Origins of Pupillary Hippus in the Autonomic Nervous System

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Purpose. The purpose of this study was to determine the relative roles of the sympathetic (SNS) and parasympathetic nervous system (PNS) in pupillary hippus.

Methods. We used a paired-eye control study design with three cohorts receiving either 1.0% tropicamide (PNS antagonist) in light (TL), 1.0% tropicamide in dark (TD), or 10% phenylephrine (SNS) in light (PL), $n = 12$ in each. Each subject received one drop to the randomly determined treatment eye, while the other eye served as control. Bilateral measures of pupil size and dynamics were made over 2.6 seconds using an infrared eye-tracker sampling at 500 Hz. Measures were taken at baseline, then every 5 minutes for 40 minutes. Hippus, analyzed in both time and frequency domains, was compared between eyes and cohorts.

Results. Pupillary hippus with a distinct dominant frequency was present in all measures at baseline (mean: 0.62 Hz, SD: 0.213 Hz), and that frequency did not change in any group ($P = 0.971$). Hippus magnitude (treatment eye relative to control eye) decreased in the TL ($-72.8 \pm 4.7\%$, $P < 0.0001$) and TD ($-71.3 \pm 2.6\%$, $P < 0.0001$) groups, but did not change in the PL ($+5.4 \pm 13.7\%$, $P = 0.173$) group, despite PL pupils dilating to a proportion similar to TD.

Conclusions. Pupillary hippus can be extinguished by antagonizing the PNS, whereas agonizing the SNS dilates the pupil without affecting hippus. This suggests that hippus originates from central PNS activity, and not from SNS activity, or oscillations in the balance between PNS and SNS at the pupil.

Keywords: pupils, parasympathetic nerves, sympathetic nerves

In the absence of changes in external influences such as luminance, mood, and fixation, the pupil is in constant motion. Such pupillary unrest is termed hippus and has long been recognized but is poorly understood.[1] Hippus is spasmodic, cyclic, bilaterally in phase, and is usually considered benign,[2,3] although exaggerated hippus has been associated with epileptic seizures[4] and increased mortality,[5] and decreased hippus may occur in myasthenia gravis.[6] Hippus lacks a precise definition,[1] and there is poor consistency in the descriptions of its parameters. This is likely due to the variety of techniques used to assess the pupil movements, wide intraindividual variation,[3] and poor understanding of its mechanism. Reported hippus frequency ranges from 0.04[7] to 2 Hz, with the majority of studies finding a peak frequency near 0.3 Hz.[8,9] The magnitude of the pupil size variations range from not detectable (usually with naked eye observation) to over 0.5 mm.[4]

The role of the parasympathetic nervous system (PNS) and sympathetic nervous system (SNS) input to the iris as a determinant of overall pupil size has been well described[10–12]; however, the mechanism of hippus remains elusive.[8,13] Some have hypothesized that as pupil diameter is determined from the relative input of the SNS and PNS, hippus is likely due to feedback or noise from this continuous neurologic antagonism.[10,14–16] However, the origin of hippus is likely in the central nervous system, rather than local to the eye, because hippus movements are bilaterally consensual. Additionally, hippus is partially correlated with respiratory[17] and cardiac activity.[18,19] Some have suggested that hippus is a function of SNS tone alone,[20] while others have suggested it is driven by the PNS input,[17,21] despite dissonance between accommodative microfluctuations and hippus.[22] Burtis et al.[23] attempted to tease out the role of the PNS and SNS in hippus by exploiting both the modest asymmetry in monocular visual field projection and inherent cortical lateralization of the autonomic nervous system.[24–26] By occluding either the left or right eye to differentially activate each cortical hemisphere, and by extension the PNS or SNS, they saw slight differences in pupil dilation based on which eye was occluded, but their study lacked sufficient power to detect a difference in hippus activity.

In this study we aimed to determine the roles of the SNS and PNS in pupillary hippus by pharmacologic means. Participants received either a PNS antagonist (tropicamide) or a SNS agonist (phenylephrine) to dilate their pupil. To control for the effect of changing the relative nervous system input to the iris due to dilation alone, a third cohort received tropicamide in darkness.

Methods

Study Design

We used a paired-eye control study design, with three cohorts of 12 subjects (36 paired-eye comparisons). Each cohort received either phenylephrine 10% or tropicamide 1% in bright indoor illumination (1200 lux) or tropicamide 1% in a darkened room (<2 lux). Subjects who participated in more than one cohort had a washout period of at least 1 week.

