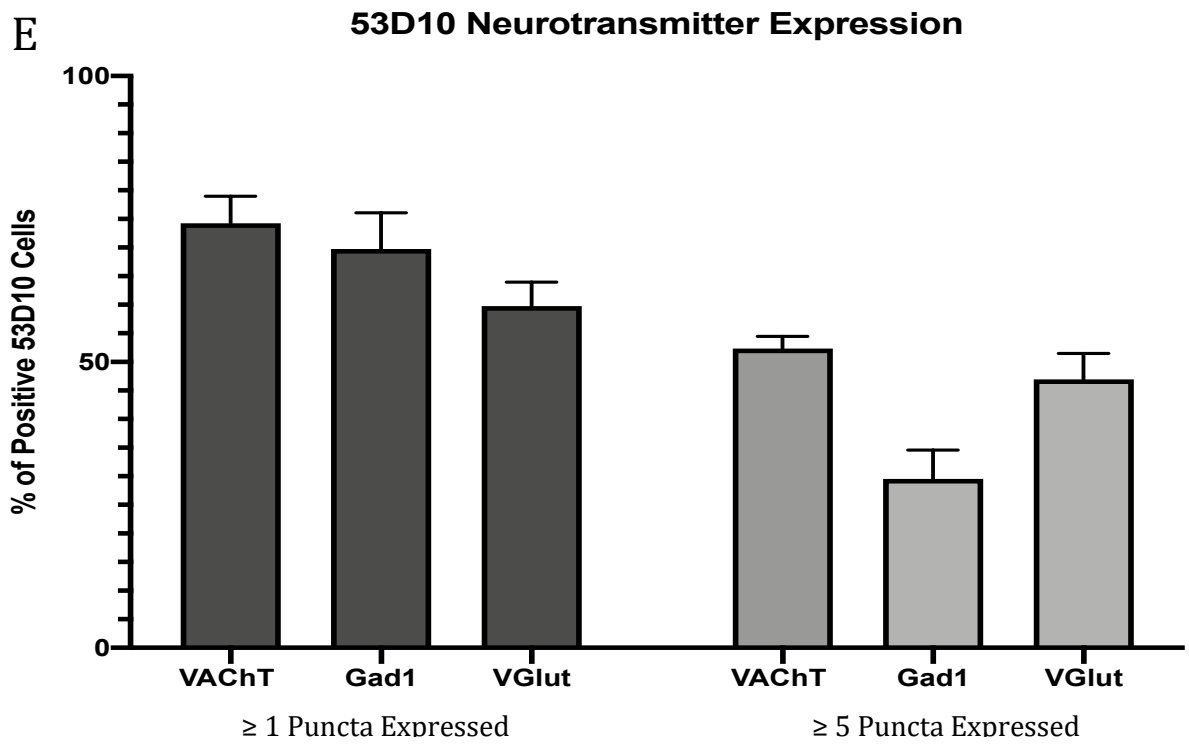
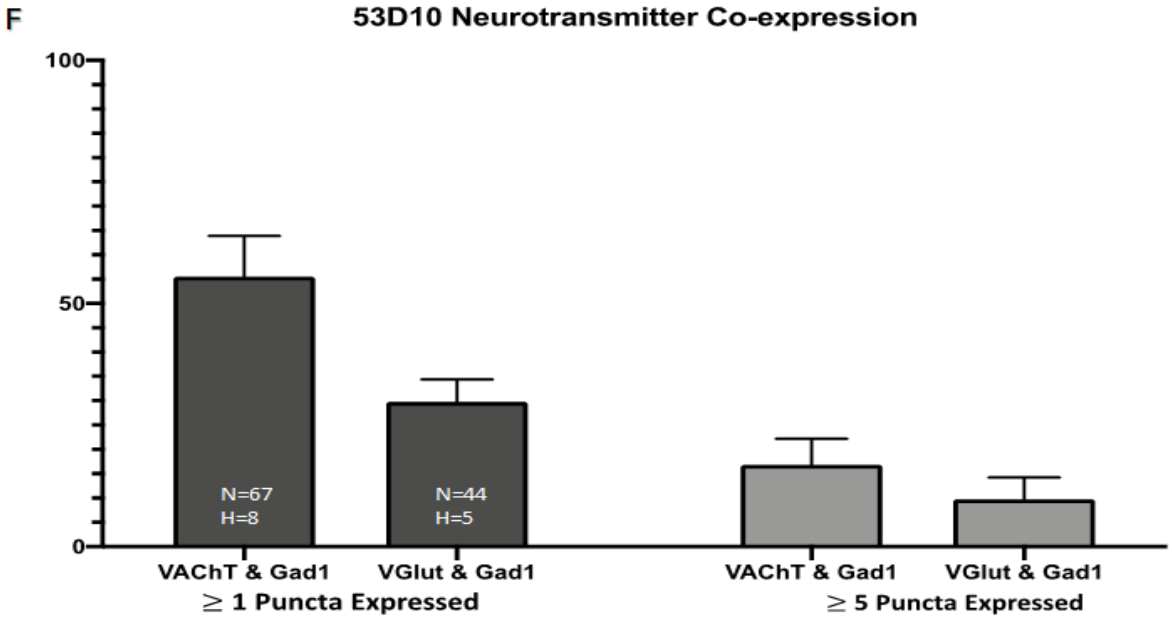


Figure 4: RNAscope data suggests medial and lateral 53D10 neurons express different combinations of neurotransmitter-specific transcripts. For all panels, ROIs are drawn and max projections of only the stacks with GFP positive neurons are shown. (A)(B) Full fly brain with 53D10 neurons labeled by GFP are shown. A white box is drawn around the hemisphere which is zoomed in to show (C) 53D10 neurons, VAChT, and Gad1 and (D) 53D10 neurons, Gad1, and VGlut. A line is drawn on all panels to distinguish between medial and lateral groups of 53D10 neurons. (E) Average percent of 53D10 neurons that express ≥1 vs. ≥5 detectable transcripts of the indicated marker. (F) Average percent of 53D10 neurons that co-express ≥1 vs. ≥5 detectable transcripts of the indicated marker. (G) Average percent of neurons that express ≥1 detectable transcripts of the indicated marker in medial and lateral 53D10 clusters. (H) Average percent of neurons that express ≥5 detectable transcripts of the indicated marker in medial and lateral clusters.

