Investigating the role of Stromalin and its potential downstream targets on seizure behavior in *Drosophila*

by

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Abstract:

The cohesin complex is a highly conserved structure that plays an important role in sister chromatid cohesion, segregating chromosomes during cell division, gene regulation, and repairing double stranded DNA breaks. The cohesin complex is comprised of four core subunits: Stromalin (STAG1/2), Rad21, SMC1 and SMC3 as well as auxiliary proteins that aid the complex in its functions and maintenance. Mutations in the subunits and auxiliary proteins have been shown to result in a group of rare, multi-organ system-wide developmental disorder known as cohesinopathies that are characterized by behavioural, neurological, and growth abnormalities. Past studies on cohesinopathies have provided evidence for dysregulated gene expression and not chromosomal segregation as a pathological mechanism underlying the disease. One of the neurological symptoms in cohesinopathies is an increased rate of seizures, particularly seen in patients with mutations in STAG1/2 and SMC1 subunits. However, the mechanisms underlying this remain unknown.

Recently, the *Drosophila* homologue of the STAG1/2 subunit, Stromalin, was found to affect learning and memory by constraining the synaptic vesicle pool sizes and synaptic communication in *Drosophila* dopamine neurons. As an imbalance in synaptic communication is typically associated with seizures, we hypothesized that the synaptic vesicle and synaptic communication increases upon Stromalin knockdown may be a potential mechanism underlying contributing to seizures in cohesinopathies. We performed a standard mechanical seizure induction assay on *Drosophila* expressing RNAi targeted against *stromalin* and *SMC1* in the brain, and found these manipulations significantly increased seizure frequencies, consistent with observations in cohesinopathy patients. We then tested 5 gene candidates, *Nep1*, *CG17698*, *Cox7c*, *Ttm2* and *Su(z)12*, that were identified to potentially regulate the cohesin complex's effects on memory, for their ability to induce seizures. Knockdown of *CG17698* and *Cox7c* in the brain using RNAi increased seizure frequencies in our animals, but not *Nep1, CG17698,* or *Ttm2*.

We then investigated the role of synaptic vesicles in the increased seizure rates we were seeing in our flies. We decreased the numbers of synaptic vesicle numbers at the synapses by impairing *unc104* function, which is known to traffic synaptic vesicles from the cell body to the synaptic terminal. We found that the pan-neuronal reduction of the synaptic vesicle trafficking protein, *unc104* failed to reduce the seizure frequency in Stromalin knockdown flies, however the positive control did not show increased seizure frequency in this experiment. Thus, this interpretation may not be accurate. Finally, we also analyzed the intensities of a synaptic vesicle marker, Synaptotagmin:GFP using fluorescence microscopy. We found inconsistent changes in Synaptotagmin:GFP across the whole-brain in our flies and upon the pan-neuronal knockdown of *unc104*, suggesting that an association between changes in synaptic vesicle numbers and seizure phenotypes in our flies could not be confidently inferred from our data.

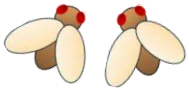
All in all, through this project we have provided evidence for a potential Stromalin based *Drosophila* cohesinopathy model that has increased seizure frequencies, consistent with the increased seizure rates seen in patients with STAG1/2 and SMC1 mutations. Reduction of Stromalin and SMC1 may induce the seizure phenotype in flies by reducing transcription of *CG17698* and *Cox7c* in neurons. Our findings suggest that certain neurological symptoms of cohesinopathies can be studied in *Drosophila*.

Preface:

The seizure behavioral experiments on *stromalin*^{RNAi} flies across age as well as the seizure rescue behavioral experiments with *unc104* whole-brain knockdown were performed and analyzed by Celina Phan for her BIOL 498 and 499 projects, and as part of her summer volunteering. I performed the remaining seizure behavioral experiments as well as the dissections and imaging experiments and analyzed them as well.

Dedication:

To my fruit flies. This is for you! Over the past 2-3 years, I have come to think of you as my children. Thank you for the sacrifices you've made, and I am sorry for the things I put you through but know that you will always be my favorite model organism. The world shall know about your greatness.



Acknowledgments:

I would like to thank Dr. Anna Phan for being a wonderful supervisor and for helping me grow both as a person and a researcher. Thank you for the constant support and guidance throughout this project. I had a great time learning all the things that I now know about *Drosophila* and have grown very fond of them during my time in the lab. Most of all, thank you for introducing me to this project-it made me appreciate certain things at the cellular level that I never really thought about earlier and had previously taken it for granted.

I would also like to thank my committee members: Dr. W. Ted. Allison and Dr. Lisa Willis for being very kind, supportive and encouraging during this journey. Their insights into the project helped me better understand the work I did and helped me expand my knowledge. I really appreciate the discussions, questions, and feedback I received from you both over the years as it opened some new directions/avenues that previously did not cross my mind.

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- B2R: B2 recombinase
- BDSC: Bloomington *Drosophila* Stock Center
- CAID: chronic atrial and intestinal dysrhythmia
- CdLS: Cornelia deLange Syndrome
- DANs: Dopamine neurons
- DSHB: Developmental Studies Hybridoma Bank
- GAL4: Galactose-responsive transcription factor 4
- GFP: Green Fluorescent Protein
- RNAi: RNA interference
- SA: Stromalin
- Scc: sister chromatid cohesion
- SMC: structural maintenance of chromosomes
- STAG: Stromal Antigen
- Syt: Synaptotagmin
- Syt:GFP: Synaptotagmin:eGFP
- TIMM50: Translocase of inner mitochondrial membrane 50
- TRiP: Transgenic RNAi Project
- ^{ts}: Temperature sensitive
- UAS: Upstream activating sequence
- VDRC: Vienna *Drosophila* Resource Center

CHAPTER 1

INTRODUCTION

1.1 Overview of the chapter:

This chapter reviews the existing literature on the cohesin complex and its functions and how mutations in the cohesin complex lead to disease in humans. I then bring the reader's attention to the known pathological mechanisms underlying cohesinopathies, and provide more insight into seizures, their prevalence and what is known about them in cohesinopathies. Finally, I introduce the usage of *Drosophila* as a seizure model and as a model to understand the mechanisms of cohesinopathy-induced seizures.

1.2 Cohesin complex:

The cohesin complex is made up of 4 highly conserved subunits that play a pivotal role in cell division, forming a ring around sister chromatids to ensure proper chromosome segregation during cell division (Figure 1) (Michaelis et al., 1997, Losada et al., 1998, and Tóth et al., 1999). Additionally, it was later found that the cohesin complex participated in repairing double stranded breaks in DNA, in looping of DNA during transcription, in long-range chromatin interactions and thus, in regulation of gene expression (Kagey et al., 2010, Zuin et al., 2013, Dorsett & Merkenschlager, 2013, and Litwin et al., 2018).

The 4 highly conserved subunits include- structural maintenance of chromosomes 1 and 3 (SMC1 and SMC3), Rad21 (Scc1 in yeast) and Stromalin, (Scc3 in yeast or STAG 1/2 in humans). Various other proteins are responsible for loading cohesin on and off the chromosome, assisting in its function, or maintenance (Peters et al., 2008, and Piché, Van Vliet, et al., 2019) (Figure 1). These include: Nipped-B (Scc2 in yeast or NIPBL in humans) and dMau-2 (Scc4 in yeast or MAU-2 in humans), two auxiliary proteins that mediate the loading of the cohesin complex onto the chromosome (Figure 1) (Ciosk et al., 2000, and Alonso-Gil & Losada, 2023), Eco and San (Eco1 in yeast or ESCO1/ESCO2 in humans), two acetyltransferases that act on the SMC3 subunit and Dmt (SORORIN in humans) to ensure proper functioning of the complex (Horsfield et al., 2012, Alomer et al., 2017, Yamada et al., 2017, Makrantoni & Marston, 2018, and Piché, Van Vliet, et al., 2019), and Pds5 (Pds5 in yeast or PDS5A in humans) and Wapl (Rad61/Wpl1 in yeast or WAPL in humans), two proteins that interact with one another to facilitate the removal of the cohesin complex from the chromosome (Figure 1) (Peters et al., 2008, Makrantoni & Marston, 2018, and Piché, Van Vliet, et al., 2019).

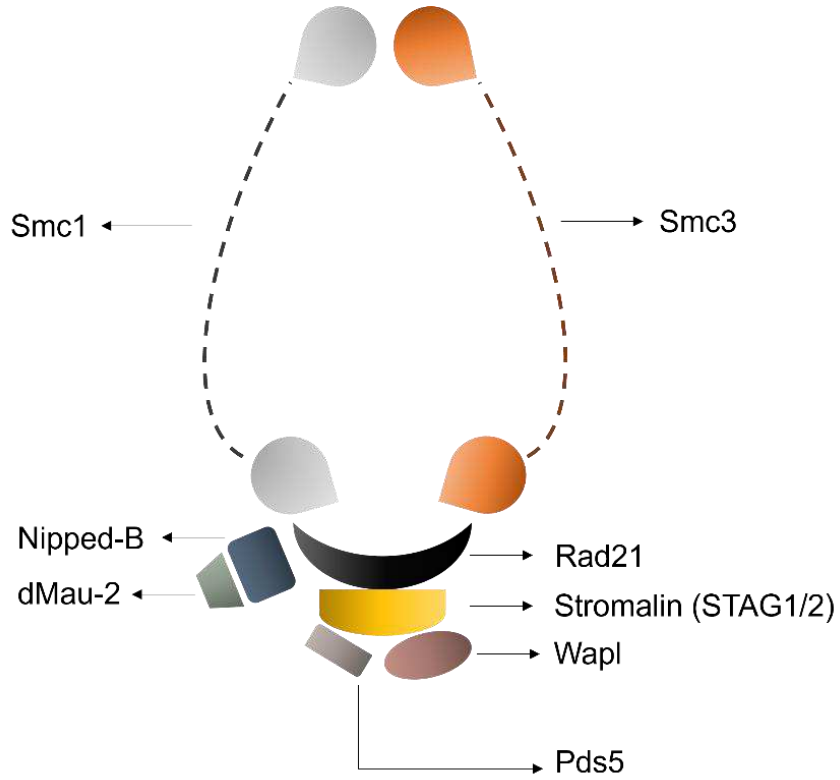

Figure 1: A representation of the cohesin complex in *Drosophila*. The diagram was adapted from Bhattacharya et al. (2023). The two structural components of the cohesin complex, SMC1 and SMC3, are connected to one another by Rad21 and Stromalin, to provide it with its signature ring-like configuration. Additionally, the cohesin complex loading proteins, Nipped-B and dMau-2 are found to be near Rad21 while the release mediating proteins, Wapl and Pds5 are found to be near Stromalin.

Table 1: Homologs of cohesin complex subunits and accessory proteins in yeast, *Drosophila*, and humans. Adapted from Horsfield et al. (2012).

Cohesin complex proteins	*Saccharomyces cerevisae* (Yeast)	*Drosophila melanogaster* (Fruit fly)	*Homo sapiens* (humans)
Core subunits	Smc1	SMC1	SMC1
	Smc3	SMC3	SMC3
	Scc1	Rad21	RAD21
	Scc3	Stromalin (SA)	STAG1/2
Loading complex	Scc2	Nipped-B	NIPBL
	Scc4	dMau-2	MAU-2
Release complex	Rad61/Wpl1	Wapl	WAPL
	Pds5	Pds5	PDS5A
Acetyltransferase	Eco1	Eco and San	ESCO1/2
Deacetyltransferase	Hos1	Unknown	HDAC8

1.3 Cohesin complex and accessory proteins:

Cohesin complex architecture and function: What have we learnt from yeast?

SMC1 and SMC3 belong to a family of large ATPases that contain specific motifs on both of their N-and C-termini, such that when the two termini of each subunit combine, it results in the formation of the ATPase domain (Strunnikov et al., 1993, Saitoh et al., 1994, and Beasley et al., 2002). The increasing proximity between the two termini while forming the ATPase domain also signifies the hinge domain that links SMC1 to SMC3 to form the cohesin complex (Melby et al., 1998, Beasley et al., 2002, and Barrington et al., 2017). While the hinge domain connects SMC1 to SMC3 on one end, the ATPase domains for the two proteins don't directly interact with one another, and instead are connected through the third subunit, Rad21 (Scc1) (Guacci et al., 1997, Haering et al., 2002, and Schleiffer et al., 2003). Furthermore, Rad21 (Scc1) also binds with the final subunit, Stromalin (Scc3) at its C-terminal to connect Stromalin with the SMC subunits and complete the ring-like structure of the complex (Tóth et al., 1999, and Haering et al., 2002).

Past work by Haering et al. (2002 and 2008) and Gruber et al. (2003) have suggested that the cohesin complex interacts with DNA by entrapping the DNA within the ring-like structure, facilitating an intimate association between the two. However, to be entrapped within the complex, it is essential for the cohesin complex to be near the DNA, and this is facilitated by the interaction of two accessory protein(s), Nipped-B (Scc2) and dMau-2 (Scc4) that join to form the cohesin loading complex (Ciosk et al., 2000). To load the complex onto the DNA, Arumugam et al. (2003) and Weitzer et al. (2003) found that the hydrolysis of the ATP molecule bound to the two SMC subunits was an important first. While Weitzer et al. (2003) suggested that the hydrolysis drove the heads of the SMC subunits away from each other to provide access to the DNA, Arumugam et al. (2003) found that the interaction between the cohesin loading complex and the cohesin subunits may point towards a role for the loading complex in the hydrolysis of ATP and opening of the cohesin complex. Furthermore, Gruber et al. (2006) found that the hinge domain that connects SMC1 to SMC3 on one side of the complex, served as an entry point for the DNA to move into the cohesin complex.

Once bound to the DNA, the cohesin complex is subject to the release process via the cohesin release or anti-establishment complex during phases prior to the 'S' phase of the cell cycle (Makrantoni & Marston, 2018). Work by Rowland et al. (2009), Sutani et al. (2009), Chan et al. (2012) and Beckouët et al. (2016) have shown that an interaction between Stromalin (Scc3), Wapl (Wpl1), and Pds5 in the absence of SMC3 acetylation leads to the detachment of the complex from

the entrapped DNA, facilitating DNA's departure through the SMC3-Rad21 (Scc1) linkage site which acts as the DNA exit. However, once the cell transitions to the 'S' phase and undergoes DNA replication (Laskey et al., 1989), the sister chromatids need to be held together, and this is ensured by stabilizing the cohesin complex through the acetylation of the SMC3 subunit at a few conserved lysine residues found at the ATP head of the subunit by an acetyltransferase known as Eco1 in yeast or Eco/Esco1/2 in mammals (Tóth et al., 1999, J. Zhang et al., 2008, Ünal et al., 2008, and Beckouët et al., 2010). In yeast, the acetylation of the lysine residues on the SMC3 subunit was initially shown to limit Wpl1's destabilizing activity (Rowland et al., 2009, Sutani et al., 2009, Chan et al., 2012 and Beckouët et al., 2016), however, work by Guacci et al. (2015) provided evidence for an additional stabilizing mechanism by Eco1 that targeted residues in addition to the lysine residues and promoted establishment even in the absence of Eco1 and Wpl1.

