Physiological Characterization of the Neural Circuits Mediating Polarization Vision in Drosophila melanogaster

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Declaration of Independence

Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me. I declare, that the thesis has not been submitted in any other examination procedure or to any other institution.

Contents

Summary

The ability of animals to navigate their environment, locate food sources, and find mating partners hinges on their capacity to process and integrate information provided by the visual system. At the heart of this complex task lies the intricate web of thousands of individual neurons, each playing a crucial role in the orchestration of sensory information. Understanding the rules and mechanisms guiding this neural computation is a profound pursuit central to the fields of neuroscience and ethology.

My doctoral research advances our understanding of navigation by delving into neural circuitry and information processing mechanisms, particularly emphasizing polarized skylight detection in insects. Focused on *Drosophila melanogaster*, a powerful model organism, the study explores the intricate visual system comprised of optically isolated unit eyes called ommatidia. Approximately 800 of these units populate the adult retina, facilitating precise spatial sampling. Within the *Drosophila* retina, different ommatidial subtypes house specialized inner photoreceptors for color perception in the central retina or the detection of skylight polarization in the dorsal rim area (DRA). Visual information undergoes complex processing in the optic lobes before being relayed to higher brain structures, such as the anterior optic tubercle (AOTU) within the visual glomeruli. My thesis contributes to understanding the less well-known ventral polarization vision, exploring local circuitries in the optic lobes, and shedding light on the less-understood aspect of this visual modality. The literature study identifies functionally specialized non-DRA detectors by examining non-celestial polarization vision across diverse insect species, including dragonflies, butterflies, beetles, bugs, and flies. Although the ventral polarization vision in *Drosophila melanogaster* presents a fascinating modality, the unknown location of the specific circuitry stays hidden. Therefore, I turned my attention to the better-known specific circuitry of skylight polarization vision in the DRA and unveiled modality-specific connectivities of local medulla neurons in the DRA. Including Mt11-like medulla tangential cells that avoid the DRA region. Despite gathering comprehensive information from the entire medulla, these cells lack inputs related to polarized light from the DRA, indicating separate processing of distinct visual attributes within the central

brain.
Finally, I characterized the anatomical and physiological properties of MeTu-types, modality-specific to the DRA, called MeTu-DRA1 and MeTu-DRA2. Using the genetic toolkit of *Drosophila melanogaster*, the study showed for the first time that both populations are modality-specific postsynaptic to DRA.R7 photoreceptors only, project to the same subunit of the AOTU and show differences in their morphology as well as connectivity. Although the morphology showed significant differences, single-cell clones revealed a topographic projection of both MeTu-DRA sub-populations from the medulla to the AOTU. Based on these findings, we hypothesized that the anatomical and connectivity differences might result in different physiological response patterns of MeTu-DRA1 and MeTu-DRA2. In order to test this theory, I implemented calcium imaging (using GCaMP) under a 2-photon microscope. I recorded the physiological response properties of Dm-DRA1 (in the medulla) and MeTu-DRA1 and MeTu-DRA2 responses in the AOTU. Interestingly, I could show for the first time that MeTu-DRA1 shows a detailed representation of different 'Angle of Polarization' (AoP) in the AOTU, and MeTu-DRA2 responses, however, split the AOTU in a dorsal or ventral half pattern. With EM reconstruction, we could identify a more detailed circuitry of the MeTu-DRAs and a new DRA-specific interhemispheric cell type called MeMe-DRAs. Additionally, I could show that only MeTu-DRA2 responds to unpolarized UV flashes presented contralaterally, which is most likely mediated by MeMe-DRAs and presents an early binocular integration of polarized skylight information.

In conclusion, the discoveries made during my doctoral research significantly contribute to our comprehension of the functional characteristics and circuitry of MeTu-DRA neurons in *Drosophila melanogaster*. This comprehensive understanding enhances our knowledge of how binocular integration plays a crucial role in the neural mechanisms guiding polarization vision and navigation.

Zusammenfassung

Die Fähigkeit von Tieren sich in ihrer Umgebung zu orientieren, Nahrungsquellen zu finden und Paarungspartner zu entdecken, hängt von ihrer Fähigkeit ab die Informationen des visuellen Systems zu verarbeiten und zu integrieren. Im Zentrum dieser komplexen Aufgabe liegt das fein verästelte Netzwerk von Tausenden einzelner Neuronen, von denen jedes eine entscheidende Rolle bei der Orchestrierung sensorischer Informationen spielt. Das Verständnis der Regeln und Mechanismen, welche die neuronale Verarbeitung lenken, ist eine tiefgreifende Untersuchung, die zentral für die Bereiche Neurowissenschaften und Ethologie ist.

Meine Doktorarbeit trägt dazu bei, unser Verständnis der Navigation zu vertiefen, indem sie sich mit der neuronalen Schaltung und den Mechanismen der Informationsverarbeitung befasst und dabei besonders die polarisierte Himmelslichterkennung bei Insekten betont. Im Fokus steht dabei *Drosophila melanogaster*, ein potenter Modellorganismus, dessen komplexes visuelles System aus optisch isolierten Einzelaugen namens Ommatidien besteht. Die Augen einer ausgewachsenen Fliege umfassen etwa 800 dieser repetitiven Einheiten und ermöglichen eine präzise räumliche Abtastung der Umgebung. In der Retina von *Drosophila* sind verschiedene Ommatidien-Untertypen, die entweder für die Farbwahrnehmung in der zentralen Retina oder für die Erkennung des polarisierten Himmelslicht im dorsal gelegenen Randbereich (DRA) angepasst sind. Visuelle Informationen durchlaufen komplexe Verarbeitungsprozesse in den Sehloben, bevor sie zu höheren Hirnstrukturen wie dem anterioren Optischen Tuberkel (AOTU) innerhalb der visuellen Glomeruli weitergeleitet werden. Meine Arbeit trägt zum Verständnis des weniger verstandenem ventralen Polarisationssehen bei, indem sie lokale Schaltkreise in den Sehloben erforscht und einen weniger bekannten Aspekt dieser visuellen Modalität beleuchtet. Die Literaturstudie identifiziert funktionell spezialisierte nicht-DRA-Detektoren, indem die nicht-himmlische Polarisationssicht in verschiedenen Insektenarten wie Libellen, Schmetterlingen, Käfern, Wanzen und Fliegen untersucht wird. Obwohl das ventrale Polarisationssehen in *Drosophila melanogaster* eine faszinierende Modalität darstellt, bleibt die spezifische Position der Schaltung unbekannt. Daher untersuchte ich spezifische synaptische Verschaltung in der schon mehr bekannten DRA bezüglich der Himmels-

lichtpolarisation. Hier konnte ich die Verschaltung diverser lokaler Medulla Neurone in der DRA zeigen. Unter anderem Mt11-ähnliche medulläre Tangentialzellen, die den DRA-Bereich explizit meiden. Obwohl sie umfassende Informationen aus der gesamten Medulla sammeln, fehlen diesen Zellen synaptische Verschaltungen, die mit polarisiertem Licht aus dem DRA zusammenhängen. Dies deutet darauf hin, dass unterschiedliche visuelle Merkmale in separaten Bereichen des zentralen Gehirns verarbeitet werden.
Schließlich charakterisierte ich die anatomischen und physiologischen Eigenschaften von MeTu-Typen, die modalitätsspezifisch für die DRA sind, nämlich MeTu-DRA1 und MeTu-DRA2. Unter Verwendung des genetischen Werkzeugs von *Drosophila melanogaster* zeigte die Studie erstmals, dass beide Populationen nur modalitysspezifisch postsynaptisch an DRA.R7-Fotorezeptoren sind, zur gleichen Untereinheit des AOTU projizieren und Unterschiede in ihrer Morphologie sowie Konnektivität aufweisen. Obwohl die Morphologie signifikante Unterschiede zeigte, enthüllten Einzelzellklone eine topografische Projektion beider MeTu-DRA-Teilpopulationen von der Medulla zum AOTU. Basierend auf diesen Erkenntnissen postulierten wir, dass die anatomischen und Konnektivitätsunterschiede zu unterschiedlichen physiologischen Reaktionsmustern von MeTu-DRA1 und MeTu-DRA2 führen könnten. Um diese Theorie zu testen, implementierte ich Calcium-Imaging (unter Verwendung von GCaMP) unter einem 2-Photonen-Mikroskop. Ich konnte die physiologischen Reaktionseigenschaften von Dm-DRA1 (in der Medulla) sowie MeTu-DRA1- und MeTu-DRA2-Reaktionen im AOTU aufzeichnen. Interessanterweise konnte ich erstmals zeigen, dass MeTu-DRA1 eine detaillierte Darstellung verschiedener 'Polarisationswinkel' (AoP) im AOTU zeigt, während MeTu-DRA2-Reaktionen den AOTU in einem dorsal-ventralen aufspalten Muster spalted. Mit EM-Rekonstruktion konnten wir eine detailliertere Schaltung der MeTu-DRAs und einen neuen DRA-spezifischen interhemisphärischen Zelltyp namens MeMe-DRAs identifizieren. Zusätzlich konnte ich zeigen, dass nur MeTu-DRA2 auf unpolarisierte UV-Blitze reagiert, die kontralateral präsentiert werden, was höchstwahrscheinlich durch MeMe-DRAs vermittelt wird und eine frühe binokulare Integration von Informationen über polarisiertes Himmelslicht darstellt.
Zusammenfassend tragen die Erkenntnisse meiner Doktorarbeit erheblich zum Verständnis der funktionalen Eigenschaften und Schaltung der MeTu-DRA-Neuronen in *Drosophila melanogaster* bei. Dieses umfassende Verständnis verbessert unser Wissen darüber, wie die binokulare Integration eine entscheidende Rolle bei den neuronalen Mechanismen spielt, die die Polarisationssicht und Navigation lenken.

Abbrivations

A anterior

AoP Angle of polarization

AOTU Anterior Optic Tubercle

AVP Anterior Visual Pathway

BDSC Bloomington Drosophila Stock Center

Brp Bruchpilot

D dorsal

Dm distal medulla cell

DoP Degree of polarization

DRA Dorsal Rim Area

DREP2 marker of putative post-synaptic membranes

eq equator of the eye

FLP Flip recombinase

GAL4 Transcription factor

GCaMP activity-dependent GFP

GECIs genetically encoded calcium indicators

GFP green fluorescent protein

GMR Glass multimeric reporter

GRASP GFP reconstitution across synaptic partners

HisCl1 histamine-chloride receptors

L lateral

La Lamina

LexA Transcriptional activator recognizing LexAop binding sites

Lo Lobula

Lp Lobula plate

L-cells lamina monopolar cells

M Medulla layer

mCD8:GFP Membrane-tagged GFP

MCFO Multi-color Flp out

Me Medulla

MEDRA DRA in the medulla

MeMe Medulla to Medulla projecting neuron

mKate Red-fluorescent protein

MeTu medulla to tubercle projecting neuron

Mt Medulla tangential cell

nCad N-Cadherin

nc82 antibody against Brp

PBS Phosphate buffered saline

PRs photoreceptor neurons

PSI Polarization Sensitivity Index

R photoreceptor

Rh rhodopsin molecules

Tm Transmedullary cells

Tom Tomato (red fluorescent protein)

TuBu Tubercle to Bulb neuron

TuTu Tubercle to Tubercle neuron

UAS Upstream activated sequences (recognized by GAL4)

UV ultraviolet light

VPA Ventral Polarization Area

Part I

Introduction & Aim

1 Introduction

In nature, every individuum is confronted with â vast number of different kinds of sensory stimuli. Using these to navigate is both a powerful and complex ability. Understanding how the brain processes and integrates different sensory stimuli to inform a specific behavioral response remains one of the primary goals of neuroscience. More specifically, the role of single cells in detecting, computing, and integrating this information are questions that have been addressed by several studies in more recent times. (Clark et al., 2013; Klapoetke et al., 2017; Longden, 2018; Maisak et al., 2013; Mauss et al., 2015; Song and Lee, 2018).

Both mammals and invertebrates provide very powerful model organisms for understanding how the brain processes visual information on a detailed level (Wernet et al., 2014). The neuronal morphology, synaptic connectivity, and therefore the computation executed by specific circuit elements can easily be manipulated and thereby understood within these models (Anderson, 2016; Moulin et al., 2021). Although the established model organisms have already provided significant insight into the computational processes across animal brains, many crucial aspects remain incompletely understood. The vinegar fly *Drosophila melanogaster* is one of the most popular molecular genetic and neurobiological models for studying and understanding neuronal circuitry. Its adult brain consists of 200,000 neurons (Raji and Potter, 2021), with roughly half of those neurons forming the fly's visual system. Based on this compact yet still significant amount of neurons, the structure and function of the fly visual system provide an excellent model for neuroscience.

1.1 The retinal basis of *Drosophila*'s visual system

Like the eyes of most insects, the adult eyes of *Drosophila melanogaster* are the so-called compound eyes, each consisting of ~800 single-unit facets for detecting different visual stimuli (Fischbach and Dittrich, 1989; Kind et al., 2020). Each facet contains a single corneal lens with eight optically isolated photoreceptor neurons (PRs) called an

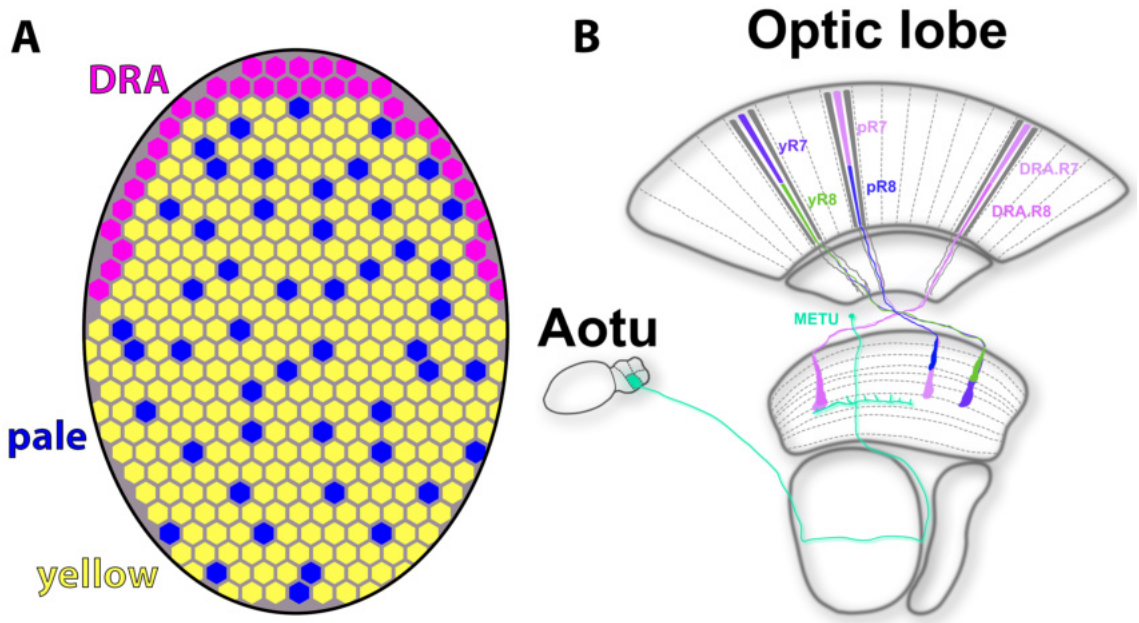

Figure 1.1: **Depiction of the mosaic eye and optic lobe of *Drosophila melanogaster*** **A** Schematic of the retinal mosaic. DRA ommatidia (pink) is located at the dorsal edge of the eye. Pale (blue) and yellow (yellow) ommatidia are stochastically distributed over the whole eye. **B** Schematic representation of the fly's optic lobe. The retinotopic arrangements of the single ommatidia are conserved from the retina upon the medulla. Each unit houses the outer photoreceptor R1-R6, which project as cartridges into the lamina (in dark grey). For color and skylight detection, ommatidium has inner photoreceptors, R7 and R8, which express specific Rhodopsins. The so-called 'yellow' ommatidium expresses Rh4 and Rh6 in the R7 and R8, respectively (here labeled as yR7, purple, and yR8, green). R7 and R8 photoreceptors of the 'pale' column express Rh3 and Rh5 (labeled as pR7, pink, and pR8, blue). The last ommatidial subtype, the DRA, expresses Rh3 in R7 and R8 (here as DRA.R7 and DRA.R8, both in pink). In addition to the columnar structures, the optic lobe contains multicolumnar neurons. One of those neurons is the so-called MeTu neuron, which projects from the Medulla to a higher visual glomerulus, the Anterior optic Tubercle (AOTU).

ommatidium. In addition to these light-gathering and light-detecting structures, each ommatidium also contains several pigment cells (for optical isolation), giving *Drosophila melanogaster* its distinct red eye color by scattering and absorbing light, as well as neuronal bristle cells (Cagan and Ready, 1989). Light enters the corneal lens and is focused onto the light-detecting apparatuses of the eight PRs – classified as six outer and two inner PRs.The light-sensitive membrane structures of each receptor are known as the rhabdomere, which is formed by microvillar protrusions containing large amounts of

light-detecting rhodopsin molecules (Rh) (Hardie, 2012). Based on differences in their amino acid sequence, different Rh molecules become sensitive to different wavelengths. These different rhodopsins are expressed in different subtypes formed by the eight PRs (Kind et al., 2020). The six outer PRs (named R1-6) of *Drosophila*always express the same broadband-sensitive Rh1, encoded by the gene *ninaE* (Zuker et al., 1985). All axons of these cells terminate in the first neuropil of the optic lobes, the lamina (Fischbach and Dittrich, 1989; Meinertzhagen and Sorra, 2001) . Outer PRs are very sensitive and known to mediate image formation and motion vision (Heisenberg and Buchner, 1977). The inner photoreceptors R7 and R8 are stacked on top of each other in the retina (Wolff and Ready, 1993) and form the color vision system for the fly (Heath et al., 2020; Schnaitmann et al., 2018; Yamaguchi et al., 2002). The axons of R7 and R8 form long visual fibers that terminate in the second neuropil, the medulla (Chotard and Salecker, 2007). The medulla can be subdivided into ten distinct layers based on the neuronal morphology of cell types found there (Fischbach and Dittrich, 1989). R8 PRs always terminate in a more distal medulla layer called M3, whereas R7 PRs always send their axons to the deeper layer M6. Notably, the axons of both inner photoreceptors originating from the same ommatidium always terminate within the same medulla column (see Figure 1.1), resulting in a retinotopic medulla map. The Rhs expressed by R7 and R8 are determined stochastically (Chou et al., 1999; Wernet et al., 2006). In the so-called 'yellow' ommatidia, R7 expresses the UV-sensitive Rh4 and R8 the green-sensitive Rh6. In contrast, 'pale' ommatidia are defined by expression of UV-sensitive Rh3 and the blue-sensitive Rh5 in R7 and R8, respectively (see Figure 1.1) (Johnston et al., 2011). Interestingly, the ratio between randomly distributed yellow and pale ommatidia is uneven (65% yellow vs 35% pale), the reason for this uneven distribution remaining obscure.

A third ommatidia, highly localized ommatidial subtype can always be found at the dorsal edge of the adult eye and is therefore named after this: Dorsal Rim Area (DRA) (Labhart and Meyer, 1999; Tomlinson, 2003; Wernet et al., 2003). DRA inner photoreceptors differ in morphology and function from both pale and yellow counterparts. Morphologically, both R7 and R8 in the DRA manifest a larger rhabdomere diameter, individual rhabdomeric microvilli being untwisted and expressing the same type of rhodopsin, the UV-sensitive Rh3. Their microvillar orientation is orthogonal to each other (Wernet et al., 2012), thereby forming the anatomical substrate suitable for polarization-opponency (Herzmann and Labhart, 1989). Indeed, these anatomical characteristics result in a physiological and behavioral sensitivity to linearly polarized UV light (Hardcastle et al., 2021;

Mathejczyk and Wernet, 2019; Weir and Dickinson, 2012; Weir et al., 2016). DRA R7 and R8 axons appear enlarged and terminate in the same M6 medulla layer; however, DRA.R8 stops just short of DRA.R7 (ref). Cell types connecting to DRA R7 and R8 PRs have been the subject of intense research over the last few years (Fischbach and Dittrich, 1989; Sancer et al., 2019), forming the neural circuits for processing skylight polarization.

1.2 Processing environmental visual cues

The fly uses all the photoreceptors described above to detect all available visual stimuli to navigate the environment. The Rhs make these photoreceptor types sensitive to different wavelengths. The exact computations of these photoreceptor responses and how they encode specific features remain at the core of many studies.

1.2.1 Color Vision

Photoreceptors and their respective postsynaptic neurons process environmental information such as color, motion, and intensity. The outer PRs R1-6, together with their main postsynaptic partners in the Lamina (the so-called lamina monopolar cells (L-cells)), the retinal basis for motion-and-contrast vision (Clark et al., 2011; Ketkar et al., 2020; Leonhardt, 2017). This system provides vital information to the fly, such as edge detection, and contributes to color vision. It was recently shown that outer photoreceptors play an indirect role in the computation of color (Li and Saha, 2021; Pagni et al., 2021). Hence, color vision appears to represent an essential modality in *Drosophila*vision and is, therefore, the focus of several studies (Chin et al., 2014; Morante and Desplan, 2008; Schnaitmann et al., 2020; Yamaguchi et al., 2010). Anatomical, physiological, and behavioral studies have contributed different aspects to our understanding of how color is processed in the fly brain. Connectomic reconstruction and genetic dissections demonstrated that the R7 and R8 within each medulla column are synaptically interconnected (Heath et al., 2020; Kind et al., 2021; Schnaitmann et al., 2020; S.-y. S. Takemura et al., 2013). They form direct connections via chemical synapses (Kind and Wernet, 2021; S. y. Takemura et al., 2017; S.-y. S. Takemura et al., 2013). Upon activation of either R7 or R8, this cell depolarizes and releases the neurotransmitter histamine (Davis et al., 2020; Heath et al., 2020; Pagni et al., 2021; Schnaitmann et al., 2020), which then binds to the histamine-chloride receptors (HisCl1) on the respective R8 or R7 counterpart, thereby hyperpolarizing it.

This intracolumnar connectivity tunes the opponent responses of R7s and R8 terminals (Davis et al., 2020; Heath et al., 2020; Pagni et al., 2021; Schnaitmann et al., 2020). This opponency between color-sensitive PRs is even further fine-tuned via intercolumnar inhibition mediated by the distal medulla cell (Dm)9 (Heath et al., 2020). These Dm9 cells are both pre-and postsynaptic to several neighboring R7 and R8 cells, leading to a complex pattern of lateral inhibition. These photoreceptor signals are then processed by the main postsynaptic target of R7 cells, another distal medulla cell named Dm8 (Li and Saha, 2021; Pagni et al., 2021; S. y. Takemura et al., 2017; S.-y. S. Takemura et al., 2013). One Dm8 cell is postsynaptic to several R7 neurons and synaptically interconnected overlapping Dm8 cells in its vicinity. Several recent studies demonstrated that each Dm8 cell has a "home " column where it gets the strongest synaptic input from R7 and indirect input from outer photoreceptors (Courgeon and Desplan, 2019; Li and Saha, 2021; Menon et al., 2019; Pagni et al., 2021). Beyond this 'home' column, neighboring Dm8 cells provide lateral inhibition via inhibitory connections through the glutamate-gated chloride channel GluClα, resulting in a center-surround stricture of Dm8 receptive fields (Li and Saha, 2021). This complexity of local processing at an early synaptic stage of color processing is fascinating. It also raises the question of how this visual modality is further processed in higher brain structures.

1.2.2 Polarized Skylight

In addition to color, many migratory and navigating insect species can detect an additional visual modality, i.e., the linear polarization of skylight. Direct sunlight is unpolarized (Wehner, 2001). The celestial pattern of polarized skylight is formed through the scattering of sunlight in the atmosphere. Unscattered sunlight, as an electromagnetic wave, oscillates randomly in different planes (Wehner, 1982). When it hits molecules in the atmosphere, it is reflected in such a fashion that the wave oscillates predominantly within one plane. The Degree of polarization (DoP)) defines the strength of the polarization, whereas the Angle of polarization (AoP) describes the orientation of the plane in which the light oscillates (see Figure 1.2). The latter depends on the position of the celestial body (the sun) in the sky in combination with the curvature of the atmosphere. To all insects, the sun presents a body that can be used for navigation. As the sun's position changes over the day, the AoPs also change over the time of day and serve as an alternative cue when the sun is obstructed from view. Previous studies have shown that many insects,

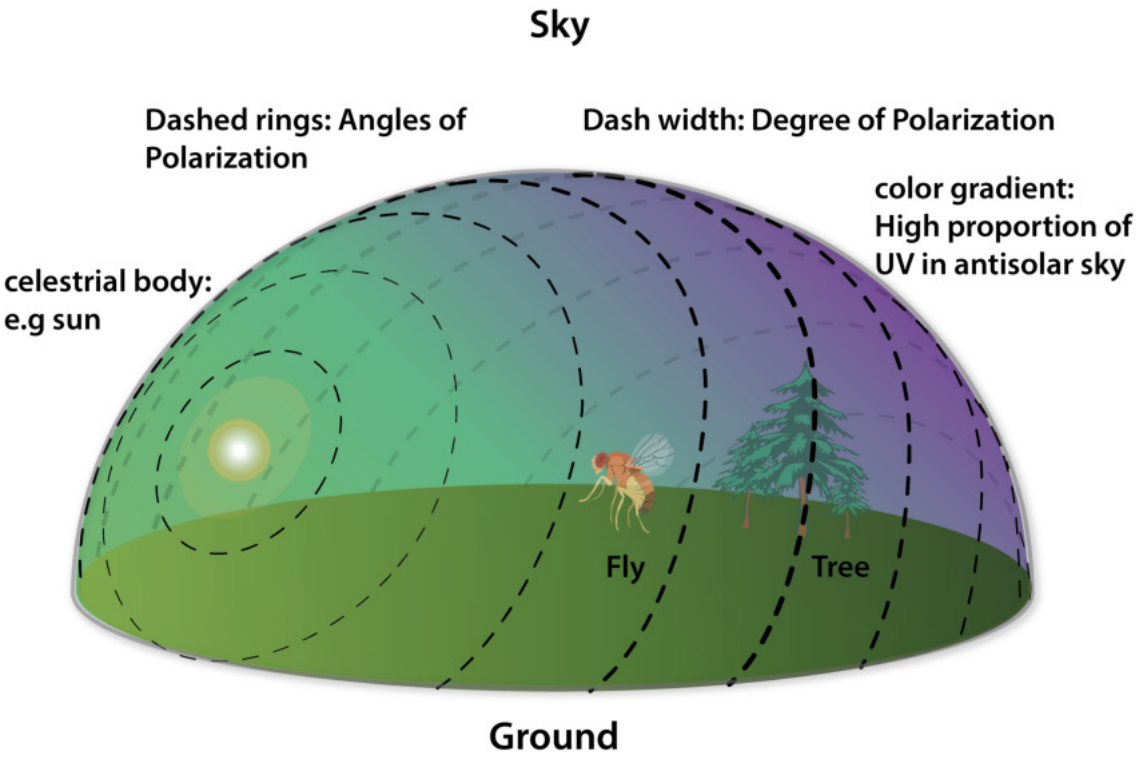

Figure 1.2: **Schematic depiction of environmental visual cues**
Reduced schematic representation of the most salient visual stimuli of a fly (center) with a celestial body (sun), color gradients (scattering of green versus UV wavelengths) and skylight polarization (defined by degree of polarization and angle of polarization). Those cues and landmarks (tree) can be used for navigation. After Kind et al., 2021.

such as locusts, honey bees, desert ants, and *Drosophila melanogaster*, can and use skylight polarization to navigate different environments (Heinze, 2017, and more).

Drosophila dorsal polarization vision

The DRA ommatidia located at the dorsal edge of the eye have the morphological characteristics necessary and sufficient to detect linearly polarized skylights. This modality has been the focus of several anatomical, physiological, and behavioral studies (Hardcastle et al., 2021; Kind et al., 2021; Mathejczyk and Wernet, 2019; Sancer et al., 2019; Weir and Dickinson, 2012; Weir et al., 2016). Different laboratory studies demonstrated that both walking and flying *Drosophila* use polarized UV light to align their body axis with specific AoPs over time (Mathejczyk and Wernet, 2019; Weir and Dickinson, 2012;

Wolf et al., 1980). In the wild, catch-and-release experiments in the desert revealed that *Drosophila* could keep a stable heading while flying over long periods of time. However, few non-visual landmarks existed (Coyne et al., 1987). These observations placed the processing of polarized skylights in the fly brain as a pressing question. This includes unraveling the modality-specific circuits downstream of DRA ommatidia, elucidating the stepwise processing of skylight polarization, and integrating with other sensory cues. Light microscopic studies, as well as electron-microscopy reconstruction, have provided insight into the anatomy and connectivity of DRA-specific circuitry (Hulse et al., 2021; Kind et al., 2021; Sancer et al., 2019; Sancer et al., 2020). In addition, few physiological studies showed how different AoPs are represented at different neuronal levels in the fly's brain, from photoreceptors to the central brain (Hardcastle et al., 2021; Weir et al., 2016). However, studies directly linking information about anatomy, physiology, and behavior for a single cell type in this modality-specific circuit remain missing.