Figure 4 continued





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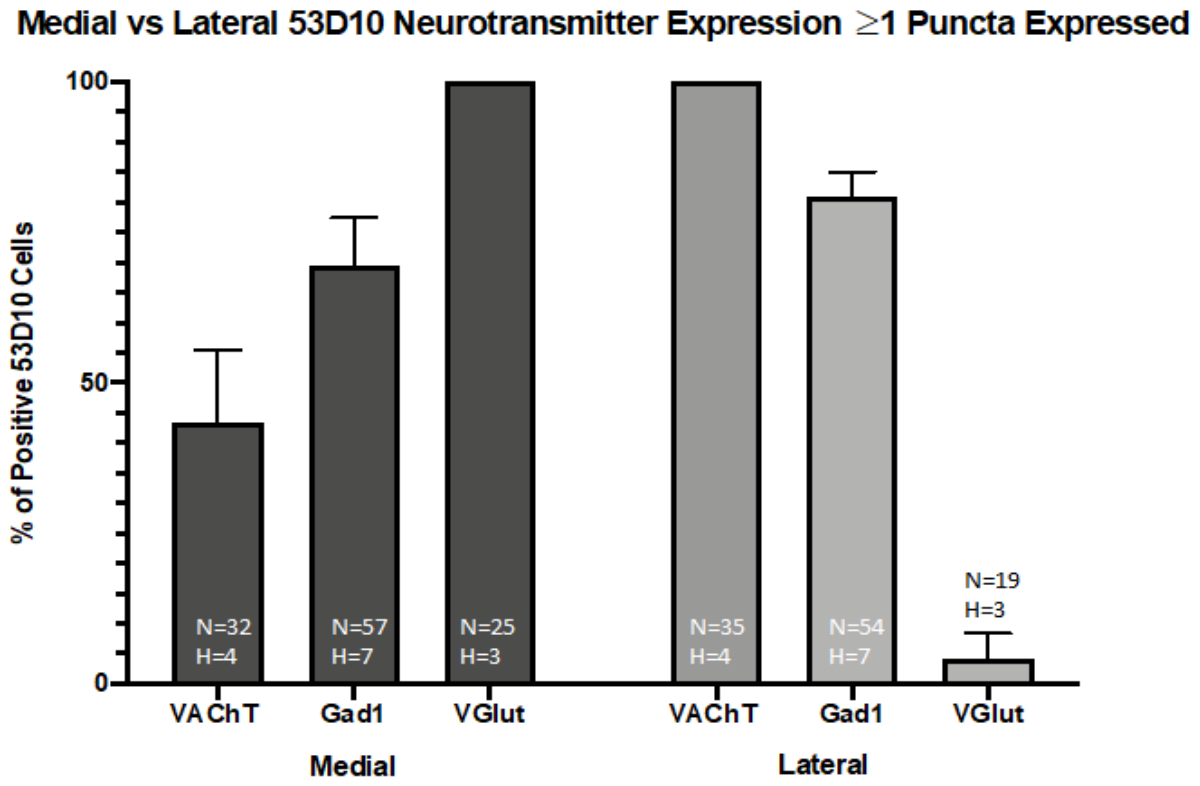

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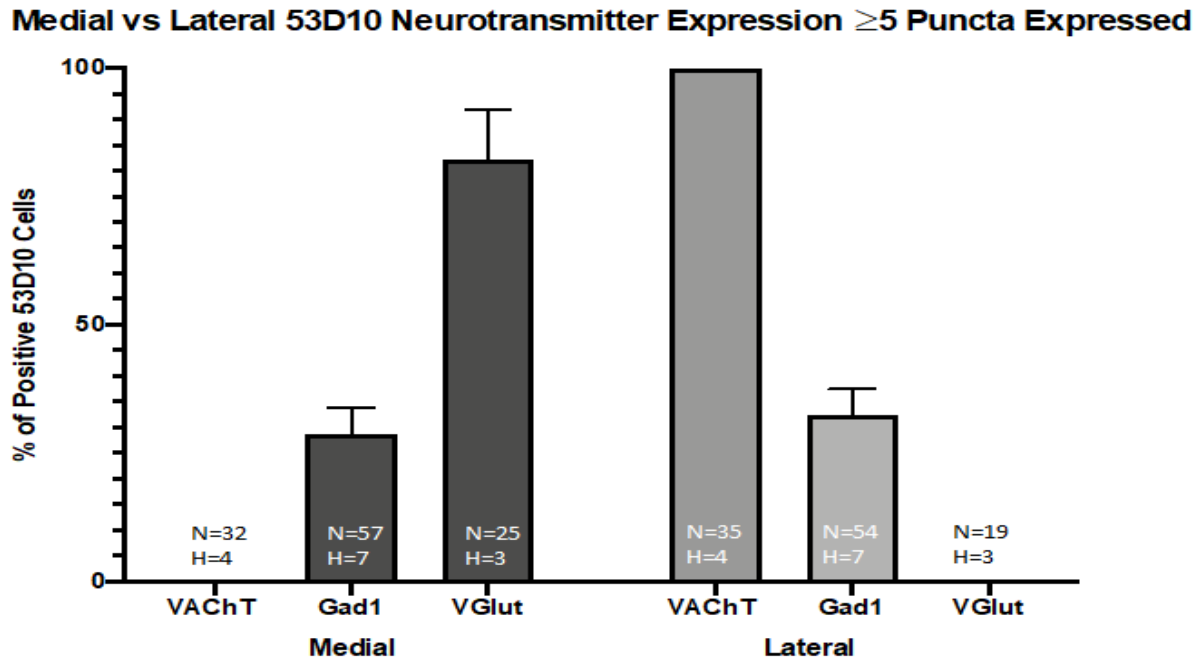

Preliminary Studies of Neurotransmitter Plasticity in the Clock Network by RNAscope.