As the cells move into the mitosis phase following DNA replication, the cohesin complex was found to detach from the chromosomes at the start of anaphase to facilitate the separation of sister chromatids (Uhlmann et al., 2000, Hornig et al., 2002 and Uhlmann, 2003). This detachment was found to be driven by Separase, a protease that had the capacity to cleave the Rad21 (Scc1) subunit, causing the cohesin complex to fall apart and set the sister chromatids free (Uhlmann et al., 2000). Finally, work by Beckouët et al. (2010) showed that as the cohesin complex falls apart, the SMC3 subunit is subject to deacetylation by Hos1 (Hdac8 in mammals) to make the subunit available for future cell cycles.

Lessons from multi-cellular organisms:

Work by Darwiche et al. (1999) and Sumara et al. (2000) were some of the first studies that provided evidence for a cohesin complex in multi-cellular organisms that was composed of the homologs of yeast cohesin complex sub-units (SMC1, SMC3 and Rad21), and enabled sister chromatid cohesion and separation. Interestingly, Sumara et al. (2000) found that the human cohesin complex was made up of two separate orthologs of Stromalin (Scc3), STAG-1 and STAG-2, while the *Xenopus* and *Drosophila* cohesin complex had one Stromalin subunit (Valdeolmillos et al., 1998).

While a large body of work (reviewed in Peters et al., 2008 and Horsfield et al., 2012) has shown the conservation of yeast cohesin complex subunits, accessory proteins and their functions in higher organisms, Darwiche et al. (1999) and Sumara et al. (2000) found that during the initial phases of mitosis, the cohesin complex was largely detached from the chromosomes. Furthermore, Waizenegger et al. (2000) showed that cohesin complex in multicellular organisms

underwent two rounds of detachment during mitosis, and that the final detachment occurred at the start of anaphase as seen in yeast. By using *Xenopus*, Sumara et al. (2002) showed that phosphorylation of the cohesin complex by polo-like kinase triggered the first round of cohesin detachment from the chromosomes in prophase. Additionally, Hauf et al. (2005), Shintomi and Hirano (2009) and Gandhi et al. (2006) found that phosphorylation of the Stromalin subunit by polo-like kinase was sufficient to promote the release complex mediated early dissociation of the cohesin complex from the chromosomes. Furthermore, Hauf et al. (2001) showed that despite the detachment of the cohesin complex subunits from the chromosomes during prophase in vertebrates, minute amounts of the complex persisted at the centromeres until sister chromatids separate during anaphase. This was possible due to the presence of another accessory protein known as Shugoshin (Sgo) that was found to block the phosphorylation of the Stromalin subunit to protect the remaining cohesin complex at the centromere (Kitajima et al., 2005 and McGuinness et al., 2005).

Cohesin complex and gene transcription:

Besides playing a pivotal role in chromosome segregation during cell division, the cohesin complex has been shown play a crucial role in regulating the expression of genes (Bose & Gerton, 2010, and Horsfield, 2022). Rollins et al. (1999) were one of first to provide evidence for a regulatory association between the cohesin complex loading protein, Nipped-B, as well as *cut* and *Ubx* in *Drosophila*, as they found dysregulated *cut* and *Ubx* and abnormal physiological phenotypes in Drosophila when Nipped-B was mutated. Furthermore, Rollins et al. (2004) found that like Nipped-B, a core subunit of the cohesin complex, Stromalin, regulated *cut* in *Drosophila*, however, the transcriptional effects of Stromalin on *cut* seemed to be inhibitory in nature. Despite the contradictory regulatory roles played by Stromalin and Nipped-B on cut in *Drosophila*, work by Horsfield et al. (2007) in zebrafish showed that in the absence of an intact cohesin complex (due to decreased expression of Rad21 or SMC3), a dysregulation of cell fate determining transcription factors was observed while cell cycle and division remained unaltered. Furthermore, work by Schuldiner et al. (2008) and Pauli et al. (2008) on the developing *Drosophila* nervous system found that mutating or severing the cohesin complex altered the expression of ecdysone receptor B1, causing neuroanatomical and mobility defects in *Drosophila*. Interestingly, all of these studies also suggested that cohesin complex's role in gene transcription was conserved between vertebrates and invertebrates.

While the consequences of dysregulated gene expression due to manipulation of the cohesin complex were seen in *Drosophila* and zebrafish, subsequent studies aimed to provide further insight into the mechanisms by which the cohesin complex regulated transcription of its targets. Work by Misulovin et al. (2007) in *Drosophila* cell lines found that the cohesin complex and Nipped-B worked together to occupy transcriptionally rich areas and regulated the expression of approximately 369 genes in the *Drosophila* genome. Interestingly, Fay et al. (2011) utilized similar *Drosophila* cell lines and showed that one of the ways by which the cohesin complex regulated its transcriptional targets was by controlling whether RNA Polymerase II could switch from a paused to an operational state in these targets. Furthermore, studies conducted on mammals by Mishiro et al. (2009) and Chien et al. (2011) revealed the existence of another mechanism by which the cohesin complex facilitated gene expression. Using human cell lines, Mishiro et al. (2009) showed that the join occupation by cohesin and CCCTC-binding factor (CTCF) at certain regions of the genomes was essential to promote long-range genetic interactions through chromatin looping as a deficit in either the cohesin complex or CTCF inhibited this long-range interaction and looping. This was further corroborated by Chien et al. (2011)'s findings in mouse cell lines.

Moreover, Kagey et al. (2010) showed that in addition to associating with CTCF, the cohesin complex also coupled with the transcriptional coactivator, Mediator at specific genomic regions in mouse embryonic stem cells to enable the expression of target genes by forming DNA loops and permitting enhancer and promoter engagement. Finally, by working with *Drosophila* embryos, and cell cultures, Strübbe et al. (2011), Schaaf et al. (2013), and Pherson et al. (2017) showed that the cohesin complex engages with the Polycomb group of proteins (PcG) to influence gene expression. Moreover, Schaaf et al. (2013) and Pherson et al. (2017) showed that the polycomb repressive complex 1 (PRC1) interacts with the cohesin complex in such a way that they oppose one another's functions at genes meant to be repressed by PcG while at transcriptionally active regions, the cohesin complex was found to introduce PRC1 to these genes wherein it regulated their transcription by influencing RNA Polymerase II.

Taken together, these studies not only shed light onto cohesin complex's regulation of transcription, but also show that the complex's role in gene expression is as crucial as its role in chromosome cohesion and segregation.

Beyond the cohesin complex:

In addition to being a part of the cohesin complex, SMC1 alone was found to associate with some DNA damage response elements like Nibrin (NBS1) and the mutated protein encoded by ataxia telangectasia (AT) and potentially complex with mediator of DNA damage checkpoint 1 (MDC1) to ensure the proper passage through S and G2 cell cycle phase checkpoints (Yazdi et al., 2002 and Stewart et al., 2003). Furthermore, SMC1 and SMC3 alone were found to complex with an isoform of a potential guanidine nucleotide exchange factor called Retinitis Pigmentosa GTPase Regulator (RPGR) in the cilia of mammalian retinal cells, suggesting the existence of additional roles for the SMC subunits besides chromosome cohesion, segregation, and gene expression regulation (Khanna et al., 2005). Thus, there is the possibility that the core cohesin complex subunits may have non-canonical functions outside of the cohesin complex that are as of yet unknown.

1.4 Mutations in cohesin complex genes leads to cohesinopathies in humans:

Since the cohesin complex and its associated proteins play fundamental roles in the cell/body, a defect or mutation in any one of the subunits and/or auxiliary proteins has been shown to cause a group of rare disorders known as cohesinopathies, resulting in a wide range of developmental symptoms across multiple organ systems, (Horsfield et al., 2012, Zakari et al., 2015, and Piché, Van Vliet, et al., 2019).

The first cohesinopathy identified was Cornelia deLange syndrome (CdLS), which had been named and described prior to identifying its cause as a mutation in the *Nipbl* gene (De Lange, C., 1933). Of the cohesinophathies, CdLS remains the most well-studied and characterized disorder to date (Piché, Van Vliet, et al., 2019). Classified as a rare disorder that affects 1 in 10,000-30,000, CdLS is marked by a series of features that include-short stature, peculiar facial features, cognitive disabilities, seizures, cardiovascular and gastrointestinal abnormalities (Mannini et al., 2013, and Kline et al., 2018). In 2004, genome-wide linkage studies on families with a predisposition towards CdLS found that alterations in the cohesin complex loader, NIPBL, that either led to protein truncation or prevented its production, caused this cohesinopathy (Krantz et al., 2004). In an independent paper, Tonkin et al. (2004) also identified mutated NIPBL as a causative agent for CdLS, and they found that heterozygous, loss-of-function mutations underlay the disease pathology in these patients. Moreover, Gillis et al. (2004) found that the onset of CdLS due to mutated NIPBL in a group of individuals was high with almost a 50% incidence rate and this was further corroborated by the investigations carried out by Bhuiyan et al. (2005) and Yan et al.

(2006). Interestingly, a majority of the NIPBL mutations uncovered by Gillis et al. (2004), Bhuiyan et al. (2005) and Yan et al. (2006) were truncating in nature as well.

While NIPBL was found to be the leading cause of CdLS, work carried out in subsequent years had shown that the two structural subunits of the cohesin complex, SMC1 and SMC3, when subjected to hypermorphic mutations or mutations affecting functional components and conserved regions could result in CdLS in individuals with non-mutated NIPBL (Musio et al., 2006, Borck et al., 2007, and Deardorff et al., 2007). Moreover, when Deardorff et al. (2012) screened a set of individuals with CdLS, they found that mutations in the RAD21 subunit of the cohesin complex served as another causal factor for the syndrome but in a small subset of the individuals. Deardorff et al. (2012) found that the Rad21 mutations either led to haploinsufficiency or manipulated its association with the STAG subunits, suggesting that like the other subunits, a change or loss in function of Rad21 may have some role in CdLS pathogenesis.

Interestingly, a few years later mutations in STAG2 were shown to be a causative agent for a previously unidentified disorder where the patients exhibited a variety of symptoms ranging from cognitive impairments to seizures to varying degrees (Leroy et al., 2015). Furthermore, Leroy et al. (2015) referred to this potentially novel cohesinopathy as Xq25 duplication syndrome and found that STAG2 had been duplicated, suggesting an increase in the expression levels of STAG2 or an impairment in cohesin complex functions in this syndrome. Furthermore, Leroy et al. (2015) suggested that these alterations in STAG2 expression and/or function may affect the transcriptional regulation of its target genes which might be the underlying mechanism for this cohesinopathy. In the same year, Kumar et al. (2015) also showed that elevated copy numbers of STAG2 in a different cohort of patients resulted in transcriptional defects that led to neurological and intellectual deficits, psychiatric symptoms, impairments in growth and a proclivity towards seizures. Subsequent studies by Mullegama et al. (2017) and Soardi et al. (2017) further corroborated the pathogenicity of mutations in STAG2 as they identified heterozygotic and germline mutations in STAG2 that led to a loss of its function and affected its interactions with other cohesin complex in cells derived from cohesinopathy patients.

Besides STAG2, Lehalle et al. (2017) identified mutations ranging from missense to microdeletions in STAG1 in individuals experiencing some of the characteristic features of cohesinopathies like intellectual disability, irregularities in facial features, epilepsy and more. The identification of these mutations in STAG1 in these symptomatic individuals prompted Lehalle et al. (2017) to suggest a causal role for dysregulated STAG1 in cognitive impairments and

cohesinopathies. Furthermore, Yuan et al. (2019) identified copy number variant deletions in STAG1 and STAG2 subunits that were predicted to result in a loss of function for STAG1/2 in their group of patients. Finally, Di Muro et al. (2021) detected a novel frameshift mutation that produced a premature stop codon in STAG1 in their cohesinopathy patient. All in all, these studies provide further evidence for alterations in STAG1 and STAG2 functions as a causative disease mechanism for cohesinopathies.

Besides the causal roles played by cohesin complex subunits and NIPBL, the cohesin complex loader, in cohesinopathies, Deardorff et al. (2012) and Kaiser et al. (2014) found loss of function mutations in HDAC8, a SMC3 deacetyltransferase, that not only affected its ability to deacetylate SMC3, but also impacted the recycling of cohesin subunits and their association with DNA, in a small number of individuals with CdLS. Moreover, work carried out on the genetic underpinnings of Robert's syndrome, another cohesinopathy, showed that mutations leading to a premature stop codon or affecting the acetyltransferase domain in ESCO2, the SMC3 acetyltransferase, was the causative factor for this syndrome (Vega et al., 2005, Schüle et al., 2005, Resta et al., 2006, Gordillo et al., 2008, and Schulz et al., 2008). Finally, Chétaille et al. (2014) identified a heritable cohesinopathy that was accompanied by impairments in cardiac and gastrointestinal functions, unlike other cohesinopathies. Chétaille et al. (2014) found that a point mutation in Shugoshin-1 or Shugoshin-like 1 (SGO1 or SGOL1), known as the 'guardian of centromeric cohesin complex' during cell division (Kitajima et al., 2006), gave rise to their newly identified cohesinopathy, CAID syndrome.

While Chétaille et al. (2014) suggested that the mutation may negatively affect SGO1's ability to guard the cohesin complex at the centromeres, they also proposed that the mutation may produce a gain of function in SGO1 which may also contribute to CAID syndrome. This proposal was further supported by Piché et al. (2019) who found upregulated expression of SGO1 and BUB1, a kinase that directs SGO1 to the centromeres during cell division (Fernius & Hardwick, 2007 and Kawashima et al., 2010), in CAID patient cell cultures. However, as insufficient evidence exists to associate SGO1's mutation to a gain of function mutation, Chétaille et al. (2014) and Piché et al. (2019)'s proposal remains to be validated.

While a gain of function mutation as the causative mechanism for CAID remains to be established, Izumi et al. (2015) identified a previously uncovered syndrome that stemmed from neomorphic mutations in AFF4, a subunit of the super elongation complex that is known to assist in initiating RNA Polymerase II's movement when it comes to a halt during transcription. Moreover, Izumi et

al. (2015) showed that the cohesin complex physically interacted with the super elongation complex through STAG1, suggesting that: a) an interaction between these complexes along with RNA Polymerase II might play a role in cohesin complex's role in transcription regulation, and b) this novel syndrome might be a cohesinopathy.

1.5 Cohesinopathies versus transcriptopathies: Do cohesinopathies arise due to impairments in chromosome cohesion and segregation or due to dysregulation of transcription?

