Behavioural State Neuromodulation of Early Visual Processing in the Dragonfly Brain

A Thesis submitted in partial fulfillment of the

HONOURS DEGREE of BACHELOR OF

HEALTH AND MEDICAL SCIENCES in

The Discipline of Physiology

Adelaide Medical School

The University of Adelaide

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June 2020

ABSTRACT

Dragonflies are apex predators capable of complex physical manoeuvres while in flight. This success can be attributed to their complex visual pathway, each layer making various contributions to visual processing. It is well known that the second neuropil, the medulla, makes substantial contributions to this process, but its response characteristics are not yet well known. Most studies have been conducted in individual neurons but there is promise in recording from the sum activity of an entire neuronal population. These population level responses, known as Local Field Potentials (LFPs), and how they are affected by the activity level (behavioural state) of the dragonfly are unknown. The aim of the study was to investigate the effect of behavioural state on population level medulla characteristics. This was achieved by using the octopamine agonist chlordimeform to mimic system arousal and by presenting full screen ON/OFF flickers and moving gratings. The recorded responses were proportional to luminance, produced a temporal frequency tuning curve, and demonstrated no directional preference. In the CDM state, luminance responses increased by between 54% and 97%, and the frequency tuning curve was shifted to higher frequency values by as much as 113%. Due to a limited sample size, statistical significance was not reported. This suggests that a behaviourally active dragonfly has increased luminance responses to faster moving stimuli, allowing faster responses and more accurate flight. This is the first evidence that neuromodulation occurs as early as the medulla and that population level responses are a useful tool for investigating neuropil function.

INTRODUCTION

Dragonflies

Surviving in a complex world requires the ability to receive information about your surroundings and from this, make behavioural decisions. One important sensory system is vision. Our understanding of vision and its processing is heavily based on evidence gathered from various insect species. One species that is most favoured is the common fruit fly, *Drosophila*, due to its fully mapped, and therefore easily manipulated, genetics. *Drosophila* have inferior visual processing qualities compared with other insect species, such as dragonflies. Dragonflies have a larger brain size and undertake more complex visual processing[1] than *Drosophila* while also having a prey catch rate of 97%, the highest of all predators[2]. As the neuronal response properties in dragonflies are comparable to other insects[3], these creatures are a logical focus for visual research.

Visual pathway

In order to interpret visual stimuli, signals undergo processing through multiple layers, known as neuropil, of a neural pathway[4], as shown in Figure 1. As the stimuli progresses from early to late neuropil, the processing becomes more complex. It is these later stages that are the most studied. One early stage neuropil that is not well characterized is the medulla. This area performs complex visual processing due to the dense neuronal arborisations[5], organised in a columnar fashion[6], that it receives from other neuropil. As the medulla and its associated accessory areas[7] are also responsible for many non-visual functions, including circadian rhythm[7] and habituation[8], it is also promising for investigating how multiple bodily processes are integrated.

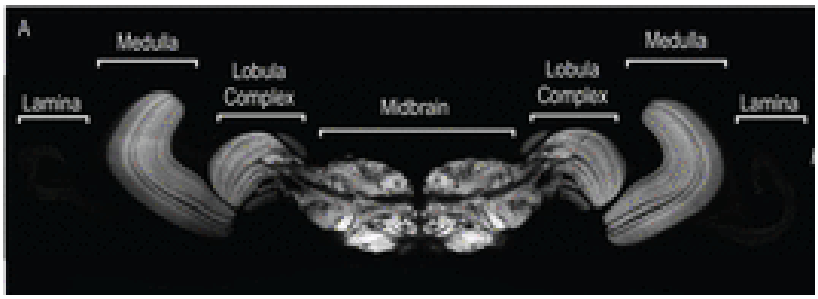

Figure 1. Dragonfly visual pathway of both eyes. Neuropil progress from early to late from lateral to medial e.g. Lamina, Medulla, Lobula Complex, Midbrain[9]

Visual functions of the medulla

Although the processing characteristics of the medulla are not well known, two important facets of vision, luminance sensitivity and movement sensitivity, are believed to rely on this area. Evidence suggests that visual processing may be separated into ON and OFF detection pathways[10]. The layer at which this separation occurs is a current area of research. The neuropil preceding the medulla, the lamina (Figure 1), demonstrates separate ON and OFF characteristics[11] in some studies, but in others is either not responsive to either[12] or always responsive to both[13]. Some studies show that this separation originates in the medulla itself[13, 14] while other evidence suggests the medulla is responsible for integrating the two pathways, thus implying that separation has already occurred[15, 16]. The evidence is consistent, however, that the outputs of the medulla and the inputs to the lobula, the following neuropil (Figure 1)[17], are divided[18, 19]. Thus the division between ON and OFF signals must occur at some stage prior, possibly the medulla.

