Metabolic effects of aneuploidy and their relevance to cancer and neurological phenotypes

By

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Thesis

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I would like to dedicate my thesis to my beloved.

wife,

Mariam Binta Rafiq and

daughter,

Ameera Juwairia Anowar

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LIST OF ABBREVIATIONS

ALL	**Acute lymphoblastic leukemia**
AML	**Acute myeloid leukemia**
AD	**Alzheimer's disease**
Ahcy	**Adenosyl-homocysteinase**
ATM	**Ataxia telangiectasia mutant**
Atgs	**Autophagy-related genes**
APC/CCdc20	**The CDC20-Bound Anaphase-Promoting Complex/Cyclosome**
BUB1	**Benzimidazoles 1**
BUB 3	**Benzimidazoles 3**
BHMT	**Betaine-homocysteine methyltransferase**
CIN	**Chromosomal Instability**
CDC20	**Cell division cycle 20**
Cdk1	**Cyclin-dependent kinase 1**
CBS	**Cystathionine-β-synthase**
CTH	**Cystathionase**
CSCs	**Cancer stem cells**
CBD	**Cannabidiol**
CIs	**Confidence intervals**
DDR	**DNA damage response**
DFMO	**Difluoromethylornithine**

DcSAM	**Decarboxy-s-adenosyl methionine**
ES	**Ewing sarcoma**
ER	**Estrogen receptor**
E2	**Estradiol**
EIMFS	**Epilepsy of infancy with migrating focal seizures**
FA	**Fanconi anaemia**
FANCD2	**Fanconi anaemia complementation group D2**
F-THF	**formyl-THF**
FOCM	**Folate-mediated one-carbon metabolism**
GIN	**Genomic instability**
GNMT	**Glycine N-methyltransferase**
GST	**Glutathione-S-transferase**
GEFS+	**Generalised Epilepsy with Febrile Seizures Plus**
GABA	**γ-aminobutyric acid**
Hcy	**Homocysteine**
MCC	**Mitotic checkpoint complex**
Mad2	**Mitotic arrest deficient 2**
m-THF	**methyl-THF**
me-THF	**methylene-THF**
MAT	**Methionine adenosyl transferase**
MTHFR	**methylenetetrahydrofolate reductase**
5-mTHF	**5-methyltetrahydrofolate**

mTORC1	**mTOR complex 1**
m6A	**N6-Methyl Adenosine**
METase	**Methioninase**
Met-s	**Methionine synthase**
MEFs	**Murine embryonic fibroblasts**
NSCLCs	**Non-small-cell lung cancers**
NACA	**N-acetylcysteine amide**
NC	**Negative control**
OSCC	**Oral squamous cell carcinoma**
ODC	**Ornithine decarboxylase**
PLK1	**Polo-like kinase 1**
3-PG	**3-phosphoglycerate**
PDAC	**Pancreatic ductal adenocarcinoma**
PDX	**Patient-derived xenograft**
PC	**Positive control**
ROS	**Reactive oxygen species**
Repo	**Reversed polarity**
SAC	**Spindle assembly checkpoint**
SMC1A	**The structural maintenance of chromosomes 1A**
SMC3	**The structural maintenance of chromosomes 3**
STAG1	**Stromal antigen 1**
STAG2	**Stromal antigen 2**

SCNAs	**Somatic copy-number changes**
SAM	**S-adenosyl methionine**
SAH	**s-adenosyl homocysteine**
SAHH	**SAH hydrolase**
SAMDC	**SAM decarboxylase**
SSP	**Serine synthesis**
SOD	**Superoxide dismutase.**
SD	**Standard deviation**
Sms	**Spermine synthase**
Spds	**Spermidine synthase**
Shmt	**Serine hydroxymethyl transferase**
SAM-s	**S-adenosyl methionine synthase**
THF	**Tetrahydrofolate**
TYMS	**Thymidylate synthase**
WT	**Wild type**

SUMMARY

Chromosomal Instability (CIN), in which cells exhibit ongoing gain or loss of complete chromosomes or significant chromosomal fragments, is present in the majority of human solid tumors. CIN is a principal cause of aneuploidy which is defined as a deviation from a normal chromosomal number, with either a gain or loss. Poor prognosis, medication resistance in cancer, and the advancement of carcinogenesis are all linked to aneuploidy. Given that aneuploidy is common in malignancies and rare in healthy cells, it has been suggested that aneuploidy may be targeted for cancer treatment. To target aneuploidy for cancer treatment, the signaling pathways that allow cells to tolerate aneuploidy must be identified. This work aims to uncover signaling pathways that are triggered by aneuploidy and may be targeted to specifically kill aneuploid cells.

I examined the **transcriptional responses (Chapter-3)** to continuous ploidy alterations (chromosomal instability, CIN) in *Drosophila* as a model organism. We observed **alterations in genes** related to one-carbon metabolism, particularly those influencing the synthesis and utilization of S-Adenosyl methionine (SAM). In CIN cells, the loss of some of these genes resulted in apoptosis, but this was not seen in normally proliferating cells. I discovered that the role of SAM metabolism in producing polyamines contributes to the heightened sensitivity of CIN cells to SAM depletion. I observed that feeding spermine to rats can prevent the cell death in CIN tissues induced by SAM synthase depletion. Reduced autophagy and susceptibility to reactive oxygen species (ROS) resulted from the loss of polyamines, and my research has demonstrated that these factors greatly contribute to CIN cell death. These results imply that CIN tumours may be targeted by a metabolic intervention that is well tolerated, such polyamine inhibition, using a mechanism that is comparatively well understood.

In addition, numerous laboratories have produced a significant body of evidence suggesting that neuronal aneuploidy and the resulting apoptosis, which may contribute to neuronal loss, are characteristics of many neurodegenerative illnesses, including Alzheimer's disease and frontotemporal dementia. I examined the impact of induced aneuploidy in GABAergic neurons using *Drosophila* **(Chapter-4)** as a model. In the brain of a third-instar larva, I observed a higher percentage of aneuploidy as a result of Mad2 depletion as well as higher cell death. A compromised climbing and seizure phenotype was also observed with depletion of Mad2 in GABAergic neurons. The climbing and seizure defects of the animals was ameliorated by feeding them an antioxidant.

Moreover, we created *Drosophila melanogaster* lines carrying human *KCNT1* **(Chapter-5)** with the patient mutations G288S, R398Q, or R928C in order to test if Drosophila may be utilised to replicate human *KCNT1* epilepsy. Each mutant channel's expression in GABAergic neurons produced a seizure phenotype that was either positively or negatively responsive to five frontline epilepsy medications that are frequently given to patients with *KCNT1*-epilepsy, frequently with little to no change in seizure frequency. While some medications increased the seizure phenotype, cannabidiol demonstrated the largest reduction in seizure phenotype. Our research indicates that *Drosophila* can serve as a model for human *KCNT1*-epilepsy and can be employed to evaluate novel therapeutic approaches for *KCNT1*-epilepsy such as inducing aneuploidy tolerance.

DECLARATION

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed....Anowarul Islam.....

Date.....March 2025.....

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LIST OF PUBLICATIONS

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1. CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Aneuploidy

The DNA molecule is packed into thread-like structures in the nucleus of each cell, called chromosomes. Every chromosome is composed of tightly coiled DNA packaged around proteins called histones that support its structure. Alterations that involve changes in chromosome numbers in the karyotype of a species are classified as either aneuploidies or polyploidies. Aneuploidy is the state in which cells carry an abnormal DNA complement, such as a human cell that has 45 or 47 chromosomes rather than the regular 46 [1]. An aneuploid is an individual organism whose chromosome number or composition varies from that of the wild type. Generally, the distribution of aneuploid chromosomes varies only by one or a small number of chromosomes from the wild form. Aneuploids may have either greater or smaller chromosome numbers than those of the wild variety. The biological effects of aneuploidy often vary significantly from those of polyploidy. An organism is said to be polyploid if its cells contain multiple pairs of homologous chromosomes. This indicates that the cells of organism contain either triploid (three sets), tetraploid (four sets), or even higher levels of ploidy than diploid (two sets of chromosomes, one from each parent). Polyploidy is commonly present in nature; it may be a part of normal plant and animal physiology, including a few forms of human cells; and usually does not result in severe defects in an organism's growth or physiology [2]. Duplications of the entire genome occurred in the evolution of many organisms such as plants and yeasts and may constitute a natural phenomenon essential for this process [3-5]. The *Drosophila melanogaster* genome normally comprises three autosomal pairs (II, III, and IV) and a pair of sex chromosomes (females are XX, males are XY). A recent study has shown that aneuploidy is a distinctive characteristic of cancer and also the leading cause of spontaneous abortion, mental retardation and growth deficiencies in humans [6]. While aneuploidy has been shown to cause stress and reduce cellular health [7-10], cancer cells have somehow found a way to cope with aneuploidy and thrive in proliferating despite the detrimental effects of aneuploidy, which is called the aneuploidy paradox [9]. Aneuploidy increases with age and is associated with shortened lifespan [11]. We know little about aneuploidy-specific responses in cancer and even less in the neurodegenerative diseases, hence the need for this study.