While previous studies (outlined above) have identified the causative agents for CdLS, Robert's syndrome and other cohesinopathies, they also investigated the potential mechanisms by which the mutated cohesin complex subunits and accessory proteins gave rise to this multi-organ system affecting disorder. While characterizing mutated NIPBL as the driving force behind CdLS, Tonkin et al. (2004) also found that the CdLS patients did not show any changes in centromeric or chromosome separation. This and the recent identification of Nipped-B's role in promoting interactions between enhancers and promoters (Rollins et al., 1999) prompted Tonkin et al. (2004) to suggest transcriptional dysregulation as a possible molecular mechanism underlying CdLS. This hypothesis was further supported by work carried out on *in-vitro* and *in-vivo* models of cohesinopathies (Horsfield et al., 2012). By working with cell lines derived from CdLS patients, Liu et al. (2009) found that mutations in NIPBL and/or SMC1 led to transcriptional dysregulation of a set of genes common to the different CdLS cell lines tested, providing further support to transcriptional dysregulation as the underlying mechanism for cohesinopathies. Additionally, by working with a few cell lines derived from patients with Robert's syndrome, Liu et al. (2009) found dysregulated gene expression in them that was not very different from their CdLS lines. Subsequent studies on CdLS animal models derived from NIPBL mutations found changes in the expression of a wide variety of genes but failed to observed defects in cohesin complex's functions, suggesting that transcriptional dysregulation participated in the cohesinopathy pathology seen in these models (Kawauchi et al., 2009, Muto et al., 2011, and Wu et al., 2015).

Additionally, by employing RNA-sequencing and pathway analyses on patient derived samples, Kumar et al. (2015) found that the increase in STAG2 copy numbers in these patients impacted the expression of genes involved in anion transport, synaptic function as well as in other neurological processes. Furthermore, Soardi et al. (2017) found alterations in gene expression in patient derived fibroblasts and HeLa cells with germline mutations in STAG2, further supporting STAG2's role in transcriptional regulation as a potential disease mechanism in cohesinopathies.

While independent studies on human/mammalian cell lines seem to suggest the presence of some functional overlap between STAG1 and STAG2 subunits with regards to their roles in sister chromatid cohesion, segregation, and gene expression regulation (Van Der Lelij et al., 2017 and Casà et al., 2020), Casà et al. (2020) showed that STAG1 and STAG2 regulated different sets of genes, suggesting that the two subunits were largely dissimilar, and may be unable to compensate for one another. This also suggests that the transcriptional dysregulation seen in cohesinopathies arising from the STAG1/2 subunits might be unique to the two subunits.

Finally, work by Piché et al. (2019) on SGO1 or SGOL1 derived cohensiopathy patients showed the presence of transcriptional dysregulation of a variety of genes and some epigenetic modifications of DNA like methylation. Taken together, the investigations not only suggest a role for transcriptional dysregulation and perturbed gene expression in cohesinopathy pathology but also suggest that dysregulation of various, potentially unrelated transcriptional targets of the cohesin complex may underlie the multi-organ system affecting nature of cohesinopathies (Horsfield et al., 2012).

1.6 Cohesinopathies and seizures:

While some of the past characterizations of cohesinopathies revealed that cognitive impairments and intellectual disability are the most commonly occuring neurological symptoms (Kumar et al., 2015 and Kline et al., 2018), other studies have shown that an increased propensity for seizures is another one of the main neurological symptoms seen in cohesinopathies (Schüle et al., 2005, Liu & Krantz, 2009, Verrotti et al., 2013, and Kumar et al., 2015). For instance, E. Pavlidis et al. (2014), Huisman et al. (2017) and Kline et al. (2018), have shown that an average of 20-26% of CdLS patients experience seizures or a form of epilepsy, and most of these seizure phenotypes arise due to mutations in the SMC1 subunit (Deardorff et al., 2007, Borck et al., 2007, Goldstein et al., 2015 and Symonds et al., 2017). Moreover, mutations in SMC3, RAD21 and NIPBL could produce seizure phenotypes in CdLS patients, however, their percentage of occurrence was not comparable to SMC1 (Deardorff et al., 2007, Borck et al., 2007, Gil-Rodríguez et al., 2015, and Krab et al., 2020).

Besides CdLS, studies characterizing cohesinopathies stemming from mutations in STAG1/2 subunits have also shown that an average of 28% of the patients experienced seizures (Leroy et al., 2015, Kumar et al., 2015, Lehalle et al., 2017, Yuan et al., 2019 and Di Muro et al., 2021). Furthermore, while characterizing the seizure phenotypes in mutated STAG1/2 and SMC1 patients, (Kumar et al., 2015, Symonds et al., 2017) found that the patients exhibited tonic-clonic

seizures. A tonic-clonic seizure phenotype is one where an individual initially experiences a "tonic" phase where their muscles tense up and rigidify leading to a collapse that lasts for a few seconds, followed by a "clonic" phase where they begin convulsing (Devinsky et al., 2018).

While the findings from these studies suggest that: a) seizures are a prevalent neurological phenotype in cohesniopathies, and b) STAG1/2 and SMC1 might be subunits of interest when investigating seizure behaviour in cohesinopathies, these studies do not provide insight into the potential molecular mechanisms underlying this phenotype.

1.7 *Drosophila* as a model for seizure behavior:

A seizure is defined as an irregulated and imbalanced synaptic transmission between neurons that results in individuals twitching and shaking their limbs continuously while experiencing periods of rigidity between the convulsions (Stafstrom & Carmant, 2015, Devinsky et al., 2018, Chen et al., 2023). When these seizure episodes cease to be less erratic and become more frequent in nature, it results in a neurological condition known as epilepsy (Beghi, 2019, Chen et al., 2023). Given the high prevalence rate and low quality of life seen with seizures and epilepsy (Baranowski, 2018, Beghi, 2019), various animal models have been used since the early 1880s to not only investigate the mechanisms underlying seizures and epilepsy but to evaluate the efficacy of potential therapeutic targets against the two (Löscher, 2017). While mammalian models have dominated the field for the most part, it is only recently that invertebrate or vertebrate models of epilepsy and seizures like *Drosophila melanogaster* and *Danio rerio*, have gained momentum (Parker et al., 2011, Grone & Baraban, 2015, Gaweł et al., 2020).

Drosophila has been a valuable model for seizure and epilepsy studies due to the existence of the versatile GAL4-UAS binary expression system that provides spatial and temporal control of transgene expression (Brand & Perrimon, 1993), an extensive genetic toolkit that is readily available, as well as a large library of RNAis that target almost all the genes in the fly (Fischer et al., 2023). These advantages are also coupled by the fact that *Drosophila* have a short-generation time, are economical to rear and have ~ 62% of their genome shared with human beings (Fortini et al., 2000, and Jennings, 2011). And so, in the last few decades, seizures have been modelled in *Drosophila* with Benzer (1971) first bringing attention to the presence of seizures in flies when they were either tapped or flicked vigorously. Flies with this behavioral phenotype were suggested to harbor mutations and were classified as 'bang-sensitive' mutants. This was followed by pioneering work by Ganetzky and Wu (1982a, 1982b), Pavlidis et al. (1994), Pavlidis & Ma, (1995), and Parker, Padilla, et al. (2011) who over the years have identified alterations in neural activity,

membrane composition and permeability towards ions, in synaptic transmission, and in voltage-gated ion channels as the causative mechanisms for the seizure phenotype seen in 'bang-sensitive' mutants. Moreover, the identification of a mutated voltage-gated sodium channel as the cause of the seizure phenotype in the bang-senseless 'bang-sensitive' mutant, not only provided evidence for shared disease mechanisms between humans and flies, but also suggested that *Drosophila* seizure mutants could serve as good and easy-to-use disease models.

1.8 Stromalin's effects on the *Drosophila* nervous system:

Recently, Phan et al. (2019) studied the mechanisms of Stromalin (STAG1/2)'s effects in the nervous system of *Drosophila*. Stromalin was identified to constrain synaptic vesicle pool sizes, limiting synaptic communication in dopamine neurons (DANs). These experiments also demonstrated the expression of Synaptotagmin:eGFP (Syt:GFP) as a reliable marker for changes in synaptic vesicle numbers *per se* when changes to other synaptic parameters such as synapse numbers or sizes can be ruled out, although electron microscopy experiments are needed to confirm alterations in synaptic vesicle numbers. Knocking down Stromalin pan-neuronally (all neurons) was found to increase Syt:GFP levels in the respective neurons, suggesting that Stromalin could also constrain vesicle pool sizes across many other neuron types in the brain, and not only in DANs (Phan et al., 2019). These effects of Stromalin reduction on the *Drosophila* nervous system are thought to be due to the impairment of the cohesin complex's functions for post-mitotic gene regulation since Stromalin was found to produce its effects in DANs at the 3rd instar-larval stage. The Phan lab has since performed DAN RNA-sequencing to identify the downstream genes responsible for mediating the effects of Stromalin on learning and synaptic vesicle pool sizes. Through this RNA-Sequencing experiment, 160 genes were found to be differentially expressed in the subset of DANs that were tested. These putative downstream targets were then subjected to a primary behavioral screen which identified genes that replicated Stromalin's learning effects. Then, these shortlisted targets were subjected to a secondary screen where their effects on Syt:GFP were investigated. The targets that were also able to mirror Stromalin's effects on Syt:GFP were identified as the potential downstream targets. As such, 5 candidate genes, Neprilysin-1 (Nep1), CG17698, Tiny tim 2 (Ttm2), Cytochrome c oxidase subunit 7c (Cox7c), and Suppressor of zeste 12 (Su(z)12) were identified as Stromalin's potential downstream targets.

Interestingly, the knockdown of Stromalin seems beneficial to learning in flies (Phan et al., 2019), conflicting with the cognitive impairments seen in cohesinopathies stemming from STAG 1/2

subunits (Leroy et al. 2015, Kumar et al. 2015, Mullegama et al. 2017, and Soardi et al. 2017). One explanation for this inconsistency is that Stromalin's effects on synaptic vesicle numbers may result in other neurological symptoms associated with cohesinopathies, such as the increased incidence of seizures which impair learning and memory in human patients (Kumar et al., 2015, Goldstein et al., 2015, Zakari et al., 2015, Symonds et al., 2017, and Schmidt et al., 2022).

Previous studies on rodent models of epilepsy have shown that changes in synaptic vesicle pool structure, in its fusion, recycling and release, as well as in active zone sizes led to the seizure and epileptic phenotype in the models (Li et al., 1995, Buckmaster et al., 2016, Tokudome et al., 2016, and Vannini et al., 2020). A few studies in *Drosophila* have shown similar associations as well. For example, Ehaideb et al. (2014) showed that mutations in *prickle*, an axonal microtubule polarity regulator that plays a role in the transport of vesicles, caused seizures in flies as the vesicular transport was impacted in such a way that the number of vesicles moving in a retrograde manner decreased. Interestingly, Kroll et al. (2015) found that impairing the recycling of synaptic vesicles in 'bang-sensitive' mutants could rescue the seizure phenotype in them, suggesting that synaptic vesicle recycling played an important role in seizures in flies as well. Kroll et al. (2015) exploited the *shibire*^{ts} mutant, a temperature-sensitive dynamin mutation that limits synaptic transmission by tampering with synaptic vesicle endocytosis (Kosaka & Ikeda, 1983) to show this rescue.

We thus hypothesized that an increase in synaptic vesicle numbers due to the knockdown of Stromalin in all neurons (Phan et al. 2019), may lead to increased synaptic communication which may induce seizures in *Drosophila* that resembled the seizure phenotype seen in cohesinopathy patients with STAG1/2 mutations. To test this, we first had to determine whether knockdown of Stromalin increased seizure rates in flies and examined this across age. Secondly, we determined the potential downstream target(s) that mediated Stromalin's seizure effects by knocking down the 5 candidate genes identified to be potential mediators of Stromalin's synaptic vesicle pool size effects. Third, we assessed whether reducing the numbers of synaptic vesicles at the terminal could reduce Stromalin's seizure phenotype. And lastly, we quantified the Syt:GFP signal pan neuronally across age to determine whether alterations in Syt:GFP levels paralleled the seizure phenotypes in the flies. This work will provide insight into cellular mechanisms underlying the neurological symptoms in cohesinopathies, and possible novel treatment avenues.

CHAPTER 2

MATERIAL & METHODS

2.1 Overview of the chapter:

To test the role of various genes in inducing seizures, we utilized inducible RNAi fly lines for all our studies. These RNAis are inducible using the *GAL4-UAS* system and a pan-neuronal *GAL4* line (*nSyb-GAL4*) was crossed with *UAS-RNAi* lines to reduce expression of our target genes in the neurons of the progeny (experimental flies). Seizures were induced using a mechanical seizure induction assay, which is the most established and common method for seizure induction in flies. Finally, to determine if synaptic vesicle levels might parallel the seizures seen in our animals, we used confocal microscopy to measure levels of a synaptic vesicle marker, *Synaptotagmin:GFP,* in the brains of animals.

2.2 Fly husbandry:

The *Drosophila* strains and crosses used in this thesis were reared on the usual cornmeal, yeast, and sugar enriched *Drosophila* media at an ambient temperature of ~25°C and a 12-hour light-dark cycle (08:00 AM-08:00 PM).

Given the pan-neuronal focus of this study, the *nSyb-Gal4* strain (gift from Julie Simpson) was used to drive the expression of our transgenes. RNAi knockdown of our targets of interest were achieved by utilizing the KK and GD RNAi collections at the Vienna Drosophila Resource Center (VDRC), which include: The KK control line containing an empty landing site (60100, VDRC), *stromalin* RNAi (KK106046, VDRC), *smc1* RNAi (KK108922, VDRC), *smc3* RNAi (KK 101501), *Nep1* RNAi (KK108660, VDRC), *CG17698* RNAi (KK105884, VDRC), *Cox7c* RNAi (KK104970, VDRC), *Ttm2* RNAi (KK100361, VDRC). The GD RNAi collection, a predecessor to the KK RNAi collection, generated RNAi lines by incorporating the RNAi construct into the fly genome in a random manner by using a P-element (Dietzl et al., 2007, VDRC). As such, an empty landing site control does not exist for GD lines. Instead, the controls used for these lines include-1) flies obtained from a cross between the Gal4 driver and the GD control (GD 60000), and 2) flies obtained on crossing the GD control (GD 60000) with the GD *Su(z)12* RNAi line (GD 42423, VDRC). We also used the TRiP RNAi collection at the Bloomington Drosophila Stock Center (BDSC), which includes- control line containing an empty insertion site (#36303, BDSC) and *unc104* RNAi (TRiP 43264, BDSC). Additionally, transgenic strains like the *UAS-Dicer2* (60008 and 60009, VDRC) and *UAS-Syt:eGFP* (6926, BDSC) were employed in our experiments.