Neurotransmitter switching has been described under several conditions in mammals, but it is unknown if a similar phenomenon can occur in Drosophila. To address this question I used RNAscope to determine if changes in external stimuli could cause changes in the neurotransmitter markers expressed by LNv neurons of the clock network. Specifically, I tested whether exposure to light vs dark in otherwise entrained flies could reveal neurotransmitter plasticity. For these experiments animals were split into two groups, DL and DD. Both groups were kept in two consecutive cycles of 12 hours of light followed by 12 hours of darkness for a total of 48 hours to entrain circadian clocks. At the end of the 48 hours, the DL group was kept in light for an additional 12 hours whereas the DD group was kept in darkness for those 12 hours. At the end of the final 12 hours, the animals were dissected and fixed. Figure 5i depicts the DL entrainment scheme and figure 5j shows the DD entrainment scheme.

Once again, I performed RNAscope on whole-mount fly brains with pairs of neurotransmitter markers, and I labeled LNv neurons with a fluorescent antibody against the cell-type specific marker PDF. I then drew ROIs around the GFP-labeled cells and quantified the fraction per hemisphere that expressed each neurotransmitter marker. Figures 5a-d show representative images of LNv neurons and their pairwise neurotransmitter marker probes for DD and DL groups.

Using this approach, I discovered that expression levels of VAChT and Gad1 remained relatively unchanged between the DL and DD groups. However, I found an increase in VGlut expression in the DD animals. This data is depicted in figures 5e-h. The increase in VGlut-positive neurons was not statistically significant but was trending towards it. However, the sample size was small. Thus, repeating the experiments will be important for determining whether glutamatergic identity is plastic within LNv neurons. I also found an increase in the

percent of LNvs that co-expressed VAChT and VGlut in DD animals. At the ≥1 stringency level, this change was not significant. However, at the ≥5 stringency level, this change was statistically significant. Because the sample size was small, the experiments need to be repeated to confirm these results. This data is depicted in figures 5i-5j.

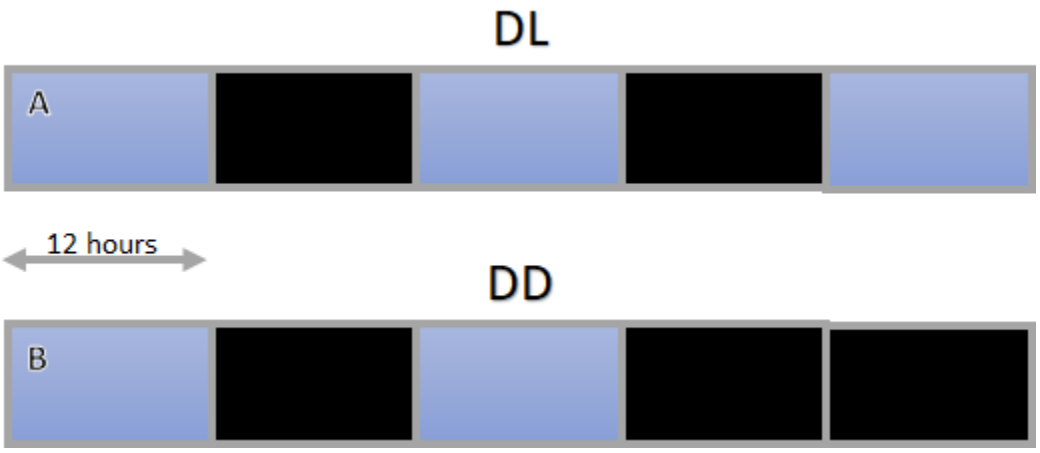

Figure 5: RNAscope preliminary data suggests changes in light/dark entrainment cycles may have an effect on neurotransmitter expression in LNv neurons. A diagram depicting the entrainment scheme for (A) DL and (B) DD flies. Dark boxes represent 12 hours of complete darkness, and light blue boxes represent 12 hours of light. Flies were dissected and fixed immediately after the last 12-hour cycle. For all panels, ROIs are drawn and max projections of only the stacks with PDF+ neurons are shown. Representative examples are shown of RNAscope and IHC being used to label LNv neurons, VAChT, and Gad1 in (C) DL conditions and (D) in DD conditions. Representative examples are shown of RNAscope and IHC being used to label LNv neurons, VAChT, and VGlut in (E) DL conditions and (F) in DD conditions. (G) Average percent of LNv neurons that express ≥1 detectable transcripts of the indicated marker in DL and DD conditions. (H) Average percent of LNv neurons that express ≥5 detectable transcripts of the indicated marker in DL and DD conditions. (I) Average percent of LNv neurons that co-express ≥1 detectable transcripts of the indicated marker in DL and DD conditions. (J) Average percent of LNv neurons that co-express ≥5 detectable transcripts of the indicated marker in DL and DD conditions. Error bars represent the standard error of the mean. N is equal to the number of neurons and H is equal to the number of hemispheres. “n.s.” is not significant, and “*” is significant by ANOVA with Tukey’s multiple comparison post-test.

