ABSTRACT

Title of Dissertation: **SEROTONIN REGULATES AN OLFACTORY CRITICAL PERIOD IN DROSOPHILA**

Ahana Mallick, Doctor of Philosophy, 2024

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Serotonin (5-HT) is known to modulate early development during critical periods when experience drives heightened levels of plasticity in sensory systems. Studies in the somatosensory and visual cortices implicate multiple target points of serotonergic modulation, yet the underlying cellular and molecular mechanisms of 5-HT modulation of critical period plasticity remain elusive. Here, we take advantage of the genetically tractable olfactory system of *Drosophila* to investigate how 5-HT modulates critical period plasticity (CPP) in the CO_2 sensing circuit of fruit flies. During the critical period, chronic exposure to CO_2 has been shown to increase the volume of the CO_2 sensing V glomerulus. We found that 5-HT release by serotonergic neurons in the antennal lobe (AL) is required for increase in the volume of the V glomerulus. Furthermore, signaling via the 5-HT1B, 5-HT2B and 5-HT7 receptors in different neuronal populations is also required during the critical period. Olfactory CPP is known to involve local inhibitory networks and consistent with this we found that knocking down 5-HT7

receptors in a subset of GABAergic local interneurons was sufficient to block CPP, as was knocking down GABA receptors expressed by olfactory sensory neurons (OSNs). Additionally, 5-HT2B expression in the cognate OSNs sensing CO_2 is also essential for CPP indicating that direct modulation of OSNs also contributes to the olfactory CPP. Furthermore, 5-HT1B expression by serotonergic neurons in the olfactory system is also required during the critical period. Our study reveals that 5HT modulation of multiple neuronal targets is necessary for experience-dependent structural changes in an odor processing circuit. Finally, we wanted to isolate the neuromodulatory effects of individual serotonergic neurons. To achieve this, we combined a state-of-the-art technique to sparsely label serotonergic neurons and a computer algorithm to search against 10,000 Gal4 promoter lines and identify candidate lines that would allow individual manipulation of the 110 serotonergic neurons.

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SEROTONIN REGULATES AN OLFACTORY CRITICAL PERIOD IN DROSOPHILA

by

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Preface

Sections of Chapter 1 have been adapted from a review written by Ahana Mallick, published in MDPI, Biology (Mallick, Dacks, and Gaudry 2024).

Chapter 2 has been published in BioRxiv (Mallick et al. 2024). This work was a shared effort between Ahana Mallick. Dr. Hua Tan, Jacob Epstein, and Clarissa Ng. Drs. Quentin Gaudry and Andrew Dacks contributed as senior authors.

Chapter 3 This work was designed and executed by Ahana Mallick and Dr. Quentin Gaudry.

Dedication

To my wife Roni. Her continuous love and support helped me overcome all the challenges I faced during my PhD journey.

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Chapter 1: Introduction

An Introduction to Critical Periods

Experience-dependent neuronal plasticity is one of the hallmarks of the nervous system wiring. Exposure to environmental stimuli induces heightened levels of circuit refinement and plasticity in response to stimuli. For example, repeated exposure to the same odor stimulus improves the robustness of the odor responses and discriminability in animals (Franco and Yaksi 2021; Cansler, Maksimova, and Meeks 2017; Berners-Lee et al. 2023; Sabrina Xu, Lee, and Holy 2016; Cheetham, Park, and Belluscio 2016). In the early postnatal life of an organism, there exists a specific time window, when neuronal circuits show heightened levels of experience dependent plasticity. This window of heightened neuronal plasticity is called the critical period (David H Hubel and Wiesel 1962). It was first defined by the Nobel prize winning work of Hubel and Wiesel in the context of development of cortical receptive fields of binocular vision (David H Hubel and Wiesel 1962; LeMasurier and Van Wart 2012; D H Hubel and Wiesel 1970; Wiesel and Hubel 1963a; 1963b). Since then, critical periods have been discovered in multiple sensory modalities across species (Hensch 2004; 2005; Reha et al. 2020, Jeanmonod, Rice, and Van der Loos 1981; Lieff et al. 1975; Ma et al. 2014; Tsai and Barnea 2014). In *Drosophila*, such critical periods have been observed in the visual and sensorimotor circuits (Fushiki, Kohsaka, and Nose 2013; Slepian et al. 2015) of the fly larvae and in the olfactory system of adult flies (J. M. Devaud, Keane, and Ferrús 2003; J. M. Devaud, Acebes, and Ferrús 2001a; J. Devaud et al. 2003; Acebes et al. 2012; J. M. Devaud et al. 2003; Sachse et al. 2007a; Chakraborty, Goswami, and Siddiqi 2009; Iyengar et al. 2010; Das et al. 2011; McCann et al. 2011a; Sudhakaran et al. 2014; Kidd and Lieber 2016; Kidd, Struhl, and Lieber 2015; Golovin

and Broadie 2016; Golovin, Vest, and Broadie 2021a; Golovin et al. 2019; Chodankar et al. 2020; Gugel, Maurais, and Hong 2023; Fabian and Sachse 2023; Fabian et al. 2023).

Experience-dependent plasticity can occur both during and after the critical period. In this study, I focus on experience-dependent plasticity during the critical period and name it critical period plasticity (CPP) with the following features (Figure 1) as reviewed in Sengpiel 2007, Cioni and Sgandurra 2013, and Knudsen 2004 (Sengpiel 2007; Cioni and Sgandurra 2013; Knudsen 2004):

1. CPP can only be induced at a specific time window in the early life of an organism in response to repeated experience dependent activity in the circuit (Sengpiel 2007; Cioni and Sgandurra 2013; Knudsen 2004).
2. It occurs when the sensory circuits are still developing but have achieved reliable and precise inputs (Knudsen 2004).
3. In addition to the presence of excitatory components, CPP onset is marked by the appearance/arrival of the inhibitory components in the circuit (Sengpiel 2007).
4. During CPP, changes occur both at the level of synaptic transmission and structure induced by activation of gene transcription and translation that ultimately lead to long-term functional changes (Knudsen 2004).

Olfactory Critical Periods

The importance of the critical period in the olfactory system can be appreciated through examples of odor imprinting across various species. For example, odor exposure during the olfactory critical period in neonatal mice increases their sensitivity to the odor. Such neonatal odor exposure resulted in increased sensitivity to the exposed odor in adult mice (Inoue et al. 2018; 2021). Similar examples of olfactory imprinting also exist in *C. elegans*, where exposure to a specific odor during

the critical period is sufficient to induce long-term behavioral changes that are observed long after the closing of the critical period (M. C. Vogt and Hobert 2017; M. Hong et al. 2017). However, the most iconic example of olfactory imprinting memory is the homing migrations of adult salmon and trout (A.T. Scholz et al. 1978; Cooper and Scholz 1976).

Salmon develops through their embryonic to juvenile stages in fresh water and return to the same freshwater stream as adults for spawning. It is believed that juvenile salmon imprints on the odors present in the freshwater stream right before leaving the stream (Simon Charles Courtenay 1989; Bodznick 1978). This helps them to navigate back towards the same stream as adults (Wisby and Hasler 2011; Allan T. Scholz et al. 1976). This form of odor imprinting occurs at various stages of development in different salmon species and starts as early as in the embryo (Quinn, Stewart, and Boatright 2006). These examples underscore the importance of circuit refinement unique to the chemical environment to which the organism is exposed.

Organization of the olfactory system in Drosophila

The olfactory system plays a crucial role in the survival of animals. It provides vital cues about the chemical environment that allows an organism to optimize feeding, reproduction, predator avoidance and social conduct. The organization of the primary olfactory processing circuits therefore shares several common themes across species, including mammals and insects (Figure. 2A) (Hildebrand and Shepherd 2003). In all species, odor information is received at the periphery by olfactory sensory neurons (OSNs) that express chemoreceptive proteins activated by different volatile chemicals. OSNs expressing the same complement of chemoreceptive proteins project into a primary olfactory processing center composed of discrete neuropil structures called glomeruli. Each OSN expresses an odorant receptor (OR) type that binds odor molecules and activates neurons downstream of it within its cognate glomeruli. Each OSN and thereby its cognate

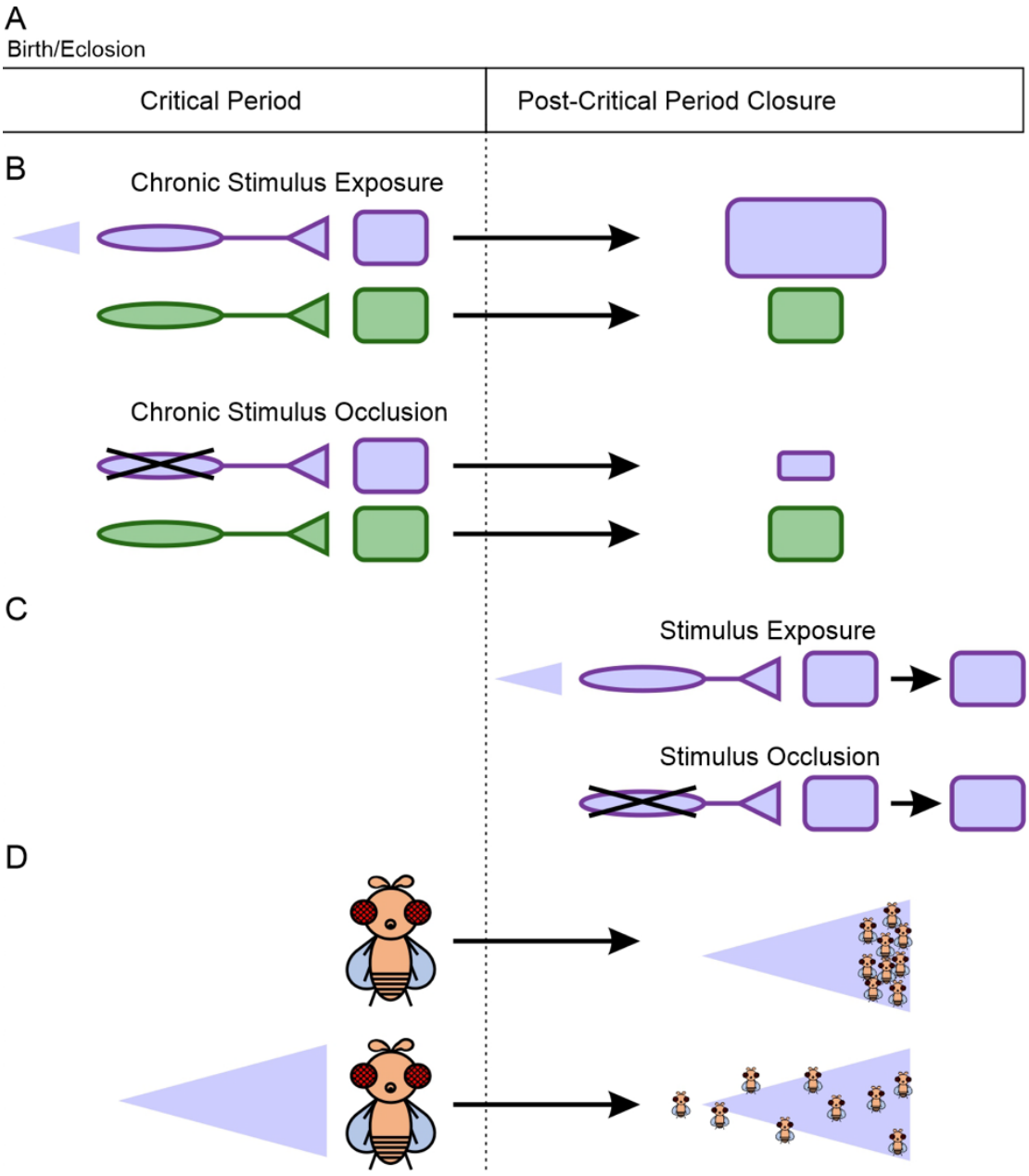

Figure 1. Core features of critical periods. (**A**) Critical periods are windows of time during which exposure to specific environmental stimuli can induce changes in the nervous system's structure and function. Once the critical period closes, the capability for plasticity is greatly reduced.

(**B**) Critical period plasticity is stimulus specific. Chronic stimulus exposure or occlusion during the critical period can induce changes in nervous system architecture and function selectively for those regions that process the stimulus (purple square), but not necessarily for other regions (green square). The purple triangle represents exposure to an odor that only activates some sensory afferents (purple) but not others (green).

(**C**) Equivalent stimulus exposure after the closure of the critical period does not induce changes equivalent to those induced during the critical period.

(**D**) The neural plasticity induced during the critical period can result in sustained differences in behavioral preferences. In this example, naïve flies distribute and move to high odor concentrations (right side of the purple triangle), while flies that experience chronic exposure to the odor do not orient themselves based on a concentration gradient for that specific odor.

OR converts an odor signal into a glomerular odor map (Malnic et al. 1999; Sakano 2020; Wilson 2013a; Hallem and Carlson 2006a; 2004; Hallem, Ho, and Carlson 2004; Imai, Sakano, and Vosshall 2010; Mori and Sakano 2011) which is thought to encode information about the type, chemical nature, concentration, duration, and directionality of the odor stimuli. Information from each glomerulus is further transmitted to various higher order olfactory processing centers by second-order neurons that ultimately give rise to olfactory perception. Nevertheless, there are striking differences in the peripheral anatomy, evolution and signaling mechanisms between *Drosophila* and mammalian olfactory systems.

In *Drosophila,* the olfactory system (Figure. 2B) starts at the antennae and maxillary palps that are covered in thousands of sensilla housing OSNs that express a diverse set of chemoreceptive proteins. In all, there are 72 chemosensory receptors and 4 co-receptors (Task et al. 2022). OSNs

that expresses the same combination of chemosensory receptors and co-receptors project to the same glomerulus and the different types of OSNs map onto distinct glomeruli like in mammals (Hallem and Carlson 2006a; 2006b; 2004; Couto, Alenius, and Dickson 2005; K. Vogt et al. 2019; De Bruyne, Foster, and Carlson 2001; K. Vogt et al. 2021; Wilson 2008a; 2013a; 2008b; Kazama and Wilson 2009; Benton et al. 2009; De Bruyne, Clyne, and Carlson 1999; E. J. Hong and Wilson 2015b; Nagel and Wilson 2011). Although the chemosensory receptors in the fruit fly are structurally similar to the mammalian ORs, containing a seven transmembrane domain, their topology is quite distinct from the rodent GPCRs. In addition, heteromeric complexes of distinct ligand binding chemosensory receptors colocalize with a single or multiple co-receptor(s) that form functional ion channels that activate OSNs upon odor binding. Each OSN projects onto odor-specific neuropil called glomeruli in the antennal lobe (AL). Within the AL, OSNs synapse with second-order projection neurons (PNs) as well as with local interneurons (LNs). The PNs in tum project onto higher order olfactory centers, including the mushroom bodies (MB) and the lateral horn (LH) (Figure 2B). Several studies allowed to define the odor specificity map for each receptor type and glomerulus in the AL (Hallem and Carlson 2006a; 2004; Hallem, Ho, and Carlson 2004; K. Vogt et al. 2019; Couto, Alenius, and Dickson 2005; Fishilevich and Vosshall 2005; Mansourian and Stensmyr 2015). Upon odor onset, multiple types of OSNs are activated at varying magnitudes whose combined activity provides information about the nature and concentration of the odorant molecule (Hallem and Carlson 2006a; De Bruyne, Foster, and Carlson 2001; Wilson 2013a; De Bruyne, Clyne, and Carlson 1999; E. J. Hong and Wilson 2015b; Nagel and Wilson 2011; Wilson 2008a).

The Critical Period of Olfaction in Drosophila

CPP is modulated by exposure to sensory stimuli, therefore, studies on critical periods are carried out using manipulations involving deprivation of or over exposure to a sensory stimulus as shown in Figure 1B. In fact, the core definition of critical periods in neural systems rests heavily upon findings from early studies in sensory deprivation experiments of the visual cortex in kittens, produced by monocular deprivation of visual inputs early in life (LeMasurier and Van Wart 2012; David H Hubel and Wiesel 1962; D H Hubel and Wiesel 1970; Wiesel and Hubel 1963b). These studies showed long-term functional changes in circuit organization and response properties in the cortex without major alterations in the peripheral visual circuits (Hooks and Chen 2020; Wiesel and Hubel 1963b; 1963a). In contrast, experience dependent changes in the visual circuit of mice

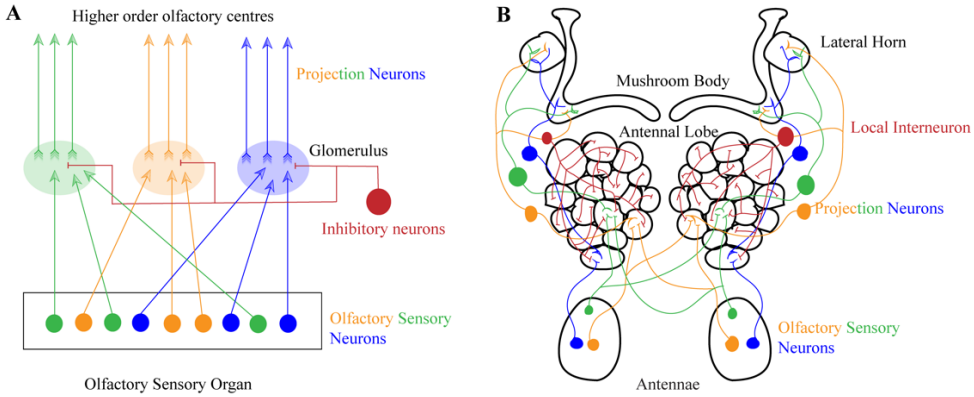

Figure 2. Organization of olfactory processing centers.

(**A**) Generic organization of the primary olfactory system in mice and fruit flies. Throughout the olfactory organ, dendrites, and cell bodies of OSNs expressing different ORs are distributed, the axons of which synapse onto distinct neuropil structures called glomeruli. Together the glomeruli make up the primary olfactory processing site. Here, projections neurons (PNs) refer to neurons with dendrites within the primary olfactory processing site and axons that project to higher-order olfactory neuropils. Within each glomerulus, inhibitory neurons also synapse with OSNs and PNs. The higher-order olfactory centers receive axonal output from the PNs.

(**B**) Organization of the primary olfactory system in Drosophila. In flies, the antennae contain OR-expressing OSNs that project onto distinct glomerular neuropil and synapse with PNs. The glomeruli together constitute the AL in the fruit fly. Within the AL, local inhibitory interneurons also synapse with OSNs and PNs. The PNs project onto higher-order olfactory centers like the mushroom body (MB) and the lateral horn (LH).

during the critical period have been observed much earlier in the retinal ganglion cells (RGCs) in response to sensory deprivation as well as over-stimulation. Dark rearing (stimulus deprived) mice during their visual critical period produces marked differences in the development of dendritic and receptive fields (Chen et al. 2021), as well as, in the thickness and length of the myelin sheath of the axons (Osanai et al. 2022) of RGCs. Daily visual stimulation in the form of optomotor response (OMR) stimulation during the critical period induces a BDNF-mediated hyperacuity in mice (Mui et al. 2018). Similar to the visual critical period in mice, olfactory critical period plasticity has been observed at the level of the OSNs in the AL of *Drosophila*. The olfactory critical period is described as the time during early life of the organisms when their olfactory circuits are refined in response to their odor environment. Reminiscent of the expansion of ocular dominance columns in the visual monocular deprivation (David H Hubel and Wiesel 1962; Wiesel and Hubel 1963b) or the expansion of the dendritic receptive fields of RGCs following eye opening during the critical period (Chen et al. 2021), the olfactory critical period in *Drosophila* and mice is marked by striking changes in the volume of the glomerulus that primarily detects the odor to which the organism is exposed (Inoue et al. 2021; Sachse et al. 2007b; J. M. Devaud, Acebes, and Ferrús 2001a). However, the cellular and molecular mechanisms underlying these structural changes in olfactory CPP are not fully understood. Here, we rely on the anatomical simplicity and the ready availability of genetic tools to define mechanisms of olfactory CPP in *Drosophila*. The fly olfactory system is

a powerful model to study critical periods because glomerulus in the AL form discrete functional units. Therefore, differential changes in synaptic transmission and structure of each glomerulus in response to an odor can be easily studied in the same identified glomerulus across animals.

The olfactory critical period in *Drosophila* begins upon eclosion of the adult fly from its pupae and lasts for 48 hours. In flies, most studies of critical periods rely on prolonged exposure to odors rather than depriving the olfactory organs (the antennae) of sensory input. This is likely because in dipterans, the vast majority of OSNs project bilaterally to the AL, thus making internal comparisons difficult (Stocker et al. 1990; Couto, Alenius, and Dickson 2005). Initial experiments on the olfactory CPP in flies (J. M. Devaud, Acebes, and Ferrús 2001a) demonstrated activity-dependent morphological changes in the cognate glomeruli as well as changes in behavior, which were observed a week after odor exposure ended. Briefly, when young flies were exposed to high concentrations of benzaldehyde and isoamyl acetate for 4 days, between 2-5 days post eclosion, the flies showed a reduced response to the exposed odors as compared to other odors. Furthermore, flies chronically exposed to benzaldehyde showed a marked decrease in the volume of specific glomeruli while the volume of the whole antennal lobe remained unchanged. Similarly, exposure to isoamyl acetate produced a significant decrease in the volume of a different glomerulus (J. M. Devaud, Acebes, and Ferrús 2001a; J. M. Devaud, Keane, and Ferrús 2003; J. Devaud et al. 2003). This anatomical change is characterized by a reduction in the synaptic density of the affected glomeruli which was dependent on cAMP signaling. Since both mutants for a phosphodiesterase and a calmodulin activated adenylyl cyclase failed to undergo these morphological and behavioral changes (J. M. Devaud, Acebes, and Ferrús 2001b; J. Devaud et al. 2003), it demonstrated a critical role for cAMP signaling. As in the mouse OB, (Inoue et al. 2021), the olfactory CPP in the fly is also characterized by synaptogenesis in the antennal lobe (AL), with a 38% increase in volume

between 1-12 days post eclosion. This increase in AL volume is due to the unique trends of volume increases in individual glomeruli (J. Devaud et al. 2003). However, we do not know if mechanisms analogous to Sem7a-PlxnC1 signaling as observed in mammalian olfactory CPP are in play behind such synaptogenesis in the fly (Inoue et al. 2018). The above experiments on CPP in the AL of *Drosophila* were performed before the odor tuning of the fly ORs were defined and before OR responses were mapped onto distinct glomeruli (Couto, Alenius, and Dickson 2005; Fishilevich and Vosshall 2005; Hallem and Carlson 2006a; Hallem, Ho, and Carlson 2004). Once the molecular map of odor coding was established, the topic of critical periods was reanalyzed independently in several studies. These studies showed that the critical period of olfaction lasts up to 48hrs post eclosion (Sachse et al. 2007a; Das et al. 2011; Chodankar et al. 2020) and the morphological and behavioral effects are reversible. Interestingly, the glomerulus responsive to geranyl acetate (GA) was an exception to this rule as it was shown that the GA-induced changes in the cognate glomerulus can occur when odor exposure starts 48hrs after eclosion (Chodankar et al. 2020; Kidd and Lieber 2016; Lieber, Kidd, and Struhl 2011) . Therefore, although the structural plasticity in response to GA is a form of experience-dependent plasticity, it does not meet our criteria for CPP. Perhaps, this is related to the ethological relevance of GA, which is found to be present in physiologically active concentrations in fruit (Dweck et al. 2018). Therefore, the high level of plasticity is probably needed to find food sources in the adult fly making it adaptable to its changing chemical environment. Prolonged exposure to fruit odors in *Drosophila* during the critical period led to reduced PN output (Pech et al. 2015). Similar experience dependent plasticity has been observed in adult foraging honeybees in response to floral odorous compounds, where prolonged exposure to such odors led to a decrease in glomerular volume (Andrione et al. 2017).

Similar kinds of ethologically relevant structural plasticity have also been reported in ants (Penick et al. 2021).

In *Drosophila*, a single odor can activate either one (private odor) or multiple (public) chemosensory receptors and their cognate glomerulus. Furthermore, at higher concentrations a

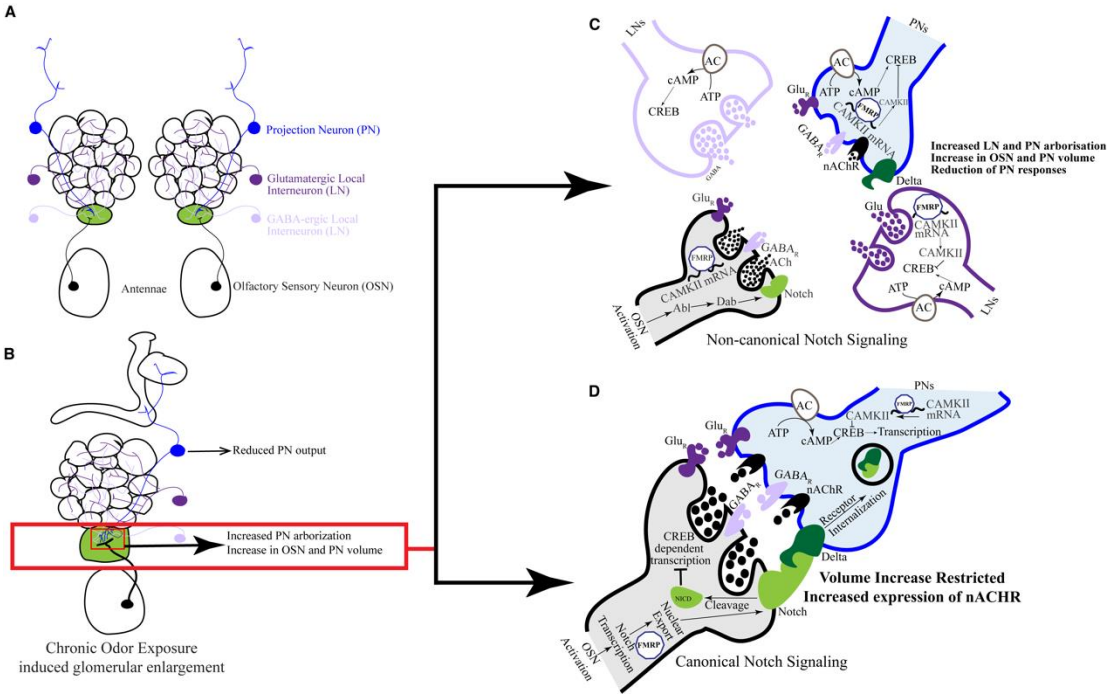

Figure 3. The olfactory circuit underlying critical period plasticity in *Drosophila*.