Experimental crosses for the seizure rescue behavioral assays were made with a 3:1 ratio of virgin females to males, respectively (30 virgin females: 10 males). The virgin females were collected by anesthetizing them with carbon dioxide (CO_2) and were stored at 18°C till the crosses were made. Virgin females older than a week were omitted from the crosses to prevent inconsistencies in seeding densities between genotypes. For the seizure rescue imaging experimental crosses, a similar virgin female to male ratio ranged between 20-30 virgins: 10 males were used to ensure equal seeding densities across all genotypes. Moreover, these crosses were housed in a 25°C incubator.

2.3 Seizure behavioral assays:

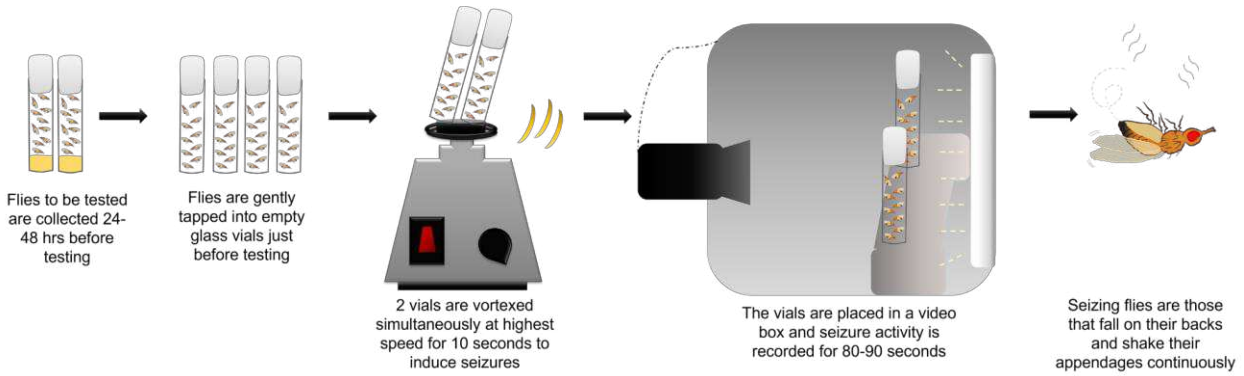

Figure 2: An illustration of the vortex-based seizure assay employed to characterize the seizure phenotype in our experimental flies. Experimental flies were collected in individual food vials a day or two before the day of the experiment to recover from CO2 anesthesia. Each food vial is housed with 10 flies of a single sex and genotype and labeled with a number from 1-4, blinding the experimenter to genotype. On the day of the experiment, flies are gently tapped into empty glass vials with corresponding numbers 1-4, and two glass vials are vortexed simultaneously at the highest speed for 10 seconds to mechanically induce seizures. This is followed by immediately transferring the glass vials into a video recording box where the seizure activity of flies is recorded for 80-90 seconds.

We tested 5, 10, 20, and 30 days old male and female flies in the field standard mechanical seizure assay (Kuebler & Tanouye, 2000, Mituzaite et al., 2021). The first generation of offspring (F1 generation) produced from crosses were anesthetized using CO2, segregated by sex, and housed in groups of 10 individuals in clean vials 24-48 hours before the testing date to allow for recovery from anesthesia. This is a population assay where one vial of 10 individuals = 1n, and each experimental group consisted of 10-20n. A seizure frequency was calculated for each vial of 10

flies, and then averaged for the group. Occasionally when flipping the flies into the vials for seizure induction some flies escaped, leaving <10 flies in the vial. The food vials were randomly labeled 1-4 to blind the experimenter to the genotype and sex of the flies, and two vials were tested at the same time in the seizure assay. On the day of testing, flies were gently flipped into an empty, clean glass vial with the corresponding number, and then subjected to high-speed vortexing for 10 seconds (Vortex-Genie 2, Scientific Industries) (Burg & Wu, 2012). Two glass vials were vortexed at the same time then immediately video recorded for 80-90 seconds.

For the time-course experiments, the F1 generation of offspring were collected 3-5 days after eclosion by anesthetizing them with CO2, separated by sexes and then collected in separate food bottles then aged to 10, 20 and 30 days old. The recorded videos of flies were visually and manually scored for seizure behavior by 2 experimenters blind to their conditions. Flies that dropped on their backs and exhibited continuous shaking of appendages (legs and wings) that resembled convulsions were identified as seizing flies (Burg & Wu, 2012). The number of flies within the vial seizing was used to calculate the seizure frequency for each vial (# of flies seized/total number of flies in vial).

All the *stromalin* knockdown and rescue related seizure experiments were carried out by Celina Phan, a talented, former undergraduate research student in the Phan lab. We employed the extensively used GAL4-UAS binary expression system (Brand & Perrimon, 1993) in our experiments. In all experiments, we used the pan-neuronal driver line, *nSyb-Gal4,* to express our transgenes.

2.4 Adult brain dissections and immunostaining:

Synaptic vesicle proteins were assessed by immunostaining for synaptotagmin conjugated green fluorescent protein (Syt:GFP). We dissected the adult brains from 5, 10, 20 and 30day old flies of each sex and genotype. The flies were anesthetized with CO2, then brains were dissected in ice cold S2 media (Schneider's insect medium, Lot# RNBJ7453, Sigma-Aldrich) (Jenett et al., 2012, Phan et al., 2019). Following dissections, the brains were stored in S2 media with 1% paraformaldehyde and were placed on a nutator at 4°C overnight. The next day, the brains were shifted to Pat3 (0.5% TritonX-100, 0.5% bovine serum albumin in 1x phosphate buffered saline) and were immunostained either immediately or stored at 4°C and immunostained a few days later.

Brains were washed in Pat3 thrice, then incubated in a blocking buffer (3% normal goat serum; NGS) in Pat3 for 1.5 hours, then incubated in primary antibody (primary antibody and 3% NGS in PAT3) for 3hr at room temperature, then shifted to 4°C overnight. A master mix of the primary antibodies were prepared for all brains. Primary antibodies were Rabbit anti-GFP at 1:1000 concentration (#A-11122, Invitrogen) and mouse anti-Brp at 1:50 concentration (#AB_2314866, Developmental Studies Hybridoma Bank, DSHB). The following day, the brains were washed 3 times in PAT3, then incubated in secondary antibody (master mix of secondary antibody and 3% NGS in PAT3). The secondary antibodies used were Alexa 488 conjugated to goat anti-rabbit IgG at 1:1000 concentration (#A-11008, Invitrogen) and Alexa 633 conjugated to goat anti-mouse IgG at 1:400 concentration (#A-21052, Invitrogen). The samples were then wrapped in foil to prevent photo-bleaching of the fluorophores, placed at room temperature for 3-hours followed by a 5 day incubation at 4°C.

2.5 Confocal microscopy:

Immunostained brain samples were prepared for mounting and imaging by washing them with Pat3 thrice at room temperature. The washes were followed by a brief mixing of the brain samples with 1x PBS by hand. Then, the brain samples were quickly rinsed in MilliQ water before mounting onto a 24 mm x 55 mm coverslip (1254418, Fisher Scientific) between 2 spacers (reinforcement stickers). The brains were mounted in Vectashield (H-1200, Vector Laboratories), and coverslipped using an 18 mm x 18 mm coverslip (12542A, Fisher Scientific), sealed with nail polish, and placed onto a 25.4 x 76.2 mm microscope slide (Sail Brand, Catalog no. 7101).

Brains were using a water immersion 25X and a dry 10X objective lens on the Leica Stellaris 5 confocal microscope (Leica Microsystems) with 488 nm and 633 nm laser. Z-stack images were taken of the whole brain at 512 x 512-pixel size and 600 Hz speed, with a step size of 1 micron. The brains were then analyzed using Fiji. To analyze the whole brain, we created a region of interest (ROI) from thresholding a maximum projection image that delineated the contours of the brain. The mean fluorescence intensity for each brain was calculated. Brains that were heavily damaged and lost their morphology as well as those that had either an air bubble, debris, or shadow on them were omitted from the analysis.

2.6 Statistical analysis:

Data was collected and sorted in MS Excel. For both seizure frequency and the Syt:GFP imaging experiments, nonparametric Mann-Whitney U or a Kruskal-Wallis test with Dunn's post-hoc was used (IBM SPSS statistics version 29, https://www.socscistatistics.com/tests/mannwhitney/default2.aspx & https://www.statskingdom.com/kruskal-wallis-calculator.html). All statistical analyses involved two tailed tests with significance level set at $\alpha = 0.05$. MS Excel was also used to graphically represent the data obtained from the seizure behavioral experiments as well as imaging experiments.

CHAPTER 3

RESULTS

3.1 Overview of the chapter:

First, we determined whether reduction of core cohesin subunit proteins Stromalin, SMC1, and/or SMC3 in all neurons could lead to increases in seizures, as seen in cohesinopathy patients. Because we hypothesize that the cohesin complex likely controls neurological activity (and therefore seizures) via regulating specific genes, we then examined 5 candidate genes for their ability to induce seizures. These candidate genes were previously identified to be potentially important in the cohesin complex's role in memory. We then test the hypothesis that increased synaptic vesicles in neurons leads to increased seizures by inhibiting their transport to the synaptic terminal by expressing *unc104 RNAi* and examine a synaptic vesicle marker to determine whether their levels parallel seizure frequencies.

3.2 The cohesin complex sub-units, Stromalin and SMC1 but not SMC3, induce a seizure phenotype in *Drosophila*.

Since seizures are defined as the excessive, uncontrolled, and synchronous firing of neurons that stem from an imbalance in excitatory and inhibitory neurotransmission in the brain (Huff & Fountain, 2011), we hypothesized that the increased synaptic vesicles and synaptic communication due to the knockdown of Stromalin in all the neurons in the brain may result in an increased seizure rates in these flies. To test the hypothesis, we knocked Stromalin down in all the neurons in the brain using *nSyb-Gal4*, the pan-neuronal driver, and tested both male and female flies at various ages, on the mechanical seizure induction assay.

We found that pan-neuronal *stromalin*^{RNAi} expression significantly enhanced seizures at all ages tested in both male and female flies (Figure 3A and B), consistent with what we expect from an animal model for cohesinopathy. Interestingly, we observed that these flies displayed a continuous convulsing phenotype which differed from the seizure followed by paralysis phenotype typically seen in the traditional *Drosophila* models for seizures and epilepsy. However, it is important to note that this was only a qualitative observation, and that quantifying this measure may be a future direction of this experiment. Furthermore, on average, the seizure rate seen in cohesinopathy patients with STAG1/2 mutations (Leroy et al., 2015, Kumar et al., 2015, Lehalle et al., 2017, Yuan et al., 2019 and Di Muro et al., 2021) is close to 28% while seizures arising from non-genetic factors like traumatic brain injury, central nervous system infections and as a side effect of medication have been found to have an incidence rate ranging from 0.08% to 41%, with 41% corresponding to seizure rates seen in various infectious diseases (Porter & Jick,

1977, Teasell et al., 2007, Singhi, 2011, Ding et al., 2016, and Larson et al., 2021). We found that the pan-neuronal *stromalin*^{RNAi} female flies at 05 days old had an average seizure rate which was close to the averages seen in cohesinopathy patients, and in patients experiencing seizures due to non-genetic factors. This suggests that the *stromalin*^{RNAi} flies have the capacity to recapitulate human disease phenotypes such as seizures.

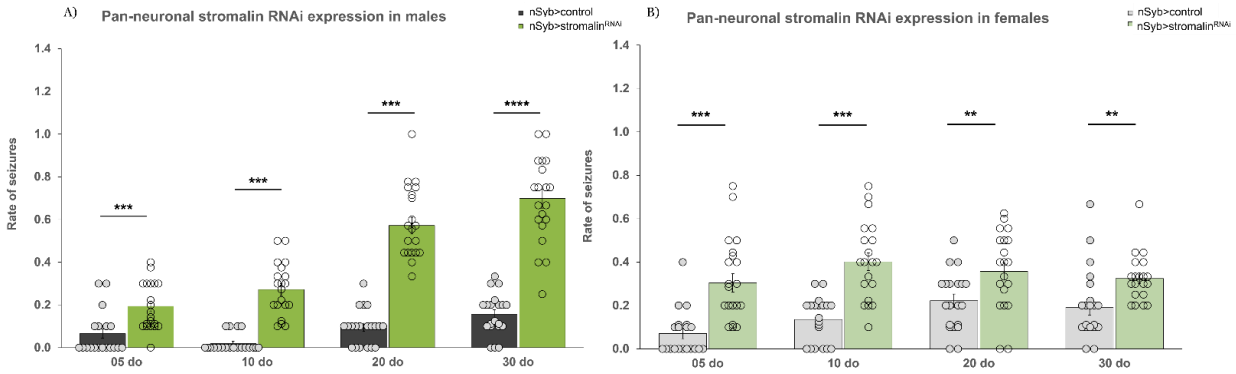

Figure 3: Pan-neuronal knockdown of Stromalin results in increased seizure rates that persist with age in both male and female flies. A) Pan-neuronal expression of stromalin^{RNAi} leads to a significant increase in seizure rates in male flies when compared to controls at 5, 10, 20 and 30 days old. B) Similarly, pan-neuronal expression of stromalin^{RNAi} leads to a significant increase in seizure rates in female flies when compared to controls at 5, 10, 20 and 30 days old. Control: nSyb-Gal4>+, UAS-Dicer2. Stromalin^{RNAi} : nSyb-Gal4>UAS-stromalin^{RNAi}, UAS-Dicer2. Two-tailed Mann-Whitney U test. *** = $p<0.001$ and ** = $p<0.01$. n=20. Graphs depict mean ± SEM.

Next, we decided to investigate whether reducing levels of other cohesin complex sub-units, SMC1 and SMC3, also increased seizure rates in our animal model of cohesinopathy. It was previously shown that silencing SMC1 with the KK RNAi line (KK 108922) in DANs resulted in a memory enhancing effect that phenocopied *stromalin* (Phan et al., 2019), while the knockdown of SMC3 (KK 101501) in the DANs did not produce an enhanced memory phenotype (unpublished data). However, pan-neuronal silencing of both SMC1 and SMC3 led to significant increases in Syt:GFP levels, although the effect of SMC3 knockdown was moderate (unpublished data), suggesting a similarity in modes of action between the three subunits in the *Drosophila* brain. Therefore, we expressed SMC1 and SMC3 RNAi pan-neuronally and tested these flies on the seizure assay. Consistent with previous behavioural memory data, we observed that at 5 days old, *SMC1*^{RNAi} female and male flies displayed a significant increase in their seizure rates compared to the controls (Figure 4A). Finally, when looking at the seizure phenotype displayed by *SMC1*^{RNAi} flies

qualitatively, we observed a resemblance to the *stromalin*^{RNAi} seizure phenotype, suggesting that the immediate onset and continuous convulsive behavior seen in these flies may be characteristic to the cohesin complex subunits. However, a pan-neuronal knockdown of SMC3 did not increase seizure rates in the flies (Figure 4B).