Evidence of the layer at which the ability to detect movement direction arises is similarly divided. Some studies suggest that movement detection occurs as early as the presynaptic medulla signals[18] while others suggest that this function is not present until after the lobula inputs (Figure 1)[14, 19, 20]. Thus, the evidence behind when movement detection arises is very unclear.

Response characteristics

It may be that some processing characteristics are not observable on the level at which these studies were conducted, the neuronal level. As the medulla is a dense structure[5], the aggregate activity of a population of neurons may be a more effective method for characterising its functions and responses. As individual neurons encode information by changing their internal ion concentrations, they also effect the ion concentrations of the extracellular space. If a large group of neurons are all responding in the same way to a certain stimulus, the effect on the extracellular space would convey enhanced information regarding the aggregate response of these neurons. This aggregate activity can be investigated by recording Local Field Potentials (LFPs) [21, 23], a measure of the changes in the extracellular space caused by the responses of neurons. It is therefore promising to use these LFPs to investigate the response properties in the medulla.

Modulation and behavioural state

Current literature suggests that neuronal activity, on both a population and a single neuron level, is affected by the metabolic activity level of the insect (e.g. resting, walking, or flying). This is known as the behavioural state[22]. The behavioural state affects many neuronal characteristics including: the magnitude of individual[23, 24] and population level[25, 26] responses, resting membrane potential and resistance[23, 27], stimuli evoked and spontaneous firing rates[28, 29], contrast/luminance sensitivity[30], velocity preference[5,6], and response latency[28]. Interestingly, some neurons[31] and entire brain regions[32] fail to respond unless the insect is behaviourally active. Thus, modulation of visual processing on the population and cellular level is strongly correlated to behavioural state.

It is believed that altering neuronal function based on behavioural state may be the result of an evolutionary adaptation[33] to decrease the energetic expense of vision[34, 35]. Reducing the activity of the visual system at rest would reduce overall energy consumption as the neuronal computation of visual processing is metabolically taxing[29]. It is believed that this system modulation is achieved by the neuromodulator octopamine[36], often referred to as the insect equivalent of noradrenaline[37], as it both circulating octopamine levels[38] and overall octopaminergic neuron activity[24] increase during the active behavioural state. Octopaminergic neurons are present throughout the insect brain[39, 40] and are known to play a role in memory[41], metabolism, and stress[42]. However, they have a uniquely high arborisation density in the centres of visual processing[27, 36], suggesting this hormone affects vision most. Pharmacological evidence supports its role in modulation as pharmacological application of octopamine to a quiescent insect can induce the neural characteristics seen in the active behavioural state[24, 27, 43]. The opposite is also true, the application of octopamine antagonists to a behaviourally active insect can reproduce the neural activity seen in the non-active state[44]. This evidence suggests that octopamine affects the neuronal properties of the visual pathway through some sort of arousal mechanism. Overall, it is believed that octopamine is the primary method for encoding behavioural state and does so through arousal. How this hormone changes the response characteristics of an entire population is not yet well known, especially in the medulla.

Pharmacology

In the metabolically active insect system, octopamine is quickly transported away from the brain and metabolized[45] and thus concentrations of experimentally applied octopamine changes with time. Therefore,

the octopamine agonist chlordimeform (CDM) is often used to test the effects of octopaminergic activity on neuronal activity. The application of CDM produces effects almost identical to that of behaviourally active flies[22, 35, 46] but is an irreversibly binding molecule[47] and thus its effects are not confounded with time.

Relevance

This research makes a relevant contribution to the wider field of insect vision as it examines an early layer of the visual pathway that is not well characterised. Additionally, it investigates whether neuromodulation occurs at this early stage of the visual pathway and what effect it has on the lesser studied population level. Wider reaching effects of the knowledge gained by this study include increased knowledge of the fundamental properties of neuronal processing and brain activity. Past perspectives on brain processes often assumed a simple feedforward mechanism and by investigating a complex pathway this study helps in shifting the perspective of the brain toward a more complex machine.