In total, 22 unique subjects completed 36 visits across the three cohorts (mean age 23.2, range 21–32 years, 12 males). Exclusion criteria included ocular or central nervous system

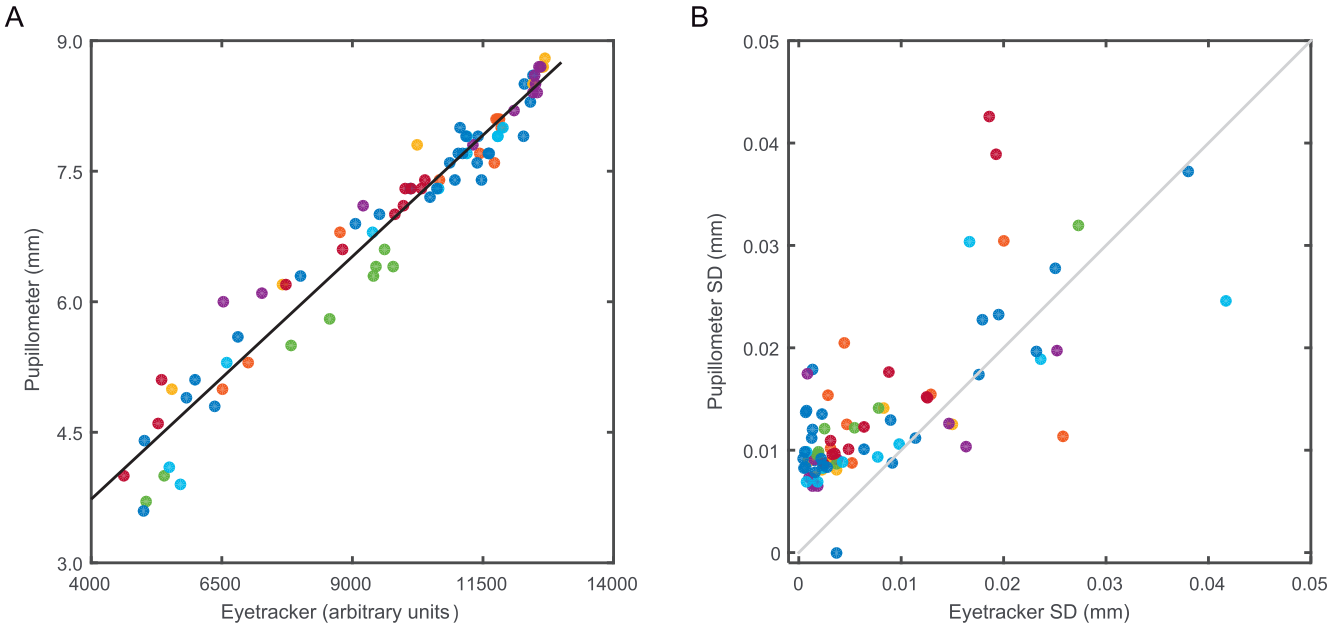

Figure 1. **(A)** Calibration of the Eyelink 1000 reported in arbitrary units against the NeurOptics pupillometer. Measurements are pupil diameter of the treated eye, colored per subject ($n = 9$), with an orthogonal regression fit with a *solid line*. The two measures were highly correlated ($n = 85$, $R^2 = 0.978$, $P < 0.0001$), with no significant difference between each subject's individual correlation ($P = 0.196$). **(B)** Comparing the single-measure standard deviation of pupil size from the Eyelink eye-tracker to the NeurOptics pupilometer. As there are a greater number of points on the pupillometer side of the *gray unity line*, this demonstrates that the pupillometer had higher variance than the eye-tracker at 86% of the points. This is significantly higher than chance ($P < 0.0001$), which means that the eye-tracker has greater precision.

pathology, mental illness, narrow anterior chamber angles (Van Herrick <0.3), intraocular pressure >21 mm Hg, or concurrent or previous hypersensitivity to any ocular or systemic medications. The study received approval from the University of Auckland Human Participants Ethics Committee (ref. no. 013672), subjects were provided informed consent in writing, and the study adhered to the tenants of the Declaration of Helsinki.

Each visit constituted 5 minutes of adaptation in either the light or dark environment, before bilateral measures of the pupil diameter using an eye-tracker were taken as a baseline. One drop of the drug was immediately instilled into the treatment eye (decided by coin toss), while the contralateral eye served as control. Repeated measurements of pupil diameter using the eye-tracker were taken every 5 minutes for 40 minutes, for a total of nine measures per eye, per subject, per visit. No masking was employed because of the monocular use of a dilating agent.

