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Cardiac-Specific Manipulation of the Trissin Signaling Pathway Affects Adult *Drosophila* Cardiac Function and Rhythm

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Abstract

The Fly Cell Atlas single-nucleus transcriptomic project observed Trissin G-protein-coupled receptor expression in cardiomyocytes for the first time. This signaling pathway is linked to intracellular calcium flux, however mainly in neurons and non-cardiac muscle tissue. Via cardiac-specific RNAi knockdown and high-speed fluorescent microscopy, we investigated the potential role(s) of Trissin signaling in our *Drosophila* cardiac model. We observed significant decreases in heart rate, diameters and cardiac output. We also observed increased arrhythmicity, primarily during relaxation. Data suggests a cardiac function and rhythm role for Trissin signaling, providing a novel target along with its downstream interactors for understanding cardiac rhythm disorders.

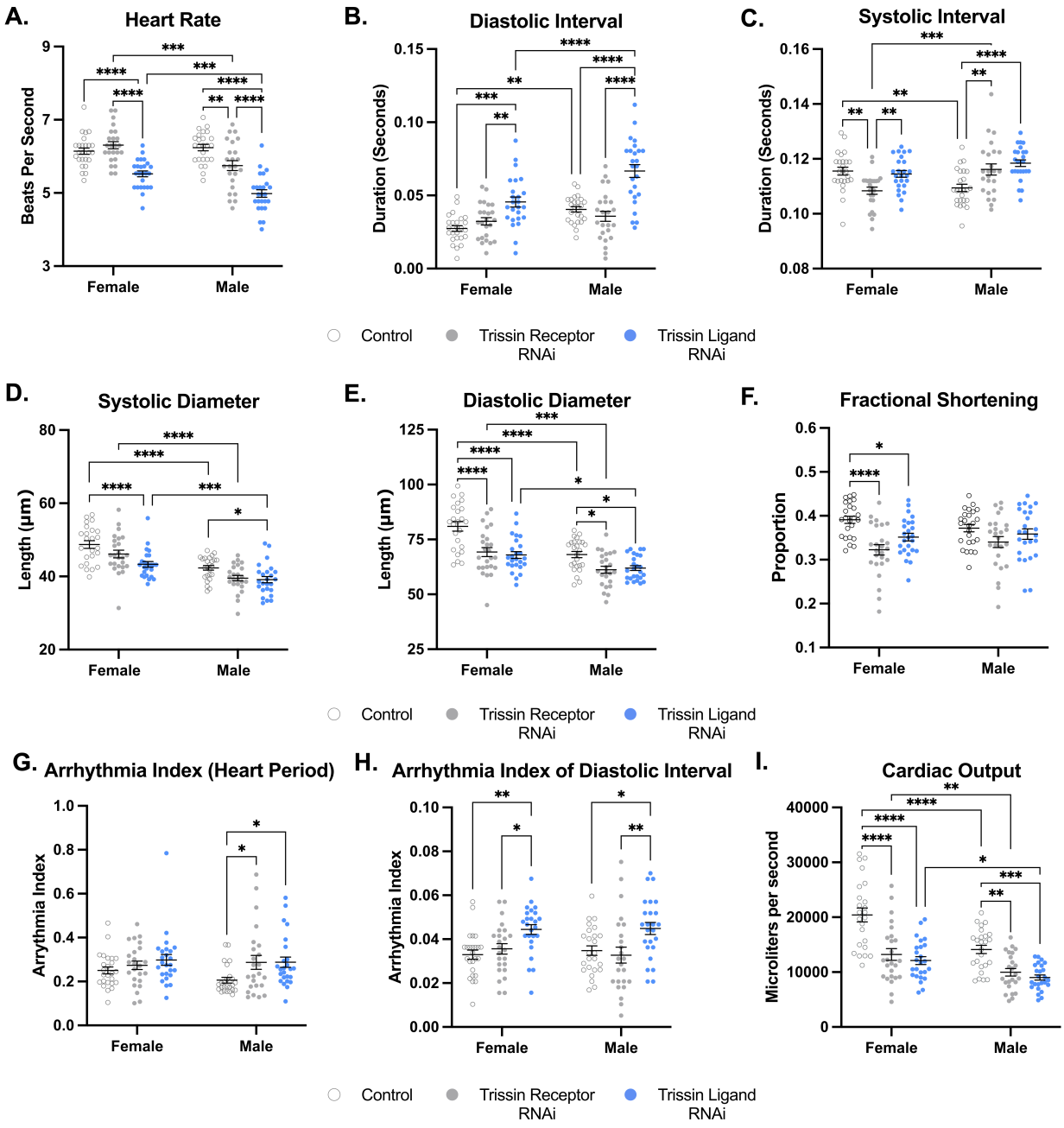


Figure 1. Effects of Reduced Trissin Signaling on *Drosophila* Cardiac Function and Rhythm:

Trissin receptor (TrissinR) and Trissin ligand expression was individually reduced specifically in the cardiac tissue via the Hand4.2-Gal4 driver line. **(A)** Heart rates were significantly slower with reduced ligand levels in both sexes, however, reducing TrissinR only affected male heart rate. **(B,C)** Trissin knockdown significantly lengthened diastolic intervals in both sexes, with minimal effects observed with TrissinR manipulation. Reducing female ligand or receptor levels caused shorter systolic intervals, whereas Trissin knockdown in males led to significantly longer systolic intervals, implying a sex-specific effect. **(D, E)** Reducing ligand expression, but not receptor expression, significantly decreased systolic diameter in both sexes. Diastolic diameter was significantly reduced in both sexes. **(F)** Female flies had significantly reduced contractility, however there were no significant changes observed in males. **(G)** Reduced Trissin signaling slightly increased arrhythmicity (heart period) in females. However, arrhythmicity was significantly increased in male flies. **(H)** The heart period, or one cardiac cycle, is comprised of the systolic and diastolic interval. Although no changes regarding arrhythmicity were observed within the systolic interval, there were significant signs of arrhythmicity as a result of reduced Trissin ligand levels. **(I)** Overall, these phenotypic changes led to a reduced cardiac output as a result of either reduced ligand or receptor expression. Video processing and data acquisition was first accomplished via R/Rstudio

analyses. For each parameter, two-Way ANOVAs with post-hoc Tukey multiple comparison testing was performed in GraphPad Prism (v10.4.1). Error bars are represented by mean +/- 1 standard error. Sample size for each cohort is either 24 or 25 flies.

Description

Atrial Fibrillation (AFib) is the most common heart rhythm disorder, with current epidemiology studies estimating ~59 million patients worldwide (Du et al., 2017, Feghaly et al., 2018; Linz et al., 2024). AFib incidence displays a strong correlation with age, affecting nearly 10% of individuals older than 65 years old. Despite the commonality, there is no single cause or driving factor behind this disease. This complex, multifactorial disorder could involve several factors such as genetic mutations, age, sex, race, diet and drug use (Campuzano and Brugada, 2009; Roselli et al., 2018., Weng et al., 2017). Current treatment options are limited and rather just temporary, failing to fix the underlying pathology. Studying AFib and other cardiac rhythm disorders tends to be challenging in vertebrate models due to their average lifespans, genetic redundancy, and the complex interrelationship with the respiratory system (Nishimura et al., 2011; Ocorr et al., 2007).

To rapidly investigate genetic candidates involved in heart function and rhythm, we can utilize the *Drosophila melanogaster* cardiac model. *Drosophila* provide the unique advantage in simplifying a mammalian cardiac system through highly conserved cardiac development mechanisms, muscular contractile structure and arrangement, regulatory ion channels and myogenic nature (Ahmad, 2017; Blice-Baum et al., 2019; Zhao et al., 2023). Lastly, the ease of population maintenance, quick generation cycles and shorter lifespans allow *Drosophila* to be an efficient research model for large-scale studies of age-related disorders (young-fly is 1-2 weeks old, old-fly is 4-5 weeks old).

Through the multi-lab Fly Cell Atlas single-cell RNA sequencing profiling of 15 different *Drosophila* tissues, (Li et al., 2022), expression of the neuroreceptor TrissinR and neuropeptide ligand Trissin were documented in cardiomyocytes for the first time. Knowledge of Trissin signaling is not well understood besides a role in intracellular calcium ion flux. Trissin is not conserved in vertebrates, and it is not universal even among invertebrates (mainly Dipteran and polyneopteran). Despite these differences, investigating Trissin signaling in the *Drosophila* heart tube can help identify affected downstream cellular and molecular cardiac mechanics, in which those may be fundamentally conserved across phyla. Identification and improved understanding of these conserved mechanisms could provide novel targets for AFib diagnostics and therapy. Therefore, because calcium, and not sodium, is central to *Drosophila* cardiac cycle, we asked whether reducing TrissinR or Trissin expression in cardiac tissue via the UAS-Gal4 system alters *Drosophila* heart function and rhythmicity.