Drosophila ventral polarization vision

Linearly polarized light is also formed via reflections from shiny, non-metallic objects and waterbodies (Horváth et al., 2008). The positioning of the reflecting surface plays an important role in influencing the AoP. When reflected off a water body, even unpolarized light becomes partially or even fully horizontally polarized (see Figure 1.3). Consequently, in nature, the angle and degree of polarization could serve as powerful indicators for identifying water bodies (Heinloth et al., 2018; Horváth et al., 2008; Mathejczyk and Wernet, 2017). As detecting water is crucial for all animals on Earth, using polarized light seems like a potentially attractive visual feature. Especially since relying solely on intensity and chromatic cues for detecting water bodies can be challenging due to the glare of water surfaces. Hence, for animals with relatively small visual systems, adding the detection and processing of reflected polarized light acts as an attractive modality that signals the presence of water. Interestingly, several semi-aquatic insect species manifest anatomical modifications in their ventral retina that most likely assist them in identifying or avoiding water surfaces, either by detecting or filtering out polarized reflections, thereby allowing them to see deeper into the water (Heinloth et al., 2018). Furthermore, reflected polarized light is used by some insects to and evaluate glossy leaves' surface as a potential oviposition substrate or to detect prey via reflections off its shiny fur (Ilić et al., 2018). Although our understanding of the neuronal mechanisms involved in ventral

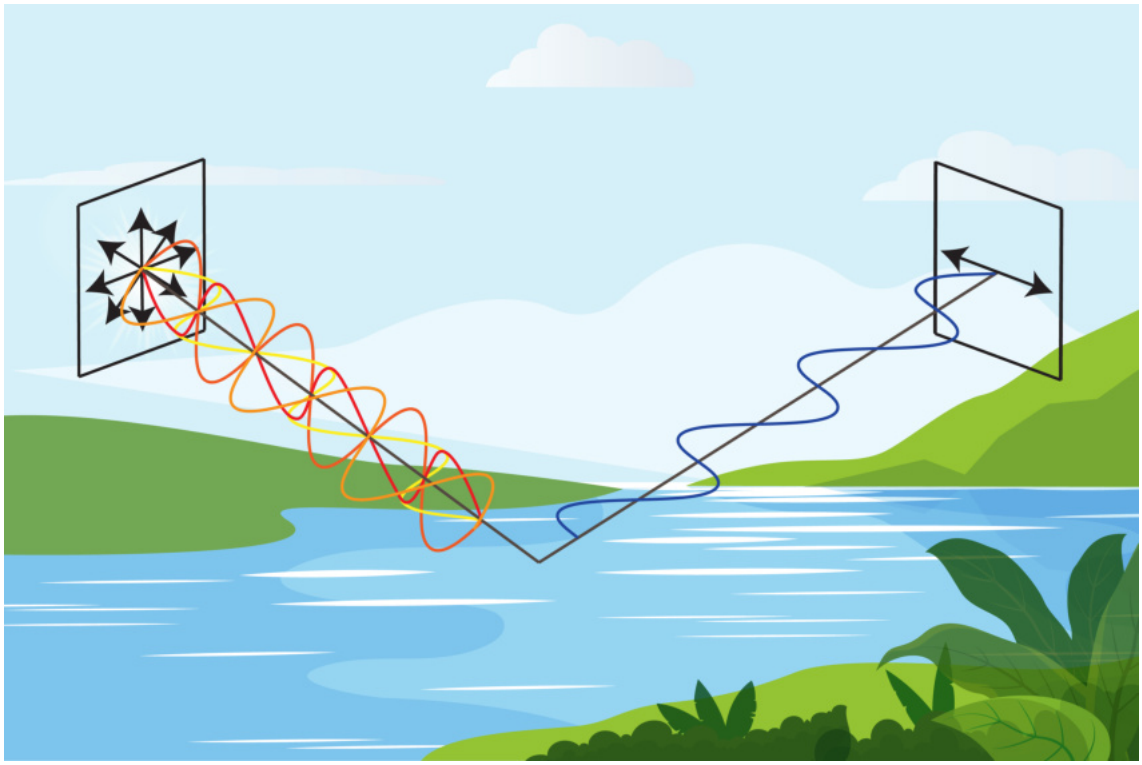

Figure 1.3: **Schematic of ventral polarized light**
Scattered, unpolarized sunlight (left panel, yellow to red sine waves) reflects from water surfaces and becomes horizontally polarized (right panel, blue sine wave). If the angle of the water/air interface is at 53° (Brewster's angle), the polarization reaches its maximum. After Wehner, 2001.

polarization vision remains limited, many mainly behavioral experiments have demonstrated that many insects are either repelled or attracted by highly polarizing surfaces (Ilić et al., 2018; Lerner et al., 2008; Lerner et al., 2011; Wernet et al., 2012). It was shown that *Drosophila* also detects polarized light when presented ventrally (Wernet et al., 2012). A combination of outer and inner photoreceptors in the ventral half of the eye appears to be necessary for detecting green or polarized UV light – potentially forming a Ventral Polarization Area (VPA). However, the exact extent of such a VPA within the *Drosophila* retinal mosaic eye remains unknown. Even more so, ventral polarization vision circuitry, integration, and processing remain highly mysterious.

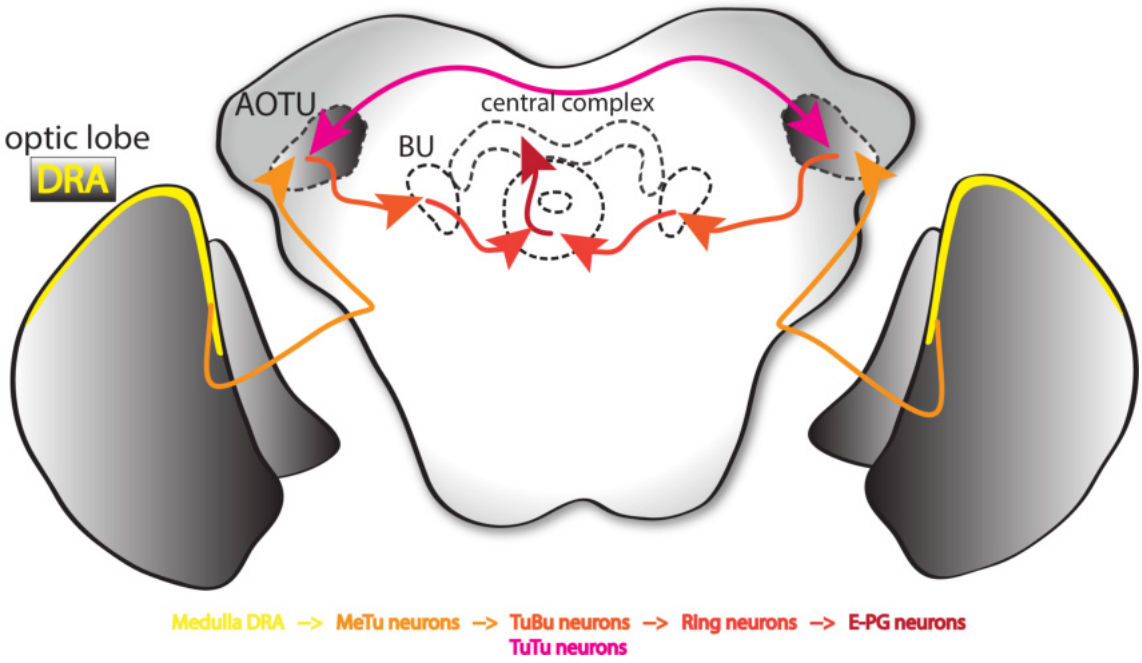

Figure 1.4: **Schematic of Anterior Visual Pathway in *Drosophila melanogaster*** The visual pathway from medulla to the central complex (AVP or sky-compass pathway) is depicted from both brain hemisphere of a *Drosophila* brain. The pathway is integrated by **DRA** (yellow), **MeTu** (orange), **TuBu** (dark orange), **Ring**- (light red), **E-PG** (burgundy) and **TuTu** (pink) neurons. Further abbreviations: **DRA** Dorsal Rim Area, **AOTU** Anterior Optic Tubercle, **BU** Bulb glomerulus. After Hardcastle et al., 2021.

1.3 The anterior visual pathway

Whereas the neuronal circuit for ventral polarization vision remains unknown, much more is known about the synaptic pathways processing linearly polarized skylight, forming the Anterior Visual Pathway (AVP). Across insect species, this pathway reaches from the photoreceptors over several glomerular relays to the central complex in the fly's brain (see Figure 1.4) (Hardcastle et al., 2021; Lovick et al., 2017; Omoto et al., 2017; Tai et al., 2021). The AVP, often called the 'compass pathway' due to its role in navigation, is the most prominent pathway connecting the eye to the central brain (Homberg, 2004). The AVP has been proposed to convey information related to several skylight cues, such as the sun's location and the pattern of the polarized skylight, all of which are required for navigation. The AVP involves two groups of interconnected glomerular systems: from the Optic lobe, the first and mostly local computation after the photoreceptor, visual projecting neurons called MeTu cells (medulla to tubercle neurons), directly connect to the Anterior Optic Tubercle (AOTU) (Hardcastle et al., 2021; Lovick et al., 2017; Otsuna

et al., 2014; Timaeus et al., 2020). From there, information is conveyed via TuBu cells (tubercle to bulb neurons) to the glomerular bulb neuropil, where ring neuron dendrites from the central complex receive information. In addition to this PR → MeTu → TuBu → Ring Neuron pathway, a binocular integration via TuTu cells (tubercle to tubercle neurons) has been described in locusts and flies (el Jundi et al., 2014; Hardcastle et al., 2021; Pfeiffer and Homberg, 2007). How binocular integration of DRA inputs shapes the perception of polarized skylight remains incompletely understood.

Several behavioral and physiological studies on different insect species have demonstrated that the AOTU is a prominent relay station for processing both skylight polarization and color (el Jundi et al., 2011; Mappes and Homberg, 2004; Mota et al., 2013). Like in other insects, cellular components of the *Drosophila* AVP respond specifically to skylight signals such as bright objects (Mota et al., 2013; Omoto et al., 2017; Sun et al., 2017). However, very little is known about the processing of polarized skylight in *Drosophila* since information about circuitry physiology within the AVP was long missing. Prior to this work, only one study described the physiological responses of DRA photoreceptors in *Drosophila* (Weir et al., 2016). Recently, a more extended physiological study revealed polarization-sensitive responses of several cell types along the AVP in *Drosophila* (Hardcastle et al., 2021). However, a detailed description of MeTu neurons, their connectivity, physiology, and their direct relevance for navigational behavior remained missing.

1.4 Physiological characterization of polarization sensitivity

Electrophysiology is a prominent and very successful method for recording the neuronal activity of circuit elements. For this technique, electrodes must be used to detect and measure the electrical activity within living cells, particularly neurons (Buzsáki et al., 2012; Mccormick et al., 1985). Their electrical activity can be recorded in order to understand any given neuron's contribution to a specific computational transformation. Electrophysiological recordings can generally be grouped into extracellular or intracellular methods (Golowasch et al., 1999). For extracellular recordings, electrodes are placed near or around neurons to detect and record electrical signals generated by neuron populations simultaneously. This technique provides information about the firing patterns of

those neurons, their local field potentials, and action potentials. Intracellular recordings, conversely, involve inserting an electrode directly into a neuron to measure its electrical activity and allow more detailed and precise measurements of single neurons (Buzsáki, 2004).

Although intracellular electrophysiological recordings from larger insect species like locusts and butterflies (Heinze, 2017; Homberg, 2015) provided detailed insight into the physiology of neuronal circuits like the AVP, they cannot easily be used in *Drosophila melanogaster*. Based on the small size of *Drosophila*, its brain, and its neurons, electrodes are too big to penetrate single neurons or produce specific extracellular population signals. Activity imaging became a standard technique in the *Drosophila* field to overcome the size issue and gain information on the physiology of specific, genetically targeted individuals. Activity imaging can visually monitor and quantify the neuronal activity within the living fruit fly brain. Using activity-dependent fluorescent proteins allows researchers to investigate patterns of neuronal activity and their dynamics, thereby aiding an understanding of how the fly's brain processes information and generates behavior (Badura et al., 2014; Grienberger and Konnerth, 2012).

A commonly used approach for activity imaging in *Drosophila* is calcium imaging, using genetically encoded calcium indicators (GECIs) (Simpson and Looger, 2018). It involves expressing fluorescent calcium indicators, such as GCaMP, in the fly's brain or, more commonly, in a specific subset of cells. Upon neuronal activation, the calcium concentration in the cytosol of the targeted cell increases (or decreases). The cytosolic Calcium level is used as a second messenger system, and its levels can, therefore, be visualized via the GCaMP tool. Upon calcium binding, the GCaMP molecule changes its confirmation, resulting in a fluorophore that can be excited (Badura et al., 2014; Grienberger and Konnerth, 2012). When calcium levels increase, the emission of these activated indicators serves as a proxy for neuronal activity. Imaging techniques like wide-field or two-photon microscopy are used to visualize and record the fluorescence signals from the brains region of interest, often reaching far into the tissue (Bilz et al., 2020; Dana et al., 2019; Reiff et al., 2010). The imaged data from activity indicators can be analyzed to study various aspects of neuronal activity, even simultaneously at subcellular resolution and in many cells. Based on this method, the spatial distribution of activity across different brain regions can be used to identify specific neurons or neuronal populations that are found to be active during specific sensory stimulation. It investigates the temporal dynamics of

activity patterns.

In combination with genetic tools, advanced imaging techniques like calcium imaging (or, more recently, voltage imaging) have greatly expanded the possibilities for recording neuronal activity in *Drosophila*. These methods provide a powerful approach for studying neuronal computation in a genetically tractable and relatively simple model organism, thereby contributing to our understanding of fundamental principles of neural circuit function.

2 Aim

To investigate identified neural circuit elements and their role in information processing, I performed some literature research, which was then used for planning and executing practical experiments.

First, I focused on conducting a literature review summarizing our understanding of the inter-species differences known for their ventral polarization vision. Gaining more insight into the differences and similarities of detecting polarized light was crucial for understanding the basic principles underlying the processing of this visual feature.

Secondly, I used molecular genetic techniques to morphologically describe distinct specialized cell types within the Dorsal Rim Area (DRA) of *Drosophila melanogaster* optic lobes to understand how the anterior visual pathway processes skylight polarization on a cellular and synaptic level.

Thirdly, I implemented an *in-vivo* calcium imaging experimental platform to study the physiological responses of specific cell types along the AVP. The goal was to gain physiological insights into how skylight polarization is processed in the adult *Drosophila* brain.

Together, these three objectives served to understand better the neuronal circuits and their physiological responses computing in skylight polarization vision and informing visual navigation in insects, using *Drosophila melanogaster* as a model.

Part II

Manuscripts

3 Manuscripts I

Insect Responses to Linearly Polarized Reflections: Orphan Behaviors Without Neural Circuits

Tanja Heinloth, Juliane Uhlhorn, and Mathias F. Wernet

Frontiers in Cellular Neuroscience Volume 12, 20 March 2018 Sec. Cellular Neurophysiology

Contribution:
I researched literature to design and generate Figure 4 and wrote the Manuscript with Tanja Heinloth and Prof. Dr. Mathias Wernet.

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REVIEW
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Insect Responses to Linearly Polarized Reflections: Orphan Behaviors Without Neural Circuits

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The e-vector orientation of linearly polarized light represents an important visual stimulus for many insects. Especially the detection of polarized skylight by many navigating insect species is known to improve their orientation skills. While great progress has been made towards describing both the anatomy and function of neural circuit elements mediating behaviors related to navigation, relatively little is known about how insects perceive non-celestial polarized light stimuli, like reflections off water, leaves, or shiny body surfaces. Work on different species suggests that these behaviors are not mediated by the "Dorsal Rim Area" (DRA), a specialized region in the dorsal periphery of the adult compound eye, where ommatidia contain highly polarization-sensitive photoreceptor cells whose receptive fields point towards the sky. So far, only few cases of polarization-sensitive photoreceptors have been described in the ventral periphery of the insect retina. Furthermore, both the structure and function of those neural circuits connecting to these photoreceptor inputs remain largely uncharacterized. Here we review the known data on non-celestial polarization vision from different insect species (dragonflies, butterflies, beetles, bugs and flies) and present three well-characterized examples for functionally specialized non-DRA detectors from different insects that seem perfectly suited for mediating such behaviors. Finally, using recent advances from circuit dissection in *Drosophila melanogaster*, we discuss what types of potential candidate neurons could be involved in forming the underlying neural circuitry mediating non-celestial polarization vision.

Keywords: insect vision, polarized light, behavior, orientation, water detection, neuroethology, visual ecology, neural circuits

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INTRODUCTION

Across insect species, a great diversity of photosensitive, image-forming structures (eyes) has been described which allow for visually guided navigation during daytime under bright illumination, as well as around dusk or dawn, or even at very low light intensities during the moonlit night (Land and Fernald, 1992). The mechanisms underlying both the sensation and subsequent integration of different aspects of the visual world, like intensity, contrast, motion, or color are crucial for shaping the specific behavioral repertoires of different animal species. One well-studied example is the perception of linearly polarized light, a sensory ability that is common to some vertebrates (birds, fishes), as well as marine invertebrates (Cephalopods, Crustaceans), and many insects (Nilsson and Warrant, 1999; Cronin et al., 2003; Mathejczyk and Wernet, 2017). Initially, sunlight (or moonlight) is unpolarized

and manifests a randomly distributed e-vector, but atmospheric scattering produces a celestial e-vector pattern that changes during the course of the day. Hence, polarized skylight represents wide-field celestial cue for navigation (for instance when the celestial body is obstructed from view), used by many insects: "Truly navigating" central place forager species like honeybees or desert ants certainly manifest the most impressive navigational skills (from their hive/nest to a food source and back), whereas other insect species appear to use celestial polarization for more basic orientation tasks (for instance crickets or dung beetles; Labhart and Wehner, 2006; Homberg, 2015). Reflection of sunlight off shiny surfaces (water, leaves, or body surfaces) represents the second important source of polarized light found in nature (Wehner, 2001). Such polarized reflections (always horizontally polarized, in the case of water bodies) can be used to either seek out or avoid localized water sources (ponds, lakes), or to follow the course of a continuous stream (creeks, rivers). Studies on many different insect species have shown that polarized reflections also provide important information for evaluating the quality of certain environments, for instance as suitable food source, or oviposition sites (Wehner, 2001). Similarly, polarized reflections off shiny body surfaces can be used to identify both conspecifics (for instance during courtship), as well as prey (in the case of certain blood-sucking insects). Conversely, the glare resulting from polarized reflections can be a nuisance to insects living on the water surface, resulting in mechanisms to specifically filter it out. In the "Behavioral Responses of Different Insect Species to Reflected Linearly Polarized Light" section we present an overview over the growing number of insect species that manifest specific behavioral responses to linearly polarized reflections.

The necessary substrate for polarization sensitivity in the insect retina is formed by a specialized ultrastructure of the photoreceptor light-gathering membranes, the so-called rhabdomeres (Wehner, 1976). Usually eight or nine photoreceptor neurons (in some species even more) are organized within stereotypical unit eyes, or ommatidia, varying numbers of which together form the insect retina (Wernet et al., 2015). Specialized ommatidia in the "Dorsal Rim Area" (DRA) of many insect eyes contain highly polarization-sensitive photoreceptors that have been identified as the substrate for detecting linearly polarized skylight (for review see Labhart and Meyer, 1999). In the DRA, two groups of photoreceptor cells from the same ommatidium have rhabdomeres with straightly aligned microvillar membranes. Such a design is crucial for achieving high polarization sensitivity, since the rhodopsin molecules appear to be anchored in a fixed orientation along the axis of these membranes, leading to preferential absorption of light of a specific e-vector orientation (Roberts et al., 2011). Outside the DRA region, polarization sensitivity is often suppressed through rhabdomere twisting (i.e., the rhabdomere orientation changes as a function of the depth through the retina), thereby avoiding mixture of color and polarization information within the same photoreceptor cell (Wehner and Bernard, 1993). In the DRA, the two groups of untwisted, polarization-sensitive photoreceptors manifest preferred e-vector orientations that are orthogonal to each other (Labhart and Meyer, 1999), a design that is optimal for polarization-opponent coding (Labhart, 1988; Heras and Laughlin, 2017). Furthermore, they always express the same Rhodopsin molecules thereby again avoiding confusion between color and polarized light information. Interestingly, the wavelength sensitivity of polarization-sensitive DRA photoreceptors varies between species, most likely reflecting their different life styles (Barta and Horváth, 2004; Hegedüs et al., 2006a): UV-sensitive receptors are found in bees, ants, flies, butterflies and some beetles (Vonhelversen and Edrich, 1974; Labhart, 1986; Fortini and Rubin, 1990); blue-sensitive rhodopsins in the DRA of crickets and locusts (Henze et al., 2012; Schmeling et al., 2014); green-sensitive DRA photoreceptors are used by cockchafers and the European corn borer moth *Ostrinia nubialis* (Labhart et al., 1992; Belusic et al., 2017). At the ventral rim of the insect retina there exists no specialized type of polarization-sensitive ommatidia analogous to the DRA, which would be common to all insects. Despite the growing list of reports describing behavioral responses to reflected polarized light, the retinal substrate mediating these responses remains elusive, for the large part. In fact, only three well-documented examples exist demonstrating the existence of photoreceptor cells with specialized rhabdomere ultrastructure at the ventral periphery of insect eyes (from water striders, back swimmers and long-legged flies). Interestingly, the organization and extent of the retinal area harboring specialized photoreceptors for ventral polarization vision differs greatly between these three examples: either (i) the entire ventral retina manifests specialized photoreceptor ultrastracture (with a sharp boundary to the rest of the eye); or (ii) discrete zones within the ventral half of the retina show different specializations; or (iii) alternating stripes of ommatidia, each containing pairs of photoreceptors manifesting two characteristic rhabdomere orientations vis-à-vis each other (either orthogonal or parallel) that are characteristic for each row. In the "Ommatidial Subtypes in the Ventral Insect Retina with Increased Polarization Sensitivity" section, we will compare these three examples and discuss the different use by the animals, as well as their relevance for a life in their respective habitats.

The signals from polarization-sensitive photoreceptors are collected and further processed by the underlying circuits within the optic lobes and the central brain. Both anatomical and electrophysiological studies in several insect species (most prominently: the desert locust) have revealed numerous cell types that show very specific responses to linearly polarized light. In the case of polarized skylight detected by the DRA, a neuronal "compass" pathway was reconstructed, leading from the DRA ommatidia to the central complex, via an optic glomerulus called the anterior optic tubercle (Homberg et al., 2011; Homberg, 2015). Over the past decades, work in this field has provided exciting insight into how e-vectors are detected and processed into polarization-opponent signals that become modulated with respect to the time of day (in a process referred to time compensation), ultimately lading to a map-like representation of different e-vectors within columnar structures of the central complex (Sakura et al., 2006; Heinze and Homberg, 2007; Kinoshita et al., 2007; Heinze and Reppert, 2011; Homberg et al., 2011; el Jundi et al., 2015). Considering this high

degree of detail, it is quite shocking that virtually nothing is known about the neural circuits processing polarized reflections detected by specialized ommatidia in the ventral periphery of the insect retina. Systematic approaches towards characterizing most, if not all neuronal subtypes in the fruit fly brain provide one attractive way towards characterizing these elusive circuit elements (Pfeiffer et al., 2008). Interestingly, two independent studies have demonstrated that fruit flies can detect linearly polarized light when presented to the ventral half of the retina, by analyzing spontaneous alignment of the body axis with respect to the incident e-vector (polarotaxis; Wolf et al., 1980; Wernet et al., 2012). Surprisingly, these responses appear to be mediated by only one of the two ommatidial subtypes that are randomly distributed throughout the fly retina. However, an incomplete ultrastructure analysis of only a small sample from this ommatidial subtype called "pale" revealed no subtype-specific rhabdomere untwisting indicative of high polarization sensitivity (Wernet et al., 2012). Nevertheless, a different study revealed a specific role for the other stochastic ommatidial subtype (called "yellow") in mediating color discrimination (Schnaitmann et al., 2013). It remains an open question whether "pale" and "yellow" ommatidia (found across fly species) could indeed serve different functions like polarization vs. color vision. Nevertheless, the fly retinal mosaic of randomly distributed ommatidial subtypes provides an attractive model for investigating differences in the cellular composition of their downstream circuits. In the "Neural Circuits Connecting to Specific Ommatidial Subtypes—Lessons from *Drosophila*" section, we will summarize the growing data on the neuronal subtypes that are specific to "pale" or "yellow" ommatidia in *Drosophila*, as well as the developmental mechanisms leading to subtype-specific connectivity. Even if serving a different function, the logic behind forming "pale" vs. "yellow" specific differences in circuitry could serve as a model for how distinct polarization vision circuit elements are specified at the ventral periphery of the insect eye.

BEHAVIORAL RESPONSES OF DIFFERENT INSECT SPECIES TO REFLECTED LINEARLY POLARIZED LIGHT

When reflected off a shiny, flat and non-metallic surface like water, sunlight becomes horizontally polarized, with the maximum degree of polarization occurring at an angle of incidence of 53° (for an air/water interface), also known as "Brewster's angle". Different flying insects appear to use polarized reflections to identify water bodies (Wehner, 2001; summarized in **Figure 1**). Depending on the species studied, such polarized reflections can be attractive, as well as repulsive, since swarms of flying desert locusts were shown to avoid flying over polarized surfaces, probably to avoid crash-landing over sea (Shashar et al., 2005). Probably the best studied example of any water-seeking insect attracted to polarized surfaces is the hemipteran back swimmer *Notonecta glauca*. This bug visually identifies water surfaces when conducting dispersal flights between water bodies, resulting in a characteristic diving reaction during which the animal raises its body axis to an angle of 53 degrees shortly before diving into the water (Schwind, 1984; Wehner, 1987). Horizontal platforms emitting linearly polarized UV light are sufficient to induce this diving reaction (Schwind, 1983a). Interestingly, Notonecta spends much of its lifetime hanging under the water surface, from where it observes the airy world above. Hence its visual system needs to accommodate both sensitivity to horizontally polarized light, as well as accurate vision through the water/air interface, which is reflected by the separation of its ventral retina into discrete zones (as discussed in the "Ommatidial Subtypes in the Ventral Insect Retina with Increased Polarization Sensitivity" section). It is known that females from many different semi-aquatic insect species erroneously lay their eggs on shiny surfaces that they seem to have mistook for water. Examples are parked cars, black gravestones, glass buildings, and sometimes even oil pits (Horváth et al., 1998, 2007; Kriska et al., 1998, 2008, 2007). One fly species, *Halaeomyia petrolei* even became adapted to a life near (or in the case of its larvae/pupae: inside) naturally occurring petroleum pools, feeding on arthropod prey that became trapped there (Thorpe, 1930). Female mayflies were shown to use horizontally polarized reflections off water to direct their so-called "compensatory upstream flights" before oviposition (Farkas et al., 2016) and this dispersion behavior is disrupted by (unpolarized) light pollution, for instance illuminated bridges (Szaz et al., 2015). In another example, female dragonflies attempted to lay eggs on an artificial, horizontally polarized surface, assuming it to be water (Wildermuth, 1998). Similarly, male dragonflies approach polarized surfaces to establish an aquatic territory, hence in this case both sexes show strong responses to reflected polarized light. At this point it remains unclear whether insects can distinguish different degrees of polarization (a stimulus that is 100% polarized virtually never occurs in nature). For instance, it was proposed that dragonflies could use such information to distinguish between habitats, for instance dark and light ponds since the degree of polarization (i.e., the ratio between reflected, polarized light and scattered, unpolarized light) is proportional to the absorbance of water in the pond and to the amount of organic nutrients suspended in water (Bernáth et al., 2002). Interestingly, visual cues like polarized reflections seem to play a rather minor role for female mosquitoes when identifying oviposition sites after a blood meal. Instead, chemical cues indicating the presence of conspecifics, eggs, or larvae appear to strongly dominate (Bernáth et al., 2008, 2012). However, one recent study confirmed polarization sensitivity of the ventral half of the retina for the Zika virus transmitting species *Aedes aegypti*. In these experiments, an optomotor reaction to rotating stripes of alternating orthogonal directions of polarization was demonstrated (Bernáth and Meyer-Rochow, 2016). Hence, it appears that mosquito polarization vision may usually be masked by the chemical senses and it remains possible that plays a role only under very specific behavioral conditions (Bernáth et al., 2012). Interestingly, the related non-biting midges (Chironomidae) which have a comparable lifestyle appear to rely more heavily on visual cues for the detection of water surfaces (Lerner et al., 2008; Horváth et al., 2011). It must be noted that polarized reflections off water can also be problematic for

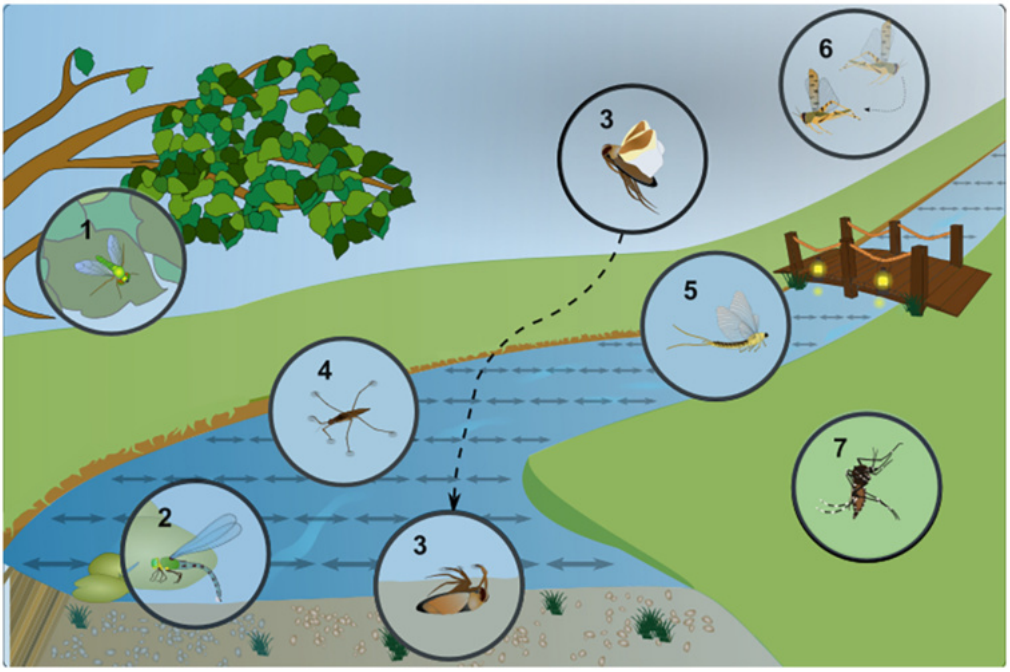

FIGURE 1 | Examples of insect responses to linearly polarized light reflected off water. Specific adaptions of different semi-aquatic insect species to a life in close proximity to water bodies and their characteristic behavior in response to linearly polarized, shiny surfaces (symbolized by double-headed arrows). (1) Different species of long-legged dipteran flies (Dolichopodidae) can be found close to the water, hunting for prey on the water surface, which produces strong glare due to polarized reflections. (2) Dragonflies are known to oviposit (lay their eggs) onto the water surface, or in some cases on any shiny surface they mistake for water. (3) The "back swimmer" *Notonecta glauca*, a hemipteran bug shows a characteristic "plunge reaction" into water (or linearly polarized surfaces). It then spends a considerable part of its life hanging under the water surface hunting for prey. (4) Another hemipteran, the water strider *Gerris lacustris* is constantly faced with the surface glare of polarized reflections, making it more difficult to identify features under water. (5) During their "dispersal flight" after copulation, female Mayflies (Ephemeroptera) are known to follow a river upstream, to find an oviposition site. Linearly polarized reflections have been identified as a major guiding cue during this process and unpolarized light pollution (for instance at illuminated bridges) forms a major obstacle. (6) Flying desert locusts (*Schistocerca gregaria*) are repelled by linearly polarized reflections, most likely to avoid crash-landing in the sea. (7) Different mosquito species, as well as certain midges (all Nematocera), seem attracted to water surfaces via their linearly polarized reflections. However, this effect seems to be rather minor in some cases, since olfactory stimuli dominate.

many (semi-)aquatic insects: for instance, the resulting glare interferes with observing underwater objects from above the water surface (Wehner, 2001). This can be particularly relevant for species living directly on the water surface, like water striders (*Gerris lacustris*), or certain flies hunting for prey living on the water surface (like Doliochopodidae). Specific retinal adaptations found in these species could therefore aim at filtering out this stimulus (as we will discuss in the "Ommatidial Subtypes in the Ventral Insect Retina with Increased Polarization Sensitivity" section).