1.2. Historical context of aneuploidy studies in cancer

Theodor Boveri proposed in the early 1900s that cellular transformation and cancer could be caused by aneuploidy [12]. His next publication, "Concerning the Origin of Malignant Tumours," published in 1914, established the first idea that aneuploidy and cancer are related [13,14]. A deeper understanding of chromosomal behaviour during cell division was provided sixteen years later when Barbara McClintock coined the terminology "laggards" or "lagging

chromosomes" to denote chromatin lagging between daughter nuclear masses during anaphase [15]. The discovery that humans contain 46 chromosomes by Tjio and Levan in 1956 marked the beginning of the science of clinical cytogenetics. In addition to disproving the theory that humans have 48 chromosomes, this discovery opened the door for research on chromosomal abnormalities in humans [16]. By utilizing laser ablation to carry out their seminal experiment in 1994, Rieder and associates were able to uncover the significance of detached kinetochores in prolonging the mitotic period, so offering vital insights into the workings of mitotic checkpoint regulation [17]. Lengauer et al. (1997) measured aneuploidy in human cancer cell lines and suggested that it was universal to all cancer types [18,19]. Meanwhile, the research conducted by Angelika Amon (1999–2010) clarified the molecular features of checkpoints critical in aneuploidy [20,21]. This investigation eventually results in the identification of aneuploidy as one of the hallmarks of cancer. The precise association between aneuploidy and cancer is still up for debate, over a century after Boveri's hypothesis [22-26]. Growing volumes of seemingly contradictory information supporting and refuting the theory that aneuploidy is a cause of cancer are fueling this debate. The findings that favour Boveri include the following: several aneuploid mice models are prone to cancer [25], the majority of malignant neoplasms in humans are aneuploid, and in some sensitised backgrounds, aneuploidy causes tumour suppressor loss of heterozygosity [27]. The findings that aneuploidy causes a range of tumor-suppressive stress responses, such as growth and cell cycle arrest [28,29], apoptosis [30-33], mitotic catastrophe [34], and senescence [35], argue against Boveri. It also delays spontaneous immortalization [29]. Furthermore, it is widely known that a number of aneuploid mouse models do not develop cancer [25]. Therefore, despite Boveri's long-standing theory, it is still unclear whether aneuploidy causes or results from transformation and in what circumstances it stimulates or inhibits carcinogenesis.

1.3. Aneuploidy and cancer

The majority of cancers exhibit several kinds of somatic copy-number alterations? (SCNAs), such as whole-chromosome aneuploidies, focal events, and/or segmental aneuploidies. Taking into account the most common cancers, 60–80% of breast tumours, 70% of colorectal tumours, 30% of prostate tumours, and 60% of non-small-cell lung cancers (NSCLCs) deviate from a diploid karyotype [36-41]. Aneuploidy and cancer have a complicated and hotly contested relationship, which stimulates a thriving study field. There is evidence that aneuploidy may have either a protumorigenic or antitumorigenic effect. Research conducted on human, mouse, and yeast strains has demonstrated that aneuploidy reduces the ability of nonmalignant cells to proliferate, and that the phenotype is not dependent on the identity of individual chromosomes but may be related to the size of the cell [29,42-46]. According to a thorough analysis by Ried et al; 2012, variations in chromosomal copy number cause transcriptome changes and gene-dosage effects at the proteomic level [47]. The cellular protein composition becomes unbalanced as a result, potentially overloading the machinery responsible for protein folding and breakdown. This can trigger a proteotoxic stress reaction and modify the balance between anabolic and redox states, ultimately resulting in a rise in reactive oxygen species (ROS) [48,49]. These results suggest that aneuploidy generally has a detrimental effect on cells, and there is a lot of evidence suggesting that aneuploidy negatively affects the fitness of nonmalignant cells. Firstly, in brain and liver, aneuploidy is a very uncommon occurrence under normal circumstances [50]. Second, after 7–10 passages in culture, trisomic murine embryonic fibroblasts (MEFs) exhibit senescence characteristics, lack of clonogenic potential, proliferation arrest in low-serum media, and contact inhibition characteristics [51]. Third, in order to increase their ability to proliferate, trisomic cells can undergo euploidization by losing excess chromosomes in both *in vitro* and *in vivo* settings [51].

Numerous research findings suggest a possible causative relationship or association between Chromosomal Instability (CIN) and aneuploidy. The two phenomena have been found to be correlated in both trisomic and monosomic models. When stimulated with phytohemagglutinin, lymphocytes from patients with Turner's syndrome (XO karyotype) or constitutional autosomal trisomy (of chromosomes 21, 18, or 13) are more likely to generate non-chromosome-specific aneuploidy. Amniocytes from trisomic foetuses also show a greater prevalence of random aneuploidy [52,53]. Furthermore, in converted Chinese hamster embryo cells, the level of aneuploidy is directly correlated with genomic instability (GIN) [54]. Two essential components of the aneuploidy-driven adaptive response that promotes malignant transformation are GIN and CIN. A "mutator

phenotype" in yeasts is caused by single-chromosome aneuploidy alone, which is also sufficient to cause CIN, an increase in double strand breaks (DSB) during DNA replication, and faulty DSB repair [55]. Cells that evolve and become more fit are positively selected, maintaining and spreading aneuploidy. In human cells, aneuploidy has also been linked to increased replication stress, genomic rearrangements, and DNA damage [56]. For example, postmitotic DNA damage can accumulate as a result of chromosome segregation mistakes (particularly, chromatin bridges, which usually result from DNA damage) because chromatin bridges can be "trapped" in the cleavage furrow [57].

1.4. Mechanisms of aneuploidy

In meiosis and mitosis, whole chromosome aneuploidy occurs due to defects in segregation. Early embryos frequently build up aneuploid cells because of mitotic errors, thereby forming a mosaic of euploid and aneuploid cells [58,59]. The aneuploid cells are eventually removed from the embryonic tissues through senescence or apoptosis or outgrown through better-proliferating euploid cells [60,61]. It is hard to estimate the frequency of aneuploidy in mammalian tissues; while single-cell sequencing shows fewer than 1% of aneuploid neurons and fibroblasts, the same tissues exhibit regular irregular chromosome numbers when tested by in situ fluorescence hybridization [50,62,63]. Moreover, tissue-specific aneuploidy variations have also been found in other animals, but the causes remain unknown [64]. Aneuploidy results from chromosome segregation defects, which can go wrong in a variety of ways. Full-chromosome aneuploidy is caused by chromosome segregation errors resulting from incorrect attachments of the microtubules of the spindle to the kinetochore, a protein complex that assembles each chromosome in the central region [65]. Due to mutations or sporadic defects that impair mitotic spindle function, kinetochore structure, sister chromatid cohesion, or spindle assembly checkpoint (SAC), chromosome mis segregation occurs. Alterations in the transcriptional regulation of Rb-E2F and Ras affect the fidelity of chromosome segregation by either the expression of SAC genes or by causing cohesion defects in sister chromatids [23,66]. Genes (*GJB3, RXFP1—also known as LGR7—and OSBPL3*) not historically related to the division of chromosomes can also cause aneuploidy, as shown by recent genetic screens [67]. Surrounding tissue can affect the incidence of chromosome segregation deficiencies as a recent study shows that epithelial tissue architecture promotes chromosome segregation fidelity [68]. Chromosome mis segregation is often accompanied by irreparable cell cycle arrest, reduced proliferation or cell death, in particular when combined with chromosome breakage [28]. The aggregation of Chromosomal instability (CIN) cells in the tissue is influenced not only by mutations that enhance mitotic errors, but also by mutations that increase the tolerance of aneuploid stresses [69-71].

Centrosome abnormalities are frequently associated with aneuploidy because they can cause inappropriate chromosomal segregation during cell division due to problems in centrosome number or function. The centrosome is a non-membraneous organelle in animal cells consisting of a pair of connected centrioles surrounded by an amorphous protein matrix called pericentriolar (PCM) material [72]. The centriole is an asymmetrical barrel-shaped array of nine sets of triplet microtubules while the PCM comprises hundreds of proteins involved in the nucleation of microtubules and other functions [72,73]. The centrosome is the nucleation and anchoring core of spindle microtubules in most animal cells, thus playing a significant role in microtubule-associated processes such as cell division [74]. Centrosome abnormality in number, structure, and size was found in virtually all human tumors and is known to be a hallmark of tumour aggression and malignancy [75]. Ganem et al. year? found that while cells with extra centrosomes may be subjected to bipolar cell divisions, in terms of lagging chromosomes, they display a significantly increased level of aneuploid cells [76].

Aneuploidy may also cause damage to DNA during cytokinesis, while DNA damage has been reported to induce centrosome amplification in human cells [77]. Thus, centrosomal abnormality and DNA damage may form a positive feedback loop that drives the development of aneuploid cells in cancers. Another explanation is that the aberrant expression of certain centrosome genes may lead to centrosome or microtubular defects which would then lead to aneuploidy. For example, the overexpression of Nek2, a serine/threonine-protein kinase that is engaged in centrosome separation [78], leads to premature centrosomes generation while depletion of Nek2 causes defects in centrosome separation [79].

Eukaryotes evolved a mechanism called the spindle assembly checkpoint (SAC) to avoid chromosome missegregation and aneuploidy to ensure the fidelity of chromosome segregation during the process of cell division [80,81]. Defects in the SAC machinery lead to aneuploidy and foster tumorigenesis in human cells [80].

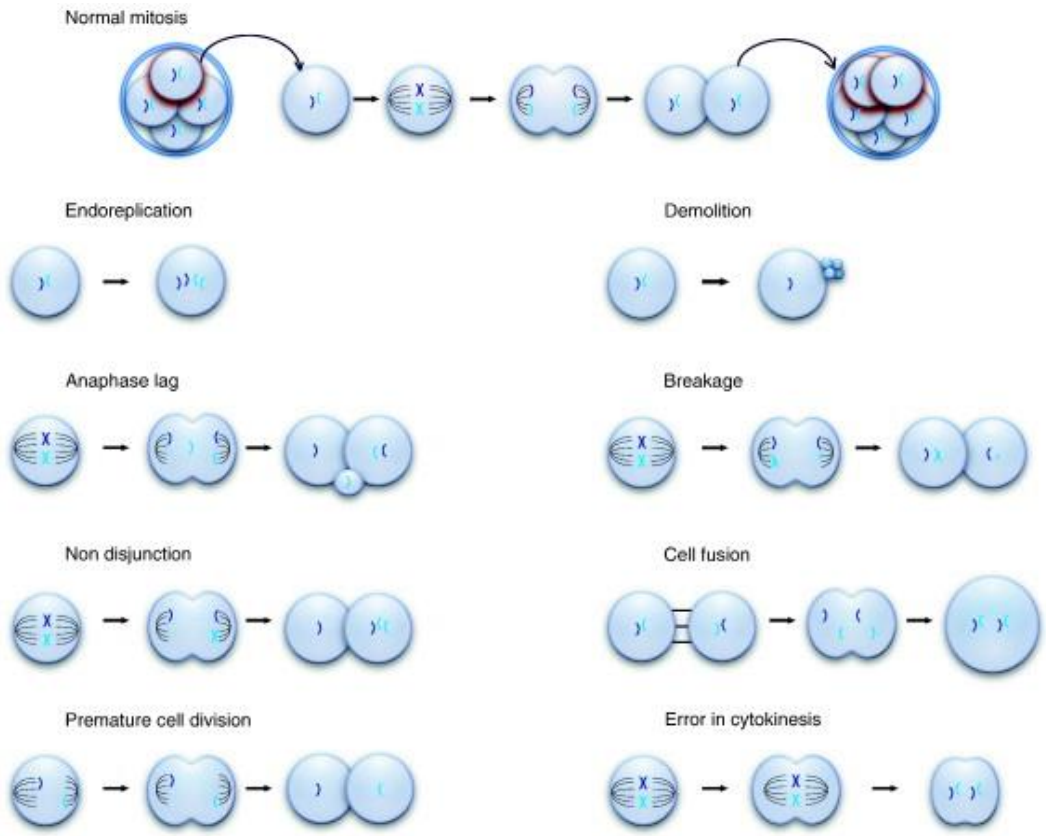

Figure 1-1.-Different mechanisms of aneuploidy [82].