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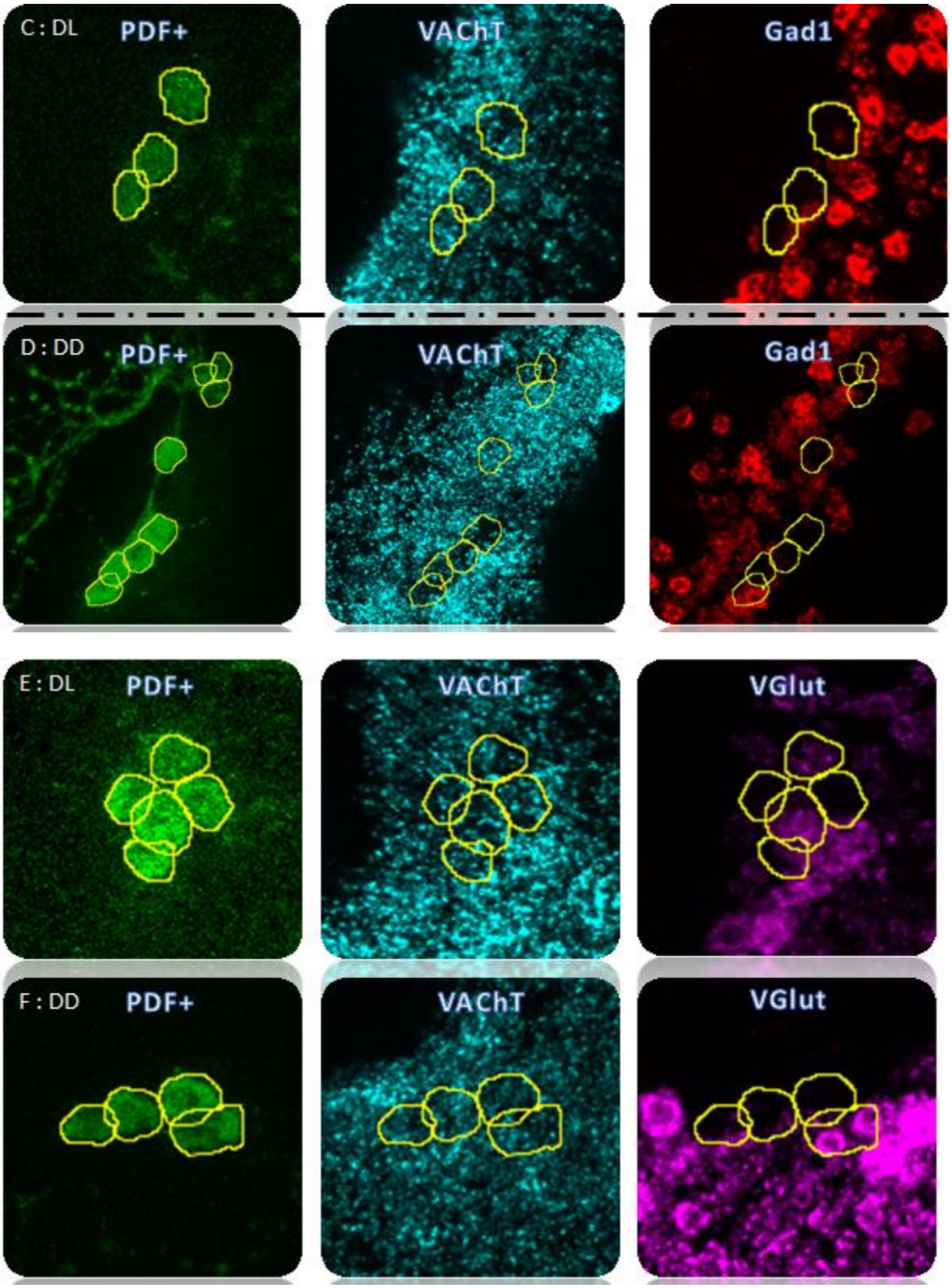

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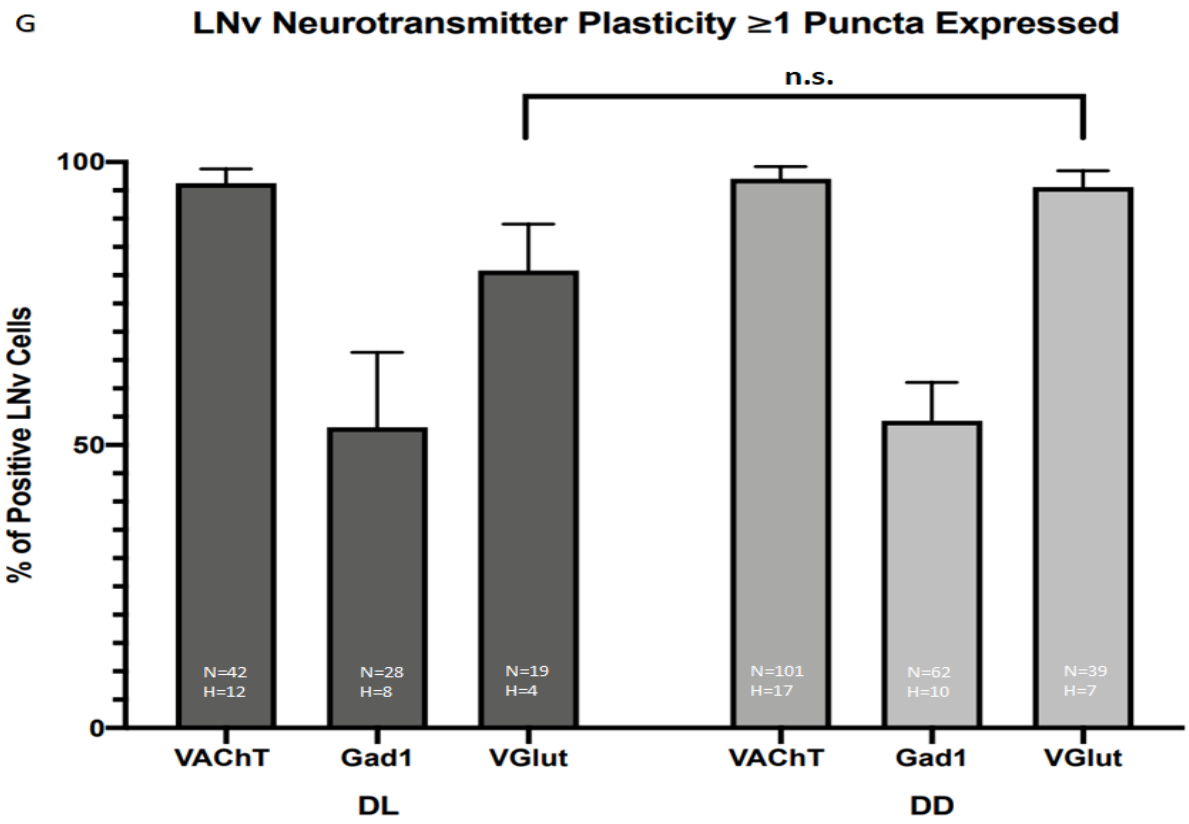