Of course linearly polarized reflections can be produced by any shiny, non-metallic object and many insects have been shown to detect such stimuli (summarized in **Figure 2**). For instance, shiny leaves are an attractive oviposition cue for certain butterfly species (Kelber, 1999a,b). Interestingly, female butterflies most likely perceive "false colors", since their visual system is mixing e-vector orientation and information about wavelength. This way, female butterflies can distinguish matte from shiny leaves by perceiving them as different colors (Kelber et al., 2001). Such a system is suitable to evaluate different features, like quality of the landing site (leaf orientation), food quality (for caterpillar offspring), or protection for the eggs. Similar mixing of linear polarization and the intensity of light was also shown in butterflies (Kinoshita et al., 2011), in this case resulting in the perception of differently polarized surfaces as differing in brightness. The wings of many butterflies also produce linearly polarized reflections that can serve as mating signals for conspecifics (Sweeney et al., 2003; Yoshioka and Kinoshita, 2007; Stavenga et al., 2012). Heliconius butterflies most likely use these reflections to increase their visibility in the midst of highly complex visual environment (Douglas et al., 2007). Hence, polarized reflections are used in this case to increase the perceived visual contrast. Some true flies (Diptera) not only show strong attraction to polarized surfaces, but also linearly polarized objects, which was demonstrated for blood-sucking horse flies (Tabanidae; Horváth et al., 2008; Egri et al., 2013). Some of these behaviors are most likely involved in prey detection since polarimetric imaging of horses and cattle reveals strong linearly polarized reflections off their fur (Horváth et al., 2010). Brown and black fur produces the strongest polarized reflections, while the scattering effect of white fur or certain fur patterns like stripes (zebras) and spots (cows) appear to be a suitable protection against horse fly attacks (Blahó et al., 2012a; Egri et al., 2012). An even more sophisticated

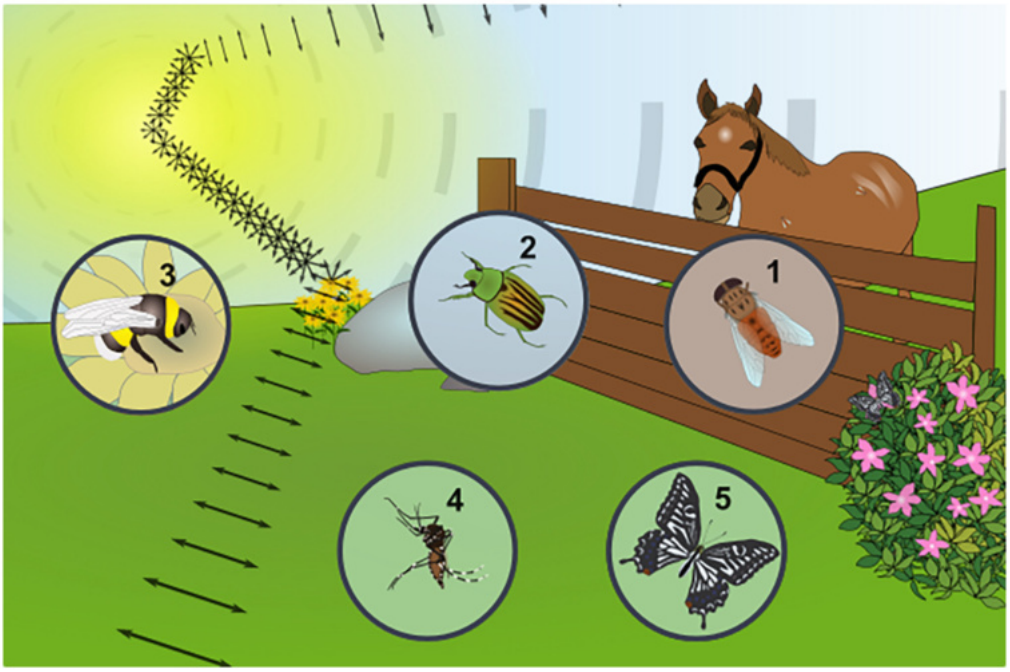

FIGURE 2 | Examples of insect responses to linearly polarized reflections from other substrates. Any shiny, non-metallic surface can produce linearly polarized reflections from unpolarized sunlight, as shown for the example of a flower, where both flowers and leaves can produce this stimulus (symbolized by the double headed arrows) and carry different kinds of information for insects. (1) Blood-sucking horse flies (*Tabanidae*) are strongly attracted by objects reflecting linearly polarized light (a fact exploited in horse-fly traps). The facts that horses and cattle reflect linearly polarized light is in agreement with these insects using this stimulus to detect their prey. (2) The exocuticle of several species of scarab beetles (*Coleoptera*) was shown to reflect circularly polarized light, yet it remains unclear whether this stimulus can be perceived by the animals (i.e., rightward- vs. leftward circularly polarized light), since contradicting behavioral studies exist. (3) Bumblebees (*Hymenoptera*) can be trained to learn different patterns of polarized light, reminiscent of the patterns that could be produced by blooming flowers, suggesting this stimulus may influence their pollenating activity. (4) It is unlikely that female mosquitoes (Nematocera) are attracted by linearly polarized reflections off the body surface of their prey and olfactory stimuli (sweat, CO_2) clearly dominate. Nevertheless, optomotor responses to alternating stripes of orthogonally oriented polarization demonstrate the existence of polarization sensitivity in the ventral half of the retina. (5) Linearly polarized reflections represent an important stimulus for different butterfly species (*Lepidoptera*): for instance, reflections off the body surface of conspecifics are an important cue for identifying potential mates in an otherwise cluttered, optically rich environment. Furthermore, reflections off leaves bear important information about how well-suited they are as an oviposition site.

example for learning to distinguish between different patterns of linearly polarized light comes from bumblebees: it appears that pollinators may also use polarized reflections to identify or evaluate floral targets (Foster et al., 2014). Finally, in a less well understood example, the body cuticle of some scarab beetles were shown to reflect circularly polarized light (Hegedüs et al., 2006b; Jewell et al., 2007; Sharma et al., 2009). This stimulus (an e-vector rotating as the beam of light propagates) would appear unpolarized to most insects, since all e-vector orientations are equally represented as they hit a photoreceptor (Labhart, 1996; Henze and Labhart, 2007). Nevertheless, one study reported specific phototactic responses to circularly polarized light were reported for the scarab beetle *Chrysina gloriosa*, whose body surface produces strong circularly polarized reflections (Brady and Cummings, 2010). Interestingly, the closely related species *Chrysina woodii*, whose cuticle manifests only weak circularly polarized reflections, exhibited no phototactic discrimination between linearly and circularly polarized stimuli. However, it must be noted that another study investigating four different scarab beetle species with well-documented circularly polarized reflections off their exocuticle found no evidence for specific behavioral responses to circularly polarized light (Blahó et al., 2012b). Taken together, a great variety of behavioral responses to reflected polarized light has been described across insect species, affecting very different aspects of their respective ecology and life cycle.

OMMATIDIAL SUBTYPES IN THE VENTRAL INSECT RETINA WITH INCREASED POLARIZATION SENSITIVITY

Insect retinas are composed of repetitive unit eyes (ommatidia) which usually contain eight or nine photoreceptor neurons (Wernet et al., 2015). In many cases, specialized ommatidia containing photoreceptors with increased polarization sensitivity can be found in the dorsal periphery, a region called the DRA (Labhart and Meyer, 1999). Only there, pairs of untwisted photoreceptor rhabdomeres within each ommatidium form orthogonal analyzers and gradual differences between neighboring DRA ommatidia are in turn forming a fan-shaped array of analyzers across the DRA. This structure acts as the

retinal substrate for detecting the e-vector orientation of the celestial polarization pattern, which the animal can use for improving its navigational skills (Blum and Labhart, 2000; Homberg and Paech, 2002; Wernet et al., 2012; Weir et al., 2016). Although both structure and function of the insect DRA, as well as its downstream circuitry have been described in great detail, much less is known about polarization-sensitive photoreceptors in the ventral periphery of the retina. Most importantly, there exists no specialized type of ommatidia at the ventral rim of the retina with polarization-sensitive photoreceptors for mediating responses to linearly polarized reflections that would be common across insects. Despite the numerous examples for behavioral responses to such stimuli, it is therefore surprising that only three retinal specializations have so far been characterized in the ventral periphery of different insect eyes (see below). For each case, a different design principle is responsible for adapting the ventral retina to the ecological needs of the animal: either specialized ommatidia can be organized as a homogeneous ventral region (*Gerris lacustris*), or subdivided into separate zones (*Notonecta glauca*), or even into alternating rows of ommatidial subtypes (Dolichopodidae).

In the retina of the hemipteran water strider *Gerris lacustris*, ommatidia in the ventral zone of the adult eye show characteristic morphological specializations: only there, one of the two central cells is lost and the proximal cell extends through the entire retina (Schneider and Langer, 1969; **Figures 3A–E**). Curiously, this single cell forms two untwisted rhabdomeres, which are both oriented along the dorsoventral axis of the animal. This unidirectional design (as opposed to orthogonal analyzers) is ideal for filtering out polarized reflections, since the resulting glare might represent a challenge for any animal living on the water surface. Hence, such a ventral adaptation forms a "matched filter" which equips the animal with an improved ability to look deeper into the water (Wehner, 1987). Alternatively, it can serve to increase contrast when observing animals against the glare that results from polarized reflections (Schneider and Langer, 1969). Hence, in analogy to the insect DRA, the ventral ommatidia from *Gerris* are morphologically specialized, forming a region with a sharp boundary to the rest of the retina. Only in this ventral region, identified photoreceptor subsets manifest important morphological features with regard to polarization sensitivity.

The second, very well-characterized example for polarization-sensitive photoreceptors at the ventral rim of the insect retina is the hemipteran back swimmer *Notonecta glauca* (**Figures 3F–I**). In this case, different zones within the ventral periphery of the retina can be distinguished, covering different areas of the visual field as the animal is flying, or when it is hanging under the water surface. Within these zones, the rhabdomeres of the two central photoreceptors of each ommatidium are untwisted (and therefore polarization sensitive), yet their microvilli orientations differ between zones: the two most ventrally facing zones are formed by ommatidia containing photoreceptor pairs with orthogonally oriented microvilli, a structure perfectly adapted for detecting polarized reflections like water surfaces in a way that is insensitive to fluctuations in radiant intensity (Schwind, 1983b). Keeping in mind the optical axes of the photoreceptors in question, it appears therefore that *Notonecta* uses orthogonal analyzers to detect water surfaces when flying. This design is therefore similar to the fan-shaped array of orthogonal analyzers in the DRA. Orthogonally oriented rhabdomeric microvilli were also proposed for the ventralmost ommatidia of the non-biting midge *Chironomus transvaalensis*, yet to our knowledge no 3D reconstruction was performed to demonstrate an increased polarization sensitivity (Lerner et al., 2008). The third, most dorsally located zone of ventral *Notonecta* ommatidia right adjacent to the "main" retina, contains photoreceptor pairs with more or less parallel rhabdomeric microvilli—a design that may increase contrast during under water vision, while the animal is hanging under the water surface (a theory supported by the optical axes of these photoreceptors; Wehner, 1987, 2001). Overall, such an interrupted design in which the ventral periphery of the retina is subdivided into discrete zones represents an ideal adaptation to the sum of its very specialized aquatic lifestyles above and below the water surface, all of which are directly affected by horizontally polarized light.

A completely different retinal design was described for long-legged flies (Dolichopodidae), which live close to water bodies, and are known to hunt smaller insects on the water surface (**Figures 3J–N**). The retina of several Dolichopodidae species consists of alternating rows of ommatidia that can be identified based on their orange/red (Type A) vs. green/yellow (Tybe B) reflecting lenses (Stavenga et al., 2017). More importantly, the rhabdomeric ultrastructure of two central photoreceptors (called R7 and R8 in related *Drosophila*) differs between alternating rows of Type A and Type B ommatidia: the rhabdomeric microvilli of "Type A" central photoreceptors are both aligned along the dorsoventral axis, whereas an orthogonal orientation is found in Type B ommatidia (Trujillo-Cenóz and Bernard, 1972). It appears therefore, that "Type A" ommatidia would be perfectly suited for detecting objects against the water surface, by filtering out the horizontally polarized glare, whereas "Type B" ommatidia could be used to detect the water bodies themselves. Additionally, the different modes of polarization sensitivity could be used for perceiving "false colors", since the two inner photoreceptors might express different Rhodopsin molecules. It remains unknown how signals from intermixed, yet alternating rows of ommatidia with different functional properties could be processed by post-synaptic units. Nevertheless, the problem is similar to the integration of signals from stochastically distributed ommatidial subtypes in other dipteran species (like *Drosophila* or *Musca*), as we will discuss in the "Neural Circuits Connecting to Specific Ommatidial Subtypes—Lessons from *Drosophila*" section. Taken together, retinal specializations in the ventral retina that are most likely related to polarized reflections (based on rhabdomeric ultrastructure) can serve very different functions, depending on their arrangement, their optical axes and the lifestyle of the insect: attraction to water via detection of horizontal e-vectors, the specific screening of such surface-reflected light, or even underwater vision. In some cases several of these functions appear to be integrated within one and the same retina.

In addition to these three specific examples for polarization-sensitive photoreceptors being organized in specific regions

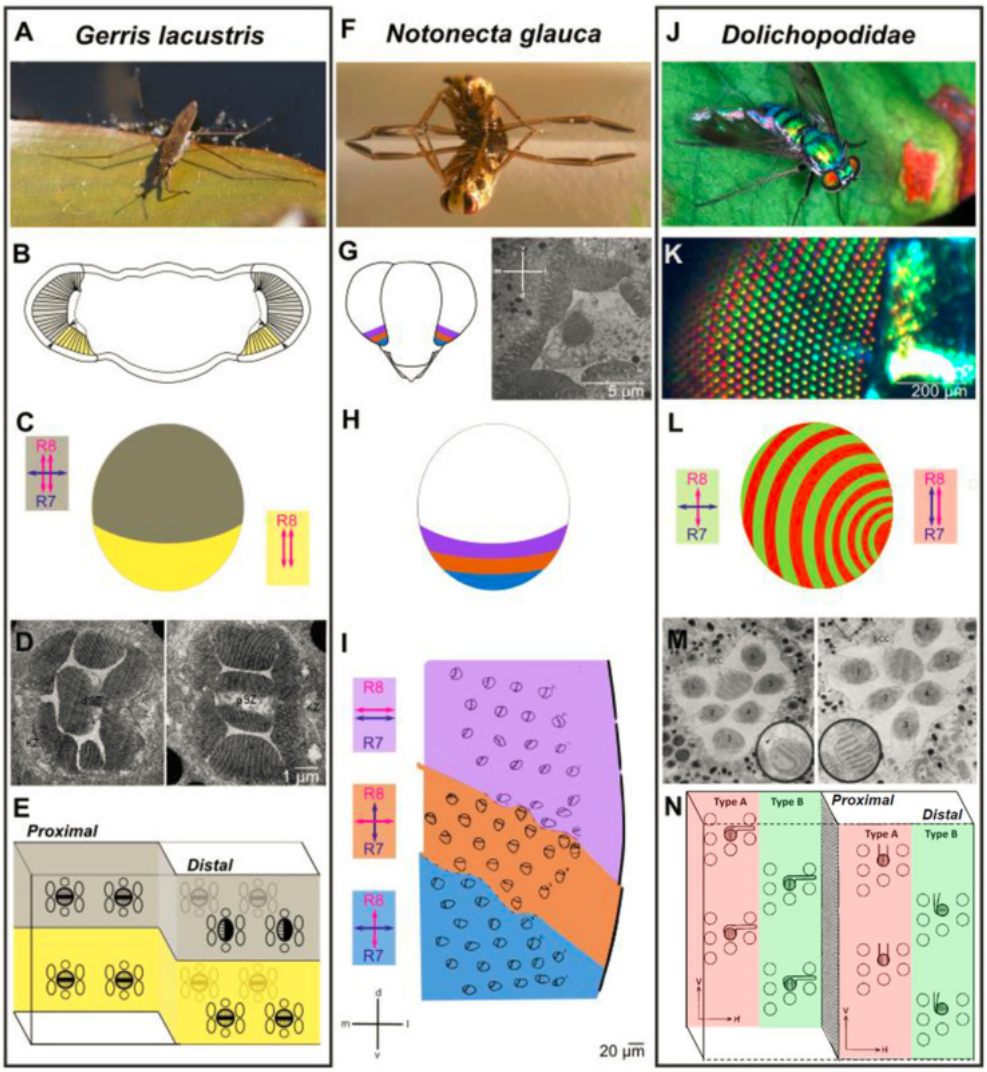

FIGURE 3 | Retinal specialization in the ventral periphery of three semi-aquatic insect species. Investigation of retinal ultrastructure using electron microscopy has revealed three very informative examples for specializations in the ventral periphery of the insect retina, each providing unique adaptations to the life close to linearly polarized water surfaces. **(A–E)** The ventral retina of the water strider *Gerris lacustris* **(A)** is perfectly adapted for filtering out horizontally polarized surface glare. A morphologically distinct ventral region is clearly visible (**B**, marked in yellow). The cellular composition of ommatidia changes drastically at the boundary between dorsomedial and ventral retina: only in the ventral part, the proximal cell with two vertically oriented rhabdomeres spans the entire thickness of the retina (double headed red arrows in **B**), whereas an additional, distal cell with one horizontally oriented rhabdomere is found on top of the distal cell across the dorsomedial retinal field (double headed blue arrow in **B**). Electron microscopy sections through the distal part of the *Gerris* retina shown in **(D)**, with a dorsal ommatidium on the left, and a ventral ommatidium on the right. Summary of cells and rhabdomere orientations at the interface of dorsal and ventral *Gerris* ommatidia shown in **(E)**. **(F–I)** Zonation of the ventral retina in the back swimmer *Notonecta glauca* **(F)** Three distinct zones can be distinguished within the ventral retina of Notonecta, based on the rhabdomere orientations of the inner photoreceptors (named R7 and R8, according to *Drosophila* nomenclature), visualized by electron microscopy **(G)**. Orientation of R7 vs. R8 rhabdomeres differ from parallel and horizontal (most dorsally), to different orthogonal configurations **(H)**. The relative position of the three zones within the ventral retina and their inner photoreceptor rhabdomere orientations are shown in **(I)**. **(J–N)** Alternating rows of ommatidial subtypes in long-legged flies (Dolichopodidae): alternating rows of shiny red and green colored facets in *Dolichopus nitidus* shown in **(K)**. Analysis of retinal ultrastrure using electron microscopy revealed specific differences in R7 vs. R8 rhabdomere orientation between "Type A" ommatidia (red) and "Type B" ommatidia (green): parallel and vertically oriented (Type A) vs. orthogonal (Type B) shown in **(L)**. This subtype-specific difference is achieved by alternating changes in R7 cell rhabdomere orientation. An electron microscopy section through a Type B ommatidium (left) and a Type A ommatidium (right) in **(M)**. **(A,F,J)** Reproduced from Wikimedia under Creative Commons licenses; **(K)** reproduced from (Stavenga et al., 2017) under Creative Commons licenses; **(D)** reproduced with permission from (Schneider and Langer, 1969); **(I)** reproduced with permission from (Schwind, 1983b); **(M)** and **(N)** reproduced with permission from (Trujillo-Cenóz and Bernard, 1972).

outside the DRA, several examples exist where insect photoreceptor subtypes throughout the retina seem to partially or completely untwist. Recently, photoreceptor cells with extreme polarization-sensitivity were characterized in the European corn borer moth *Ostrinia nubialis* (Belusic et al., 2017). In this case, each ommatidium contains one or two

blue-sensitive photoreceptors with straight rhabdomeric microvilli manifesting polarization-sensitivities far greater than those measured in the DRA of the same animal. Interestingly, a very similar retinal design seems to have evolved independently in some scarab beetles (Gokan, 1989). Although the functional role of these extremely polarization-sensitive cells is not yet understood, the orientation of their rhabdomeric microvilli along the dorsoventral axis has led to the hypothesis that they could be used for filtering out horizontally polarized reflections, or for detecting vertically polarized skylight patterns in the north and south at sunset or sunrise. Less dramatic examples where photoreceptor subtypes manifest only partial untwisting of their rhabdomeric microvilli exist for several species. Such a design must result in mixing of e-vector information with the perception of either color or intensity. For instance, the "false color" detection system of the Australian orchard butterfly *Papilio aegeus* results from blue- and green-sensitive photoreceptors outside the DRA retaining polarization sensitivity due to insufficient rhabdomere twist (Arikawa and Uchiyama, 1996). Hence, the polarized reflections from different leaves (and therefore potential oviposition sites) will be perceived as different colors as the animal flies by. Another example is from blood-sucking horse flies (Tabanidae): electron microscopy revealed that in the mid region of the retina, both R7 and R8 cell rhabdomeres are largely untwisted, a design that should also function as a "false color" system. It is therefore possible that tabanid inner photoreceptors could be used for finding prey via the polarized light reflected off their fur (Wunderer and Smola, 1986; Smith and Butler, 1991). Interestingly, very similar studies also identified a subtype of untwisted R8 photoreceptor in blow flies, yet no specific function could be attributed to it (Wunderer and Smola, 1982). Similarly, systematic analysis of rhabdomere twist in *Drosophila melanogaster* revealed a low number of untwisted, UV-sensitive R7 cells in the ventral fly retina (Wernet et al., 2012). Together with low twisting R1–6 photoreceptors within the same ommatidia, these cells could provide the retinal substrate for *Drosophila*'s polarotactic responses to linearly polarized stimuli presented (Wolf et al., 1980; Wernet et al., 2012; Velez et al., 2014a,b). The exact number and distribution of untwisted R7 cells remains unknown and additional studies are needed for a complete description of a putative "ventral polarization area" formed by these cells somewhere in the fly retina. It must be noted that the analysis of rhabdomere twist is tedious and labor intensive, due to the need for 3D reconstruction of serial electron microscopy sections. It is therefore possible that polarization-sensitive photoreceptors might exist in the ventral periphery of the retina of many insect species, yet it is likely that they have been overlooked in the past.

NEURAL CIRCUITS CONNECTING TO SPECIFIC OMMATIDIAL SUBTYPES—LESSONS FROM *DROSOPHILA*

The ommatidial mosaic of the fruit fly *Drosophila melanogaster* has long served as a powerful genetic model system for the dissection of cell-cell interactions during photoreceptor cell fate specification, revealing a long list of molecular key players involved in this process (Johnston, 2013; Wernet et al., 2014). Of particular interest are transcription factors expressed in very restricted groups of cells where they induce specific cell types while repressing other fates. For instance, the homeodomain transcription factor Homothorax (Hth) is expressed specifically in developing polarization-sensitive central photoreceptors R7 and R8 exclusively within prospective DRA ommatidia which form a narrow band of ommatidia along the dorsal margin of the fly eye (Wernet et al., 2003; Wernet and Desplan, 2014). Genetic manipulations revealed that Hth is both necessary and sufficient to induce the DRA fate when (mis-) expressed in any inner photoreceptor (Wernet et al., 2003). Importantly, Hth is usually not expressed at the ventral margin of the retina, nor anywhere else in the retina where one could suspect polarization-sensitive photoreceptors. The rest of the fly retina consists of two randomly yet unevenly distributed ommatidial types called "pale" and "yellow" (summarized in **Figure 4A**). The main difference between these two subtypes lies in the identity of the Rhodopsin molecules expressed by the central photoreceptors R7 and R8, resulting in subtype-specific pairing of the Rh3/Rh5 gene products in "pale" ommatidia and Rh4/Rh6 in "yellow" ommatidia (where both Rh3 and Rh4 are UV-sensitive Rhodopsins, Rh5 is blue-sensitive, and Rh6 is UV+green-sensitive; Johnston, 2013). Due to this mosaic of randomly distributed chromatic sensitivities it was long assumed that pale and yellow ommatidia serve color vision in *Drosophila*, and several recent studies have supported this hypothesis (Yamaguchi et al., 2010; Schnaitmann et al., 2013, 2018; Melnattur et al., 2014). Importantly, very similar ommatidial mosaics with two or three randomly distributed ommatidial subtypes have been described for many different insect species (Diptera, Hymenoptera, Lepidoptera, Hemiptera, Orthoptera; reviewed in: Wernet et al., 2015). More importantly it was recently shown that the same transcription factor is responsible for establishing the pale/yellow mosaic between central photoreceptor cells, both in flies and butterflies: using the Crispr/Cas9 technique in *Papilio* butterflies to produce large patches of retina lacking the Dioxin receptor (called Spineless in *Drosophila*), the butterfly retinal mosaic was disrupted in a predictable manner (Perry et al., 2016). Like in the *Drosophila* retina, loss of Spineless led to a complete loss of one ommatidial subtype ("yellow" in *Drosophila*, Wernet et al., 2006). It appears therefore, that the molecular mechanisms shaping the stochastic retinal mosaic are conserved between these distantly related species.

A rather unexpected potential function of the randomly distributed "pale" and "yellow" ommatidia as separate input channels for polarization vision vs. color vision was revealed by two independent studies both presenting visual stimuli to isogenic populations of walking *Drosophila*. In both cases sufficiency of "pale" and "yellow" ommatidia was investigated by rescuing phototransduction in a cell-type specific manner in blind flies lacking an eye-specific isoform of Phospholipase C (called NorpA in *Drosophila*). One study found that "yellow" ommatidia were sufficient to mediate color discrimination in an

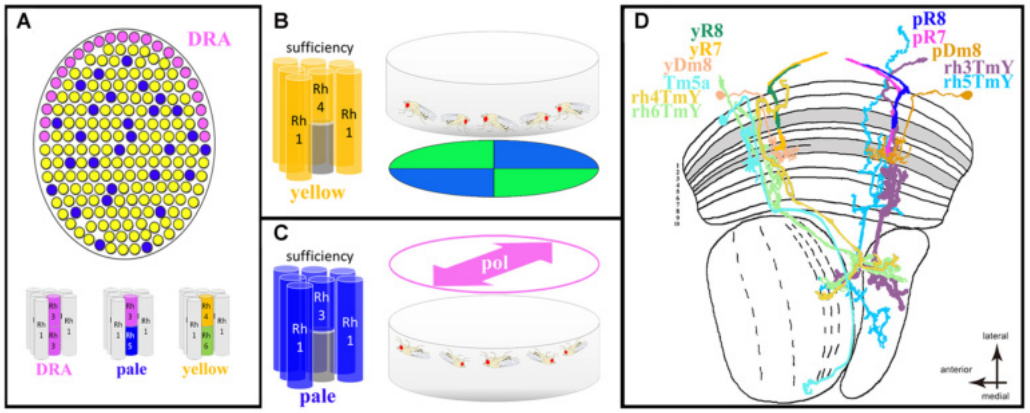

FIGURE 4 | *Drosophila* ventral polarization vision and ommatidial subtype-specific circuitry. **(A)** Schematic description of the *Drosophila* retinal mosaic: specialized, polarization-sensitive ommatidia for detecting celestial cues are found in the dorsal rim area (DRA; pink), where both inner photoreceptors (R7 and R8) express the UV Rhodopsin Rh3. The remaining retina is populated by randomly distributed omatidial subtypes named "pale" and "yellow" in which R7 and R8 cells always express specific Rhodopsins. All short visual fiber photoreceptors R1–6 express the same Rhodopsin (Rh1) across all ommatidial subtypes. **(B)** Summary of population responses of walking *Drosophila* trained to discriminate between colors (blue and green quadrants) observed with the ventral half of the retina. Sufficiency experiments using cell-type specific restoration of the phototransduction cascade component NorpA (Phospholipase D) revealed a specific role for "yellow" ommatidia (more specifically: the combination of Rh4-containing yR7 cells and R1–6 photoreceptors). **(C)** Summary of spontaneous alignment behavior of (upside-down) walking *Drosophila* populations in response to linearly polarized UV light perceived with the ventral half of the retina. In this case, sufficiency experiments using cell-type specific NorpA rescue revealed a specific role for "pale" ommatidia (more specifically: the combination of Rh3-containing pR7 cells and R1–6 photoreceptors). **(D)** Summary of our current knowledge about ommatidial subtype-specific neural circuit elements within the optic lobes of *Drosophila*. In the distal medulla neuropile, both pR7 and yR7 cells connect to the amacrine-like cell type Dm8. Results obtained from RNA profiling of inner photoreceptors and Dm8 cells suggests expression of specific cell-surface molecules in yR7 cells (expressing Dpr11) and their Dm8 connections (termed here yDm8, expressing DIP-γ) are important for establishing pale vs. yellow specific circuits. Furthermore, anatomical studies have identified the transmedullary (Tm) cell type Tm5a (sending projections to the lobula neuropile) as being specific to columns connected to yellow ommatidia (hence missing from those columns connected to pale ommatidia). Finally, a study using a trans-synaptic tracer technique identified four distinct classes of bifurcated TmY cell types (sending projections to both the lobula and lobula plate neuropiles), each type being specifically connected to pR7, yR7, pR8, or yR8 cells, respectively (hence termed rh3Tmy, rh4Tmy, rh5TmY and rh6TmY).

assay where the flies were presented blue and green quadrants (more specifically: the combination of *rh4*-expressing yR7 cells in combination with *rh6*-expressing yR8 or in combination with R1–6, the short visual fiber photoreceptors; Schnaitmann et al., 2013). In this assay, "pale" ommatidia were not sufficient to mediate color discrimination (**Figure 4B**). Another study presented isogenic populations of walking *Drosophila* with linearly polarized light of different, fixed e-vector orientations (Wernet et al., 2012). Strikingly, in this case only rescue of "pale" ommatidia was sufficient to mediate a polarotactic orientation response in which the flies oriented their body with the incident e-vector (more specifically: the combination of *rh3*-expressing pR7 cells with R1–6 photoreceptors). However, "yellow" ommatidia were not sufficient to mediate such a response (**Figure 4C**). These genetic experiments indicate that under certain conditions "pale" and "yellow" ommatidia in the ventral half of the retina may serve two separate functions: color vision (yellow) vs. polarization vision (pale). If this was the case, one would predict differences in rhabdomeric twist between pR7 (not twisting) and yR7 (twisting) cells. However, the analysis of rhabdomeric twist within a randomly chosen region of the fly retina revealed no difference between the two (both twisting) and the low-twisting R7 cells that were identified could not be attributed to a specific subtype (Wernet et al., 2012). A possible functional specialization therefore cannot apply to all "pale" vs. "yellow" ommatidia. However, there may exist a region within the ventral half of the fly retina where pR7 cells are specifically untwisted while yR7 cells remain twisted. An example for such subtype-specific untwisting of photoreceptor rhabdomeres was described for R8 cells in *Calliphora*, a functional significance for this anatomical substrate has yet to be demonstrated (Wunderer and Smola, 1982). Taken together, groups of ommatidia from different subtypes may form segregated input channels mediating distinct behavioral responses (color vs. polarization vision), yet more data is needed to understand their relative contribution. Strikingly, functionally specialized ommatidial subtypes could be distributed randomly (as in the case of *Drosophila*), or in alternating rows (as shown for *Dolichopodidae*)—two fundamentally different design principles that could be viewed as alternative solutions for spatially separating these inputs without sacrificing too much of the visual field to either one modality (while neglecting the other). Interestingly, similar segregation of color- and polarization sensitive pathways has recently been proposed for a vertebrate retina (Novales Flamarique, 2017).