Gap

The gap in the field addressed in this study is the characteristics of the population level neuronal responses of the early dragonfly neuropil, the medulla, and whether these responses are affected by neuromodulation. Response properties of interest include behavioural state differences in luminance encoding, frequency tuning, and directional sensitivity. These properties will be elucidated by presenting stimuli varying in luminance, frequency, and direction properties, in both a control (resting) and an active (neuromodulated by CDM) condition. Population level responses will be measured in microvolts.

Hypothesis

The application of the octopamine agonist chlordimeform will modulate the visual sensitivity and temporal responsiveness of neuronal activity in the medulla of the dragonfly.

Aims

1. To determine the characteristic properties of population level neuronal responses of the dragonfly medulla in response to visual stimuli.

Stimuli include full screen luminance ramped ON/OFF flicker and full screen luminance ramped directional gratings. All stimuli will be presented at various temporal frequencies using MATLAB. Responses are to be measured as LFPs (in microvolts), as recorded by extracellular tetrodes.

2. To determine the characteristic properties of population level neuronal responses of the dragonfly medulla in response to visual stimuli in the active behavioural state.
   Stimuli and methodology as above. The active behavioural state will be induced using the octopamine agonist chlordimeform, applied in liquid form to the brain using pipettes.

METHODOLOGY

Electrophysiology

Recordings were taken from a total of 6 male, wild caught (Adelaide Botanical Gardens, SA) Hemicordulia sp. (n=3) and A Brevistyla sp. (n=3) dragonflies. Insects were stored in the laboratory fridge, temperature approx. 10ºC, for up to 4 days. As the medulla is known to interact with circadian rhythm[7], the time of day of each experiment was varied to ensure there was no significant effect of time of day on responses. Additionally, the laboratory temperature was closely monitored and controlled (range: 1.4°C). As dragonflies do not require any ethical approval for science, no restrictions on handling and procedures needed to be observed. Insects were immobilized using a wax rosin mixture and fixed to an articulating magnetic stand. Dragonflies were positioned with the head tilted ventrally so the back of the head was exposed. A minor dissection was conducted on the left posterior surface of the head to expose the optic lobe. The reference wire was placed on the posterior surface of the opposite (right) eye and the site of tetrode recording was determined visually using anatomical landmarks.

LFP recordings (µV) were taken using NiChrome wire tetrodes[48] housed in two layers of polyamide tubing and a glass capillary. Acceptable tetrode resistances were between 0.2 and 5 MOhm as tested by an impedance apparatus (NANO – Z)[49]. Resistances were adjusted by plating with a PEG and Gold solution (1% PEG and Gold solution 3:1, NEURALYNX)[49]. Tetrodes were controlled using two micromanipulators (MAHZHUSER: MM3301R and SENSAPEX) for macro and micro movements, respectively. Experimentation occurred on a gas suspended table (NEWPORT) and room temperature was monitored.

Pharmacology

A biological ringer (contents: 140mM NaCl, 5mM KCl, 5mM MgCl, 5mM CaCl, 6.3mM HEPES, 4mM Sodium Bicarbonate, 73mM Sucrose)[50] was applied ad libitum to the head capsule to prevent neuron death and promote the longevity of recording. A CDM mixture (ringer, average 14µM[35, 46] Chlordimeform (Sigma-Aldritch (n=2)) was applied using a disposable dropper, average quantity applied was 0.13ml (n=3), suitable to elicit responses for over an hour[29].

Screen and Stimuli

Stimuli were presented on an LCD monitor (frame rate: 164.4Hz) and were distorted such that they would appear curved and mimic natural optic flow. The screen was placed 20cm from insect eyes then angled and centred to ensure optimal placement within the visual field. Extracellular responses were digitized at 32 kHz and acquired using a Neuralynx amplifier for analysis in MATLAB (R2019a and R2020a).