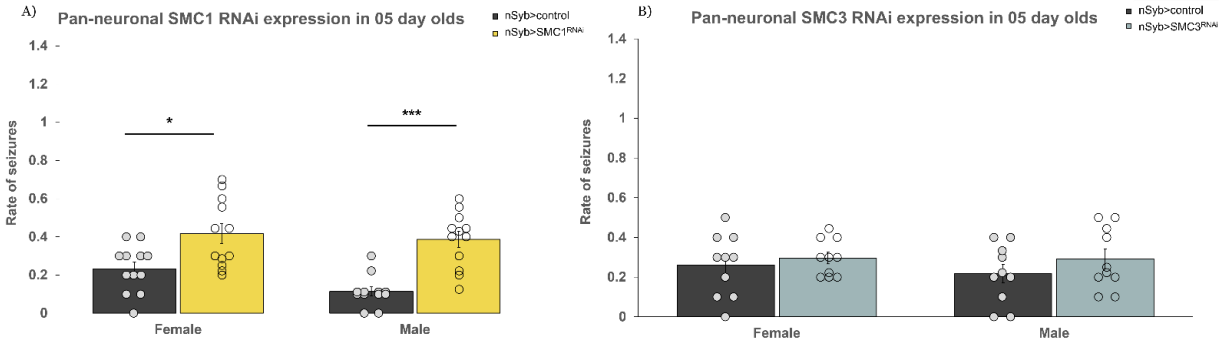

Figure 4: Pan-neuronal knockdown of SMC1 results in increased seizure rates in 5 day old male and female flies. A) Pan-neuronal expression of SMC1^{RNAi} leads to a significant increase in seizure rates in male and female flies when compared to controls at 5 days old. B) Contrastingly, the pan-neuronal expression of SMC3^{RNAi} does not lead to a significant change in seizure rates in male and female flies when compared to controls at 5 days old. Control: nSyb-Gal4>+, UAS-Dicer2. SMC1^{RNAi} : nSyb-Gal4>UAS-SMC1^{RNAi}, UAS-Dicer2. SMC3^{RNAi} : nSyb-Gal4>UAS-SMC3^{RNAi}, UAS-Dicer2. Two-tailed Mann-Whitney U test. *** = $p<0.001$, * = $p<0.05$, and $p>0.05$. n=10-12. Graphs depict mean ± SEM.

3.3 *CG17698* and *Cox7c* potentially mediate Stromalin's seizure phenotype in *Drosophila*.

As mentioned earlier, one of the biological roles undertaken by the cohesin complex is the regulation of gene expression (Dorsett & Merkenschlager, 2013, and Horsfield, 2022). Furthermore, past work on cohesinopathies has provided evidence for impairments in cohesin complex's role in transcriptional regulation and not chromosomal cohesion or segregation as the underlying pathological mechanism in cohesinopathies (Bose & Gerton, 2010, Horsfield et al., 2012 and Zakari et al., 2015).

This prompted us to hypothesize that the seizure effect found with Stromalin and SMC1 knockdown is the result of impairing cohesin's gene regulatory effects. As mentioned earlier, a separate but related project in the Phan lab has used DAN RNA-Sequencing to identify genes that are dysregulated upon Stromalin knockdown in DANs. From these efforts, 5 gene candidates were

identified as being the likely downstream effectors responsible for the synaptic vesicle constraints seen previously (Phan et al., 2019 and unpublished data). These include: Neprilysin1 (*Nep1*), *CG17698*, Cytochrome c oxidase subunit 7c (*Cox7c*), Tiny tim 2 (*Ttm2*) and Suppressor of zeste 12 (*Su(z)12*). From our RNA-Sequencing work, we found that reducing *stromalin* levels results in reduced transcript levels for all 5 candidate genes. Thus, if these candidate genes mediated the effects of Stromalin and SMC1 on seizures (Figure 3 and 4A), then reducing them using RNAi should also cause increased seizure rates in flies. We expressed RNAis targeted against *Nep* 1, *Ttm2*, *Cox7c*, *CG17698* and *Su(z)12* using the same pan-neuronal driver we used previously (*nSyb-GAL4*) and tested them in our seizure assay when flies were 5 days old.

We found knocking down *Nep1*, *Ttm2* and *Su(z)12* did not affect seizure rates in 5 day old males or females (Figure 5A, B and E). For reasons that are unclear at this point, the genetic controls: nSyb-Gal4>+, UAS-Dicer2 and +>UAS-su(z)12^{RNAi}, UAS-Dicer2 had unusually high seizure rates than typically observed (Figure 5E). However, pan-neuronal knockdown of *CG17698* and *Cox7c* did significantly increase seizure rates in both male and female flies at 5 days old when compared to the controls (Figure 5C and D). Thus, our data suggests that *CG17698* and *Cox7c* are responsible for the seizure phenotypes seen on reducing Stromalin levels in all the neurons.

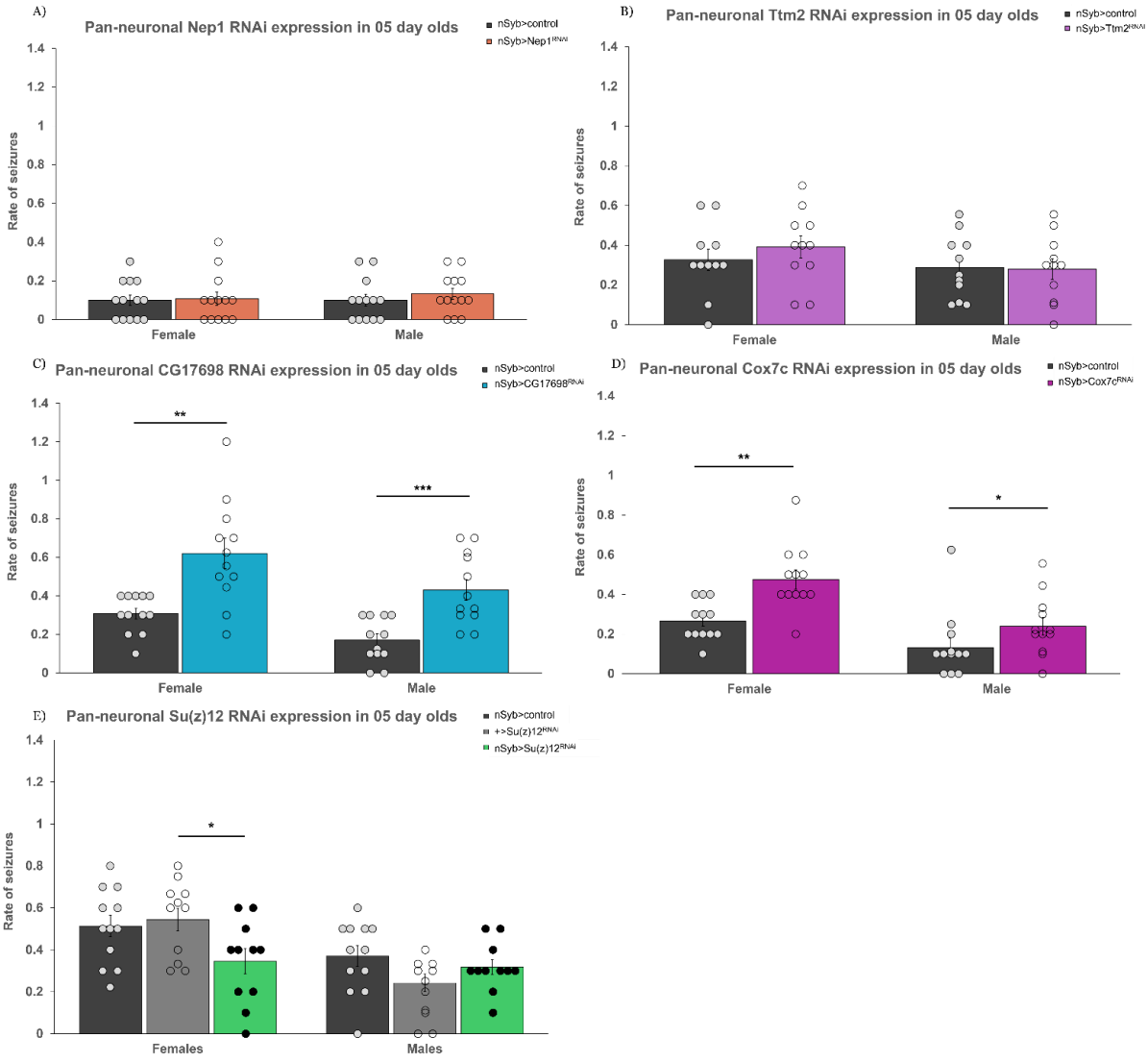

Figure 5: Pan-neuronal knockdown of CG17698 and Cox7c results in increased seizure rates in 5 day old male and female flies. A) Pan-neuronal expression of Nep1^{RNAi} does not lead to a significant increase in seizure rates in male and female flies when compared to the controls at 5 days old. B) Similarly, the pan-neuronal expression of Ttm2^{RNAi} fails to produce a significant increase in seizure rates in male and female flies when compared to the controls at 5 days old. C) Meanwhile, the pan-neuronal expression of CG17698^{RNAi} leads to a significant increase in seizure rates in male and female flies when compared to controls at 5 days old. D) Similarly, the pan-neuronal expression of Cox7c^{RNAi} produces a significant increase in seizure rates in male and female flies when compared to controls at 5 days old. E) Finally, the pan-neuronal expression of Su(z)12^{RNAi} fails to produce a significant increase in seizure rates in male and female flies when compared to controls at 5 days old. Control: nSyb-Gal4>+, UAS-Dicer2. GD Control: +>UAS-su(z)12^{RNAi}, UAS-Dicer2. Nep1^{RNAi}: nSyb-Gal4>UAS-nep1^{RNAi}, UAS-Dicer2.

Ttm2^{RNAi}: nSyb-Gal4>UAS-ttm2^{RNAi}, UAS-Dicer2. CG17698^{RNAi}: nSyb-Gal4>UAS-CG17698^{RNAi}, UAS-Dicer2. Cox7c^{RNAi}: nSyb-Gal4>UAS-cox7c^{RNAi}, UAS-Dicer2. Su(z)12^{RNAi}: nSyb-Gal4>UAS-su(z)12^{RNAi}, UAS-Dicer2. Two-tailed Mann-Whitney U test, Kruskal-Wallis test with Dunn's post hoc for su(z)12^{RNAi}. *** = $p<0.001$, ** = $p<0.01$, and * = $p<0.05$. n=11-12. Graphs depict mean ± SEM.

3.4 Pan-neuronal knockdown of *unc104* does not decrease the *stromalin*^{RNAi} driven seizure phenotype in *Drosophila* at various ages.

Phan et al., (2019) found that reducing levels of *unc104* (the *Drosophila* homolog of KIF1A) that is responsible for anterograde transport of synaptic vesicles to the synaptic terminal (Hall & Hedgecock, 1991, Yonekawa et al., 1998, and Van Den Berg & Hoogenraad, 2012) rescued the memory enhancement of *stromalin*^{RNAi} expression in DANs. Thus, by normalizing the synaptic vesicle content at the dopaminergic synaptic terminal, they normalized learning in the flies. This prompted us to hypothesize that if increased synaptic vesicles and synaptic communication were the cause of the increased seizures in our flies, then reducing synaptic vesicle numbers at the termini through the pan-neuronal knockdown of *unc104* in *stromalin*^{RNAi} flies may decrease the increased seizure rates observed in these flies.

For this experiment, we had four groups of flies: the control group that contained both empty landing site controls for the KK and TRiP RNAi libraries (KK 60100 and TRiP 36303). The other 2 control groups included flies that contained the KK *stromalin*^{RNAi} construct with the TRiP empty landing site control, TRiP 36303, and the TRiP *unc104*^{RNAi} construct coupled with KK empty landing site control, KK 60100. Our final group was the experimental flies that contained both KK *stromalin*^{RNAi} and TRiP *unc104*^{RNAi} constructs. We then aged our experimental and control flies to the previously used age points: 5 days old, 10 days old, 20 days old and 30 days old, to investigate the extent to which the pan-neuronal knockdown of *unc104* could decrease the seizure rates in *stromalin*^{RNAi} flies.

We found that pan-neuronal expression of both *stromalin*^{RNAi} and *unc104*^{TRiPRNAi} led to a significant increase in seizure frequency rates when compared to *unc104*^{TRiPRNAi} male and female flies at all ages (Figure 6A and B). However, when compared to *stromalin*^{RNAi} male and female flies at all ages, *stromalin*^{RNAi} and *unc104*^{TRiPRNAi} flies showed no significant differences in seizure frequency rates, suggesting the absence of a rescue phenotype (Figure 6A and B). Surprisingly, we observed that only *stromalin*^{RNAi} male flies at only 20 and 30 days old showed a significant

increase in seizure frequency rates when compared to controls, which was inconsistent with our previous findings. Taken together, our data suggests that knocking down *unc104* along with Stromalin in all neurons may be insufficient to decrease the seizure phenotype induced by the pan-neuronal knockdown of Stromalin. However, as we were unable to replicate our previous findings of increased seizure rates in *stromalin*^{RNAi} flies in these experiments, our current data cannot robustly suggest that pan-neuronal knockdown of *unc104* has no effect on seizure behavior in *stromalin*^{RNAi} flies.

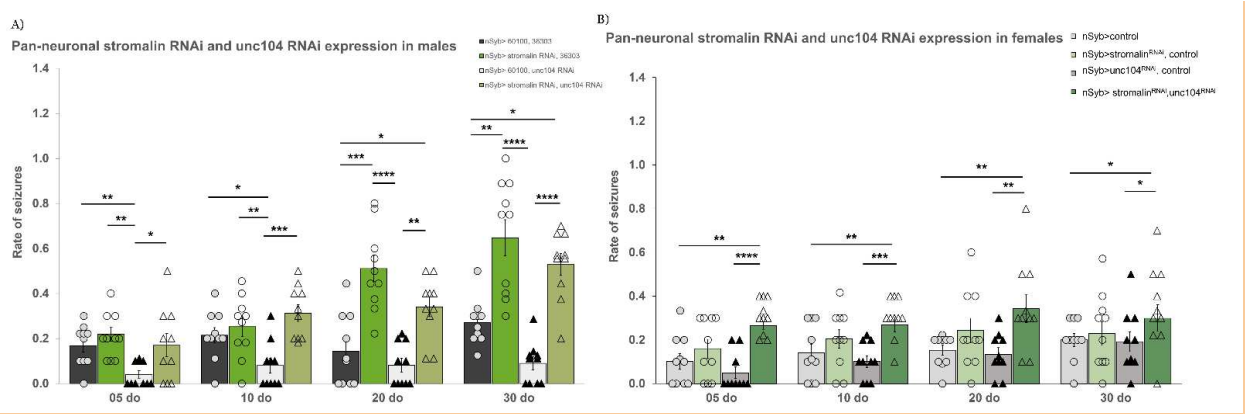

Figure 6: Pan-neuronal knockdown of Stromalin and *unc104* fails to rescue the effects of *stromalin*^{RNAi} on seizure behaviour in male and female flies at all age points. A and B) The pan-neuronal expression of stromalin^{RNAi} and unc104^{TRiPRNAi} does not lead to a significant change in seizure rates when compared to stromalin^{RNAi} male and female flies at all age points. However, the pan-neuronal expression of stromalin^{RNAi} and unc104^{TRiPRNAi} produces a significant increase in seizure rates when compared to unc104^{TRiPRNAi} male and female flies at all ages. Finally, and unexpectedly, the pan-neuronal expression of stromalin^{RNAi} produces an inconsistent seizure phenotype when compared to most male and female control flies at all age points. Control: nSyb-Gal4>+, UAS-Dicer2. Stromalin^{RNAi}: nSyb-Gal4>UAS-stromalin^{RNAi}, UAS-Dicer2. Unc104^{RNAi}: nSyb-Gal4>UAS-unc104^{TRiPRNAi}, UAS-Dicer2. Stromalin^{RNAi} and Unc104^{RNAi}: nSyb-Gal4> UAS-stromalin^{RNAi}, UAS-unc104^{TRiPRNAi}, UAS-Dicer2. Kruskal-Wallis test with Dunn's post-hoc. **** = p< 0.0001, *** = p<0.001, ** = p<0.01, and * = p<0.05. n=10. Graphs depict mean ± SEM.