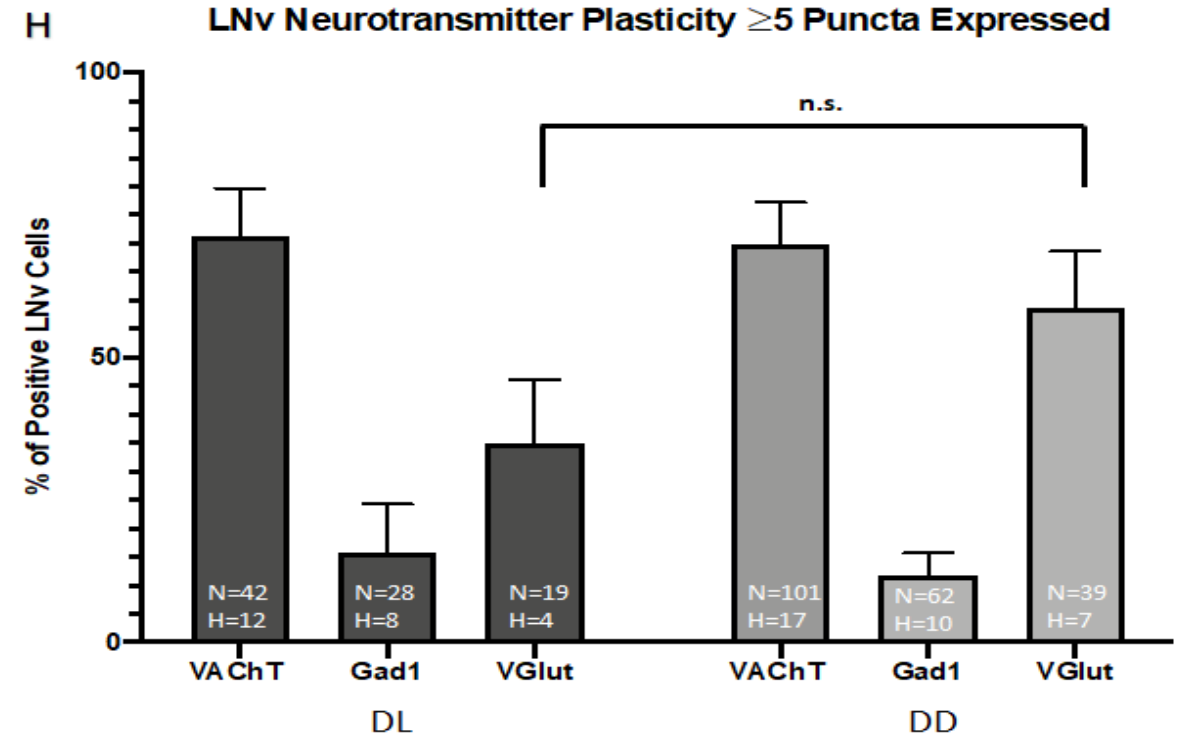

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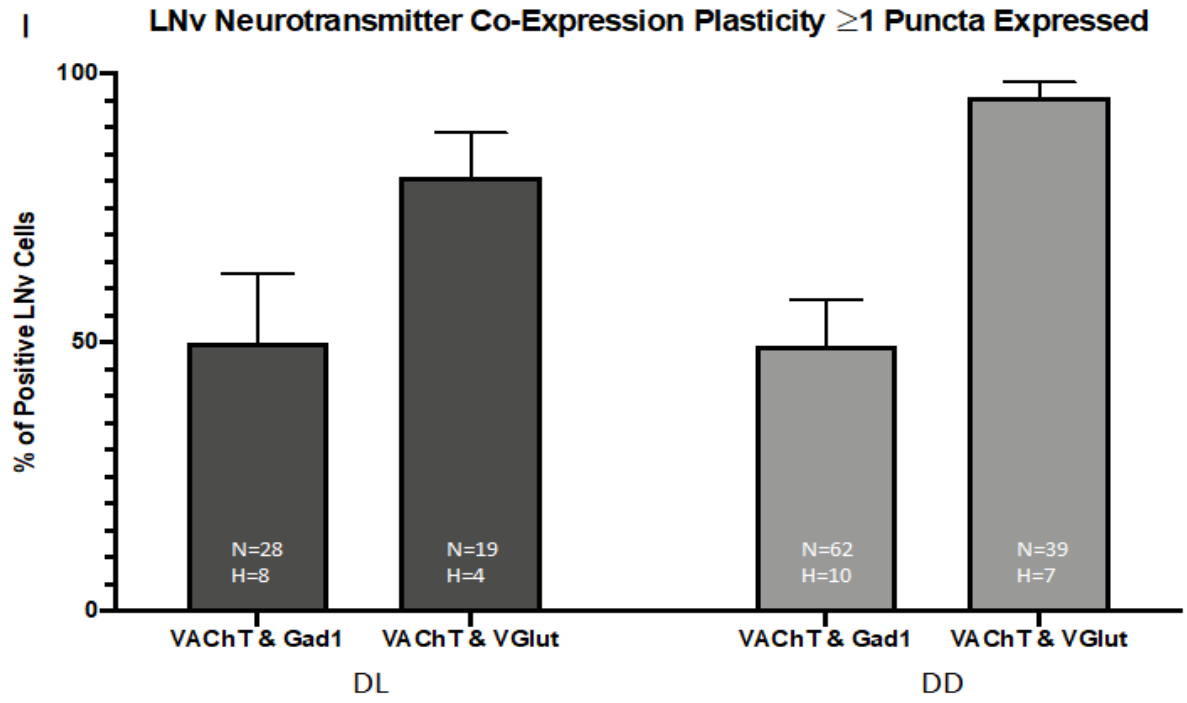