(A) The olfactory circuit in the fruit fly starts at the periphery in the antennae that houses the cell bodies of the OSNs. Discrete AL glomeruli are formed by OSN axons, PN dendrites and LN processes.

(B) Upon chronic odor exposure during the critical period, the cognate glomerulus that responds to the odor, increase in volume (shown in green).

(C) Volume increase of cognate glomeruli is a result of Notch dependent increase in volume of PN and OSN arbors(Lieber, Kidd, and Struhl 2011; Kidd, Struhl, and Lieber 2015; Kidd and Lieber 2016) and increase in the number of PN processes (Chodankar et al. 2020).

(D) OSN activation during the critical period following chronic odor exposure activates PNs and LNs. In the OSNs, FMRP upregulates transcription of calcium calmodulin dependent kinase II (CAMKII) that inhibits the transcription factor cAMP response binding element (CREB). Non-canonical Notch signaling pathways mediate an increase in volume of the OSN and PN arbors(Kidd and Lieber 2016; Kidd, Struhl, and Lieber 2015; Lieber, Kidd, and Struhl 2011). Calcium calmodulin dependent adenylate cyclase mediates CREB dependent gene transcription in the LNs which is required for the increase in the number of PN arbors. Notch-Delta interaction between OSNs and PNs respectively through the Canonical Notch Signaling pathway limits the extent of volume increase of OSN and PN arbors. FMRP aids in the nuclear export of Notch within the OSNs.

private odor may activate multiple non-cognate glomeruli. However, both public and private odors have been tested at high concentrations and each of them were shown to have unique effects on their cognate glomeruli and behavioral responses. An exception to this rule is CO_2 which activates a single glomerulus even at higher concentrations. Exposure to CO_2 during the critical causes an increase in the volume of the cognate glomerulus (Figure. 3 A, B). In contrast, a public odor, ethyl butyrate (EB) causes an increase in volume of two of its cognate glomeruli (Sachse et al. 2007a; Das et al. 2011; Sudhakaran et al. 2014; Chodankar et al. 2020) and a decrease in the volume of another cognate glomerulus (Golovin, Vest, and Broadie 2021a; Chodankar et al. 2020). This increase in volume is resultant of increased number of synapses between a subclass of LNs and PN dendrites (Chodankar et al. 2020). Behaviorally, a reduced responsiveness was observed to these aversive odors, while physiologically, odor induced activation of a subset of inhibitory local interneurons (LNs) were shown to inhibit PN activity following critical period odor exposure

(Sachse et al. 2007a; Das et al. 2011). In comparison, similar experiments with attractive odors led to physiologically contradictory observations. While exposure to some attractive odorants reduced OSN activity and increased PN responses (Kidd, Struhl, and Lieber 2015), exposure to other attractive odorants improved the sensitivity (Iyengar et al. 2010) or response rate of their cognate OSNs (Chakraborty, Goswami, and Siddiqi 2009). Behaviorally, such an increase in OSN activity led to increased attractiveness in the exposed flies. Collectively, these studies reveal a high degree of heterogeneity in the impact of CPP on olfactory network structure.

Mechanisms Regulating Olfactory Critical Period Plasticity in Drosophila

Glomerulus-specific volume increase can be attributed to expansion of the pre-existing neuronal arbors and to an increase in the number of arborizations (Figure. 3B). Both mechanisms are involved during the critical period. Expansion of OSN-PN synapses was shown to be regulated by the Notch-Delta signaling pathway (Kidd, Struhl, and Lieber 2015; Kidd and Lieber 2016). Briefly, Notch is expressed by OSNs in an activity dependent manner, that in turn activates Delta in the PNs that synapse onto these OSNs, which leads to an increase in glomerular volume through non-canonical mechanisms (Figure. 3C). However, the extent of increase in volume is regulated by canonical Notch mechanisms through feedback from Delta on PNs (Kidd, Struhl, and Lieber 2015) (Figure. 3D). The increase in glomerular volume is also driven by the increase in the number of PN arborizations and postsynaptic contact sites, as well as increased number of inhibitory synapses between an LN subset and PN dendrites (Chodankar et al. 2020), however the number of viable PNs, OSNs and LNs remain unchanged following critical period odor exposure. Further, cAMP dependent mechanisms in a subset of local interneurons (LNs) are required for the increase in PN arborizations and inhibitory synapses between LNs and PNs. Specifically, knockdown of a specific adenylyl cyclase encoded by *rutabaga* in an LN subset was sufficient to prevent increase

in PN arborizations following critical period odor exposure (Chodankar et al. 2020). In addition, expression of an inhibitory form of the transcription factor cAMP response element binding protein (CREB) was also able to prevent such plasticity (Das et al. 2011). These results are consistent with previous observations about the importance of cAMP in glomerular volume changes (J. M. Devaud, Acebes, and Ferrús 2001b; J. Devaud et al. 2003).

However, it is important to note that these LNs in these studies are, as a population, pan-glomerular, thus questions remain about the exact mechanisms that impart glomerulus-specific plasticity. Hence, how cAMP dependent transcription in the LNs leads to structural and physiological changes only in the affected glomeruli is unclear. One line of evidence showed that knocking down *Ataxin 2* (Atx2) and the *Drosophila* homologue of the Fragile-X mental retardation protein (dFMR1) in the CO_2 sensing PNs impaired physiological, behavioral, and structural plasticity during the critical period (Sudhakaran et al. 2014; McCann et al. 2011b). The proposed mechanism for this posits that both dFMR1 and Atx2 are required for microRNA dependent translational repression during critical period plasticity at the local LN-PN synapse (McCann et al. 2011b). However, the exact mechanisms by which Atx2 might promote glomerular specific translational repression to give rise to structural, physiological, and behavioral changes remain unknown. One explanation could be that Atx2 and dFMR1 regulate expression of calcium-calmodulin dependent protein kinase II (CAMKII) at synapses because knockdown of Atx2 and dFMR1 upregulate CAMKII expression (Sudhakaran et al. 2014). It is interesting to note that, CAMKII is a known regulator of plasticity in visual critical periods (Reha et al. 2020; Hensch 2005; 2004; Dehorter and Del Pino 2020) .

In addition to their role in the PNs, dFmr1 has been shown to be involved in OSN axon remodeling during the critical period. OSN-specific knockdown of dFmr1 prevents critical period

OSN retraction whereas overexpression of dFmr1 in the VM7 glomerulus enhances OSN retraction when exposed to ethyl butyrate (EB) during the critical period (Golovin, Vest, and Broadie 2021a). However, optogenetic activation of OSN-specific dFmr1 knockdown flies still showed OSN retraction which implies EB-specific activation is required for this type of remodeling in the OSNs (Golovin, Vest, and Broadie 2021a).

In addition to the activity dependent transcriptional and translational control mechanisms in place, we cannot rule out the role of neurotransmitters like GABA and Glutamate in shaping critical periods. Previous studies have shown that silencing glutamatergic LNs could prevent OSN remodeling during the critical period in an NMDAR independent manner as NMDAR mutants do not show any defects in OSN remodeling (Golovin, Vest, and Broadie 2021a). However, OSN-specific knockdown of NMDAR1 and GABA-A showed impaired OSN remodeling (Golovin et al. 2019). In addition, GABA-A and NMDAR receptors were shown to be required in the PNs for both structural and behavioral plasticity. Thus, glomerulus-specificity may arise from the combination of excitatory and inhibitory interactions that occur within a given glomerulus during odor stimulation.

In conclusion, during the olfactory critical period, chronic odor exposure induces odor specific structural, physiological, and behavioral plasticity in the AL of the fruit fly. These physiological changes are driven by local changes in activity of the OSNs, LNs and PNs within the cognate glomerulus. Several transcriptional and translational mechanisms are in place that induce structural plasticity in the glomerulus by changing the volume and number of processes of these neurons. Apart from these, neurotransmitters like GABA and Glutamate shape intercellular signaling mechanisms during the critical period. However, much remains to be understood regarding the interdependence of these cellular mechanisms to bring about odor specific changes

in the glomeruli. Another avenue not explored yet is if and how neuromodulators like serotonin (5-HT), dopamine, oxytocin modulate the olfactory critical period.

Interestingly, evidence from studies on the critical period in the visual circuit in mammals have suggested that serotonin (5-HT), a neuromodulatory transmitter, can have an important role in CPP. Studies in the kitten visual cortex showed the differential expression of serotonin receptor 2C (5-HT2C) influenced the location and type of plasticity that were induced during the critical period (Kojic et al. 2000; Gu and Singer 1995). Other studies showed that increasing 5-HT brain levels by administering selective-serotonin reuptake inhibitors (SSRIs) led to the reopening of critical period for ocular dominance plasticity in the visual cortex. This effect was due to reduction in inhibition by GABAergic interneurons (Teissier, Soiza-Reilly, and Gaspar 2017a). Also, enhancing or reducing the GABA function could modulate the duration and closing of critical periods (Hensch 2005). Future experiments should test the possibility that the opposite holds true, that is, that reducing serotonin activity could reduce GABA function and delay maturation of inhibitory circuits and thereby extend the critical period.

Serotonergic Neuromodulation of the Drosophila Olfactory Circuit

Neuromodulators are responsible for efficient signal transmission and circuit function in the central nervous system (CNS), influencing behavior and cognition in almost all animal species. A dysregulation in neuromodulation leads to psychiatric disorders like schizophrenia, anxiety, depression, anorexia, etc., and often neuromodulatory systems are directly targeted for treating the symptoms of these disorders (Temel et al. 2012). The repertoire of genetic tools and optical imaging techniques available today have produced an attractive opportunity to explore interactions between the different neuromodulatory and sensory circuits in the central nervous system (CNS).

Hence, to study how 5-HT might affect CPP in *Drosophila* olfaction, it is important to understand the functional organization of serotonergic neuromodulatory circuits in relation to olfactory information processing centers. Here, I highlight recent neurophysiological evidence concerning modulation of olfactory processing by 5-HT in *Drosophila*.

Serotonin (5-HT) is a universal neuromodulator found to actively modulate neural processes of cognition, motor control, appetite, and sensation. It is an evolutionarily conserved neuromodulator and in *Drosophila*, it performs crucial roles in feeding (Albin et al. 2015; Al-Anzi and Zinn 2018), learning and memory (Sitaraman et al. 2012; 2017), aggression (Alekseyenko and Kravitz 2014; Alekseyenko, Lee, and Kravitz 2010; Alekseyenko et al. 2019; 2014) vision (Sampson et al. 2020), mating (Pooryasin and Fiala 2015) and olfaction (Gaudry 2018; Zhang and Gaudry 2016; Suzuki et al. 2020; Zhang et al. 2019; Coates et al. 2017; Sizemore and Dacks 2016; Lizbinski and Dacks 2018; Sizemore, Hurley, and Dacks 2020; Dacks, Christensen, and Hildebrand 2006; Dacks et al. 2009; Coates et al. 2020). Studying neuromodulatory systems in mammals becomes a challenge due to the inherent complexity of the brain which limits the number of simultaneous manipulations that can be performed. The 100,000-neuron rich *Drosophila* CNS is an attractive model to study 5-HT neuromodulation because there are approximately 106 serotonergic neurons in the adult fly (Pooryasin and Fiala 2015). In addition, optogenetic tools enable researchers to visualize and conveniently track neuronal activity associated with specific behaviors at single cell resolution (Yoshihara and Ito 2012; Jesse Isaacman-Beck, Kristine C. Paik, , Carl F. R. Wienecke, Helen H. Yang, Yvette E. Fisher, Irving E. Wang, Itzel G. Ishida, Gaby Maimon, Rachel I. Wilson 2019; Zheng et al. 2018; Batelli et al. 2017; Nern, Pfeiffer, and Rubin 2015). Importantly, *Drosophila* and the vertebrate olfactory system are significantly similar which

allows the study of the general principles that govern neuromodulation in olfactory circuits across species (Wilson 2013b; Wilson and Mainen 2006).

Importance of a Comprehensive Understanding of Neuromodulation on Neuronal Circuits

To gain a comprehensive understanding of how the activation sensory circuits result in complex behaviors, one must understand how a stimulus is encoded within the sensory circuit, how this information is modulated by external and internal states during neural processing as it moves from the periphery to the higher regions of the brain and finally the behavioral repercussions of such modulation. Simple studies of the anatomical connectome cannot capture such diverse network effects on multiple circuit components because it fails to report the molecular events critical to understanding neuromodulation. This creates the need to map the neuromodulatory network and its interaction with the sensory circuit to understand how such anatomically constrained networks give rise to complex and diverse behavioral patterns (Marder 2012). Therefore, it is imperative to study neuromodulation in a model system where the anatomical connectome is already well characterized. *Drosophila* is such a model system. Not only are the neurons in the olfactory circuit exhaustively identified (Wilson and Mainen 2006; Wilson 2013b), but recent technological advances have also enabled scientists to generate an atlas of the expression patterns of the neuromodulator 5-HT and its receptors (5-HTR) in the fly brain (Coates et al. 2017; 2020; Pooryasin and Fiala 2015; Sitaraman et al. 2012; Sizemore and Dacks 2016; Zhang et al. 2019; Lizbinski and Dacks 2018; Giang et al. 2011).

The role of the neuromodulators like 5-HT in olfactory circuits is to bring about dynamicity to circuit excitability and function through differential modulation of neuronal activity. The neuromodulator 5-HT is delivered to the olfactory regions either through conventional synapses

to modulate local circuits or via the extracellular space (ECS) as a paracrine signal to modulate distal targets. These two distinct delivery modes bring about diverse and often opposing modulatory effects in fly olfaction. This has been clearly observed during ethanol attraction in flies where, a pair of serotonergic neurons, the contralaterally-projecting serotonin immunoreactive deuterocerebral neurons (CSDns) deliver 5-HT synaptically within the AL and counteract the inhibitory effects of paracrine 5-HT (Xu et al. 2016).

The Serotonergic System in Drosophila

Serotonin is a biogenic amine, synthesized from the essential amino acid tryptophan in a two-step reaction by the serotonergic cells. In the adult fly, the 110 serotonergic neurons are distributed into 10 clusters consisting of 1 to 5 neurons each (Sitaraman et al. 2017; Pooryasin and Fiala 2015; Sitaraman et al. 2012). These neurons supply 5-HT either via direct synaptic connections at specific synapses or as a paracrine signal to reach distal targets.

Serotonin (5-HT) Receptors (5-HTRs) in Drosophila

The 5-HT responsive cells are characterized by the presence of G protein-coupled 5-HT receptors (5-HTRs). In Drosophila, five G-protein coupled receptors: 5-HT1AR, 5-HT1BR, 5-HT2AR, 5-HT2BR, and 5-HT7R are expressed. These receptors are homologous to the mammalian 5-HT receptors both in sequence and function. They also signal through distinct second messenger pathways. The 5-HT1R type is inhibitory and negatively coupled to adenylate cyclase (Saudou et al. 1992), the 5-HT7R type is excitatory and positively coupled to adenylate cyclase (Witz et al. 1990a). The 5-HT2R type is excitatory and positively coupled to phospholipase C (Saudou et al. 1992; Colas et al. 1995; Gasque et al. 2013). Although specific antibodies against the receptors have not been characterized, several genetic manipulations and emerging techniques enable the visualization of these receptors *in situ.*

Expression of Serotonin Receptors in the antennal lobe

"Protein trap" and "gene trap" expression studies have been used to directly ascertain the distribution of the serotonin receptors within the antennal lobe, the first olfactory relay station (Sizemore and Dacks 2016). While OSNs exclusively express 5-HT2BRs, the ventral PNs and LNs express any one of the 5 serotonin receptor subtypes. The lateral PNs express any one of the 5-HT1ARs, 5-HT2ARs, 5-HT2BRs, and 5-HT7Rs serotonin receptor subtypes and the anterodorsal PNs express one of the 5-HT7R, 5-HT1AR and 5-HT2BR serotonin receptor subtypes. Also, OSNs aside the other olfactory neurons express distinct 5-HTRs. For example, only a small population of the PNs in the lateral and anterodorsal cell clusters express inhibitory 5-HTR1Rs while most of them expresses the excitatory 5-HTRs. This expression pattern tells us that 5-HT neuromodulation acts through diverse receptor types to differentially affect specific features of the olfactory network. Knowing which 5-HT receptors are expressed within each of the 50 distinct olfactory neuropils arising from OSNs expressing different olfactory receptors, will provide a foundation for making predictions about the mechanism of neuromodulation within each pathway. This way, we can also identify how distinct stimuli is modulated within the olfactory circuit to give rise to characteristic behavioral outcomes.

A single pair of serotonergic neurons make synaptic connections in the antennal lobe.

A central focus in neuroscience has been on studying neuromodulation at synapses because of the ease with which they can be imaged using electron microscopy or studied physiologically. In Drosophila, a single pair of 5-HTergic neurons innervate the olfactory system, the "contralaterally projecting, serotonin-immunoreactive deuterocerebral neurons" or CSDns (Coates et al. 2017; Zhang and Gaudry 2016; Coates et al. 2020). The CSDns innervate almost all olfactory glomeruli and are the only serotonergic neurons that maintain synaptic connections throughout the olfactory system in AL, LH, MB calyx and superior lateral protocerebrum. Like most other

neurons in the olfactory circuit of the AL, CSDns are subject to inhibition in response to most odorants (Zhang and Gaudry 2016; Zhang et al. 2019; E. J. Hong and Wilson 2015a). Using synaptobrevin GFP Reconstitution Across Synaptic Partners (syb:GRASP) GFP reconstitution technique as well as electron microscopy data Coates et al., showed that the CSDns receive synaptic inputs from the OSNs, LNs and PNs (Coates et al. 2017; 2020). The LNs provide a homogeneous inhibitory output while the OSNs and PNs provide glomerulus and organism specific excitatory outputs onto the CSDns. It is also interesting to note that the CSDns make more uniform reciprocal connections with the LNs than with the PNs and OSNs. The LNs being inherently inhibitory in nature, the reciprocal connection may serve as a way for LNs to modulate olfactory signal processing and transmission upstream in response to global network states. Future work will be needed to characterize the role of CSDns in modulating LN and PN responses under different olfactory coding contexts for example during the critical period.

The CSDns receive at least two glomerular specific inputs from the OSNs but do not maintain outputs onto OSNs. This implies that the widespread distribution of the 5-HT2BRs on OSNs are solely to detect 5-HT as a paracrine signal and are probably excitatory in nature. It is to be seen if paracrine 5-HT stimulation of OSNs can also induce excitation in the CSDNs in these specific glomeruli. While the CSDns receive excitatory input from only a specific subset PNs that innervate the DM5 glomerulus, they project onto PNs throughout the AL (Coates et al. 2017; 2020). It remains to be seen how CSDns differentially modulates other PNs as they express both inhibitory and excitatory 5-HT receptors. Also, we do not know how synaptic or paracrine 5-HT differentially modulates the DM5 PNs.

Isolation of paracrine effects of Serotonin

Paracrine neuromodulation has proven harder to study because of the slow diffused mode of transmission, the multiple receptors 5-HT can activate, and the lack of anatomical correlate of

functional connections. However, to gain a comprehensive understanding of how the CNS functions, we need to understand the neuromodulatory effects of both synaptic and paracrine signals and how they are interpreted in neuronal circuits. Drosophila becomes an attractive model to study this because manipulations are easier to perform in the fly due to readily available genetic tools. Since CSDns are the only pair of 5-HT neurons that make synaptic connections with the olfactory system, the remaining ~104 5-HT neurons might contribute to neuromodulation in a paracrine fashion. Hence, ablating the CSDns will allow the isolation of global paracrine 5-HT effects in the fly (Suzuki et al. 2020).

Studies by Suzuki et al., 2020 involving the pheromone sensitive DA1 glomerulus sheds some light onto how paracrine 5-HT modulates olfactory processing. They demonstrated for the first time that the effects of paracrine 5-HT are mediated via the 5-HT7Rs and blocking 5-HT7R signaling increases global PN responses. Also, LNs are the only neuron type which expresses 5-HT7R in this glomerulus. Hence a probable mechanism of neuromodulation by paracrine 5-HT on this glomerulus would involve 5-HT mediated excitation of LNs via the excitatory 5-HT7Rs. Excitation of these GABAergic LN subset would then inhibit either OSN or PN responses in this glomerulus. Interestingly antagonist screens, optogenetic stimulation, RNAi and blocking the LN-PN synapse via application of tetrodotoxin (TTX) studies showed that excitation of the 5-HT7R LNs postsynaptically inhibits PN responses but had no effect on the OSNs. Thus, a subset of GABAergic LNs which express 5-HT7Rs are responsible for sensing paracrine 5-HT and sets an overall inhibitory tone on global PN responses, postsynaptically. It is also important to note here that although the DA1 PNs lack 5-HT7Rs, it is not necessarily true for all other PNs. Indeed, as stated above, several PNs express 5-HT7Rs (Sizemore and Dacks 2016) and it would be interesting to see how paracrine 5-HT affect these PNs.

From the DA1 PN responses, we see that paracrine and synaptic 5-HT has opposing effects on olfactory responses. Basal 5-HT levels excite the GABAergic 5-HT7R expressing lateral LNs which inhibits these PN responses (Sizemore and Dacks 2016; Suzuki et al. 2020). Another point to be noted here is that some 5-HT7R expressing LNs are glutamatergic in nature and are located ventral to the antennal lobe (Sizemore and Dacks 2016). Glutamate is a known inhibitory neurotransmitter in the *Drosophila* antennal lobe (W. W. Liu and Wilson 2013). While the GABAergic LNs act within glomeruli and the glutamatergic LNs act between glomeruli, paracrine 5-HT might act on different spatial and temporal scales to set a net inhibitory tone throughout the AL. It is also known that the glutamatergic LNs might inhibit GABA LNs thereby indirectly upregulating PN responses (W. W. Liu and Wilson 2013). It would be of therapeutic interest to explore the nature of these bidirectional effects and their necessity.

To better understand the mechanisms of paracrine neuromodulation, future work needs to focus on isolating the effects of each of the 10 identified serotonergic neuron subsets and characterize their specific effects in the olfactory centers. Employing a 2-promoter based intersectional strategy, each expressing half a Gal4 molecule that dimerizes in the cell, we can restrict expression to only the target neurons where both promoters are active. This might help to genetically isolate all the 10 serotonergic clusters and study their effects on the olfactory system in isolation. Identification of such promoters will be possible by using a stochastic labeling approach like the MultiColor FlpOut (MCFO) (Batelli et al. 2017) or the Sparse Predictive Activity through Recombinase Competition (SPARC) (Isaacman-Beck et al. 2020) to parse out individual serotonergic neurons with their unique arborizations. Once this is achieved, we can employ search algorithms to identify unique promoter lines to perform cell specific manipulations using the split-Gal4 approach (Luan et al. 2020).

Research Topics Covered

In this work, I present unique roles of specific 5-HTRs in specific neurons in the olfactory system of *Drosophila* during the critical period. I show that release of 5-HT from the CSDns that maintain synaptic projections within the AL, the primary olfactory processing center is essential to induce CPP in the fly. Further, I show that the specific subset of 5-HT7R expressing LNs that were shown to respond to paracrine serotonin in earlier studies modulates CPP through cAMP dependent mechanisms. Within the OSNs, I show that the expression of 5-HT2BRs is differentially controlled during the critical period and their expression on OSNs is crucial for CPP. I also show that 5-HT1BRs may act on serotonergic cells to maintain optimum levels of 5-HT conducive to structural plasticity during the critical period. Lastly, to isolate the paracrine effects of serotonin on the olfactory system, I attempted to stochastically label individual serotonergic neurons and identifying unique Gal4 lines that could be used to make split-Gal4 reagents to manipulate those individual neurons. I show that it is possible to identify such Gal4 lines and provide a proof of principle of how this method can be used in the future to isolate individual cells of interest.