1.5. Chromosomal Instability

The term "chromosomal instability" (CIN) describes an ongoing process of duplicating or deleting entire chromosomes or specific regions of chromosomes, which can result in changes over time to the number and structure of chromosomes. About 1% of cell divisions in normal cells result in mistakes in chromosomal segregation, whereas 20% of cell divisions in CIN-affected cells experience this error. It is believed that chromosomal aberrations suggestive of CIN are present in 60–80% of human tumours [37,83]. Relapsed and metastatic tumour specimens have higher incidence of CIN, which has a positive correlation with the stage of the tumour [84,85]. It is frequently observed in solid tumours and many haematological malignancies [18,86]. CIN has significant effects on drug resistance, carcinogenesis, cancer cell evolution, and metastatic potential. It is also a primary cause of aneuploidy [87,88]. Due to comparable diagnostic markers, CIN and aneuploidy were often considered same in the past, however now they are considered as two separate but interlinked processes [89]. Aneuploidy is the potentially stable condition of having an abnormal karyotype, while CIN is unstable aneuploidy. Human cancers frequently contain both CIN and aneuploidy, which are associated with tumorigenesis and poor clinical outcomes [24,90]. CIN has the ability to

induce the genetic rearrangements necessary for a tumour to spread to other areas of the body [91]. In mouse models, CIN induction doubles the likelihood of spontaneous tumour formation [92], and in human cancer, CIN is linked to a markedly worse prognosis [93]. It has been proposed that because CIN creates genetic variation in tumours, it encourages medication resistance and relapses after chemotherapy [94]. Despite the discovery of multiple mechanisms capable of producing CIN, a thorough understanding of CIN's molecular basis in the majority of malignancies remains elusive. It is believed that CIN is caused by mutations in genes necessary for chromosome stability, taking into account additional clinical characteristics of tumours [24]. Nevertheless, relatively few human malignancies have been shown to contain the particular gene alterations already known to induce CIN. There are two types of CIN: whole chromosome instability (W-CIN), which results from faulty whole chromosome segregation, and structural chromosome instability (S-CIN), which results from chromosomal alterations such as deletions, duplications, and translocations [95].

1.6. Mechanisms or causes of CIN.

The etiology of CIN is complex and has multiple contributing components. Fundamentally, chromosome segregation errors during cell division, which can result in the formation of cells with an abnormal number of chromosomes, and errors in DNA replication, which can result in the formation of cells with incomplete or excess genetic material, are the main causes of CIN [22,96]. Chromosome missegregation may be caused by abnormal spindle assembly checkpoint (SAC) activity, defective sister chromatid segregation, aberrant centrosome number, and microtubule-kinetochore attachment error [97-110]. Numerical CIN may also result from chromosome missegregation, replication stress, sister chromatid defects, and aberrant centrosome numbers [111-115]. Chromosome bridges, micronuclei, aneuploidy, polyploidy, and lagging chromosomes are some of the main indicators of CIN [25,56,89,116-124].

Chromosome bridges are structures created when parts of sister chromatids intertwine and fail to fully segregate, Unresolved DNA replication intermediates or insufficient double-strand break repair may be the cause of this. Conversely, chromosomes that are unable to correctly align and segregate during cell division are known as lagging chromosomes [112,113]. A micronucleus is a tiny, extra-nuclear entity that comprises chromosomal

fragments or entire chromosomes that are not incorporated into the main nucleus, and may arise as a result of trailing chromosomes and chromosome bridges [117,125-127].

While errors in chromosome segregation can result from defects in the machinery that facilitates chromosome segregation, errors in DNA replication can arise from abnormal replication or fidelity as well as replication stress, such as DSBs and stalled replication forks. Additional, chromosomal instability may be further triggered by some CIN-induced events. For example, chromothripsis is both a result of CIN (micronucleus formation from lagging chromosomes) and causes CIN (chromosome fragmentation) [125,128-137].

Centrosome abnormality both in number and function is considered to be another possible cause of CIN. Centrosome amplification can occur in cells by a variety of pathways, including cell fusion, cytokinesis abnormalities, and disruptions in the centrosome biogenesis process. It has been shown that numerous spindle poles during mitosis caused by supernumerary centrosomes can result in chromosomal missegregation and CIN [76]. According to research by Basto et al; 2008 in flies, metastatic tumours can be induced by centrosome amplification [138]. Centrosome amplification is also caused by a number of methods, including viral-based cell fusion, high expressions of Eg5, and cytokinesis failure that results in tetraploidy [139]. Tetraploid cells are therefore predicted to be more vulnerable to CIN.

1.6.1. Defects in SAC components and CIN

A cell going through mitosis requires its replicated chromosomes to be separated into two new daughter cells. Chromosomes are attached to the microtubules of the mitotic spindle machinery to do this [140]. At kinetochores, which are specific protein structures that bind to the chromatin centromere, chromosomes are attached to the ends of microtubules [140]. Every chromosome normally contains two kinetochores, and bi-orientation formation is necessary for mitotic cells. When every sister kinetochore connects microtubules pointed to opposing spindle poles, this condition is achieved.

When kinetochores are either disconnected to spindle microtubules or attached in a way that prevents normal tension between sister kinetochores, the SAC plays a critical role in proper chromosomal segregation by preventing cells from entering anaphase [80,141,142]. This guarantees that every pair of duplicated chromatids in metaphase is properly aligned. By observing appropriate kinetochore attachment and chromosomal alignment,

the SAC serves as a safety measure to ensure chromosome bi-orientation on the mitotic spindle [80,141,142] . The components of the SAC that migrate to unattached kinetochores and form the mitotic checkpoint complex (MCC) along with cell division cycle 20 (CDC20) are mitotic arrest deficient 2 (MAD2) [109,143-146] , budding uninhibited by benzimidazoles 1 (BUB1) [147-150], budding uninhibited by benzimidazoles 1 beta (BUBR1) [149,151,152], and budding uninhibited by benzimidazoles 3 (BUB3) [147,148,153-157]. One important SAC effector is MCC, which prevents the activation of the CDC20-bound anaphase-promoting complex/cyclosome (APC/CCdc20), an E3 ubiquitin ligase that targets securin and cyclin B for proteasome destruction [80,141,142,158-166]. After sister chromatids have correctly aligned and connected, the MCC can separate due to the inactivation of the SAC, which frees up CDC20 to activate the APC/C (34210579). The degradation of securin and cyclin B is initiated by the activation of APC/CCDC20 [158,162,164,167]. Sister chromatid separation and the start of anaphase are made possible by separase, an enzyme that is released when securin is destroyed and cleaves and inactivates the cohesin complex [158]. Cell division and mitotic exit are made possible by the inactivation of cyclin-dependent kinase 1 (Cdk1) caused by cyclin B degradation [80,141,168,169].

The SAC is essential for maintaining genomic integrity in eukaryotic cells, and abnormalities in it result in errorsin chromosome segregation [108,170]. Deleting a single *Mad2* allele is sufficient to induce a spindle checkpoint malfunction in both human cancer cells and mouse primary embryonic fibroblasts. This deficit causes an increased rate of uneven chromosomal segregation as well as early sister chromatid separation [109]. Without *MAD2*, a SAC component, cells can multiply both in vivo and in vitro, but they will produce more CIN [171,172]. Furthermore, as previously mentioned, the chromosomal bridges and lagging chromosome may generate micronuclei, a structure resembling a nucleus made of a bilayer membrane enclosing an extrachromosomal DNA fragment [173].

Spindle checkpoint problems typical of yeast *Mad2* mutants and *Drosophila Bub1* mutants include early segregation of sister chromatids [174,175]. Due to excessive non-disjunction, *Mad2* null mice are not viable and rapidly suffer apoptosis [176]. However, animals with CIN can be produced by depleting the *Mad2* protein in *Drosophila melanogaster*. This shortens metaphase, allowing cells less time to align their chromosomes prior to the start of anaphase, leading to chromosomal bridges and lagging chromosomes [177].

Surprisingly, CIN can also be brought on by overactivity of the checkpoint, which can be brought on by, for instance, overexpression of SAC genes like *MAD2* or reduction of genes implicated in SAC silencing pathways like TRIP3 or p31/comet [178-181]. A number of investigations showed that the overexpression of *Mad2* results in polyploid cell formation, increased mitotic inaccuracy, and a delay in mitosis in B-cell lymphoma, lung adenocarcinoma, and hepatocellular carcinoma [179,182-184]. SAC hyperactivation may result in an increase in micronuclei, lagging chromosomes, and chromosome bridges, much like SAC depletion [178-181,185]. SAC hyperactivation, however, postpones the commencement of anaphase and extends mitotic arrest, in contrast to a weaker SAC, which speeds up mitotic progression and tumour cell proliferation [178-181,185]. So, the SAC is essential for proper chromosomal segregation during mitosis, and paradoxically, CIN can result from both its failure and hyperactivation.