Three stimuli were presented to dragonflies, all written using MATLAB's Psych Toolbox. Stimuli were run for 7 seconds, with 1 second pre and post stimulus intervals. The monitor was flickered between black and white using a sinusoidal luminance ramp, e.g. the screen would change from black to white but the extent of black and white would increase (sinusoidally) as time progressed (see Figure 2A, monitor luminance). These sinusoidal flickers were displayed at 30 different temporal frequencies (from 0.5 Hz to 40Hz) e.g. different amounts of peaks and troughs. These frequencies were logarithmically distributed. Full screen sinusoidal moving gratings (see Figure 2B) were displayed in 8 directions (at 5Hz) to assess direction preference. These same gratings were then displayed in the preferred direction at the 30 temporal frequencies as described above.

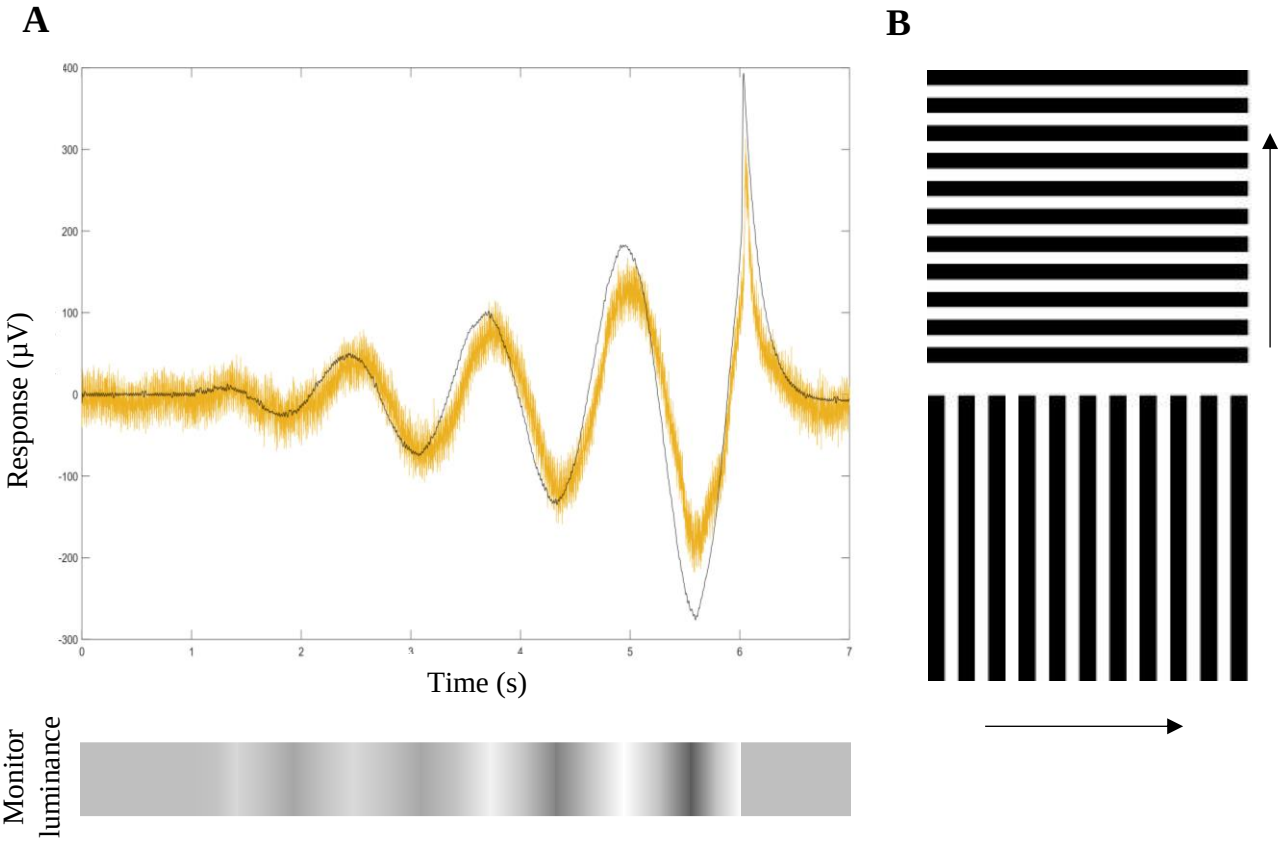

Figure 2. Stimuli examples. ***A.*** *Example sinusoidal flicker at 0.8 Hz over time with black trace showing monitor luminance (as indicated by monitor luminance panel) and orange trace showing LFP.* ***B.*** *Example gratings and directions (as indicated by arrows)*

Data Analysis and Statistics

Data exclusion criteria included pathological damage to the insect, excessive system noise, faulty tetrodes, or expired pharmacological agents. LFPs were filtered using a bandpass filter of 0.1 to 50Hz. Each biological replicate value represents the average of between 2 and 5 technical replicates. As the amplitude of recorded LFPs is dependent on the distance of the wire from the origin of the response, the overall magnitude of response was not comparable between biological replicates and data were instead normalized to the sum of all responses. Significance values were not included owing to an in inadequate sample size. If conducted, significance values and statistical tests would need to be generated using non-parametric tests.