The effect(s) of knocking down Trissin ligand expression or the Trissin receptor (TrissinR) expression in the *Drosophila* heart was documented via high-speed video fluorescent microscopy. Results will be reported as means ± 1 standard error (sample size = 24 or 25 flies per condition per sex). Reduced expression of TrissinR showed no significant changes in female heart rate (6.31 ± 0.1 beats per second (bps)) compared to controls (6.14 ± 0.09 bps). However, male flies had significantly decreased heart rate via TrissinR knockdown (KD; 5.75 ± 0.14 bps) compared to controls (6.24 ± 0.09 bps) (Fig 1A). Reducing Trissin ligand expression significantly lowered heart rate in both 2-week old male and female flies. Female heart rates decreased to 5.52 ± 0.08 bps whereas male hearts rates decreased to 4.99 ± 0.1 bps. These effects are further supported by changes in the systolic intervals (heart movement) and diastolic intervals (heart fully relaxed). Trissin KD significantly lengthened diastolic intervals in both sexes, with minimal effects observed after reducing TrissinR expression (Fig 1B). Diastolic intervals increased from 0.027 ± 0.002 s in control females to 0.046 ± 0.004 s in Trissin ligand KD females. In males, the diastolic interval increased from 0.41 ± 0.002 s in controls to 0.067 ± 0.004 s in Trissin ligand KD males. Reducing female receptor levels caused shorter systolic intervals, whereas Trissin KD (ligand or receptor) in males led to significantly longer intervals, implying a sex-specific effect (Fig 1C). Female systolic interval decreased from 0.116 ± 0.001 s to 0.108 ± 0.001 s as a result of TrissinR KD. In males, the average systolic interval rose from 0.109 ± 0.001 s in control flies to 0.116 ± 0.002 s with TrissinR KD and 0.118 ± 0.001 s with Trissin ligand KD.

Reducing ligand expression, but not receptor expression, significantly decreased systolic diameter in both sexes (Fig 1D). Female systolic diameter decreased from 48.72 ± 1.03 µm to 43.31 ± 0.76 µm. Male systolic diameter decreased from 42.33 ± 0.63 µm to 39.12 ± 0.86 µm. Regarding diastolic diameter, reducing either the ligand or the receptor levels significantly decreased the diameters in both sexes (Fig 1E). Female diastolic diameter decreased from 80.97 ± 2.18 µm to 69.28 ± 2.04 µm (TrissinR KD) and to 67.92 ± 1.57 µm (Trissin ligand KD). Male diastolic diameter decreased from 68.13 ± 1.42 µm to 61.23 ± 1.57 µm (TrissinR KD) and to 61.96 ± 1.1 µm (Trissin ligand KD). Altering cardiac diameters can also affect the fractional shortening or in other words, the ratio of diameter reduction during contraction. This essentially represents contractile ability of the cardiac tube. Female flies had significantly reduced contractility (Fig 1F), however there were no significant changes observed in males. As a result of Trissin ligand KD, the fractional shortening of control females decreased from 0.39 ± 0.008 to 0.35 ± 0.008, whereas TrissinR KD reduced fractional shortening even further to 0.32 ± 0.01. Although Trissin signaling is mainly involved in calcium regulation, disruption in this aspect could prevent the cardiac tube from maintaining basal tone through IP3-mediated pathways (Capel et al., 2021; Piamsiri et al.,

2024). Additionally, calcium dysregulation could lead to systemic stress, disrupting myofibrillar alignment during remodeling and leading to a constricted cardiomyopathy (Bare and Ai, 2025; Wang et al., 2025).

Increased arrhythmicity during overall heart period, and specifically within the diastolic phase, was observed as a result of reduced Trissin ligand concentration (Fig. 1G-H). Regarding heart period, Trissin ligand KD only significantly increased arrhythmicity in the male flies (0.21 ± 0.013 to 0.288 ± 0.024) with insignificant increases observed in females (Δ +0.048). As a response to reduced receptor expression, only males exhibited significantly increased arrhythmicity (Fig 1G). Male heart period arrhythmicity increased from 0.21 ± 0.013 to 0.29 ± 0.032. As mentioned above, the contraction cycle can be split between systolic and diastolic intervals, and therefore, so can the arrhythmicity measures. We saw negligible changes in systolic interval arrhythmicity. However, we did observe significant increases in diastolic interval arrhythmicity in both sexes (Fig 1H). Female arrhythmicity rates increased from 0.033 ± 0.002 to 0.044 ± 0.002 as a result of Trissin ligand KD. Male arrhythmicity rates increased from 0.035 ± 0.002 to 0.045 ± 0.003 as a result of Trissin ligand KD. Overall, the cardiac effects seen as a result of reduced Trissin signaling (either ligand or receptor) have led to significantly reduced cardiac output in both sexes (Fig 1I). Cardiac output in females reduced from 20,430.99 ± 1266.54 µL/s in controls to 13,214.52 ± 1062.73 µL/s (TrissinR KD) or 12,108.34 ± 713.96 µL/s (Trissin ligand KD). In male flies, cardiac output went from 14,132.35 ± 749.29 µL/s in controls down to 9,972.13 ± 682.5 µL/s (TrissinR KD) or 9,025.28 ± 467.86 µL/s (Trissin ligand KD). The reduced output is evidence that Trissin signaling is not necessarily just associated with rhythm. The structural and functional effects imply that multiple cardiac mechanisms are being affected by the disruption of calcium flux.