Over the past few decades, the neural circuits mediating polarization vision downstream of DRA ommatidia have been described in great detail, across species. The circuit diagram deduced from these studies reveals how celestial e-vectors are represented in the central brain, how they are integrated with other positional cues like the sun, and how the compass system is compensating for the changes in e-vector orientation as the

sun moves across the celestial hemisphere (Homberg et al., 2011; Homberg, 2015). In contrast, next to nothing is known about the neural circuits underlying ommatidial specializations in the ventral periphery of the insect retina, like those described for *Gerris*, *Notonecta* and *Dolichopodidae*. In recent years, powerful molecular genetic tools have been developed for the cellular dissection of neural circuits across the *Drosophila* visual system, with a special emphasis on the optic lobes (Takemura et al., 2015). One first step towards addressing the neural circuitry of non-celestial polarization vision therefore lies in identifying optic lobe cell types that make ommatidial subtype-specific connections. At first glance, it seems hard to imagine how such connections could be wired during development of the visual system, given that *Drosophila* "pale" and "yellow" ommatidia are specified in a stochastic and therefore non-deterministic manner. Nevertheless, examples for pale- vs. yellow-specific optic lobe cell types exist and are currently increasing. For instance, anatomical characterization of the transmedullary cell type Tm5 (connecting the medulla neuropile with the lobula neuropile) revealed three subtypes termed Tm5a, Tm5b and Tm5c (Meinertzhagen et al., 2009). Interestingly, Tm5a cells were found to specifically arborize dendrites in single medulla columns containing yR7 terminals (Karuppudurai et al., 2014), whereas Tm5b and Tm5c are not subtype-specific (summarized in **Figure 4D**). Another study revealed subtype-specific circuit elements using a transgenic approach for trans-synaptically labeling optic lobe cell types that are connected to specific photoreceptor subtypes (Jagadish et al., 2014). In this case, four similar yet different cell types of so-called TmY cells with bifurcated axons (connecting the medulla neuropile with both the lobula and lobula plate neuropiles) were identified. Each of the four TmY subtypes appeared to specifically connect to either pR7, yR7, pR8, or yR8 cells and they were therefore termed rh3-TmY, rh4-TmY, rh5-TmY and rh6-TmY. So far, the existence of these cells and their subtype specific synaptic connections remain to be confirmed by EM reconstruction (Takemura et al., 2015). If confirmed, it is not known how these cell types would establish specific connections with photoreceptor cells that were specified stochastically. However, an important first step towards understanding how such wiring could be achieved came from two studies investigating the development of R7 connections with their most important synaptic partners, a distal medulla cell type called Dm8. Roughly every medulla column contains one Dm8 cell that receives inputs from ∼10–16 neighboring R7 cells (Gao et al., 2008; Karuppudurai et al., 2014; Ting et al., 2014). Assuming that each Dm8 cell receives preferential synaptic input from the R7 terminal located within its "home cartridge", one can therefore deduce the existence of Dm8 cells that receive predominant "pale" vs. "yellow" input (hence termed pDm8 and yDm8, in **Figure 4D**). How "pale" and "yellow" information is then processed further is currently not well understood and made more difficult by the fact that Dm8 cells appear to be locally processing units without a clear directed axonal output (Gao et al., 2008; Karuppudurai et al., 2014; Ting et al., 2014; Lin et al., 2016). Via profiling of the RNA transcriptome of R7 vs. R8 cells, recent studies studying the role of two classes of immunoglobulin transmembrane proteins (called DIPs and Dpr's) identified one protein that is specifically expressed in developing yR7 cells (Dpr11). More importantly, its ligand DIP-γ, the protein that specifically binds to Dpr11, is expressed in developing Dm8 cells (Carrillo et al., 2015; Tan et al., 2015). It appears therefore that the specific interaction between these transmembrane proteins could be the key to establishing subtype-specific connectivity between stochastically specified photoreceptor subtypes and their specific post-synaptic targets, thereby shaping distinct input pathways with different properties. Although still being in a very early stage, these experiments on ommatidial subtype-specific wiring could serve as a model system for understanding how neural circuits in the ventral periphery of the insect retina are shaped in order to result in functionally specialized channels.

CONCLUDING REMARKS

Different visual responses of insects to linearly polarized reflections have been described. Given the general importance of water bodies as habitats for insects, as well as the well-described adaptation of many species to a (semi-)aquatic lifestyle, a multitude of such behaviors could have been expected. That makes it even more surprising that only few examples exist for the retinal detectors responsible for processing linearly polarized reflections. The most fascinating aspect of these retinal detectors remains their developmental Bauplan: specialized ommatidia are found either restricted locally at the ventral edge, or subdivided into zones or even alternating stripes. Some retinal designs are capable of detecting linearly polarized reflections (the zonated ventral retina of Notonecta, or those ommatidial rows of Dolichopodidae with crossed polarizers), whereas others most likely serve to filter out linearly polarized light, like glare at the water surface (for instance the ventral retina of the water strider and potentially the retina of the corn borer moth). In the future, new studies should focus on analyzing the retinal ultrastructure from additional (semi-)aquatic insect species to deepen our understanding of how linearly polarized reflections are being detected.

What are the neural circuits processing the information from these ommatidial subtypes? To our knowledge, nothing is known about the underlying circuits in (semi-)aquatic insects. Using electrophysiology, many of the underlying circuit elements can be characterized. We expect that future studies on different species like tabanids will reveal important insight into the functional properties and the anatomy of the underlying circuits. Alternatively, we have shown how the investigation of photoreceptor subtype-specification in the molecular genetic model organism *Drosophila melanogaster* can provide insight into how the establishment of ommatidial subtype-specific circuitry may be regulated. A growing number of studies demonstrates the existence of neural circuit elements whose identity or morphology are specific to either one of the two stochastically distributed ommatidial subtypes. Combining the molecular genetic toolkit of *Drosophila* with behavioral paradigms for quantifying the behavioral response to linearly polarized reflections therefore presents another

attractive approach for studying how subtype-specific cell types might specifically alter the function of repetitive, retinotopic micro-circuits.

AUTHOR CONTRIBUTIONS

MFW wrote the manuscript. TH and JU assisted with the writing and provided all the figures.

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4 Manuscripts II

Cellular and synaptic adaptations of neural circuits processing sky-light polarization in the fly

Gizem Sancer, Emil Kind, Juliane Uhlhorn, Julia Volkmann, Johannes Hammacher, Tuyen Pham, Haritz Plazaola-Sasieta, Mathias F. Wernet

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Contribution:
I designed, conducted, and analyzed all experiments for Figure 6 and supported analysis for experiments in Figures 3 and 4 under the supervision of Prof. Dr. Mathias Wernet. The Manuscript was written by me, Gizem Sancer, Emil Kind, and Prof. Dr. Mathias Wernet.

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5 Manuscripts III

Colour Vision: Self-Centered Fly Photoreceptors Communicate over Distances

Juliane Uhlhorn, Mathias F. Wernet

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Contribution:
I designed and generated the figures under the supervision of Prof. Dr. Mathias Wernet and wrote the Manuscript with Prof. Dr. Mathias Wernet.

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6 Manuscripts IV

Early binocular processing of skylight polarization by specialized visual projection neurons in the fly brain

Juliane Uhlhorn, Gizem Sancer, Thomas F. Matheczyk, Emil Kind, and Mathias F. Wernet

Contribution:
Anatomical Studies were done by Gizem Sancer and me. Scholl analysis was done by Gizem Sancer, and extended, re-analyzing and re-plotting was done by me. All physiological experiments were planned, performed, and analyzed by me. Thomas Matheczyk performed and analyzed all behavioral experiments. Connectomic Data was reconstructed and analyzed by Emil Kind. Figures were composed by me under the supervision of Mathias Wernet. The manuscript was written by me together with Prof. Dr. Mathias Wernet.

Manuscript to be submitted.

Early binocular processing of skylight polarization by specialized visual projection neurons in the fly brain

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The celestial pattern of polarized skylight is an important visual cue that many animals use to navigate and forage in their environments. Across the compound eyes of insects, polarized skylight is detected in morphologically and molecularly specialized unit eyes (or ommatidia) at the dorsal most periphery, a region called the 'dorsal rim area' (DRA). In the brain of *Drosophila melanogaster*, a synaptic pathway known as the 'anterior visual pathway' (AVP) connects both eyes with the central complex, where heading decisions are encoded. In the first stage of processing skylight polarization along the AVP, here we describe two similar yet distinct types of specialized visual projection neurons (named MeTu-DRA1 and MeTu-DRA2), connecting the DRA area of the medulla neuropil in the optic lobes with the same optic lobe target region in an optic glomerulus, named 'anterior optic tubercle' (AOTU). Using a multidisciplinary approach combining anatomical analyses, physiological recordings, and behavioral assays, we show that (i) both MeTu-DRA cell types together are necessary for polarotactic navigation behavior. (ii) Both subtypes display similar synaptic inputs, along with similar morphological characteristics and a similar topographic exon projection pattern from the DRA to the AOTU. (iii) Their physiological responses to polarized UV light differ considerably. As a basis for these differences, we show that four interhemispheric neurons (named MeMe-DRA) interconnect MeTu-DRA cells in a subtype-specific manner, resulting in two distinct channels for the binocular processing of polarized skylights signals for navigation.

Introduction

The ability to perceive the pattern of polarized skylight is a fascinating sensory modality employed by numerous organisms in order to improve their orientation and to forage in their environments (Heinloth et al., 2018; Held et al., 2016; Kraft et al., 2011; Heinze, 2017). Since the pattern or polarized skylight forms symmetrically around the celestial body, it is perfectly suited as an alternative reference for setting a course during the navigation of both walking and flying insects (Rossel, 1993; Wehner, 2001; Labhart and Meyer, 2002; Warren et al., 2018; Mathejczyk and Wernet, 2019; Dacke et al., 2021; Homberg et al., 2023). Across insect species, the neuronal substrate for detecting skylight polarization (more specifically, the angle of polarization, AOP) is found in morphologically and molecularly specialized unit eyes (or ommatidia) in the dorsalmost periphery of the adult compound eye, a region therefore termed 'dorsal rim area' (DRA) (Labhart and Meyer, 1999; Wernet et al., 2015). In these DRA ommatidia, two groups of photoreceptors always form monochromatic pairs with straightly aligned (or untwisted) rhabdomeric microvilli, an ultrastructure that conveys high polarization sensitivity (Israelachvili and Wilson, 1976), and which are orthogonally oriented between the two groups (Labhart and Meyer, 1999), an arrangement that is ideal for opponent processing of skylight polarization in analogy to color (Labhart, 1988). In flies, these opponent pairs are formed by the long visual fiber photoreceptors R7 and R8 (Wada, 1974; Labhart and Meyer, 1999; Wernet et al., 2003), which form inhibitory synapses onto each other (Weir et al., 2016; Schnaitmann et al., 2018; Kind et al., 2021), and project axons to the medulla neuropil of the optic lobe (Fischbach and Dittrich, 1989).

In recent years, research on different model species has unveiled an evolutionarily conserved synaptic pathway from the DRAs of both eyes to the central brain, which includes specialized neuronal cell types for processing skylight information (Omoto et al., 2017; Grob et al., 2019; Homberg et al., 2023). This so-called 'anterior visual pathway' (or, alternatively, compass pathway) includes visual projection neurons, connecting the medulla with an optic glomerulus in the central brain, called the anterior optic tubercle (AOTU) (el Jundi et al., 2011; Pfeiffer and Kinoshita, 2012; Otsuna et al., 2014; Hardcastle et al., 2021). Here, we will refer to these visual projection neurons as MeTu (medulla-to-tubercle) cells, according to the *Drosophila* nomenclature (Omoto et al., 2017; Timaeus et al., 2020; Kind et al., 2021; Tai et al., 2021). In the AOTU, these cells synapse on TuBu (tubercle-to-bulb) cells, which project axons into the bulb (BU) region of the central complex, where they synapse onto visual ring neurons (or ER neurons) in the ellipsoid body of the central complex (Shiozaki and Kazama, 2017; Sun et al., 2017; Hardcastle et al., 2021; Hulse et al., 2021). These cells, in turn, are directly presynaptic to famous head-direction-like cells (so-called EPG neurons), which encode the heading direction of the flies in relative coordinates (Seelig and Jayaraman, 2015; Kim et al., 2017). The AVP, therefore, appears to be the perfect neuronal substrate for processing different visual cues that the fly can use for informing the head direction system during navigation.

Evidence for a specific role of distinct cell types within the AVP for mediating behavioral

responses to polarized light is sorely missing, yet cutting the axonal connections between the eye and the AOTU in locusts was shown to abolish their behavioral responses to polarized light (Mappes and Homberg, 2007). A potential solution could be using the molecular-genetic toolkit available in *Drosophila melanogaster* for the reversible, cell-type specific silencing of neuronal activity (Kitamoto, 2001; Pfeiffer et al., 2008). Several studies have demonstrated that *Drosophila* exhibits robust behaviors that rely on detecting and interpreting polarized light cues, such as spontaneous responses during walking or flying (Wolf et al., 1980; Weir and Dickinson, 2012; Wernet et al., 2012), as well as maintaining straight flight headings (Warren et al., 2018; Mathejczyk and Wernet, 2019). A variety of virtual flight arenas now allows for their combination with genetic perturbations (Mathejczyk and Wernet, 2020).

The physiological properties of polarization-sensitive cell types within the AVP have probably been most extensively studied in locusts (Homberg et al., 2023), and more recently in *Drosophila* (Hardcastle et al., 2021). Early processing of skylight polarization in DRA photoreceptors R7 and R8 has also been studied in *Drosophila* using calcium imaging (Weir et al., 2016), demonstrating opponent signals within presynaptic terminals. Downstream of DRA R7 and R8 photoreceptors, modality-specific distal medulla neurons (called Dm-DRA1) were described both anatomically (Sancer et al., 2019, 2020; Kind et al., 2021), and physiologically (Hardcastle et al., 2021). Their post-synaptic targets are DRA-specific MeTu subtypes (called MeTu-DRA) (Kind et al., 2021), which are the first polarization-sensitive cell type to send axons into the central brain. Two different types of presumable MeTu-DRA cell types were recently shown to be specifically tuned to polarized light signals (Hardcastle et al., 2021). Importantly, axon projections of MeTu cells with dendrites along the anterior-posterior axis of the MEDRA were shown to project topographically to the AOTU, terminating along the dorsoventral axis there, while respecting their neighboring relationships (Hardcastle et al., 2021). Due to their anatomy and their physiology, MeTu neurons, therefore, represent potential key players within the fly AVP, suggesting an important role in the neural processing of navigational information.

To understand the role of MeTu-DRA neurons in *Drosophila*, we decided to explore their light microscopic anatomy, their synaptic connectivity within the AVP, their necessity for producing behavioral responses to polarized light, as well as their physiological properties. Here we describe two overlapping sets of genetically distinguishable MeTu-DRA subtypes (MeTu-DRA1 and MeTu-DRA2) with very similar single-cell morphology while manifesting different synaptic output connectivity within the AVP. Both MeTu-DRA types together are necessary for the fly's ability to select a stable heading using polarized light, while their physiological responses are surprisingly different, given virtually identical synaptic inputs. We show that the major difference between these two cell types lies in exclusively ipsilateral processing of skylight information (MeTu-DRA1) versus the combination of ipsi+contra-lateral inputs (MeTu-DRA2) via four interhemispheric neurons (named MeMe-DRA). Our findings provide the first demonstration of an early combination of both mono- and binocular synaptic pathways processing polarized skylight cues, bearing important similarity

in other insect species, while providing important new anatomical, physiological, and behavioral insight.

Results

Two types of modality-specific MeTu neurons in the DRA

Using a connectomic resource, we recently described modality-specific MeTu neurons that received synaptic inputs from R7-DRA photoreceptors, while avoiding inputs from non-DRA counterparts (Kind et al., 2021) (Figure 1A). We therefore tested available MeTu drivers from previous studies proposing parallel MeTu channels from the medulla to the AOTU (Otsuna et al., 2014; Omoto et al., 2017; Timaeus et al., 2020; Hardcastle et al., 2021; Tai et al., 2021) for modality-specific morphology. However, neither of them specifically labeled MeTu-DRA neurons, including the ones used for characterizing polarization-sensitive responses in the AOTU (R73C04-Gal4 and R56F07-Gal4) (Hardcastle et al., 2021) (Supplemental Figure 1). In parallel, we screened all available resources (Pfeiffer et al., 2008; Gohl et al., 2011; Kvon et al., 2014) and identified two Gal4 driver lines (InSITE 0871 and GMR18G04, see materials and methods), both covering the dorsal half of the medulla (Figure 1B-D) manifesting modality-specific contacts exclusively with DRA photoreceptors (Figure 1D-D") and sending axons into the same central-posterior (cp) area of the small unit of the AOTU (Timaeus et al., 2020), where their terminals intermingle (Figure 1C-C"). To our surprise, these two lines appeared to label two mutually exclusive groups of cells when labeled with two different binary expression systems (Figure 1E). Since both of these populations manifested modality-specificity photoreceptor contacts, we will now refer to them as putative MeTu-DRA1 and MeTu-DRA2 subtypes (Figure 1D-D"). Interestingly, only one of these subtypes (MeTu-DRA2 / GMR18G04) co-labeled with the previously published line labeling polarization-sensitive MeTus (R56F07) in cell bodies distal to the medulla as well as in the AOTU (Supplemental Figure 1B), whereas the other published line projected axons to the anterior areas of the AOTU (Supplemental Figure 1A). We then proceeded to closely characterize any potential morphological differences between MeTu-DRAs in order to better understand any putative subtypes they may form. First, we tested synaptic connectivity with DRA inner photoreceptors. GFP reconstitution across synaptic partners (GRASP) between rh3-expressing photoreceptors and both MeTu-DRA driver lines a positive GRASP signal only in the DRA region (Figure 1F and 1G, 3A and 3B). However, GRASP with an R8-DRA driver resulted in no signal, thereby revealing a synaptic connection only between DRA.R7 and both MeTu-DRA cell types, but not R8-DRA. This was in good agreement with our previous connectomic analysis (Kind et al., 2021).

In order to investigate potential morphological differences between MeTu-DRA1 and MeTu-DRA2, we generated single-cell clones using MCFO and extracted individual medulla profiles for reconstruction and analysis (Figure 1H,I). The overall number of contacted DRA.R7 photoreceptors per cell was not significantly different between the two subpopulations (mean: 10.63 ± 0.7934 for MeTu-DRA1; mean: 9.714 ± 0.7934 for MeTu-DRA2; Figure 1J). However, MeTu-DRA2 cells

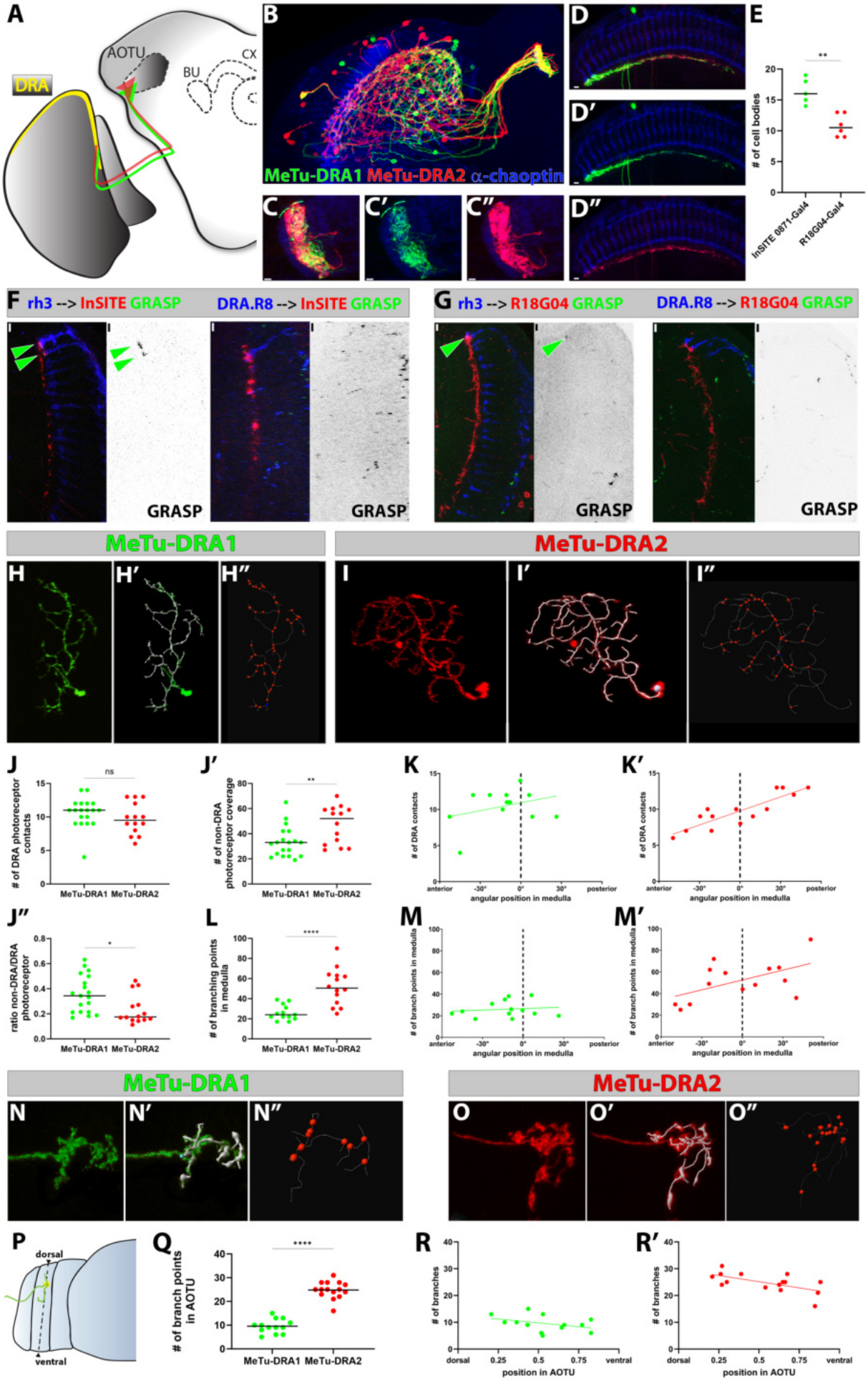

Figure 1: Morphological characterizations of MeTu-DRA modality specific cell types

Figure 1: A Schematic representation of MeTu neurons connecting DRA of medulla and AOTU **B** Co-labeling of 2 MeTu-DRA sub-cell populations. MeTu-DRA1 (green) and MeTu-DRA2 (red) with photoreceptors (blue). **C-C"** Co-labeling of MeTu-DRA1 and MeTu-DRA2 in small subunit of AOTU. **D-D"** Side view of MeTu-DRA1 and MeTu-DRA2 co-labeling and single channels with photoreceptors. Only DRA photoreceptors are in close contact with MeTu-DRA cells. **E** Counted number of cell MeTu-DRA cell bodies labeled by InSITE0871-Gal4 and R18G04-Gal4 driver line (unpaired t-test: **p=0.0011). **F** GRASP of photoreceptors to MeTu-DRA1. Rh3 shows GRASP signal in DRA (green arrows). Presynaptic cells (blue), postsynaptic cells (red), GRASP signals (green/greyscale). No GRASP signal could be detected between DRA.R8 and MeTu-DRA1. **G** GRASP of photoreceptors to MeTu-DRA2. Rh3 shows a GRASP signal in DRA (green arrows). Presynaptic cells (blue), postsynaptic cells (red), GRASP signals (green/greyscale). No GRASP signal could be detected between DRA.R8 onto MeTu-DRA2. **H-H"** Single cell clone (MCFO) of MeTu-DRA1 cell. Light microscopic data, Imaris surface reconstruction, and filament tracing with marked branching points. **I-I"** Single cell clone (MCFO) of MeTu-DRA2 cell. Light microscopic data, Imaris surface reconstruction, and filament tracing with marked branching points. **J-J"** Quantification of the number of DRA photoreceptors contacted by one for MeTu-DRA1 (green) or MeTu-DRA2 (red) (**J**: unpaired t-test: ns p=0.2564), number of photoreceptor columns which are covered by one MeTu-DRA cell (**J'**: unpaired t-test: **p=0.0066), and the ratio of one cell of contacted DRA photoreceptors in comparison to the columnar spread of non-DRA reach (**J"**: unpaired t-test: **p=0.0014). **K-K'** Number of contacted DRA photoreceptor columns per cell, dependent on angular position of MeTu-DRA cell in the medulla. **L** Number of dendritic branching points in the medulla (unpaired t-test: ****p=0.0014). **M-M"** Number of branching points per cell, dependent on the angular position of MeTu-DRA cell in the medulla. **N-N"** Single cell clone (MCFO) of MeTu-DRA1 cell in AOTU. Light microscopic data, Imaris surface reconstruction, and filament tracing with marked branching points. **O-O"** Single cell clone of MeTu-DRA2 cell in AOTU. Light microscopic data, Imaris surface reconstruction, and filament tracing with marked branching points. **P** Schematic of MeTu cells terminating in AOTU. **Q** Number of axonal branching points in AOTU (unpaired t-test: ****p<0.0001). **R-R'** Number of axonal branching points per cell, dependent on the dorsoventral position of MeTu-DRA cell in the AOTU. All scales in medulla 10µm. Scales in AOTU 3µm.

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covered a significantly larger area of non-DRA columns when compared to MeTu-DRA1 (33.32 ± 4.724 for MeTu-DRA1; 47.07 ± 4.724 for MeTu-DRA2; Figure 1J'), resulting in a smaller ratio of DRA vs non-DRA columns covered by this cell type (0.3573 ± 0.04786 for MeTu-DRA1; 0.2343 ± 0.04786 for MeTu-DRA2; Figure 1J"). Correlation of the number of contacted DRA photoreceptor columns to the position of MeTu-DRA dendrites in the medulla revealed that both MeTu-DRA1 and MeTu-DRA2 cells on the anterior side of the medulla contacted slightly fewer photoreceptors than cells on the posterior side (see Figure 1K). Interestingly, the dendritic morphology of MeTu-DRA1 and MeTu-DRA2 single-cell clones was drastically different. MeTu-DRA1 manifested an elongated morphology with about 26.00 ± 5.430 branch points per cell, somewhat independent of the cell's position (Figure 1L,M). MeTu-DRA2 cell, in contrast, has a larger dendritic spread (Figure 1J,M') with a significantly higher amount of dendritic branching (51.71 ± 5.430), which ranged from 20 to 90 branches, increasing in number along the anterior-posterior axis. These findings raised the question of whether single-cell profiles of MeTu-DRA1 and MeTu-DRA2 presynaptic terminals in the AOTU echo such differences in branching (Figure 1N-M). Indeed, we observed that the MeTu-DRA2 single-cell clones also manifested significantly more presynaptic branch points in the AOTU (9.538 ± 1.252 for MeTu-DRA1; 24.80 ± 1.252 for MeTu-DRA2; Figure 1P,Q). Both MeTu-DRA types manifested only a slight trend towards fewer branching along the dorsoventral axis (Figure 1R). Despite these morphological differences, no obvious differences in the coverage of the cp area of the AOTUsu were observed between the two cell types. These morphological differences of MeTu-DRA cells in both medulla and AOTU support the idea of both cell types processing polarized lights in parallel. Nevertheless, only MeTu-DRA2s were previously described

physiologically.

