UNIVERSITY OF CALIFORNIA SAN DIEGO

Development of RNAscope for Multiplexed Imaging of Multiple Transcripts and Immunohistochemistry in Whole Mount Fly Brains

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

in

Biology

by

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The thesis of Vanessa Caguioa Lambatan is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-chair

Chair

University of California San Diego

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ABSTRACT OF THE THESIS

Development of RNAscope for Multiplexed Imaging of Multiple Transcripts and Immunohistochemistry in Whole Mount Fly Brains

by

Vanessa Caguioa Lambatan

Master of Science in Biology

University of California San Diego, 2020

Professor William Joiner, Chair
Professor Chih-Ying Su, Co-Chair

Understanding the molecular mechanisms responsible for various biological phenomena often involves demonstrating necessity or sufficiency of gene function in specific cells. Such studies gain strength from confirmation that molecules of interest are normally expressed where

they are hypothesized to function. However, such efforts are often limited by the availability of high quality probes. To overcome this problem in vertebrates, RNAscope was recently developed as a high-sensitivity alternative to *in situ* hybridization. In the present study I describe the development of RNAscope for use in whole mount adult fly brains. I demonstrate that RNAscope can achieve high signal, low background and good spatial resolution of mRNAs. I further demonstrate that RNAscope can be used in multiplex format for simultaneous detection of up to three types of transcripts, or for a single type of transcript combined with immunohistochemistry. Lastly, I show that RNAscope can be used to assay the spatial distinctness and plasticity of neurotransmitter identity in the nervous system. Collectively these results should open up new opportunities for quantitative gene expression studies in the adult fly brain.

INTRODUCTION

Common methods for spatial resolution of protein and gene expression include immunohistochemistry (IHC) and *in situ* hybridization (ISH), respectively (Ramos-Vara and Miller, Vet Pathol, 2014). Though these methods can be effective, there can also be limitations to them (Komminoth and Werner, Histochemistry, 1997). With IHC, non-specific or unavailable antibodies can preclude localization of certain proteins. With ISH, poor expression of certain transcripts or limitations to the design of specific probes can make it difficult to obtain selective, high intensity signal for certain mRNAs. Recently RNAscope has been developed as an alternative to ISH to obtain high signal, low noise, and good spatial resolution of mRNAs (Kersigo et al., Cell Tissue Res, 2018; Gross-Thebing et al., BMC Biol, 2014). RNAscope achieves these advantages in several ways. First, selectivity is attained by requiring two high-affinity probes to bind side-by-side in order to produce signal, thus reducing off-target effects of individual probes. Second, amplification is achieved by binding of primary probe pairs to branched secondary and tertiary adaptors, leading to expanded surface areas to which fluorescent dye can be conjugated. As a result, an individual mRNA molecule of interest can be visualized as a single puncta under a fluorescent microscope with little to no background signal. (Wang et al., J Mol Diagn, 2012).

RNAscope has been optimized for use in a multitude of organisms (Kann et al., Development, 2019; Grabinski et al., Plos One, 2015; Ledwon et al., Gene Expr Patterns, 2018), but it has not yet been successfully applied to the central nervous system of adult flies. This shortcoming limits the ability of fly researchers to confirm the expression of genes in specific cells. It also limits the ability of researchers to determine how genetic and environmental factors contribute to changes in expression of specific genes across the nervous system of fruit flies. Here

I describe a protocol to overcome these deficits and several ways in which RNAscope can be applied to address biological questions in adult fly brains. I demonstrate the sensitivity and selectivity of RNAscope by using the protocol I developed to contrast the high levels of several transcripts in wildtype control brains with low levels of the same transcripts in mutants. I demonstrate the utility of RNAscope for spatial resolution of up to 3 different types of mRNAs simultaneously or for fewer transcripts combined with IHC. In the former case I show that markers of cholinergic, GABAergic and glutamatergic signaling segregate into non-overlapping populations of neurons. In the latter case I confirm the expression in the dorsal fan-shaped body of several genes purported to function in those structures to regulate sleep. Finally, I use RNAscope to examine whether select markers of neurotransmitter identity change in response to severe, chronic reductions in sleep.

In summary, I demonstrate that RNAscope is a useful technique for quantitative measurement of mRNA expression across the adult fly brain. Furthermore, the protocol I developed for RNAscope can be performed on simple whole mount preparations rather than having to resort to more complicated and tedious tissue sectioning. Thus, this technique is likely to expand the types of questions that can be addressed in the fruit fly nervous system, and it should improve standards of reproducibility by making quantitative, spatial measurements of mRNA expression routine.

RESULTS

RNAscope is sensitive and specific to target transcripts

I first established the robustness and sensitivity of the RNAscope protocol I developed by using it to detect changes in specific transcripts in the *Drosophila* nervous system. In the first such experiment I assayed for *Dop1R1* mRNA in whole mount fly brains of wildtype animals and in negative controls in which *Dop1R1* had been knocked down throughout the nervous system. As shown in Fig 1a, *Dop1R1* signal was abundant and widespread throughout the brains of wildtype animals. Importantly, this signal was specific to *Dop1R1* since it was reduced in the brains of knockdown animals (Fig 1a, c). This difference could not be attributed to larger ROIs in wildtype animals since the estimated volume under each ROI did not differ between the two groups (Fig 1b).

In the second experiment I assayed for *Shaker* (*Sh*) mRNA in whole mount fly brains of wildtype animals and negative controls in which the *Sh* locus had been deleted (Cirelli et al., Nature, 2005). As shown in Fig 1d, using my RNAscope protocol I observed abundant, widespread expression of *Sh* expression across the brains of wildtype animals. Moreover this signal was specific since it was completely absent, with no background signal, in mutants lacking the *Sh* locus (Fig 1d). Although results are shown for individual animals for each condition in Fig 1, there was little variability between brains. Thus, the results of both experiments confirm that my RNAscope protocol is consistent, sensitive, and specific to the targeted transcript, leading to images with a high signal:noise ratio.