Chapter 2: Serotonin acts through multiple cellular targets during an olfactory critical period

Introduction

In early postnatal life of animals, all sensory systems exhibit heightened levels of plasticity and circuit refinement in response to environmental stimuli in a specific time window called critical periods. Critical period plasticity provides (CPP) an excellent readout to assess how sensory experiences shape circuits early in life at the cellular and molecular level (Mallick, Dacks, and Gaudry 2024; Reha et al. 2020; Hensch 2005). Along with sensory experiences, neuromodulators like 5-HT also play an important role in shaping CPP. Initial experiments indicating the role of 5-HT in modulating sensory critical periods was observed in both the visual and somatosensory cortices (Wang, Gu, and Cynader 1997; Gu and Singer 1995; Jitsuki et al. 2011; Dyck and Cynader 1993). Similarly, 5-HT has also been shown to modulate the early postnatal development of limbic circuits such as the pre-frontal cortex and disruption in serotonergic signaling in this circuit have been linked to an increased risk for behavioral and cognitive deficits in adults (Teissier, Soiza-Reilly, and Gaspar 2017b; Suri et al. 2014; Higa et al. 2022; Ogelman et al. 2024). Thus, 5-HT targets multiple effector regions during early development to facilitate proper brain development and function in adults. However, 5-HT can activate several receptor subtypes expressed by distinct cell types within a network, so the cellular mechanisms by which 5-HT impacts CPP can be difficult to identify.

We took advantage of the wealth of transgenic tools and foundational work in the olfactory system of *Drosophila* to determine how 5-HT can impact different circuit mechanisms within an olfactory critical period. The organization of the fruit fly olfactory network is similar to that in mammals (Hildebrand and Shepherd 1997; Mallick, Dacks, and Gaudry 2024) in that olfactory processing begins upon odor binding chemoreceptor proteins localized at the dendrites of olfactory

sensory neurons (OSNs) (Hallem and Carlson 2006b; Hallem, Ho, and Carlson 2004; Benton et al. 2006; 2009). All OSNs expressing the same complement of chemoreceptive proteins project to a distinct glomerulus to form an olfactory map within the primary olfactory center, the antennal lobe (AL) (Wilson 2013b). OSNs synapse upon second-order projection neurons that project onto higher order olfactory centers in the mushroom body (MB) and lateral horn (LH). The cellular and molecular mechanisms of structural plasticity observed in olfactory CPP in the fruit fly is well known.(Fabian and Sachse 2023; Fabian et al. 2023; Das et al. 2011; Chodankar et al. 2020; Golovin, Vest, and Broadie 2021b; Golovin et al. 2019; Kidd and Lieber 2016; Kidd, Struhl, and Lieber 2015; Lieber, Kidd, and Struhl 2011; Sachse et al. 2007b) Most CPP studies in *Drosophila* were conducted using either CO_2 or ethyl butyrate exposure as sensory stimuli, or both. With either of the odors, chronic exposure during the critical period induced structural plasticity in the relevant glomerulus causing a change of either an increase or a decrease in the glomerular volume. At the circuit level, the structural plasticity resulting in an increase in glomerular volume is manifested through an increase in the number of LN and PN arbors innervating the glomerulus while the total number of neurons remains intact.(Sachse et al. 2007b; Das et al. 2011; Chodankar et al. 2020; Fabian et al. 2023) Glomerular volume decrease, on the other hand is caused by retraction of the OSN axon fibers upon chronic ethyl butyrate exposure in a specific glomerulus.(Golovin et al. 2019) These studies demonstrated a strong resemblance to visual CPP mechanisms in mammals.(Sachse et al. 2007b; Das et al. 2011; Chodankar et al. 2020; Golovin, Vest, and Broadie 2021b; Golovin et al. 2019) In both cases, CPP involves GABAergic and glutamatergic signaling, Ca2+/Calmodulin dependent adenylate cyclase and cAMP response element binding protein (CREB) dependent gene transcription.(Berardi et al. 2003; Das et al. 2011; Chodankar et al. 2020) Although 5-HT neuromodulation has been shown to be required for visual critical periods in

mammals(Kirkwood 2000; Kojic et al. 2000; Gu and Singer 1995; Maya Vetencourt et al. 2011; Vetencourt et al. 2008), it has not been studied with respect to CPP in any olfactory system.

Previous studies in mammalian models have established that 5-HT signaling plays a key role in shaping plasticity of sensory circuits during the CP and altering 5-HT levels in adults could reinstate CPs(Jitsuki et al. 2011; Vetencourt et al. 2008; Dyck and Cynader 1993; Maya Vetencourt et al. 2011). To investigate how 5-HT modulates olfactory critical periods, we focused on the behaviorally relevant (Suh et al. 2004; Faucher et al. 2006) CO_2 sensing circuit in *Drosophila*. Since the V glomerulus is exclusively dedicated to respond to CO_2 and the CPP mechanisms is already known for this glomerulus, it is ideal for studying the effect of serotonergic modulation. We performed cell-type specific genetic manipulations of the serotonergic system to identify where 5-HT is required during odor-evoked structural plasticity in the olfactory circuit. Our results show that during the critical period, 5-HT modulates distinct cell types in the AL via activation of different 5-HT receptor subtypes. We thus identified cell types where serotonergic modulation may be interacting with the previously described mechanisms of CPP to modulate structural plasticity during CPs.

Materials and Methods

Fly rearing and maintenance

All *Drosophila* lines were raised in sparse cultures on cornmeal, yeast, dextrose medium (Schenk and Gaudry 2023) at 25°C in a 12hour light/dark cycle unless otherwise noted. The fly lines used in this study are listed in Table 1.

We used female flies for our studies, except where specifically mentioned. Flies used in the CO_2 and air exposure experiments were raised at 25°C until the 4-day old pupae stage and at 23°C until they were sacrificed.

Odor exposure

We employed previously established odor exposure protocol to induce critical period plasticity in the flies (Golovin et al. 2019; Chodankar et al. 2020). Briefly, 4-day old pupae of relevant genotype were collected into separate vials based on odor-exposure. A fine mesh cheesecloth was secured at the opening of the fly vial to ensure free gaseous exchange. Fly vials were placed in either a temperature-controlled CO_2 or regular incubator at 23°C on 12-hour light/dark cycles. Eclosed flies were transferred into clean vials 18-21 hours after the start of odor exposure until day 5 post eclosion when they were collected for dissection and immunohistochemistry. For each of the odor exposure experiments, pupae for all the genotypes including control and Canton-S were collected on the same day and odor exposure started at the same time. Pupae of the same genotype were collected from the same stock bottle and separated into odor exposed (CO_2) control (air) vials to ensure consistency in food composition, availability, and conditions. During odor exposure both male and female flies of a specific genotype present in each vial and female flies were collected by cold anesthetizing the flies at the end of the experiment right before dissection.

Plasmid preparation for CRISPR/Cas9 knock-in

The GFP11-HA was synthesized de novo by Twist Bioscience, CA and the coding sequence was knocked-in the fly genome using CRISPR/Cas9 via homology-directed repair (HDR). Two plasmid DNAs were prepared for each knock-in preparation, the guide RNA (gRNA) plasmid and the donor plasmid. The gRNA plasmid was prepared following a previously established protocol(Ran et al. 2013). Briefly, a pair of 24-nt oligos were commercially synthesized as for normal unsalted primers (Eurofins Genomics). For each oligo, the first 4 nucleotides (TTCG for the sense strand and AAAC for the antisense strand) at the 5' end formed

the overhanging sequence after reannealing. These overhanging nucleotides were compatible with the Bbs I digestion sites of the plasmid pU6b, while the remaining 20 nucleotides carried the sense or antisense strand of the target sequence for the insertion site in the genome. The 20-nt target sequences (sense) were identified close to the C-terminus of the 5-HTR coding region using an online tool FlyCRISPR (Gratz et al. 2014) for 5-HT receptor genes, as listed in **Table 1**. The backbone for gRNA expression, pU6b, was digested with Bbs I and ligated with the reannealed 24-nt oligo pairs with T4 DNA ligase. To prepare the donor plasmid, approximately one kilo basepair of DNA upstream of the insertion site was PCRed as the left arm for homology directed repair (HDR). Similarly, about one kilo basepair of 5-HTR DNA in the downstream of the insertion site was PCRed as the right arm for HDR. The encoding sequence of GFP11-HA was then inserted in between both arms via overlap extension PCRs. The resultant PCR product was then inserted into pTwist Amp plasmid (High copy, Twist Bioscience HQ, CA) via restriction digestion and ligation with Rapid DNA Ligation Kit (Thermo Fisher Scientific, Cat. # K1422). For both plasmids, the ligation mixture was then used for transformation of *E. coli* strain DH5α (Thermo Fisher Scientific, Cat. # EC0112). Single colonies were used for inoculation of 10 mL mini cultures with LB medium supplemented with 100 μg/mL ampicillin (Thermos Fisher Scientific, Cat. # J60977.06). After overnight incubation with vigorous shaking, the plasmid DNA was prepared from the culture using the NucleoSpin Plasmid Mini kit (MACHEREY-NAGEL, Cat. # 740588.250). The purified DNA was digested with restriction enzymes for quality control and subject to sequencing analysis for verification.

Extraction of genomic DNA

To extract the genomic DNA from adult flies or larvae for PCR templates, 2 flies were smashed in 50 μL of Squishing buffer (10 mM TrisCl with pH 8.2, 1 mM EDTA, 25 mM NaCl

and 200 µg/mL freshly added Proteinase K) with a 200-µL pipette tip in a PCR tube. The mixture was incubated at 50 degrees Celsius for 30 minutes before inactivated at 95 degrees Celsius for 5 minutes. After it cooled down, 0.5 to 1 uL of the supernatant was used for the PCR template.

Generation of fly lines

To generate the 5-HTR-GFP11-HA transgenic lines, the gRNA plasmid and the donor plasmid was co-injected with a 1:1 mass ratio into fly embryos by Rainbow Transgenic Flies, Inc. (California, USA). The genotype of the embryos was *y,sc,v; {nos-Cas9}attP2* for 5-HT1A and -1B, or *y,sc,v; {nos-Cas9}attP40/CyO* for 5-HT2A, -2B and -7. Depending on which chromosome the 5-HTR gene is on, the candidate flies were individually balanced with Cyo (for 5-HT1A and -1B) by crossing with *w1118; Cyo/Sco*, or *Tm6b, Tb, Hu* (for 5-HT2A, -2B and -7) by crossing with *w1118; Tm6b, Tb, Hu/MKRS*. The nos-Cas9 transgene was finally removed by selecting progeny against red eyes.

Screening for successful knock-in was performed using PCR with a pair of primers targeting respectively the upstream and the downstream of the inserted sequence in the genomic DNA extracted from the candidate transformants. For PCR template, the genomic DNA was extracted from the daughter flies of individual injected embryos. Immunostaining against the HA-tag was used as the second step of verification.

Immunostaining and confocal imaging

All immunohistochemistry was performed using a standard protocol as previously described unless otherwise noted (Schenk and Gaudry 2023; Suzuki et al. 2020). For volume measurement experiments, the brains were incubated in the mounting medium for 1 hour before imaging to allow equilibration (Ostrovsky, Cachero, and Jefferis 2013). For imaging HA and split-

GFP, the immunohistochemistry protocol was slightly modified. Lp,.Briefly, the flies were anesthetized in a glass vial on ice for 1 min. The brains were dissected in PBS and fixed in 4% formaldehyde (37%, diluted in PBS) at room temperature. After three times of washing each for 15 min with 0.2% PBST, i.e., PBS supplemented with 0.2% (v/v) Triton X-100 (Thermo Fisher Scientific, Cat. # A16046.AE), the brains were blocked in 10% normal goat serum (NGS; Thermo Fisher Scientific, Cat. # PCN5000) at room temperature for 1 - 2 hours. Next, the brains were incubated with primary antibodies diluted in 0.2% PBST supplemented with 5% NGS at 4° for 3 to 4 days. Then the brains were washed with 0.2% PBST three times each for 15 min, and subject to the incubation with the secondary antibodies diluted in 0.2% PBST supplemented with 5% NGS in a dark environment at 4° for 1 day. Table 2 lists all the antibodies used in this study. After washing with 0.2% PBST four times each for 10 min, the brains were mounted on glass slides (VWR, Cat. # 16004-422) with VECTASHIELD Antifade Mounting Medium (Vector Laboratories, Cat. # H-1000-10) covered with glass coverslips (VWR, Cat. # 48366-089). To prevent the brains from being smashed, two smaller coverslips (VWR, Cat. # 48366-045) were placed as spacers between the glass slide and the covering coverslip with one on each side. The coverslips were fixed with a few drops of nail polish on the edge. All samples were scanned with a confocal microscope in the institution imaging facility: ZEISS LSM980, ZEISS LSM 710 or PerkinElmer Spinning disk. Brains used in the same experiment were imaged on the same day, under the same microscope and imaging settings.

Image Analysis

For measuring the volume of the glomerulus, we performed image segmentation by specifying the borders of the specific glomerulus across the Z-stack of the confocal image based on n-cad staining

using the segmentation plugin in Image J. The volume of the selected glomeruli was obtained by 3-D analysis of the segmented image using the MorphoLibJ (Legland, Arganda-Carreras, and Andrey 2016) plugin in ImageJ. Absolute volume is computed in this plugin by multiplying the number of voxels comprising the selection with the volume of individual voxel. The resultant glomeruli volumes were found to be consistent with those previously reported (Sachse et al. 2007b; Chodankar et al. 2020).

The fluorescence intensity measurements in Fig 4J were normalized for each glomerulus against the fluorescence intensity of the VL1 glomerulus. This was done to show the fold-difference in 5-HT2B expression in the selected glomeruli in comparison to a glomerulus of average 5-HT2B expression. The fluorescence intensity measurements reported in Fig. 3F, were obtained using previously reported described methodology in ImageJ (Fitzpatrick 2014). The corrected total cell fluorescence for each sample reported was calculated by subtracting the product of the area of the AL and mean background fluorescence of the brain outside the AL from the integrated density of the AL.

More than 15 samples were analyzed for each genotype, age, or odor exposure unless otherwise noted. An unpaired Student's t-test was used to compare the difference in glomerular volumes of air and CO_2 exposed flies or fluorescence intensity between sexes or across fly of different ages. The p-values are indicated as **$p < 0.05$ and as N.S for nonsignificance ($p > 0.05$) unless otherwise noted.

Table 1. Coding sequences of guide RNA (gRNA) spacers for CRISPR/Cas9 knock-in of GFP11-HA in 5-HTR genes.

Receptor name	Open reading frame	Gene ID	gRNA sequence (With thymine)

5-HT1A	CG16720	37196	gaccagtccactaccgcagc
5-HT1B	CG15113	37191	aatttcgacgggccttcaag (gRNA1) gaaaatttgatttcaactga (gRNA2)
5-HT2A	CG1056	40575	catattcaatcgcacgttcc (gRNA1 for isoform A) ttcgaggtgccttcgtcggt (gRNA2 for isoform A) tccttctggcgcaaacacgg (gRNA1 for isoform D) ctgaagacataattacgtgg (gRNA2 for isoform D) cgctatcggtctgtgacaga (gRNA1 for isoforms B, F and H) ggaaaagccgctaattacag (gRNA2 for isoforms B, F and H)
5-HT2B	CG42796	41017	aggcactcgtgctcgaatag (gRNA1) ttcagtttgcccggtttaac (gRNA2)
5-HT7	CG12073	43669	ggcgagggagagctttctct

Table 2. List of Antibodies used in this study.

Name	Type	Species of origin	Dilution	Supplier	Cat. #
anti-HA	Primary	Mouse	1:500	Thermo Fisher Scientific	26183
anti-rec.GFP*	Primary	Mouse	1:1000	Sigma	G6539
anti-GFP	Primary	Chicken	1:1000	Abcam	ab13970

nc82 Antibody	Primary	Mouse	1:50	Developmental Studies Hybridoma Bank (DSHB)	nc82
N-Cadherin	Primary	Rat	1:50	Developmental Studies Hybridoma Bank (DSHB)	ncad
Anti-chicken Alexa Fluor 488	Secondary	Goat	1:400	Life Technologies	A-11039
Anti-mouse Alexa Fluor 488	Secondary	Goat	1:400	Life Technologies	A-11004
Anti-mouse Alexa Fluor 633	Secondary	Goat	1:400	Life Technologies	A-21050

* rec.GFP: reconstituted GFP.

Table 3. List of Flies used in this study.

Figure	Genotype	Source
1A and 1B	Gr21a-Mmus\Cd8a.GFP	BDSC #52619
1C	R60F02-Gal4/10X-UAS-IVS-mCD8::GFP	R60F02-Gal4 (BDSC #48228) 10X-UAS-IVS-mCD8::GFP (BDSC #32185)
1D	Left to right: Canton-S R60F02-Gal4/UAS-Vmat-RNAi w1118;;UAS-Vmat-RNAi P{y[+t7.7]=CaryP}attP2/R60F02-Gal4	Canton-S (BDSC #64349), UAS-Vmat-RNAi (BDSC #44471) w1118 (BDSC#5905) P{y[+t7.7]=CaryP}attP2 (BDSC #36303)

2A	Canton-S	BDSC #64349
2B	TI{GAL4}5-HT1A[Gal4] / TI{GAL4}5-HT1A [Gal4]	BDSC#86275
2C	TI{GAL4}5-HT1B[Gal4]/ TI{GAL4}5-HT1B [Gal4]	BDSC #86276
2D	TI{GAL4}5-HT2A[Gal4]/ TI{GAL4}5-HT2A [Gal4]	BDSC #86277
2E	Left to right: UAS-5-HT2B-RNAi; TI{GAL4}5-HT2B[Gal4] w1118; UAS-5-HT2B-RNAi P{y[+t7.7]=Cary P [attp40]; TI{GAL4}5-HT2B - [Gal4]	UAS-5-HT2B-RNAi (BDSC #60488) TI{GAL4}5-HT2B[Gal4] (BDSC #86278) P{y[+t7.7]=CaryP[attp40] (BDSC #36304)
2F	TI{GAL4}5-HT7[Gal4]	BDSC #86279
3A	w1118; 5-HT1A-7×GFP_{11}-HA/5-HT1A-(MI1140)-T2A-GAL4,10×UAS-mCD8-GFP	5-HT1A-7×GFP_{11}-HA(this study) 5-HT1A-(MI1140)-T2A-GAL4(Gnerer, Venken, and Dierick 2015)
3B	10×UAS-mCD8-GFP/ w1118; 5-HT1B-(MI5213) -T2A-Gal4/5-HT1B-7×GFP_{11}-HA	10×UAS-mCD8-GFP (BDSC #32189) MiMIC 5213 HT1B T2A Gal4(Gnerer, Venken, and Dierick 2015) 5-HT1B-7×GFP_{11}-HA (this study)
3C	w1118; 10×UAS-mCD8-GFP/+; 5-HT2A-(MI459)-T2A-Gal4/5-HT2A(BFH)-7×GFP_{11}-HA	10×UAS-mCD8-GFP (BDSC #32186) 5-HT2A-(MI459)-T2A-Gal4(Gnerer, Venken, and Dierick 2015)

		5-HT2A(BFH)-7×GFP_{11}-HA (this study)
3D	w1118; 10×UAS-mCD8-GFP/+; 5-HT2B-(MI6500)-T2A-Gal4/5-HT2B-7×GFP_{11}-HA	5-HT2B-(MI6500)-T2A-Gal4(Gnerer, Venken, and Dierick 2015) 5-HT2B-7×GFP_{11}-HA (this study)
3E	w1118;+/+;5-HT7-(MI215)-T2A-Gal4,10×UAS-mCD8-GFP/5-HT7-7×GFP_{11}-HA	5-HT7-(MI215)-T2A-Gal4(Gnerer, Venken, and Dierick 2015) 5-HT7-7×GFP_{11}-HA (this study)
4A	R70A09-GAL4}attP2/10XUAS-IVS-mCD8:: GFP	R70A09-GAL4}attP2 (BDSC #47720)
4B	10xUAS-sfGFP1-10; R70A09-GAL4/ 5-HT7-7xGFP_{11}-HA (this study)	10xUAS-sfGFP1-10 (VK00037) - Gift from J. Wildonger, University of California, San Diego
4C	NP1227-Gal4 (LN1) / 10XQUAS-6XmCherry -HA; R70A09Q/ 10XUAS-IVS-mCD8::GFP	NP1227-Gal4 (LN1) (DGRC #103945) 10XQUAS-6XmCherry-HA} (BDSC #52269) GMR70A09Q(Suzuki et al. 2020)
4E	NP2426-Gal4 (LN2); UAS-mCherry/QUAS-mCD8-GFP; R70A09Q	NP2426-Gal4 (LN2) (DGRC #104198) UAS-mCherry (BDSC #59021) QUAS-mCD8-GFP (BDSC #30002)
4G	Left to right: Canton-S R70A09-Gal4/UAS-5HT7-RNAi w1118;;UAS-5HT7-RNAi P{y[+t7.7] =CaryP}attp2/R70A09 -Gal4	UAS-5HT7-RNAi (BDSC #32471) P{y[+t7.7] =CaryP}attp2 (BDSC #36303)

4H	Left to right: Canton-S UAS-dunce;;R70A09-Gal4, w1118 /UAS-dunce	w, UAS-dunce; +; + was a gift from B.White Lab at NIH. The fly was first described in Cheung et al., 1999(Cheung et al. 1999)
4I	Left to right: Canton-S Peb-Gal4; UAS-GABAB-RNAi; UAS-$GABA_B$-RNAi Peb-Gal4; UAS-Rdl-RNAi Orco-Gal4/ UAS-GABAB-RNAi; UAS-$GABA_B$ -RNAi, Orco-Gal4/UAS-Rdl-RNAi Peb-Gal4; P{y[+t7.7]=CaryP}attp40 Peb-Gal4;; P{y[+t7.7]=CaryP}attp2 Orco-Gal4/P{y[+t7.7]=CaryP}attp40 Orco-Gal4;P{y[+t7.7]=CaryP}attp2 w1118; UAS-GABAB-RNAi; UAS-$GABA_B$-RNAi w1118; UAS-Rdl-RNAi	UAS-GABAB-RNAi; UAS-$GABA_B$-RNAi: Gift from Jing Wang Peb-Gal4 (BDSC #80570) UAS-Rdl-RNAi (BDSC #52903) Orco-Gal4 (BDSC #26818)
5A	UAS-5-HT2B-RNAi; Gr21a-Gal4/5-HT2B-7x-GFP_{11}-HA.	Gr21a-Gal4 (BDSC #23890)
5B	UAS-5-HT2B-RNAi; Gr63a-Gal4/5-HT2B-7x-GFP_{11}-HA.	Gr63a-Gal4 (BDSC #9943)

5C	Peb-Gal4; UAS-5-HT2B-RNAi; 5-HT2B-7x-GFP_{11}-HA.	
5D	Left to right: Canton-S UAS-5-HT2B-RNAi;Gr21a-Gal4 w1118;UAS-5HT2B-RNAi P{y[+t7.7]=CaryP}attp40;Gr21a-Gal4	
5E	Left to right: Canton-S UAS-5-HT2B-RNAi/Gr63a-Gal4 w1118;UAS-5HT2B-RNAi P{y[+t7.7]=CaryP}attp40/Gr63a-Gal4	
5F	Left to right: Canton-S Peb-Gal4; UAS-5HT2B-RNAi UAS-5-HT2B-RNAi/Orco-Gal4 w1118;UAS-5HT2B-RNAi Peb-Gal4;P{y[+t7.7]=CaryP}attp40 P{y[+t7.7]=CaryP}attp40/Orco-Gal4	
6(A-B)	5-HT2B-7x-GFP_{11}-HA	This study
7A	Left to right: Canton-S R60F02-Gal4/UAS-5-HT1B-RNAi Trh-T2A-Gal4/UAS-5-HT1B-RNAi w1118;;UAS-5-HT1B-RNAi R60F02-Gal4/ P{y[+t7.7]=CaryP}attp2 Trh-T2A-Gal4/ P{y[+t7.7]=CaryP}attp2	UAS-5-HT1B-RNAi (BDSC # 27635) Trh-T2A-Gal4 (BDSC #84694)

7B	Left to right: Canton-S UAS-5-HT1B;R60F02-Gal4 UAS-5-HT1B;+ +;R60F02-Gal4	UAS-5-HT1B (BDSC #27632)

All MiMIC lines were a gift from Dr. Herman A. Dierick, Baylor College of Medicine. The 10X-UAS-sfGFP$_{1-10}$ lines were a gift from Dr. Jill Wildonger at University of California, San Diego.

Results

Olfactory CPP requires the release of 5-HT by the serotonergic neurons

In *Drosophila*, the olfactory CPP manifests as a change in the volume of the glomerulus innervated by OSNs responsive to the odor used as a stimulus (Figure 1A-B)(Sachse et al. 2007b; Das et al. 2011; Chodankar et al. 2020). Consistent with previous reports,(Sachse et al. 2007b; Das et al. 2011; Chodankar et al. 2020) in flies where 5-HT transmission is intact, the V-glomerulus increased in volume in flies exposed to CO_2 compared to air exposed (Figure 1B). In *Drosophila* and other holometabolous insects, a single pair of serotonergic neurons called the Contralaterally projecting Serotonin immunoreactive Deuterocerebral neurons (CSDns) innervate the AL (Dacks, Christensen, and Hildebrand 2006; Coates et al. 2017; 2020) (Figure 1C) and supply serotonin (Zhang and Gaudry 2016; Coates et al. 2017; 2020; X. J. Sun, Tolbert, and Hildebrand 1993). Previous work from our lab has shown that expression of the Vmat-RNAi transgene in the CSDns successfully eliminates 5-HT mediated responses in the AL(Zhang and Gaudry 2016; Suzuki et al. 2020). We thus assessed the role of 5-HT in CPP in the *Drosophila* olfactory system by measuring the structural plasticity induced in the CO_2 sensitive V- glomerulus upon chronic exposure to 5% CO_2 (Sachse et al. 2007b; Das et al. 2011; Chodankar et al. 2020). We employed the R60F02-Gal4 promoter line that labels the CSDns (Jenett et al. 2012) to prevent serotonin release by knocking

down Vmat in these cells via expression of Vmat RNAi. Chronic CO_2 exposure to flies deprived of CSDn serotonin output failed to undergo structural plasticity in the V (Figure 1D), suggesting that serotonin release is required for structural CPP.