MeTu connectivity in the medulla

As Dm-DRA1 cells are the main postsynaptic partners of DRA.R7 cells and were previously proposed to be presynaptic to MeTu-cells (Hardcastle et al., 2021), we tested for potential differences in connectivity between Dm-DRA1 and the two MeTu-DRA populations using activity-dependent GRASP (Figure 2A,B). Interestingly, we did not find any differences when Dm-DRA1 was tested presynaptically, and MeTu-DRA1 or MeTu-DRA2 were postsynaptic (Figure 2C,D). To our surprise, a clear difference in connectivity became apparent when testing MeTu-DRA1 or MeTu-DRA2 cells as being presynaptic to Dm-DRA1 cells (Figure 2E,F): the activity GRASP signal suggests that only MeTu-DRA1 → Dm-DRA1 form a synaptic feedback loop in the medulla, as opposed to MeTu-DRA2, suggesting a more complex interaction between Dm-DRA1 and MeTu-DRA1 cells. It must be noted, however, that such synaptic feedback is not clearly apparent in the current connectomic data (Garner, Kind et al, in preparation).
The previously mentioned GRASP signal between DRA photoreceptors and MeTu-DRA1 appeared stronger than the one obtained for MeTu-DRA2, suggesting potential differences in connectivity between the two cell types in the DRA region. To test this further, we labeled the postsynaptic membranes of MeTu-DRA1 or MeTu-DRA2cells, specifically using UAS-DREP2::GFP (Figure 2G-M). We did not observe a significant difference in the amount of DREP2 puncta (unpaired t-test: ns p=0.2171; Figure 2M). However, due to the difference in the total number of cells labeled by each driver line (unpaired t-test: ****p<0.0001; Figure 2M'), a significant difference in the postsynaptic sites as labeled via DREP2 puncta per cell arose (unpaired t-test:*p=0.0108; Figure 2M"). We also identified the MeTu-DRA1 and MeTu-DRA2 cells in the connectome, allowing us to compare our light microscopic findings with the connectome. Importantly, the difference in the count of postsynaptic sites in the connectome is in good agreement with our light microscopic data (data not shown). In order to investigate potential differences in the distribution of postsynaptic sites between MeTu-DRA1 and MeTu-DRA2 cells, we quantified the spread of their DREP2 signals across the medulla (Figure 2G-K). To do this, we extracted those DREP2 puncta located exclusively within layer M6 to normalize and bin the distribution of the DREP2 signal. The distribution plots of MeTu-DRA1 and MeTu-DRA2 DREP2 signals revealed different patterns: for MeTu-DRA1 cells, over 60% of the total signal was located close to the DRA region with a drastic drop in postsynaptic signal into the medulla (Figure 2G,I,J). On the other hand, the MeTu-DRA2 signal was more evenly distributed all over the medulla with no drastic peak in the DRA region (Figure 2H,K,L). These differences in DREP2 distribution could reflect differences in DRA- versus non-DRA inputs between these cell types, bearing potential consequences for their physiological responses.

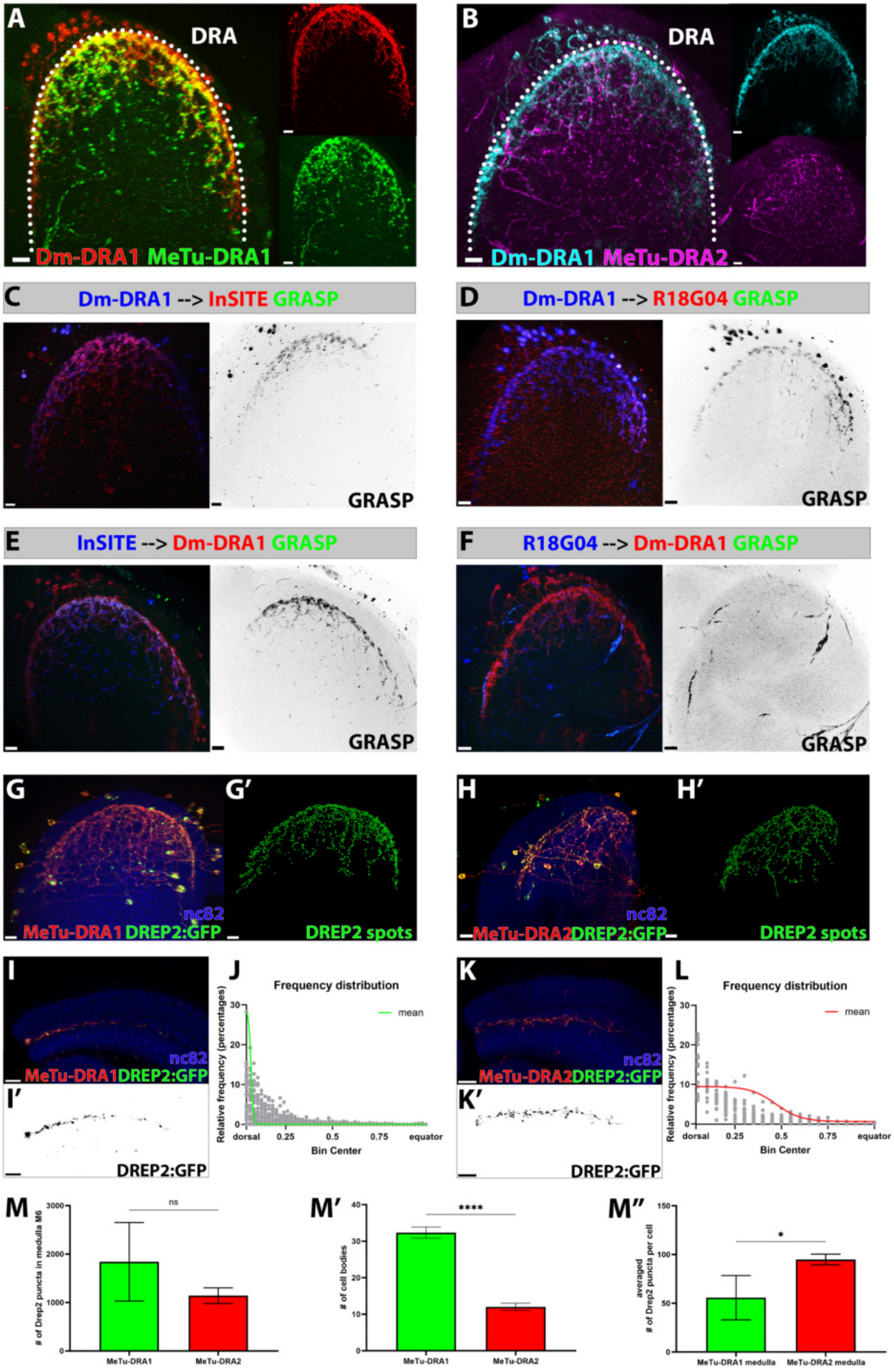

Figure 2: Wiring differences of MeTu-DRA1 and MeTu-DRA2 in the medulla

Figure 2: **A** Co-labeleing and single channels of Dm-DRA1 (red) cells and MeTu-DRA1 (green). **B** Co-labeleing and single channels of Dm-DRA1 (cyan) cells and MeTu-DRA2 (magenta). **C** Co-labeling of GRASP (green) between Dm-DRA1 (presynaptic, blue) and MeTu-DRA1 [InSITE-Gal4] (postsynaptic, red). GRASP signal (grey). **D** Co-labeling of GRASP (green) between Dm-DRA1 (presynaptic, blue) and MeTu-DRA2 [R18G04-Gal4] (postsynaptic, red). GRASP signal (grey). **E** Co-labeling of GRASP (green) between MeTu-DRA1 (presynaptic, blue) and Dm-DRA1 (postsynaptic, red). GRASP signal (grey). **F** Co-labeling of GRASP (green) between Dm-DRA2 (presynaptic, blue) and Dm-DRA1 (postsynaptic, red). GRASP signal (grey). **G-G'** Labeling of postsynaptic sides (DREP2:GFP, green) in MeTu-DRA1 (red), nc82(blue) and extracted DREP2 spots in medulla M6 layer only). **H-H'** Labeling of postsynaptic sides (DREP2:GFP, green) in MeTu-DRA2 (red), nc82(blue) and extracted DREP2 spots in medulla M6 layer only). **I-I'** Sectional side view of G and DREP2:GFP signal measured for frequency distribution in **J** binned DREP2:GFP signal and plotted distribution from dorsal edge to equator region. **K-K'** Sectional side view of H and DREP2:GFP signal measured for frequency distribution in **L** binned DREP2:GFP signal and plotted distribution from dorsal edge to equator region. **M-M"** counted number of DREP2 spots in M6 layer (G', H') (unpaired t-test: ns p=0.2171), counted number of cell bodies labeled (unpaired t-test: ****p<0.0001), ratio of estimated DREP2 spots per cell based on number of total spots and number of CB (unpaired t-test: *p=0.0108). Scales all 10µm.

Topographic representation of Skylight polarization in the AOTU

In analogy to the previous section, we used MCFO single cell clones to correlate the dendritic position of a given MeTu cell in the medulla to the termination point of its axon along the dorsoventral axis within the cp region of the AOTU (Figure 3A-D). We found that MeTu-DRA1 cells with dendrites on the posterior side of the medulla always terminate in the dorsal part of the AOTU. In contrast, clones with dendrites located closer to the anterior edge of the DRA always terminated towards the ventral edge of the AOTU, thereby revealing that the DRA's posterior-anterior axis within the medulla becoming topographically transformed into dorsoventral representation within the AOTU (Figure 3C). We then compared our measured clone positions to the published data relating the topography of DRA.R7 terminals (Weir et al., 2016) to their preferred 'Angle of Polarization' (AOP) sensitivity (as determined physiologically)(Figure 3E,F). This comparison further supports the hypothesis of a topographic representation of preferred AOPs from the DRA region of the medulla to the AOTU. We then tested whether MeTu-DRA2 cells also project topographically from the DRA into the AOTU, performing the same MCFO experiments (Figure 3B,D). Indeed, MeTu-DRA2 clones close to the posterior edge of the medulla terminated dorsally in the AOTU (Figure 3D) and more anterior clones projected more ventrally. Hence, like the MeTu-DRA1 cells, MeTu-DRA2 also transforms a posterior-anterior signal from the MEDRA into a dorsoventral slope in the AOTU. The correlation of this topographic projection pattern with previously measured AOPs of the DRA.R7 inputs is summarized in Figure 3F, revealing a great resemblance of MeTu-DRA1 and MeTu-DRA2 topography within the AOTU.
Since both MeTu-DRA subtypes manifested a topographic representation of the MEDRA within the AOTU, we wondered whether the higher number of MeTu-DRA2 branches within the AOTU also resulted in a higher number of presynaptic sites there. We, therefore, generated single-cell clones where we labeled the presynaptic sites with UAS-BRP:GFP (Figure 3G). Surprisingly, the high number of branches of MeTu-DRA2 cells did not correlate with a higher number of presynaptic sites (Figure 3G,H). Instead, our analysis revealed that single MeTu-DRA1 and MeTu-DRA2 terminals have roughly the same number of presynaptic sides in the AOTU. This could be confirmed by using

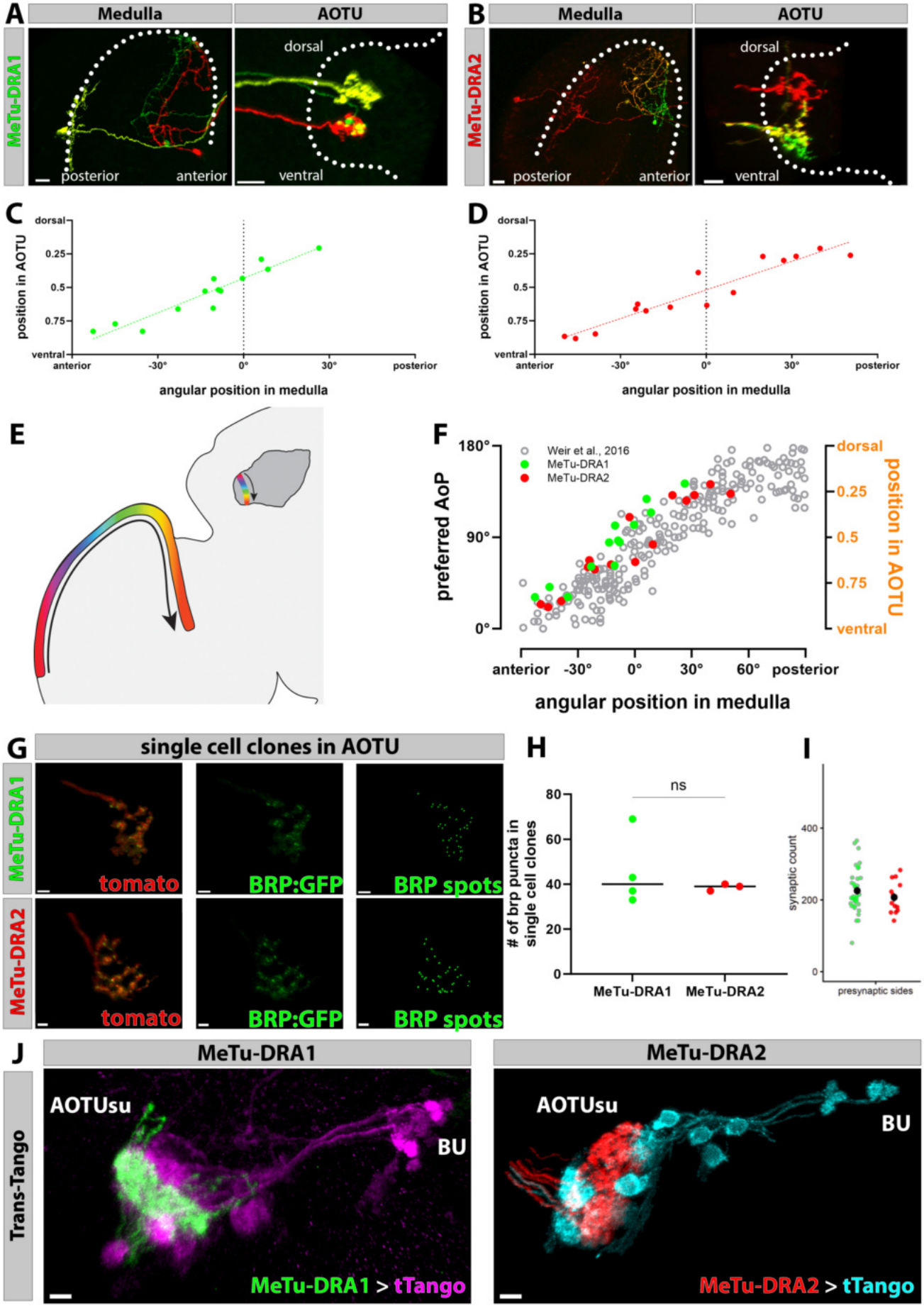

Figure 3: Morphological characterizations of MeTu-DRA modality specific cell types

Figure 3: **A** Single cell clones (MCFO) of MeTu-DRA1 cells in medulla and their terminals in AOTU. Cells branching in a posterior-anterior gradient in medulla project dorsoventrally in AOTU. The dashed line indicates edge of medulla and AOTU. **B** Single cell clones (MCFO) of MeTu-DRA2 cells in medulla and their terminals in AOTU. Cells branching in a posterior-anterior gradient in medulla project dorsoventrally in AOTU. The dashed line indicates edge of medulla and AOTU. **C** Measured position of single cell clones of MeTu-DRA1 terminals in AOTU based in its angular position in the medulla. **D** Measured position of single cell clones of MeTu-DRA2 terminals in AOTU based in its angular position in the medulla. **E** Schematic representation of MeTu-DRA DRA topography from medulla to AOTU. **F** Comparision of measured position in AOTU (right y-axis) to angular position in medulla as in C and D to published data of preferred AoP from Weir et al. (2016). **G** single cell clones of MeTu-DRA1 and MeTu-DRA2 cells with presynaptic sides (BRP:GFP). **H** Quantification of light microscopic data of pre-synaptic sides in single cell clones (unpaired t-test: ns p=0.5088). **I** Pr-synaptic sides of single cells based on connectomic data. **J** Trans-Tango labeling postsynaptic cells for MeTu-DRA1 and MeTu-DRA2. Scale for medulla 10µm. Scale for AOTU 3µm.

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the currently existing connectomic data (Figure 3I). Finally, we wondered whether MeTu-DRA1 and MeTu-DRA2 would connect to the same or different targets in the AOTU. Previous studies identified TuBu neurons as the major target of MeTu cells (Omoto et al., 2017; Hulse et al., 2021; Timaeus et al., 2020). These highly polarized neurons form dendrites in the AOTU and form short axons to the bulb region of the central complex, where they connect to different ring neuron types of the ellipsoid body. In order to gain insight into which TuBu neurons both MeTu classes could be connected to, we performed trans-synaptic labeling using the transTango tool (Talay et al., 2017) (Figure 3J). For both MeTu types, a small number of TuBu cells was labeled, yet their light microscopic morphology was too similar to reliably tell them epart. Current connectomic data suggests that both MeTu types converge on TuBu6 neurons, while only MeTu-DRA1 forms additional connections with TuBu1, thereby revealing clear differences in downstream connectivity between the two MeTu-DRA types (Garner, Kind et al, in preparation).

Physiological responses of MeTu cells to linearly polarized UV stimuli

The observed differences in both morphology and connectivity between MeTu-DRA1 and MeTu-DRA2 cells suggested they could potentially differ in their physiological responses. We, therefore used genetically encoded calcium sensors to study their physiological responses to a linearly polarized UV stimulus, similar to what had previously been done (Weir et al., 2016; Hardcastle et al., 2021) (Figure 4A,B). First, to specifically visualize the responses in the AOTU, we used the synaptically targeted syt:GCaMP6s indicator in combination with a split Gal4 driver line labeling all MeTu-DRA1 cells (Supplemental Figure 2). We presented 365nm UV light through a polarization sheet to imitate polarized skylight, moving the filter in 30° steps while recording the populational response in the AOTU (see materials & methods). As previously described, we first calculated the Polarization Sensitivity Index (PSI) for each recorded pixel and calculated a mean of chosen ROIs. Both MeTu-DRA lines showed strong PSI values (Figure 4A-B and E-F). We then proceeded to determine the preferred AOP for all pixel with high PSI, resulting in AOP tuning maps for Dm-DRA1 in the medulla (Figure 4C), as well as both MeTu-DRA1 and MeTu-DRA2 in

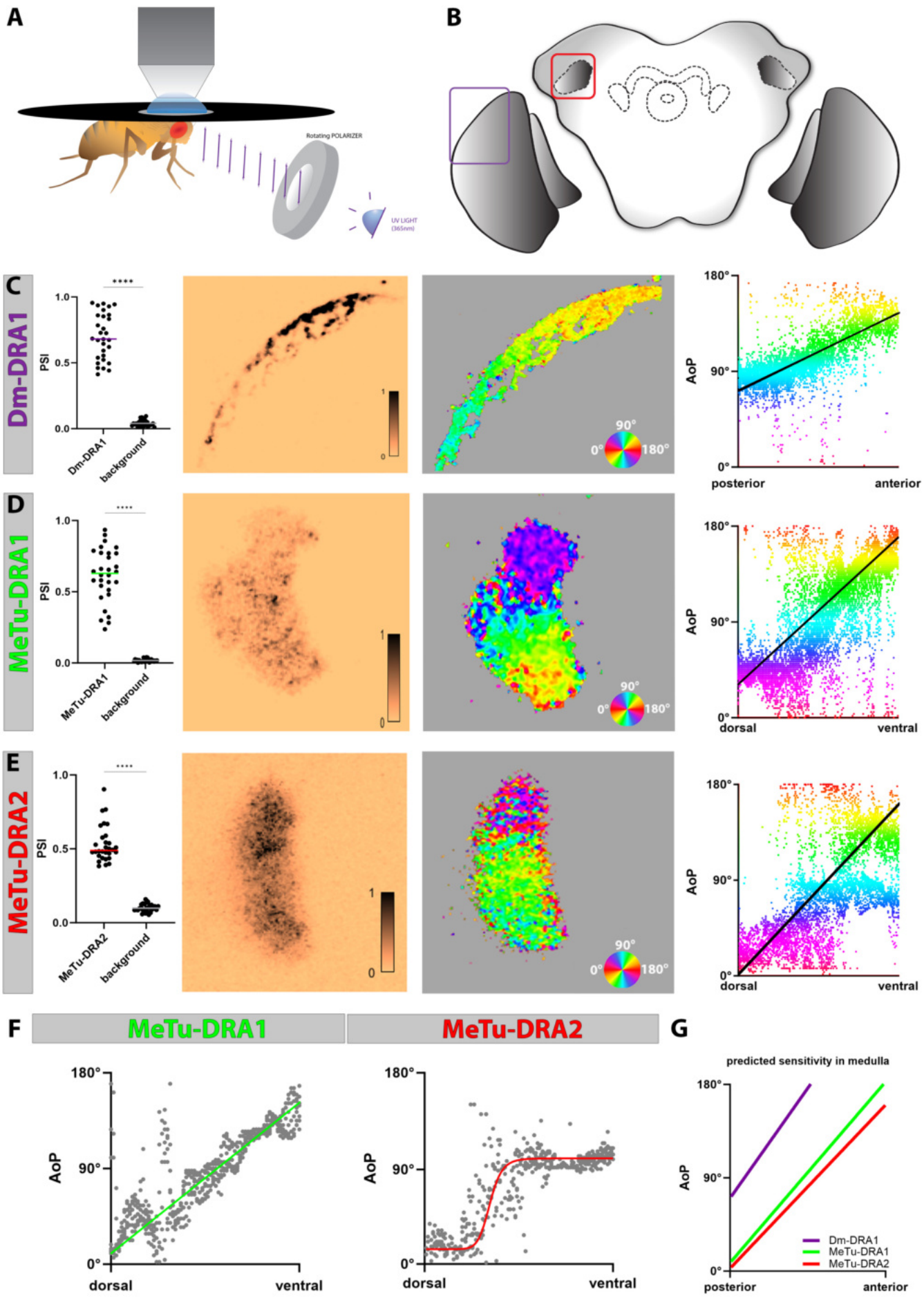

Figure 4: Physiological characterization of MeTu-DRA specific cells to polarized UV light

Figure 4: **A** Schematic representation *in-vivo* setup experiments. Fly is mounted in a custom-designed holder position under the microscope. UV light (365nm) is presented through a rotating polarizer. **B** Schematic representation of imaged areas. Imaged Dm-DRA in medulla (purple box) and MeTu-DRAs in the AOTU (red box). **C** Measured PSI (3 ROIs per recording, unpaired t-test: ****p<0.0001) and background values for Dm-DRA1 cells. LUT for PSI values of Dm-DRA1 in the medulla, sensitivity of AoP for same PSI. Averaged preferred AoP from posterior to anterior edge. The black line indicates fit for pooled data for Dm-DRA1 (N=10 recordings). **D** Measured PSI and background values for MeTu-DRA1 cells in AOTU. LUT for PSI (3 ROIs per recording, unpaired t-test: ****p<0.0001) values of MeTu-DRA1 in AOTU, the sensitivity of AoP for same PSI. Averaged preferred AoP from dorsal to posterior edge. The black line indicates fit for pooled data for MeTu-DRA1 (N=10 recordings). **E** Measured PSI and background values for MeTu-DRA2 cells in AOTU. LUT for PSI (3 ROIs per recording, unpaired t-test: ****p<0.0001) values of MeTu-DRA2 in AOTU, the sensitivity of AoP for same PSI. Averaged preferred AoP from dorsal to posterior edge. The black line indicates fit for pooled data for MeTu-DRA2 (N=10 recordings). **F** Measured AoP in MeTu-DRA1s (green) in the AOTU for one example fly and Measured AoP in MeTu-DRA2s (red) in the AOTU for one example fly. **G** Predicted sensitivity of MeTu-DRA1 (green) and MeTu-DRA2 (red) dendrites in the medulla in comparison to Dm-DRA1 (purple) sensitivity.

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the AOTU (Figure 4D,E).

The AOP tuning map obtained for Dm-DRA1 cells nicely reproduced previously published fan-like representation of AOPs along the MEDRA (Figure 4C)(Hardcastle et al., 2021). Interestingly, we failed at recording MeTu-DRA1 and MeTu-DRA2 responses in the medulla as well, the reason remaining unclear (low GCaMP6 expression being one possibility, or the absence of AOP modulation in MeTu dendrites being another). In the AOTU, MeTu-DRA1 responses manifested clear PSI values that were stronger than those of MeTu-DRA2 cells. In addition, both DRA-specific MeTu cell types revealed topographic responses within the AOTU, similar to what had previously been published (Figure 4D,E)(Hardcastle et al., 2021). These responses are in good agreement with the topographic projection pattern from MEDRA to the AOTU, meaning that the fan-like arrangement of AOPs from posterior to anterior within the MEDRA is represented along the dorsoventral axis in the AOTU. Interestingly, our experiment revealed that MeTu-DRA2 responses were rather different from MeTu-DRA1 cells: Within the latter population, we observed a smooth topographic representation of AOPs within the AOTU, roughly covering 180° of AoPs (Figure 4D,F). In contrast, MeTu-DRA2 responses consisted of two preferred AOPs separating the dorsal half from the ventral half of the AOTU, with a rather sharp transition between the two (Figure 4E,F). Hence, despite their topographic projection pattern, MeTu-DRA2 cells appeared to fall into two functionally rather homogeneous classes (posterior versus anterior, in the medulla), responding to roughly orthogonal AOPs. Further, when taking the measured AOPs of MeTu-DRA cells in the AOTU and projecting it into a sensitivity into the medulla, a sensitivity shift of MeTu-DRA cells to Dm-DRA1 arises (Figure 4G), suggesting a difference in cellular processing of the same signal. These results and the few differences between the DRA-specific MeTu cells we characterized morphologically hint at different roles in the perception and processing of polarized UV light.

MeTu-DRA1 are necessary for polarotactic behavior

Next, we decided to investigate the importance of both MeTu-DRA subpopulation for the fly's ability to use polarized skylight for navigation, We, therefore, tested single flies during tethered flight in a custom-made virtual flight arena (Mathejczyk and Wernet, 2020)(Figure 5). In the past, we and others have shown how flies can use a moving polarized UV stimulus presented from above as a reference for setting a stable heading, referred to here as polarotactic orientation behavior (Warren et al., 2018; Mathejczyk and Wernet, 2019). As a population, control genotypes (driver vs effector) consistently manifested robust orientation responses (including the occasional flies not caring about the stimulus, as previously reported). In all cases, we could not detect any statistically significant differences between both control lines. Flies expressing the temperature-sensitive, dominant negative effector $shi^{[ts]}$ at 30°C in MeTu-DRA1 manifested a very mildly reduced polarotactic performance, meaning these flies were still mostly able to use the polarized stimulus to select a stable heading (Figure 5A). Similarly, expression of $shi^{[ts]}$ under the same conditions in MeTu-DRA2 cells resulted in no detectable decrease in behavioral performance (Figure 5B). In contrast, when silencing both MeTu-DRA1 and MeTu-DRA2 cell populations together, we observed a highly significant loss in the polarotactic behavior, resulting in a loss of the flies' ability to perform polarotaxis (Figure 5C). This significant decrease in orientation behavior indicates both MeTu-DRA subpopulations together are necessary for navigation behavior, irrespective of whether they process skylight polarization information differently or in the same way. Given our behavior findings, our physiological data, and the morphological differences we had characterized, we wondered how such minor morphological differences between MeTu-DRA1 and MeTu-DRA2 subtypes could result in physiological and behavioral differences.