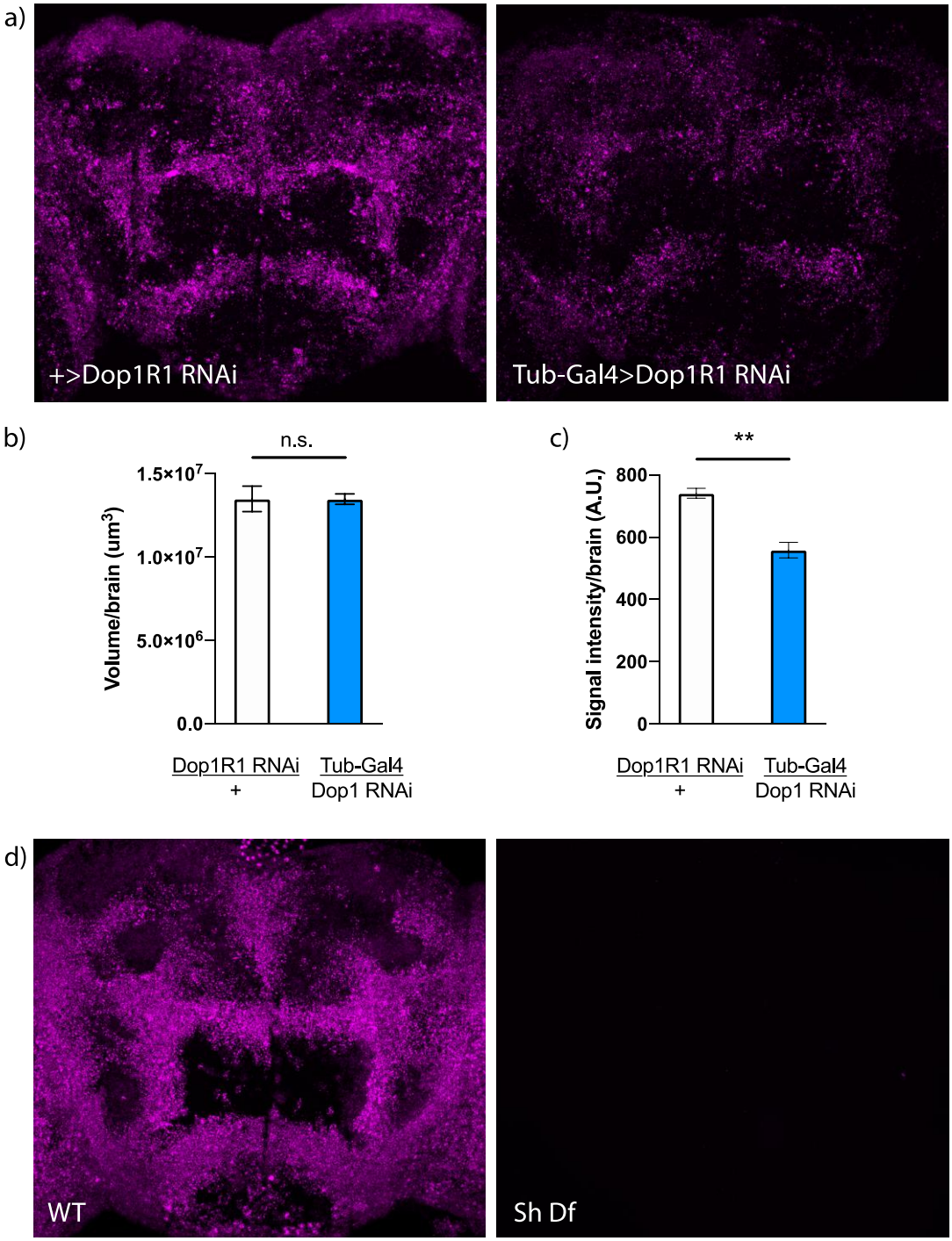
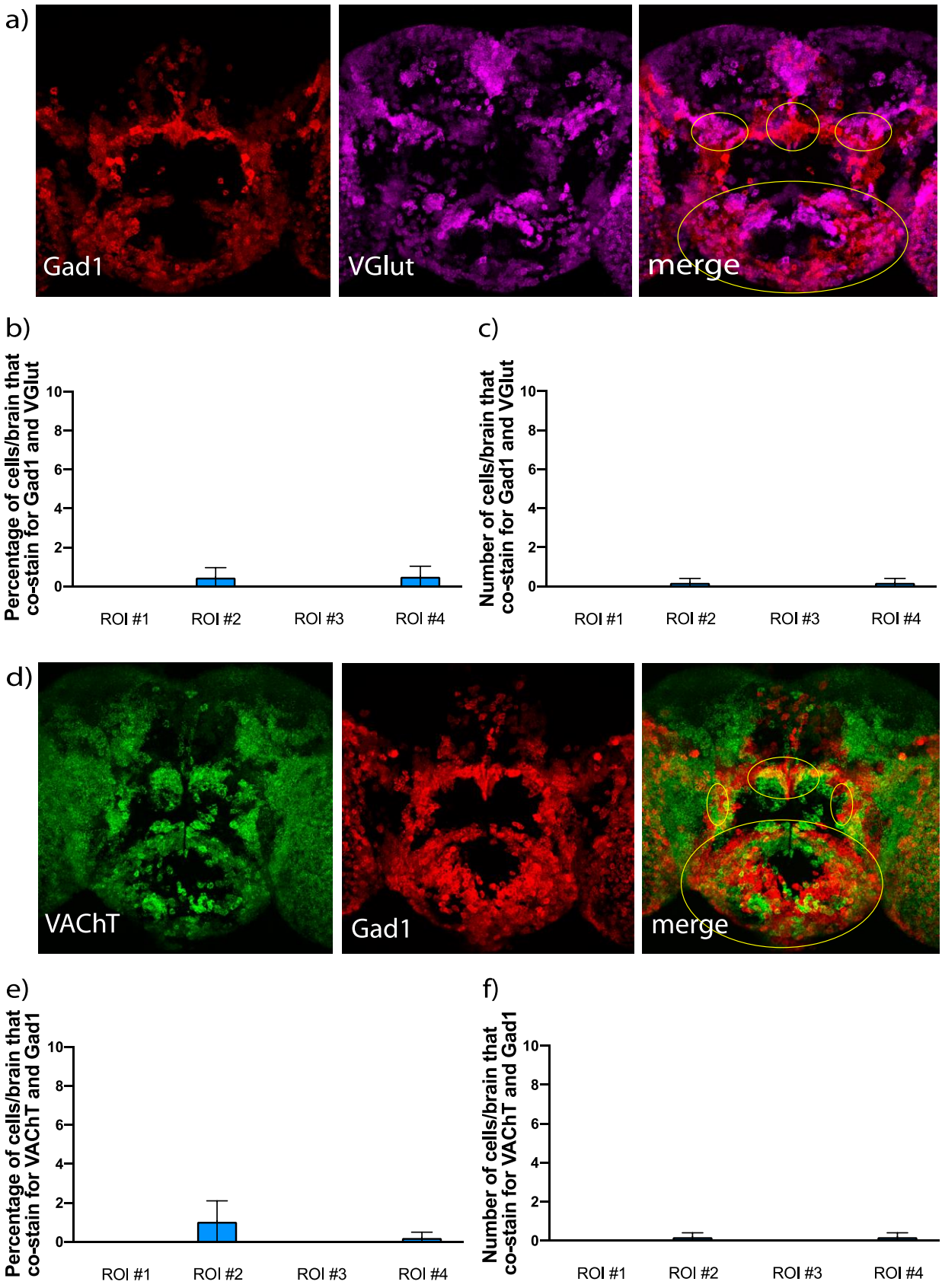


Figure 1. RNAscope is sensitive and specific to target transcripts in the fly brain. (a) Representative example of RNAscope using *Dop1R1* probe in adult brains of control and *Dop1R1* RNAi knockdown animals. (b) Calculation of average volume for each brain. (c) Calculation of average signal intensity for each brain. (d) Representative example of RNAscope with *Sh* probe in adult brains of wildtype animals and mutants lacking *Sh* expression.