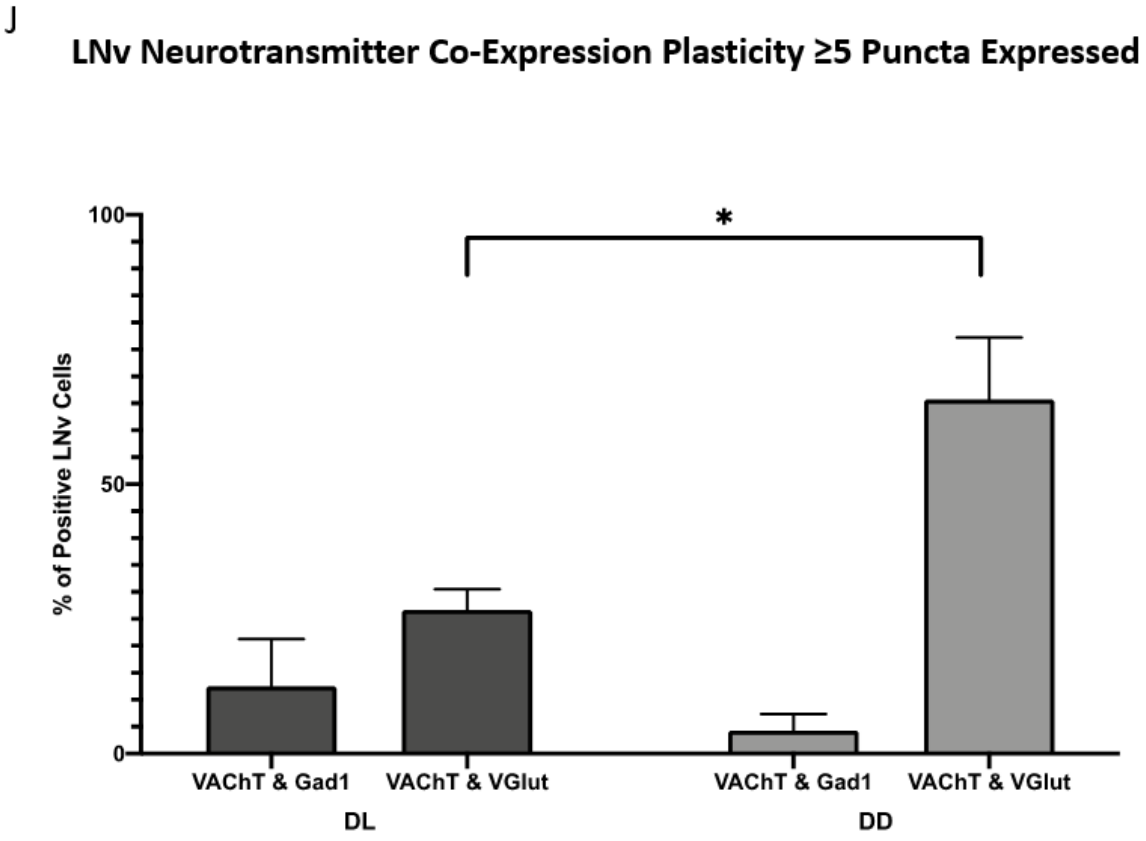

Discussion:

In this thesis I used RNAscope to conduct experiments that can provide us with a better understanding of the neurotransmitter identity of neurons that regulate the sleep/wake cycle. Specifically, I wanted to investigate the neurotransmitter identity of two populations of neurons in the clock network, LNv and DN1p, and one set of neurons in the AMMC called 53D10. The LNv neurons were targeted because all three neurotransmitters I examined have been suggested to be present in the clock network, though their precise sources and functions have not been thoroughly investigated. For example, LNv neurons are known to have glutamate and GABA receptors and are responsive to ACh (Hamasaka et al., 2007; Lelito et al., 2012). Some of their glutamatergic inputs are believed to come from the DN1p neurons (He et al., 2017); however, the origins of the cholinergic and GABAergic signals are still being explored. I also tested whether LNv and DN1p neurons could be the origin of cholinergic or GABAergic signaling in the clock network.

Using IHC to label specific cell types within the circadian clock network, and RNAscope to lab VAChT, VGlut, and Gad1 across the brain, I found that LNv neurons express markers for all three classical small molecule neurotransmitters. VAChT and VGlut transcripts were expressed at high levels while Gad1 transcripts were expressed at lower levels. This suggests that the LNv neurons are primarily cholinergic and glutamatergic, but also a substantial minority are GABAergic. These results suggest that there may be functional subsets of LNv neurons that have yet to be discovered. For example, we know that LNv neurons project to at least two places: medially across the brain and dorsally towards the DN1ps. There exists the possibility that these different projections utilize different neurotransmitters to perform unique roles. Even within a specific projection, there may be heterogeneity in neurotransmitter release and function. Follow up experiments will have to be conducted which investigate this more directly. One way to do this might be to use RNAscope to determine if corresponding neurotransmitter receptors

are expressed in downstream targets of LNvs. However, this would be complicated by the possibility that the receptors might be present to respond to inputs from neurons other than LNvs.