1.6.2. Merotelic Kinetochore Attachment and CIN

Centrosome amplification dramatically raises merotelic binding at the kinetochore, at least under some conditions [186]. As demonstrated in and frequently observed in CIN cells [89], merotelic abnormalities occur when the same kinetochore concurrently attaches to microtubules emanating from both spindle poles and hence cannot be segregated [83]. Kinetochore merotelic problems can result from mutations in centromere and kinetochore structural proteins, while kinetochore–microtubule attachment defects are partly caused by mutations in centromeric heterochromatin [83]. For instance, retinoblastoma protein and histone deacetylase mutations result in centromeric chromatin that is poorly organized, which produces lagging chromosomes [66,187]. Furthermore, merotelic and lagging chromosomes at an increased rate in anaphase are caused by changes in microtubule-depolymerizing enzymes such as the Ndc complex, CENP-E, CENP-F, MCAK, and Kif2b. These findings demonstrate the significance of these proteins in rectifying faults in kinetochore-microtubule interaction [139,188]. In particular, disruption of these proteins significantly stabilizes the binding between kinetochores and microtubules, decreasing the ability to repair merotelic attachments and leading to CIN.

1.6.3. DNA damage and Chromosome Bridges

A condition known as structural chromosomal instability (S-CIN) occurs when the rate of subchromosomal alterations rises and leads to the loss or modification of small chromosomal areas through insertions, deletions,

translocations, and DNA amplification. Translocation and S-CIN can be caused by mutations in the machinery that repairs double-strand DNA damage [189,190]. Non-homologous end joining, an error-prone DNA damage repair mechanism can join two non-specifically damaged ends of DNA to produce S-CIN [190]. Non-specific chromosomal fusions can result from double strand breaks, especially at dysfunctional telomeres [191]. Short nucleotide repeat segments; for example, TTAGGG found in eukaryotic telomeres (DNA-protein complexes) normally stop chromosomal ends from breaking and joining during segregation. Telomere length normally shortens with each replication as a result of the end-replication problem, in which DNA polymerase fails to fully synthesise the 3' end of chromosomes. Fusion at telomeres produces di-centric or ring chromosomes, which, as a result of incorrect microtubule attachment to these di-centric chromosomes, result in the development of chromatin bridges during anaphase. These chromosomal bridges result in fusion and breakage during cytokinesis, a process that is repeated throughout the next mitosis [192]. This breakage-fusion-bridge cycle can result in significant genomic reorganization and is frequently observed across numerous cell generations. Tumour initiation rates may rise as a result of S-CIN abnormalities [193].

1.6.4. Impaired sister chromatid cohesion

During mitosis, the chromatin-associated cohesin protein physically joins sister chromatids [194]. Sister chromatid cohesiveness must be lost in a timely way for the chromosomes to separate during anaphase. The structural maintenance of chromosomes 1A (SMC1A), 3 (SMC3), RAD21 cohesin complex component (RAD21), and either stromal antigen 1 (STAG1) or stromal antigen 2 (STAG2) are the four core subunits that make up the cohesin complex, a multiprotein complex [195-200]. The majority of the cohesin complex, forms a ring-shaped structure around the sister chromatids and is eliminated from chromosome arms during prophase [201,202]. PDS5 cohesin-associated factor (PDS5), polo-like kinase 1 (PLK1), and WAPL cohesin release factor (WAPL) are among the proteins involved in this process. These proteins aid in the cohesin ring's opening, which makes it easier to remove from the chromosomal arms [203]. In human malignancies, CIN could be induced by deficiencies in cohesin or cohesion regulators [204-206]. Human colorectal tumours have been found to contain somatic mutations in genes governing chromatid cohesion; the majority of these malignancies exhibit CIN. RNA interference-induced depletion of these genes caused chromatid cohesion abnormalities and CIN in a human chromosomally stable cell line [204]. Increased separase levels may cause an early loss of sister chromatid cohesion, which in turn may cause chromosomal

mis-segregation by obstructing the normal back-to-back orientation of sister kinetochores [207]. Separase is a cysteine protease that cleaves sister chromatid cohesion to initiate anaphase. This is consistent with the possibility that aberrant securin, a protein that deactivates separase, could cause chromosomal loss in human cells to occur often [206]. In *Drosophila* cells, RNAi-induced cohesin depletion results in a significant level of CIN and aneuploidy [208].

1.7. CIN speeds up therapeutic resistance and evolution.

Experimental findings from several preclinical models demonstrate that CIN gives cancer cells an effective way to respond to different selective pressures [43,209-211]. In karyotypically heterogeneous populations, rare clones frequently only outcompete other cells when they are subjected to selective pressures [43]; tetraploidization, in particular, makes it easier for cells to quickly acquire copy number alterations and mutations in response to stressful situations, which increases cell fitness [209,210]. Because CIN and tetraploidization increase genetic variation and encourage the formation of drug-resistant clones, they also confer multidrug resistance, including resistance to some of the most widely used chemotherapeutic drugs [212,213]. Continuous CIN can potentially get around the fundamental idea underlying the efficacy of molecularly targeted therapies [214], which is oncogene addiction. Clever studies with inducible mouse models have demonstrated that CIN (caused by overexpression of the mitotic spindle assembly checkpoint protein MAD2A) can reliably help to bypass oncogene addiction upon oncogene withdrawal, which in turn facilitates tumor recurrence and persistence, when combined with expression of the KRASG12D or HER2 oncogenes [178,215]. Therefore, CIN shows that the loss of oncogenic driver mutations after a copy-number-altering event would not be as harmful in cancer cells with continuous CIN and provides an escape mechanism for the tumour after treatment with targeted medicines.

1.8. Aneuploidy and metabolic signaling

Aneuploidy and CIN frequently cause a disruption in metabolic balance through the dysregulation of metabolic signaling [29,45,172,216]. Oncogenesis is closely linked to altered metabolic signaling, especially in tumours that exhibit hypoxia, glycolysis, and changed amounts of carcinogenic metabolites [193,217]. In fact, a dysregulated metabolism is a common feature of many malignancies, particularly those that have aneuploidy, which promotes the growth and increased energy requirement of these tumours [218]. Oral squamous cell carcinoma (OSCC), in which genetic abnormalities in both normal and neoplastic cells promote metastasis through dysregulation of metabolic pathways, is an example of the link between aneuploidy and cellular metabolism [219]. Comparably, aneuploidy-

induced hypoxia in Ewing sarcoma (ES) sets off mechanisms that lead to further genomic changes, bone loss, and medication resistance [220]. Lactic acidosis, low glucose, and hypoxia can cause or worsen aneuploidy phenotypes in cancer cells [193,221]. How does this changed cellular metabolism result from aneuploidy? One crucial element is that the ensuing aneuploidy will impact the gene copy number of metabolic enzymes and, as a result, change how these genes are expressed, leading to abnormalities in a variety of metabolic pathways [29,222]. Additionally, it was discovered that metabolic dynamics were reprogrammed by aneuploidy-induced activation of oncogenes like c-Myc or loss of tumour suppressors like p53 [223,224]. Moreover, mitochondrial activity may be compromised by oxidative stress brought on by aneuploidy, which will impact cellular metabolism [225,226]. Lastly, aneuploidy may have an impact on the copy number of genes that are involved in growth factor signaling, which includes crucial regulators of cellular metabolism like insulin and IGF-1 signaling [227]. When taken as a whole, these elements emphasize the significant impact that aneuploidy has on cellular metabolism [218,228].

1.9. One carbon and polyamine metabolism in cancer

All cells receive the energy they need to function through a variety of interrelated cellular pathways that make up the metabolic process [229]. For cancer cells to multiply quickly and unchecked, altered metabolism is essential; hence, cancer cells modify their metabolism to enable increased survival and multiplication. The folate cycle and methionine cycle join forces to create a bicyclic metabolic pathway that circulates carbon atoms, often known as the C1 metabolism [230]. One-carbon moieties are produced by the global biochemical process, which is necessary for the biosynthesis of compounds such as purine, dTMP, methionine, methylmethionine, and formylmethionine in mitochondria [231]. The trans-sulfuration pathway, the methionine cycle, and the folate cycle are three crucial reactions in one-carbon metabolism that provide methyl groups for the creation of DNA, amino acids, creatine, polyamines, and phospholipids, which are essential for cellular activity [232]. Nucleotide metabolism and epigenetic regulation of DNA and histones, whose aberrant expression is a distinguishing feature of tumour cells, both depend on C1 metabolism (methylation) to maintain genomic integrity. The study and regulation of C1 metabolism impacts precision medicine's basis for disease prevention, the discovery of biomarkers, the diagnosis, and treatment of different illnesses, including cancer [230,233]. The relevance of C1 metabolism to aneuploidy was not previously known but came out of my work.

1.10. How aneuploidy arises in neurons

Brains contain aneuploid cells; the most important question is where do they originate from? Generally, aneuploidy occurs when something goes wrong with replication of DNA or in the mitosis process. Aneuploid cells may be produced at an early stage of development or later during usual or unusual cell division. Generally, growing brains show an increased rate of aneuploid cells, so defective clearance of these cells could explain their existence in the adult brain [234,235]. Since neurons have historically believed to be post-mitotic [236], how severe mosaic aneuploidy can occur in neurodegenerative or neurodevelopmental disorders has been unclear. Three mechanisms that may produce the neuronal aneuploidy found at autopsy in patients with Alzheimer's disease (AD), FTLD-MAPT and other neurodegenerative and neurodevelopmental disorders have been described throughout the adult brain.