RNAscope reveals non-overlapping expression patterns for markers of distinct neurotransmitter systems

Next, I asked whether RNAscope could be used to confirm the expected broad, non-overlapping expression patterns of transcripts used as markers of distinct neurotransmitter systems. For this purpose I used probes against *Gad1*, *VGlut*, and *VAChT*, which are thought to express selectively in GABAergic, glutamatergic and cholinergic neurons, respectively (Lacin et al., Plos One, 2015; Maruska et al., J Comp Neurol, 2017). As shown in Figs 2a, d and g, merged images from labeling with any two probes revealed patterns of transcripts that were largely non-overlapping. However, in some locations within each brain I observed regions of potential overlap. To distinguish between legitimate sites of co-expression and artifacts derived from flattened z-stacks, I first drew regions of interest (ROIs) around each of four areas of potential overlap. These included the SEZ/AMMC, two regions immediately lateral (and sometimes just dorsal) to the antennal lobes, and a central region of the brain containing the ellipsoid body (Fig 2a, d, g). Within each ROI I then examined each stack of images and counted how many cell bodies contained two labeled probes. Although I found a few examples containing at least one molecule of each labeled transcript, these cells were exceedingly rare and showed little consistency between brains (Figs 2b-c, e-f, h-i). Thus, unlike in mammals (Zhang et al., Neuron, 2019), neurons in the fly brain seem to have distinct, singular classical neurotransmitter identities, at least under basal conditions.

Figure 2. Multiplexed RNAscope shows non-overlapping expression of *Gad1*, *VGlut* and *VAChT* in adult fly brains. (a) RNAscope labeled probes for *Gad1* (red) and *VGlut* (purple). (b) The percentage of cells that show overlapping expression for *Gad1* and *VGlut*. (c) Number of neurons that show overlapping expression of both *Gad1* and *VGlut* transcripts. (d) RNAscope labeled probes for *VAChT* (green) and *Gad1* (red). (e) The percentage of cells that show overlapping expression for *VAChT* and *Gad1*. (f) Number of neurons that show overlapping expression of *VAChT* and *Gad1* transcripts. (g) RNAscope labeled probes for *VAChT* (green) and *VGlut* (purple). (h) The percentage of cells that show overlapping expression of *VAChT* and *VGlut*. (i) Number of neurons that show overlapping expression of *VAChT* and *VGlut* transcripts.


a)
Gad1
VGlut
merge
b)
Percentage of cells/brain that co-stain for Gad1 and VGlut
10
8
6
4
2
0
ROI #1
ROI #2
ROI #3
ROI #4
c)
Number of cells/brain that co-stain for Gad1 and VGlut
10
8
6
4
2
0
ROI #1
ROI #2
ROI #3
ROI #4
d)
VAChT
Gad1
merge
e)
Percentage of cells/brain that co-stain for VAChT and Gad1
10
8
6
4
2
0
ROI #1
ROI #2
ROI #3
ROI #4
f)
Number of cells/brain that co-stain for VAChT and Gad1
10
8
6
4
2
0
ROI #1
ROI #2
ROI #3
ROI #4

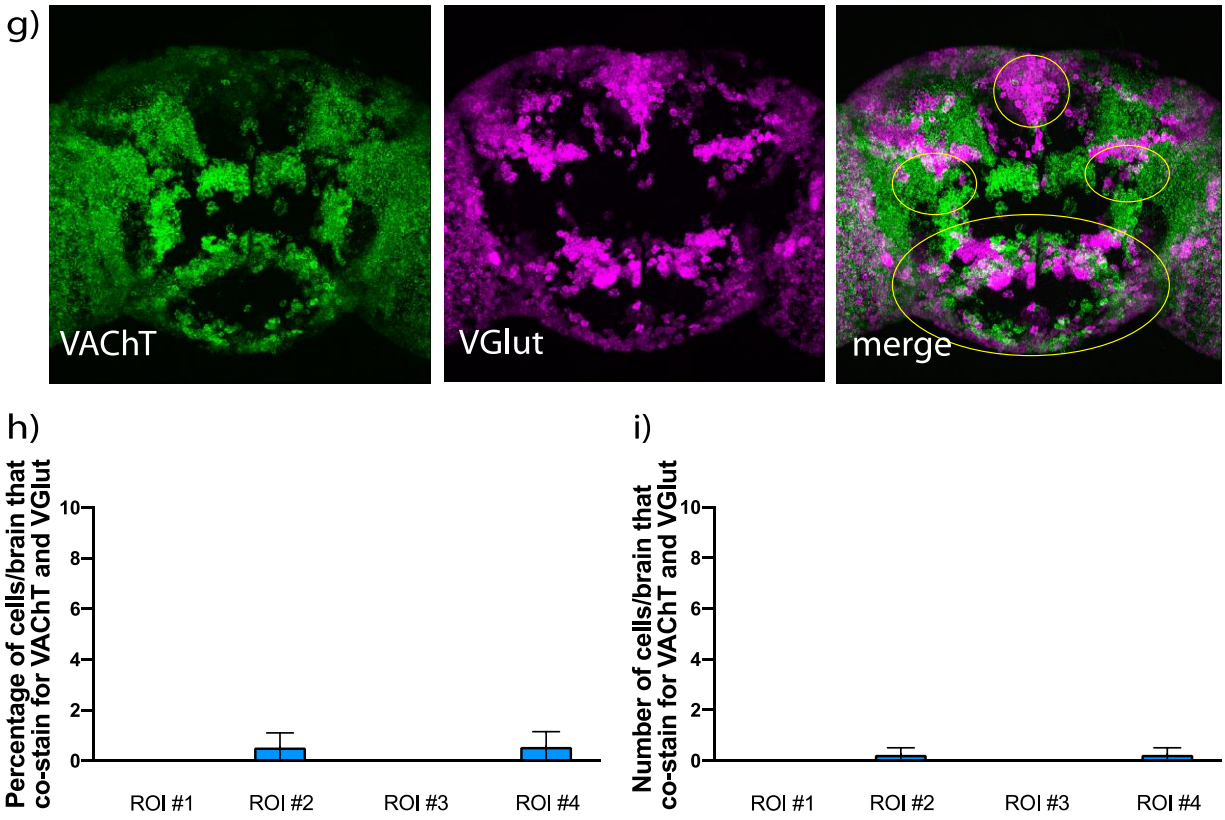


Figure 2. Multiplexed RNAscope shows non-overlapping expression of *Gad1*, *VGlut* and *VAChT* in adult fly brains (continued). (a) RNAscope labeled probes for *Gad1* (red) and *VGlut* (purple). (b) The percentage of cells that show overlapping expression for *Gad1* and *VGlut*. (c) Number of neurons that show overlapping expression of both *Gad1* and *VGlut* transcripts. (d) RNAscope labeled probes for *VAChT* (green) and *Gad1* (red). (e) The percentage of cells that show overlapping expression for *VAChT* and *Gad1*. (f) Number of neurons that show overlapping expression of *VAChT* and *Gad1* transcripts. (g) RNAscope labeled probes for *VAChT* (green) and *VGlut* (purple). (h) The percentage of cells that show overlapping expression of *VAChT* and *VGlut*. (i) Number of neurons that show overlapping expression of *VAChT* and *VGlut* transcripts.