Another result that stood out from the above experiment was that a substantial percentage of LNvs expressed markers for two different neurotransmitters. Although VAChT and VGlut represented the bulk of dual labeling, Gad1 was also detected in VAChT-positive and with VGlut-positive neurons. The relevance of this dual labeling is unclear. However, it could represent a shift in neurotransmitter identity across the circadian cycle in animals that were unentrained and therefore sampled at random subjective times of day. To address this possibility it would be worthwhile to conduct follow up experiments that repeat the measurements I made but in entrained animals sampled at different subjective times of day (e.g. ZT or CT0, 6, 12, and 18).

RNAscope also helped clarify the neurotransmitter identity of DN1p neurons of the clock network. Previous data suggests that DN1p neurons are primarily glutamatergic (Hamasaka et al., 2007). However, I found good representation of VAChT in these neurons, suggesting that some of them may also be cholinergic. These results are surprising for two reasons. Firstly, previous studies have not suggested that DN1p neurons are cholinergic, and secondly, this is the first time DN1p neurons have been shown to have the capacity to use more than one small molecule neurotransmitter. As with the LNvs, the detection of both VAChT and VGlut in individual DN1ps could reflect a physiological function for two neurotransmitter systems in clock network function or in clock output. Alternatively, it could reflect a shift in neurotransmitter identity across the circadian cycle in unentrained animals whose clocks are therefore in mixed phases. Further experiments would have to be conducted to answer these questions more directly. In particular, the experiments I performed should be repeated at different times of the day with animals on fixed light:dark cycles.

RNAscope also helped clarify the neurotransmitter identity of 53D10 neurons of the AMMC. The data show that all three of the neurotransmitter markers were well represented in these neurons. Overall, the 53D10 neurons expressed VAChT, VGlut, and Gad1 relatively evenly. However, it became clear that there were two distinct clusters of 53D10 neurons. The medial 53D10 neurons expressed VGlut and Gad1 whereas the lateral 53D10 neurons expressed VAChT and Gad1. VGlut and VAChT were almost exclusively localized in medial and lateral clusters respectively. Gad1 was evenly localized between the two clusters. This level of heterogeneity suggests that these two clusters may serve different functions and have different downstream targets. Additionally, even within a single cluster, the neurons showed dual neurotransmitter identity which raises the possibility that the relative amounts of each neurotransmitter marker might be defined by activity of the cell's inputs. As indicated in the Introduction, these cells may be effectors of sensory arousal as well as the circadian clock. Thus, it is possible that these signals modulate neurotransmitter identity in 53D10 neurons. Related to this idea, it will also be interesting to determine if 53D10 neurons are subject to activity dependent changes in the neurotransmitter expression levels as has been shown to occur in some mammalian neurons (Spitzer, 2012).

Finally, I used RNAscope to test the possibility of neurotransmitter switching in Drosophila in response to light, which is arguably the most important environmental signal to flies. I found that animals entrained to the same light:dark cycle but exposed to different amounts of light for 12 hours of their last subjective day had different numbers of VGlut-positive neurons. Although the results were not significant, this may have been due to the limited sample size and therefore the experiments need to be repeated. Additionally, at the ≥5 stringency level, I found a statistically significant increase in the percent of LNvs that co-expressed VAChT and VGlut. The trend towards significance suggests that environmental stimuli may be able to induce neurotransmitter plasticity in flies. If this could be demonstrated conclusively it would be

very exciting since it would open up the possibility of using flies to tease apart the genetic underpinnings of neurotransmitter plasticity, which are currently unknown.

While my results using RNAscope provide support for neurotransmitter co-expression and plasticity in Drosophila, there are a few limitations that I would like to acknowledge. Firstly, I couldn't measure actual neurotransmitter levels. Instead, I used RNAscope to measure mRNA transcripts of common markers of neurotransmitters such as VAChT, Gad1, and VGlut. While I expect that expression of these markers translates well into expression of their respective neurotransmitter, I would have to confirm this through a more direct approach. Other members of my lab are currently working on using antibodies against proteins encoded by the markers I assayed, or against the neurotransmitters themselves, to confirm that RNAscope is a good proxy for neurotransmitter identity in flies. Another confounding variable in all but the last experiment of my thesis was the lack of entrainment of my animals. Thus, the LNvs, DN1ps and 53D10 neurons were assayed at mixed times in the animals' subjective time of day. This could be a potential issue since neurotransmitter levels may change throughout the day in relation to the circadian cycle. However, animals in the final experiment of my thesis were in fact entrained to a light:dark cycle prior to dissection. Importantly, the results from this experiment (Figures 5e and 5f) appeared to be qualitatively similar to results in other experiments in which neurotransmitter identity was examined in unentrained animals (Figure 2d). This suggests that time of day may not play a major role in reprogramming neurotransmitter identity. However, additional experiments that directly test this hypothesis will need to be performed. This limitation in my approach does not mean that neurotransmitter co-expression does not exist. While the exact expression levels may vary with the circadian cycle, the expression of multiple markers was still identified using RNAscope. This suggests that co-expression does in fact occur in the neurons that I investigated.

Materials and Methods:

Animal Husbandry and Entrainment

For neurotransmitter identity experiments (Figure 2-4), flies were grown at room temperature and dissected 2 to 4 days after eclosion. Only female flies were dissected.