Luminance response data were generated by averaging between specimens and plotted suing MATLAB. Frequency tuning curves were generated by first parameterizing using a Fast Fourier Transform. These were then normalized by converting each temporal frequency response value to a proportion of the sum of responses to all temporal frequencies. These values were then plotted using Excel. Average data curves were then generated by averaging across insects. The peak frequency value was identified as the temporal frequency at which the highest proportion of response occurred, while the cut-off value was identified as the temporal frequency that elicited half of this same proportion. Directional sensitivity was assessed by normalizing each direction response to the sum of responses in all directions. These values were then averaged between insects and plotted in MATLAB.

RESULTS

Luminance Response

In order to observe the effects of neuromodulation on the medulla, the control state response characteristics were recorded in response to stimuli designed to show a range of screen luminances. In the control state, the medulla produced responses proportional to the luminance of flicker (Figure 3, blue traces), e.g. as luminance increased, so too did medulla responses. This response conveyed luminance information in both the positive and negative direction (Figure 3, blue traces). This was observed across the flickers of all temporal frequencies and examples are shown in Figure 3. These frequencies elicited the peak, or saturated, responses in the control (Figure 3A) and CDM (Figure 3B) states. In the CDM condition, responses proportional to luminance were also observed but the magnitude of responses to a given luminance value were increased in both the positive and negative direction (Figure 3, red traces). These responses were increased by 55% at the one second mark and 54% at the four second mark for the control peak (Figure 3A) and by 97% at the one second mark and 80% at the four second mark for the CDM peak (Figure 3B). Thus the CDM effected the responses to faster frequencies more than lower frequencies (Figure 3A vs 3B), a difference of 42% at the one second mark and 26% at the four second mark. These results imply that in the active behavioural state the system both responds more and responds with greater scope than the control state.

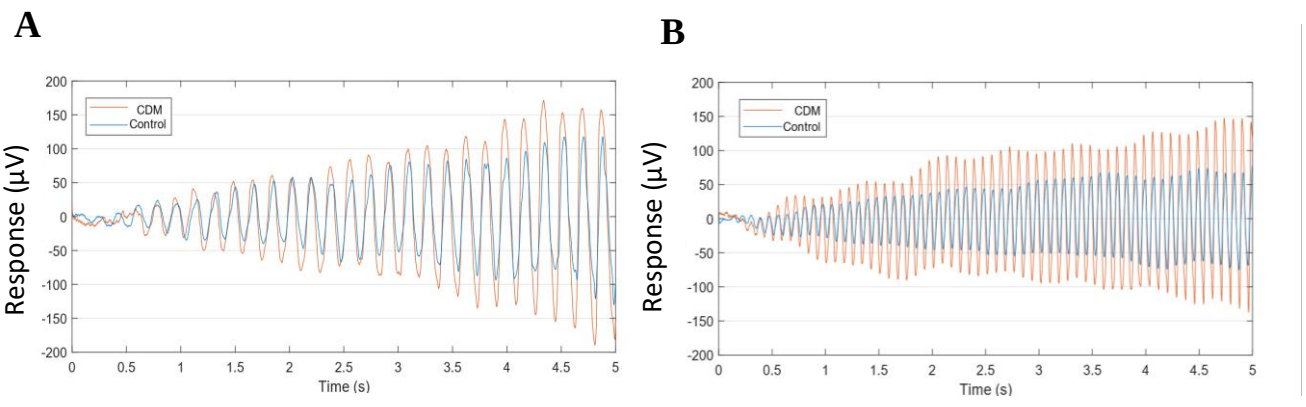

Figure 3. Local Field Potential activity in microvolts in the dragonfly medulla against time in the control state (blue, n=4) and CDM state (red, n=2) in response to a sinusoidal temporal frequency of ***A****. 5.6Hz and* ***B.*** *11.9Hz*