RNAscope can be combined with immunohistochemistry in the fly brain

Next I asked whether my RNAscope protocol could be used for multiplexed labeling of transcripts together with immunohistochemistry (IHC). This question is particularly relevant to studies in which neurons of interest are labeled with GFP using the binary Gal4/UAS system (Brand and Perrimon, Genes Dev, 1994). In the present context I asked whether I could reliably use IHC to label GFP targeted to glutamatergic neurons with OK371-Gal4 while simultaneously

using RNAscope to label the same neurons with a probe targeting the glutamatergic marker *VGlut*. As shown in Fig 3a, the two techniques allowed for simultaneous labeling of neurons. These results led me to use the same approach to ask whether the OK371 driver, which is based on a Gal4 insertion in the *VGlut* gene, is a reliable proxy for glutamatergic expression. To address this question I quantified the percentage of GFP-positive cell bodies that were labeled with *VGlut* probe by RNAscope in OK371>GFP animals. As show in Fig 3b, this value exceeded 99%. Although it is possible that overlap between processes and cell bodies may have led me to slightly underestimate the number of the latter, at least at first approximation my results suggest that OK371 expression very accurately reflects the identity of glutamatergic neurons in the adult fly brain. My results also highlight the ability of RNAscope in general to validate the expression of genes purported to function in specific populations of neurons.

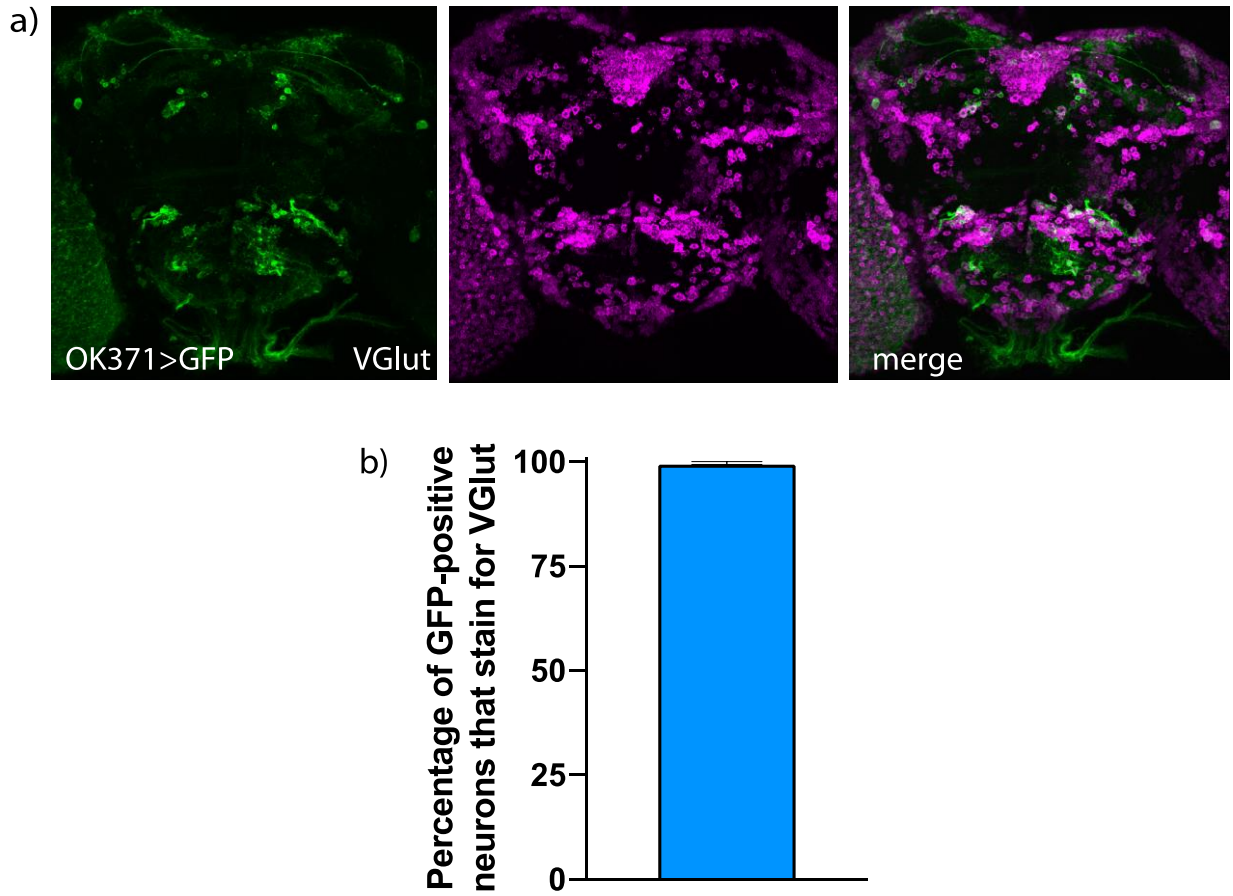


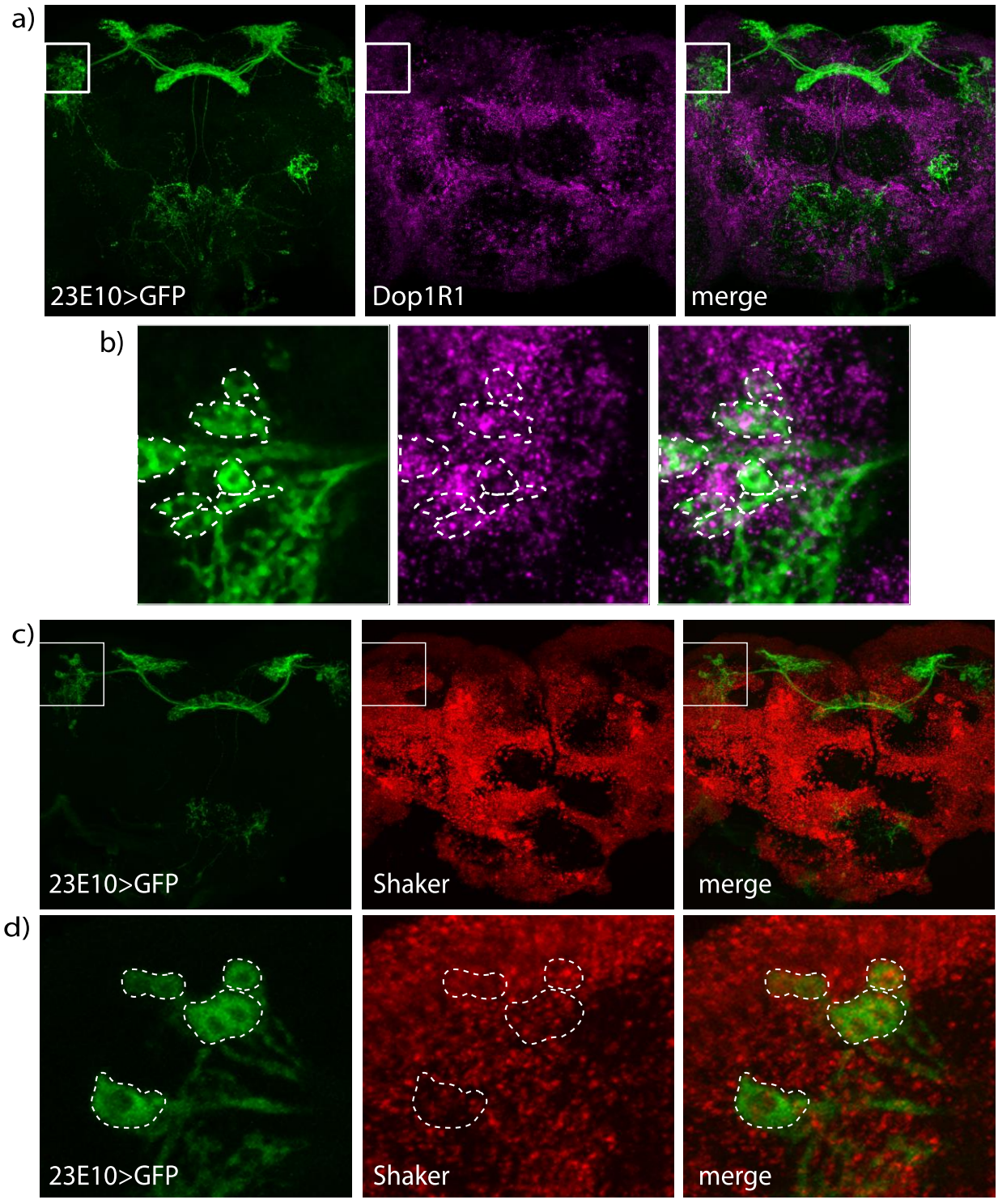
Figure 3. Colocalization of *VGlut* and GFP targeted to glutamatergic neurons. (a) RNAscope with *VGlut* probe (purple) in brains from *OK371*>GFP flies co-labeled with anti-GFP (green). (b) Percentage overlap of GFP positive cell bodies that contain *VGlut* probe.