Firstly, it has become clear that adult neurogenesis (cell division that produces new neurons) is more frequent than commonly assumed, and that the neurogenesis potential persists into old age, even though it is not usually used [237-240]. There is good evidence that neurogenesis can arise in many areas of the brain during life. In general, genetic, and environmental stressors can produce and accumulate aneuploidy in the division or regeneration of cell populations at any time in life [7,241]. In addition, evidence from several studies indicate that neurogenesis in many brain regions can be induced in adult mice and rats in response to brain injury and attempted self-repair by the brain [242-244].

Aβ has also been shown to trigger the expression of mitotic proteins and the reentry of the cell cycle into mature ne urons in culture [245-247]. Amyloid-beta (Aβ) is associated with Alzheimer's disease because the accumulation of Aβ plaques in the brain disrupts cell function, leading to inflammation, neuronal damage, and the progressive cognitive decline characteristic of the disease. New aneuploid neurons may be formed as a result of neuronal damage, mitotic defects in AD, FTLD-MAPT and other neurodevelopmental and neurodegenerative disorders. The other possible mechanism for generating neuronal aneuploidy in neurodegenerative disease is re-entry of the cell cycle. The main evidence for this hypothesis is that neurons of AD brain showed cyclin B1, cyclin D1, cdc2, and Ki67 phospho-proteins expression which are normally identified during mitosis [248-252].

Secondly, striatal astrocytes may transdifferentiate into new neurons able to form active neuronal circuits with pre-existing neurons following ischemic brain injury, which is the basis of an alternative potential process of neuronal

aneuploidy formation [253,254]. This result suggests that in AD and FTLD-MAPT brains, some of the aneuploid neurons may originate from glia.

Thirdly, defective clearance of aneuploid cells could account for their presence in the adult brain, since significant frequencies of aneuploid cells have been found, particularly in the developing brain [234]. Many more cells are generated throughout brain development than become adult brain tissue, indicating that specific cell types are strongly selected for [255]. The significantly lower rate of aneuploidy recorded in the adult brain compared to the developing brain may be explained by the possibility that this process involves negative selection for aneuploid cells. There is a chance that the adult brain contains aneuploid cells if the selection process fails to select diploid cells [256,257]. This has been observed in vitro: increasing levels of aneuploidy and micronuclei are associated with the differentiation of pluripotent stem cells into neural progenitor cells by retinoic acid treatment [258]. Increased total exposure to environmental stressors is related to aging, which can enhance missegregation of chromosomes and neuronal aneuploidy [241,259]. Age may be triggering all other mechanisms mentioned to form neuronal aneuploidy because evidence has shown that neuronal and non-neuronal aneuploidy increased with age [260-264]. Neurodegenerative diseases such as Alzheimer's and FTLD-MAPT are associated with aneuploidy in neurones, which can result from a variety of processes, including errors during neurogenesis, cell cycle re-entry, and improper clearance of aneuploid cells. Neural aneuploidy is further aggravated by ageing and environmental stresses, and it plays a major role in the development of these disorders.

1.11. Aneuploidy in neurodegeneration

Understanding the role of aneuploidy in the progression of neurodegeneration is important for successful therapeutic interventions. Owing to the *in vivo* inaccessibility of human brain tissue, several researchers used peripheral cells such as lymphocytes and fibroblasts to examine the association between genomic damage and neurodegenerative diseases such as AD. Several studies have been published with a link between AD and enhanced peripheral aneuploidy [265-269]. Since then, a connection between aneuploidy and AD has been confirmed by various studies that show a role for aneuploidy, particularly hyperploidy, early in family and sporadic AD [270-272]. Alzheimer's brain disease has been shown to have elevated levels of spontaneous aneuploidy. [271,273-275]. Besides, Alzheimer's disease genes are involved in molecular mechanisms, causing changes in chromosome missegregation and aneuploidy [276]. Additionally, it has been shown that submicroscopic CIN-generating structural rearrangements of

the APP gene (21q21.3) participate in neurodegenerative pathways to Alzheimer's disease [277]. Deficiencies in DNA repair [278] and DNA replication stress [279] were identified as potential mechanisms for neurodegeneration. Two to threefold increase in variability in DNA content was seen in patients with AD relative to elderly controls [280]. Other neurodegenerative diseases, including Lewy body disease, have also found alterations in DNA [281]. Moreover, in many experimental systems, aneuploidy has been shown to promote cell death like neurodegeneration [7,282,283].

1.12. Consequences of aneuploidy in the brain

Aneuploidy in the brain may theoretically lead to functional variability, for instance learning and behaviour; the most obvious result would be a cognitive deterioration and disease predisposition. Accordingly, the most common reason of dementia among aging population has involved aneuploidy: Alzheimer's disease. Lymphocytes and splenocytes isolated from AD patients show mitosis and chromosomal segregation defects [284,285], demonstrating AD patients' vulnerability to aneuploidy and their predisposition to produce aneuploid cells. Aneuploidy rates for Chromosome 21 (HSA 21) in the hippocampus, cortex and cerebellum of brain was studied by Iourov and coworkers, relating AD patients with a group of unaffected aged-matched controls [63,286]. They noticed a minimal percentage of aneuploidy (0.7 per cent) for each area analysed in the controls, proposing that aneuploidy affects all brain areas in normal physiological conditions similarly. However, in affected individuals, the frequency and distribution of aneuploidy showed 29.3% in hippocampal cells, 20.7% in cerebral cortex and 1.7% in cerebellum and they suggested that the increased levels of aneuploidy in AD were due to aberrant adult neurogenesis resulting from mitotic non-disjunction in neurons that have re-entered the cell cycle. Recent study shows that neurogenesis in the adult brain is an indicator for initial identification of AD [287]. This is based on evidence that re-entering into an aberrant cell cycle may result in aneuploidy.

1.13. Metabolic disturbances in diseases with neurological involvement

Neurodegenerative diseases are characterised by deterioration of neurons and resulting nervous system dysfunction. These diseases can also be known as disorders of protein aggregation or catabolism [288]. In the neurodegenerative diseases, mitochondrial changes also play a role, triggered by either genetic changes or exogenous toxins influence on mitochondrial activity [289]. In this scenario the mutations of mitochondrial DNA affect enzymatic defects of complexes of the respiratory chain that make cells' energy; these modifications appear to predominantly affect high-

energy systems, including brain, skeletal muscle, and heart [290,291]. Disturbance of key energy metabolism steps has been noted previously in neurodegenerative diseases [292-296]. However, it should be emphasised that the mitochondrial involvement associated with the pathophysiology of neurodegenerative disorders is not restricted to defects in the energy metabolism and DNA alterations, but also redox stress [297-299] and changes in the mitochondrial form and distribution [300-302] as well as metabolic contact with other organelles [303]. Numerous genetic disorders also involve neurodegeneration and mitochondrial dysfunction, including inborn metabolism errors for instance glutaric aciduria type I, phenylketonuria, methylmalonic acidemia, and Type C Niemann-Pick disease.

1.14. The effect of age on metabolic alterations and neurodegenerative diseases

Oxidation of cellular elements by free radicals, catalysed by oxidative enzymes and traces of metal ions is the main cause of age-related neurodegeneration according to the Free Radical Theory of Aging [304]. Bioenergetic modifications, primarily alterations related to mitochondria, are a current focus for research that may better explain the ageing progression. Mitochondria are the organelles accountable for producing ATP, regulating Ca^{2+} and processing and detoxifying reactive oxygen species (ROS). These functions are affected by the potential of the mitochondrial membrane ($\Delta\psi m$), while cytochrome c from mitochondria activates several apoptotic signals [305]. Based on putative ROS generation by mitochondria, the mitochondrial ageing theory was established. Mitochondria's net ROS development may be the main cause of oxidative damage that accumulates with ageing, impairing several mitochondrial functions. With aging, mitochondrial membrane potential, respiratory control ratio, cellular O_2 uptake [306,307] and mitochondrial complex I, III, and IV enzyme action [308] are reduced. Calorie restriction has been shown to be a successful method to postpone the age-related cognitive decline and mammalian diseases [309]. The respiratory capacity of the cerebral mitochondria is increased by calorie restriction [310]. In addition, multiple studies have documented a positive association between physical activity and cognitive function and activity can decrease the risk of elderly-linked neurodegenerative diseases [311,312]. It has been shown that physical activity increases the mitochondrial number and dendritic spine synapses in the hippocampus [313], stimulates neurogenesis and enhances long-term potential in the hippocampus [314-318], leading to improved learning and memory in ageing [319]. It is not yet known how the metabolic consequences of aneuploidy interact with these disease processes.

1.15. *Drosophila* as model organism to investigate human genetic diseases.

Due to its considerable genetic homology to mammals and the abundance of available genetic resources, the fruit fly, *Drosophila melanogaster*, has been identified as a key model organism for human disorders. For the study of molecular and cellular processes underlying human disease, modelling human brain disorders in *Drosophila melanogaster* provides many advantages. In *Drosophila*, functional research, including high-throughput pharmacological screens as well as behavioural assays, of human disease genes has become available. The convenient features of *Drosophila* as a model organism are a short generation time, their small physical size (2–3 mm), cost effectiveness to culture in laboratory, many offspring, multiple genetic techniques, a well-known anatomical situation, and a wide range of readily-available mutant lines. There are lines available with temporal and tissue specific inducible gene promoters for driving gene expression [320-322]. *Drosophila* genome sequencing revealed about 13,600 genes [323]. The elucidation of the entire *Drosophila* genome sequenced has been helpful to study human diseases [323] because studying and manipulating a single gene is now relatively simple. 60% of the *Drosophila* genome is homologous to humans and 75% of genes that contribute to human diseases are found in flies [324] which show that *Drosophila* can be a good model to investigate the role of genes involved in human diseases.