Pupil Measurement

Pupil diameter was recorded with an infrared eye-tracker (Eyelink 1000, SR Research, Mississauga, Ontario, Canada) running in binocular mode at 500 Hz. Subjects placed their head in a headrest positioned 25 cm from the eye-tracker camera, and the camera was focused so that the corneal reflex appeared crisp. Subjects did not wear optical correction during the measurements in order to eliminate lens magnification effects and to reduce reflections for the eye-tracker camera. Subjects were asked to fixate directly at the camera and minimize ocular movement during measurements. This presented a near perpendicular pupillary plane to the camera, while the near fixation target increased the likelihood of hippus being detected.[9] Misalignment of the camera and ocular axis, either due to fixation disparity or movement, were automatically accounted for in real time through gaze position tracking. After a 1-second stabilization period, bilateral pupil hippus movements were simultaneously recorded for 2.6 seconds. The short capture window minimized changes in SNS tone, reduced the likelihood of capturing a spasmodic change in hippus activity within a capture period, and eliminated the need to interpolate missing data due to blinking. If pupil detection failed during the capture period (e.g., due to a blink), the current trial data was discarded and the capture window restarted once both pupils were visible for at least 1 second.

Measures of pupil diameter were directly recorded into Matlab software (2015b; The Mathworks, Natick, MA, USA). In the time domain, pupil diameter and its variation within the 2.6-s measurement period (intrameasure variation), coefficient of variation, and the paired-eye pupil size correlation was computed. The raw pupil diameter data were then processed with a fast Fourier transform (FFT) algorithm (frequency resolution 0.244 Hz, Nyquist = 250 Hz), which decomposes the pupil diameter changes over time into a series of summed periodic functions of different frequencies. This allows comparisons of rhythms of variation between the unsynchronized time-domain measures. The total FFT energy (the sum across the whole frequency spectrum), as well as the dominant FFT frequency and its magnitude, were computed.

Eye-Tracker Calibration

To calibrate our eye-tracker (from arbitrary units to millimeters), each subject had the treated eye pupil size measured with an infrared pupillometer (NeurOptics; BD Ophthalmic Systems, Heidelberg, Germany) at 40 minutes, when the pupil was largest. The NeurOptics pupilometer has previously been shown to be accurate to within 0.1 mm, with good repeatability.[27] In order to validate the eye-tracker measures across the full range of pupil sizes, a subset of nine subjects had pupillometry performed on the treated eye immediately after each eye-tracker measurement. Orthogonal regression (Fig. 1A) showed high overall correlation between the eye-tracker and