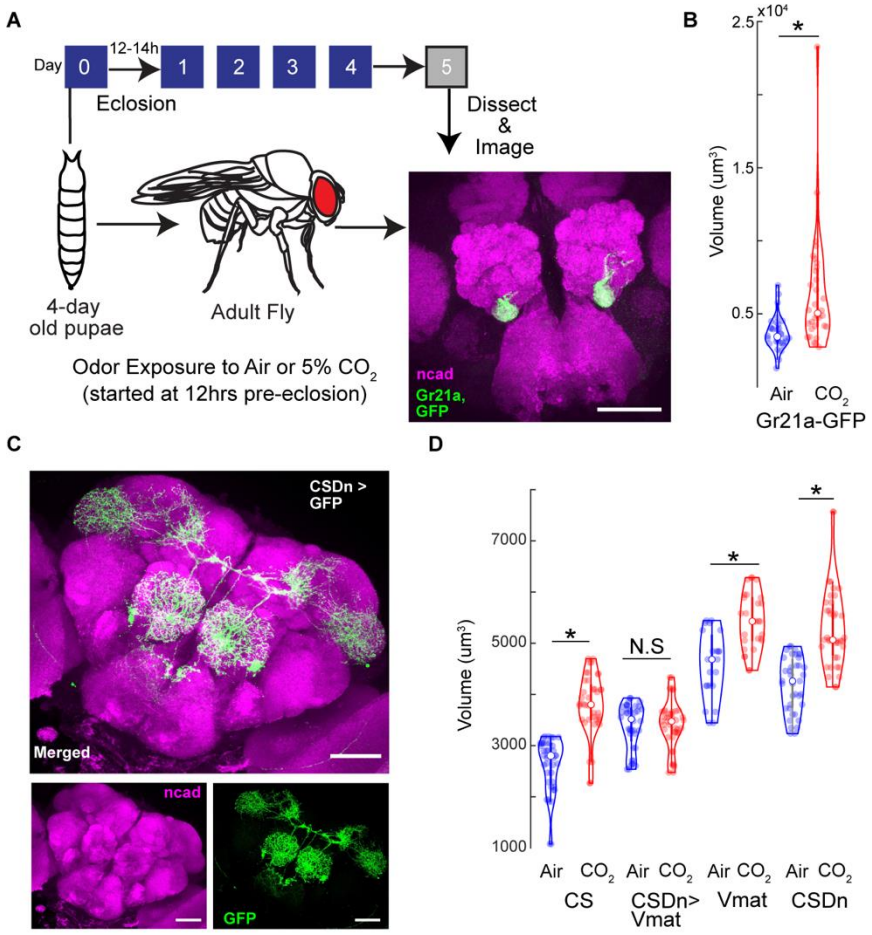

Figure 1. Blocking serotonin release from CSDns prevents structural plasticity during the critical period.

(A) Schematic of the experimental protocol. 4-day old pupae are collected and subject to 5% CO_2 for 5 days. On day 5 after eclosion, flies are collected and stained with n-cadherin and imaged under a confocal microscope to analyze structural plasticity. Confocal maximum intensity projection of AL by CO_2 responsive V-glomerulus in green co-labeled for n-cadherin (magenta).

(B)Quantification of V-glomerulus volumes comparing air (right) and 5% CO_2 (left) exposed flies during the critical period. Genotype shown here is endogenously expressed GFP under the Gr21a promoter (Gr21aGFP).

(C) Confocal maximum intensity projections of AL innervation by CSDns (CSDn-Gal4 > UAS-mcd8::GFP, green) co-labeled for n-cadherin (magenta).

(D) Quantification of V glomerulus volumes comparing air and 5% CO_2 exposed flies during the critical period. Four genotypes are shown here from left to right : Canton-S (wildtype), CSDn-Gal4>UAS-Vmat-RNAi (CSDn targeted Vmat knockdown), w1118;;UAS-Vmat-RNai (background control for Gal4) and y,v;; CSDn-Gal4 > RNAi background (background control for RNAi).

* indicates $p < 0.05$; N.S indicates $p > 0.05$; n >=15

The scale bar indicates 50 μm in all cases.

Serotonin modulates the olfactory CPP via multiple receptor targets

Having established that 5-HT plays a role in the olfactory CPP in *Drosophila*, we wished to determine the cellular and molecular targets by which 5-HT was exerting its impact. 5-HT mediates its effect in cells by concentration dependent activation of its cognate receptors. In *Drosophila*, there are 5 serotonin receptors (5-HTRs): 5-HT1AR, 5-HT1BR, 5-HT2AR, 5-HT2BR and 5-HT7R (Witz et al. 1990b; Saudou et al. 1992; Colas et al. 1995; Qian et al. 2017). We systematically interrogated 5-HTR signaling to determine which receptors are involved in mediating structural plasticity during the critical period (Figure 2). For this, we used the same experimental paradigm as before and employed the null mutants of the 5-HTRs generated by a CRISPR knock in strategy to replace all or parts of the gene encoding the 5-HTRs with the GAL4 gene.(Qian et al. 2017) Chronic exposure of CO_2 in flies with mutations in the 5-HT1BR and 5-

HT7R (Figures 2 C&F) failed to demonstrate structural plasticity in the V-glomerulus during the critical period. Since the 5-HT2BR homozygous mutants were not viable in our hands, we

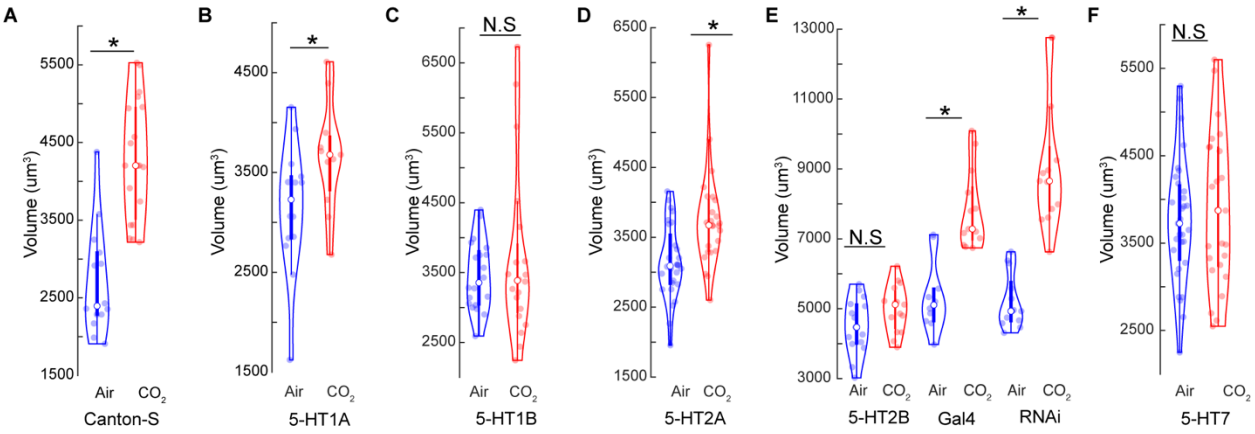

Figure 2. Serotonin acts through multiple receptors during the critical period

(A) Comparison of V-glomerulus volumes in air and 5% CO_2 exposed brains in wildtype Canton-S flies.

(B-D, F) Comparison of V-glomerulus volumes in air and 5% CO_2 exposed brains of 5-HT1AR (B), 5-HT1BR (C), 5-HT2AR (D) and 5-HT7R (F) knockout flies.

(E) Comparison of V-glomerulus volumes in air and 5% CO_2 exposed brains of flies with genotypes (left to right): 5-HT2B knockdown in 5-HT2B heterozygous mutants, UAS-5HT2B-RNAi in Gal4 knock in background, heterozygous 5-HT2B mutant with Gal4 knock in RNAi background (y,v;5HT2B[Gal4] > TRiP background).

* indicates $p < 0.05$; N.S indicates $p > 0.05$; n >= 15 in all cases.

employed a slightly modified strategy to investigate its effects on CPP. We crossed the heterozygous 5-HT2BR mutants expressing Gal4 under the 5-HT2BR promoter to induce expression of 5-HT2B-RNAi. This 5-HT2BR deficient state was sufficient to block structural plasticity in the V-glomerulus during the critical period (Figure. 2E). In contrast, we still observed

structural plasticity in 5-HT1AR and 5-HT2AR mutants upon chronic CO_2 exposure during the CP (Figures 2 B&D) indicating that these two receptors do not underlie the effects of 5-HT on early life olfactory plasticity. Together, these results indicate that 5-HT1BR, 5-HT2BR and 5-HT7R are required during the critical period.

Previous studies demonstrated that all five 5-HT receptors are expressed by distinct neuron types in the AL.(Sizemore and Dacks 2016) These studies relied on GFP expression induced by the Gal4 protein expressed from the endogenous promoter. While this approach reveals which cells classes express which 5-HTRs, the method does not easily identify where and when the receptors are trafficked within the neurons. We therefore generated flies with an endogenous HA-tag and the GFP_{11} fragment on 5-HTRs. (Vicario et al. 2019; Pedelacq and Cabantous 2019; Kamiyama et al. 2021) We employed a CRISPR-Cas9 based strategy(Gratz et al. 2014; Ran et al. 2013) to generate these flies. The split-GFP is an elegant tool that consists of splitting the superfolder GFP (sfGFP) between the beta-strand 10 and 11 to generate two non- fluorescing, self-complementing fragments: GFP_{1-10} and GFP_{11}.(Cabantous, Terwilliger, and Waldo 2005; Romei and Boxer 2019) Only cells that would simultaneously express both fragments will be able to form the complete sfGFP molecule that would fluoresce (Figure S1A). Additionally, the HA tagged 5-HTRs would enable us to locate 5-HTRs expression in all cells by immunolabeling against HA (Figure S1B). Together, this strategy allows us to simultaneously visualize the localization of 5-HTRs and determine the cell types that express them (Figure S1C- F). We found that the expression patterns of these 5-HTR lines labeled using the MiMIC-5HTR-Gal4 drivers to be consistent with previously reported expression patterns of the 5-HTRs(Sizemore and Dacks 2016; Gnerer, Venken, and Dierick 2015) using more traditional GAL4/UAS approaches (Figures 3A-E). Next, we

investigated the differential expression patterns of the 5-HTRs in the olfactory processing centers of the brain. The 5-HT1Rs are expressed in varying degrees within the AL and MBs (Figure 3A).

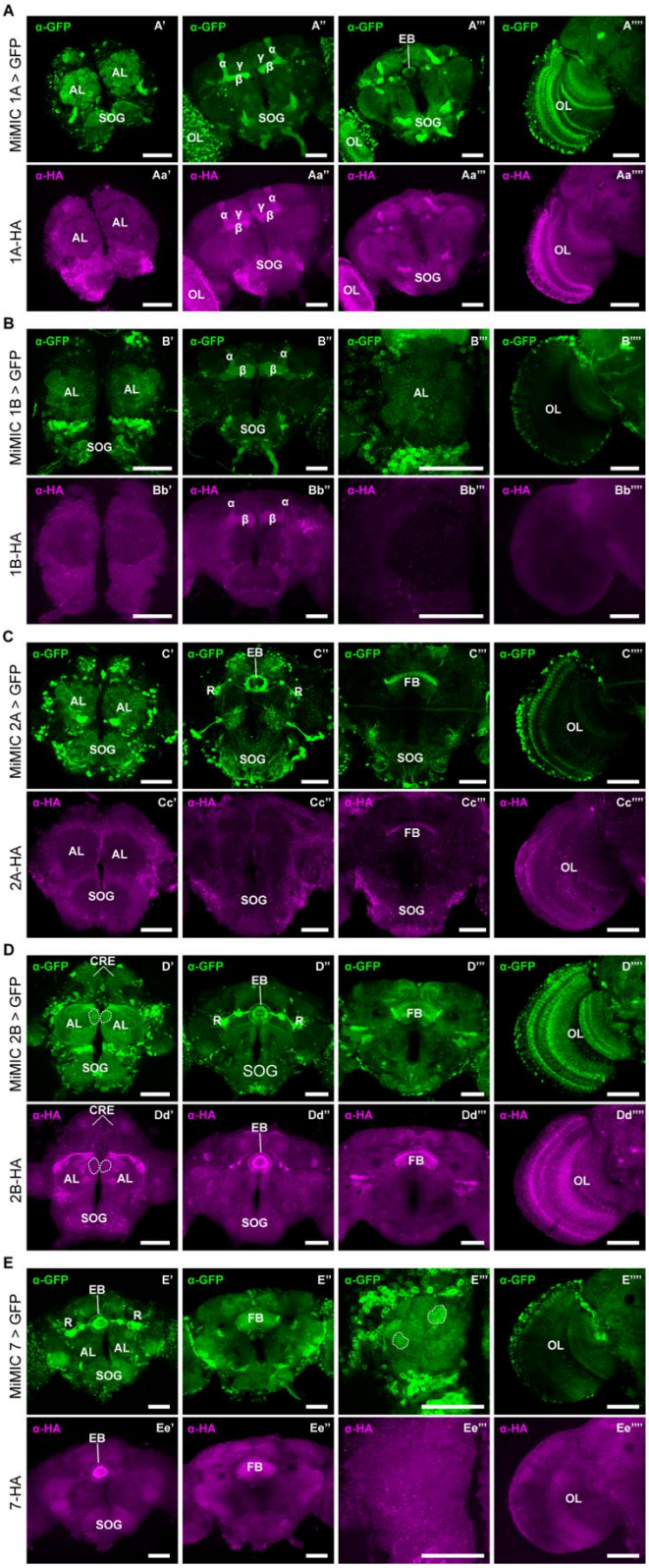

Figure 3. Distribution of 5-HTRs in adult *D. melanogaster* brains

(A) Brain sections indicate 5-HT1A expression in the AL, suboesophageal ganglion (SOG), MB, ellipsoid body (EB), and optic lobe (OL). As shown in (A'') and (Aa''), both α-HA and α-GFP indicate expression in all three lobes of MB, including α, β, and γ lobes. Note, as shown in (Aa'''), 5-HT1A expression in EB is largely devoid revealed by α-HA, contrary to the α-GFP staining pattern shown in (A''').

(B) Brain sections indicate 5-HT1B expression in SOG, AL, MB and OL. In contrast to the MiMIC approach, which labels cell bodies broadly in the brain, immunostaining against the HA tag show significant distribution of the receptor only in the α and β lobes of MB.

(C) Brain sections indicate 5-HT2A expression in many areas such as AL, SOG, EB, the fan-shaped body (FB), and optical lobe (OL). Among all these areas the staining by α-HA shows weak signals in FB (Cc''') and probably in OL (Cc'''', indicated by the arrow heads). The contrast of the images from the α-HA channel were elevated to see weak signals, resulting in irregular signal presentation which might be artefacts, as indicated by the arrow heads in (Cc'-Cc''').

(D) Brain sections indicate 5-HT2B expression in AL, crepines (CRE), SOG, EB, FB, and OL. In ALs, the MiMIC approach indicates a relatively high expression in a medial glomerulus, as emphasized by the white dashed circle, which is not consistent in the α-HA channel (Dd'). It is noticeable that localization of this receptor in the R neurons (R) and their arborizations toward EB (D'', arrow heads) is missing in the staining pattern revealed by α-HA (Dd''). Arrow heads in (D''') and (Dd''') indicate some unknown structures stained in both channels.

(E) Brain sections indicate 5-HT7 expression in SOG, EB, FB, and (E''''/Ee'''') OL. In the antennal lobe labeled with the MiMIC approach, extraordinary signals were observed in an anterior dorsal glomerulus as indicated by dashed circle on the right side, and a posterior lateral glomerulus

as indicated by dashed circle on the left side (E’’’). In contrast, no prominent signals showed in these two glomeruli as indicated by the α-HA approach (Ee’’’). Signals were observed in the optical lobe (OL) revealed by both approaches (E’’’’/Ee’’’’). For both (E’’’/Ee’’’) and (E’’’’/Ee’’’’), the brain was oriented with the lateral toward left and the dorsal upward.
The scale bar indicates 50 μm in all cases.

The 5-HT2ARs are mostly expressed in the cells surrounding and innervating the AL, most likely the LNs and PNs (Figures 3C’ and Cc’). Remarkably 5-HT2BRs is not uniformly expressed throughout the AL (Figures 3D’,Dd’ and S2A-I). This indicates varying levels of 5-HT2BR mediated serotonergic modulation in the OSNs. Consistent with prior reports(Sizemore and Dacks 2016), we found most of the 5-HT2BR expression in the AL to be in the OSNs. When we removed the antennae or the maxillary palps that houses the cell body and dendrites of OSNs, 5-HT2BR expression in the related AL region the OSNs project to is eliminated (Figure S2J). Similarly, we were able to selectively knockdown 5-HT2BRs expression using Gal4 drivers for OSN subtypes using an RNAi against 5-HT2BR (Figures S2K,L). The AL neuropil is innervated by various cells including OSNs, PNs, LNs and CSDns. We found most of the 5-HT7R expression in the cells surrounding the AL, most likely in the LNs and PNs (Figures 3E’,E’’’). We also found unusually high GFP labeling in two glomeruli in the AL (Figure 3E’’’) but no corresponding HA labeling (Figure 3Ee’’’) for 5HT7R expression using the MiMIC-5HT7R-Gal4 promoter line. Taken together, these results show that 5-HT targets multiple components of olfactory processing through distinct receptors and therefore it was unclear where and how serotonergic receptors mediate their effects during the olfactory critical period.

Serotonin modulates distinct components of sensory processing during the critical period

Next, we wanted to isolate the neuronal basis of 5-HTR signaling that modulates CPP. Earlier studies have identified a crucial role of inhibitory, GABAergic LNs, namely LN1 and LN2 during the olfactory critical period.(Das et al. 2011; Chodankar et al. 2020; Sachse et al. 2007b) Previous work in our lab has identified a distinct population of 5-HT7R expressing GABAergic LNs (R70A09-Gal4) that are responsive to low 5-HT concentrations and modify odor coding in the AL.(Suzuki et al. 2020) As a population, these LNs innervate all glomeruli including the V-glomerulus (Figure 4A). We also found that R70A09-GAL4 LNs express 5-HT7Rs (Figure 4B) and show almost a complete overlap with LN1 neurons (Figures 4C,D) and a partial overlap with the LN2 neurons (Figures 4E,F). The LN1 neurons have been previously implicated to induce an increase in the number of PN arbors leading to structural plasticity during the critical period.(Chodankar et al. 2020; Das et al. 2011) We therefore sought to determine if these LNs are the target for 5-HT modulation via the 5-HT7Rs and found that knocking down 5-HT7Rs in the R70A09 LNs was sufficient to prevent CPP in the V-glomerulus (Figure 4G). In *Drosophila*, 5-HT7Rs are known to activate an adenylate cyclase that results in an increase in cytosolic cAMP.(Witz et al. 1990a; Sizemore, Hurley, and Dacks 2020) When we overexpress the cAMP specific phosphodiesterase *dunce* to deplete cAMP selectively in the R70A09 LNs keeping the 5-HT7Rs and adenylate cyclase intact, CPP in the V-glomerulus is abolished (Figure 3H). These results indicate that both 5-HT7R signaling and cAMP in R70A09-GAL4 LNs play an important role during the CP that ultimately permit the induction of structural plasticity in the cognate glomerulus. The R70A09 LNs that express 5-HT7 receptors are GABAergic in nature and release GABA upon activation.(Suzuki et al. 2020) The pan-glomerular innervation of these LNs implies

that they release GABA all over the AL. Apart from these LNs, other GABAergic LNs also exist in the AL that can be sensitive to serotonergic modulation. Previous work has shown that GABA released from the GH298 LNs which are distinct from the R70A09 LNs mediate glomerulus selective presynaptic divisive gain control in Or83b expressing OSNs in adult *Drosophila* but do not affect CO_2 responses in the Gr21a expressing OSNs.(Root et al. 2008) Consistent with these studies, knocking down $GABA_B$ and $GABA_A$ receptors respectively in the CO_2 sensing OSNs and PNs was not sufficient to block structural plasticity during the CP.(Golovin et al. 2019; Das et al. 2011) However, 5-HT7R mediated activation in the R70A09 LNs and thereby GABA release is important for inducing CPP. Therefore, it is likely that R70A09 LN activation linked GABA release during the critical period could lead to network level changes in the AL that ultimately facilitate structural plasticity in the cognate glomerulus. We asked if the two GABA receptors expressed in the fly, the $GABA_A$ and $GABA_B$ receptors are required for global inhibition in the OSNs during the critical period. When we knock down $GABA_B$ receptors in all OSNs we saw no structural plasticity in the V-glomerulus (Figure 4I). In contrast, knocking down $GABA_A$ receptors in all OSNs did not hinder CPP in the V (Figure 4I). Finally, we targeted Or83b OSNs in the AL using the Orco-Gal4 promoter line. This enabled targeting multiple OSNs responsive to different odors(Root et al. 2008; Suzuki et al. 2020) but not the CO_2 sensing ones. Surprisingly, flies expressing $GABA_A$ RNAi in Or83b OSNs failed to undergo structural plasticity in the V upon CO_2 exposure (Figure 4H). However, knocking down $GABA_B$ in the Or83b OSNs was not sufficient to prevent structural plasticity in the V in response to CO_2 (Figure 4I). This shows that GABAergic inhibition during the critical period is required in a broader sub-population of OSNs in the AL but not selectively in the cognate glomerulus. In fact, GABA targets distinct OSNs through $GABA_A$ and $GABA_B$ receptors to facilitate CPP in the V glomerulus.

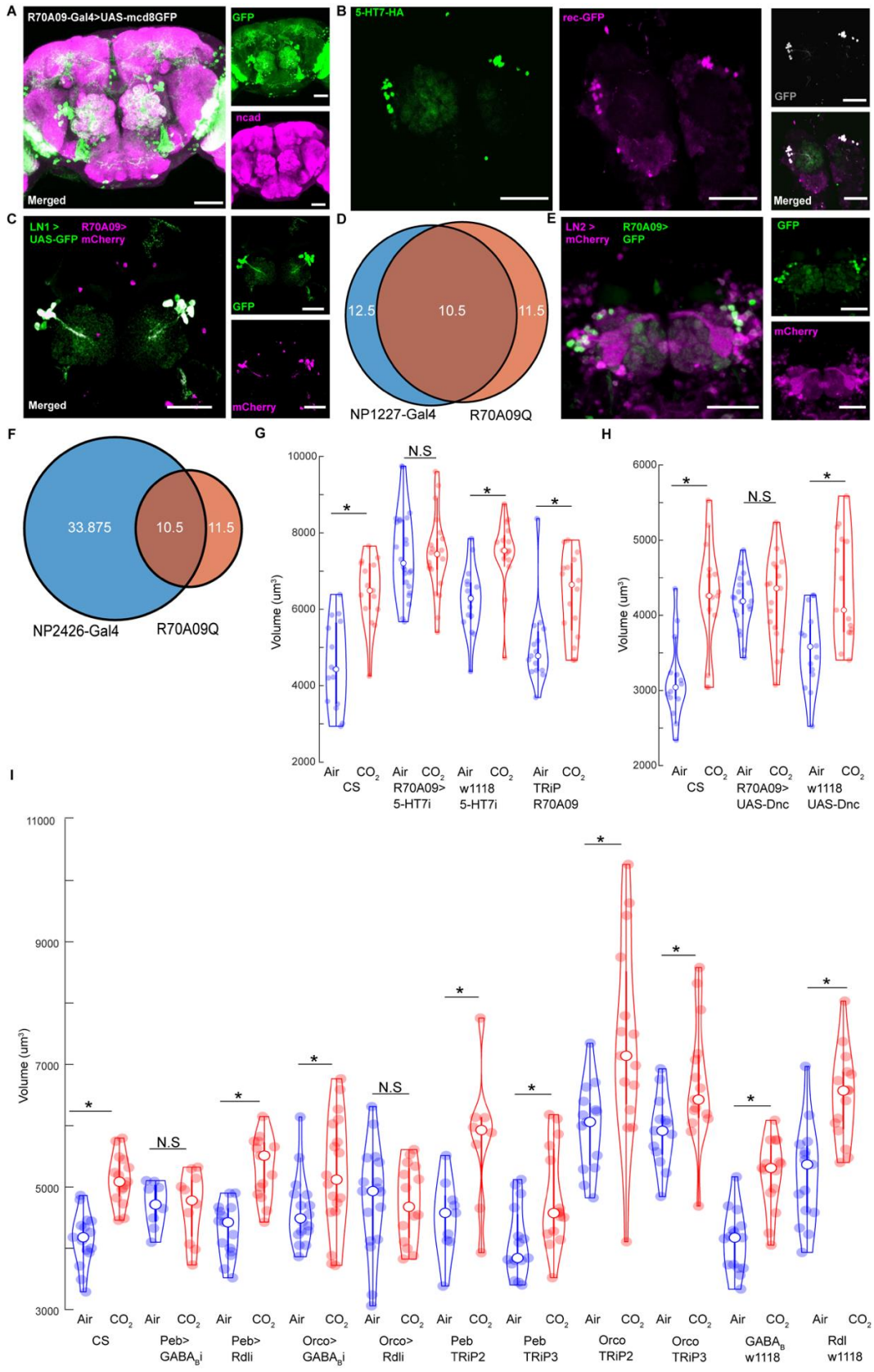
A
R70A09-Gal4>UAS-mcd8GFP
GFP
ncad
Merged
B
5-HT7-HA
rec-GFP
GFP
Merged
C
LN1 > UAS-GFP
R70A09> mCherry
GFP
mCherry
Merged
D
12.5
10.5
11.5
NP1227-Gal4
R70A09Q
E
LN2 > mCherry
R70A09> GFP
GFP
mCherry
F
33.875
10.5
11.5
NP2426-Gal4
R70A09Q
G
N.S
Volume (um³)
Air CO2
CS
R70A09> 5-HT7i
w1118 5-HT7i
TRiP R70A09
H
N.S
Volume (um³)
CS
R70A09> UAS-Dnc
w1118 UAS-Dnc
I
N.S
Volume (um³)
CS
Peb> GABAB i
Peb> Rdli
Orco> GABAB i
Orco> Rdli
Peb TRiP2
Peb TRiP3
Orco TRiP2
Orco TRiP3
GABAB w1118
Rdl w1118

Figure 4. 5-HT7 targets GABAergic inhibition in the primary olfactory circuit.