Frequency Response

In order to investigate the temporal response properties of the medulla, full screen flickers were shown at 30 different temporal frequencies. In the control state the medulla responded to flickers in a frequency dependent manner (e.g. responses to some frequencies were higher than to others) and this resulted in a frequency tuning curve (Figure 4A). The CDM condition produced a frequency tuning curve (Figure 4B) which was shifted to the right, i.e. to higher frequencies, compared to the control state (Figure 4C). This frequency shift is more easily seen in the increase in the values of the peak and cut-off frequencies as shown in Figure 4D and E. There was a shift from 5.4Hz to 11.5Hz in the peak values and a shift from 24.6Hz to 31.8Hz in the cut-off values. This shows an increase in the peak frequency response by 113% and the cut off frequency by 29%. This implies that in the active behavioural state, the medulla responds preferably to higher frequency values, i.e. faster moving stimuli.

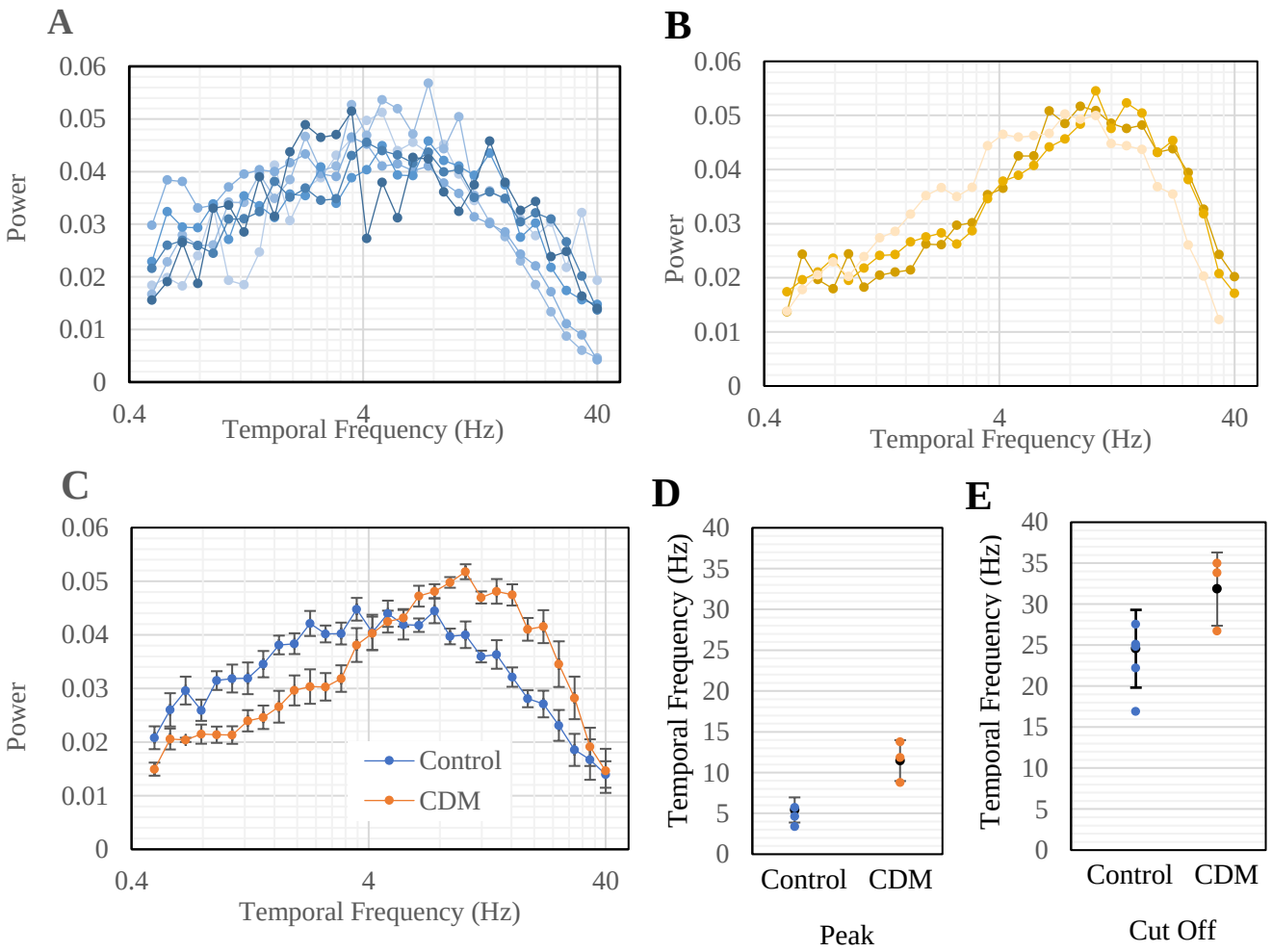

*Figure 4. Dragonfly medulla activity by temporal frequency (Hz) in response to sinusoidal full screen flicker in **A.** Control condition (n=6) and **B**. CDM condition (n=3). Each colour shade trace represents a biological replicate. **C**. Average response curves in the control (blue) **D.** Temporal frequencies of the peak responses in the control (blue (n=6)) and CDM (orange (n=3)) including averages (black, S.D.) **E.** Temporal frequencies of the half peak responses in the control (blue (n=5)) and CDM (orange (n=3)) including averages (black, S.D.)*

Directional Grating

In order to investigate the directional responsiveness and/or preference of the medulla, moving gratings were displayed in 8 different directions. In the control state, the medulla produced responses to gratings in all directions (Figure 5A). This response may simply reflect the change in the overall luminance of the screen from the pre stimulus display to the gratings. There was no difference between any of the 8 directions, implying a lack of directional preference. The CDM condition produced the same responses (Figure 5A) with no difference observed.

A

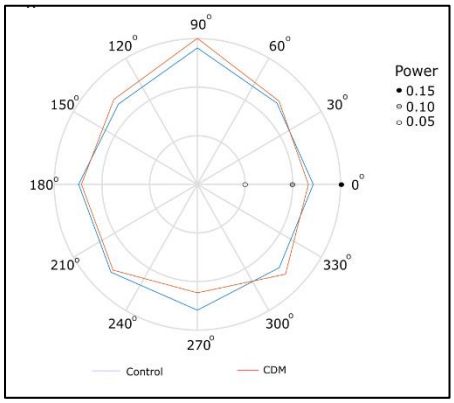

Figure 5. Power of activity in the dragonfly medulla in response to full screen gratings moving in 8 directions at a spatial frequency of 0.1 Hz in the control state (blue trace, n=4) and CDM state (red trace, n=2).

DISCUSSION

Luminance Response