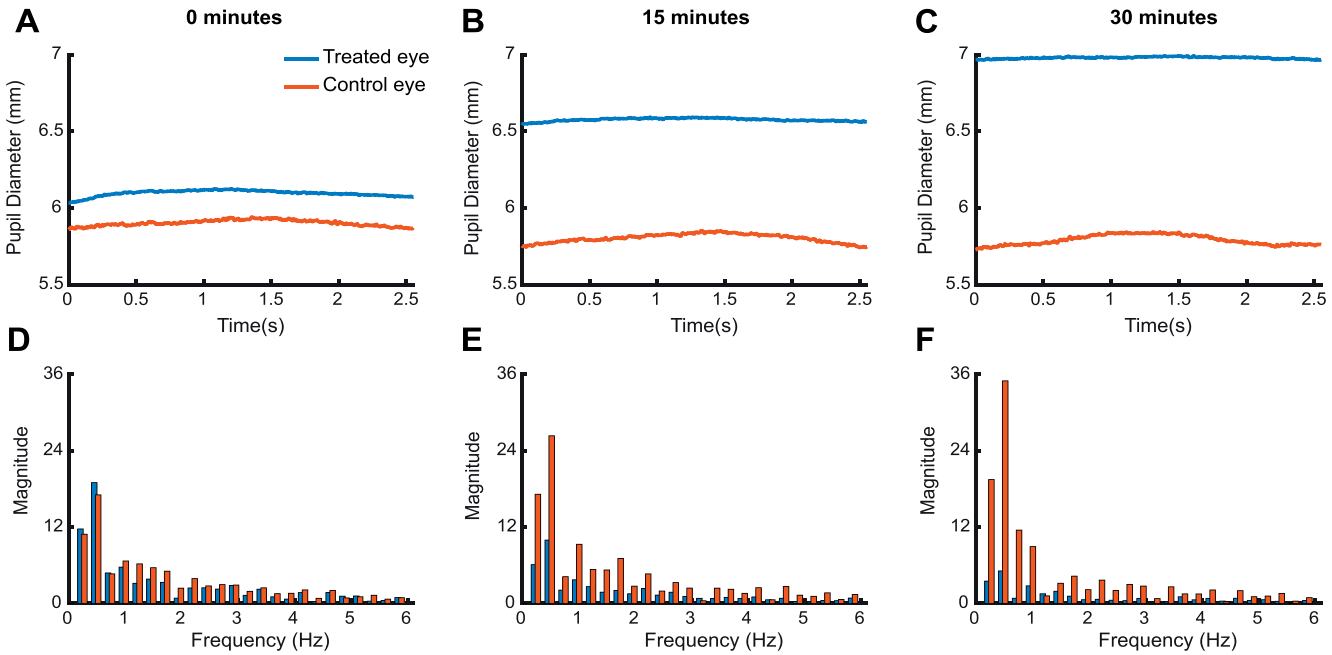

Figure 2. Example eye-tracker output from a single subject from the tropicamide dark (TD) group at baseline and at 15 and 30 minutes. (**A**) At baseline, the control eye (*orange*) pupil diameter is slightly smaller than the treated eye (*blue*), demonstrating physiological anisocoria, and pupil movements within this sample were highly correlated ($r = 0.921$, $P < 0.0001$). (**D**) Fast Fourier transform (FFT) analysis shows a dominant hippus frequency at 0.49 Hz in both eyes, and a similar FFT spectrum at baseline (frequency axis clipped at 6 Hz for clarity). After 15 minutes, the treated pupil diameter has increased (**B**), and the changes in pupil size are less correlated ($r = 0.719$, $P < 0.0001$). The corresponding FFT (**E**) shows a reduction in FFT energy in the treated eye, while the control eye remains similar to baseline. After 30 minutes (**C**) the treated eye is close to full dilation and shows very little variation in diameter compared to either the control eye or baseline, and the corresponding FFT (**F**) has much less energy in the treated eye compared to the control eye, as well as compared to baseline values.

pupillometer values, with no obvious bias across the range of pupil sizes ($n = 85$, $R^2 = 0.978$, $P < 0.0001$). Individual subjects' R^2 values were all highly correlated and ranged from 0.981 to 0.996 (all $P < 0.0001$). The mean conversion factor was 1412 eye-tracker arbitrary units per pupilometer millimeter, and there was no significant difference between the calibration factors between subjects ($F_{(8,76)} = 1.425$, $P = 0.196$).

Due to the arbitrary measures obtained from the eye-tracker, mean-difference analysis of pupil diameter measurements between the two methods could not be conducted. Instead, we plotted the standard deviation of every pupil measurement obtained by each instrument against each other. If the variance in pupil diameter measurement (intrameasure variance) was solely due to pupil diameter fluctuations, and both instruments recorded them accurately, then the variance associated with each measurement from the two instruments would be highly correlated. However, if instrument measurement error contributes to the variance, then the instrument with the higher measurement error would have higher variance. Plotting the intrameasure variance of pupil size from the two instruments against each other (Fig. 1B) shows the eye-tracker to have significantly less variance, and therefore greater precision, for 86% of the paired measurements ($t = -7.41$, $P < 0.0001$; Fig. 1B). Figure 2 shows examples of eye-tracker output from a single subject.

Statistical Analysis

Measurement of hippus was completed successfully every 5 minutes for 40 minutes, for 12 individuals, in each of the three cohorts, for a total of 324 treated to control comparisons (12 eye pairs × 3 cohorts × 9 time points). Primary outcome measures were changes in pupil diameter and paired-eye correlation in the time domain, and changes in total FFT energy, peak frequency, and peak frequency magnitude in the frequency domain. Data were analyzed in Matlab using repeated-measures 2-way ANOVA, using condition (tropicamide light [TL], tropicamide dark [TD], and phenylephrine light [PL]) and eye (treated and control) as factors, repeated across time (0–40 minutes). Post hoc analysis of significant differences was done with 1-way ANOVA as required. Equality of baseline measures between treated and control eyes were compared with paired *t*-tests. Validation of the eye-tracker measurements and correlations used Pearson's *r* and paired *t*-tests. Other correlations between nonnormally distributed variables were conducted with Spearman's rho (ρ). Values were considered significant at $P < 0.05$.

Because hippus is yoked between the eyes and hippus energy can vary over time, many measures are presented as a ratio of treated eye to control eye. As the ratio of FFT energy was not normally distributed, nonparametric tests were used when comparing FFT ratio over time and between groups. Akaike[28] information criteria, corrected for small sample size,[29] were used to determine the best model for the change in FFT ratio over time for the three cohorts and was compared between linear and one- and two-term exponential, Gaussian, polynomial, and power models.