3.5 Pan-neuronal knockdown of Stromalin has inconsistent effects on the Syt:GFP levels in *Drosophila* brains across age.

Phan et al. (2019) have previously shown that the knockdown of Stromalin in the DANs leads to an increase in the levels of Syt:GFP, which was shown to be caused specifically by an increased

number of synaptic vesicles using electron microscopy. They also previously showed that pan-neuronal knockdown of *stromalin* caused a similar increase in Syt:GFP levels, suggesting that there may be increased synaptic vesicles across many neurons of the brain, although this has not been directly assessed (Phan et al., 2019). Thus, if synaptic vesicle increases are the causal mechanism for the Stromalin knockdown induced seizure phenotypes, the Syt:GFP changes in whole brain should parallel the seizure effects we see. So, we first aimed to replicate the previous Syt:GFP increases seen in Phan et al. (2019) at 5 days old, then measure Syt:GFP across age and in genotypes tested in the seizure assay.

We expressed *stromalin*^{RNAi} and simultaneously expressed Syt:GFP using the *nSyb-Gal4* pan-neuronal driver. We dissected adult brains from these flies at various age points, and immunostained the brains using a GFP antibody (green), and NC82 antibody as a background stain (magenta) to visualize changes in synaptic vesicle proteins. Firstly, we were able to replicate the Syt:GFP changes seen in whole *Drosophila* brain in female 5 day old flies (Figure 7B) as shown previously (Phan et al., 2019). We also show for the first time this increase in whole brain Syt:GFP occurs also in male fly brains (Figure 7A). This suggests that many neurons across the whole brain may have increased synaptic vesicle numbers which may play a role in Stromalin's seizure effects.

However, when we looked at Syt:GFP changes in male and female flies at other age points, we found that the Syt:GFP changes in the whole brain were not significantly different from the controls except in female flies at 30 days old (Figure 8A and B). Moreover, when we again looked at Syt:GFP changes in 5 day old flies, we found that Syt:GFP changes in the whole brain were only significantly different from the controls in male flies which was inconsistent with our previous finding and the findings by Phan et al. (2019) (Figure 8A and B). The inconsistencies in our Syt:GFP data suggests that we cannot confidently determine whether Syt:GFP parallels the seizure phenotypes in *stromalin*^{RNAi} flies. As such, the potential effects of Stromalin on synaptic vesicle numbers in a wide range of neurons cannot be inferred with confidence from our data. Furthermore, our data suggests that the inconsistent changes in Syt:GFP may point towards the need to consider possible factors that may impact Syt:GFP. Moreover, it suggests the need to investigate other presynaptic protein markers as well as synaptic vesicle proteins to assess whether changes in synaptic vesicle numbers and Syt:GFP in the whole brain of *stromalin*^{RNAi} flies parallels its seizure behavior.

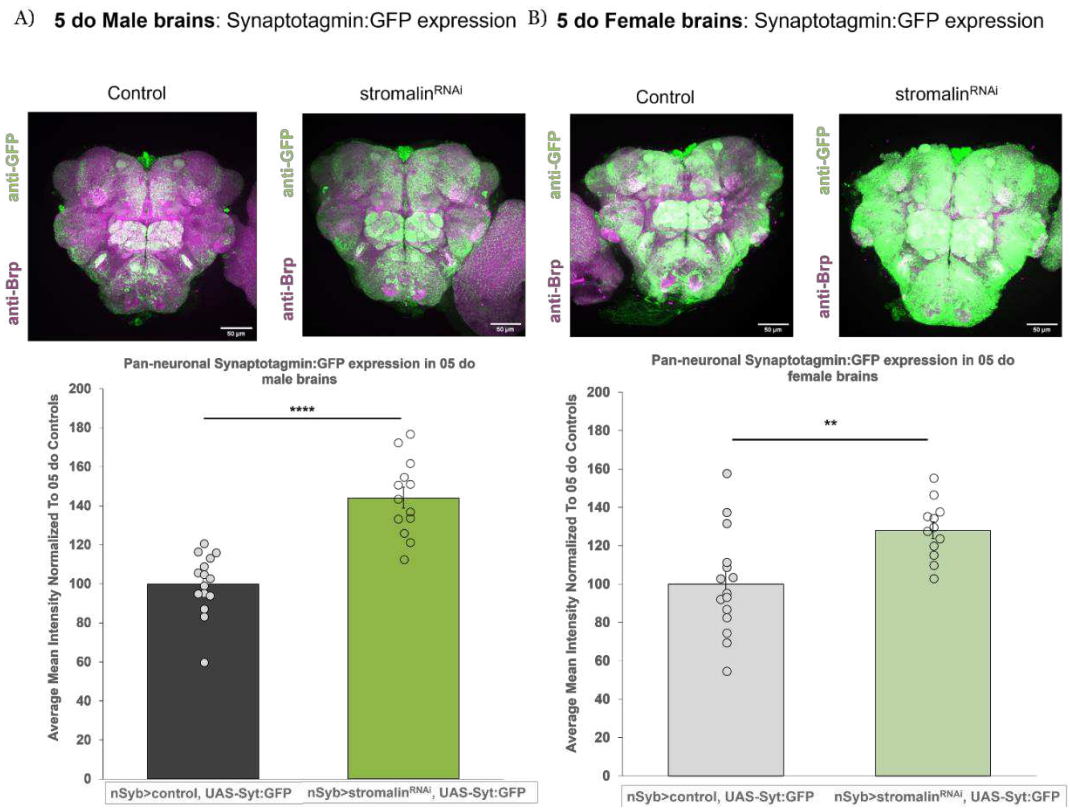

Figure 7: Pan-neuronal knockdown of Stromalin significantly enhances Syt:GFP in both male and female flies at 5 days old. A and B) Pan-neuronal expression of stromalin^{RNAi} leads to a significant increase in Syt:GFP when compared to control male and female flies at 5 days old. Quantification of the average mean intensities for Syt:GFP are shown in the bar charts below. Control: nSyb-Gal4>+, UAS-Dicer2 and UAS-Syt:GFP. Stromalin^{RNAi}: nSyb-Gal4>UAS-stromalin^{RNAi}, UAS-Dicer2 and UAS-Syt:GFP. Two-tailed Mann-Whitney U test. **** = $p < 0.0001$ and ** = $p < 0.01$. n=13-16. Graphs depict mean ± SEM. Scale bar = 50-60µm.

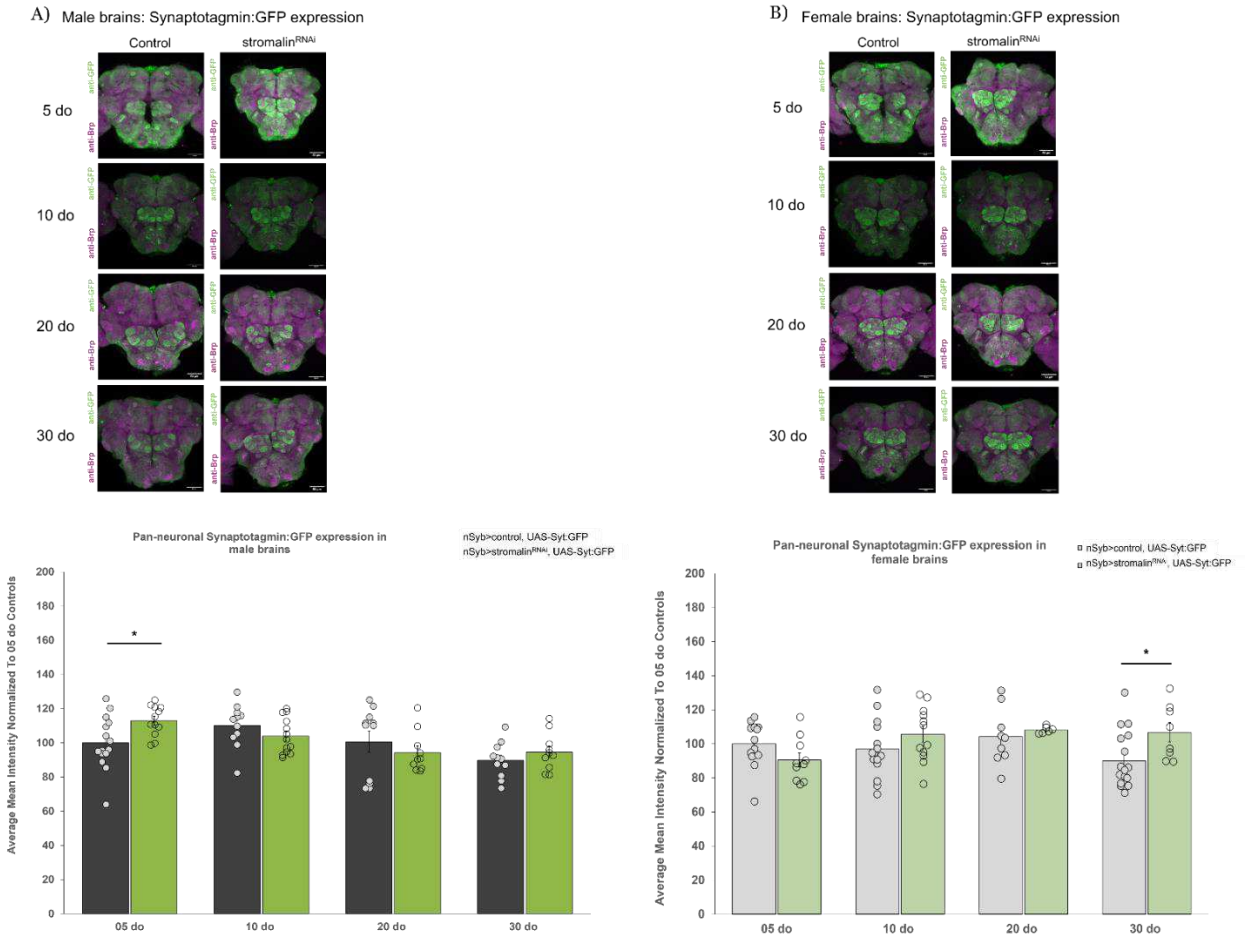

Figure 8: Pan-neuronal silencing of Stromalin has inconsistent and non-significant effects on Syt:GFP in male and female flies at almost all age points. A and B) Besides 5 day old male and 30 day old female stromalin^{RNAi} flies, the pan-neuronal expression of stromalin^{RNAi} leads to changes in Syt:GFP that are not significant from controls in male and female flies at almost all age points. Quantification of the average mean intensities for Syt:GFP are shown in the bar charts below. Control: nSyb-Gal4>+, UAS-Dicer2 and UAS-Syt:GFP. Stromalin^{RNAi}: nSyb-Gal4>UAS-stromalin^{RNAi}, UAS-Dicer2 and UAS-Syt:GFP. Two-tailed Mann-Whitney U test. * = $p<0.05$. n=6-17. Graphs depict mean ± SEM. Scale bar = 50-60µm.

3.6 Pan-neuronal knockdown of *unc104* does not decrease Syt:GFP levels in *stromalin*^{RNAi} *Drosophila* brains across age.

In addition to identifying Syt:GFP and synaptic vesicle pool size changes due to knockdown of Stromalin in DANs, Phan et al. (2019) have also shown that knocking down *unc104* along with *stromalin* in the same subset of neurons not only led to a significant decrease in memory acquisition but also significantly decreased the levels of Syt:GFP that resulted from *stromalin*^{RNAi}

expression. This further supported their notion that Stromalin's effects on synaptic vesicle pool sizes could underlie its learning phenotype. Given that we previously showed that the pan-neuronal knockdown both of *unc104* and *stromalin* failed to significantly decrease the seizure phenotype produced by of *stromalin*^{RNAi}, we speculated that the pan-neuronal knockdown of *unc104* may have failed to alter synaptic vesicle numbers in the whole brain. As such, we hypothesized that the pan-neuronal knockdown both of *unc104* and *stromalin* would not significantly change Syt:GFP in the whole brain, in line with the seizure phenotype seen in these flies.

We tested this hypothesis by expressing Syt:GFP with the four groups of flies tested previously in our seizure assay, pan-neuronally and across age. Similar to our previous experiments, we dissected adult brains at all the age points tested in our seizure assay, immunostained them with the same antibodies as before (GFP and NC82 antibodies) and visualized changes in synaptic vesicle proteins. We found that pan-neuronal expression of both *stromalin*^{RNAi} and *unc104*^{TRiPRNAi} did not produce a significant difference in Syt:GFP when compared to *stromalin*^{RNAi} male and female flies at almost all age points (Figure 9A and B). The absence of a significant difference in Syt:GFP between the two groups mirrored the seizure phenotype previously seen in them (Figure 8A and B), suggesting that the whole brain expression of *unc104*^{TRiPRNAi} may have failed to alter synaptic vesicle numbers in these flies. The lack of a Syt:GFP effect in these flies also provides support to the notion that Stromalin's manipulation of synaptic vesicle numbers may induce the seizure phenotype, however, further investigations are required to validate this speculation.

Lastly, we found that the pan-neuronal expression of *stromalin*^{RNAi} did not lead to significant change in Syt:GFP levels when compared to the control group in male and female flies at almost all age points (Figure 9A and B), similar to our previous Syt:GFP findings in flies with whole brain *stromalin*^{RNAi} expression (Figure 8A and B) and contradictory to our initial observations (Figure 7A and B) and the findings by Phan et al. (2019). Finally, for reasons addressed in the discussion section, the pan-neuronal expression of *unc104*^{TRiPRNAi} led to inconsistent changes in Syt:GFP when compared to the other groups, and non-uniform staining patterns when compared to other brains of the same genotype (Figure 9A and B). Taken together, our data suggests that: a) the validity of *unc104*^{TRiPRNAi} expression might be questionable, b) the effects of pan-neuronal knockdown of unc104 on Stromalin's Syt:GFP and synaptic vesicle number phenotype cannot be determined with confidence, nor can we robustly show that Syt:GFP levels and by extension synaptic vesicle numbers parallel seizure phenotypes, and c) alternate trafficking proteins and/or

synaptic vesicle cycle related proteins should be considered to associate presynaptic markers and synaptic vesicle numbers to *stromalin*^{RNAi}'s seizure phenotype.