Although luminance responses are seen at all stages of the visual pathway, this is the first evidence that they are observable in the medulla at the population level, as seen in Figure 3. As the responses to luminance occur in both a positive and negative direction, there is no evidence in this study to support the theory that the separation into ON and OFF channels occurs in the medulla, as other studies suggest[18, 19]. It may be that this separation is only observable on the single neuron level and not the aggregate activity recorded here. The finding of a response increase, of between 26% and 42%, between conditions is the first evidence that CDM affects population level responses. As CDM was chosen in this study design to mimic the arousal properties of the naturally occurring hormone octopamine, this finding supports existing literature that octopamine acts as a neuromodulator of the visual system[36]. Interestingly, this increase in both the positive and negative directions also results in an increased ability to encode differences in luminance. This makes for an increase in system resolution thus allowing the neuronal system a wider value range to encode the same luminance levels. On the behavioural level these findings indicate that the dragonfly would have a greater ability to detect luminance differences in the visual field in the active behavioural state. This means that during flight, the insect would have increased visual processing power. This would likely lead to better object discrimination, an advantageous ability when moving at high speed through a cluttered visual field.

Frequency Response

As dragonflies require fast movements to avoid objects and capture prey, these insects need the ability to encode different visual velocities of both themselves and other moving objects. The medulla accomplishes this by responding to frequencies in a preferential manner and creating a frequency tuning curve, as shown in Figure 4. This finding supports the idea that velocity encoding occurs on the medulla level and is the first evidence that this response can be measured on the population level. From a behavioural perspective this could suggest that the dragonfly is tuned to perceive objects or scenery moving at medium temporal frequencies when at rest. In the CDM condition this tuning curve is shifted to the higher temporal frequencies (Figure 4C) by 113% for the peak frequency and 29% for the cut off frequency. Thus, the visual system is tuned to substantially faster frequencies in the active behavioural state than in the control state.