Early binocular integration in MeTu-DRA cells

In order to identify potential differences in medulla connectivity between MeTu-DRA1 and MeTu-DRA2 that could explain the apparent differences of their physiological responses, we turned to the currently existing connectomic data (Garner, Kind et al, in preparation). Upon classifying their synaptic inputs, we discovered a rather difference between these cells. To our surprise, MeTu-DRA1 cells are ipsilaterally presynaptic to a specific interhemispheric cell type that directly connects the DRA regions of both medulla neuropiles without making any synaptic connections in the central brain, hence called a MeMe-DRA (Kind et al., 2021)(Figure 6A-C). Two MeMe-DRA cells exist in each hemisphere, where both their ipsilateral dendrites, as well as their presynaptic terminals, cover either the anterior or posterior half of the DRA, with one given cell connecting the anterior half of one DRA with the posterior half on the other side, and vice versa (Figure 6B). Interestingly, we found MeTu-DRA2 cells on the contralateral side to be post-synaptic to MeMe-DRAs, identifying this cell type as binocular units potentially integrating information from both DRAs, whereas MeTu-DRA1 receives exclusively ipsilateral DRA input. Using the neurotransmitter prediction tool from FlyWire, the MeTu-DRA1 neurotransmitter was predicted to

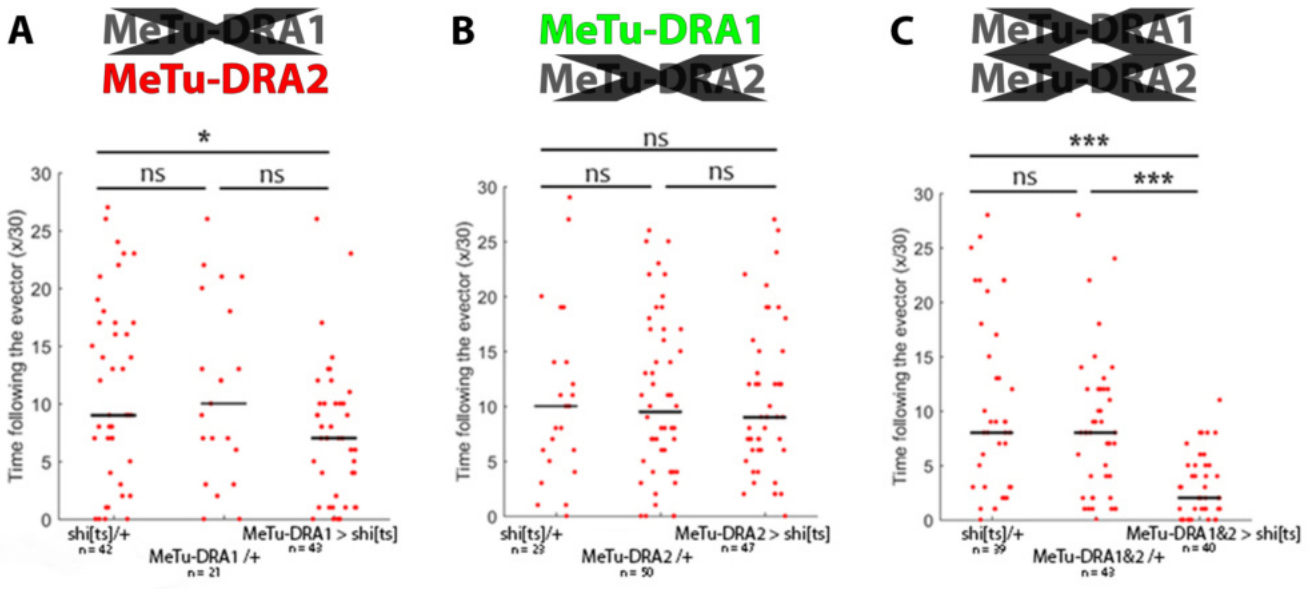

Figure 5: MeTu-DRA1 is necessary for polarotactic behavior
A Quantified performance index for single flies in free-flying setup. Silenced MeTu-DRA1 (MeTu-DRA1 > shi$^{[ts]}$) showed significantly reduced performance index for polarotactic behavior. **B** Quantified performance index for single flies in free-flying setup. Silenced MeTu-DRA2 (MeTu-DRA2 > shi$^{[ts]}$) showed no significant changes in polarotactic behavior. **C** Quantified performance index for single flies in free-flying setup. Silincing MeTu-DRA1 and MeTu-DRA2 together (MeTu-DRA1, MeTu-DRA2 > shi$^{[ts]}$) showed significant changes in polarotactic behavior.

be cholinergic, whereas the prediction for MeTu-DRA2 was more variable (Supplemental Figure 4A,B). Light microscopic techniques for investigating the MeTu neurotransmitter were unsuccessful since immunohistochemical stainings against the vesicular Acetylcholine transporter vAChT were inconclusive (Supplemental Figure 4C). Therefore, The current connectomic predictions represent the most robust predictions and hint at MeTu-DRA1 outputs onto MeMe-DRA ipsilaterally, as well as MeTu-DRA1 and MeTu-DRA2 outputs onto TuBu neurons in the AOTU to be excitatory.
Via four MeMe-DRA cells, polarized skylight information, is integrated binocularly at a very early stage processing along the 'anterior visual pathway,' including a very specific exchange of information along the anterior-posterior axis of both eyes. Unfortunately, we could not further characterize either morphology, functional properties or behavior relevance of MeMe-DRA cells since we did not have access to a specific driver line. Connectomic neurotransmitter prediction revealed that MeMe-DRA cells are most likely gabaergic (Figure 6D). Hence, upon stimulation on the ipsilateral side, MeMe-DRA should specifically inhibit MeTu-DRA2 cells located in the contralateral optic lobe. Although calcium imaging using GCaMP-style indicators is considered not ideal for visualizing inhibitory signals, we decided to test this prediction. Using fine light guides, we presented unpolarized UV light flashes to either the fly's ipsilateral or contralateral eye while imaging only on the ipsilateral side (Figure 6E). We expected cells that only receive information in the ipsilateral eye (e.g., Dm-DRA1 and MeTu-DRA1) to respond exclusively to UV light which is presented ipsilaterally but not contralaterally. However, MeTu-DRA2 cells, which the connectome predicts to differ from these cells in that they receive contralateral information via MeMe-DRA,

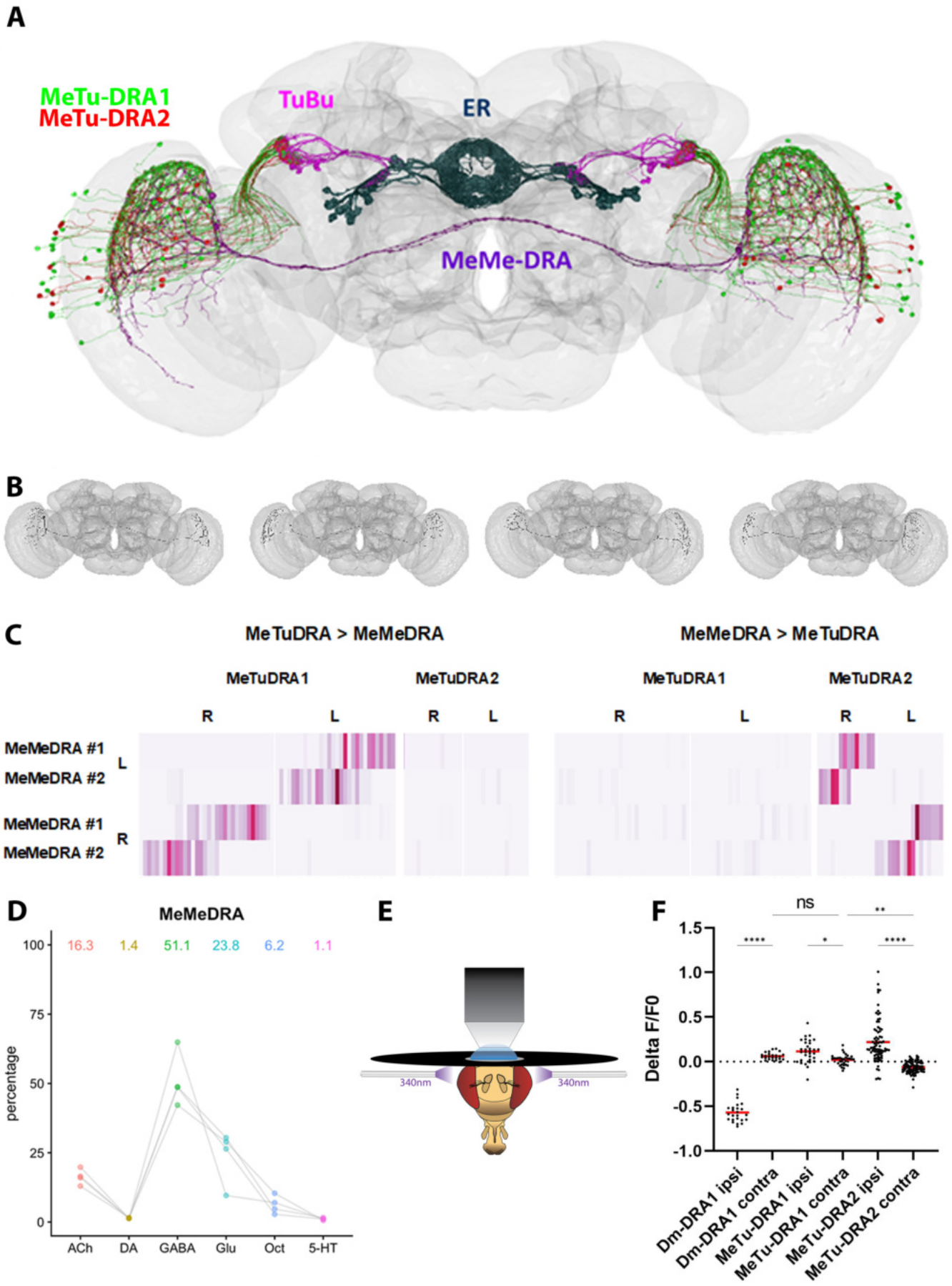

Figure 6: DRA specific, contralateral connecting Medulla neurons

Figure 6: **A** Connectomic reconstruction neurons in the Anterior Visual Pathway for navigation. Medulla to Medulla (MeMe, purple) connecting neurons are reconstructed for both optic lobes. MeTu-DRA1 (green) and MeTu-DRA2 (red) are reconstructed in both optic lobes. Post-synaptic neurons to MeTu-DRAs are connected to Tubercle to Bulb (TuBu, pink) and finally projecting to Ring neurons (ER, dark green). *B* Reconstruction of all MeMe-DRA neurons as single examples. MeMe projecting: anterior Medulla right ⇒ posterior Medulla left, posterior Medulla right ⇒ anterior Medulla left, posterior Medulla left ⇒ anterior Medulla right, anterior Medulla left ⇒ posterior Medulla right, respectively. **C** Synaptic connectivity between MeTu-DRAs and MeMe-DRAs. MeMe-DRAs receive input from MeTu-DRA1 and project on to MeTu-DRA2. **D** Neurotransmitter prediction of MeMe-DRA cells, based on connectome prediction. **E** Schematic of physiological SetUp for Contra-Ipsi-lateral stimulation. **F** Quantification of Ipsi-Contra-Lateral UV flashes. (Dm-DRA1 ipsi - Dm-DRA contra: ****p<0.0001, MeTu-DRA1 ipsi - MeTu-DRA1 contra: *p=0.0102, MeTu-DRA2 ipsi - MeTu-DRA2 contra: ****p<0.0001, Dm-DRA1 contra - MeTu-DRA1 contra: ns p=0.2970, MeTu-DRA1 contra - MeTu-DRA2 contra: **p=0.0073)

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should also manifest (inhibitory) responses upon contralateral stimulation. We therefore presented the UV stimulus for 4sec each to both eyes consecutively and then calculated the mean $\Delta F/F$ (Figure 6F). As expected, we observed that Dm-DRA1 cells show a drastic decrease in $\Delta F/F$ when stimulated ipsilaterally but no response when stimulated contralaterally. This result agrees with previous data showing that Dm-DRA1 cells are direct targets of histaminergic DRA photoreceptors (Hardcastle et al., 2021). In contrast, MeTu-DRA1 cells manifested a robust increase of $\Delta F/F$ upon stimulation with ipsilateral UV light, thereby differing significantly from Dm-DRA1 cells. However, MeTu-DRA1 cells did not manifest any significant change in fluorescence when the contralateral eye was stimulated with UV light. When imaging MeTu-DRA2 cells, we could observe a wide variation of responses: First, when presented with ipsilateral UV light, we did observe an increase in fluorescence, similar to what we had observed for MeTu-DRA1. Fascinatingly, once we presented the fly with UV light from the contralateral side, we observed a small but significant decrease in fluorescence in MeTu-DRA2 cells, which presents a significant difference to Dm-DRA1 and MeTu-DRA1 responses. It appears, therefore, that MeTu-DRA2 integrate DRA signals from both eyes via MeMe-DRA, resulting in a deterioration of their topographic representation of AOPs in the AOTU, whereas MeTu-DRA1 act as a purely ipsilateral units that faithfully represent the topography of the DRA into the AOTU.

In summary, our light microscopic, connectomic, and physiological data together allowed for a clearer insight into the binocular processing of polarized skylight. Here, we show how two types of visual projection neurons in the periphery of the visual system differentially connect to an interhemispheric neuron, thereby resulting in different forms of integration of the same celestial cue (Figure 7). These results provide a first description of how binocular integration of skylight contributes to the parallel processing of different celestial cues on their way from the eye to the central brain via the AOTU.

Discussion

Polarization vision is a remarkable sensory capacity utilized by numerous organisms for navigation, communication, and foraging in their environments (Nilsson and Warrant, 1999; Wehner,

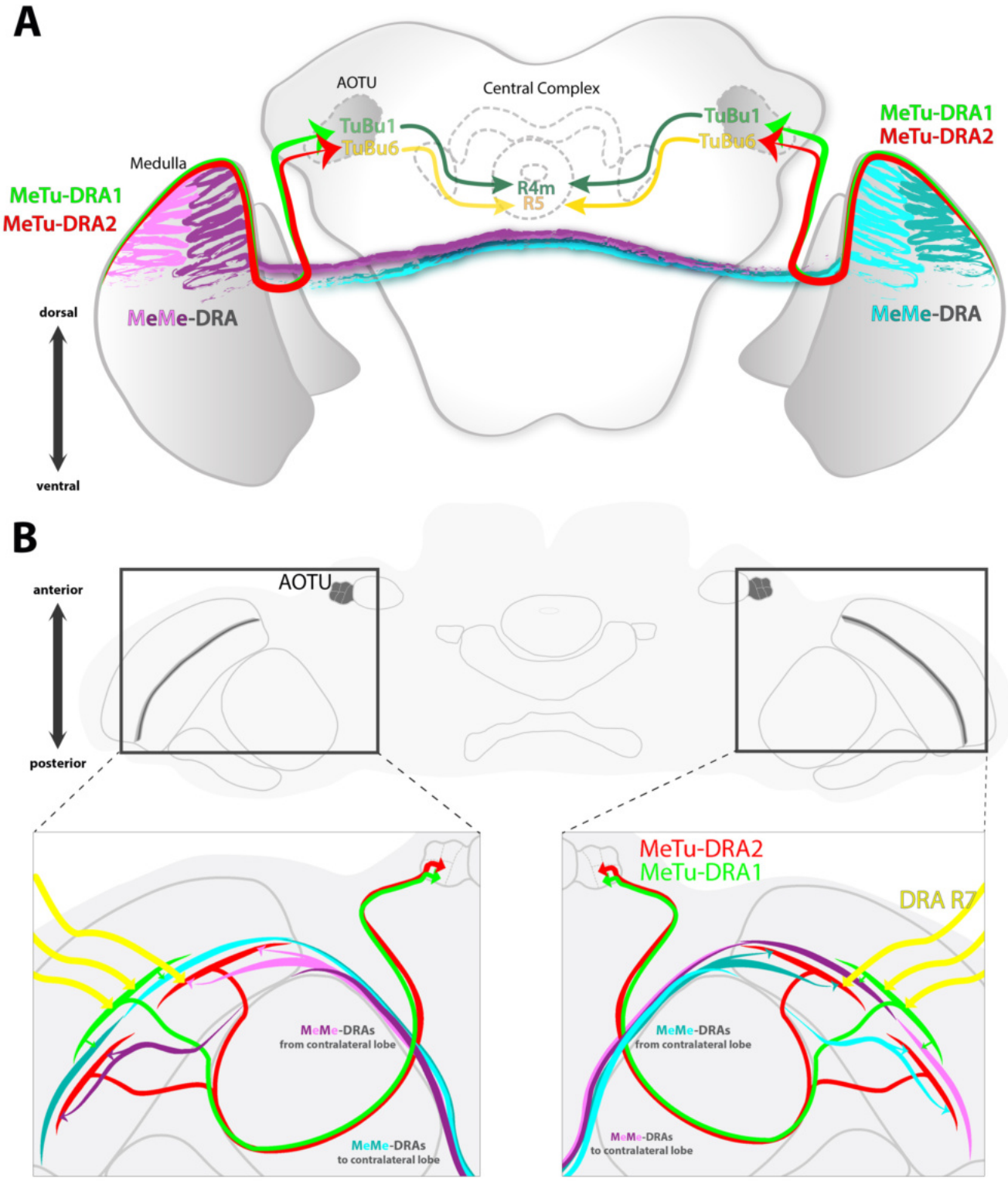

Figure 7: Early binocular integration of polarized skylight
A Anterior view onto adult *Drosophila melanogaster* brain. MeMe-DRA neurons (pink, purple, cyan, turquoise) project to the contralateral Medulla. MeTu-DRA1 (green) and MeTu-DRA2 (red) project ipsilateral from Medulla to AOTU. MeTu-DRA1 connects to TuBu1 (dark green) and TuBu6 (yellow) neurons, and MeTu-DRA2 connects only to TuBu6. TuBu1 and TuBu6 project finally onto Ring neurons R4m (dark green) and R5 (yellow), respectively. **B** Dorsal view of adult *Drosophila melanogaster* brain. Highlighted in the dark are the Medulla M6 layers in the optic lobes and a small unit of AOTUs. Boxes indicate the area where the DRA-specific neurons integrate. Detailed neuronal connectivity of boxed areas are depicted below. DRA.R7 (yellow) connect mainly to MeTu-DRA1 (green) ipsilateral. MeTu-DRA1 connects to MeMe-DRAs ipsilateral (MeMe-DRA left: cyan and turquoise; MeMe-DRA right: purple and pink). MeMe-DRAs project contralateral with anterior-posterior flip on to MeTu-DRA2 (red). MeTu-DRA2 project ipsilateral to small unit of AOTU.

2001). Recent research using *Drosophila melanogaster* as a model system has unveiled the existence of specialized visual projection neurons called MeTu neurons (Omoto et al., 2017; Timaeus et al., 2020; Hardcastle et al., 2021; Tai et al., 2021). Some of these neurons are specifically tuned to polarized light signals and project axons to a discrete region in the AOTU. Here, we have shown that a specific sub-class named MeTu-DRA neurons with dendrites in the MEDRA play a crucial role in the fly's utilization of polarized light for navigation, making them essential study subjects to understand the neural basis of insect orientation. We adopted a multidisciplinary approach, integrating anatomical analyses, physiological recordings, and behavioral experiments to investigate the role of MeTu-DRA neurons in polarization vision and navigation. Our light microscopic investigations revealed the existence of two quite similar-looking subtypes of MeTu-DRA, named MeTu-DRA1 and MeTu-DRA2, each exhibiting specific morphological characteristics and differences in connectivity upon closer investigation. Importantly, both subtypes displayed an innervation pattern into the AOTU that results in a topographic representation of the anterior-posterior axis of the dorsal rim area (DRA) along the dorsoventral axis of the anterior optic tubercle (AOTU).

Here, we show that the physiological responses of MeTu-DRA1 and MeTu-DRA2 cells to polarized UV light are different. In the AOTU, MeTu-DRA2 cells showed a broader response compared to MeTu-DRA1 cells, their presynaptic terminals clustering into two groups representing largely two orthogonal AoPs. We wondered how minor differences in morphology and input connectivity could result in different physiological responses. Although our behavioral studies underlined the critical role of both MeTu-DRA cell types in the fly's navigation ability, they also hinted towards potential differences in their necessity for mediating these responses since inactivation of MeTu-DRA1 alone (but not MeTu-DRA2 manifests a mild phenotype). So far, a functional role for MeTu cells (called MC61 or MC64) was demonstrated in color vision: Inactivation of MeTu neurons results in a wavelength-specific loss of phototaxis when testing flies in a choice assay using different combinations of light (Otsuna et al., 2014). However, these experiments did not differentiate between different MeTu types, as they were later described (Omoto et al., 2017; Timaeus et al., 2020; Tai et al., 2021). Only more recently, MeTu-DRA neurons were characterized for the first time (Kind et al., 2021), and despite their modality-specific morphology suggesting a role in skylight navigation, their functional role has so far not been addressed. Anatomical differences between MeTu types suggest parallel processing of different skylight cues along the 'anterior visual pathway', leading to the representation of different features in different ring (ER) neuron types (Hulse et al., 2021). The discovery of MeTu-DRA cells now allows for assigning specific functional features to one of these parallel streams of information. In fact, this pathway appears to be bifurcated into MeTu-DRA1 and MeTu-DRA2 sub-channels with different properties when it comes to binocular integration.

Binocular integration is a vital mechanism that allows insects to combine and compare visual input from both eyes, extracting comprehensive and presumably more precise information in their environment (Linneweber et al., 2020). For accurate depth perception, binocular vision is essential

to an animal (Rosner et al., 2019). In ants, the transfer of learned information between the two eyes, so-called inter-ocular transfer' has also been investigated in the context of navigation (Wehner and Müller, 1985). Combined with polarized light cues, binocular vision is considered crucial for navigation and orientation tasks, such as determining the position of the sun or skylight polarization patterns (Heinze and Homberg, 2007; Labhart and Meyer, 1999). By comparing slightly different visual scenes detected from each eye, insects might be able to more accurately infer the position of celestial bodies (Read, 2021; Rosner et al., 2020). This could be vital for maintaining a specific course during time-compensated navigation. On one hand, binocular integration could enhance the sensitivity of polarized UV light detection. Since each eye contributes to the overall visual input, insects could detect weaker polarized light signals more effectively, which is particularly beneficial in challenging or low-light environments (Warrant and Dacke, 2018; Warrant, 2017). More importantly, binocular integration increases the robustness when interpreting polarized skylight cues, allowing insects to compensate for environmental variations such as changes in skylight polarization patterns due to atmospheric conditions or cloud cover (Homberg et al., 2023; Horváth, 2014). Despite behavioral and physiological data dealing with binocular integration during navigation keeps increasing, the underlying circuit mechanisms remain poorly understood. Part of the problem lies in the fact that not too many binocular neuron types connecting both optic lobes have so far been described in detail. One exception is 'dorsal cluster neurons' with direct connections between the medulla and/or lobula neuropiles of the two sides. Inactivation of these neurons results in walking flies losing the ability to fixate a black stripe (Linneweber et al., 2020). Large-scale connectomic efforts now allow for a systematic characterization of all binocular neuron types (Dorkenwald et al., 2023), which will enable similar characterization of their functions.

Here we show that MeMe-DRA binocular neurons are connected to both MeTu-DRA1 (their input) and MeTu-DRA2 (their output) in a very specific manner. In fact, this difference in connectivity is one major connectomic difference between these neurons, in addition to differences in TuBu connectivity (Garner, Kind et al, in preparation). So far, MeMe neurons have been described in locusts, where they were shown to be sensitive to both skylight polarization as well as an artificial celestial body, suggesting that these cells could be integrating these navigational stimuli (el Jundi et al., 2011). The FlyWire neurotransmitter prediction, together with our physiological responses to ipsi- versus contralaterally presented UV flashes, indicate an inhibitory influence of MeMe-DRA cells onto MeTu-DRA2 cells (but not MeTu-DRA1) in the medulla. Both connectomic and light microscopic anatomy revealed that MeMe-DRA cells in one optic lobe cover either the anterior or posterior dorsal half and project to the contralateral side with a flip along the anterior-posterior axis Kind et al. (2021). Anatomy of MeMe-DRA cells suggests that they should average the angles of polarizations (AoPs) from either the anterior and posterior halves of one optic lobe's DRA and then integrated with the contralateral side, representing early binocular integration of polarized skylight information. As a result, a MeTu-DRA2 cell in the contralateral medulla must receive averaged information from either the anterior or posterior half of the ipsilateral side. This should result in a deterioration of MeTu-DRA2's topographic signals,

which result from their ipsilateral inputs (DRA-R7 and Dm-DRA1). Indeed, we measured exactly such a split of MeTu-DRA2 cells responses into two predominant AoPs in the dorsal vs the ventral half of the AOTU, whereas MeTu-DRA1 displayed smooth topography there. Hence, in the AOTU, MeTu-DRA2 cells represent either posterior or anterior MEDRA signals, respectively. When considering the rather elaborate organization of visual fields and preferred AoPs of DRA ommatidia within one DRA (Weir et al., 2016; Hulse et al., 2021), such a binocular integration pattern could be beneficial for detecting the plane of symmetry of the surrounding skylight polarization pattern and by this way, the position of the sun. Hence, MeMe-DRA cells might play a role similar to what has been proposed for MeMe cells in locusts, where these cells were proposed to detect solar elevation and azimuth in order to provide a differentiated zeitgeber signal (el Jundi et al., 2011, 2014).

In conclusion, our findings provide critical, important new insights into the functional properties and circuitry of MeTu-DRA neurons in Drosophila melanogaster, contributing to our understanding of how binocular integration contributes to the neural mechanisms underlying polarization vision and navigation. The distinct characteristics of MeTu-DRA1 and MeTu-DRA2 cells suggest separate, similar yet distinct computations of polarized light signals, resulting in a smoothly topographic ipsilateral channel (MeTu-DRA1) and a second channel where ipsi- and contralateral DRA-information are mixed, thereby breaking the smooth topography into anterior versus posterior signals (MeTu-DRA2). Moreover, their topographic representation in the AOTU demonstrates how insects process and integrate visual information for navigation. Future studies investigating neural computations and synaptic interactions between MeTu-DRA and MeMe-DRA cells and their connections with other visual circuit elements will deepen our understanding of polarization vision and its significance in insect behavior. Additionally, the parallel processing and early binocular integration observed mediated by different MeTu neuron types may reveal shared mechanisms have broader implications for sensory processing amongst other organisms and offer valuable insights into navigation and orientation principles across diverse species.

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Material and Methods

Immunohistochemistry

All dissected flies were between 3-7 days old and were raised at 25°C with 12/12h light/dark. Flies were dissected in ice-cold Schneider's Medium, fixated in 4% PFA for 20min RT. Followed by extensive washing with 0.4% PBS-T [PBS with 0.5% (v/v) Triton X-100 (Sigma Aldrich, # X100)] fixed. Fixated brains were incubated in primary solution containing 10% Normal Donkey Serum (NDS) and different combinations of goat anti-GFP 1:1000 (abcam, ab6673), rat anti-GFP 1:1000 (BioLegend, 338002), rabbit anti-CD4 1:600 (Novus Biotech, NBP1-86143), rb anti-HA 1:500 (BioLegend, 902302), chicken anti-FLAG 1:1000 (Novus Biotech, NB600-343), rb anti-V5 1:500 (BioLegend, 903801), rat anti-OLLAS 1:30 (Novus Biotech, NBP1 06713), rb anti-DsRed 1:500 (Clontech, 632496), ms nc82 1:30 (DSHB), ms 24B10 1:100 (DSHB), rat anti nCad 1:100 (DSHB). Incubation was done for 2x O/N at 4°C, followed by extensive washing with 0.4% PBS-T at RT. According to primary antibodies, the secondary antibodies (all Jackson Immuno Research; 1:500) were chosen from D anti-goat Alexa488, D anti-rat Alexa488, D anti rb Alexa488, D anti-rabbit Cy3, D anti-rat Alexa594, D anti-ms Cy5, D anti-chicken Cy5 or, D anti-goat Cy5. Incubation was performed O/N at 4°C and followed by extensive washing. For MCFO, additional staining with rabbit anti-V5-Dylight 549 1:1000 (Rockland, 600-442-378) at RT for 2h was followed by washing. All confocal microscopy was performed with Leica SP8-X white light laser and SPE-5 microscopes. The 63x objectives were used to acquire image stacks in the resolution of 1024x1024 for imaging in the optic lobe or 1024x510 for images of the AOTU.

MCFO experiments:

To acquire single-cell clones MultiColor FlpOut experiments were performed as described by Sancer et al. (2019). Summarized, 3d-old flies were shocked in a 37°C water bath for 30 minutes to induce the flippase (FLP). Afterward, the flies were kept for 3 days at 25°C 12h/12h light/dark incubator to allow the expression of the reporter. Dissection, fixation, and staining were performed as described above.

GRASP:

GRASP experiments were performed as described by Sancer et al. (2019). In short, flies for activity GRASP were raised at 25°C 12h/12h light/dark incubator and transferred to custom-made UV-transparent Plexiglas tubes before light induction. To ensure that photoreceptors in the DRA are activated sufficiently, 1d old flies were kept in a custom-made lightbox (at 25°C, 10h/4h light/dark). Dissection, fixation, and staining were performed as described above. To label the GRASP signal, as well as presynaptically cells, monoclonal GFP antibody (anti-GFP rat mAB) and polyclonal GFP (anti-GFP goat pAB), were used, respectively. CD4 antibody was used to visualize the postsynaptic cells.

BRP single-cell clones:
<1d old flies containing hs-flp, Tub-FRT-Gal80-FRT-stop, UAS-BRPD3::GFP were shocked in 37°C water bath for 10-30min. Shocked flies were incubated at 25°C 12/12 l/d. 3d old flies were dissected, fixated, and stained as described above.

DREP2 quantification:
To extract the DREP2 signal specific to the signal in MeTu-DRA branching in the medulla, the surface function of IMARIS was used. The surface function was used on the Tomato signal in the medulla. Projections from the cell body into the medulla were cut right above the branches in the medulla, as well as projections from the medulla branching to the AOTU right below the branches. The masking function was used to duplicate the DREP2::GFP signal only in the masked area. The image was transferred to ImageJ by using the in-build function of IMARIS. In ImageJ, the segmented line tool was used to follow the Tomato signal in the medulla from dorsal edge to the equatorial region. The covered region for Tomato and DREP2::GRP signal was extracted using the plot profile function. Extracted values were normalized and plotted in PRISM. Test for normal distribution and binned frequency distribution was performed in PRISM as well.

Topographic quantification (MCFO):
Single cells were extracted by using IMARIS and Matlab Channel arithmetic code. IMARIS was used to produce Skeletons based on the single cell clones. Scholl analyzes was used to extract positioning, angles, number of branches and PR connection.

BRP single-cell clones quantification:
To extract BRP puncta, IMARIS surface and spot function was used. A surface channel for the Tomato signal in the AOTU was created (Surface Grain Size =0.200 um), and by masking a new channel with BRP::GFP signal in the covered area was duplicated. To find the BRP::GFP puncta, the SPOT function of IMARIS was used (Estimated XY Diameter =0.400 um, Estimated Z Diameter =0.800 um). All spots were afterwards manually confirmed or rejected. By the spot function detected and counted number of BRP puncta was transferred to PRISM and plotted.

Calcium Imaging
All used flies were between 3-7 days old and female. They were raised at 25°C in 12/12h, light/dark. Flies were collected while 1d old and kept in non-crowded housing. For preparation, flies were anesthetized on ice and mounted in a custom-made holder built from a metal plate attached to a 3D-printed holder. Flies were glued to the plate with Bondic UV glue, and preparation was performed using breakable blades and forceps. The proboscis was immobilized by cutting the muscle. Calcium imaging was performed with oxidized 3mM solution after Weir et al. (2016).
To fit the rotating polarizer for stimulation under the stage, The Leica SP8 MP was modified. The

bright light condenser lens and holder were removed. In the resulting space, a custom 3D-printed housing for the motorized polarizer sheet and UV lamp could be positioned. UV light (Roschwege Star-UV365-01-00-00) was presented through diffusing paper, and a 50mm wide polarizer filter (HNPB replacement from Knight Optical) and positioned 50mm in front of the fly. Rotations of the polarizer sheet were driven by a Dynamixel MT28-T motor operated through an Arbotix-M. Stimulation was performed with in 30°/4sec steps. The recordings were performed using the Leica 25x/0.95 water immersion objective and a Coherent tuneable Laser set to 920nm to excite tomato and GCaMP simultaneously. The Tomato channel was recorded as a reference channel and used for post-recording movement correction. Images were 256x256 pixels, and volumes were recorded with a z-step of 2.5µm - 4.5µm depending on the imaged area, trying to keep a speed of 20Hz.