RNAscope identifies relevant signaling molecules in putative sleep/wake-controlling neurons

The sensitivity of RNAscope and its compatibility with both the Gal4/UAS system and IHC make it ideal for validating the presence of specific transcripts in brain regions implicated in behaviors. Such a determination would be particularly useful in studies whose conclusions rely heavily on RNAi knockdown approaches, considering that these can lead to off-target effects. To test the utility of RNAscope in complementing behavioral studies, I examined the expression pattern of *Dop1R1* and *Sh*, whose expression in the dorsal fan-shaped body (dFB) has been proposed to suppress and promote sleep, respectively (Liu et al., Curr Biol, 2012; Ueno et al., Nat

Neurosci, 2012; Pimentel et al., Nature, 2016). To label dFB neurons I expressed GFP under the control of the 23E10 driver. To determine whether GFP labeling coincided with *Dop1R1* or *Sh* labeling of cell bodies I then performed multiplexed IHC and RNAscope on dissected brains (Fig 4a-d). In these experiments I first identified putative cell bodies based on their distinctive morphology. I also asked a partner who was blinded to my data to independently verify my choices. Our consensus was then used to determine the percentage of dFB neurons that express *Dop1R1* or *Sh*. To arrive at this number I drew an ROI around each identified cell body in each plane of each z-stack, and I marked it as positive for a given RNAscope probe if I observed at least one cluster of fluorophore inside. As shown in Fig 4e-f I found that *Dop1R1* and *Sh* were expressed in >95% and >100% of dFB neurons, respectively. These data are consistent with proposed sleep-regulating functions for *Dop1R1* and *Sh* in the dFB (Liu et al., Curr Biol, 2012; Ueno et al., Nat Neurosci, 2012; Pimentel et al., Nature, 2016).

Figure 4. RNAscope identifies relevant signaling molecules in a putative sleep locus.
(a) RNAscope with *Dop1R1* probe (purple) in brains from *23E10*>GFP flies co-labeled with anti-GFP (green). (b) Insets: cell bodies labeled with GFP (green) and *Dop1R1* (purple) to show co-localization of transcript with GFP signal. (c) RNAscope with *Shaker* probe (red) in brains from *23E10*>GFP flies co-labeled with anti-GFP (green). (d) Insets: cell bodies labeled with GFP (green) and *Shaker* (red) show co-localization of transcript with GFP signal. (e) Percentage of *23E10*>GFP cell bodies that are positive for *Dop1R1* probe. (f) Percentage of *23E10*>GFP cell bodies that are positive for *Shaker* probe.


a)
23E10>GFP
Dop1R1
merge
b)
c)
23E10>GFP
Shaker
merge
d)
23E10>GFP
Shaker
merge

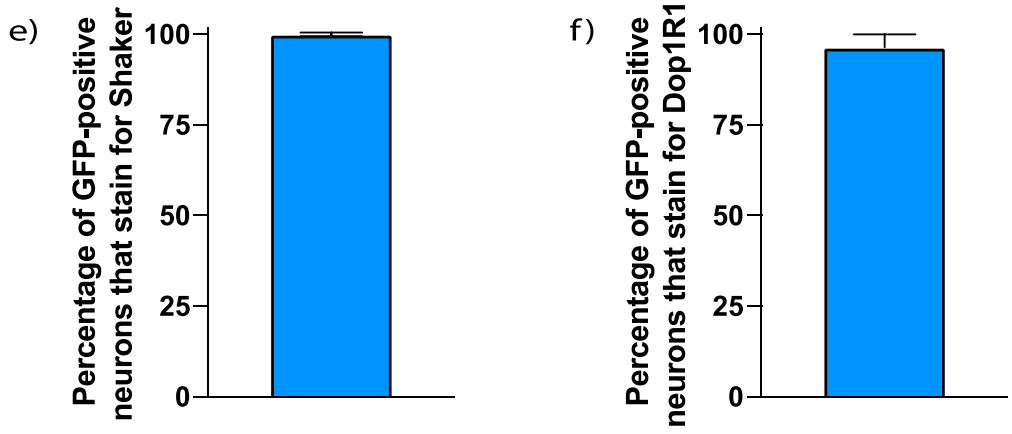


Figure 4. RNAscope identifies relevant signaling molecules in a putative sleep locus (continued). (a) RNAscope with *Dop1R1* probe (purple) in brains from *23E10*>GFP flies co-labeled with anti-GFP (green). (b) Insets: cell bodies labeled with GFP (green) and *Dop1R1* (purple) to show co-localization of transcript with GFP signal. (c) RNAscope with *Shaker* probe (red) in brains from *23E10*>GFP flies co-labeled with anti-GFP (green). (d) Insets: cell bodies labeled with GFP (green) and *Shaker* (red) show co-localization of transcript with GFP signal. (e) Percentage of *23E10*>GFP cell bodies that are positive for *Dop1R1* probe. (f) Percentage of *23E10*>GFP cell bodies that are positive for *Shaker* probe.