Results

Baseline Comparisons

At baseline, the treated and control eyes' mean pupil diameter, hippus energy, dominant hippus frequency, and dominant frequency magnitude were not significantly different within each cohort. Instead, pupil diameter changes within each 2.6-

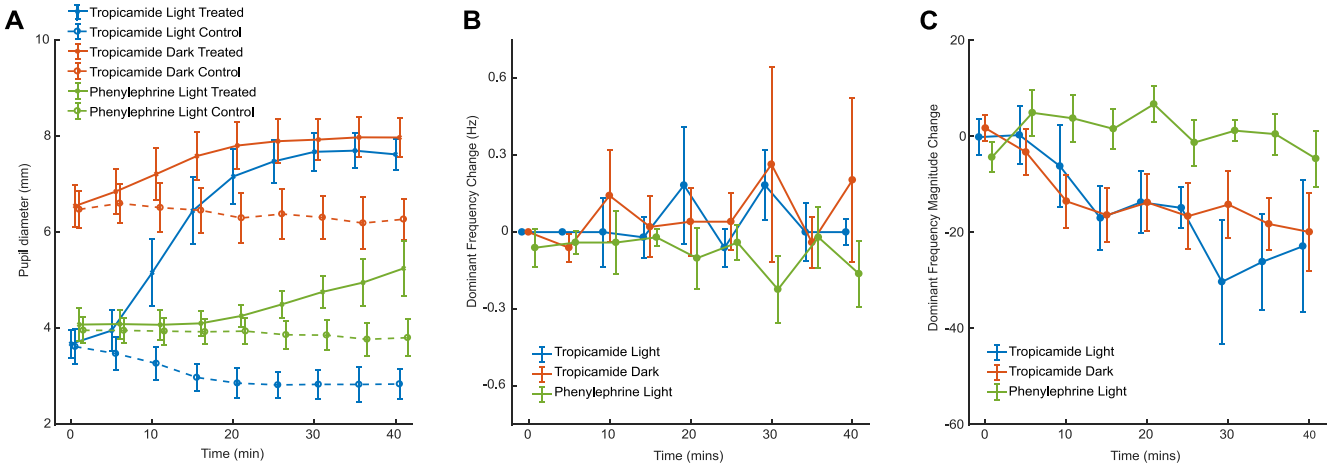

Figure 3. (**A**) Tropicamide and phenylephrine dilated all treated eyes; however, the onset was shorter in eyes treated with tropicamide. At baseline, the TD group had larger pupil diameters, but by 20 minutes, there was no significant difference in pupil diameter of the treated eyes of the two tropicamide groups. The phenylephrine-treated eye was significantly less dilated than the tropicamide-treated eyes throughout. Note the consensual miosis of the control eye due to the increased light capture of the dilated eye, which was significant in the TL group ($P = 0.001$, *blue dashed line*). (**B**) There was no change (treated–control eye) in FFT frequency over time in any of the groups ($P = 0.971$). The mean frequency throughout the experiment was also not significantly different between the three groups (TL: 0.60 ± 0.0 Hz, TD: 0.62 ± 0.09 Hz, PL: 0.62 ± 0.04 Hz, $P = 0.878$). (**C**) The change in magnitude of the peak hippus frequency decreased in both the TL ($P = 0.001$) and TD ($P = 0.003$) groups, but was unchanged in the phenylephrine group ($P = 0.523$). Note in both charts higher fluctuations from 30 minutes; this reflects the dominant hippus frequency becoming increasingly difficult to isolate from noise as the energy of the FFT decreases. *Error bars* are 95% confidence intervals.

second measurement period were highly correlated between the treated and control eyes (all $r > 0.88$, $P < 0.0001$). There was no difference in the number of right eyes (total: 17/36) used between groups ($F_{(2,33)} = 2.2$, $P = 0.127$). As the pupil was examined while the subject was instructed to focus at 25 cm (+4.00 diopter [D]) with refractive correction removed, there was a range of true accommodative demands (mean +2.82, SD 2.52 D). However, there was no difference in the refractive errors of the groups ($F_{(2,33)} = 1.7$, $P = 0.200$) nor between treated and control eyes ($t_{(20)} = 1.03$, $P = 0.311$). Contrariwise, refractive error, and therefore the accommodative demand during fixation, was very highly correlated between each subject's treated and control eye ($r = 0.994$, $P < 0.0001$).

Comparing baseline values between the three cohorts showed pupil diameter to be larger at baseline (as expected) in the TD group (6.51 ± 0.72 mm) than both the light groups (PL: 4.02 ± 0.53 mm, TL: 3.64 ± .057 mm, both $P < 0.0001$; Fig. 3A). The pupil diameters of the two light-condition cohorts were not different from each other at baseline ($P = 0.10$). The FFT energy was lower in the TD group (0.876 ± 0.363) than the PL (1.944 ± 1.137, $P = 0.003$) and TL (2.530 ± 1.465, $P < 0.0001$) groups, while the two light groups were not different from each other ($P = 0.157$). The dominant FFT frequency was correlated between the eyes at baseline ($r = 0.914$, $P < 0.0001$) and was not significantly different between paired eyes across the three groups ($F_{(2,35)} = 0.47$, $P = 0.969$; Fig. 3B). The magnitude of the dominant hippus frequency was similar between eyes and groups at baseline ($F_{(2,35)} = 0.95$, $P = 0.549$; Fig. 3C), and this was also significantly correlated between eyes ($r > 0.99$, $P < 0.0001$).