For neurotransmitter plasticity experiments (Figure 5), age controlled (2-4 day old), female flies were placed into two groups: DL and DD. The two groups were placed in separate incubators with identical 12 hr:12 hr light:dark settings for the first 48 hours. After the 48 hours, the DL group had lights on whereas the DD group was kept in complete darkness. The animals were dissected and fixed on the 60th hour.

LNv neurons were identified by using fluorescent antibodies against the cell type specific marker PDF. DN1p and 53D10 neurons were identified by using the UAS Gal4 system. Clock 4.1m Gal4 (36316), 53D10 Gal4 (45347), and UAS GFP (32188) were obtained from Bloomington Stock Center.

Tissue Processing and Dehydration:

Once the fly brains were dissected, they were fixed in a solution of 4% formaldehyde for 18-24 hours at room temperature (RT) on a rocker at low speed. The brains were then dehydrated by adding different methanol/PBST solutions at RT in the following order: 25% methanol, 50% methanol, 75% methanol, and 100% methanol. The brains were then stored in -20 degrees Celsius until they were needed.

Rehydration and Target Retrieval:

The samples were thawed and rehydrated in different methanol/PBST solutions at RT in the following order: 100% methanol,75% methanol, 50% methanol, 25% methanol. They were

then washed in PBST + 1% BSA for 10 minutes at RT. Afterwards, warm Target Retrieval buffer (ACD) was added, and the samples were placed in a heating block at 100 degrees Celsius for 5.5 minutes. Samples were then washed at RT in PBST + 1% BSA for 1 minute, 100% methanol for 1 minute, PBST + 1% BSA for 10 minutes, and 4% formaldehyde for 25 minutes. The samples were then washed between 1 and 3 times in PBST + 1% BSA at RT for 10 minutes. Afterwards, 2 drops (50 uL) of protease plus (ACD) were added and the samples were incubated at 40 degrees Celsius for 10 minutes. Samples were then washed in PBST + 1% BSA for 10 minutes at RT. 2 drops (50 uL) of probe diluent (ACD) was added for 20 seconds. Finally, the probes were added, and the samples were incubated overnight at 40 degrees Celsius. ACD provided and designed the probes for each targeted sequence. The probes were provided as Channel 1 (C1), Channel 2 (C2) or Channel 3 (C3) depending on the sequence.

Signal Amplification

Samples were washed 2 times in RNAscope Wash Buffer (RWB) (ACD) for 5 minutes at RT and then washed in 2 drops (50 uL) of AMP 1 (ACD) for 30 minutes at 40 degrees Celsius. Samples were then washed 2 times in RWB for 5 minutes at RT and then washed in 2 drops (50 uL) of AMP 2 (ACD) for 30 minutes at 40 degrees Celsius. Samples were then washed 2 times in RWB for 5 minutes at RT and then washed in 2 drops (50 uL) of AMP 3 (ACD) for 15 minutes at 40 degrees Celsius. Finally, the samples were washed in RWB twice for 5 minutes at RT.

HRP Reaction

2 drops (50 uL) of channel specific RNAscope Multiplex FL v2 HRP reagent (ACD) was added for 15 minutes and the samples were incubated at 40 degrees Celsius. The samples were then washed twice with RWB for 5 minutes at RT. The respective wavelength specific opal dye (Akoya Bioscience) (1:2000) was then added and the samples were incubated at 40 degrees Celsius for 30 minutes. Samples were then washed twice with RWB for 5 minutes at

RT. 2 drops (50 uL) of Multiplex vs2 HRP-Blocker (ACD) was then added and the samples were incubated at 40 degrees Celsius for 15 minutes. Finally samples were washed twice with RWB for 5 minutes at RT. This process was repeated for all other probes used by using the probe channel specific Multiplex FL v2 HRP reagent and opal dyes.

Immunohistochemistry

Samples were then washed 3 times in PBST for 10 minutes at RT and blocked in 10% normal goat serum + 1% BSA/0.3% Triton X-100 for at least one hour in RT. After blocking, a solution of primary antibody diluted in blocking solution was added to the samples. For Goat anti GFP the dilution was 1:1000 and for Goat anti PDF the dilution was 1:100. The samples were incubated for at least 18 hours in 4 degrees Celsius. Samples were then washed 4 times in PBST for 15 minutes and then incubated in a solution of secondary antibody diluted in blocking solution. Goat anti Rabbit 1:1000 was used for GFP staining and Goat anti Mouse 1:650 used for PDF staining. Samples were incubated for at least 18 hours at 4 degrees Celsius. Samples were then washed 2 times in RWB for 5 minutes at RT. The respective wavelength specific Opal dye (Akoya Bioscience) (1:1000) was then added and the samples were incubated for 30 minutes at 40 degrees Celsius. Samples were then washed 2 times in RWB for 5 minutes at RT. 2 drops (50 uL) of Multiplex v2 HRP-Blocker (ACD) were then added and the samples were incubated at 40 degrees Celsius for 15 minutes. Finally samples were washed 2 times in RWB for 5 minutes at RT and then mounted on slides.

Imaging

Brains were imaged using a Leica Confocal Microscope. All laser settings and gains were consistent between experiments. The lasers used were Argon, DPSS561, HeNe594, and HeNe 633. Magnification was 40x.

Analysis

All analysis was done on FIJI and was conducted by hemisphere. Only hemispheres with at least 3 neurons were used. ROIs were drawn around cell bodies labelled with GFP or PDF. Only the stacks with IHC signals were used for analysis. The number of puncta within a given ROI for its respective sub-stack was counted; this provided the percent of neurons within a hemisphere that expressed at least 1 puncta and at least 5 puncta of the given probe. Statistical comparisons were made between groups by ANOVA with Tukey's multiple comparison post-test.

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