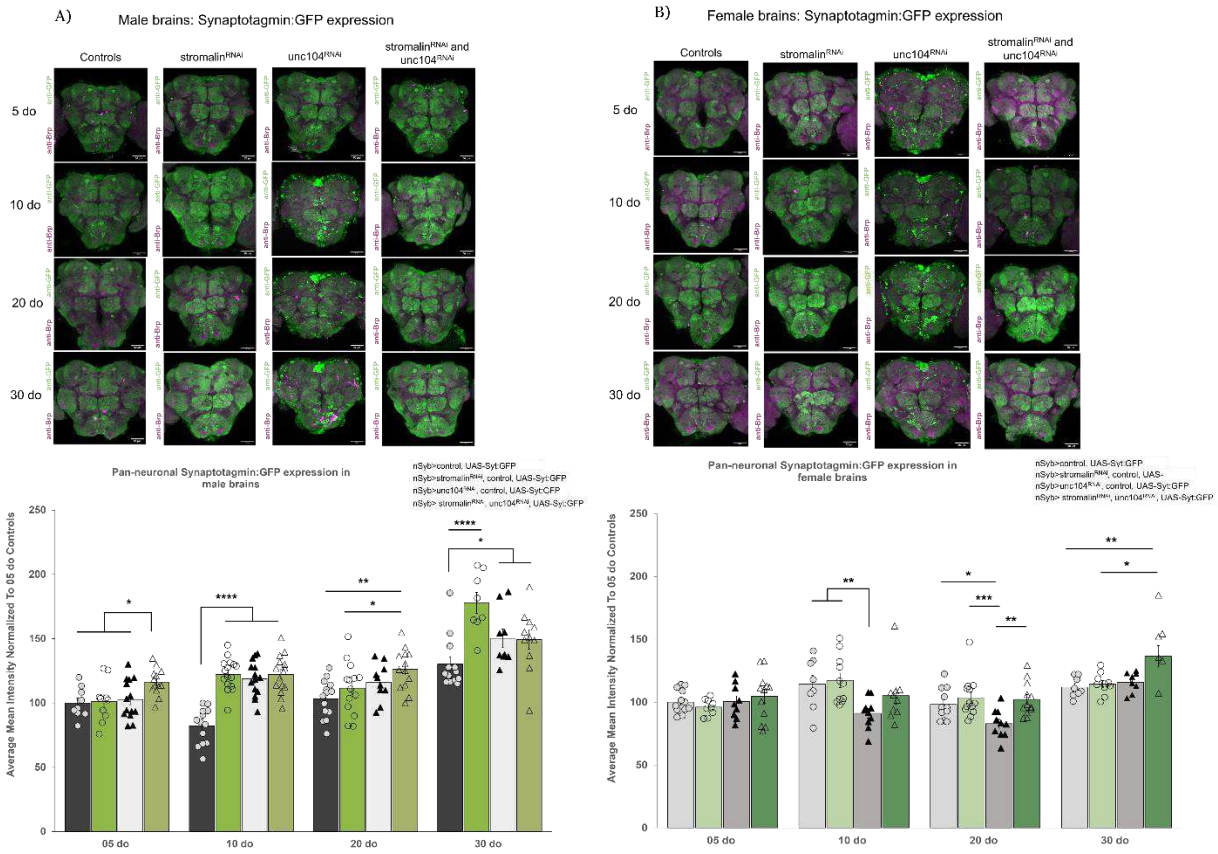

Figure 9: Pan-neuronal knockdown of *unc104* along with *stromalin* does not significantly decrease Syt:GFP levels in males and female flies across all age points. A and B) Pan-neuronal knockdown of *unc104* and *stromalin* does not significantly reduce Syt:GFP levels when compared to stromalin^{RNAi} male and female flies at all age points. A significant reduction in Syt:GFP levels due to pan-neuronal expression of unc104^{TRiPRNAi} and stromalin^{RNAi} when compared to controls and unc104^{TRiPRNAi} male and female flies is absent at all age points, as well. Quantification of the average mean intensities for Syt:GFP are shown in the bar charts below. Control: nSyb-Gal4>+, UAS-Dicer2 and UAS-Syt:GFP. Stromalin^{RNAi}: nSyb-Gal4>UAS-stromalin^{RNAi}, UAS-Dicer2 and UAS-Syt:GFP. Unc104^{RNAi}: nSyb-Gal4>UAS-unc104^{TRiPRNAi}, UAS-Dicer2 and UAS-Syt:GFP. Stromalin^{RNAi} and Unc104^{RNAi}: nSyb-Gal4> UAS-stromalin^{RNAi}, UAS-unc104^{TRiPRNAi}, UAS-Dicer2 and UAS-Syt:GFP. Kruskal-Wallis test with Dunn's post-hoc. **** = p<0.0001, *** = p<0.001, ** = p<0.01 and * = p<0.05. n=6-16. Graphs depict mean ± SEM. Scale bar = 50 or 60 µm.

CHAPTER 4

DISCUSSION

4.1 Overview of the chapter:

We found that reduction of core subunits *stromalin* and *SMC1,* but not *SMC3,* in neurons increased seizure frequencies in flies, and that these increases in seizures may result from the downstream reduction of *CG17698* and *Cox7c* gene expression. We attempted to rescue the higher seizure rates in our Stromalin knockdown flies by inhibiting synaptic vesicle localization to the synapse, however, we failed to see an effect. However, these experiments are inconclusive, as the positive control (Stromalin knockdown) did not show an increased seizure frequency in these experiments. Lastly, we did not observe consistent effects of our synaptic vesicle marker across the brains of our animals. At the end of this chapter, I will discuss and speculate my findings in the context of existing invertebrate and vertebrate literature, and outline some of the limitations of the behavioral assay and experiments performed.

4.2 Knocking down Stromalin, the *Drosophila* homologue of STAG1/2 results in a seizure phenotype in fruit flies.

Characterization of the symptoms displayed by cohesinopathy patients with STAG1 mutations have shown that almost half of the patients exhibited a seizure phenotype (Lehalle et al., 2017, Yuan et al., 2019 and Di Muro et al., 2021) while STAG2 mutations led to a seizure phenotype in almost one-third of the patients assessed (Kumar et al., 2015). Furthermore, a high frequency of the seizure phenotype was also observed in patients with SMC1 mutations as opposed to SMC3, Rad21 and NIPBL mutations (Deardorff et al., 2007, Borck et al., 2007, Goldstein et al., 2015, Gil-Rodríguez et al., 2015, Symonds et al., 2017, and Krab et al., 2020). While vertebrate and invertebrate models of cohesinopathies have recapitulated some aspects of the disease phenotype like growth, cardiovascular, gastrointestinal and cognitive defects as well as perturbed gene expression (Xu et al., 2010, Remeseiro et al., 2013, Muto et al., 2014, and Kamel et al., 2022), only one haploinsufficient *Nipbl* mouse model has been shown to exhibit the seizure phenotype associated with the disease (Kawauchi et al., 2009). Interestingly, the invertebrate *Drosophila* Nipped-B based model of cohesinopathies recapitulated some of the physiological and cognitive symptoms of cohesinopathies but failed to display a seizure phenotype (Wu et al., 2015).

The lack of invertebrate models exhibiting cohesinopathy induced seizures further supports our investigations into Stromalin and its effects on seizure behaviour. Through our experiments, we show that whole-brain knockdown of Stromalin, a subunit of the cohesin complex, can produce a seizure phenotype in *Drosophila* that is significantly higher than controls at early and late age

points in both sexes (Figure 3A and B). Furthermore, our experiments reveal that at 5 days old, pan-neuronal *stromalin*^{RNAi} expressing female flies have an average seizure rate similar to the average seizure rate seen in cohesinopathy patients with STAG1/2 mutations (Leroy et al., 2015, Kumar et al., 2015, Lehalle et al., 2017, Yuan et al., 2019 and Di Muro et al., 2021). Moreover, we have also shown that whole-brain knockdown of the *SMC1* subunit results in a similar seizure phenotype in male and female flies at 5 days old (Figure 4A). Our data not only seems to be in line with the case studies on cohesinopathy patients with STAG1/2 and SMC1 mutations (Borck et al., 2007, Deardorff et al., 2007, Kumar et al., 2015, Goldstein et al., 2015, Lehalle et al., 2017, Symonds et al., 2017, Yuan et al., 2019 and Di Muro et al., 2021), but it also suggests that like the pre-existing vertebrate and invertebrate models (Kawauchi et al., 2009, Xu et al., 2010, Remeseiro et al., 2013, Muto et al., 2014, Wu et al., 2015, and Kamel et al., 2022), certain neurological symptoms of cohesinopathies can be recapitulated in fruit flies as well, making them a potential platform in which cohesinopathies can be studied. Furthermore, mirroring the increased seizure rates associated with the STAG1/2 and SMC1 subunits in pan-neuronal *stromalin*^{RNAi} and *SMC1*^{RNAi} flies suggests that STAG1/2 and SMC1's roles in seizures might be conserved across species. However, it is important to note that as a variability was seen in the seizure phenotypes in pan-neuronal *stromalin*^{RNAi} expressing flies when tested in other experiments (Figure 6A and B), our data also suggests that additional replication experiments need to be run to validate the seizure phenotype seen in pan-neuronal *stromalin*^{RNAi} expressing flies.

In addition to observing a seizure phenotype, we were able to show that Stromalin induced seizure phenotype persists with age in both male and female flies (Figure 3A and B). Prior work on vertebrate and invertebrate models have been on animals that were in the embryonic stages or were young adults (Kawauchi et al., 2009, Mönnich et al., 2011, Muto et al., 2011, Chétaille et al., 2014, Wu et al., 2015, and Lopez-Burks et al. 2016). While Rao et al. (2016 and 2018) provided some evidence for cerebral and colonic transcriptional changes as well as amyloid-beta accumulation in aged rodent models of cohesinopathies, much remains to be known about the symptoms and pathology of cohesinopathies at older age points. The dearth of cohesinopathy characterizations in aged vertebrate models might be because aging in these models is time-consuming and laborious, as it takes months-year(s) to obtain aged animals (Piper and Partridge 2016 and 2018, and Clancy et al., 2023). And so, fruit flies due to their short lifespan, high reproduction rates and ease of maintenance (Piper and Partridge 2016 and 2018, and Clancy et al., 2023) allowed us to study the effects of Stromalin on seizure behavior in flies as young as 5

days old to as old as 30 days old. The later time point corresponds to roughly 50-66% the lifespan of *Drosophila melanogaster*.

A wide-spread evaluation of multiple older CdLS patients performed by Kline et al. (2007) found that some of the physiological and neurological symptoms associated with CdLS exacerbated with age. Moreover, through their extensive evaluation Kline et al. (2007) suggested that CdLS patients aged faster than normal, implying that aging plays a role in CdLS pathology. Our data hints towards the possibility that the seizure phenotype induced by the pan-neuronal expression of *stromalin*^{RNAi} may enhance with age, particularly in pan-neuronal *stromalin*^{RNAi} male flies. However, it is imperative to note that this enhanced seizure phenotype with age is speculative, as a statistical comparison of seizure rates in *stromalin*^{RNAi} male and female flies across age was not performed. Furthermore, during their assessment of older CdLS patients, Kline et al. (2007) did not separately assess their patients by sex. Taken together, we cannot say with certain whether the elevated seizure frequency with age in male *stromalin*^{RNAi} flies is consistent with what is found in humans or is a by-product of the *Drosophila* model system.

4.3 CG17698 and/or Cox7c may act downstream of Stromalin and SMC1 to regulate seizures in cohesinopathies.

After establishing Stromalin and SMC1's effects on seizure behavior in fruit flies, we then investigated the effects of the 5 potential downstream targets of Stromalin identified by the Phan lab: *Nep1*, *CG17698*, *Ttm2*, *Cox7c*, and *Su(z)12* on seizure behavior in male and female flies at 5 days old. Our experiments showed that the pan-neuronal knockdown of *Nep1*, *Ttm2* and *Su(z)12* failed to produce an effect on seizure behavior while knocking down *CG17698* and *Cox7c* pan-neuronally significantly increased seizure rates in male and female flies at 5 days old, mirroring Stromalin and SMC1's seizure phenotype. This allowed us to narrow our targets to *CG17698* and *Cox7c* as the potential downstream mediators of Stromalin and SMC1's seizure phenotype in cohesinopathies. *CG17698* as a potential downstream target and mediator of Stromalin is further supported by the findings by Arruda et al. (2020) who found that eliminating the STAG1/2 subunits in mouse embryonic stem cells led to the downregulation of multiple genes of which calcium/calmodulin dependent protein kinase kinase 1 (*Camkk1*), the mammalian homolog of *CG17698*, was one of them. Besides being a potential downstream target for STAG1/2, Zhu et al. (2020) utilized a human neutrophil derived cell line to show that calcium levels regulated the presence of NIPBL on gene promoters and/or enhancers. Zhu et al. (2020) found that in the presence of calcium, the amount of NIPBL found at active enhancers of various targets including

Camkk1/2, increased. Taken together, these studies suggest that *Camkk1/2* is a downstream transcriptional target for the cohesin complex and that the transcriptional control of *Camkk1/2* by the cohesin complex seems to be conserved in other species such as *Drosophila*. Additionally, the increased seizure phenotype we observed in pan-neuronal expressing CG17698^{RNAi} flies (Figure 5C) is corroborated by Bronstein et al. (1988)'s findings as they observed reduced calmodulin kinase activity in rat models of status epilepticus, providing further support for the conservation of *Camkk1/2* functions in other species.

Similarly, *Cox7c* as a potential downstream transcriptional target and mediator of Stromalin is also supported by findings in mammalian cell culture models as Casà et al. (2020) found *Cox7c* to be one of the many genes that were downregulated upon STAG1 impairment in a human cancer cell line. However, unlike *CG17698*, *Drosophila Cox7c*'s role in seizure behavior lacks robust support from mammalian or vertebrate seizure studies as the evidence is limited and predictive in nature (Raviglione et al., 2021, and Hammer et al., 2024). Nevertheless, future studies should aim to validate the roles of *CG17698* and *Cox7c* in Stromalin and SMC1 knockdown induced seizure phenotype by overexpressing the two putative downstream targets in pan-neuronal expressing *stromalin*^{RNAi} and *SMC1*^{RNAi} flies. As potential downstream mediators, overexpressing *CG17698* and *Cox7c* should rescue the increased seizure phenotype and normalize the seizure behavior observed in *stromalin*^{RNAi} and *SMC1*^{RNAi} flies. A successful rescue of the seizure phenotype will suggest *CG17698* and/or *Cox7c* as potential downstream mediators of Stromalin and SMC1's effects on seizure behavior in *Drosophila*.