Time Domain

The pupil diameter of the treated eye increased over time compared to both the paired control eye and baseline values in all three groups ($F_{(2,16)} = 174.90$, $P < 0.0001$; Fig. 3A), confirming that both drugs modified the relative input of the SNS and PNS in the treated eye relative to the control eye. However, likely due to increased light capture by the dilated eye (stimulating consensual miosis), there was an overall *decrease* in the control eye pupil diameter ($F_{(8,11)} = 2.21$, $P = 0.035$); however, this was only significant in the TL control eye ($F_{(8,97)} = 3.59$, $P = 0.001$). Following instillation of tropicamide, the treated eye pupil diameter became significantly larger than the paired control beyond 10 minutes in the light ($F_{(1,22)} = 23.13$, $P < 0.0001$) and after 15 minutes in the dark ($F_{(1,22)} = 10.44$, $P = 0.004$). In the PL group, the treated eye was significantly larger than the control eye after 25 minutes ($F_{(1,22)} = 9.56$, $P = 0.005$). While the TL-treated eye pupil was smaller than the TD group at baseline, it was no longer smaller after 20 minutes ($P = 0.140$) following the application of tropicamide.

The dispersion of measures relative to the mean pupil size within each sample measurement (the coefficient of variation: SD/mean pupil size, of each sample) decreased in the treated eye in both tropicamide groups (TL: $F_{(8,97)} = 14.56$, $P < 0.0001$; TD: $F_{(8,99)} = 10.71$, $P < 0.0001$), but not in the PL group ($F_{(8,99)} = 1.3$, $P = 0.255$). The control eyes in all three groups showed no change in the coefficient of variation over time ($F_{(2,107)} = 0.87$, $P = 0.780$).

Frequency Domain

The dominant hippus frequency was not different between the treated and control eye in any of the three groups over time ($F_{(2,8)} = 0.26$, $P = 0.971$; Fig. 3B) nor between groups (TL: 0.60 ± 0.09 Hz, TD: 0.62 ± 0.09 Hz, PL: 0.62 ± 0.04 Hz; $F_{(2,24)} = 0.13$, $P = 0.8781$). However, there was a significant change in the magnitude of this dominant frequency between the groups ($F_{(2,24)} = 10.37$, $P = 0.001$), with the change in magnitude in the PL group (+0.8692 ± 3.9007) significantly less than both TL (–14.6188 ± 10.9254, $P = 0.001$) and TD (–12.7556 ± 3.9007, $P = 0.003$; Fig. 3C). The reduction in magnitude in TL and TD were not significantly different from each other ($P = 0.871$), while the change in PL was not different from zero over the 40-minute period ($t_{(8)} = 0.6685$, $P = 0.523$).

There was considerable variation in FFT energy over time in all eyes, reflecting the spasmodic nature of hippus and reinforcing the requirement to measure FFT energy as a ratio between the treated and control eyes to remove the temporal