(A) Confocal maximum intensity projection of the R70A09 LNs expressing GFP in green, co-labeled for n-cadherin in magenta. The V-glomerulus is circled in the merged and GFP channels.

(B) Confocal maximum intensity projection of the AL showing R70A09 LNs co-labeled for 5-HT7-HA in green, reconstituted GFP in magenta, GFP in grey and the merged channel.

(C) Confocal maximum intensity projection of the AL showing the overlap between R70A09 LNs in magenta and LN1 neurons in green.

(D) The R70A09 line labels 11-12 LNs, LN1 labels 12-15 LNs per hemisphere. There is a total overlap of 10-11 cells between R70A09 and LN1 population per hemisphere.

(E) Confocal maximum intensity projection of the AL showing the overlap between R70A09 LNs in green and LN2 in magenta.

(F) The R70A09 line labels 11-12 LNs, LN2 labels 33-44 LNs per hemisphere. There is a total overlap of 10-11 cells between R70A09 and either LN1 or LN2 population per hemisphere.

(G) Quantification of V glomerulus volumes comparing air and 5% CO_2 exposed flies during the critical period. Four genotypes are shown here from left to right: CS (Canton-S wildtype), R70A09>5-HT7i (R70A09 targeted 5-HT7 knockdown), w1118 5-HT7i (background control for Gal4) and TRiP R70A09 (background control for RNAi).

(H) Comparison of V-glomerulus volumes in air and 5% CO_2 exposed brains of flies with overexpression of *dunce* in the R70A09 LNs. 3 genotypes are shown here from left to right: CS (Canton-S wildtype), R70A09>UAS-Dnc (*dunce* overexpression in R70A09 LNs), w1118 UAS-Dnc (background for Dnc and R70A09).

(I) Comparison of V-glomerulus volumes in air and 5% CO_2 exposed brains of flies with GABA receptor knockdown in OSNs. 11 genotypes are shown here from left to right: CS (Canton-S wildtype), Peb>$GABA_Bi$ ($GABA_B$ knockdown in all OSNs), Peb>Rdli ($GABA_A$ knockdown in all OSNs), Orco>$GABA_Bi$ ($GABA_B$ knockdown in Or83b OSNs), Orco>Rdli ($GABA_A$ knockdown in Or83b OSNs), Peb TRiP2 (background control for Rdl RNAi crossed with Peb-Gal4), Peb TRiP3 (background control for $GABA_B$ RNAi crossed with Peb-Gal4), Orco TRiP2 (background control for Rdl RNAi crossed with Orco-Gal4), Peb TRiP3 (background control for $GABA_B$ RNAi crossed with Orco-Gal4), w1118 $GABA_Bi$ (background for Gal4 crossed with $GABA_B$-RNAi), w1118 Rdli (background for Gal4 crossed with Rdl-RNAi).

* indicates $p < 0.05$; N.S indicates $p > 0.05$. n>=15.

The scale bar indicates 50 μm in all cases.

Next, we asked which cells projecting to the AL could be the source of 5-HT2BR mediated serotonergic modulation of the olfactory CPP. We hypothesized that 5-HT targets 5-HT2BRs on OSNs during the critical period. Previous research has shown that the 5-HT2BRs are expressed by all OSNs and a few LNs and PNs in the AL (Sizemore and Dacks 2016). We showed earlier in Figure S2J that the majority of the 5-HT2BR expression in the antennal lobe is due to their expression by the OSNs. Therefore, we selectively knocked down expression of the 5-HT2BRs in the V-glomerulus OSNs (Figure 5).In the CO_2 detecting OSNs, two chemoreceptors, Gr21a and Gr63a are co-expressed to form a functional CO_2 responsive odor receptor(Kwon et al. 2007). However, in our 5-HT2BR knockdown experiments, we observed residual 5-HT2BR expression in the V-glomerulus using the Gr21a-GAL4 driver line (Figure 5A). Therefore, 5-HT2BR knockdown driven by the Gr21a-Gal4 line was not sufficient to prevent CPP in the V-glomerulus

(Figure 5D). In contrast, driving the 5-HT2BR RNAi using Gr63a-Gal4 significantly reduced 5-HT2BR expression in the V-glomerulus without impacting expression in the rest of the AL (Figure 5B). This selective knockdown of the 5-HT2BRs by the Gr63a-Gal4 line was sufficient to prevent the induction of structural plasticity in the V-glomerulus (Figure 5E) suggesting that 5-HT2BR expression is required by OSNs within the glomerulus expanding during the CP. To determine if 5-HT2BR expression by OSNs in other glomeruli is required for CPP in the V-glomerulus, we next extended the 5-HT2BR knockdown using drivers expressed broadly in all OSNs (Peb-Gal4) or in many OSNs except those projecting to the V and a few other glomeruli (Orco-Gal4). We found that flies that expressed 5-HT2B RNAi in all OSNs (Figure 5C), failed to undergo structural plasticity in the V-glomerulus in response to chronic CO_2 exposure (Figure Figure 5F). In contrast, 5-HT2B knockdown in multiple OSNs using the Orco-Gal4 driver line did not show any deficits in structural plasticity in response to CO_2 (Figure 5F). Together, these results show that the 5-HT2BR expression is required in cognate ORNs of the V glomerulus for proper expression of CPP. We also observed glomerulus specific differences in the expression levels of the 5-HT2BRs. (Figure S2A-I). Therefore, to determine if expression of the 5-HT2BRs within the AL varies post-eclosion, we employed the 5-HT2BR-HA tagged recombinant flies. We found that the expression of the 5-HT2BRs increases significantly post-eclosion and reaches its peak at day 2 or 48-hour post eclosion (Figures 6A,B), which coincides with the closing of the critical period. (Sachse et al. 2007b; Chodankar et al. 2020) After day 2, the 5-HT2BR expression does not vary significantly as we saw no difference in the corrected total fluorescence between day 2, day 4 and day 5 post eclosion. Taken together, these results show that 5-HT targets both excitatory and inhibitory neurons within the antennal lobe via distinct receptors.

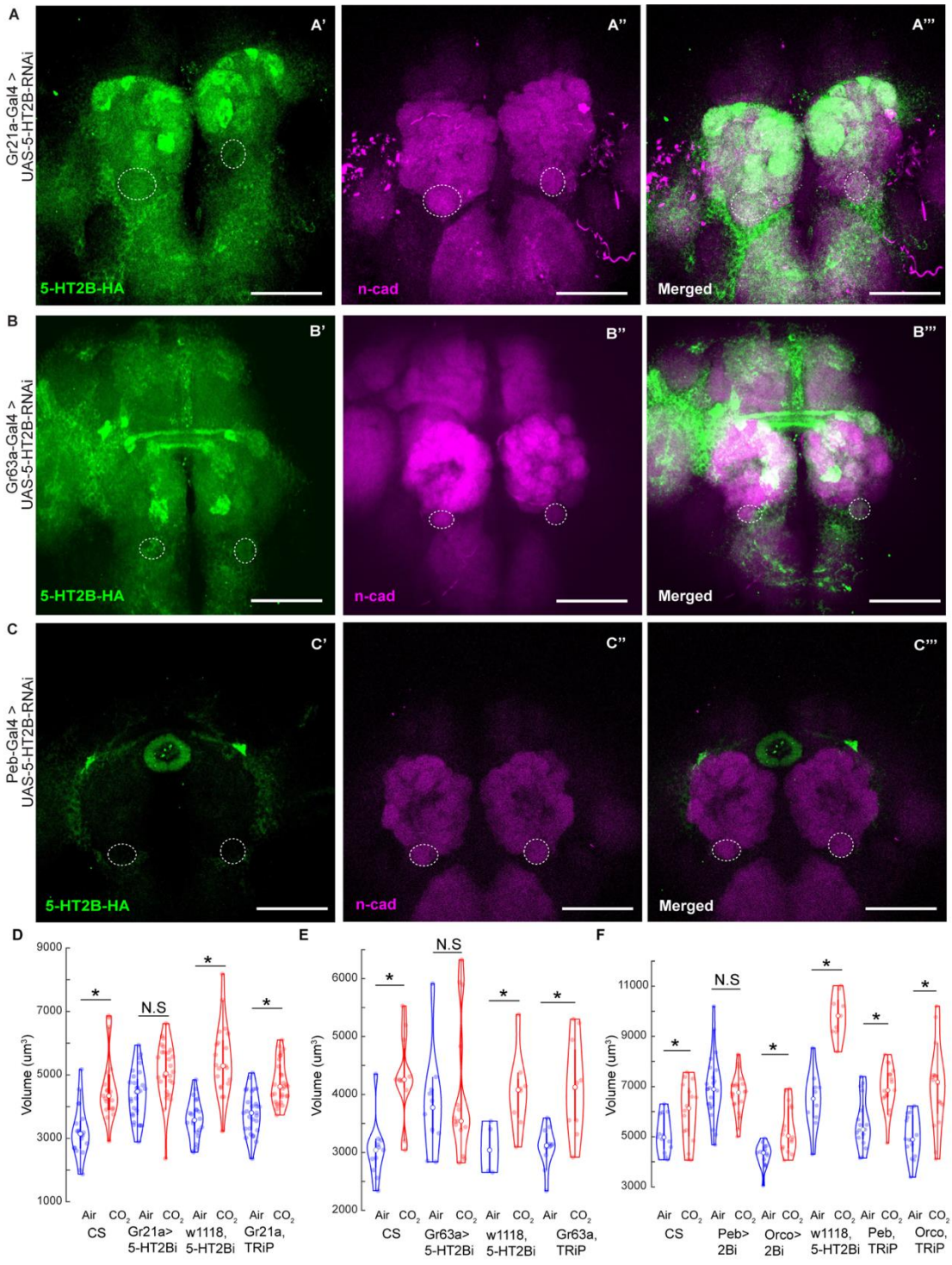

Figure 5. 5-HT2BRs are required in OSNs during the critical period.

(A) Insufficient 5-HT2B knockdown in the CO_2 sensing OSNs by Gr21a-Gal4. The V glomerulus is circled on all three channels.

(B) 5-HT2B knockdown in the CO_2 sensing OSNs by Gr63a-Gal4. The V glomerulus is circled on all three channels.

(C) 5-HT2B knockdown in all OSNs but no other brain regions by Peb-Gal4. The V glomerulus is circled on the n-cad (magenta) and merged channels.

(D) Quantification of V glomerulus volumes comparing air and 5% CO_2 exposed flies during the critical period. Four genotypes are shown here from left to right: CS (Canton-S wildtype), Gr21a>5-HT2Bi (5-HT2B knockdown in CO_2 OSNs), w1118 5-HT2Bi (background control for Gal4) and Gr21a, TRiP (background control for RNAi).

(E) Quantification of V glomerulus volumes comparing air and 5% CO_2 exposed flies during the critical period. Four genotypes are shown here from left to right: CS (Canton-S wildtype), Gr63a>5-HT2Bi (5-HT2B knockdown in CO_2 OSNs), w1118, 5-HT2Bi (background control for Gal4) and Gr63a, TRiP (background control for RNAi).

(F) Quantification of V glomerulus volumes comparing air and 5% CO_2 exposed flies during the critical period. Six genotypes are shown here from left to right: CS (Canton-S wildtype), Peb>5-HT2Bi (5-HT2B knockdown in all OSNs), Orco> 2Bi (5-HT2B knockdown in Or83b OSNs), w1118,5-HT2Bi (background control for Gal4), Peb, TRiP (background control for 5-HT2B-RNAi) and Orco,TRiP (background control for 5-HT2B-RNAi).

* indicates $p < 0.05$; N.S indicates $p > 0.05$. n>=15. The scale bar indicates 50 μm in all cases.

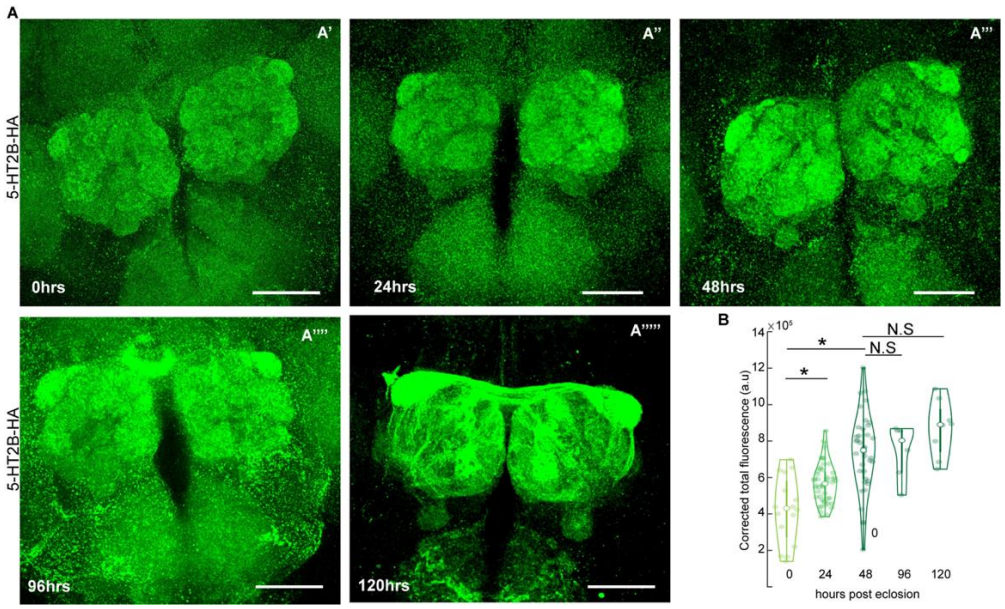

Figure 6. 5-HT2BR expression in the AL varies during the critical period.

(A) 5-HT2B expression levels in the AL as indicated by HA staining in green at different life stages of the fly post eclosion: 0 hrs or freshly eclosed (A') 24hrs (A''), 48hrs (A'''), 96hrs (A'''') and 120hrs (A''''').

(B) Total corrected cell fluorescence indicating 5-HT2B expression levels in the AL of the fly at different ages from left to right: 0hrs or freshly eclosed, 1 day or 24 hrs post eclosion (p.e.), 2 days or 48hrs p.e., 4 days or 96hrs p.e., and 5 days or 120 hrs p.e. ** indicates $p < 0.05$; N.S indicates $p > 0.05$. The scale bar indicates 50 μm in all cases. $n >= 15$.

Autoregulation of serotonergic neurons during the critical period

Finally, we sought to determine the neurons for whom expression of the 5-HT1BR is required for the olfactory CPP. Within serotonergic neurons, the 5-HT1BRs often act as auto receptors by either inhibiting the release of 5-HT(Tiger et al. 2018; Middlemiss and Hutson 1990; Brazell et al. 1985; Barnes and Sharp 1999) or by

modulating serotonin reuptake by upregulating SERT activity and clearance rate.(Hagan et al. 2012) Therefore, we knocked down 5-HT1B receptors in the CSDns, release of 5-HT from which is required during critical period (Figure 7A). We found that 5-HT1B signaling in CSDns is required for glomerular specific volume increase during the critical period. Similarly, knocking down 5-HT1BRs in all serotonergic neurons in the brain using a Gal4 promoter line Trh-Gal4, was able to block CPP in the V-glomerulus (Figure 7A). The CSDns release 5-HT upon activation. Since the 5-HT1BRs are inhibitory in nature, activation of 5-HT1BRs on CSDNs will inhibit 5-HT release from them. We also know that release of 5-HT from the CSDns is important during the critical period. Therefore, if we overexpress 5-HT1BRs in the CSDns there will be a stronger inhibition in the CSDns most likely preventing CPP.

Consistent with our hypothesis, we saw that 5-HT1BR overexpression on CSDNs prevents CPP in the V-glomerulus following CO_2 exposure (Figure 7B). Together these results indicate that 5-HT levels need to be tightly controlled to induce CPP.

Discussion

There are many cellular and molecular components that contribute to CPP. Serotonin is elegantly positioned to affect CPP because a diverse set of 5-HT receptors are broadly expressed throughout the network. The genetically accessible olfactory circuit of *Drosophila* allowed us to isolate the effects of serotonergic modulation in the critical period exclusively within the olfactory circuit by regulating serotonin release from the CSDns and selectively knocking down 5-HTRs in specific cell types

within the olfactory circuit. Our results indicate that 5-HT modulates both excitatory and inhibitory elements in the olfactory circuit during the critical period.

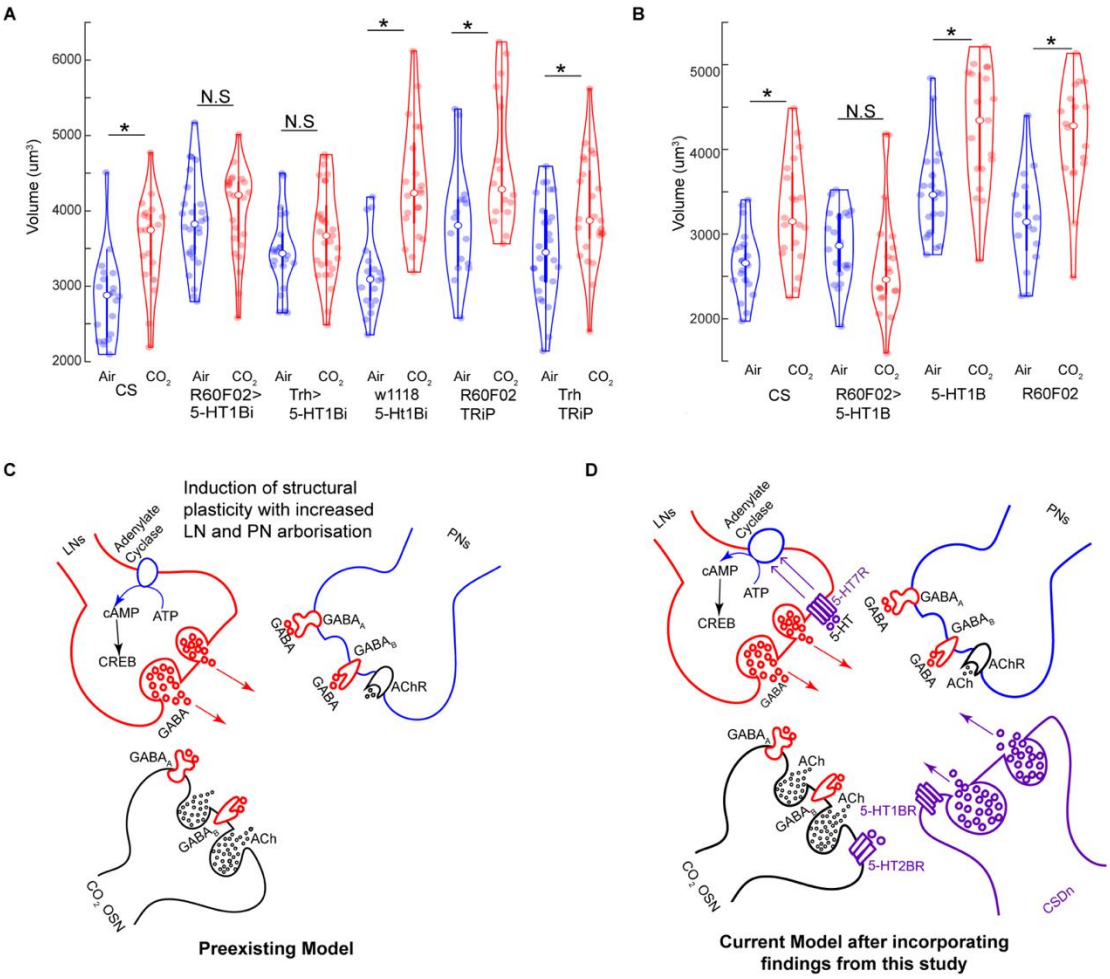

Figure 7. Serotonergic signaling during the critical period.

(A) Quantification of V glomerulus volumes comparing air and 5% CO_2 exposed flies during the critical period. Six genotypes are shown here from left to right: CS (Canton-S wildtype), R60F02>5-HT1Bi (RNAi knockdown in CSDns), Trh>1Bi (5-HT1B knockdown in all serotonergic cells), w1118,5-HT1Bi (background control for Gal4), R60F02, TRiP (background control for 5-HT1B-RNAi crossed with CSDn line) and Trh,TRiP (background control for 5-HT1B-RNAi crossed with Trh line).

(B) Quantification of V glomerulus volumes comparing air and 5% CO_2 exposed flies during the critical period. Four genotypes are shown here from left to right: CS (Canton-S wildtype), R60F02>5-HT1B (Overexpression of 5-HT1B in CSDns), 5-HT1B (control for UAS), R60F02 (control for Gal4).

(C) During the critical period, chronic odor exposure leads to OSN dependent activation of LNs and PNs. Activation of GABAergic LNs induces GABA release that modulates OSN and PN responses. cAMP dependent mechanisms in GABAergic LNs lead to CREB dependent gene transcription that promotes to structural plasticity in the LN and PN arbors resulting in glomerulus specific volume increase.

(D) Our results (in pink) show 5-HT also plays a role within the existing model of CPP. 5-HT is released from the CSDNs and is tightly regulated by 5-HT1BRs during the critical period. Differential expression of 5-HT2B neurons on the OSNs regulates structural plasticity in the LNs and PNs. 5-HT7 mediated GABAergic LN activation interacts with the preexisting model of cAMP dependent gene transcription to facilitate CPP. GABAergic signaling from the LNs modulate global OSN activation levels during the critical period.

* indicates $p < 0.05$; N.S indicates $p > 0.05$. n>=15

5-HT modulates inhibitory LN circuits that underlie critical period plasticity

Critical periods are known to be tightly regulated by the maturation of inhibitory circuits. In fact, the emergence and maturation of GABAergic inhibitory

local interneurons (LNs) in mammals(Toyoizumi et al. 2013; Larsen et al. 2022) are known to improve the signal to noise ratio by improving their excitation/inhibition balance(Toyoizumi et al. 2013) in visual(Levelt and Ḧubener 2012), auditory(Takesian et al. 2018), and somatosensory(Erzurumlu and Gaspar 2012) cortices during the critical period. Previous investigations of the olfactory critical period in *Drosophila* have identified a key role of two distinct inhibitory, GABAergic LN populations (LN1 and LN2) in modulating PN output and structural plasticity upon chronic odor exposure during the critical period.(Sachse et al. 2007b; Das et al. 2011) The LN1 sub-population labelled by the NP1227 Gal4 line showed small, statistically insignificant increments in its dose-response curve during chronic CO_2 exposure. In contrast, the LN2 LNs labelled by the NP2426-Gal4 line showed significant increases in its cytosolic Ca^{2+} upon chronic CO_2 exposure during the critical period.(Sachse et al. 2007b) We identified that 5-HT7R mediated serotonergic modulation within this circuit activates cAMP dependent mechanisms of gene expression in LN1 neurons that then induces the volume changes.

The CSDns maintain reciprocal connections with the inhibitory, GABAergic, and glutamatergic LNs within the AL.(Coates et al. 2017) These LNs are critical for network level inhibition in the AL as they tone down PN output before it reaches the MB and LH.(Olsen and Wilson 2008; Root et al. 2008; Yaksi and Wilson 2010; Nagel, Wilson, and Nagel, Katherine I;Wilson 2011; W. W. Liu and Wilson 2013; Hong and Wilson 2015a; Nagel, Hong, and Wilson 2015) Following odor exposure, CSDn inhibits some LN types via 5-HT. In turn, the CSDns are inhibited by both GABAergic and glutamatergic inhibition.(Zhang and Gaudry 2016) Thus, the CSDns could

modulate network level inhibition in the AL via serotonergic modulation and can themselves undergo inhibition based on the network wide inhibitory dynamics established by the LNs. While blocking 5-HT release from the CSDns prevented the availability of synaptic 5-HT and thereby CPP, the AL neurons also had access to basal 5-HT levels released by the remaining 108 serotonergic neurons in *Drosophila*. A prime candidate of basal 5-HT modulation is the 5-HT7R expressing R70A09 GABAergic LNs we identified to be required during the critical period.(Suzuki et al. 2020) These R70A09 LNs mediate subtractive gain control in the PNs and thereby downregulate global PN responses(Suzuki et al. 2020). Our results indicate that although the basal 5-HT levels are trivial during the critical period, 5-HT7R mediated modulation of R70A09 is required during the critical period. This implies that these cells are most likely playing a crucial role during the critical period in maintaining the inhibitory tone in the AL that is conducive to structural plasticity during the critical period.