Finally, the absence of a seizure phenotype seen upon the whole brain knockdown of *Nep1* (Figure 5A) seems to correlate with mammalian findings. When assessing the expression levels of certain proteins in their samples from temporal-lobe epilepsy patients, Gourmaud et al. (2019) found that *Nep1* was not decreased in these samples. While these experiments did not utilize the same approach as us (RNAi methods) to investigate *Nep1*'s role in seizures, their findings seem to also suggest that a decrease in *Nep1* may not be associated with temporal-lobe epilepsy in patients. Furthermore, work by De Gortari et al. (2007) on rats subjected to the kindling paradigm to study temporal lobe epilepsy showed that mammalian *Nep1* was downregulated in certain regions of the brain during the initial stages of the paradigm. However, at later stages of the paradigm where a seizure phenotype was observed, De Gortari et al. (2007) found that mammalian *Nep1* was upregulated in the same brain regions, suggesting that *Nep* expression may alter as a seizure phenotype manifests. Taking this information into consideration, it is possible that maybe the over-expression of N*ep1* rather than its knockdown may produce seizures in *Drosophila*.

Interestingly, the absence of seizures in male and female Nep1^{RNAi} flies suggests that a role for decreased Nep1 in seizures may be conserved between mammals and invertebrates like *Drosophila*.

However, the absence of an increased seizure rate phenotype in pan-neuronally expressing Su(z)12^{RNAi} and Ttm2^{RNAi} flies (Figure 5B and E) may not correlate with their mammalian counterparts due to their complex roles in epilepsies and seizures. For instance, through their bioinformatics analyses on gene expression data obtained from rodent models of status epilepticus, Khan et al. (2019) suggested that an upregulation in the Enhancer of Zeste Homolog 2 (EZH2), a subunit of the polycomb repressive complex 2 might be associated with dampening the seizure burden in these models. Furthermore, through their analyses Khan et al. (2019) suggested that EZH2 and the other PRC2 subunit, SUZ12 may work together to regulate the expression of a common set of genes following status epilepticus. While Khan et al. (2019)'s findings seem to suggest a beneficial role for SUZ12 in status epilepticus, work by Miller-Delaney et al. (2012) and Reynolds et al. (2015) seems to suggest the opposite as they not only found a differentially expressed SUZ12 in neural regions susceptible to status epilepticus but also the hypomethylation of SUZ12's promoter in mouse models of status epilepticus, suggesting that an increased expression of SUZ12 may be associated with seizures. In a similar vein, mutations in TIMM50, the mammalian homolog of Ttm2, were found to give rise to a mitochondrial encephalopathy that was characterized by seizures amongst other neurological symptoms (Shahrour et al., 2016, and Tort et al., 2019). While these findings contrast our data, it is possible that the difference in phenotypes may arise due to the mutations altering TIMM50's structure and function (Shahrour et al., 2016, and Tort et al., 2019) as opposed to its tissue-specific knockdown in our flies. Thus, unless we investigate other possibilities such as mimicking the TIMM50 mutations in Ttm2 or testing the effects of overexpression of Su(z)12 in flies, we cannot disregard the roles of Nep1, Ttm2 and Su(z)12 in *Drosophila* seizure behavior.

4.4 Limitations of the *Drosophila* seizure assay

By utilizing the standard mechanical seizure induction assay, we successfully established a seizure phenotype in *stromalin*^{RNAi} and SMC1^{RNAi} male and female flies, as well as identified putative downstream targets of Stromalin and SMC1 that may potentially mediate their seizure effects in cohesinopathies. However, our data from these seizure behavioral assays also revealed an inconsistency in the seizure rates displayed by our controls (Figures 3-5). We found that our controls displayed seizure rates ranging from 10% to 31% in our experiments. Furthermore, we

observed that this inconsistency was apparent in both male (9%-29%) and female control flies (10%-33%) (Figures 3-5). It is important to note that the seizure behavioral experiments with Stromalin, its sister subunits and potential downstream targets were performed by two separate individuals. As the flies experience a mechanical stimulus to evoke seizures in them, it is possible that differences in handling and mechanically stimulating the flies could contribute to the discrepancy in seizure rates in control flies. Furthermore, the manual assessment of seizure behavior in flies might differ from person to person despite the existence of a stringent criterion. Taken together, our observations suggest that the mechanical seizure induction assay as well as its analysis needs improvement to identify seizure phenotypes effectively and confidently.

Besides possible differences in executing the mechanical seizure induction protocol, environmental factors such as ambient temperatures that have been shown to have a profound effect on *Drosophila* life cycle, behavior, and neuroanatomy (Miquel et al., 1976, Kiral et al., 2021, and Mollá-Albaladejo & Sánchez-Alcañiz, 2021) could have contributed to the differences in seizure frequency rates seen in controls. While all our experimental crosses were placed in the same growth chamber to rear flies under similar environmental conditions, the growth chamber was subjected to fluctuations in temperature due to a variety of reasons that were out of our control. Even though the effects of rearing temperatures on seizure behavior are yet to be shown, there is a possibility that the temperature fluctuations seen in the growth chamber may have influenced our flies, leading to an inconsistency in seizure frequency rates between controls.

In addition to temperature, another environmental factor such as the fly diet/fly food has also been shown to have an impact on *Drosophila* growth, development, behavior, and life expectancy (Ormerod et al., 2017, and Kruger and Denton, 2020). Differences in the type of yeast and sugar, lipid, moisture, and possibly nutrient content in fly food have been shown to impact various fly behaviors such as geotaxis capabilities, egg-laying behavior, life expectancies, developmental windows and more (Bass et al., 2007, and Ormerod et al., 2017). Like housing conditions, all our experimental crosses and flies were reared on fly food that was produced by the same fly kitchen. While visually the fly food used for all our experiments looked the same, there could have been some differences in the fly food composition as the moisture content in the food was found to vary with different batches. As such, the absence of moisture or presence of excess moisture in the fly food could have impacted our control flies' development and seizure behavior, resulting in inconsistencies in seizure behavior in control flies.

To prevent variability in seizure rates in flies in the future, certain strategies can be implemented such as: a) housing experimental crosses in environment-controlled incubators, b) preparing fly food in the lab and using in-house fly food to rear experimental flies, c) increasing the number of 'n' for each sex and genotype (Parker, Padilla, et al., 2011, Howlett et al., 2013, Saras & Tanouye, 2016 a and b) to assess a larger population of flies to reduce variability in seizure rates, and finally d) implementing the seizure behavior video recording and analysis protocol by Stone et al. (2014) that combines time-lapse images of seizing flies with ImageJ's image analysis functions, or using the machine-learning algorithm developed by Sleep et al. (2024) to eliminate the need for manual assessment of seizure behavior.

4.5 Reducing synaptic vesicle numbers at the synaptic terminal may not prevent seizures.

While characterizing the memory suppressive effects of Stromalin, Phan et al. (2019) showed that knocking down Stromalin in DANs led to an increase in synaptic vesicle numbers and synaptic communication in these neurons, which suggested that this might be a potential mechanism by which Stromalin knockdown produced an enhancement in learning. Given that Phan et al.'s (2019) previous findings and our initial experiments with Syt:GFP showed an increase in Syt:GFP levels, which indirectly suggested a possible increase in synaptic vesicles across many neurons of the brain, we thought that the seizure phenotype we observed in our *stromalin*^{RNAi} flies were due to Stromalin's effects on synaptic vesicles and synaptic communication in all neurons.

Since Stromalin affected synaptic vesicle numbers, Phan et al. (2019) exploited this mechanism to show that reducing synaptic vesicle numbers at presynaptic termini by knocking down *unc104* could rescue Stromalin's effects on learning and synaptic vesicles. As the seizure phenotype in *stromalin*^{RNAi} flies was thought to arise from an increase in synaptic vesicle numbers and synaptic communication in all the neurons of the brain, we utilized the same strategy as Phan et al. (2019) to try decreasing the seizure rates in these flies. We found that the pan-neuronal knockdown of *unc104* in *stromalin*^{RNAi} flies did not produce a seizure phenotype that was significantly different from *stromalin*^{RNAi} male and female flies at almost all age points (Figure 6A and B).

Interestingly, recent case studies on different cohorts of individuals with different backgrounds experiencing epileptic phenotypes have identified structure-and function altering mutations in the motor domain of KIF1α as the causative agent for the seizure phenotypes (Lee et al., 2014, Nieh et al., 2015, Muir et al., 2019, Guo et al., 2020, and Kurihara et al., 2020). While these studies

suggest a role for KIF1α, the mammalian homolog of *unc104*, in seizure and epileptic phenotypes, our data suggests that *unc104*^{TRiPRNAi} male and female flies displayed seizure frequency rates that were either equal to or lower than controls (Figure 6A and B). This discrepancy in seizure phenotypes between mutated KIF1α and the pan-neuronal knockdown of *unc104* in our flies may probably arise due to the restriction of *unc104*^{TRiPRNAi} expression to the brain compared to the wide-spread expression of mutated KIF1α. Moreover, through their work on mouse embryonic stem cells, Arruda et al. (2020) also showed that the absence of STAG1/2 subunits led to the upregulation of certain genes one of which was KIF1α. As such, it is possible that the absence of decreased seizure rates in pan-neuronal expressing *unc104*^{TRiPRNAi} and *stromalin*^{RNAi} flies may be due to some yet to be discovered transcriptional effects of pan-neuronal Stromalin knockdown on targets like *unc104*.

4.6 Inconsistent Syt:GFP levels provide an unclear parallel between seizure phenotypes and synaptic vesicles.

Through electron microscopy experiments, Phan et al. (2019) showed that the increase in Syt;GFP upon Stromalin knockdown in DANs was indicative of increased synaptic vesicles and synaptic communication at the presynaptic termini of DANs. Moreover, they also showed that pan-neuronal knockdown of Stromalin caused a similar increase in Syt:GFP levels across all neurons but whether this was indicative of increased synaptic vesicle numbers across the whole brain remains undetermined (Phan et al., 2019). However, the evidence Phan et al. (2019) provide for Syt:GFP as a marker of synaptic vesicle numbers upon *stromalin* knockdown in DANs are compelling, and so, we visualized Syt:GFP in our pan-neuronal expressing *stromalin*^{RNAi} flies as well as in *unc104*^{TRiPRNAi} and *stromalin*^{RNAi} flies to determine whether Syt:GFP and by extension synaptic vesicle numbers paralleled the seizure phenotypes in these flies. We found that pan-neuronal expression of *stromalin*^{RNAi} did not significantly change Syt:GFP in male and female flies at almost all age points (Figure 7 and 8). Mirroring their seizure phenotypes, the whole brain knockdown of *unc104* in *stromalin*^{RNAi} flies produced no significant difference in Syt:GFP levels when compared to *stromalin*^{RNAi} flies at almost all age points (Figure 9A and B).

The inability to normalize Syt:GFP levels by the pan-neuronal knockdown of *unc104* in *stromalin*^{RNAi} flies might be due to compensatory effects. Furthermore, the inconsistency in Syt:GFP changes in pan-neuronal expressing *stromalin*^{RNAi} flies as well as in pan-neuronal expressing *unc104*^{TRiPRNAi} and *stromalin*^{RNAi} flies hints towards the unreliability of the Syt:GFP overexpression system (Zhang et al., 2002) as well as the image analysis method we employed.

While testing their novel conditional, endogenous synaptic marker, Williams et al. (2019) found that the pan-neuronal expression of Syt:GFP in larvae produced a staining pattern that did not completely coincide with the staining pattern of endogenous Syt, suggesting that the Syt:GFP overexpression system might produce some false-positive staining patterns. Williams et al. (2019) observed that the Syt:GFP expression in cell bodies was not replicated when endogenous Syt was stained, suggesting that Syt:GFP expression in cell-bodies may not always be an accurate presentation of synaptic vesicle numbers. This observation is important because while analyzing whole-brain Syt:GFP levels, we included Syt:GFP signals in the cell bodies which may have- a) provided us with an inaccurate indirect representation of synaptic vesicle numbers, and b) does not provide us with an accurate picture of whether the pan-neuronal unc104 knockdown in *stromalin*^{RNAi} flies prevented synaptic vesicles from reaching the synapses. In the future, we can prevent this by- a) utilizing a robust and endogenous synaptic vesicle marker such as the B2 recombinase dependent GFP tagged Rab3 (B2R, Rab3-GFP) that strictly restricts itself to synaptic vesicles (Williams et al., 2019) in addition to Syt:GFP, and b) eliminating GFP signals from cell bodies during whole-brain image analysis.

Finally, it is also important to note that during the final leg of our experiments, we observed that our *unc104*^{TRiPRNAi} fly line had flies of varying eye colors in them suggesting a contamination of the fly line. The possible contamination of our *unc104*^{TRiPRNAi} fly line not only explains the inconsistent Syt:GFP staining patterns we observed in the brains of *unc104*^{TRiPRNAi} flies, but also provides an alternate explanation for the seizure phenotypes seen in these flies. When we looked at the progeny of this fly line crossed to the Gal4 driver, we observed a variation in eye color in female flies. In the field of *Drosophila* research, eye color has and continues to serve as an excellent and obvious marker for the presence or absence of a transgene in a fly (Klemenz et al., 1987). The TRiP RNAi library was constructed such that the presence of the RNAi construct in the fly could be determined by the vermillion eye color of the flies (Perkins et al., 2015). Unfortunately, as we did not test to confirm the contamination, our suspicion remains to be validated. Furthermore, as we were short on time, we could not repeat the seizure behavior and imaging experiments with corrected lines.

4.7 Conclusion

In conclusion, our work suggests that the knockdown of Stromalin at a pan-neuronal level can induce an increased seizure rate phenotype in *Drosophila*, as seen in cohesinopathy patients. We also show that the pan-neuronal knockdown of SMC1 produces an increased seizure rate phenotype that is similar to the *stromalin*^{RNAi} phenotype in fruit flies. Additionally, we show that Stromalin and SMC1 may induce the seizure phenotype in flies by influencing the transcription of *CG17698* and *Cox7c* at the pan-neuronal level, while the pan-neuronal knockdown of *unc104* in *stromalin*^{RNAi} flies does not seem to decrease the seizure phenotype and Syt:GFP effects produced by Stromalin. However, as we observed some reproducibility issues with seizure phenotypes and Syt:GFP levels across experiments, our data are currently inconclusive, and our data is insufficient to support our hypothesis. From a disease perspective, we show that certain neurological symptoms of cohesinopathies can be studied in *Drosophila*, making our *stromalin*^{RNAi} flies a potential and continuously evolving disease model for further studies into the genetic underpinnings of seizure behavior in cohesinopathies.

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