Overall, RNAi KD of either the ligand or receptor appears to alter cardiac function, with reduced ligand levels appearing to have stronger effects in most cardiac parameters. It must be stated that these conclusions are preliminary findings as we have not yet verified/quantified KD efficiency. Validating KD efficiency of the Trissin receptor RNAi line and the Trissin ligand RNAi line will help verify the results, but also provide additional context for certain parameters with differing results. Based on Vienna Drosophila Research Center, the Trissin ligand RNAi line (KK 105016) has 0 off target effects, but the Trissin receptor RNAi line (KK 107943) does have 2 off target effects, however those details are not available. In addition to tissue-specific qPCR for KD verification, additional experimentation in our lab will include: 1) use of GCaMP reporters to follow changes in calcium availability due to Trissin signaling manipulation, 2) visualize presence of Trissin receptors in *Drosophila* cardiomyocytes via immunohistochemistry, and 3) investigate a potential age-specific role for Trissin signaling by analyzing 4-week old fly cardiac data. Lastly, to fully understand how manipulating Trissin signaling is altering cardiac performance, completing RNA sequencing in Trissin manipulated cohorts is a logical future experiment as it would provide insight into affected downstream targets and molecular mechanisms.

Methods

Fly Husbandry

Flies were kept in an incubator at 25°C and fed a standard banana – molasses medium. UAS-RNAi lines and their respective genetic background control lines were acquired from the Vienna Drosophila Resource Center (VDRC, Vienna, Austria): Trissin Receptor UAS-RNAi line – KK 107943; Trissin Ligand UAS-RNAi line – KK 105016.

Cardiac-Specific Knockdown

Cardiac-specific gene knockdown (KD) was achieved using a Gal-4 based system that drives expression of dsRNAi in a cardiac-specific manner. For *in vivo* studies the cardiac-specific Hand4.2-Gal4 driver was recombined with a transgenic fluorescent reporter expressing tdTomato specifically in the heart. Virgin females were crossed to male flies from UAS-RNAi lines for Trissin ligand or Trissin receptor. A control line for the KK genetic background from VDRC was used as the control. Crosses resulted in Hand4.2-Gal4, tdTomato/+ ; UAS-RNAi progeny. Adult female and male progeny for all crosses were collected upon eclosion, separated by sex, and raised at 25°C on a 12-hour light-dark cycle. Flies were fed a standard banana-molasses diet, with food replaced every other day.

Filming and Analysis:

Cardiac phenotypes of 2-week old female and male flies from each cross were characterized using a high-throughput (HT) *in vivo* fluorescent platform (Kervadec et al. 2023). Adult flies were anesthetized using Fly Nap® (Carolina, NC, USA), a triethylamine-based anesthetic, and fixed on a microscope coverslip using the UV-curable, Norland Optical adhesive #61 (Norland Products, NJ, USA). High-speed video microscopy was performed using a modified fluorescent microscope and a Hamamatsu EM-CCD camera, and HC Image capture software (Hamamatsu Corp). Hearts from 20-25 flies per sex per genotype were imaged for 5s at ~285 frames/s with 555nm illumination. Movie analysis was automated using a custom R script (Vogler 2021) that traces the heart wall movements and returns heart wall displacement over time. Individual beats were identified and automatically segmented into discrete contraction and relaxation events. Multiple morphological and functional spatiotemporal parameters were calculated from the segmented heartbeat waveforms including: end-diastolic and end-systolic diameter, fractional shortening, systolic and diastolic interval, heart period, maximum speed of

contraction and relaxation, and multiple arrhythmicity parameters. GraphPad Prism and Adobe Illustrator were used to statistically analyze and visualize data. Two-Way ANOVA tests (post-hoc Tukey testing) were used to determine statistical significance between specific comparisons (n = 24 or 25 flies per sex per group). Scatter dot plots are plotted with error bars representing mean values +/- 1 standard error.

Reagents

Trissin Receptor UAS-RNAi line – KK 107943 (Vienna Drosophila Resource Center, Vienna, Austria)

Trissin Ligand UAS-RNAi line – KK 105016 (Vienna Drosophila Resource Center, Vienna, Austria)

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