In *Drosophila*, the Ca^{2+}/calmodulin sensitive adenylate cyclase rutabaga (*rut*) acts as a coincidence detector for cytosolic Ca^{2+} increase and GPCR activation(Gervasi, Tché, and Preat 2010; Das et al. 2011) and converts ATP to cAMP. Rescuing (*rut*) in LN1 or GABA expressing glutamic acid decarboxylase (GAD-1) positive neurons in rut^{2080} mutants was sufficient to reinstate CPP in those flies(Das et al. 2011; Chodankar et al. 2020). We found almost a complete overlap between 5-HT7 expressing R70A09 LNs and the LN1 neurons. Therefore, we can presume a serotonergic modulation in LN1 and consequently in the R70A09 LNs to be acting via 5-HT7 mediated cAMP increase that results in CREB dependent gene transcription. Our results show that

depleting cAMP while keeping the 5-HT7Rs intact in the R70A09 LNs was sufficient to block CPP. The known organisms across phyla that express 5-HT7 all employ an adenylate cyclase dependent mechanism to increase cytosolic cAMP.(Witz et al. 1990a; Stam et al. 1997; Hobson et al. 2006; Omar et al. 2009; Shen et al. 1993; Ruat et al. 1993; Qi et al. 2017a; Dacks et al. 2013; Qi et al. 2017b; Schlenstedt et al. 2006; Pietrantonio, Jagge, and McDowell 2001; Röser et al. 2012; Lee and Pietrantonio 2003; Vleugels et al. 2014; Sizemore, Hurley, and Dacks 2020) Within the LN1 neurons, the adenylate cyclase *rutabaga* is required for CPP.(Das et al. 2011; Chodankar et al. 2020) It is likely that 5-HT7Rs expressed in these LNs modulate cAMP dependent gene transcription to facilitate structural plasticity during the critical period. This is also consistent with the known mechanism of 5-HT7R activity which increases intracellular cAMP levels in *Drosophila*.(Sizemore, Hurley, and Dacks 2020) Future work is required to identify if 5-HT7Rs in *Drosophila* acts through the adenylate cyclase *rutabaga* to induce CREB dependent structural plasticity in the R70A09/LN1 neurons.

5-HT directly modulates excitatory OSNs during the critical period

In addition to impacting local interactions, serotonin also directly impacts 5-HT2BRs on OSNs during the critical period suggesting that there is direct modulation of primary sensory afferents by 5-HT. The differential expression levels exhibited by 5-HT2BRs following eclosion and until the end of the critical period (2 days post eclosion) in the AL is reminiscent of the patchy temporal expression of 5-HT2CRs in the kitten striatal cortex during the visual critical period.(Kojic et al. 2000) Since the ORNs are the primary source of 5-HT2BR expression in the AL, it is likely that the 5-HT2BR mediated serotonergic modulation adapts specifically to the odor environment

presented to the fly during the critical period. This explains why knocking down 5-HT2BRs in the CO_2 responsive OSNs prevents CPP in its cognate V-glomerulus. These results indicate that lower levels of 5-HT2BR expression are permissive to CPP while higher levels of 5-HT2BR signaling beyond the critical levels achieved at day 2 prevent CPP. Additionally, we found differential, patchy expression patterns of the 5-HT2BRs in distinct glomeruli of the antennal lobe during the critical period and in adults, which could also indicate odor dependent differential serotonergic modulation within distinct glomerulus in the AL. Future work directly correlating 5-HT2BR expression in individual glomerulus with the induction of CPP can shed light on the exact levels of 5-HT2BR modulation required during the critical period. Since the OSNs do not undergo structural plasticity during the critical period, downstream pathways by which 5-HT2BRs modulate OSNs during the critical period might shed light on how they indirectly affect structural plasticity in the LNs and PNs. The most likely mechanism could be via NMDA receptor dependent coincident detection that plays an important role in mediating glomerulus specific volume increase during the critical period (Sachse et al. 2007b; Das et al. 2011; Chodankar et al. 2020). Thus, 5-HT can differentially modulate distinct glomeruli based on their 5-HT2BR expression levels upon a specific odor encounter during the critical period.

Maintenance of optimum serotonin levels during the critical period

The balance of excitation and inhibition (E/I) within a network is fundamentally important for facilitating critical period plasticity (Hensch and Fagiolini 2005; Hunter et al. 2024a). Intracellular electrophysiological recordings indicate 5-HT acts by

differentially modulating relevant excitatory and inhibitory synapses during the critical period (Carlos-Lima et al. 2023). This indicates that 5-HT levels can play an important role in controlling permissive levels of excitation and inhibition during the critical period. Therefore, 5-HT levels and thereby 5-HT release permissive to CPP needs to be tightly controlled. Serotonin neurons are known to be modulated by 5-HT itself via expression of 5-HTRs (Sizemore, Hurley, and Dacks 2020). We show direct evidence of how serotonergic neurons modulate its own release to achieve this. In *Drosophila* larvae, the inhibitory 5-HT1BRs are expressed in a pair of serotonergic neurons in the nociceptive circuit that directly inhibit sensory afferents and facilitate a form of experience dependent plasticity in this larval circuit (Kaneko et al. 2017). Similarly, the 5-HT1BRs are expressed on CSDns (Sizemore, Hurley, and Dacks 2020) and knocking them down or overexpressing them prevents CPP. Additionally, knocking down 5-HT1B neurons globally in all serotonergic neurons also has the same effect. The inhibitory nature of the 5-HT1BRs imply that they inhibit serotonin release from the serotonergic neurons which is required to facilitate CPP. Knocking down 5-HT1BRs on CSDns or all serotonergic neurons promotes 5-HT release. On the other hand, overexpressing 5-HT1BRs on CSDNs ensures less 5-HT release. Combining these results with the fact that 5-HT release from the CSDns is also required during the critical period, we can conclude that 5-HT levels are carefully regulated during the critical period to maintain permissive levels of E/I balance. This concentration dependent, bi-directional control allows for the maintenance of an optimal 5-HT level above or below which CPP is hindered.

An alternate mode of action of the 5-HT1BRs on serotonergic cells could be the localization of the serotonin transporters (dSERT) that promote serotonin reuptake thereby reducing extracellular 5-HT levels (Tiger et al. 2018; Middlemiss and Hutson 1990; Barnes and Sharp 1999). Further studies are required to confirm if this mechanism holds true for serotonergic modulation of serotonin neurons during the critical period.

Neuronal mechanism of serotonergic modulation in CPP

During the olfactory critical period, chronic activation of the OSNs by an odor, leads to activation dependent structural plasticity in the cognate glomerulus. This increase in volume can be attributed to the increase in PN and LN arborizations in an odor specific manner (Chodankar et al. 2020; Fabian et al. 2023; Sachse et al. 2007b; Das et al. 2011). A specific subpopulation of GABAergic LNs, the LN1 neurons, the adenylate cyclase *rutabaga* increases cAMP levels to promote CREB dependent gene transcription. This facilitates the structural plasticity observed in both the LNs and PNs (Das et al. 2011). Functionally, it leads to an increase in inhibitory output and a decrease in excitatory PN output onto the higher order olfactory centers. This mechanism suggests a way by which the primary olfactory center modulates sensory output before it can reach the second-order olfactory centers (Figure 7C). Our observations show that serotonergic modulation integrates at multiple levels of this model to modify the output from the AL. Firstly, 5-HT release from the CSDns within this circuit is required for the structural plasticity. Additionally, 5-HT levels in the extracellular space are maintained by 5-HT1BR mediated inhibition of serotonergic neurons. Similarly,

serotonergic modulation on the OSNs is tightly controlled where lower levels of 5-HT2BR expression permits structural plasticity while higher levels of 5-HT2BR expression achieved 2 days post eclosion coincides with the end of the critical period. The 5-HT7Rs on the R70A09 and therefore the LN1 neurons likely activates cAMP dependent gene transcription that ultimately leads to the increase in LN and PN arborizations resulting in glomerular volume increase (Figure 7D). Future experiments are required to examine this model at a greater detail at the cellular and molecular level using pharmacology, and electrophysiology.

In conclusion, our work provides novel insight into how 5-HT modulates structural plasticity at multiple sites of primary olfactory processing during the olfactory critical period. Specifically, we show that 5-HT directly affects the stimulus specific circuit via 5-HT2BRs on the OSNs and 5-HT7Rs on LNs which indirectly modulates GABAergic inhibition throughout the AL. Finally, we show that 5-HT release from the CSDns is carefully controlled during the critical period, disruption of which hinders CPP. This supports the view that neuromodulators affect different components of sensory processing to facilitate structural plasticity during the critical period.

Supplementary Information

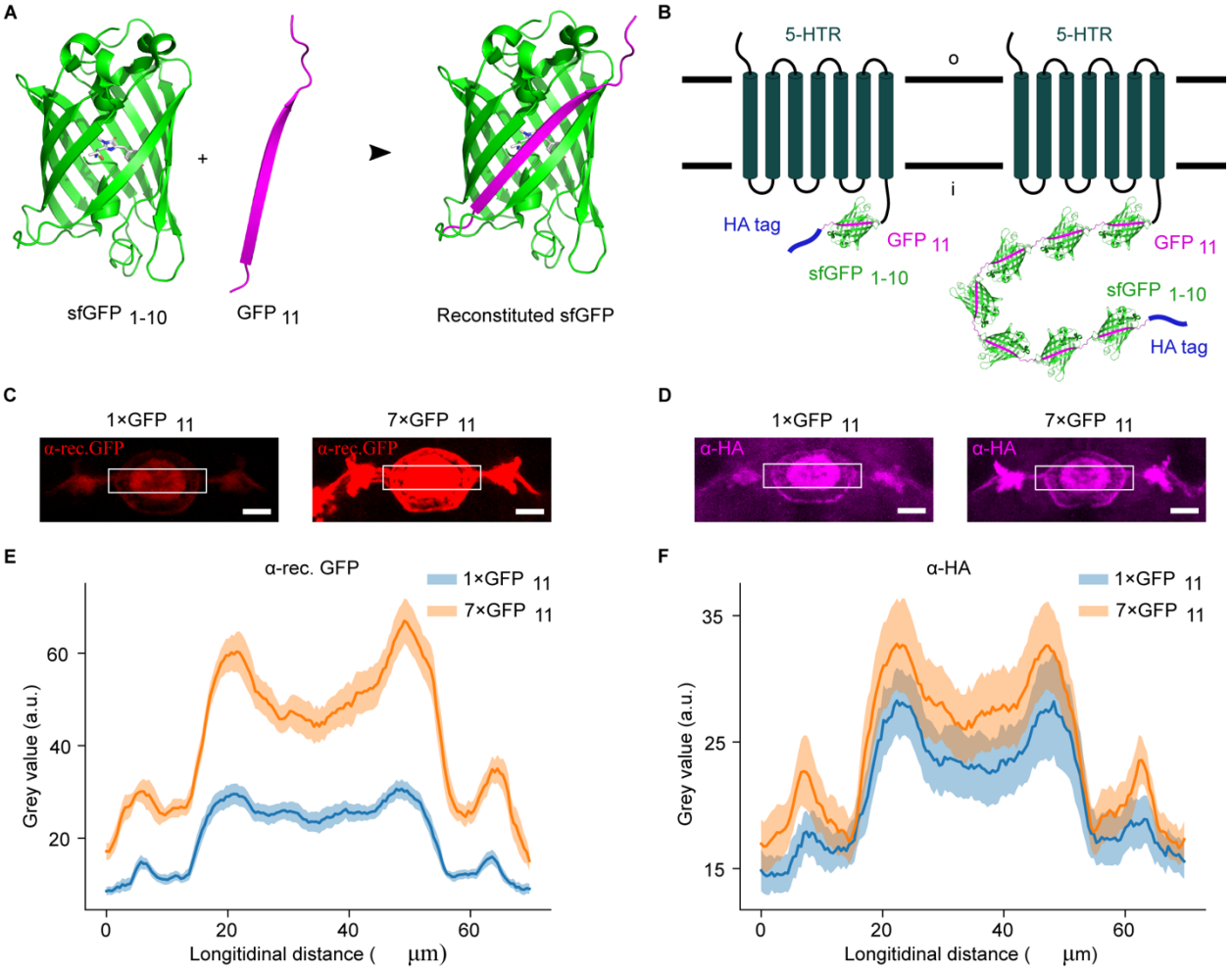

Figure S1. The principle of using the split-sfGFP approach to label 5-HTRs in a cell type-specific manner.

(A) sfGFP$_{1-10}$ (green) and GFP$_{11}$ (magenta) reconstitute and fluoresce. PDB code: 2B3P.

(B) One or seven tandem copies of GFP$_{11}$ fragments with an HA tag are tagged to the intracellular C terminus of 5-HTRs. sfGFP$_{1-10}$ is introduced via the binary expression system to achieve cell type specificity.

(C) Reconstitution comparison between 5-HT7R-GFP_{11}-HA (5-HT7 receptor with one copy of GFP_{11} fragment plus an HA tag fused to the C-terminus of the receptor) or 5-HT7R-7×GFP_{11}-HA (5-HT7 receptor with seven copies of GFP_{11} fragments plus an HA tag fused to the C-terminus of the receptor) in the area of the ellipsoid body. sfGFP_{1-10} was brought in via 5-HT7-Gal4 (Gifted by Charles D. Nichols). The reconstitution was detected with an antibody that specifically targets the reconstituted GFP, denoted as α-rec. GFP. An 8 by 70 μm rectangle was drawn over the ellipsoid body, in which the longitudinal accumulated fluorescence intensity was collected for comparison. Scale bar, 20 μm.

(D)Similar to (C), except that an antibody that targeted the HA-tag (α-HA) was used in the immunostaining for comparison. Flies with the same genotypes as in (C) were used. Scale bar, 20 μm.

(E-F) The longitudinal accumulated fluorescence intensities collected from the rectangular regions shown in (C) and (D) were respectively plotted to compare for the α-rec. GFP channel (E) and the α-HA channel (F). In (E), N = 10 for both 1×GFP_{11} and 7×GFP_{11}; in (F), N = 9 and 10 for 1×GFP_{11} and 7×GFP_{11}, respectively.

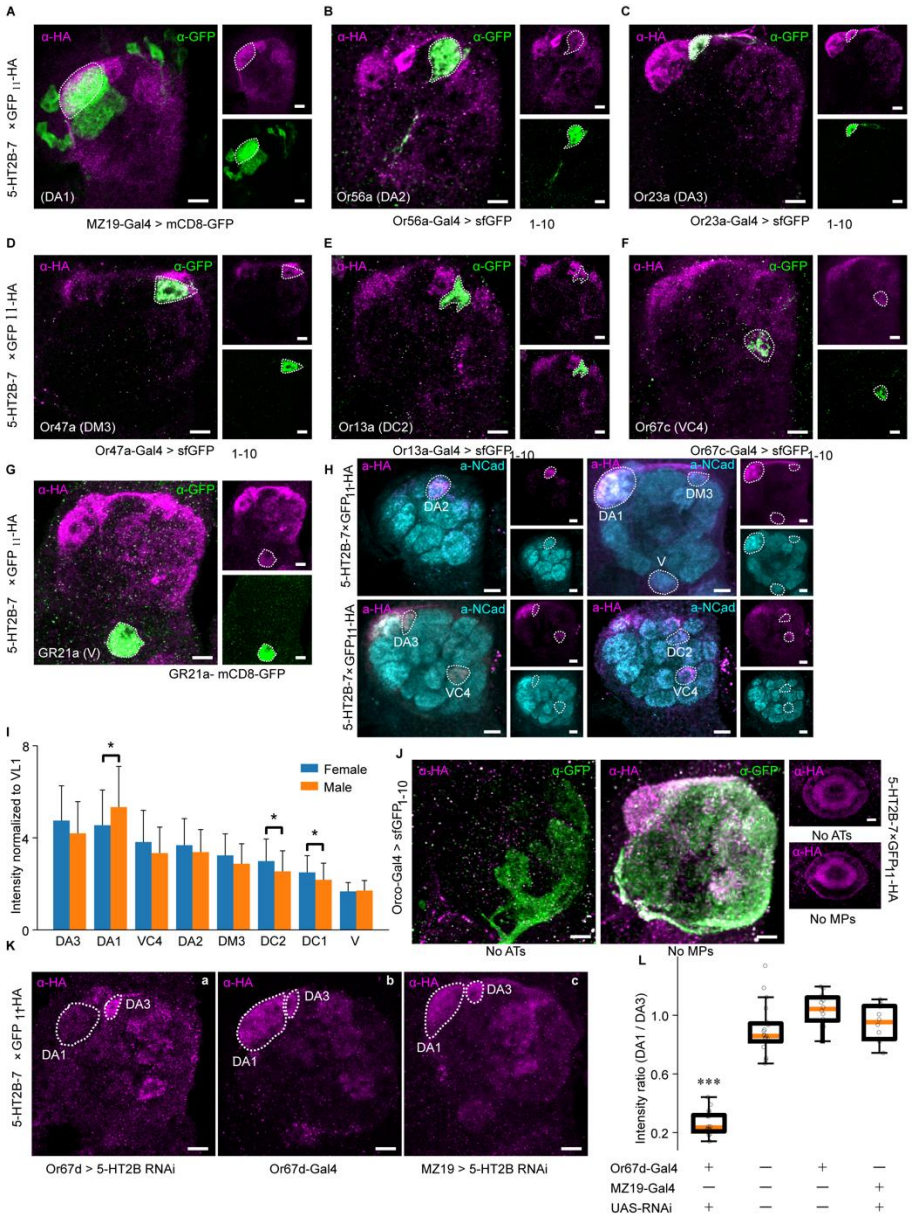

Figure S2. 5-HT2B receptor is expressed in multiple glomeruli in antennal lobes of Drosophila.

(A-G) Confirmation of the identity of OSNs expressing 5-HT2B. Overlap of GFP11-HA (magenta) and sfGFP1-10 were used to identify specific glomeruli. Dotted lines

demarcate the glomerulus of interest in each image. All images are organized with lateral towards left and dorsal upwards.

(H) The seven glomeruli that have a relatively high expression level of 5-HT2B revealed by the α-HA antibody (magenta) in the background staining with α-nCad (N-Cadherin, cyan). Dotted lines indicate the glomeruli of interest.

(I) In male and female brains, mean α-HA fluorescence intensity in each of the eight glomeruli was normalized to that mean signal intensity in VL1 glomerulus. Student's t-test was performed to compare the intensities in each glomerulus between males and females. Asterisks indicate significant difference for DA1, DC1 and DC2 glomeruli, for which, $p = 0.029$, 0.041 and 0.027, respectively. No significant difference was found in other glomeruli. N = 43 brains for females and 44 brains for males.

(J) Four days old of female flies were severed antennae (ATs) or maxillary palps (MPs). Six days later, their brains were dissected and immunostained with α-HA (magenta) and α-GFP (green) antibodies. In addition to antennal lobes, the fluorescent state in the ellipsoid body was also examined (right).

(K) RNAi targeting 5-HT2BRs was introduced to Or67d-Gal4 labeled OSNs or MZ19-Gal4 labeled. In (a), compared to (b), the transgene for RNAi was omitted. Dotted lines indicate the DA1 glomerulus. Magenta indicates the α-HA.

(L) Under different conditions in terms of 5-HT2BR RNAi expression, the fluorescence intensity within the DA1 glomerulus in the α-HA channel was collected and normalized to that within the DA3 glomerulus. n = 9 for Or67d with RNAi, 19 for RNAi alone, 14 for Or67d-Gal4 alone and for MZ19 with RNAi.

Scale bar, 10 μm.

Table S1. List of flies used in the supplementary figures.

Figure	**Genotype**	**Source**
Fig. S1C – F	w1118; 10×UAS-sfGFP$_{1-10}$/+; 5-HT7-7×GFP$_{11}$-HA/5-HT7-Gal4 and w1118; 10×UAS-sfGFP$_{1-10}$/+; 5-HT7-GFP$_{11}$-HA/5-HT7-Gal4	5-HT7-Gal4(Becnel et al. 2011) Gift from Dr. Charles D. Nichols, LSU Health Sciences Centre in New Orleans
Fig. S2A	w1118 ; MZ19-Gal4, UAS-mCD8-GFP/+; 5-HT2B-7×GFP$_{11}$-HA/+	MZ19-Gal4 (BDSC # 34497)
Fig. S2B	w1118; 10×UAS-sfGFP$_{1-10}$/+; 5-HT2B-7×GFP$_{11}$-HA/Or56a-Gal4	Or56a-Gal4 (BDSC #23896)
Fig. S2C	w1118; 10×UAS-sfGFP$_{1-10}$/Or23a-Gal4; 5-HT2B-7×GFP$_{11}$-HA/+	Or23a-Gal4 (BDSC #9956)
Fig. S2D	w1118; 10×UAS-sfGFP$_{1-10}$/+; 5-HT2B-7×GFP$_{11}$-HA/Or47a-Gal4	Or47a-Gal4 (BDSC #9982)

Fig. S2E	w1118; 10×UAS-sfGFP$_{1-10}$/Or13a-Gal4; 5-HT2B-7×GFP$_{11}$-HA/+	Or13a-Gal4 (BDSC #9945)
Fig. S2F	Or67c-Gal4/W1118; 10×UAS-sfGFP$_{1-10}$/+; 5-HT2B-7×GFP$_{11}$-HA/+	Or67c-Gal4 (BDSC #24856)
Fig. S2G	w1118; Gr21a-Mmus\Cd8a.GFP /+;5-HT2B-7×GFP$_{11}$-HA/+	
Fig. S2H	5-HT2B-7xGFP$_{11}$-HA	
Fig. S2J	w1118; 10×UAS-sfGFP$_{1-10}$/Orco-Gal4; 5-HT2B-7×GFP$_{11}$-HA/TM2	
Fig. S2K, left panel	Or67d-Gal4/ w1118; UAS-5-HT2B-RNAi/Pin; 5-HT2B-7×GFP$_{11}$-HA/+	Or67d-Gal4 (BDSC 23906)
Fig. S2K, middle panel	Or67d-Gal4/ W1118; +/+; 5-HT2B-7×GFP$_{11}$-HA/+	
Fig. S2K, right panel	w1118;10×UAS-5-HT2B-RNAi/MZ19-GAL4; 5-HT2B-7×FP$_{11}$-HA/+	
Fig. S2L, 1 – 4 from left to right	1: Same to Fig. S2K, left panel	

	2: w1118; +/+; 5-HT2B-7×GFP$_{11}$-HA/5-HT2B-7×GFP$_{11}$-HA 3: Same to Fig. S2K, middle panel 4: Same to Fig. S2K, right panel	

Sequence of pU6b (circular) backbone.

GTTCGACTTGCAGCCTGAAATACGGCACGAGTAGGAAAAGCCGAGTCAA
ATGCCGAATGCAGAGTCTCATTACAGCACAATCAACTCAAGAAAAACTCG
ACACTTTTTTACCATTTGCACTTAAATCCTTTTTTATTCGTTATGTATACTT
TTTTTGGTCCCTAACCAAAACAAAACCAAACTCTCTTAGTCGTGCCTCTAT
ATTTAAAACTATCAATTTATTATAGTCAATAAATCGAACTGTGTTTTCAAC
AAACGAACAATAGGACACTTTGATTCTAAAGGAAATTTTGAAAATCTTAA
GCAGAGGGTTCTTAAGACCATTTGCCAATTCTTATAATTCTCAACTGCTCT
TTCCTGATGTTGATCATTTATATAGGTATGTTTTCCTCAATACTTCGGGGTC
TTCGTAGAGTCTAGAAAACATCCCATAAAACATCCCATATTCAGCCGCTA
GCATGGATGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTCTTAA
GCTCGGGCCCCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTCGTT
GCAACAAATTGATGAGCAATGCTTTTTTATAATGCCAACTTTGTACAAAA

AAGCAGGCTCCGCGGCCGCCCCCTTCACCACTAGAGGAGAGCAACTGCAT

AAGGCTATGAAGAGATACGCCCTGGTTCCTGGAACAATTGCTTTTACAGA

TGCACATATCGAGGTGGACATCACTTACGCTGAGTACTTCGAAATGTCCG

TTCGGTTGGCAGAAGCTATGAAACGATATGGGCTGAATACAAATCACAGA

ATCGTCGTATGCAGTGAAAACTCTCTTCAATTCTTTATGCCGGTGTTGGGC

GCGTTATTTATCGGAGTTGCAGTTGCGCCCGCGAACGACATTTATAATGA

ACGTGAATTGCTCAACAGTATGGGCATTTCGCAGCCTACCGTAGTGTTTGT

TTCCAAAAAGGGGTTGCAAAAAATTTTGAACGTGCAAAAAAAATTACCAA

TAATCCAGAAAATTATTATCATGGATTCTAAAACGGATTACCAGGGATTT

CAGTCGATGTGAATTCGAGAAGACCTGTTTTAGAGCTAGAAATAGCAAGT

TAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGT

GCGATCCATTTTTTTGCTCACCTGTGATTGCTCCTACTCAAATACAAAAAC

ATCAAATTTTCTGTCAATAAAGCATATTTATTTATATTTATTTTACAGGAA

AGAATTACTAGTGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGC

GCGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCC

GCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT

GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTG

CCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGG

CCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCT

Notes: U6b promotor; gRNA scaffold; The two BbsI cutting sites are located at the end of the U6b promotor and the beginning of the gRNA scaffold sequence, respectively. After cutting with Bbs I and re-ligated with the re-annealed primer pair,

the chunk in between the two Bbs I sites will be replaced by the coding sequence of the gRNA spacer.

References in Table S2

1. Becnel, J., Johnson, O., Luo, J., Nässel, D.R., and Nichols, C.D. (2011). The Serotonin 5-HT7Dro Receptor is Expressed in the Brain of Drosophila and is Essential for Normal Courtship and Mating. PLoS One 6, e20800. 10.1371/JOURNAL.PONE.0020800.