1.16. The UAS-Gal4 system in Drosophila for RNA interference (RNAi)

Double-stranded RNA (dsRNA) is used in RNA interference (RNAi), a powerful technique for gene silencing, to target particular mRNA molecules for breakdown. This effectively "silences" the target gene's expression. The UAS-*GAL4* system is frequently utilized in *Drosophila melanogaster* to drive the expression of genes in a tissue-specific or temporal manner. A particular promoter is used to create flies that produce the *GAL4* transcription factor. This promoter may be inducible, unique to a certain developmental stage, or both. For instance, *GAL4* expression in neural tissues is made possible by using a promoter specific to neurons (such as *elav*). An RNA interference (RNAi) construct intended to silence a particular gene is generated with a UAS sequence upstream of the gene of interest. *GAL4* binds to the UAS sequence and promotes the expression of the RNAi construct when it is crossed with a *GAL4* driver line. Following the expression of the UAS-RNAi transgene, the dsRNA is broken down into small interfering RNAs (siRNAs) by the enzyme Dicer. These siRNAs are integrated into the RNA-induced silencing complex (RISC), which uses sequence complementarity to direct the complex to the target mRNA. Reduced gene expression and results from the target mRNA degradation by the RISC complex [325,326].

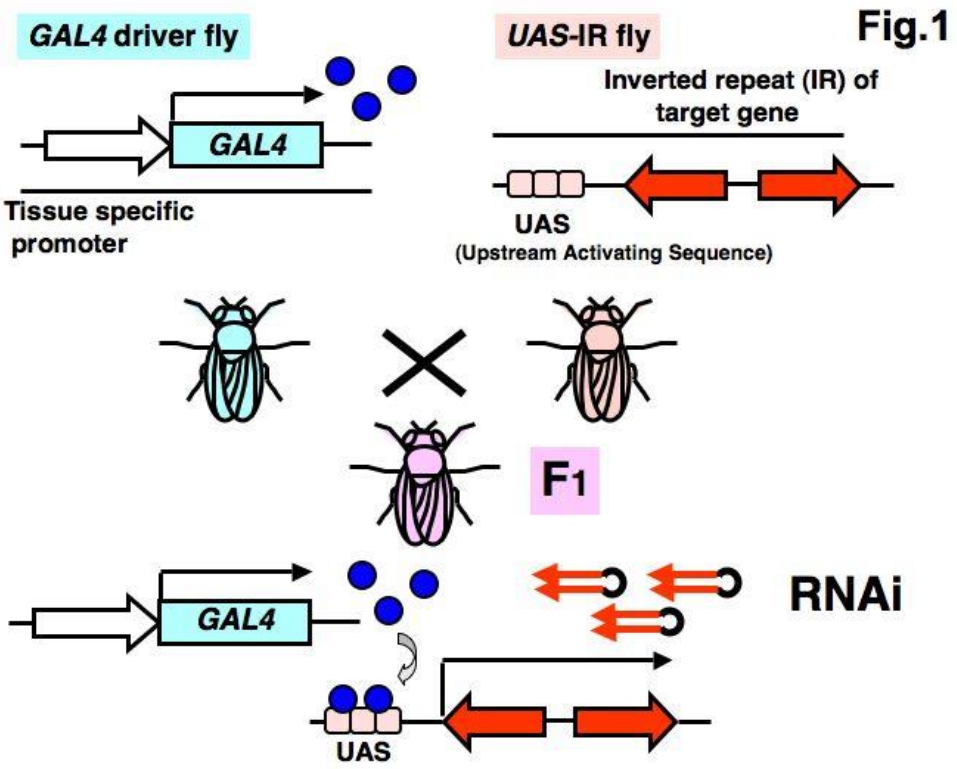

Figure 1-2. UAS-Gal4 system for RNAi in our laboratory. This figure is re-printed from "Heritable and inducible RNAi knockdown system in *Drosophila*" written by Nishihara Shoko (Ver 1), 2007.

1.17. Established aneuploidy models.

A feature of aneuploidy responses is that they do not depend on the gain or loss of a specific locus. We have established induced instability models in *Drosophila* that allow us to generate random segregation errors in a proliferating epithelium [208,327]. Depletion of *Mad2* gives karyotype defects in 25% of cells in the targeted tissue without compromising viability (Figure 4). Alternatively, we can deplete cohesin (*Rad21*) to generate a high rate of aneuploidy with extensive cell death [208]. These well-established models allow us to generate a diverse population of aneuploid cells in a common genetic background.

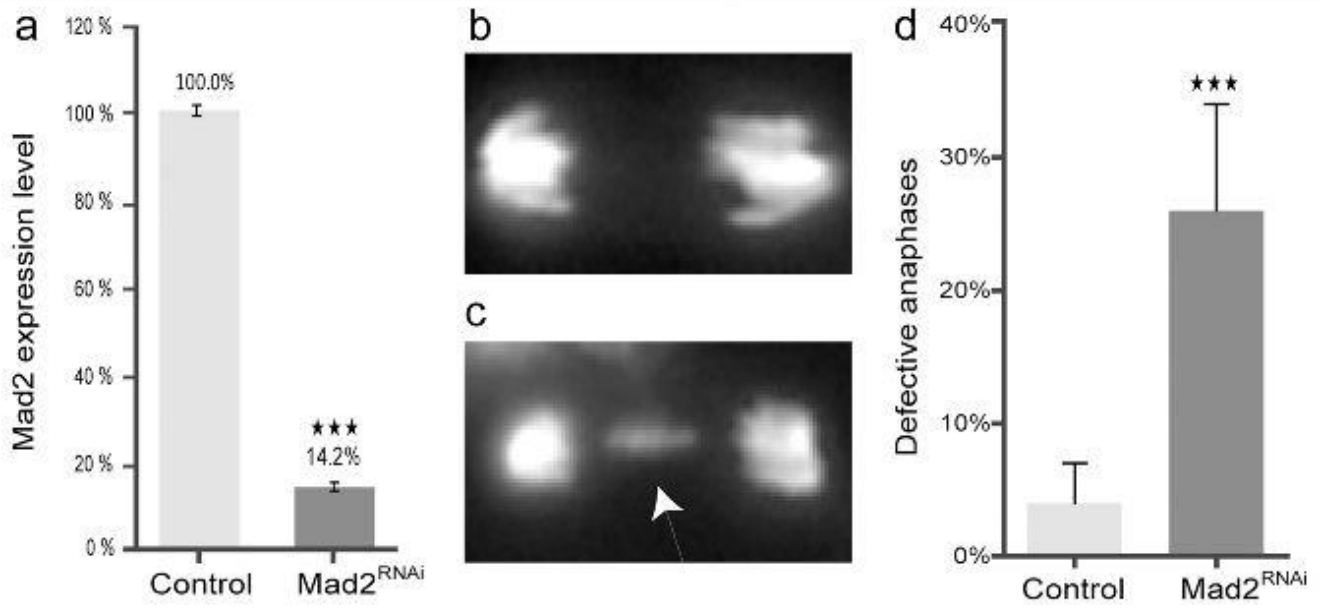

Figure 1-3.- (a) Reverse transcriptase-qPCR demonstrates that the extensive expression of UAS-mad2 RNAi led to an approximately 85% reduction in the level of mad2 expression (black bar), which is significantly lower than the level of mad2 in the UAS-LacZ RNAi control (grey bar). The error bars show SD. P-values are determined using the two-tailed student's t-test: $p<0.001$ = ★★★. (b–c) Hoechst 33342-stained larval brain cells from the third instar, used to identify DNA. (b) Typical segregation in an anaphase of the wild type. (c) A lagging chromosome due to defective anaphase in an induced-CIN brain cell (da>mad2) (arrowed). (d) The percentage of defective anaphases (lagging chromosomes or bridges), seen in brain squashes with Mad2 knockdown (black bar) and wild type controls (grey bar). 95% CIs are shown by error bars. The two-tailed Fisher's exact test yields P-values when $p<0.001$ = ★★★★. This data reprinted from "Zeeshan Shaukat, A screen for selective killing of cells with chromosomal instability induced by a spindle checkpoint defect, PLoS One. 2012;7(10), Fig-1".

1.18. Aneuploidy and *KCNT1* mutants

Our laboratory and others have shown that a stereotypical set of stresses is induced in aneuploid cells which is dependent on the magnitude of aneuploidy, but independent of the specific chromosomal aberration [44,45,328-330]. Oxidative stress occurs in response to aneuploidy in all eukaryotes that have been tested, including yeast, plants, *Drosophila*, mouse, and humans. Our laboratory has shown an increase in repair mechanisms [331], autophagy [332] and antioxidant levels [330] in response to aneuploidy which are required to tolerate the deleterious effects of aneuploidy shown in Figure 1-4. Our laboratory has revealed that the induction of aneuploidy increases cells metabolic rate, making them vulnerable to oxidative stress so they show DNA damage and apoptosis in response to metabolic challenges that do not damage normal cells [330]. Moreover, aneuploid cells are metabolically different, with overactive mitochondria giving elevated ROS levels [330], which causes damage to macromolecules, resulting in protein folding defects and ER stress [333]. It is observed that increased chaperone levels and ER stress markers in aneuploid cells are dependent on ROS levels.

Aneuploidy has also been linked to an increased incidence of neurodegenerative diseases [334] and with our aging population these diseases are becoming more common. Aneuploidy is well tolerated in the young brain, but aneuploid neural cells are sensitive to age related metabolic disorders and senescence that impact motor function and lifespan [335] as well as mental health [336]. This is accentuated in diseases such as epilepsy, in which recurrent seizures cause additional oxidative stress [337]. About a quarter of children with epilepsy continue to have seizures despite medication, resulting in ongoing mitochondrial disruption, further seizures and a progressive cognitive loss in severe forms such as Dravet syndrome or *KCNT1* encephalopathy [338]. Further advances in the treatment of associated serious seizure disorders is the main purpose of using *KCNT1* mutants to generate a greater understanding of the mechanisms underlying the *KCNT1*-related disease.