Behaviourally, this implies that when active, for example during flight, the dragonfly responds preferably to faster moving stimuli. As the optic flow surrounding the dragonfly would be much faster while flying than at rest, an arousal mechanism that prioritizes faster moving stimuli, such as these findings suggest, would be advantageous.

Direction Response

As dragonflies move in all three dimensions simultaneously, the visual system must encode movements of objects and optic flow in many different directions. To determine what contributions the medulla makes to this processing, moving gratings in different directions were displayed. As the medulla showed a response to these gratings it is tempting to infer that these results show directional response properties in the medulla. However, the response seen in this study is most likely due to the overall luminance change of the screen when switching from the grey pre stimulus background to the black and white grating. The lack of preferred direction also supports this interpretation of the results. However, as the response properties of the medulla have been shown to be column specific[6], it may simply be that any directional response is not discernible in an LFP reading. LFPs may be recording the integration of inhibitory and excitatory responses from multiple columns and thus directional information is averaged out on the population level. This explanation would help to explain why no difference was seen in the CDM state. If the trend of response magnitude increase seen in Figure 3 is an accurate representation of neural responses, then both the positive and negative direction responses would be enhanced. As LFPs show the integration of both the positive and negative responses across columns (which may act in opposing manners), the effect of CDM would not be observable on the aggregate population level. Therefore these findings should not be interpreted to mean the medulla lacks directional information, as some studies suggest[14, 19, 20] but instead suggests that these responses, and any effect of behavioural state, should not be investigated on the population level.

Study Limitations

The primary method of identifying recording location was via anatomical landmarks. The medulla is easily identified by its characteristic shape (Figure 1), but this method lacks precision. An instantaneous flicker was presented to infer location based on response latency, but the monitor used lacked the precision required to

calculate time differences on this scale. Strategies for locating recording sites that would increase precision include biological dyes and electrical marking[49].

Due to reference wire placement limitations, it was not viable to include a non-ringer condition in this study design. Although ringer is designed to be a replicate of natural biological solutions[50], it would have been beneficial to include this control. Additionally, as CDM is an irreversible binder, it was not possible to include a CDM wash out condition. This could be mitigated by including a CDM only condition, not included in this study due to expired CDM solutions[47] which caused these data sets to be discarded.

Summary

The aims of the study were to characterize the population level responses in the dragonfly medulla to visual stimuli in a control and an active behavioural condition. This was addressed and achieved through the analysis of luminance, frequency, and directional sensitivity. The hypothesis that the application of the octopamine agonist CDM modulates the sensitivity and temporal responsiveness of resting state neuronal activity to visual stimuli in the medulla of the dragonfly was not confirmed nor disproven owing to limited sample sizes. The overall trend of data suggests that the active behavioural state increases the temporal and luminance sensitivity of the medulla.

This research contributes to the current knowledge gap surrounding the roles of the medulla in visual processing, the effect of neuromodulators, and the effectiveness of population level recordings. The wider significance of this research is that the brain is a much more complex system than was once believed. There is no simple feedforward explanation for the diversity of responses observed in the visual pathway. Evidence from this study suggests that complex processing occurs at all stages of the pathway, not just later stages. The evidence generated here of the effectiveness of population level recordings on characterizing response properties helps overcome the historical difficulties in investigating the dense structure[5] that is the medulla.

Future studies would be best suited to first addressing the current study's limitations by increasing recording location precision, increasing sample size, and including two additional conditions ('ringer' and 'CDM only'). Logical extensions of this work would be investigation of various CDM concentrations or quantities.

This would allow the analysis of the many different behavioural states that dragonflies can exhibit, anywhere from perching still, to hovering, to flying quickly. Overall, the findings of this study contribute to the field by outlining the functions of a lesser known brain area, using an emerging technique, under the effect of a neuromodulator, and enhancing our knowledge of the complexity of neuronal systems.

PROFESSIONAL & FUNDING ACKNOWLEDGEMENTS

The author would like to acknowledge Matthew Schwartz and Benjamin Lancer for their significant assistance in procedure training, specimen collection, and data analysis.

This research was supported by funds from an Australian Research Council Future Fellowship (FF180100466).

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