RNAscope as an assay for sleep-dependent neurotransmitter switching

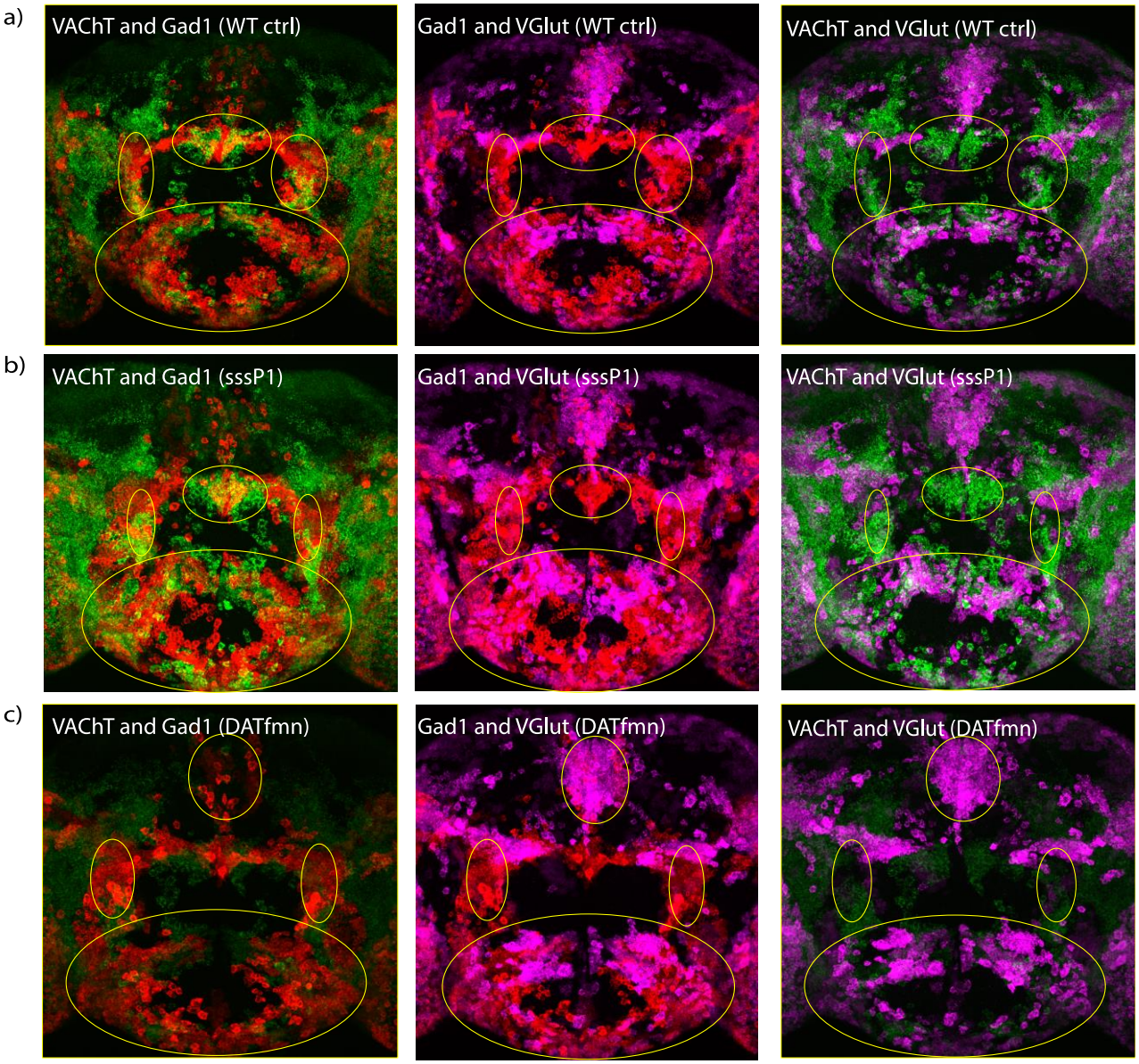
It has previously been demonstrated that neurotransmitter identity is sensitive to neuronal activity in the mammalian brain (Dulcis et al., Science, 2013). These conclusions were based in part on using RNAscope to detect changes in markers of neurotransmitter systems in specific neurons (Li and Spitzer, Nat Commun, 2020). Here, I used RNAscope to test if neurotransmitter identity is also sensitive to changes in the sleep/wake cycle in flies. In these experiments I compared brains from wildtype animals with brains from sss^{P1} and DAT^{fmn} mutants, which have severe chronic sleep deficits (Koh et al., Science, 2008; Kume et al., J Neurosci, 2005). As in earlier experiments, I used expression of *VAChT*, *Gad1*, and *VGlut* as proxies for cholinergic, GABAergic and glutamatergic neurons, respectively.

I looked for changes in neurotransmitter identity in two ways. In the first, I used the same approach as in Fig 2. I.e. I used RNAscope to label each brain with each of my three probes, drew

ROIs around areas that appeared to contain overlapping signals in flattened two-dimensional images, and then quantified the percentage overlap within each ROI by examining every neuron in an entire stack of images. Regardless of which probes I assayed, I did not measure any clear shifts in neurotransmitter identity in sleep mutants relative to wildtype controls (Fig 5a-f).

In a second approach I looked for widespread changes in *intensity* of signals for neurotransmitter identity that were dependent on sleep. I quantified total intensity of each probe across the entire brain, and I calculated the ratio of this value for each pair of probes. Although I measured no change in *Gad1* relative to *VGlut*, intriguingly I measured a statistically significant global decrease in levels of *VAChT* relative to the other two markers in DAT^{fmn} mutants (Fig 5g). However, I did not measure any significant changes in sss^{P1} mutants. Thus, it seems likely that decreased *VAChT* in DAT^{fmn} mutants is caused by increased dopamine signaling in DAT^{fmn} mutants rather than by reduced sleep that accompanies that increased dopamine signaling.

Figure 5. RNAscope as an assay for sleep dependent neurotransmitter switching in adult fly brains. (a) RNAscope labeled probe pairs for *VAChT* (Green), *Gad1* (red) and *VGlut* (purple) in wildtype control brains. (b) RNAscope labeled probe pairs in sss^{P1} mutant brains. (c) RNAscope labeled probe pairs in DAT^{fmn} mutant brain. 4 ROIs are indicated for each condition. (d) The percentage of cells that show overlapping expression in *VAChT* and *Gad1* transcript for control, sss^{P1}, and DAT^{fmn} mutants. (e) The percentage of cells that show overlapping expression in *Gad1* and *VGlut* transcript. (f) The percentage of cells that show overlapping expression in *VAChT* and *VGlut* transcript. (g) Mean global intensity for wildtype, sss^{P1}, and DAT^{fmn} mutants by *VAChT*, *Gad1*, and *VGlut* pairs.


a)
VAChT and Gad1 (WT ctrl)
Gad1 and VGlut (WT ctrl)
VAChT and VGlut (WT ctrl)
b)
VAChT and Gad1 (sssP1)
Gad1 and VGlut (sssP1)
VAChT and VGlut (sssP1)
c)
VAChT and Gad1 (DATfmn)
Gad1 and VGlut (DATfmn)
VAChT and VGlut (DATfmn)