The *KCNT1* subunit gene has recently emerged as a cause of a variety of multiple epileptic conditions. Human *KCNT1,* was first described molecularly in 2000 [339]. A subunit of the sodium-gated potassium channel, also known as Sequence Like a Calcium Activated K+ Channel (SLACK), KCa4.1 or Slo2, is encoded by *KCNT1* `[340]. The gene is strongly expressed in the nervous system, and after

repeated firing of the potential for action, the encoded ion channel is thought to modulate hyperpolarization [341,342]. Studies revealed that the *KCNT1* channel is expressed in neurons, interneurons of the cortex and also in the hippocampus of the CA3 region [343,344]. K+ currents in neurons and interneurons are raised by *KCNT1* mutations resulting in disparity between neuronal excitation and inhibition [345,346]. Two phenotypes are most associated with *KCNT1*-related epilepsy: childhood epilepsy with migrating focal seizures (EIMFS) and autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). Tests for KCNT1 mutations in patients with Malignant Migrating Focal Seizures of Infancy (MMFSI) showed 39% positive [346-351]. Our ultimate goal is to investigate how metabolic stress and oxidative stress imposed by aneuploidy is linked to the severity of neurodegeneration.

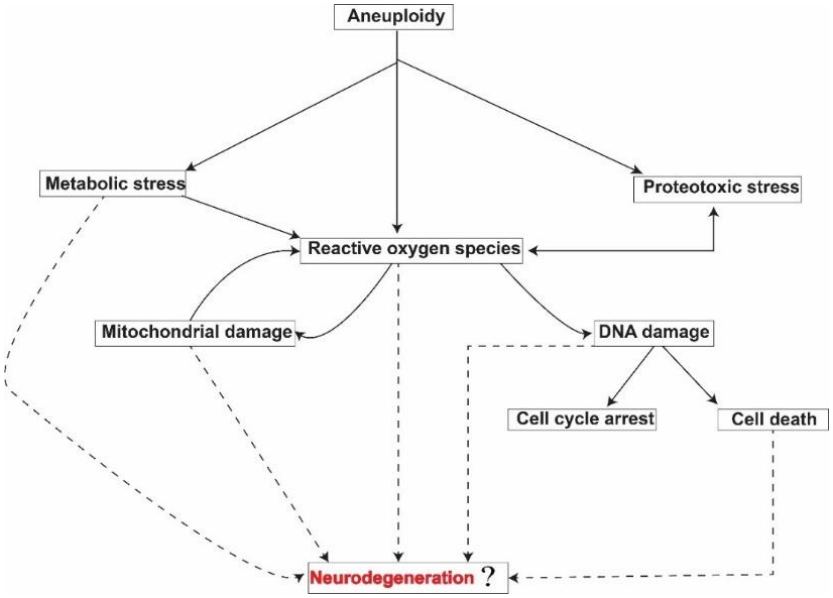

Figure 1-4. Aneuploidy responses may impact the progression of neurodegeneration. The black arrows indicate prior work in our laboratory. Dashed lines indicate areas that we are currently investigating.

1.19. Summary

Aneuploidy is associated with a shorter lifespan [11] and a higher risk of neurological diseases [352]. It also increases with age. Surprisingly little is known about aneuploidy-specific cell responses, despite this major influence on our ageing population. According to current models, stress responses are triggered by aneuploidy [29,45]. An up-regulation of stress response genes occurs in response to

aneuploidy in most organisms, including yeast, plants, *Drosophila,* mouse and humans. This stress response is not a gene-specific, or even chromosome-specific effect, but rather a global response to aneuploidy [328]. The stress is typically proportional to the size of the aneuploid region and does not depend on any specific genomic location being affected. However, the mechanism producing this aneuploidy response is not known.

There is now abundant evidence that neurodegenerative diseases are exacerbated by reactive oxygen species [353,354]. Because it is problematic to replace defective neurons, they must be able to last a lifetime. Metabolic stresses like reactive oxygen species (ROS) lead to accumulation of damage to DNA, proteins and membranes, inflammatory responses and eventually to loss of cells (i.e. neurodegeneration). Reactive oxygen species are generally increased in aneuploidy and exacerbate neurodegenerative diseases; but the effect of metabolic aneuploidy responses on neurodegeneration has not been studied.

1.20.Hypotheses

In *Drosophila,* aneuploidy causes changes in expression of genes in metabolic pathways. By depleting genes in these altered pathways we may be able to preferentially kill aneuploid cells and identify promising therapies for advanced stage cancers.

Aneuploidy affects progression of neurodegenerative phenotypes and will exacerbate the severity of *KCNT-1* epileptic neurological disease. The fundamental mechanisms of this disease can be studied through *Drosophila* modelling.

Finally, Mutant human *KCNT1* transgene-expressing *Drosophila* models faithfully reproduce the main phenotypic characteristics of *KCNT1*-epilepsy, making them a dependable and efficient whole-animal platform for evaluating possible treatment agents. These models will pave the way for the development of targeted therapies for *KCNT1*-related epileptic illnesses by making it possible to identify medications that can modify the abnormal neuronal activity brought on by *KCNT1* mutations.

1.21.Aims of this thesis

The main objective of this study is an investigation of the role of metabolic pathways in response to aneuploidy and the identification of potential interventions in aneuploid cells that amplify small metabolic changes to the point where they affect the survival of aneuploid cells.

Aim 1: To find new metabolic pathways altered in response to aneuploidy in *Drosophila.*

Aim 2: To investigate the mechanism of sensitivity of aneuploid cells to depletion of metabolic candidates of the one-carbon and polyamine pathways in *Drosophila.*

Aim 3: To find the role of aneuploidy in the development of neurological abnormalities in *Drosophila.* The ultimate target, to generate a *Drosophila* model to investigate the involvement of aneuploidy in the severity of *KCNT-1* epileptic neurological disease.

Aim 4: To screen for potential effective treatments for human *KCNT-1* epilepsy by using *Drosophila* models.

Aims 1, 2, and **3** is described in **Chapter 3 and 4**. **Aim 4** is described in **Chapter 5**.

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CHAPTER 2: ONE-CARBON AND POLYAMINE METABOLISM AS CANCER THERAPY TARGETS

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2.1. Abstract

Cancer metabolic reprogramming is essential for maintaining cancer cell survival and rapid replication. A common target of this metabolic reprogramming is one-carbon metabolism which is notable for its function in DNA synthesis, protein and DNA methylation, and antioxidant production. Polyamines are a key output of one-carbon metabolism with widespread effects on gene expression and signaling. As a result of these functions, one-carbon and polyamine metabolism have recently drawn a lot of interest for their part in cancer malignancy. Therapeutic inhibitors that target one-carbon and polyamine metabolism have thus been trialed as anticancer medications. The significance and future possibilities of one-carbon and polyamine metabolism as a target in cancer therapy are discussed in this review.

2.2. Introduction

For cancer cells to multiply quickly and unchecked, altered metabolism is essential, and a range of modifications to their metabolism are known to enable increased survival and multiplication. A key element in this is the generation of sufficient nucleotides and lipids, both of which are dependent on the availability of methyl groups from the one carbon metabolic pathways. These methyl groups are necessary for the biosynthesis of compounds such as nucleic acids, amino acids, and the major membrane lipid phosphatidyl choline [1,2], among many others. The methionine and the folate cycles (Figure 2-1) are crucial interrelated pathways in one-carbon metabolism that provide methyl groups for the creation of DNA, amino acids, creatine, polyamines, and phospholipids [3]. Nucleotide metabolism and epigenetic regulation of DNA and histones, whose aberrant expression is a distinguishing feature of tumor cells, both depend on one-carbon metabolism to maintain genomic integrity. Studying one-carbon metabolism offers the prospect of precision medicine intervention for disease prevention, the discovery of biomarkers, and the diagnosis and treatment of different illnesses, particularly cancer [1,4].

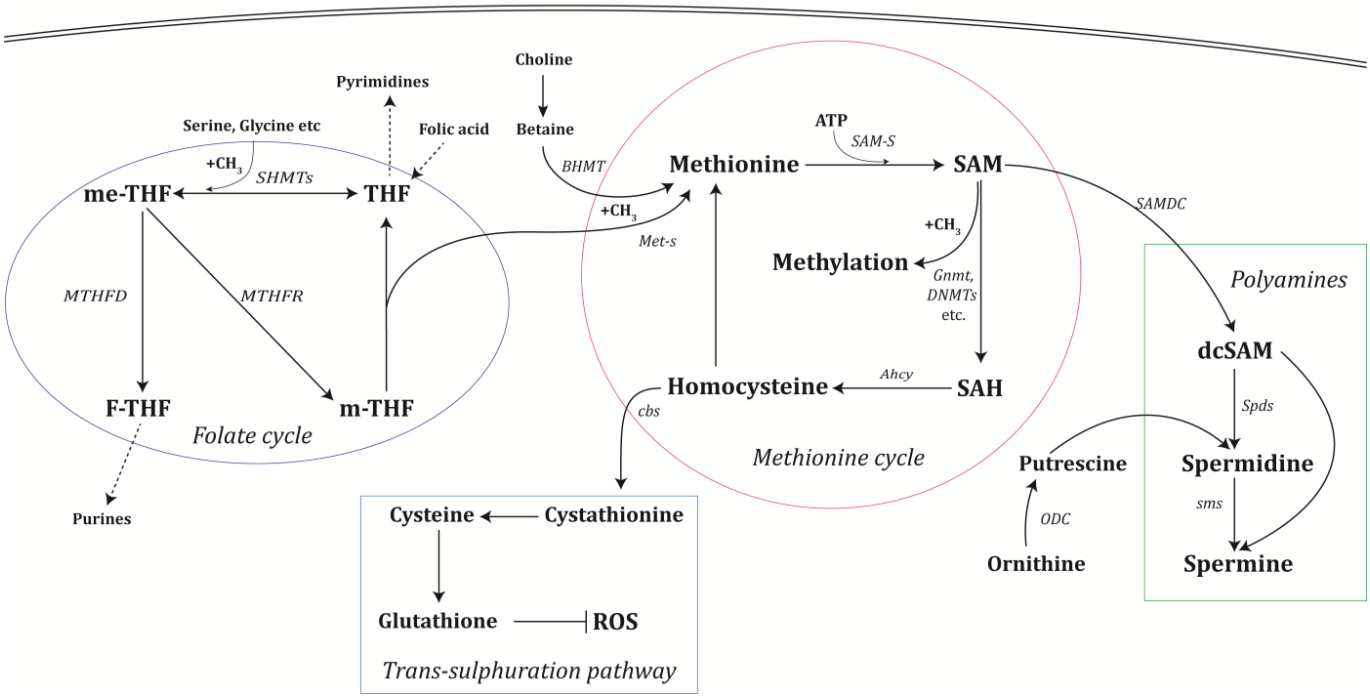

Figure 2-1. One-carbon metabolism includes the methionine cycle, which is linked to the folate cycle, polyamine synthesis, and the trans-sulphuration pathway. Enzymes catalyzing significant reactions are shown in italics. Metabolite abbreviations are: SAM: S-adenosyl methionine; SAH: s-adenosyl homocysteine; dcSAM: decarboxy-s-adenosyl methionine; ROS: reactive oxygen species; THF: tetrahydrofolate; m-THF: methyl-THF; me-THF: methylene-THF; F-THF: formyl-THF.