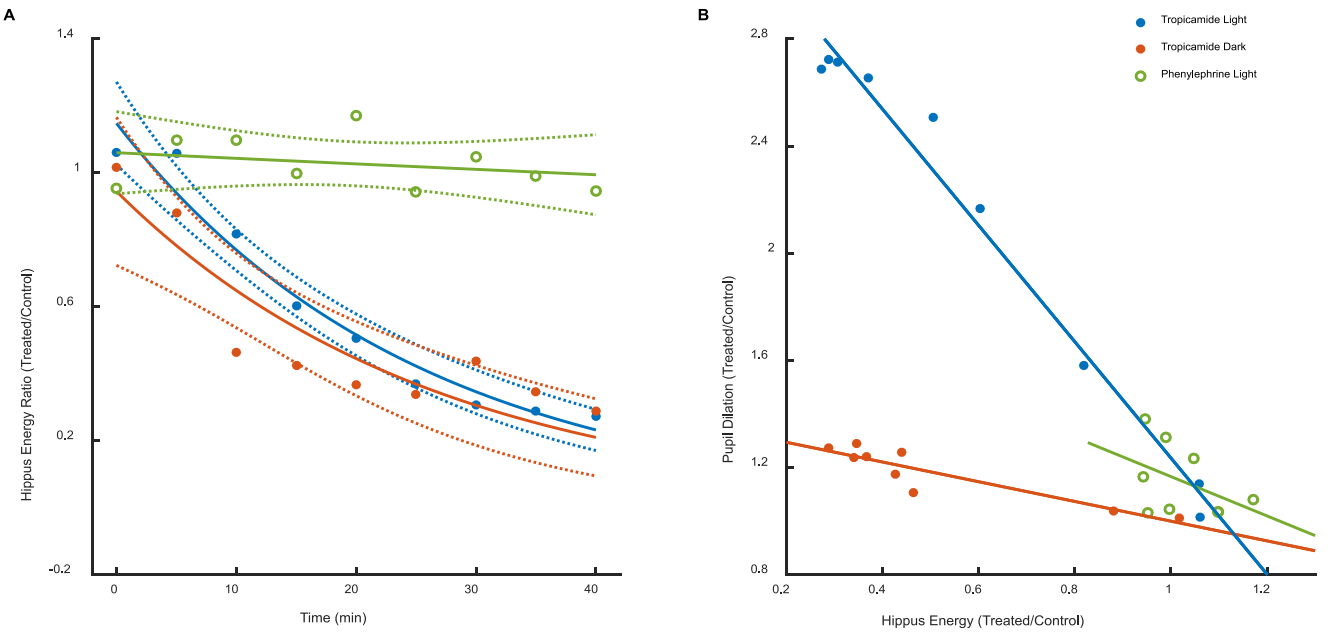

Figure 4. (**A**) The change in FFT energy ratio (i.e., the amount of hippus movement in the treated eye compared to the control eye) in each group, fitted with decay functions and 95% CI. Both tropicamide groups had a significant reduction in FFT energy ratio in the treated eye (both ~70% from baseline, $P < 0.0001$), while the FFT energy ratio of the phenylephrine group did not change over time ($P = 0.173$). The decrease in FFT energy ratio in the two tropicamide groups were not different from each other ($P = 0.768$), but both were significantly less than the phenylephrine group (versus TL: $P = 0.002$; versus TD: $P = 0.0004$). (**B**) When the FFT energy ratio is plotted against the dilation ratio, there is a significant negative correlation in the tropicamide groups (TL: $\rho = -0.633$, TD: $\rho = 0.542$; both $P < 0.0001$), suggesting that hippus decreases as a function of pupil dilation. However, despite a greater dilation ratio in the PL group (≈1.5× control eye) than the TD group (≈1.35×), the phenylephrine group dilation was not correlated with a change in FFT energy ratio ($\rho = -0.022$, $P = 0.830$). This suggests that the reduction in hippus following tropicamide is related to blocking the parasympathetic input to the pupil rather than the change in relative SNS and PNS input that occurs during pupil dilation.

fluctuations (for example, see Figs. 2D–F). The change in FFT energy over time was most appropriately fit with decay functions (using the minimum sum of corrected Akaike information criterion for the three groups[29]). This permitted direct comparison between groups (Fig. 4A). The β coefficient (i.e., the exponent of the initial ratio value of 1) was significantly less in the PL group than both tropicamide groups (TL mean: –0.040 [95% confidence interval (CI), –0.0481 to –0.0318], $R^2 = 0.957$; TD mean: –0.038 [–0.0547 to –0.021], $R^2 = 0.760$; PL mean: –0.002 [–0.007 to 0.003], $R^2 = -0.052$; Fig. 4A). There were significant differences in the FFT energy ratio both over time ($F_{(11,8)} = 12.439$, $P < 0.0001$) and between groups ($F_{(2,16)} = 3.93$, $P < 0.0001$). The FFT energy ratio decreased by over 70% in both TL and TD groups (both $P < 0.0001$), and the decrease was not significantly different between these two groups ($P = 0.768$). However, the FFT energy ratio of the PL group was not different over time ($P = 0.173$) and was not significantly different from 1.0 throughout ($t_{(8)} = 0.992$, $P = 0.350$).

In both tropicamide groups, there was a strong negative correlation between the pupil size and hippus in the treatment eye (TL: $\rho = -0.633$, $P < 0.0001$; TD: $\rho = -0.542$, $P < 0.0001$; Fig. 4B). However, despite a greater proportional increase in pupil size occurring in the PL group than the TD group (the y-height in Fig. 4B), there was no corresponding correlation between pupil dilation and FFT energy ($\rho = -0.022$, $P = 0.830$). This suggests that it is not pupil dilation itself, but rather the blocking of the PNS signal with tropicamide that causes the reduction in hippus.

Effect of Lateralization and Refractive Error

Individual lateralization asymmetries in the cortical innervation of the PNS and SNS may inadvertently bias monocular tasks,[23–25] so despite our treatment eye being randomly determined, the effect of eye was also investigated. However, the allocation of treatment eye did not affect the amount of dilation, relative to the paired control eye ($t_{(16)} = -0.048$, $P = 0.962$), nor the change in hippus energy ($t_{(16)} = 0.165$, $P = 0.871$). As mydriasis and cycloplegia took hold monocularly, the eye that was driving accommodation may have changed during the course of the experiment. However, the accommodative demand of the control and treatment eyes were not different ($t_{(20)} = 1.03$, $P = 0.311$), and the control eye accommodative demand was not different between groups ($F_{(2,20)} = 1.72$, $P = 0.196$). Further, the accommodative demand was not correlated with the pupil dilation ratio ($n = 36$, $\rho = 0.139$, $P = 0.420$) nor hippus energy ratio ($n = 36$, $\rho = 0.119$, $P = 0.489$), meaning lateralization and refractive error was not a major factor in our experiment.