Chapter 3: Dissecting serotonin neuromodulation at single-cell resolution

Introduction

Neuromodulators like 5-HT are responsible for proper signal transmission and circuit function in the central nervous system (CNS) where they influence behavior and cognition in almost all animal species. A dysregulation in neuromodulation can lead to disorders like schizophrenia, anxiety, depression, and anorexia. Often serotonergic systems are directly targeted for treating the symptoms of these disorders. We also know that 5-HT plays an important role during embryonic development of the CNS and postnatally during the critical period (Gaspar, Cases, and Maroteaux 2003; Teissier, Soiza-Reilly, and Gaspar 2017b; Laurent et al. 2002; Salichon et al. 2001). 5-HT is delivered either at conventional synapses or into the extracellular space (ECS) as a paracrine signal to reach distal targets. A central focus in neuroscience has been on studying neurotransmission at synapses because of the ease at which they can be imaged using electron microscopy or studied physiologically. Paracrine neuromodulation has proven harder to study because of the slow diffused mode of transmission, the multiple receptors a single neuromodulator can activate, and the lack of anatomical correlate of functional connections. However, to gain a comprehensive understanding of how the CNS functions, we need to understand the neuromodulatory effects of both synaptic and paracrine 5-HT signals and how they are interpreted in neuronal circuits.

The overarching goal of this study was to determine how individual modulatory neurons contribute to the serotonin levels in the extracellular space. Our lab studies

how the serotonergic (5-HT) system modulates olfaction in *Drosophila* because manipulations are easy to perform in the fly due to readily available genetic tools. Using such reagents, our lab can ablate the synaptic 5-HT neurons projecting into olfactory regions, and thus isolate the paracrine effects of 5-HT in the olfactory system (See Introduction: Isolation of paracrine 5-HT effects) (Zhang and Gaudry 2016). Although the mechanism by which paracrine 5-HT is detected has been identified by our lab (Suzuki et al. 2020), the neurons supplying 5-HT in a paracrine fashion to olfactory centers have not been defined. Fortunately, there are only 10 serotonergic neuron clusters constituting a total of 106 neurons in the adult fly, making *Drosophila* a suitable model for this study.

To genetically isolate distinct serotonergic neurons (Figure 1), our goal was to first generate images where only one serotonergic neuron is labelled per brain. Using these images of neurons as a mask, we would next run a search algorithm that may identify Gal4 lines that label the neuron of interest. Then, using Gal4 lines unique to a single serotonergic neuron subtype, we planned to generate reagents employing an intersectional strategy based on two promoters each expressing half a Gal4 molecule that dimerizes in the cell to restrict expression only to neurons where both promoters are active. This would allow us to genetically isolate individual classes of serotonergic neurons that will allow their direct manipulation to study how they contribute to serotonergic signaling in the olfactory centers. For this, we first employed a stochastic labelling technique called SPARC (Sparse Predictive Activity through Recombinase Competition) (Isaacman-Beck et al. 2020) to sparsely label serotonergic neurons in the brain. But how is this sparse labelling achieved only in a small fraction of cells within

a large population of cells of the same genetic identity? The conventional Gal4-UAS binary expression system enables labelling of all cells within the same genetically

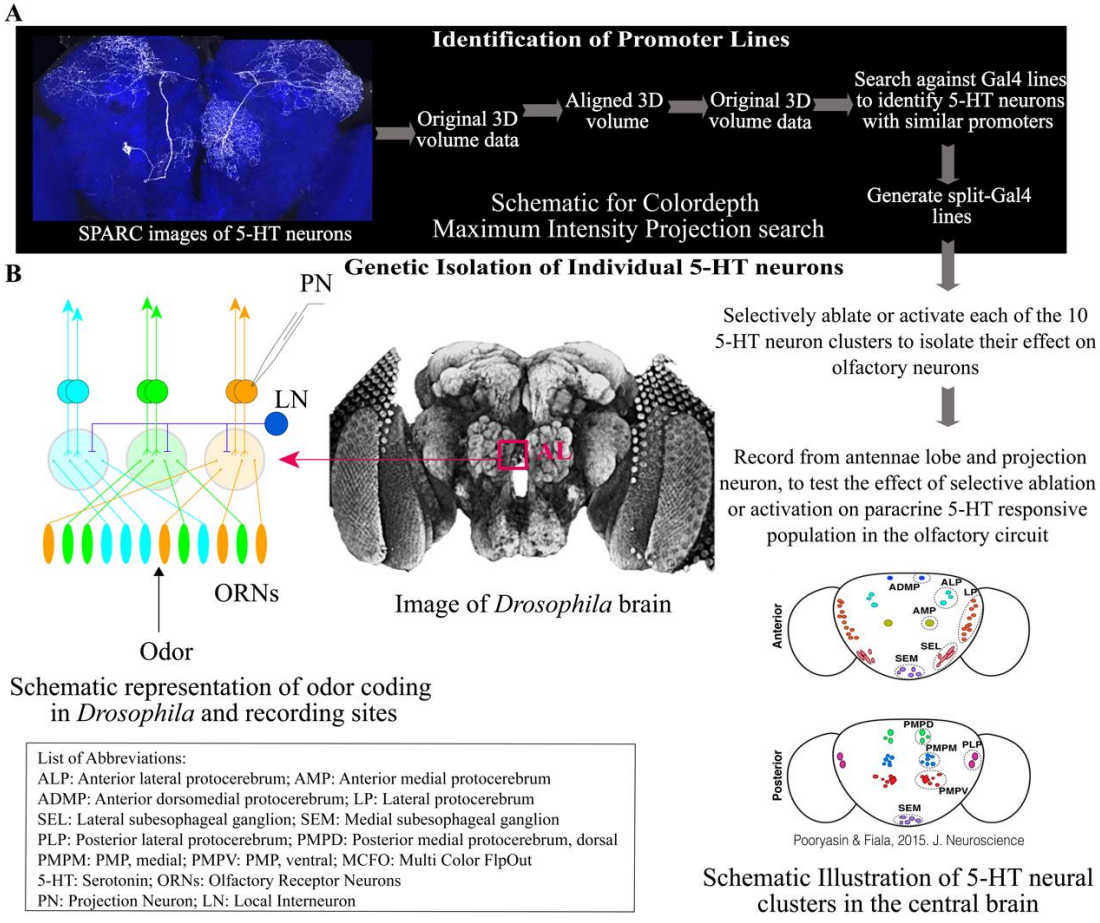

Figure 1. Schematic showing the steps involved in this study.

(A) Workflow to identify unique Gal4 promotors for genetic isolation of serotonergic neuronal subsets. First, we would randomly label 5-HT expressing cells in the fly brain. Trh or tryptophan hydroxylase is a 5-HT synthesis pathway gene and therefore its expression would enable visualization of individual cell morphologies of 5-HT expressing cells at high resolution.

(B) Identification and/or generation of the split-gal4 lines. This will be a useful tool not only for the current study in the olfactory circuit, but also to study global 5-HT neuromodulation. In the future, these reagents will serve to enrich our understanding of the neuronal circuitry with the goal to find common principles in neurobiology across species.

defined cell population as shown in Figure 2A. The SPARC technique builds up on this principle with a few modifications. First, it broadly expresses the recombinase PhiC31 in a broad population of cells of a certain type. For example, to restrict recombination strictly within neurons, the PhiC31 was expressed under n-synaptobrevin that is broadly expressed in all neurons. The SPARC reagent itself consists of a bi-stable UAS-construct that consists of two attP sequences flanking a stop cassette followed by an attB sequence and the effector gene. This allows expression of effector gene only in cells expressing Gal4. A further level of restriction in effector expression applies at the level of the UAS construct itself. Expression of the effector is only possible in cells expressing Gal4 when recombination between the attP upstream of the stop cassette and attB downstream excises out the stop cassette (Figure 2B, Reaction II). Recombination between the attP downstream of the stop cassette and attB retains the stop cassette and the effector gene is not expressed. An additional level of sparseness is achieved by using one of the three different variants of attP constructs: canonical attP (60 base pairs) or truncated attP (38 or 34 base pairs) with varying recombination efficiencies. Thus, 3 different SPARC-UAS contructs are available with decreasing levels of recombination efficiency: Dense (D), Intermediate (I) and Sparse (S). For all

our experiments we used the sparsest SPARC-S reagent. However, when we used SPARC-S reagent to label serotonergic neurons, we observed that too many serotonergic neurons were labelled. This is because the driver line that labels serotonergic neurons is too strong and labels more than one neuron per brain. Generating a mask from such images meant that arbors from multiple neurons could be present in the same mask yielding Gal4 lines that might not label our neuron of interest. Next, as a proof of principle, we used a different Gal4 line that labels a pair of serotonergic neurons, the CSDns. This promoter line only labels two neurons and we could successfully register the confocal stack and generate Color-depth Maximum Intensity Projections (CD-MIPs) of the individual neurons (Otsuna, Ito, and Kawase 2018). The CD-MIPS technique assigns a distinct color to an element (neurons and its arbors) based on its unique x, y, and z positions. This enables comparison of the same neurons in multiple brains as neurons with the same color have the same x, y, and z coordinates. Using such CD-MIPs image of the CSDNs we could generate a mask that was sufficiently unique to yield resultant Gal4 lines that selectively labels the CSDns. Taken together, in this study, I show that it is possible to identify unique candidate Gal4 lines that can be used to uniquely label classes of specific serotonergic neurons. Once such promoter-Gal4 lines that label each of the 5-HT neuron classes are identified, we can use standard molecular biology approaches to rapidly generate split-Gal4 (Pfeiffer et al. 2010; Luan et al. 2020) lines for each serotonergic neuron class in the entire *Drosophila* brain. We can next use these split-Gal4 lines to genetically ablate or activate each serotonergic neuron class and record their effect on the olfactory system by a variety of techniques, including whole-cell patch clamp recordings, optogenetics

etc. of the neurons in the antennae lobe. In the future, this will be an important tool to study the neuromodulatory effects of 5-HT across all neuronal circuits in the fly as this would enable us to manipulate one specific serotonergic neuron cluster at a time.

Materials and Methods

Fly rearing and maintenance

All Drosophila lines were raised in sparse cultures on cornmeal, yeast, dextrose medium at 25°C in a 12hour light/dark cycle. The fly lines used in this study can be found in Table 4. We used female flies for our studies.

Immunostaining and confocal imaging

The immunohistochemistry protocol was adapted from Ostrovsky et al., 2013 (Ostrovsky, Cachero, and Jefferis 2013). Briefly, flies were cold anesthetized and treated with 100% ethanol for 30-60 secs and the brains were dissected in ice cold PBS. Next, the brains were fixed in 4% paraformaldehyde in PBS for 25 mins at room temperature, washed five times with PBS for 15 mins and blocked in 5% normal goat serum (NGS) in PBST for 1 hour. After blocking, the brains were treated with primary antibodies in 5% NGS in PBST for 48 hours at 4°C. Then the brains were washed five times in PBST and incubated with secondary antibodies in 5% NGS in PBST for 48 hours at 4°C. After this, brains were washed for five times in PBST for 15 mins and then before mounting on a glass slide for imaging. Next, the brains are incubated in Vectashield mounting medium (Vector Laboratories, Cat. # H-1000-10) for 1 hour and mounted. To prevent the brains from being smashed, two smaller coverslips (VWR, Cat. # 48366-045) were placed as spacers between the glass slide and the covering

coverslip with one on each side. The coverslips were fixed with a few drops of nail polish on the edge. The brains were imaged at 40X in a Zeiss LSM 710 confocal microscope.

Identifying Candidate Gal4 lines

The confocal stacks of the fly brain were aligned using CMTK (Rohlfing and Maurer 2003) and registered against the Janelia brain template (Bogovic et al. 2020). The CMTK registration was run in Fiji using a github code published by Greg Jefferis (https://github.com/jefferis/fiji-cmtk-gui). The resultant 2D image after CMTK registration was used to generate a Color Depth MIPS map (Otsuna, Ito, and Kawase 2018) of the given brain. This map is created based on the x,y and z position of a neuron and its arbors in the brain.

Next, from the resultant MIPS images we created a mask by selecting the cell body and arborizations of a neuron of interest. This resultant mask was used to search against the available Color Depth MIP image repository of candidate Gal4 lines that labels the neuron. The search yields around 100-200 images of relevant Gal4 lines that best matched the mask or the neuron of interest. Following this, we can select out the best matched Gal4 lines manually.

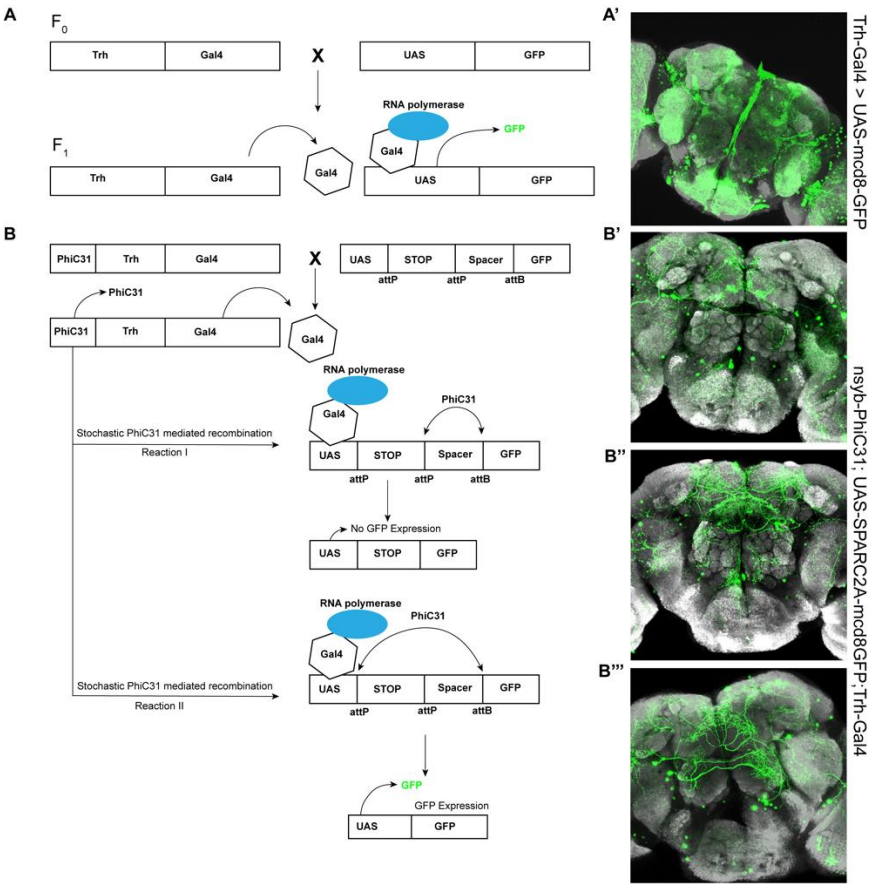

Figure 2: Strategy for labelling serotonergic neurons.

(A) Conventional strategy to label serotonergic neurons in the brain using a Gal4 protein expressed under the Trh- promoter labels all serotonergic neurons.

(A') Confocal maximum intensity projection of the brain labelling all serotonergic neurons in the brain does not allow the dissection of arborizations of single serotonergic neurons.

(B) Stochastic labeling strategy using the SPARC reagent to sparsely label serotonergic neurons in the brain. Successful labelling of serotonergic neurons only occurs in cells where PhiC31 mediated recombination can remove the stop codon to enable GFP expression downstream of the upstream activation sequence (UAS).

(B’ – B’’’) Confocal maximum intensity projection of brains with sparsely labelled serotonergic neurons in each brain using the SPARC strategy.

Table 1. List of Flies used in this study

Figure	**Genotype**	**Source**
1A’	Trh-T2A-Gal4/UAS-mcd8-GFP	Trh-T2A-Gal4(BDSC #84694) 10X-UAS-IVS-mCD8::GFP (BDSC #32185)
1B’-B’’’	nSyb-PhiC31;20X-UAS-SPARC -2S-mCD8::GFP; Trh-T2A-Gal4	n-Syb-PhiC31 (BDSC #84151) 20XUAS-SPARC2-S-mCD8::GFP} (BDSC #84148)
2A-D’	nSyb-PhiC31;20X-UAS-SPARC -2S-mCD8::GFP; Trh-T2A-Gal4	
3A	nSyb-PhiC31;20X-UAS-SPARC -2S-mCD8::GFP; Trh-T2A-Gal4	
4A-B	R60F02-Gal4/10X-UAS-IVS-mCD8::GFP	R60F02-Gal4 (BDSC #48228)

Results

Manipulating neurons in *Drosophila* entails identifying Gal4 promoter lines that express in a cell of interest and crossing those flies with another line containing an effector gene. A limitation of this approach is that a given promoter line will often label many neurons. In *Drosophila*, there are a total of 106 serotonergic neurons. When we use a traditional Trh-Gal4 promotor line that labels all serotonergic neurons and use it to drive a Green Fluorescent Protein (GFP) the resultant images label all serotonergic neurons, and it becomes difficult to isolate single neurons from these images due to their overlapping arbors. To overcome this limitation, we can employ an intersectional strategy based on two promoters each expressing half a Gal4 molecule that dimerizes in the cell to restrict expression only to neurons where both promoters are active (Luan et al. 2020; Pfeiffer et al. 2010). To identify such promoters for the serotonergic neurons, we first generated images of individual 5-HT neurons via a stochastic labeling approach called Sparse Predictive Activity through Recombinase Competition (SPARC) (Isaacman-Beck et al. 2020). The SPARC reagent we used in this case is a GFP codon with an upstream UAS followed by stop codon and 2 recombination sites. As a result, GFP is expressed upon Gal4 binding the UAS sequence and only if PhiC31 mediated recombination removes the preceding stop codon before the GFP. This ensures that most cells expressing the Trh-Gal4 do not express GFP. Using the SPARC reagent, we were able to generate brain images where no more than 4-5 neurons were labelled at a time. These generated images were next used for registration against a common brain template. Accurate image alignment and registration is the first important step towards accurately identifying the 5-HT neurons. Through our immunostaining protocol we aimed to capture 2 things:

a. adequate SPARC labelling that is sparse enough to identify individual neuronal arborizations. b. bright pan-neuronal background staining demarcating clear anatomy in the fly brain for proper image registration. While the first one depends on rapid immunostaining technique, image registration requires antibody incubation steps that lasts up to several days. Optimizing and combining them has been a challenge as you see in the first figure, prolonged incubations (> 4 days) although resulted in lighter staining and the resultant registration suffered. While in the image below, a shorter incubation period (48 hours) resulted in good staining and therefore good image registration.

The CMTK registration protocol performs automatic alignment of our confocal stacks onto a provided template by geometrically aligning one image to another. As a result, neurons in different brain images sharing the same x, y, z position are usually the same neuron. Now, from the registered images, we get the accurate x, y positions of the neurons. For absolute determination of position of a neuron and its arbors, we need a way to distinguish between the depth of each fragment neuropil. This is where we used Color Depth Maximum Intensity Projection images of our confocal images. This technique allowed us to generate 2D color coded maximum intensity projections of 3D

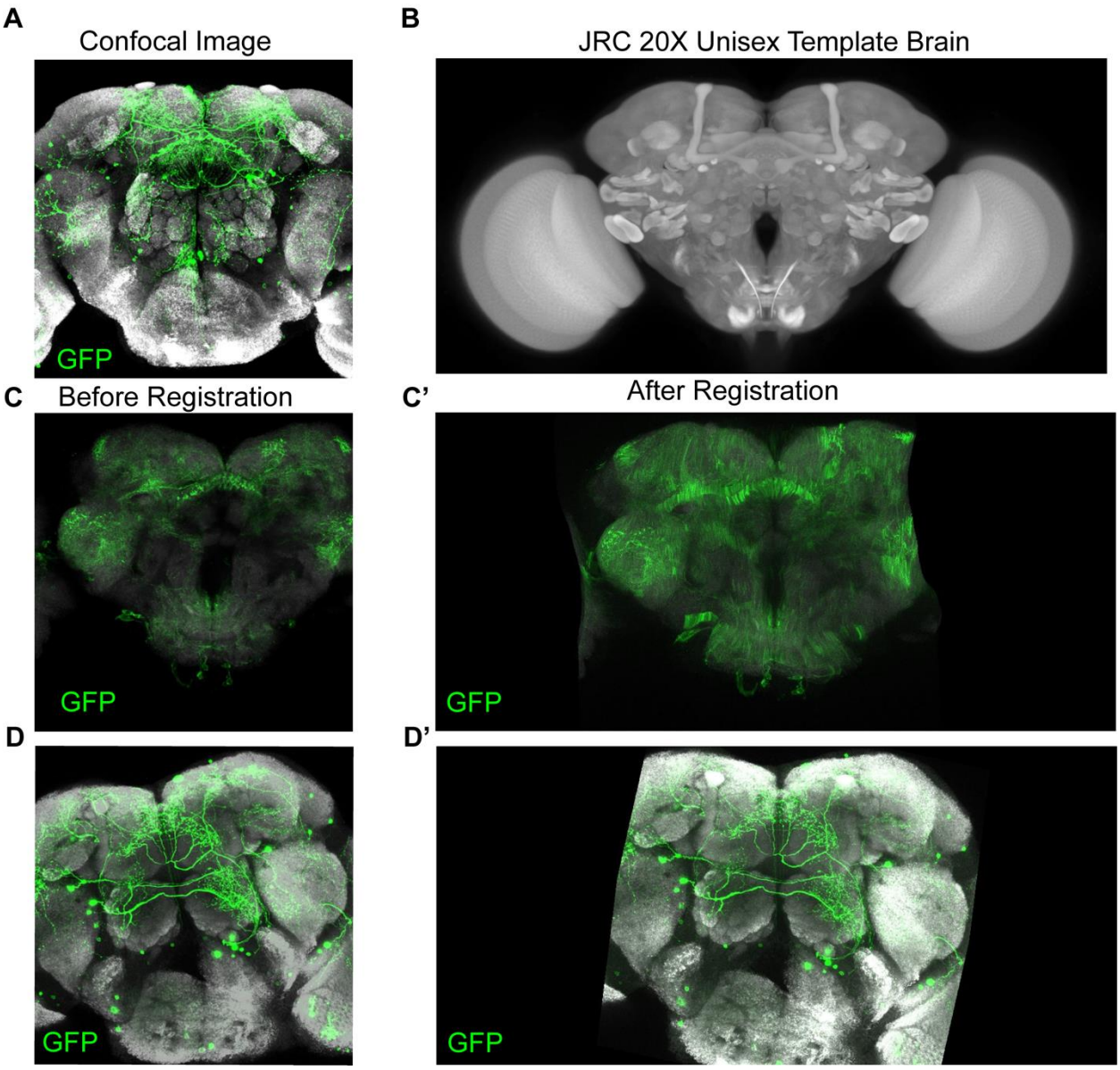

Figure 3: Successful CMTK Image Registration requires high quality of confocal images.

(A) Confocal maximum intensity projection with sparsely labelled serotonergic neurons (in green) used as the input of an image registration.

(B) Template brain that is used to register the obtained confocal image.

(C) Confocal maximum intensity projection image of inferior quality with sparsely labelled serotonergic neurons (in green) used as the input of an image registration.

(C') Resultant image after image registration of (C) against the template brain in (B). Low quality of confocal image resulted in a blurry image after registration.

(D) Confocal maximum intensity projection image of good quality with sparsely labelled serotonergic neurons (in green) used as the input of an image registration.

(D') Resultant image after image registration of (D) against the template brain in (B). Good quality of confocal image resulted in a better-quality image after registration.

confocal stacks. Using such 2D images we were able to search against a library of Gal4 candidates to identify lines that best label our neuron of interest. By using the Trh-Gal4 line to sparsely label 5-HT neurons, the neurons mask we generated was noisy and intermixed with arborizations from another neuron. As a result, the MIPS mask search yielded 400-500 Gal4 lines which were not the best match with our neuron of interest. This implies that although the SPARC reagent was successful to sparsely label serotonergic neurons, it was not sparse enough to allow labeling of one neuron per brain. Therefore, next we selected the R60F02-Gal4 line that labels a single pair of serotonergic neurons in the fly brain. Following successful image registration and Color Depth Maximum Intensity Projection image generation of the confocal stacks,

we were able to run a MIPS mask search. Once the MIP approach identifies promoters that label each of the 5-HT neuron classes, we can use standard molecular biology approaches to rapidly generate split-Gal4 (Luan et al. 2020; Pfeiffer et al. 2010) lines for each serotonergic neuron class in the entire *Drosophila* brain. We can next use these split-Gal4 lines to genetically ablate or activate each serotonergic neuron class and record their effect on the olfactory system by whole-cell patch clamp recordings of the neurons in the antennae lobe. In the future, this will be an important tool to study the neuromodulatory effects of 5-HT across all neuronal circuits in the fly as this would enable us to manipulate one specific serotonergic neuron cluster at a time.