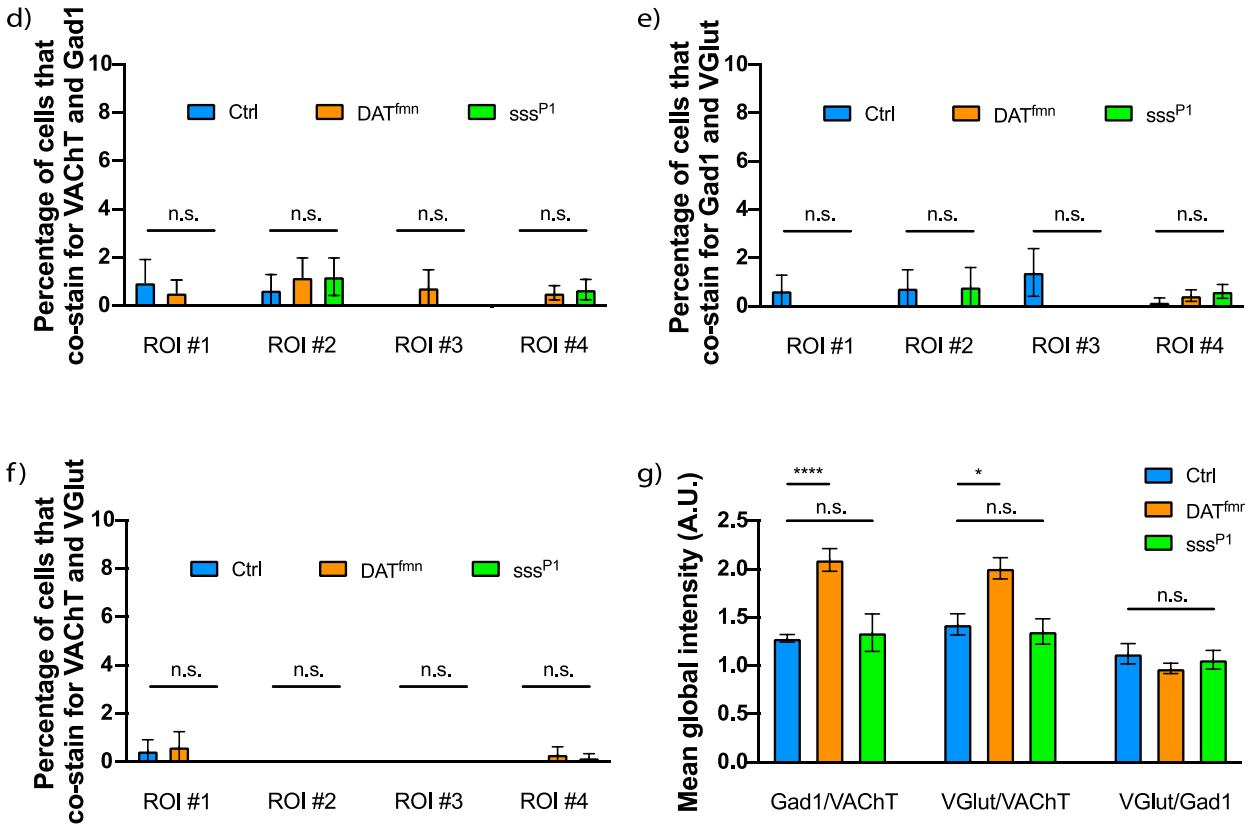


Figure 5. RNAscope as an assay for sleep dependent neurotransmitter switching in adult fly brains (continued). (a) RNAscope labeled probe pairs for *VAChT* (Green), *Gad1* (red) and *VGlut* (purple) in wildtype control brains. (b) RNAscope labeled probe pairs in sss^{P1} mutant brains. (c) RNAscope labeled probe pairs in DAT^{fmn} mutant brain. 4 ROIs are indicated for each condition. (d) The percentage of cells that show overlapping expression in *VAChT* and *Gad1* transcript for control, sss^{P1}, and DAT^{fmn} mutants. (e) The percentage of cells that show overlapping expression in *Gad1* and *VGlut* transcript. (f) The percentage of cells that show overlapping expression in *VAChT* and *VGlut* transcript. (g) Mean global intensity for wildtype, sss^{P1}, and DAT^{fmn} mutants by *VAChT*, *Gad1*, and *VGlut* pairs.

DISCUSSION

In this thesis I describe a protocol I developed for use of RNAscope in the adult fly nervous system. Using this protocol, I was able to routinely obtain confocal images with high signal:noise and quantifiable, comparable results between batches of animals in less than a week. This technique should be useful to many researchers in various experimental contexts.

In my initial experiments I confirmed the specificity of RNAscope in whole mount fly brains. I showed that signals for *Dop1R1* and *Sh* are found at high levels throughout the brains of wildtype animals but not in animals in which expression of the genes is reduced. Thus, RNAscope signal appears to be proportional to the amount of transcript to which a given probe is targeted and does not arise from spurious binding of probe to unintended targets. This specificity offers an advantage over classical *in situ* hybridization, in which off-target signal can arise (Chu et al., Semin Diagn Pathol, 2019).

Another advantage of the RNAscope protocol I developed is that it can be used in combination with the Gal4/UAS system and IHC for multiplexed imaging of transcript expression in populations of neurons labeled transgenically with a marker such as GFP. I demonstrated the utility of this approach in determining the reliability of the OK371-Gal4 driver as a marker of glutamatergic neurons. I also demonstrated that this approach can be used to validate expression of genes in the specific neuroanatomical context in which they have been proposed to regulate behaviors. Specifically I demonstrated that *Dop1R1* and *Sh* are indeed expressed in the dFB, where they are thought to function to regulate sleep (Liu et al., Curr Biol, 2012; Ueno et al., Nat Neurosci, 2012; Pimentel et al., Nature, 2016). Combining RNAscope and IHC will be especially helpful in

future studies that depend on phenotypes resulting exclusively from RNA knockdown, in which off-target effects can arise.

I also used RNAscope to demonstrate that markers of cholinergic, GABAergic and glutamatergic signaling are expressed in distinct populations of neurons. Notably these results contrast with findings in mammals, where a given neuron may transcribe molecules involved in synthesis, packaging or reuptake of multiple neurotransmitters (Vaaga et al., Curr Opin Neurobiol, 2014). It is unclear if this difference arises from a bona fide lack of plasticity in neurotransmitter identity in the adult fly brain, from a lack of sensitivity of RNAscope in the protocol I developed, or from the limited number of markers of neurotransmitter identity I assayed for.

In mammals neurotransmitter identity often appears to be controlled at least partly by the activity patterns of neurons (Dulcis et al., Science, 2013). I thus reasoned that I might be able to reveal plasticity in neurotransmitter identity in the adult fly brain if I could compare animals with widespread expected differences in neuronal activity. The context I chose for this was sleep, which transforms electrical activity across the brain in mammals and flies (Ly et al., Neurosci Biobehav Rev, 2018). Specifically I used RNAscope to compare expression of three markers of cholinergic, GABAergic and glutamatergic signaling in adult fly brains of wildtype animals and two mutants, DAT^{fmn} and sss^{P1}, which have large, chronic reductions in sleep. What I found did not confirm my hypothesis, though. Just like in wildtype animals, neurons in DAT^{fmn} and sss^{P1} mutants showed negligible co-expression of markers of ACh, GABA or glutamate signaling. Furthermore, although DAT^{fmn} mutants exhibited what appeared to be a widespread reduction in the cholinergic marker *VAChT*, sss^{P1} mutants lacked any significant brain-wide changes in levels of any marker I assayed for. Thus, drastic changes in sleep *per se* do not appear to alter markers of cholinergic, GABAergic and glutamatergic identity in the adult fly brain. However, I cannot rule out the possibility that

plasticity exists for these neurotransmitter systems in other contexts. I also cannot rule out the possibility that sleep affects neurotransmitter identity in neurons that produce dopamine, octopamine, serotonin or neuropeptides.