Ipsi-Contra-later stimulation:

GCaMP flies were mounted as described above. Due to the lag of space under the Leica SP8 MP, Fiber Optic Cannulae (Thorlabs CFMLC21L20) were positioned on both sides of the fly. Each cannulae was attached via a ceramic mating sleeve to a light guide and a Fiber-Coupled LED light (340nm) (Thorlabs M340F4f). Stimulation of ipsi or contralateral side was done for 4sec.

Calcium imaging analysis:

Recordings were movement corrected using Fiji Image stabilizer. And the background was subtracted. Images according to the polarizer position were extracted based on a feedback protocol of motor position. According to the image sequence, each pixel was fitted to a sinusoidal wave using custom code in MatLab (Ilić et al., 2018). Based on the fitted sinusoidal curve, a max and min fluorescence intensity was determined, which are in 90° intervals. based on this Intensity, the PSI was determined afterHardcastle et al. (2021) with as (F_{max}-F_{min})/(F_{max}+F_{min}). From pixel-wise PSI calculation, 3 ROIs along the dorsoventral axis were chosen, and the averaged PSI was plotted. For pixels over a chosen threshold of 0.2, the AoPs were calculated. Maximum sensitivity for AoPs of regions was color-coded and plotted with MatLab as well. AoP along the D-V axis of AOTUs were extracted from the AoP matrix, normalized, and plotted for with R. Figures were compiled with Photoshop.

Ipsi-Contra-later quantification:

The recordings for ipsi-contra lateral stimulations were movement corrected and background subtracted as above. ROIs of the same size were chosen manually along the DRA and one randomly in the background of the image for background verification. Custom-written code in R was used to calculate F0 as the mean of the values in each ROI in the first 'off' phase before stimulation. ΔF/F0 was calculated based on said F0 for each ROI, respectively. The temporal recording was plotted as an example fly. For each fly, the mean during the different stimulation phases was calculated. These means were accumulated and plotted. One-way ANOVA was performed to test

for differences in GCaMP signal between different stimulation phases.

Free flying fly:

It was mainly performed as in Mathejczyk and Wernet (2019) (Mathejczyk and Wernet, 2019).

Fly rearing:

Flies were crossed and grown at 25°C, 60% relative humidity in a 12h/12h light/dark cycle on standard cornmeal agarose food. To limit the population densities, vials were flipped daily.

Fly preparation:

All Experiments were performed at 30°C and 50% relative humidity. The experiment time was chosen to correlate with the evening activity peaks until one hour into the dark period within the respective rearing incubators. The flies were cooled on ice for the experiment and glued to a 10mm long, 100µm diameter steel pin so that a vertical positioning resulted in a natural flying angle. Before the actual experiments, the flies were allowed to recover for at least 20 min from the gluing procedure with a small piece of tissue to prevent flies from starting flying. The flies were air-puffed from below to initiate flying and start the experiment. This air puffing was performed up to three times per experiment when the fly stopped flying.

Flight simulator setup:

Virtual flight arena from Mathejczyk and Wernet (2019) was used. In short, flies glued to a pin were mounted between magnets to fly freely at 360°. A polarized UV light was presented from above through a rotatable filter cassette. This cassette holds a 50mm × 50mm sheet linear polarizer (OUV5050, Knight Optical, UK) with 13 layers of non-fluorescent diffuser paper. Polarized UV light of a collimated UV LED (365 nm: LCS-0365-13-B) was presented to the flies through the filter cassette with the polarizer sheet at the bottom. Rotation of the cassette holder resulted in a controlled change of different AoPs. Recordings were done for 5 minutes with a constant angular velocity of 5.97 deg/s while the fly was recorded. Control flies were tested first, followed by experimental flies.

Extraction of flight performance:

As described in Mathejczyk and Wernet (2019), a custom-written macro script for the open-source software Fiji was used to extract the heading of each fly in each acquired video frame. Summarized: each video was binarized, and an ellipse was fitted around the fly's body to extract the heading (range from 0° to 180°). The results from Fiji tracking were analyzed in MatLab. The circular statistics were used to quantify the polarotatic behavior of a fly's heading. The behavior was quantified if the mean difference between the angular velocity and the AoP angular velocity was smaller than 3°/s for a given 10s time window. The amount of 10s time windows in the 5min experiment that a fly followed the AoP was plotted for each individual.

Connectome reconstruction:

EM reconstruction

Reconstruction was performed as described in Kind et al. (2021). In short, The dataset by Zheng et al. (2018) comprises a serial section transmission electron microscopy (EM) volume of a female *Drosophila melanogaster* brain. The two auto-segmentation methods, FAFB-FFN1 (Li et al., 2020) and FlyWire (Dorkenwald et al., 2021), were employed to identify neurons, and if needed, certain fragments from the FAFB-FFN1 auto-segmentation were imported into the CATMAID (Saalfeld et al., 2009) environment, streamlining the identification process for a subset of neurons.
Locations (in MeTu-DRA1, MeTu-DRA2, and MeMe-DRAs) with a T-bar structure were annotated as pre-synaptic sides. The post-synaptic neurons with the main connection were identified if needed. The synaptic connectivity between MeTu-DRAs and MeMe-DRAs was reconstructed and plotted as heat maps.

Neurotransmitter prediction:

The neurotransmitter prediction for MeMe-DRAs, MeTu-DRA1, and MeTu-DRA2 was performed using FlyWire. Predictions were performed by using scribes from and as described in Eckstein et al. (2023).

Supplement

Table 1: Antibodies used

Reagent/Resource	Source	Identifier
Mouse anti-chaoptin (24b10)	DSHB	Cat# 24B10
Mouse anti-brp (nc82)	DSHB	Cat# nc82
Rat anti-HA	Biolegend	Cat# 902301
Rabbit anti-CD4	Atlas Antibodies	Cat# HPA0045252
Chicken anti-FLAG	Novus	Cat# NB69-343
Rat anti-GFP mAB	BioLegend	Cat# 338002
Rabbit anti-GFP pAB	Thermo Fisher Scientific	Cat# A-11122
Goat anti-GFP pAB	Abcam	Cat# ab6673
Rat cadherin, DN- (extracellular domain) antibody	DSHB	Cat# DN-EX#8
Rabbit anti-V5 Epitope Tag Antibody Dylight 549 conjugated	Rockland	Cat# 600-442-378
Donkey anti-Chicken Cy5	Jackson ImmunoResearch Labs	Cat# 703-175-155
Donkey Anti-goat Alexa Fluor 488	Jackson ImmunoResearch Labs	Cat# 705-545-147
Donkey anti-goat Cy5	Jackson ImmunoResearch Labs	Cat# 705-175-147
Donkey Anti-Rabbit Cy3	Jackson ImmunoResearch Labs	Cat# 711-165-152
Donkey Anti-Mouse Alexa Fluor 594	Jackson ImmunoResearch Labs	Cat# 715-585-151
Donkey Anti-Mouse Cy5	Jackson ImmunoResearch Labs	Cat# 715-175-151
Donkey Anti-Rabbit Alexa Fluor 488	Jackson ImmunoResearch Labs	Cat# 711-545-152

Table 2: Fly strains used

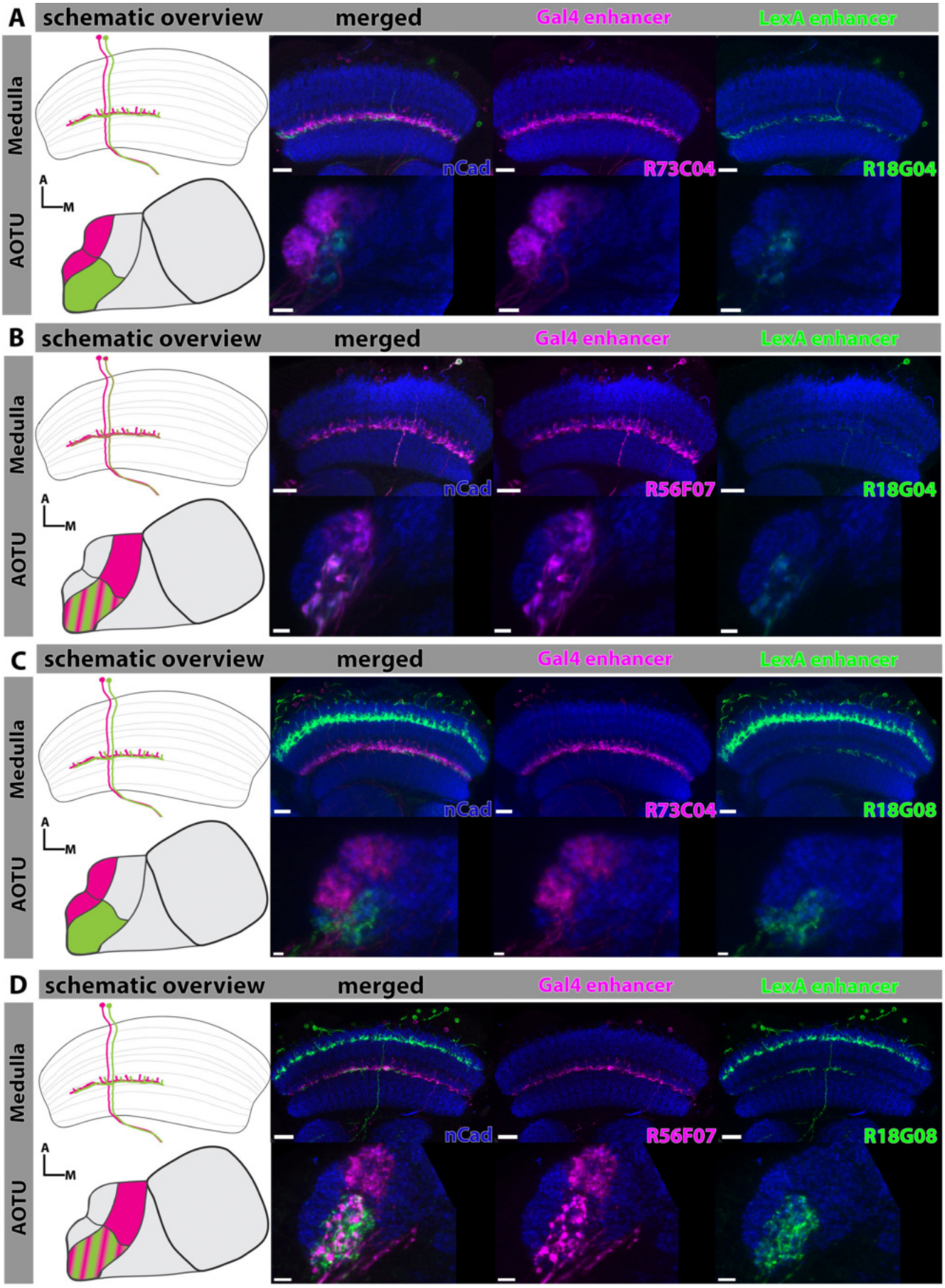
Genotype	Reference	Figure panel
Drosophila: longGMR-LexA	Sancer et al. (2019)	S.3A,B
Drosophila: rh3-lexA	Sancer et al. (2019)	Fig.1F,G
Drosophila: DRA.R8-LexA	Sancer et al. (2019)	Fig.1F,G
Drosophila: GMR18G04-Gal4	Bloomington Drosophila Stock Center	Fig.1G,I,O; Fig.2B,D,F,H,K; Fig3B.G,J; Fig.4E,F; Fig.5B,C; Fig.6F; S3B; S4C
Drosophila: GMR18G04-LexA	Bloomington Drosophila Stock Center	Fig.1B-D; S1A,B; S2A,D

Drosophila: GMR18G08-LexA	Bloomington Drosophila Stock Center	S1C,D; S2B,C
Drosophila: GMR56F07-Gal4	Bloomington Drosophila Stock Center	S1B,D
Drosophila: GMR73C04-Gal4	Bloomington Drosophila Stock Center	S1A,C
Drosophila: GMR13E04-Gal4	Bloomington Drosophila Stock Center	Fig.4C
Drosophila: GMR13E04-LexA	Bloomington Drosophila Stock Center	Fig.2A-F
Drosophila: InSite0871-Gal4	Gohl et al. (2011)	Fig.1B-F,H,N; Fig.2A,C,E; Fig.3A,G,J; Fig.5A,C; S.3A
Drosophila: SS00336	Gift from A. Nern	Fig.2G,I; Fig.4D,F; Fig.6F; S.2C,D; S.4C
Drosophila: MCFO-1	Nern et al. (2015)	Fig.1H,I,N,O; Fig.3A,B
Drosophila: hsFLP; tub-FRT-Gal80-FRT; UAS-brpD3::GFP	Sancer et al. (2019)	Fig.3G
Drosophila: UAS-nSyb-spGFP1-10, lexAop-CD4-spGFP	Bloomington Drosophila Stock Center	Fig.1F-G; S.3
Drosophila: lexAop-nSyb-spGFP1-10, UAS-CD4-spGFP11	Bloomington Drosophila Stock Center	Fig.1F-G; S.3
Drosophila: UAS-myrTom	Bloomington Drosophila Stock Center	Fig.1B-D; Fig.2A,B,G-K; Fig.3G; S.2A-D; 2A-D; S.4C
Drosophila: lexAop-mCD8::GFP	Bloomington Drosophila Stock Center	Fig.1B-D; Fig.2A,B,G-K
Drosophila: LexAop-tdTomato	Bloomington Drosophila Stock Center	S.2A-D; 2A-D
Drosophila: UAS-IVS-myr::smGdP-V5	Bloomington Drosophila Stock Center	S.2A-D; 2A-D
Drosophila: w+; UAS-GCaMP6f,UAS-TdTomato;+	gifted by Silies lab	Fig. 4C; Fig.6F
Drosophila: UAS-syt::GCaMP6s	Bloomington Drosophila Stock Center	Fig.4C-F
Drosophila: UAS-DREP2::GFP	Andlauer et al. (2014)	Fig.2G-K

Drosophila: trans-Tango	Talay et al. (2017)	Fig.3J
Drosophila: UAS-myr::GFP, QUAS-mtdTomato(3xHA)	Talay et al. (2017)	Fig.3J
Drosophila: UAS-shi$^{[ts]}$	Bloomington Drosophila Stock Center	Fig.5A,C
Drosophila: LexAop-shi$^{[ts]}$	Fisher et al. (2015)	Fig.5B,C

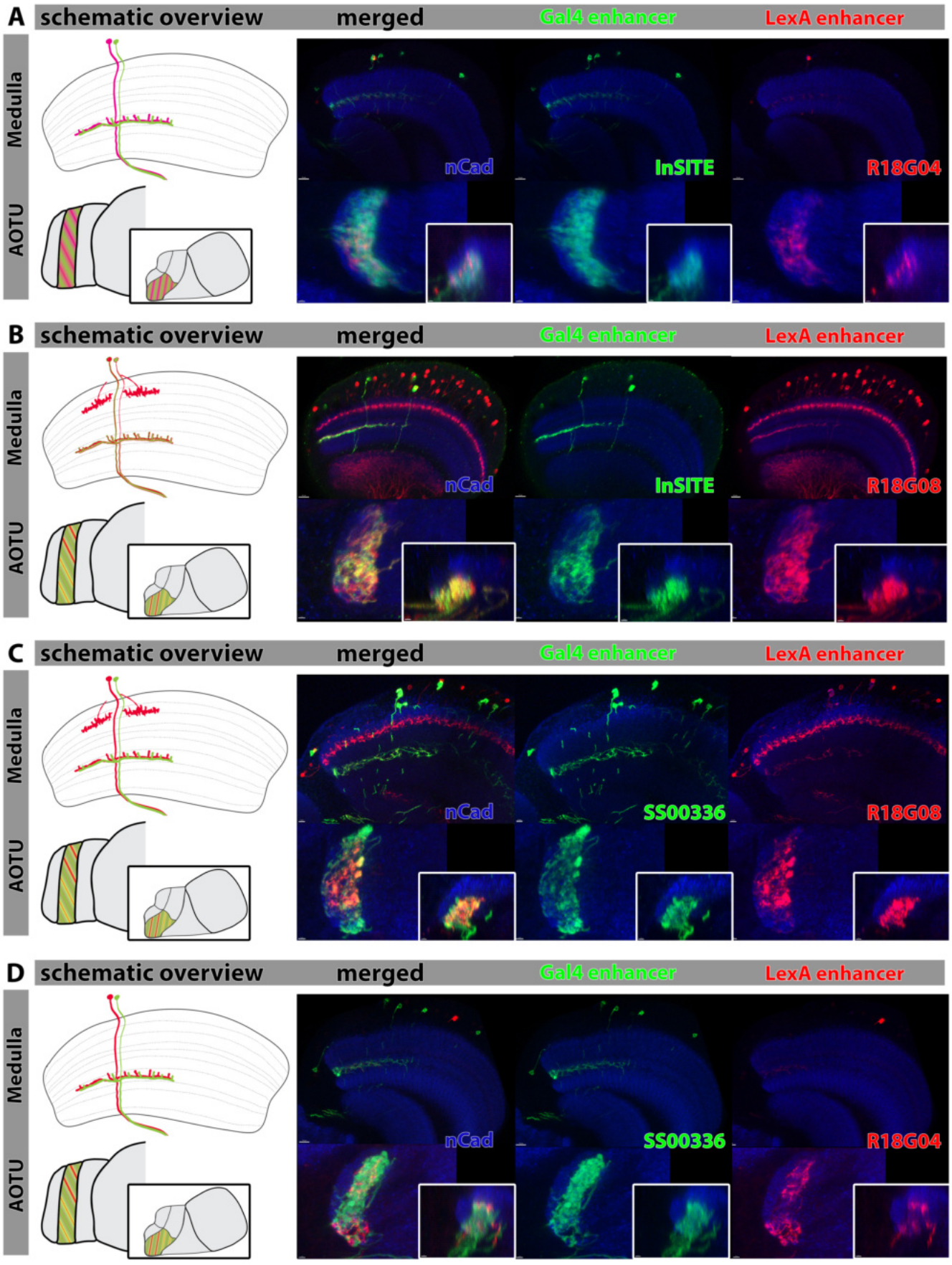
Table 3: Software used

Software	**Reference**
Leica Application Suite X	Leica Microsystems
Fiji	http://fiji.sc
R Project for statistical Computing	https://www.r-project.org/
MatLab R2020b	MathWorks
IMARIS	Bitplane AG
GraphPad Prism	GraphPad Software



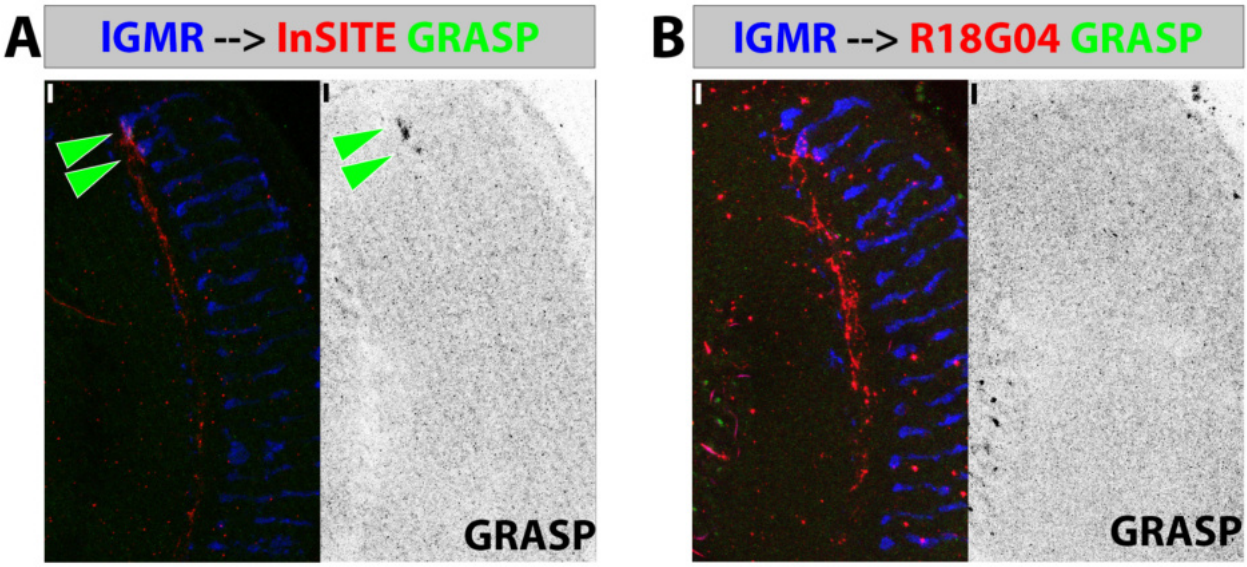
Supplement 1: Co-Labeling of different MeTu driver lines

Supplement 1: Schematic representation of real labeling pattern from medulla (blue) and AOTU (blue) of different Gal4 (red) and LexA (green) driver lines. **A** Co-labeling of R73C04 and R18G04-LexA (MeTu-DRA2). **B** Co-labeling of R56F07 and R18G04-LexA (MeTu-DRA2). **C** Co-labeling of R73C04 and R18G08-LexA (MeTu-DRA1). **D** Co-labeling of R56F07 and R18G08-LexA (MeTu-DRA1). Scales in medulla 10µm. Scales in AOTU 3µm.

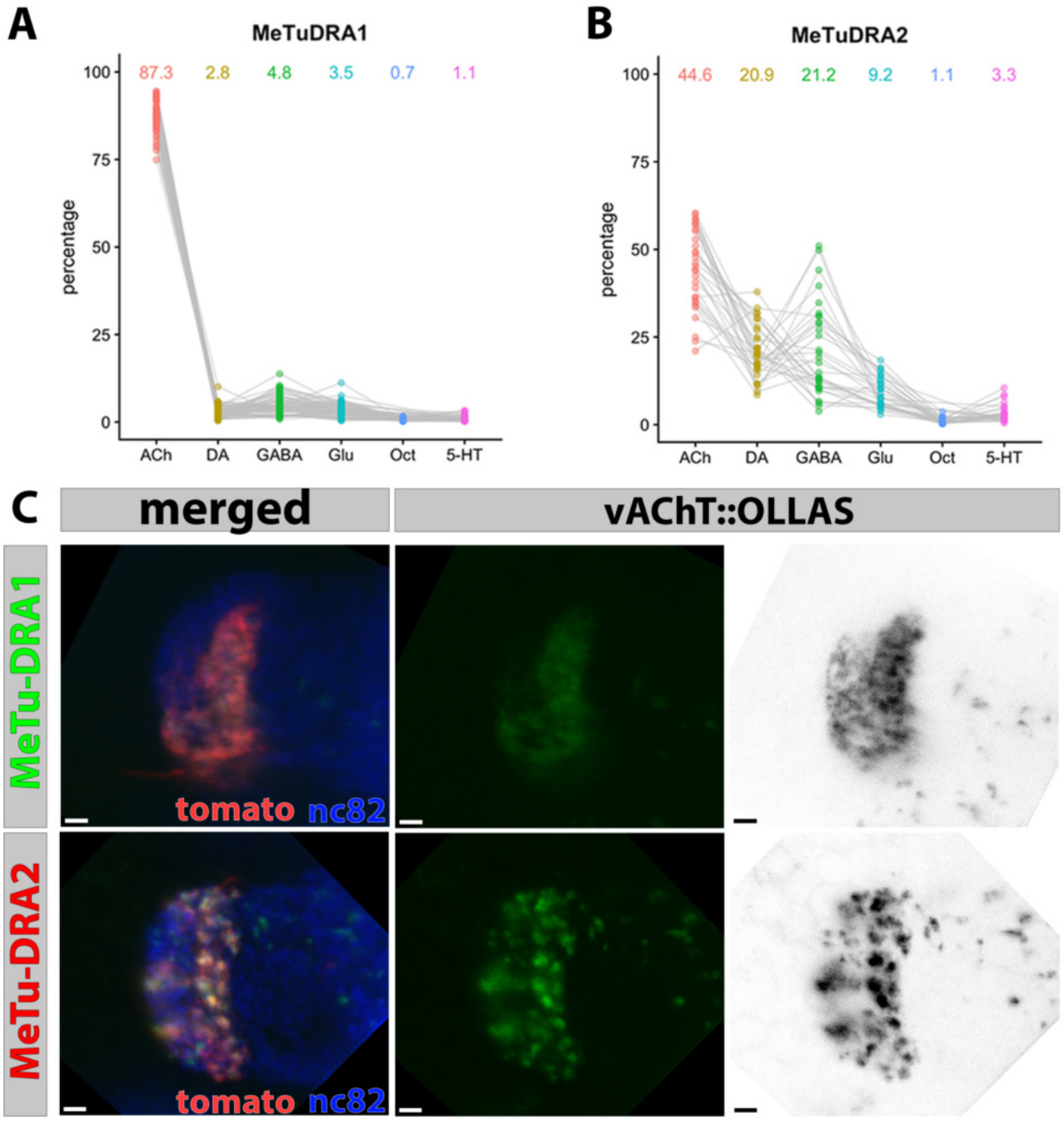


Supplement 2: Co-Labeling of MeTu-DRA specific driver lines

Supplement 2: Schematic representation of real labeling pattern from medulla (blue) and AOTU (blue) of different Gal4 (red) and LexA (green) driver lines. **A** Co-labeling of InISTE0871 (MeTu-DRA1) and R18G04-LexA (MeTu-DRA2). **B** Co-labeling of InISTE0871 (MeTu-DRA1) and R18G08-LexA (MeTu-DRA1). **C** Co-labeling of SS00336 (MeTu-DRA1) and R18G08-LexA (MeTu-DRA1). **D** Co-labeling of SS00336 (MeTu-DRA1) and R18G04-LexA (MeTu-DRA2). Scales in medulla 10µm. Scales in AOTU 3µm.



Supplement 3: GRASP connectivity of photoreceptors and MeTu-DRAs
A GFP reconstitution across synaptic partners. GRASP (green/grey) between lGMR presynaptically (blue) and MeTu-DRA1 (InSITE 0871, red) postsynaptically. Arrowheads highlighht DRA photoreceptors. **B** GFP reconstitution across synaptic partners. GRASP (green/grey) between lGMR presynaptically (blue) and MeTu-DRA2 (R18G08, red) postsynaptically. Scales 5µm.



Supplement 4: Neurotransmitter prediction for MeTu-DRA neurons
A Neurotransmitter prediction for MeTu-DRA1 by connectome. **B** Neurotransmitter prediction for MeTu-DRA2 by connectome. **C** Labeling of vAChT in MeTu-DRA terminals in AOTU. Top row MeTu-DRA1, bottom MeTu-DRA2. Labeled MeTu-DRA cells (red), neuropil (blue), and vAChT::OLLAS (green/grey). Scales 3µm.

Part III

Discussion & Future Direction

7 General Discussion

Animals need to navigate the environment to find new food sources or mating partners. To do that, their brains must process and integrate information the visual system provides so the animal can behave appropriately. As brains are built up by thousands of single neurons, understanding how the rules and mechanisms underlying this neuronal computation is of particular interest. For this thesis, the ability of most insects to detect polarized skylights for navigational purposes provided a relatively straightforward platform for studying important aspects of this process.

The visual system of *Drosophila melanogaster* consists of optically isolated unit eyes called ommatidia, which are repeated approximately 750 times across the adult retina, allowing the sampling of individual points in space. Within the *Drosophila* retina, different ommatidial subtypes with specialized inner photoreceptors (R7 and R8) are adapted for detecting either color (in the central retina) or skylight polarization (in the dorsal rim area (DRA). Visual information processing occurs in the optic lobes before it gets further relayed to higher brain structures, such as the visual glomeruli – among them the anterior optic tubercle (AOTU). Processing in the optic lobes involves repetitive microcircuits arranged in columns and layers, receiving input from photoreceptor cells directly or indirectly. In addition to local circuits in the optic lobes, visual projection neurons and their post-synaptic partners form the central circuitries in the AOTU and beyond.

Prior to this thesis, modality-specific circuits downstream of the dorsal rim area (DRA) were starting to be described, both in the medulla (Sancer et al., 2019), as well as parallel projections from the DRA to the AOTU (Hardcastle et al., 2021; Omoto et al., 2017; Tai et al., 2021; Timaeus et al., 2020). However, these circuit elements' detailed morphological description, physiological characterization, and behavioral relevance remained missing. The main objectives of this thesis were to (i) review our current knowledge of less-well-understood aspects of polarization vision, (ii) to provide a better insight into modality-specific circuit elements in the DRA region of the optic lobes, (iii) to summarize current progress on understanding visual field properties in the main synaptic targets of

R7 photoreceptors, and (iv) to characterize the anatomical similarities and differences in two specific MeTu-type visual projection neurons along the parallel synaptic circuit from the DRA region of the medulla neuropil to AOTU in the central brain, including the physiological exploration of their functional properties. Together, this research aimed to understand better the modality-specific pathways processing skylight polarization and to compare them to color-sensitive counterparts.

7.1 Distinct insect behavioral responses to polarized reflections

In Manuscript 1, the article "Insect Responses to Linearly Polarized Reflections: Orphan Behaviors Without Neural Circuits" (Section 3), the growing list of intriguing insect responses to linearly polarized reflections were reviewed. It discusses the challenges and prospects in understanding the orphan behaviors observed in response to linearly polarized reflections. Unlike many other sensory modalities, the neural circuits responsible for processing and interpreting polarized reflections across insect species remain largely elusive. We, therefore, explored possible underlying mechanisms that could govern these behaviors. Furthermore, this review aimed at different aspects of insect polarization vision (DRA-mediated navigation circuitry versus non-DRA-mediated responses to reflections).