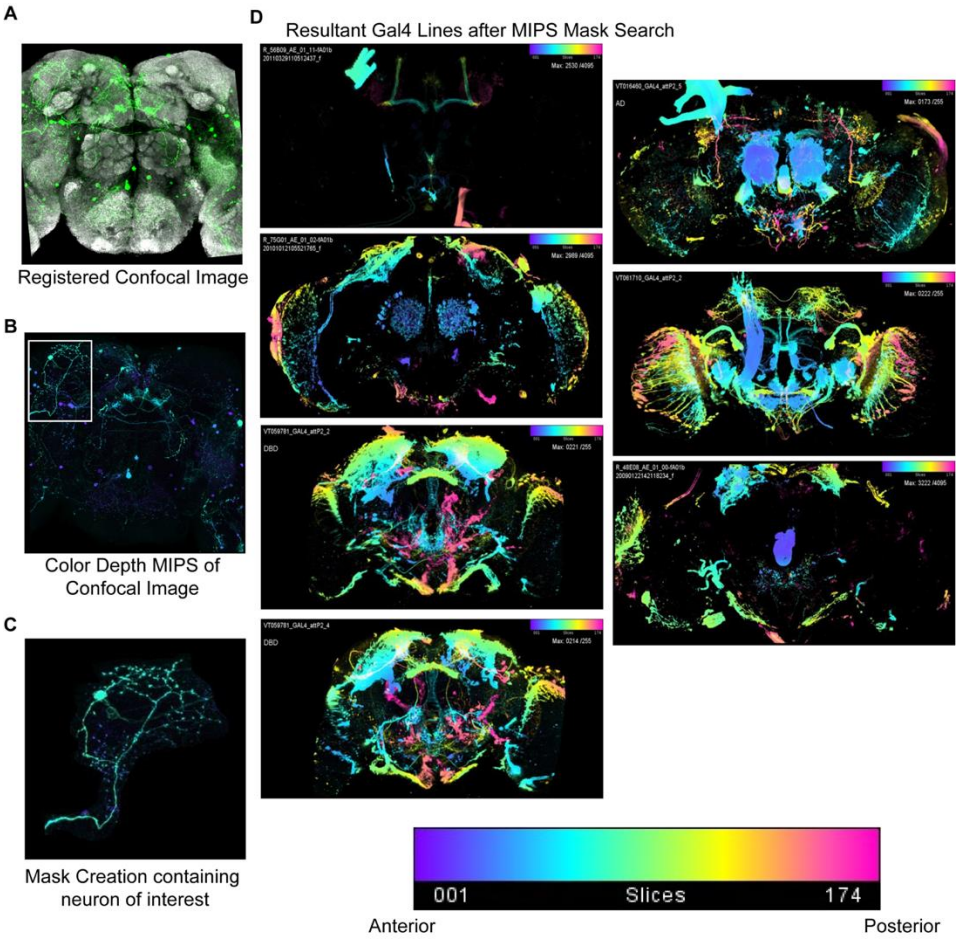

Figure 4: Color Depth MIPS mask search from a serotonergic neuron labelled using SPARC.

(A) Confocal maximum intensity projection of a registered brain with spase labelling of serotonergic neuron using the SPARC strategy.

(B) Color Depth Maximum intensity projection (MIP) of the confocal image in (A). A single neuron to be used as a mask for MIPS mask search is identified by the rectangular selection.

(C) Mask of the neuron indicated in (B) used for MIPS mask search.

(D) Resultant Color Depth MIPs of Gal4 lines where the neuron of interest is also labelled.

Discussion

Our result shows that combining sparse labelling techniques with CD-MIPS is a useful pipeline to generate sparsely labelled images of neurons using the SPARC approach and identify potential split-Gal4 candidate lines. However, the resultant images generated using the Trh-Gal4 driver line was not sparse enough to label less that 2 neurons per brain. This created a challenge when we wanted to create a mask for MIPS search as neuronal arborizations from surrounding labelled neurons also showed up in our region of interest. This yielded Gal4 lines in the MIPS mask search

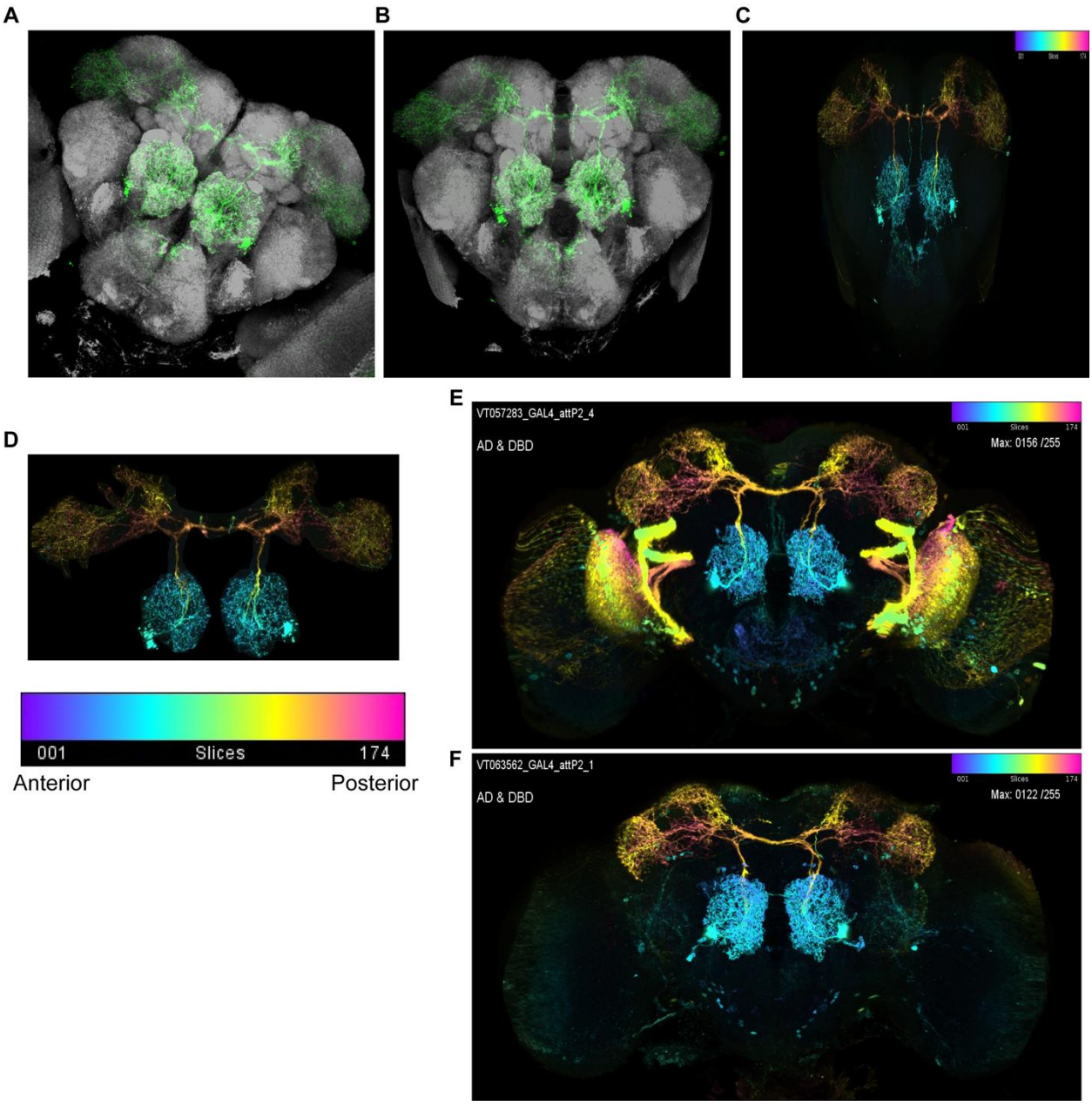

Figure 5: Color Depth MIPs mask search of the CSDNs labelled by R60F02-Gal4.

(A) Confocal maximum intensity projection with the CSDns labelled in green.

(B) Registered confocal maximum intensity projection of the image in (A).

(C) Color Depth MIPs of the registered image in (B).

(D) MIPs mask of the CSDNs.

(E and F) Resultant Gal4 lines from MIP mask search that identified 2 split-Gal4 lines that labels our neuron of interest.

that were not the best match to label our neuron of interest. However, when we used a Gal4 line that label two serotonergic neurons, the resultant MIPS mask search yielded appropriate Gal4 lines that best matched our neurons of interest. Taken together, these results show that in the future, we need to generate sparser labeling techniques to generate masks containing arbors from a single neuron subtype only. This would in turn allow for narrower search results containing Gal4 lines that best matches our neuron of interest. Once we can create such masks containing arborizations from a single neuron, the MIPS mask search will yield more accurate matches of Gal4 lines containing the neuron of interest.

Chapter 4: Conclusion

Concluding remarks

Neuromodulators like 5-HT exist to impart a flexibility to an anatomically static circuit to meet the ever changing internal and external demands of an organism. This is crucial for critical period plasticity as environmental stimuli has a greater effect of inducing plasticity in the developing neuronal circuits in response to experience. In this work, I have identified distinct serotonergic receptors that are required during the critical period in the olfactory system. I achieved this through targeted RNAi mediated knockdown of the 5-HTRs in distinct neurons within the primary olfactory circuit. I have also determined the possible mechanism by which serotonin interacts with the current model of critical period plasticity. Lastly, I have begun to investigate ways in which we can parse out serotonergic subpopulations in the *Drosophila* brain and study their individual contribution to neuromodulation.

Olfaction as a Model System to study Critical Period Plasticity

The olfactory system is unique in that it is composed of organized neuropil structures that encode distinct odor cues in the early processing stages like the antennal lobe (AL) in the fly. The olfactory circuit provides an ideal model to study how distinct environmental cues represented at the periphery during the critical period leads to complex behavior and perception through further processing at the higher centers in the brain. Employing such a highly defined circuit to uncover mechanisms of serotonin neuromodulation during critical period plasticity is therefore highly advantageous for several reasons. First, as the discrete neuropil structures that encode specific odors are predefined at the periphery, it is possible to track morphological, physiological, and

behavioral changes to each odor in the repertoire back to distinct circuits in the brain. This also allowed us to perturb serotonergic signalling on discreet neuronal subtypes without affecting serotonergic components on other neuronal projections within the neuropil. Second, the critical period of olfaction overlaps with postnatal development of the immature olfactory circuit. During the critical period, while the circuit is still developing, environmental factors like experience modulates the genetic programming of the circuit as observed in the case of dendrite selection by OSN axons in the mouse olfactory bulb (Inoue et al. 2018; 2021). Hence, the olfactory critical period posits a unique opportunity to study how genetic programs and environmental cues interact to shape brain circuits during development and how serotonin modulates this interaction. Finally, the olfactory critical period in fruit flies thus far matches the features of critical periods observed in other sensory systems in being restricted to a specific time window, guided by the onset of sensory input, exhibiting high levels of structural and functional plasticity that modifies behavior and perception in adults and being modulated by serotonin.

Development of the Olfactory Circuit coincides with the Critical Period

A central question in neuroscience research has always been how neurons develop to form specialized synapses with each other. To unravel the logic behind this wiring specificity researchers have investigated olfactory circuits, because each olfactory sensory neuron (OSN) subtype is unique in their expression of odorant receptors (ORs) and make highly specialized connections with distinctly identifiable second order neurons. Initial experiments where cognate ORs were swapped with a different functional OR (Bozza et al. 2002) or a mutated OR (Imai, Suzuki, and Sakano

2006) that could not activate upon odor binding compromised the wiring specificity of the OSNs in the OB of mammals during development. Similar receptor swap experiments in *Drosophila* however did not compromise glomerular map formation in the antennal lobe (AL). Instead, swapping cognate ORs in the fly only shifted the response properties of the OSNs to match the response of the new OR (Hallem, Ho, and Carlson 2004). It becomes evident from these experiments that the developmental program and wiring logic in mammals and flies are quite divergent. Indeed, we see ORs and therefore OSNs playing a central role in the development of glomerular map formation and refinement in the OB of mice whereas the same does not hold true for the fly. However, once the initial topographic map is formed, experience dependent activity is known to refine the olfactory circuit during a critical period in both. Another striking similarity is the development of the local inhibitory interneurons during this critical period in both mammals and Drosophila.

Drosophila passes through 3 main metamorphic stages before the adult fly ecloses out of its pupa. In this section we will limit our discussion to the development of the olfactory system of the adult fly. The adult OSNs in the fly emerges right after puparium formation (APF) and fully develop within 72hrs APF(Jefferis et al. 2002; Jefferis and Hummel 2006). However, the odorant receptors (ORs) are not detectable until 60 -90 hours APF. In contrast, the adult PNs are born 48h after larval hatching (ALH) (RF Stocker 1997). The developmental events that give rise to the distinct olfactory map are pre-determined by the sequential emergence of PNs that innervate their cognate glomeruli by 48h APF (RF Stocker 1997). OSNs make synapses with their cognate PNs later during development after PNs have specified distinct glomeruli

(Jefferis et al. 2001). Principles and mechanisms of this development have been previously reviewed (Jefferis and Hummel 2006; Jefferis et al. 2001; 2002; RF Stocker 1997). Such hard-wiring of the olfactory map by the PNs enabled scientists to create a receptor-to-neuron map in the fly by swapping cognate ORs in OSNS with other ORs (Hallem, Ho, and Carlson 2004). These experiments further proved that the only distinguishable factor between two distinct OSNs are in the expression of the OR subtype. The LNs develop at a much later time at or after eclosion which coincides with the critical period of the olfactory circuit in the fly (Jefferis and Hummel 2006; Chodankar et al. 2020; Sachse et al. 2007a; Das et al. 2011). Maturation of inhibitory circuits in the mammalian sensory cortices also coincides with the closing of critical period in the relevant sensory circuits (Toyoizumi et al. 2013; Lo, Sng, and Augustine 2017). Future work needs to confirm if the closing of the critical period coincides with maturation of the inhibitory neurons in their respective olfactory circuits. Such experiments will provide us an opportunity to understand common principles that are at play during the critical period in sensory circuits.

Serotonin Targets both Excitatory and Inhibitory Elements during the Olfactory Critical Period

Co-recruitment of excitation and inhibition by 5-HT adds a further level of complexity and flexibility to the fly olfactory circuit. This is not unique to the fly serotonergic system (Marder 2012). In fact, co-occurrence of excitation and inhibition is common in vertebrate sensory cortices (Isaacson and Scanziani 2011). A similar pattern of modulation also exists during the critical period as shown in this study where serotonin targets both the excitatory OSNs via 5-HT2B receptors and the inhibitory 5-

HT7 LNs via 5-HT7 receptors. Since both serotonergic receptors are excitatory in nature, we can argue that 5-HT mediates activation of both excitation and inhibition in the antennal lobe and thereby achieving an average balanced state of activity during the critical period. This bi-directional modulation is essential during the critical period as knocking down either 5-HT2B on OSNs or 5-HT7 on LNs impairs critical period plasticity. Such optimum balance of activity was also found to be crucial during the critical period of locomotion in Drosophila larvae (Hunter et al. 2024b). Further, in the visual critical period, 5-HT is known to help in the maintenance of excitation-inhibition balance during the critical period by decreasing both excitatory and inhibitory inputs in the visual cortex (Carlos-Lima et al. 2023). Future work needs to confirm if 5-HT plays a direct role in maintaining excitation-inhibition balance during the critical period of olfaction.

Basal versus Synaptic Serotonergic Neuromodulation during the Critical Period

Intensive studies of the CSDns have yielded significant knowledge about synaptic transmission of 5-HT in Drosophila olfaction. During the critical period, release of 5-HT from the CSDns promote structural plasticity. Previous studies in our lab showed that synaptic and paracrine 5-HT has opposing effects on olfactory processing in the adult fly (Suzuki et al. 2020). Similar bi-directional effects of 5-HT have been seen in olfactory attraction to ethanol in *Drosophila* (Xu et al. 2016). Using the UAS-Gal4 system and optogenetics, it was shown that the CSDns counteract the inhibition to ethanol attraction by four 5-HT neurons. It is interesting to note that external olfactory stimulus about presence of ethanol counteracts the internal inhibitory

tone set by paracrine 5-HT. A probable mechanism for this would be strengthening of olfactory PN responses by the CSDns. However, the distinct olfactory network elements involved in this behavior remains unidentified. The authors also could not rule out the possibility of involvement of other neuromodulatory systems as well. Future research can answer these questions by delineating the distinct olfactory network elements involved to regulate this behavior. Such bi-directional neuromodulation has also been demonstrated in several other model systems by dopamine, another monoamine neuromodulator (Rodgers et al. 2011) and serotonin (Teshiba et al. 2001). This suggests a universal theme for neuromodulation in neural circuits.

It can also be argued that the basal levels of paracrine 5-HT acts to moderate neural activity postsynaptically in the PNs to probably suppress effects from spontaneous firing of neurons or probably to increase sensitivity and perception to a specific subset of odors in response to information from other sensory modalities or neural circuits. Evidence shows that 5-HT modulates such cross-modal plasticity between the visual and somatosensory cortices during the critical period via glutamatergic signalling. Here, visual deprivation was shown to increase 5-HT and facilitate synaptic strengthening levels in the barrel cortex during the critical period via glutamate receptor GluR1 (Jitsuki et al. 2011). Similarly, glutamatergic inhibition of GABAergic LNs in the antennal lobe might prove useful to boost PN responses in the olfactory circuit during the critical period. In fact, glutamatergic LNs have been implicated to play a role during the olfactory critical period in *Drosophila* (Das et al.

2011). Hence, the role of paracrine 5-HT during the critical period cannot be completely dismissed and needs to be investigated further.

Methods for Investigating the Role of Paracrine Serotonin

Although volumetric transmission was implied by Golgi (Fuxe et al. 2007), the paracrine effects of 5-HT have been broadly ignored. Initial work on vertebrate volumetric signaling sheds some light into how neuromodulators might affect circuits over long distances (Agnati et al. 2010; Agnati, Bjelke, and Fuxe 1995; Borroto-Escuela et al. 2015). For example, there have been evidence showing the presence of extrasynaptic diffusion of 5-HT in both rat and mice models (Doly et al. 2004; Ridet et al. 2000; Bunin and Mark Wightman 1999; Bunin and Wightman 1998; Ciranna 2006; Benussi et al. 2019). Efforts were made by Agnati and Fuxe et al., to define volume transmission and its principal mechanisms starting in the late 90s (Agnati et al. 2010; Borroto-Escuela et al. 2015; Agnati, Bjelke, and Fuxe 1995). However, a lot remains to be known about the paracrine mode of neuromodulation. One reason behind why paracrine neuromodulation has not been studied as extensively as synaptic neuromodulation could be the lack of appropriate techniques to study paracrine effects. Paracrine effects being a diffused mode of transmission acting at lower concentrations, it sometimes becomes difficult to detect *invivo* using the tools available to us. The innovation of the genetically encoded GPCR-activation based (GRAB) (F. Sun et al. 2018) sensors might have the potential to generate a renewed interest to study paracrine and volumetric signaling. The GRAB sensors would enable detection of minute changes in neuromodulators like 5-HT and dopamine with subcellular resolution with nanomolar to micromolar affinities. However, it will be challenging to detect the basal

concentration of the neuromodulators below nanomolar levels. This might prove to be a challenge specifically when we are studying the effects of basal concentrations of paracrine neuromodulators which work at very low concentrations. In addition, there is a possibility that a single neuromodulatory neuron might act at synapses in its local vicinity and simultaneously contribute neuromodulators into the extracellular space for paracrine transmission. For example, during ethanol attraction, the inhibitory 5-HT neurons might synaptically modulate the regions they innervate in the protocerebrum while simultaneously mediating their paracrine effects by dumping 5- HT into the extracellular space. Although this would be challenging to prove, the GRAB method might just provide us with a tool to spatiotemporally measure neuromodulator levels while the 5-HT neurons are activated individually using optogenetic tools. Despite efforts from the fly community to map a connectivity diagram at synaptic resolution, we cannot completely understand how circuits work without a complete description of the chemical modulators that affects each neuron. Recent efforts have been made in the fruit fly to generate a chemoconnectome (CCT), which basically includes all neuro-transmitters, modulators, neuropeptides, and their receptors (Deng et al. 2019). Such chemical connectome studies reveal that neurons might be modulated by the action of multiple neuromodulators simultaneously.

Finally, paracrine and synaptic serotonin broadly targets nearly all cell types of the olfactory circuit through both inhibitory and excitatory 5-HT receptors. Studying these effects in isolation seems to be an indomitable task but could be possible by using mosaic multicolor labeling techniques like SPARC (Isaacman-Beck et al. 2020) as shown in this study in combination with GRAB. While it is beneficial to study the

effects on the target cells, it is equally important to study the conditions that regulate the release of 5-HT from the 5-HT cells. Another area of focus should be to see how 5-HT modulates higher order regions of the brain responsible for integration of multisensory input. In fact, we know that the 5-HT LP1 neurons innervate the ventral lateral protocerebrum in *Drosophila*, a region where nearly all sensory stimuli including olfactory information is integrated (Xu et al. 2016). Future work in this direction might add to our current understanding of mechanisms of integration of multisensory stimuli and ultimately understand how brain circuits work.

Role of Higher Order Olfactory Centers during the Critical Period

Literature on olfactory critical period in fruit flies has mainly focused on the mechanisms at play within the primary olfactory processing centers, i.e., the antennal lobe. However, the behavioral changes are not likely to be solely due to the changes in these primary olfactory centers. Higher centers of the brain that drive behavior are most likely to modulate these changes. How these mechanisms finally give rise to behavioral responses is yet to be seen. This raises the motivation to see if higher order centers act through hormones and neuromodulators to modulate the critical period. For example, defective social interactions in oxytocin KO mice are rescued when these mice are administered oxytocin as neonates during the olfactory critical period (Inoue et al. 2018). Initial experiments examining the branching patterns of projection neurons in the lateral horn of *Drosophila* showed no visible changes following odor exposure during the critical period (Fabian and Sachse 2023). Experiments from this current study also show that knocking down 5-HT1B receptors in the mushroom body Kenyon cells did not influence critical period plasticity. However, we did not test the role of

serotonergic modulation in the output neurons of the mushroom body on critical period plasticity. Therefore, we cannot rule out the contribution of the lateral horn and mushroom body in modulating the behavioral changes seen following critical period odor exposure.

The studies on *Drosophila* olfactory critical period mentioned above all relied on chronic odor exposure at a high concentration to study the changes induced during the critical period. However, such high concentrations could lead to activation of multiple glomeruli (Hallem and Carlson 2006b; Yaksi and Wilson 2010) and it is unclear then how such glomerular specific changes were seen during the critical period. It is known that in flies, lower odor concentration recruits lateral excitation to promote sensitivity in PNs via excitatory LNs (eLNs). At higher odorant concentrations, these eLNs activate inhibitory GABAergic LNs to induce gain control mechanisms and regulate global AL responses (Yaksi and Wilson 2010). In the absence of ORN-PN excitation, odorants can still invoke excitatory responses through lateral excitation (Olsen, Bhandawat, and Wilson 2007). Indeed, it was seen that chronic odor exposure at naturally occurring or low concentrations during the critical period induced limited changes in the cognate PNs and differentially affected the activity of surrounding PNs via lateral excitation (Gugel, Maurais, and Hong 2023). Similarly, in mice chronic exposure to food odor during the olfactory critical period led to differential activation of different M/T cells in the olfactory bulb (A. Liu and Urban 2017). These findings underscore the need to explore how such mechanisms modulate the critical period and if higher order centers in the brain modulate such lateral activation through feedback loops i.e., via output neurons in the mushroom body and lateral horn.

Reintroducing Critical Periods in Adults

During the critical period, the sensory systems exhibit heightened levels of plasticity and circuit refinement in response to environmental stimuli. These heightened levels of critical period plasticity provided an easy readout to assess how sensory experiences shape the early postnatal brain at the cellular and molecular level (Mallick, Dacks, and Gaudry 2024; Reha et al. 2020; Hensch 2005). A comprehensive understanding of these mechanisms allowed the discovery of pharmacological interventions to reintroduce critical period plasticity for therapeutic purposes in amblyopia and mental health disorders (Lepow, Morishita, and Yehuda 2021; Nardou et al. 2023; Hensch and Quinlan 2018; Schneider et al. 2021; Barber and Aaronson 2022; Vetencourt et al. 2008; Sale et al. 2007; Maya Vetencourt et al. 2011). The most common mode of action for these therapeutic interventions were modulating serotonergic signaling via selective serotonin reuptake inhibitors (SSRIs) that prevent 5-HT reuptake into cells (Schneider et al. 2021; Maya Vetencourt et al. 2011; Sale et al. 2007; Vetencourt et al. 2008) or via psychedelics that act as serotonin receptor agonists (Barber and Aaronson 2022; Lepow, Morishita, and Yehuda 2021; Nardou et al. 2023). However, treating with SSRIs or serotonin receptor agonists and antagonists is a generic therapy that has a trophic effect. This means that such therapies have off target effects often leading to various side effects ranging from nausea and vomiting to suicidal thoughts, anhedonia etc. (Ferguson 2001). Therefore, the enormous potential of serotonin can be better utilized if we develop targeted therapies that only modulate specific neurons to alleviate the relevant symptoms of the problem without having too many side effects. Although previous research showed that 5-HT modulates the critical period, we did not know where and how it modulates a specific circuit during the

critical period. In this study, I show that 5-HT targets the excitatory neurons via 5-HT2B, inhibitory neurons via 5-HT7 and serotonergic neurons via 5-HT1B receptors in the olfactory circuit. Such distinct receptor mediated modulation can act as therapeutic targets of intervention. In the future, we can modulate the excitation-inhibition balance within a given network by cell specific targeting of serotonergic receptors, to reintroduce critical period like plasticity in adults. One way to do this would be via cell specific expression of designer serotonin receptors that are activated by a specific designer drug using a technique akin to DREADDS (designer receptor exclusively activated by designer drugs) (Armbruster et al. 2007) and activates the usual downstream pathways that are activated upon endogenous serotonin receptor activation.

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