Discussion

While the PNS and SNS act antagonistically in the establishment of overall pupil diameter, our data show that instillation of a parasympatholytic (tropicamide) reduces pupillary hippus, while installation of a sympathomimetic (phenylephrine) has no effect. Our findings challenge the assumption of PNS versus SNS rivalry as a cause of pupillary hippus, as hippus decreased with increased pupil size following installation of tropicamide but was unchanged after dilation with phenylephrine. This suggests that hippus is neither related to the absolute level of SNS activity (which was modified with phenylephrine) nor to the relative PNS-to-SNS input at the pupil (modified by light and dark environments), but is simply related to the level of PNS input (modified by tropicamide).

Our findings also support the idea that the PNS origin of hippus is not local to the eye. If hippus frequency were determined by a temporal integration at the neuromuscular junction in the pupil, then we would have expected a shift in hippus frequency as the time to reach a given threshold would be affected by the proportion of drug binding.[30] Instead, we found no change in hippus frequency during any period in any of the groups, suggesting tropicamide exerted its effect by simply blocking an upstream hippus signal from reaching the iris.

Our findings expand upon to the relationship others have found between hippus and both central and ocular PNS activity. Hippus has been shown to increase with heightened PNS tone during sleepiness,[31,32] accommodation,[9] and with increasing light level.[33] However, there are some seemingly conflicting reports in the literature. Opioids also increase the PNS tone to the iris,[34] but *decrease* hippus activity.[35] Additionally, hippus dynamics are too rapid to be caused by feedback from the parasympathetic light reflex,[30,36] and when the reflex loop is closed using a fixed-size artificial pupil, hippus persists.[37] However, these findings are consistent with our suggestion of a nonocular origin of the PNS hippus signal, whose magnitude, but not frequency, may be modulated by overall PNS tone in the case of light intensity, while opioids have complex central nervous system effects[38] and may influence the upstream origin of hippus.

Our experiment only examined hippus after perturbing the balance between PNS and SNS activity to produce mydriasis (i.e., with a PNS antagonist or an SNS agonist). We cannot rule out the possibility that similar effects might result from upsetting the innervation balance in the opposite direction, that is, using PNS and SNS miotics while in the dark. However, in either case, if the hippus signal originates centrally, instillation of local agonists or antagonists might produce paradoxical effects. For example, the addition of a PNS agonist would increase PNS tone and cause pupil miosis, but paradoxically it may potentially decrease hippus energy because of reduced acetylcholine receptor availability at the pupil.

We were able to detect a dominant hippus frequency within the commonly reported range (0.2–2 Hz) in all individuals, but this may have been due to our measurements requiring fixation on the camera, which would have stimulated accommodation in most individuals.[9] However, we found no relationship between the accommodative demand and the amount of hippus present, so our finding may just reflect increased sensitivity of our experimental setup. Unfortunately, our results are not strong enough to conclude that the *entirety* of hippus is due to PNS activity. While the eye-tracker appeared precise in its measure (Fig. 1B), we cannot determine how much of the residual hippus signal is due to incomplete PNS antagonism as compared to measurement noise. As the Fourier transform captures all variance in the pupil size, including that from machine measurement error (e.g., camera sensor noise) and other biological effects (e.g., tear film changes), it is likely that our measure of FFT energy is overestimating pupil movements and would never truly reach zero, even with a completely rigid biological pupil. This likely explains why the total reduction in FFT energy in both tropicamide groups was approximately 70% rather than a complete reduction, as the energy was simply summed over the entire spectral distribution and no assumptions about the properties of hippus were made. However, what is striking is the reduction of energy in the previously reported hippus range of 0.24 to 1 Hz (which accounted for 96.5% of all sample energy) after the application of tropicamide. In both the light and dark tropicamide conditions, the low-frequency FFT amplitude reduced to approximately 5% of the control eye levels, such that the FFT appeared relatively flat across the entire spectrum, making the residual signal indistinguishable from noise, while the PL group's FFT amplitude was unaffected.

While the sympathetic tone contributes to overall pupil diameter, our results suggest investigating hippus could provide insight to the PNS and add detail to the pupillary evaluation of brain function.[12,39] Improving our understanding of the mechanisms of pupil dynamics, in combination with the rapid advancement in cost-effective imaging technologies, make pupillary examination a useful means to gain insight to underlying central nervous system activity.

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