Many insects have evolved the ability to perceive polarized skylights and polarized reflections from non-metallic, shiny surfaces like cuticles, plant leaves, or water. Together, the detection of this vast array of signals allowed insects to occupy diverse ecological niches and optimize their survival strategies (Foster et al., 2014; Giraldo et al., 2018; Homberg, 2015; Ogawa et al., 2017). More specifically, this unique sensory capability, which has been observed across various insect species, is crucial for navigation, mate selection, water detection and/or avoidance, oviposition, and foraging tasks (Horváth, 2014; Horváth et al., 2008; Kelber et al., 2001). Despite the rich body of described behaviors by many insect species in response to polarized reflections, the neural circuits and mechanisms underlying these behaviors remain poorly understood. The main focus of most studies remains understanding the use of polarized skylights for navigation. Even for *Drosophila melanogaster*, a well-studied model organism, research on polarization vision focuses mainly on navigation (Mathejczyk and Wernet, 2019, 2020; Weir and Dickinson, 2012; Wernet et al., 2012; Wolf et al., 1980). However, *Drosophila*'s ability to detect polarized light when presented ventrally could also be described, involving a genetic dissection of the necessity and sufficiency of specific photoreceptor subtypes (Wernet et al., 2012). Despite this progress, the underlying circuits were not investigated any further. Ventrally localized Photoreceptors necessary for detecting polarized light ventrally were proposed to be localized within a putative 'ventral polarization area' (VPA) (Velez et al., 2014; Wernet et al., 2012). Whether this VPA is connected to a discrete

modality-specific circuit similar to the DRA, whether it extracts information from other circuits like the motion vision system, or whether it connects to the anterior visual pathway remains unknown. Hence, Behavioral responses to polarized reflections are robust, yet they lack clear neural pathways or dedicated circuits for their execution, even in *Drosophila*. Ventral polarization vision to date remains "orphan" or "de-coupled" from all known neural circuits within the insect brain. Therefore, understanding the mechanisms behind such orphan behaviors poses a significant challenge to researchers in the field. The functional dissection of 'poorly understood orphan behaviors across insects, raises intriguing questions about the flexibility and plasticity of neural processing in response to specific cues like polarized light. While some behaviors are associated with well-known neural circuits - such as the navigational behavior using polarized skylight, which is mediated by DRA circuitry, the existence of orphan behaviors suggests the possibility of alternative, yet unidentified, circuitry or parallel processing pathways in the insect brain. Unraveling the adaptive potential of these orphan behaviors could provide novel insights into the complex nature of insect sensory processing and behavioral decision-making. Aquatic insects appear to have developed specific forms of polarization vision to be perfectly adapted to the marine environment, dealing with glare and reflections (Horváth et al., 2008; Lerner et al., 2011; Sharkey et al., 2015) A very recent study using free-flying *Drosophila* as a model demonstrated that an aerial insect's responses to polarized reflections can also depend on the internal state (Mathejczyk et al., 2023). This behavioral study showed how the thirstiness of the animal influences the level of attractiveness of reflected polarized light ventrally (Mathejczyk et al., 2023). The attractiveness of polarizing surfaces had previously been demonstrated using horseflies (Horváth et al., 2020; Ilić et al., 2018; Meglič et al., 2019). In this case, a directional polarotaxis was shown to be mediated by morphological specializations within the ventral retina (Ilić et al., 2018; Meglič et al., 2019), something that remains unknown in *Drosophila*. Hence, the ability of free-flying flies to adjust their flight maneuvers around shiny surfaces appears to be shared across species and now provides a fascinating platform for studying both the retinal detectors and the downstream circuitry. Even more so, neurophysiological studies investigating the neural responses to polarized skylights have revealed complex neuronal properties in *Drosophila* (Hardcastle et al., 2021; Weir et al., 2016). However, a similar study investigating neuronal responses to linearly polarized reflections remains missing. The lack of direct correspondence between neural activation patterns and observed behaviors highlights the need for comprehensive investigations to bridge the gap between sensory input and behavioral output. Cutting-edge tools such as optogenetics

and genetic dissection will be instrumental in identifying and manipulating specific neural populations involved in ventral polarization vision. Moreover, comparative studies across diverse insect species may provide valuable insights into polarization vision's evolution and functional adaptations, similar to what has been done for the detection of skylight polarization (el Jundi et al., 2014; Heinze, 2017; Homberg, 2004).

Taken together, the review article "Insect Responses to Linearly Polarized Reflections: Orphan Behaviors Without Neural Circuits" (Manuscript 1) sheds light on the intriguing yet puzzling phenomenon of ventral polarization vision across insect species. This review highlights the gaps in our understanding by examining orphan behaviors that lack clear neural circuits. It emphasizes the importance of future research endeavors to uncover the underlying neural mechanisms, which is supported by the most recent advances in current research. As progress delves deeper into the study of modality-specific circuits, especially in polarization vision, the remaining mysteries for this sensory ability come within grasp, leading to new perspectives on the complexities of visual processing.

7.2 Modality-specific cells in and outside of the DRA

The modality-specific circuits for detecting and processing polarized skylights are much better understood than what we know about the detection of polarized reflections. Several studies from *Drosophila* and other insect species have described a synaptic pathway called the 'anterior visual pathway' (or 'compass pathway') leading from the DRA region in the retina into the central brain (Hardcastle et al., 2021; Homberg et al., 2011; Lovick et al., 2017; Omoto et al., 2017; Paulk et al., 2009; Tai et al., 2021). Given the evident differences in modality-specific physiological responses of R7/R8 photoreceptors within the dorsal rim area (DRA) (Weir et al., 2016) versus those located outside it (Schnaitmann et al., 2018), we investigated the potential existence of modality-specific cell types within the DRA region of the medulla neuropil, in comparison to other neuron types previously characterized within the central medulla region (Fischbach and Dittrich, 1989). Our previous study indeed pointed in that direction (Sancer et al., 2019). Hence, this endeavor culminated in the publication "Cellular and Synaptic Adaptations of Neural Circuits Processing Skylight Polarization in the Fly" (Manuscript II; Section 4). In this Manuscript, we quantified the morphological attributes and connectivity patterns within the DRA region of diverse medulla neuron types, categorized as downstream elements of color-sensitive photoreceptors, along with various neuromodulatory and some visual projection neuron types. This study aimed to reveal similarities and/or differences between circuits serving skylight polarization (i.e., circuit elements located within the DRA) versus color vision (i.e., circuit elements located in the non-DRA regions). It, therefore, aimed to push the modality-specific characterizations beyond the confines of the previously found Dm-DRA cell types (Sancer et al., 2019).

In analogy to what had been shown for Dm-DRA cells, investigation from Manuscript II identified the distal medulla cell type Dm2 as being potentially modality-specific: Within the DRA region of the medulla, Dm2 exclusively formed connections with DRA photoreceptors and one given cell never mixed DRA and non-DRA inputs. Furthermore, in contrast to their non-DRA counterparts, Dm2 cells exhibited differences in synaptic connectivity and the distribution of pre- and post-synaptic membranes within the DRA. This observation, therefore, added crucial new insight into potential modality-specific roles of Dm2, whereas other reports identified Dm2's role as a post-synaptic partner of color-sensitive R8 photoreceptors (Kind et al., 2021; S.-y. Takemura et al., 2015).
Curiously, the multi-columnar Dm9 cell type, known for its reciprocal connections with

both R7 and R8 photoreceptors in the main part of the medulla neuropil (Heath et al., 2020; Kind et al., 2021; Nern et al., 2015; Sancer et al., 2019), displayed no modality-specific morphology (Sancer et al., 2020). Strikingly, a single Dm9 cell spanned both DRA and non-DRA columns without any discernible preference for any of the modalities associated with them (polarization vs. color). This observation led to the hypothesis that either Dm9 cells play no role in the computation of modality-specific signals or Dm9 cells situated at the boundary of DRA and non-DRA regions could play a pivotal role in integrating color and skylight polarization information. However, it is important to note that Dm9 cells exhibited divergent patterns of pre-and post-synaptic membrane distribution within DRA columns as compared to non-DRA columns, maybe simply reflecting the distinct synaptic profiles of DRA.R7 and DRA.R8 (Sancer et al., 2019). Alternatively, these synaptic differences within Dm9 cells could very well have a modality-specific impact on circuit function, although the precise functional implications of such modality-specific synaptic variations remain unknown.

Interestingly, the well-known R7 target cell type(s) Tm5a/b (Kind et al., 2021; Melnattur et al., 2014) were found to be conspicuously absent from DRA columns. Moreover, other previously identified columnar downstream elements of color-sensitive R8 (Kind et al., 2021; Melnattur et al., 2014) also displayed distinct or weakened synaptic connectivity to inner photoreceptors within DRA columns. These findings suggest that, unlike color information, skylight polarization may not involve significant computations in the lobula neuropil where columnar Tm-cell types project their axons (Melnattur et al., 2014). However, it is important to acknowledge that the study summarized in Manuscript II focused primarily on a limited subset of Tm cells known to be post-synaptic partners of R7 and R8, leaving room for the possibility that other specialized Tm cells may exist within DRA columns.

Moreover, it is reasonable to speculate that the distinctive morphological and synaptic distribution differences observed within DRA columns extend beyond the post-synaptic partners of R7 and R8 photoreceptors. For instance, we observed that dendrites of an Mt11-like tangential cell type projecting to the ventrolateral protocerebrum (VLP) specifically avoided DRA columns. Additionally, octopaminergic cells (e.g., Tdc2 cells), characterized by their extensive dendritic trees, similarly exhibited a marked avoidance of DRA columns. These findings suggest the influence of neuromodulation on visual circuits differs between the circuit elements processing different modalities, such as color and skylight polarization. Interestingly, previous reports have indicated opposing effects of octopamine and dopamine on distinct visually guided behaviors (Gorostiza and Colomb,

2016).

In conclusion, the investigation summarized in Manuscript II delved into the intricate neural adaptations that facilitate the processing of skylight polarization in *Drosophila melanogaster*. By exploring specialized neural populations, distinctive connectivity patterns, and the potential impact of neuromodulation, this study contributes to a more nuanced comprehension of the mechanisms underlying polarization vision. The interplay between modality-specific adaptations and circuitry connectivity paves the way for future research elucidating the functional significance of modality-specific neural arrangements.

7.3 The opponent organization of R7/R8 photoreceptor receptive fields

The dispatch "Colour Vision: Self-Centered Fly Photoreceptors Communicate over Distances" aimed at providing a discussion of the publication from Heath et al. in order to reveal more insight into the synaptic communication mechanisms existing between R7/R8 photoreceptor cells in flies. Their mechanism of computing color information between adjacent medulla columns over varying distances results in complex photoreceptor responses that change our current view of how different modalities are encoded (Manuscript III; Section 5). This discussion aimed to highlight the study's significance, contextualize them within the broader field of color vision (and polarization vision) research, and elucidate potential implications for understanding sensory perception and neural processing in insects.

The phenomenon of color vision has intrigued researchers for decades, with insects providing critical model systems (Behnia and Desplan, 2015; Kelber et al., 2003; Longden, 2016; Menzel and Backhouse, 1991). The study of Heath et al. significantly advanced the mechanistic understanding of insect color vision by describing the dynamic physiological interactions between R7/R8 photoreceptors (Hardie, 1984; Schnaitmann et al., 2013; Schnaitmann et al., 2018). The utilization of intracellular recording, calcium imaging, pharmacological, and genetic manipulations provide a comprehensive approach to dissecting the surprisingly complex synaptic pathways through which photoreceptors influence each other's ability to convey color information. In this multi-faceted methodology lies the strength and the key to the robustness of the reported results.
The discovery of multi-columnar synaptic communication mechanisms between *Drosophila* R7/R8 photoreceptors, resulting in kind of a center-surround organization of their receptive fields, marks somewhat of a paradigm shift in the current understanding of sensory processing (Heath et al., 2020). The presynaptic activity levels of a given photoreceptor cells being influenced by neighboring ones in an opponent way challenge traditional notions of sensory processing, which suggested that photoreceptors prioritize local interactions and optimize energy expenditure by selectively transmitting color information from one point in space only. This newly described mechanism now raises intriguing questions regarding the evolutionary advantages of such a strategy. Is multi-columnar opponency an adaptation specific to the ecological niche of flies, aiding in efficient navi-

gation and acquisition of resources? Does this strategy confer a significant advantage when operating in complex visual environments, enabling rapid and context-specific responses to color cues?

The study of Heath et al. prompts a reevaluation of the functional significance of photoreceptor interactions in color perception. While conventional signal transmission models emphasize central processing and integration (Buchsbaum and Gottschalk, 1983; Schnaitmann et al., 2018), the multi-columnar communication model highlighted here underscores the active role of individual photoreceptor cells in shaping color perception. This finding challenges the prevailing notion of the photoreceptor as a passive conduit of sensory information, suggesting that these cells play a more intricate and dynamic role in color vision than previously thought.

From a broader perspective, the study's insights into multi-columnar opponency have implications for sensory processing in other organisms and across different modalities. Could similar principles also underly other sensory systems, such as olfaction, mechanosensation, or even navigation? What about R7/R8 interactions for the computations of skylight polarization in the DRA region? With the knowledge that Dm9 provides the cellular substrate for the center-surround tuning of *Drosophila* color vision, the function of Dm9 in the DRA seems even more interesting. As connectivity between DRA and non-DRA photoreceptor columns by one Dm9 cell was shown (Kind et al., 2021; Sancer et al., 2020), it remains to be seen whether a similar center-surround tuning also happens there. If such a fine-tuning were to happen, then it would be of particular interest to study whether the computations of the DRA are fine-tuned by color vision or the other way around. The Dm9 cells reside in a unique position for playing such an integral role.

However, several questions remain unanswered. While the study discussed in Manuscript III provides evidence for multi-columnar opponent interactions between photoreceptors, the exact mechanisms underlying this phenomenon warrant further investigation. Are specific signaling neurotransmitters involved in mediating this local communication? How do photoreceptors prioritize and regulate color information transmission based on the self-centered perspective? Unraveling these mechanistic intricacies will offer deeper insights into the neural circuitry underpinning color perception in flies.

In summary, the dispatch "Colour Vision: Self-Centered Fly Photoreceptors Communicate over Distances" advances our understanding of color vision and sensory communication in insects. It adds to traditional paradigms and enriches our conceptual framework

of sensory processing by highlighting the multi-columnar opponent signals generated in fly photoreceptor terminals. The implications of such a 'self-centered' communication extend beyond fly photoreceptors, inspiring new research and technological innovation avenues. As we continue to explore the complexities of sensory perception, this study serves as a starting point for deciphering the remarkable adaptations that enable organisms to make sense of their complex and dynamic environments.

7.4 The binocular integration of polarized skylight within the medulla neuropil

Various skylight cues such as celestial bodies (sun, moon), changes in light intensity (when the celestial body is occluded), variations in chromatic composition (same as before), and the polarization of skylight play a crucial role in aiding insects in enhancing their orientation and navigation behaviors (Heinze, 2017). Skylight polarization vision is an extraordinary sensory modality that enables organisms to navigate, communicate, and forage effectively in their environments (Mathejczyk and Wernet, 2019; Wehner, 2001). Although meaningful progress has been made using different insect species such as locusts, crickets, and monarch butterflies, significant gaps in understanding persist regarding the detection of skylight cues, their processing from the eye to the central brain, and their ultimate spatial representation and integration in these organisms (el Jundi et al., 2014; el Jundi et al., 2011; Homberg et al., 2011; Warren et al., 2019). In recent years, significant progress has been achieved in understanding the representation of visual landmarks in the central complex of *Drosophila* (Hulse and Jayaraman, 2020; D. B. Turner-Evans and Jayaraman, 2016). This progress has been facilitated by using genetically encoded activity indicators (Simpson and Looger, 2018; Zhang and Looger, 2023). A series of publications have unveiled how the central complex receives visual input through retinotopically organized ring neurons (Fisher et al., 2019; Hardcastle et al., 2021; Seelig and Jayaraman, 2013, 2015). The study presented here under the title 'Early binocular processing of skylight polarization by specialized visual projection neurons in the fly brain' (Manuscript IV, Section 6) now provides a comprehensive exploration of the neural basis of polarization vision and navigation in the fruit fly, *Drosophila melanogaster*, focusing on the specialized MeTu visual projection neurons that process polarized light signals downstream of the DRA (Hardcastle et al., 2021; Kind et al., 2021; Lovick et al., 2017; Omoto et al., 2017; Timaeus et al., 2020). This research contributes significantly to our understanding of the complex interplay between sensory processing, neural circuits, and behavior in the context of polarized light cues.

The investigations conducted and described in Manuscript IV encompassed the various facets of MeTu neurons, including their anatomical organization, synaptic connectivity, physiological responses, and behavioral implications. The research revealed the existence of two distinct subtypes of MeTu neurons connected to DRA photoreceptors, MeTu-DRA1

and MeTu-DRA2, both characterized by specific morphological features. The distribution of these subtypes within the medulla and their connectivity and branching patterns provided only weak insight into their potential roles in processing polarized light information. Our morphological characterizations showed that only DRA photoreceptors provide direct input into these modality-specific cells. This modality-specificity is directly projected into the same area of the AOTU, a higher visual glomeruli. The projecting patterns of both MeTu-DRA subpopulations into the AOTU followed a topographic pattern. Hence, anatomical characterization was not sufficient to understand the role of two distinct MeTu-DRA subtypes since they appeared nearly identical.

Additionally to our anatomical findings, we therefore demonstrated the physiological responses of MeTu-DRA1 and 2 to polarized UV light. We revealed that the fan-shaped sensitivity to different AoPs of DRA photoreceptors (Weir et al., 2016) is not only conserved in the Dm-DRA1 cells (Hardcastle et al., 2021) but also represented in MeTu-DRA cells within the AOTU. A similar study recently showed a virtually identical topographic representation for putatively MeTu-DRA-like cells (Hardcastle et al., 2021). Hardcastle et al. proposed a circuit involving parallel channels from the DRA in the medulla (MEDRA) to AOTU in this study. In our work, we could confirm that such parallel processing exists within MeTu-DRA1 and MeTu-DRA2. To our surprise, however, our findings revealed that only the physiological response of MeTu-DRA2 cells was reported in this previous study. Instead, we found that the second driver line used by Hardcastle et al. does not include MeTu-DRA cells since the labeled cells project to another subunit of the AOTU, yet they manifest polarization sensitivity. This points towards a more complex processing of polarized UV light, including additional, non-DRA-specific MeTu neurons. Importantly, our optophysiological imaging experiments demonstrated that the newly discovered MeTu-DRA1 cells represent the AoP of the DRA similarly to the MeTu cells labeled by R73C04-Gal4 in Hardcastle et al., 2021. Interestingly, these responses differed quite significantly from those of MeTu-DRA2 in that they maintained a more precise topographic signal in the AOTU, whereas MeTu-DRA2 responses seemed to represent only two orthogonal AOPs dorsally and ventrally (corresponding to the posterior and anterior halves of the DRA, respectively). Despite their virtually identical DRA inputs, this physiological observation was the first apparent difference between MeTu-DRA1 and MeTu-DRA2.

These differences in their physiological response raised the question of how MeTu-

DRA1/2 influences navigational behavior. Therefore, the significance of MeTu-DRA neurons in the fly's navigation abilities was tested using quantitative behavioral assays (Mathejczyk and Wernet, 2019, 2020). Silencing MeTu-DRA1 neurons led to a noticeable strong reduction in polarotactic behavior (depending on how many cells were silenced), emphasizing their essential role in using polarized light cues for navigation. Interestingly, this effect was enhanced by additionally silencing MeTu-DRA2 cells, but no phenotype was observed when only MeTu-DRA2 cells were silenced alone. The crucial role of MeTu neurons complements previous studies that have shown that maintaining straight flight paths and orienting toward attractive visual landmarks are vital for navigation (Giraldo et al., 2018; Mappes and Homberg, 2004; Mathejczyk and Wernet, 2019; Seelig and Jayaraman, 2013), yet the role of MeTu-DRA2 cells remained obscure.

One of the most intriguing discoveries in this study was identifying the connectivity between MeTu-DRA cell types and previously found MeMe-DRA cells (Kind et al., 2021). Using EM reconstruction, we found that MeMe-DRA cells connect the ipsilateral DRA region of the medulla to the contralateral medulla, yet an intriguing flip along the anterior-posterior axis (Kind et al., 2021). Therefore, the contralateral information is indirectly represented within the ipsilateral AOTU since MeTu-DRA1 cells feed into MeMe cells, which project contralaterally and feed into MeTu-DRA2 cells. This particular wiring pattern can then influence the specific topographic organization of skylight information within the AOTU, for instance, by deteriorating the topography of MeTu-DRA2 signals there (without affecting MeTu-DRA1), as observed in our optophysiological experiments. Furthermore, this circuitry suggested a novel mechanism for binocular integration for polarized light cues emanating from two mirror-symmetrical DRA regions. So far, binocular integration has mainly been known to exist only on the level of neurons directly connecting AOTU to AOTU, so-called TuTu neurons (Hardcastle et al., 2021; Kind et al., 2021; Pfeiffer and Homberg, 2007; Tai et al., 2021). This mechanism likely contributes to the robustness of polarized light detection and enhanced sensitivity to environmental variations. MeMe-DRA cells now represent a second site for binocular integration of polarization vision in *Drosophila* (Kind et al., 2021), and similar cells were described in locusts (el Jundi et al., 2014; el Jundi et al., 2011). Studies in locusts found that the MeMe cells are sensitive to polarized UV light and unpolarized green and UV light (el Jundi et al., 2014; el Jundi et al., 2011). As MeMe cells in locusts appear to provide information about solar elevation and solar azimuth (el Jundi et al., 2014; el Jundi et al., 2011), MeMe-DRA cells in *Drosophila* might provide similar, essential information for navigational behavior.

Based on neurotransmitter prediction from the fly connectome and studies in locusts, we hypothesized that inhibition by unpolarized UV light in the contralateral eye might also elicit in *Drosophila* (el Jundi et al., 2011). Our physiological experiments with unpolarized UV flashes demonstrated that only MeTu-DRA2 responds to a contralateral stimulus with a weak yet significant inhibition in the medulla, which supports the hypothesis. Basil et al. additionally found an intriguing connection of MeMe neurons with the presumable circadian clock of the locust brain (el Jundi et al., 2011). Therefore, Basil et al. hypothesize that the MeMe neurons, with their sensitivity to the solar azimuth and their connectivity to the circadian clock circuitry, might provide highly differentiated zeitgeber signal to the circadian clock (el Jundi et al., 2011). As our physiological data regarding MeMe-DRA influence suggest a similar physiological function as in locust, one has to wonder if MeMe-DRA neurons in *Drosophila* are also connected to the circadian clock circuitry and might provide similar zeitgeber signals (el Jundi et al., 2011).

How are the signals of MeTu-DRA1/2 processed by downstream partners? The TuBu neuron subtypes downstream of MeTu-DRAs have been identified as TuBu01 and TuBu06 (Hulse et al., 2021, Garner, Kind et al, in preparation). These TuBu cells are known to be connected to specific subpopulations of Ring neurons, which are part of the network for integrating the heading direction of the animal (Hardcastle et al., 2021; Hulse et al., 2021; Seelig and Jayaraman, 2013; Timaeus et al., 2020; D. Turner-Evans et al., 2017). Different subpopulations have been proposed to integrate particular kinds of information within all of the ring neurons. For instance, R4m neurons are known for conveying navigational information, most prominently skylight polarization (Pan et al., 2009; Varga et al., 2017). Since MeTu-DRA1 cells feed into TuBu01, which projects onto R4m neurons, it can be speculated that the topographic information from the DRA contributes to determining the fly's heading direction. On The other hand, MeTu-DRA1 and MeTu-DRA2 are connected to TuBu06 neurons, which project onto R5 neurons (Garner, Kind et al, in preparation). However, R5 neurons have been proposed to play a role in the sleep regulation of *Drosophila* (Lamaze et al., 2018). It can, therefore be speculated that the MeTu-DRA1 → MeMe-DRA → MeTu-DRA2 → Central complex pathway could represent a zeitgeber signal promoting the wake/sleep rhythms.

Our findings offer valuable insights into the functional properties and circuitry of MeTu neurons, shedding light on the neural mechanisms underlying polarization vision and navigation in *Drosophila melanogaster*. These findings advance our understanding of

insect sensory processing and behavior and have broader implications for neuroscience. Identifying modality-specific neurons, early binocular integration mechanisms, and the interplay between distinct cell populations provide a foundation for future research in diverse areas, including sensory integration, neural computation, and the quantification of behavior. Therefore, our multidisciplinary approach employed in this study exemplifies integrative neuroscience's power in unraveling complex neural circuits and their functional significance. By characterizing the structure and function of MeTu neurons and their connections, this research contributes to the specific field of polarization vision. It offers a model for studying sensory processing and behavior in other organisms.

In conclusion, Manuscript IV contributes to a better understanding of polarization vision as a critical sensory modality for insects, with implications for navigation. The research provides a foundation for future investigations into the complex neural mechanisms underlying polarized skylight processing and insect navigation. Moreover, the study's insights into early binocular integration and the roles of distinct cell populations open new avenues for further interdisciplinary research, bridging the fields of sensory neuroscience, behavior, and ecology. As progress adds to our understanding of polarization vision and its significance, this study serves as a platform for uncovering the mechanisms underlying how organisms perceive and interact with the world around them.

8 Future Direction

In this cumulative thesis, I have investigated different anatomical circuit elements and their roles for color and polarization vision by reviewing the literature (Manuscripts I & III) or conducting experiments myself (Manuscripts II & IV). By doing this, I focused my efforts on understanding the processing of either of these modalities in particular and gaining more insight into the processing of any single visual cue in general. I focused on studying and describing circuit elements specialized for processing polarization patterns, most prominently skylight (Manuscripts II & IV), but also reflected polarized light (Manuscript I). Although a synaptic pathway from MEDRA to AOTU was previously proposed to contain parallel channels (Omoto et al., 2017; Tai et al., 2021; Timaeus et al., 2020), amongst them at least one for skylight polarization (Hardcastle et al., 2021), I could identify a previously unknown, binocular circuit architecture that adds important new aspects to the proposed circuit function. Moreover, my experimental work was embedded in a multi-integrative approach that included light microscopic studies, EM reconstruction, physiological responses, and behavioral experiments to understand the depth of these circuit elements for orientation behavior and navigation.

As shown in manuscript IV, both MeTu-DRA1 and MeTu-DRA2 cells are connected to DRA.R7 directly. Like Dm-DRA1 cells, the MeTu-DRA cells exclusively receive input from several adjacent DRA.R7 cells (Kind et al., 2021). With light microscopic techniques such as GRASP, MCFO, and single-cell analyses, we found intriguing differences between both subpopulations of MeTu-DRA cells. These findings could be confirmed by EM reconstruction. Due to EM reconstruction, it is possible to identify the post-synaptic partner of MeTu-DRA cells, revealing the targets of this pathway in the R4m and R5 Ring neurons of the central complex (Garner, Kind et al, in preparation). The potential role of MeTu-DRA2 in promoting sleep leads to many future experiments. As our behavioral experiments with silenced MeTu-DRA2 cells did not show any significant effects on polarotactic behavior, testing these flies in an activity tracker will be interesting. If MeTu-DRA2 provides a zeitgeber signal for the fly, one would expect a significant difference in sleep-related behavioral activity compared to wild-type flies. Also, the physiological responses of

MeTu-DRA1/2 to polarized UV light in the medulla, or even responses to unpolarized blue and green light, might provide important new insight about integrating other visual stimuli. It will be exciting to test the responses to a single wavelength and map the response to different elevations of those stimuli. One could expect responses similar to what has been shown in locusts (el Jundi et al., 2011; Zittrell et al., 2020), thereby revealing similar information on the stimuli integration across insect species.

In Manuscript IV, we could show that the DRA has two modality-specific MeTu subtypes, which we described morphologically, physiologically, and behavioral. Interestingly, I found that the driver line R73C04-Gal4 does not contain MeTu-DRA cells and instead projects to an entirely different subarea in the AOTU. However, a previous study demonstrated that cells labeled by this driver are sensitive to polarized UV light (Hardcastle et al., 2021). In light of these results, it is plausible to hypothesize that integration of different modalities might happen on the dendritic level of MeTu cells in the medulla. Especially in MeTu cells, which are not directly connected to polarization-sensitive photoreceptors, they could become sensitive to polarized UV light and whatever their non-DRA inputs make them sensitive to. This effect can only be explained if MeTu cells in the R73C04 driver are downstream of other DRA-connected cells. Possible candidates would be Dm-DRA1 cells. Future experiments will reveal whether different modalities are integrated within the medulla according to this predicted mechanism or whether other/additional mechanisms exist.

Although sleep and navigation are two prominent research areas involving polarized skylights, it is doubtful that these are the only areas of integration. It seems plausible that additional visual modalities are processed over the anterior visual pathway, and it is, therefore, to be expected that more integration happens along the path toward the CX. But which and where those computations happen remains to be decrypted. Additionally, Kind et al., 2021 described several additional cell types, such as Mt-DRAs, which we could not yet be identified by light microscopic techniques so far (Kind et al., 2021). The role of these cell types in shaping the responses of MeMe-DR, MeTu-DRAs, and the whole AVP will be interesting to study further. As all of the medulla-specific cells in the DRA mentioned above manifest rather broad dendritic branches, not only in the DRA but also in the non-DRA part of the medulla, it is doubtful that no other (i.e., non-DRA) information is processed within these cells.

Finally, we propose that MeMe-DRA cells represent a central component of binocular integration, which has not been solved yet. A significant step in studying and understanding the computation and integration of binocular vision for polarized skylights would be finding a specific driver line labeling MeMe-DRA cells specifically. With this line, it would be possible to study the MeMe-DRA physiologically and behaviorally more closely. With optophysiological experiments, one could explore the response pattern of MeMe-DRA cells to polarized UV, unpolarized UV, blue and green light. These experiments could give more information about *Drosophilas* processing coding am azimuth or elevation. Even more, using the Chrimson technique, the direct influence of MeMe-DRA on MeTu-DRA2 could be tested. Similarly, silencing MeMe-DRA cells could give exciting information about the impact these cells have on the tuning of MeTu-DRA2 cells in the AOTU. Finally, silencing MeMe-DRA cells could also be of great interest in combination with behavioral experiments testing for changes in polarotactic behavioral or overall activity.

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