Lastly, my results in the previous section were based on measurements of absolute integrated signal intensity across the fly brain as well as ratios of such intensity for two neurotransmitter-specific markers. In future studies I recommend performing related experiments with a control probe such as GAPDH, which would not be expected to change with genetic or behavioral manipulations, and to which signals of interest could be normalized. This additional step would ameliorate confounding variables such as changes in levels of both markers that are being compared or factors that could affect signal intensity independent of transcriptional changes, such as variable permeability of brains to probes.

MATERIALS AND METHODS

Animal husbandry

Male flies were used for the WT and *Shaker (Sh)* deficiency experiments. Female flies were used for the rest of the experiments. The *Sh Df* line was obtained by from Barry Ganetzky (U. Wisconsin); the DAT^{fmn} mutant was obtained from K. Kume (Kumamoto University); and the sss^{P1} mutant was obtained as previously described (Koh et al., Science, 2008). Tub-Gal4 (#5138), OK371-Gal4 (#29160), 23E10-Gal4 (#49032), and Dop1R1 RNAi (#62193) were obtained from the Bloomingdale Stock Center. All flies were aged 3 days - 2 weeks immediately prior to testing.

RNAscope

Tissue processing and dehydration: Immediately after dissection flies were fixed for 18-24 hrs at room temperature (RT) on a rocker at low speed in freshly prepared 4% formaldehyde solution. After the fixation period, the samples were washed for 10 min 2 times in 650 μL PBST (no BSA) at RT. The samples were then dehydrated in 500 ul methanol/PBST in 10 min increments at RT as follows: 25% methanol, 50% methanol, 75% methanol and 100% methanol. Following dehydration, samples were stored at -20 C until they were ready to be used.

Rehydration and target retrieval: Samples were thawed and rehydrated in 500 ul MeOH/PBST in 10 min increments at RT as follows: 100% methanol, 75% methanol, 50% methanol and 25% methanol. Samples were then transferred to 500 uL PBST + 1% BSA and incubated for 10 min at RT. Samples were then transferred to 500 ul 1x Target Retrieval buffer (ACD) and incubated for 5.5 min at 100°C. Samples were then sequentially washed at RT with 650 ul of: PBST + 1% BSA

for 1 min, 100% methanol for 1 min, PBST + 1% BSA for 10 min, and 500 ul post fix (4% formaldehyde solution) for 25 min. Samples were then removed and incubated in 2 drops of Protease Plus (ACD) for 10 min at 40°C. Afterward samples were washed with 650 µL PBST + 1% BSA for 10 min at RT, followed by a wash with 2 drops (50 µL) of probe diluent (ACD) for 20 seconds, and finally transferred to probes for overnight incubation at 40°C. ACD provided and designed 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 removed from probes and washed 2 times for 5 min at RT with RNAscope 1X Wash Buffer (ACD), followed by incubation in 2 drops of AMP 1 Amplifier (ACD) for 30 min at 40°C. Samples were then washed 2 times for 5 min at RT with RNAscope 1X Wash B, followed by incubation in 2 drops of AMP 2 (ACD) for 30 min at 40°C. Samples were then washed 2 times for 5 min at RT with RNAscope 1X Wash B, followed by incubation in 2 drops of AMP 3 (ACD) for 15 min at 40°C. Samples were then removed and washed 2 final times for 5 min at RT with RNAscope 1X Wash B.

HRP reaction: Wash buffer was removed and 2 drops of RNAscope Multiplex FL v2 HRP-C1 reagent (ACD) was added to samples for 15 min at 40°C. Samples were then washed 2 times for 5 min with RNAscope 1X Wash Buffer at RT, followed by incubation in Opal dye (Akoya Bioscience) for 30 min at 40°C. Samples were then washed 2 times for 5 min at RT with RNAscope 1X Wash B, followed by incubation in 2 drops Multiplex FL vs2 HRP-Blocker (ACD) for 15 min at 40°C. Samples were then washed 2 times for 5 min at RT with RNAscope 1X Wash B. This protocol was repeated for experiments involving additional probes, except that HRP-C1 was

replaced with HRP-C2 and HRP-C3 reagents (ACD) for probes 2 and 3, respectively. Samples were then mounted onto slides for imaging or treated as described below for immunohistochemistry.

Immunohistochemistry (IHC): Samples were washed with PBST 3 times for 10 min at RT and then blocked in 10% normal goat serum + 1% BSA/0.3% Triton X-100 for 1-2 hours at RT or overnight at 4°C. Samples were then transferred to fresh blocking solution + 1:1000 rabbit anti-GFP antibody (A11122 from Life Technologies) and incubated overnight at 4°C. Samples were then washed 4 times for 15 min at RT in PBST and then incubated overnight at 4 C in fresh blocking solution + 1:1000 goat anti-rabbit antibody fused to HRP. Samples were then washed 2 times for 5 min at RT in RNAscope 1X Wash B. For conjugation by HRP, Opal dyes (Akoya Bioscience) were diluted 1:2000 in TSA buffer (ACD) and incubated with samples for 30 min at 40°C. Samples were then washed 2 times for 5 min at RT in in RNAscope 1X Wash B and then incubated in 2 drops of Multiplex FL v2 HRP-Blocker (ACD) for 15 min at 40°C. Samples were washed 2 more times for 5 min at RT in RNAscope 1X Wash B before mounting on slides.

Imaging

Brains were imaged using a Leica Confocal Microscope. For head to head comparisons (sleep-dependent neurotransmitter switching) I kept all settings constant including laser settings and gain. The gain for *VAChT* was 620, *Gad1* was 480 and *VGlut* was 520. Signal from all of the imaged brains was quantified using FIJI.

Analysis

Brain volume associated with an ROI for *Dop1R1* knockdown was measured by first outlining the entire brain as an ROI and multiplying the area by the total number of slices to get volume. Global measurements of *Dop1R1* and neurotransmitter markers were performed by first outlining the entire brain as an ROI and summing the average intensity across all of the slices. This sum was then averaged within a given genotype. For colocalization of OK371>GFP with *VGlut*, ROIs were generated for cell bodies containing GFP, and the number of these that also contained signal for *VGlut* was counted to arrive at a percentage overlap. For colocalization of neurotransmitter markers, ROIs were chosen across brains based on apparent overlapping signals within maximum projections. Subsequently, however, actual overlap was ascertained in single optical planes, rather than throughout an entire z-stack. For all experiments, statistical comparisons were made between groups by ANOVA, and neurons were counted manually.

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