2.3. One-Carbon and Polyamine Metabolism

2.3.1. The Methionine Cycle

The first phase of the methionine cycle is the synthesis of S-adenosylmethionine (SAM) from methionine using the enzyme methionine adenosyl transferase (MAT) [5,6]. While MAT II (a dimer) is expressed in the majority of other cell types and is encoded by MAT2A, MAT I (a tetramer) and MAT III (a dimer) are often expressed in the liver, where substantial SAM synthesis occurs [7,8]. Then, SAM is used by numerous methyl transferases to donate a methyl group to their diverse targets. This loss of a methyl group changes SAM into S-adenosylhomocysteine (SAH). To complete the methionine cycle, SAH is hydrolyzed to homocysteine by the enzyme SAH hydrolase (AHCY or SAHH) [5,6]. Homocysteine can then be re-methylated to methionine by the enzymes methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase; MTR or MS) betaine-homocysteine methyltransferase (BHMT). Alternatively, cystathionine-β-synthase (CBS) can divert homocysteine into the transsulfuration route to become cystathionine, which is subsequently changed into cysteine by cystathionase (CTH) for use in the synthesis of glutathione and the preservation of redox equilibrium [5,9]. To summarize, SAM is primarily used to donate a methyl group, then is either recycled to methionine by receiving a methyl group from the folate cycle or is converted to cysteine/glutathione. Methyl groups, a single carbon plus three hydrogens, are usually stable and unreactive, so the use of SAM and methyl transferases is essential for a wide range of biosyntheses and modifications that regulate gene expression, epigenetics, detoxification, and more [10]. To maintain metabolite levels, the methionine and folate cycles are closely connected [11]. SAM inhibits the enzymes methylenetetrahydrofolate reductase (MTHFR) and (betaine-homocysteine methyltransferase) BHMT to limit the conversion of homocysteine to methionine, allowing homocysteine to be diverted for transsulfuration when SAM is abundant, a sign of high methionine levels [8,11]. Additionally, SAM stimulates CBS, which directs homocysteine into transsulfuration [11,12]. Low methionine levels cause SAM levels to drop, freeing inhibition of MTHFR and BHMT and restraining activation of CBS to keep the methionine cycle in flux and regenerate SAM. 5-methyltetrahydrofolate (5-mTHF) builds up as a result of low methionine synthesis, and it inhibits glycine N-methyltransferase (GNMT), which would otherwise be a significant sink for SAM [11]. These feedback inhibitions act to maintain homeostasis in SAM levels.

2.3.2. The Folate Cycle

The water-soluble B vitamin folic acid is obtained from food and transformed by the body into tetrahydrofolate (THF). Through folate-mediated one-carbon metabolism (FOCM), THF can provide the necessary nucleotides for replication and one-carbon groups for DNA methylation, which are important for epigenetic gene regulation [13]. Serine is a key methyl donor in the folate cycle, though there are many other ways that cells can obtain one-carbon groups, including choline, betaine, glycine, histidine, and sarcosine [14,15]. THF can either be used for nucleotide synthesis or regenerate methionine from homocysteine in the one-carbon cycle (Figure 1). So, it can be seen that FOCM regulates the production of S-adenosylmethionine (SAM), nucleotides, certain amino acids, glutathione, and other cellular processes critical for the proliferation of cancer cells [15]. FOCM distributes carbon atoms among the various acceptor molecules required for biosynthesis in addition to controlling the nutritional status of cells through their redox and epigenetic states.

2.3.3. Polyamine Synthesis

The other major metabolic pathway that relies on SAM is the synthesis of polyamines. Spermidine, putrescine, and spermine are polycationic alkylamines that interact with negatively charged macromolecules [16] because they have protonated amino groups at physiological pH levels. They are involved in a number of cellular processes, such as chromatin organization, cellular proliferation, gene regulation and proliferation, immune system function, and cell death [17-20]. All cells produce polyamines in their cytoplasm, and their synthesis requires SAM plus ornithine, an amino acid from the urea cycle [21]. SAM is decarboxylated by SAM decarboxylase (SAMDC) to generate s-adenosyl methioninamine or dcSAM, which is a key aminopropyl donor used to form spermidine (Figure 2-1). The other part of spermidine comes from ornithine via ornithine decarboxylase (ODC), which generates putrescine. Putrescine plus dcSAM is used by spermidine synthase to generate spermidine. A further aminopropyl group from dcSAM can be added to spermidine by spermine synthase to generate spermine, the final product in this pathway. We do not address the interesting topic of polyamine degradation in this review; it is covered in detail elsewhere [16,17]. To this point, we have considered the three main outputs of one-carbon metabolism: methyl groups, cysteine/glutathione, and polyamines. We now move to examine how these pathways impact carcinogenesis.

2.4. The Implications of One-Carbon and Polyamine Metabolism for Cancer

2.4.1. Folate Metabolism and Cancer

Due to its range of roles in protein and DNA synthesis, methylation processes, and redox homeostasis, folate metabolism can contribute to oncogenesis. In tumor treatment, drugs that specifically target folate metabolism have been employed frequently, particularly against dihydrofolate reductase (DHFR) [22]. These inhibitors stop the growth of cancer by preventing the production of nucleic acids, which are needed for DNA replication and cell proliferation. DHFR inhibitors block the production of tetrahydrofolate, which thus inhibits purine and thymidylic acid synthesis [22]. However, antifolate medications have an adverse effect on normal cells when used to treat cancer because one-carbon metabolism is also required for healthy cells, particularly in the immune system. Nonetheless, numerous cancers have been treated with DHFR inhibitors, such as methotrexate, which was introduced in 1947 but is still very widely prescribed. Like other chemotherapeutic treatments, these drugs may fail because cells develop resistance by, for instance, impairing drug absorption, decreasing drug retention inside the cell, and decreasing drug affinity [23]. There is a need to develop further therapies that specifically target folate metabolism. In a review of the mRNA profiles of 1981 tumors, MTHFD2 and SHMT2 were shown to be among the top five genes with the highest levels of expression, demonstrating the carcinogenic influence of mitochondrial folate metabolism [24,25]. Similar studies on the mitochondrial folate metabolism enzymes revealed a link between cancer and aberrant SHMT2 and MTHFD2 expression [26,27]. Aberrant SHMT2 and MTHFD2 expression might impair DNA synthesis and damage redox balance, which is important for cancer cell survival [28]. Other folate metabolism enzymes, such as SHMT1 and MTHFD1L, have also reportedly been linked to cancer. Disrupting SHMT1 interferes with the incorporation of dUMP into DNA, causing DNA double-strand stability to be disturbed [29]. Additionally, ovarian cancer is prevented from spreading and growing by SHMT1 knockdown [29]. Lung cancer cells are also affected by SHMT1 knockdown [30]. According to a recent study, MTHFD1L knockdown caused tongue squamous cell carcinoma cells to die under redox stress via lowering the concentration of NADPH [31]. These results suggest that folate metabolism is a desirable target for the therapy of cancer if the problems of toxicity and resistance can be overcome.

2.4.2. Serine Metabolism and Cancer

Changes in serine metabolism may have significant consequences that may lead to the development of cancer as well as other illnesses [32,33]. Serine can be absorbed by the cell or produced by the serine synthesis pathway from

glycolytic intermediates. It has long been recognized that serine, whether from diet or generated endogenously, is linked to cancer, and actively promotes its growth [34,35]. Serine can also be produced by breaking down cell proteins, such as through autophagy, and by converting glycine [36]. The process of serine synthesis (SSP) is one of numerous glycolysis side branches that allows carbons obtained from glucose (or pyruvate under gluconeogenic circumstances) to be redirected to the production of serine and is upregulated in many cancers [37]. Glucose is the primary source of carbons for de novo serine synthesis in people and rats that are well-fed, but under starving conditions, gluconeogenesis can contribute up to 70% of the total serine produced [38]. Serine is necessary for the creation of phospholipids such as sphingolipids and phosphatidylserine, as well as other amino acids like cysteine and glycine. Serine is a key methyl donor, though there are many other ways that cells can obtain one-carbon groups, including choline, betaine, glycine, histidine, sarcosine, and the formate that is produced when tryptophan is broken down [14,15]. Studies in yeast and mammalian cells revealed that serine catabolized in the mitochondria is the source of the majority of the cytosolic one-carbon units [29,39,40], and blocking one-carbon metabolism in both the mitochondria and cytoplasm precludes cell growth [37]. Serine's role in generating methylene-THF makes it a key contributor to avoiding the toxic consequences of homocysteine build-up. Homocysteine is the link between the transsulfuration pathway and the methionine cycle, and the building blocks for the synthesis of cysteine are homocysteine and serine. Serine depletion results in lower amounts of glutathione [41] because glycine and cysteine are by-products of serine degradation, whereas activation of serine synthesis enables glucose-derived carbon to be channeled towards glutathione synthesis for antioxidant defense [32,42]. This has implications for tumor oxidative stress tolerance that have not been fully examined.

2.4.3. SAM-S Metabolism in Cancer

Methionine, which makes up half of the body's daily requirement for amino acids, is the primary amino acid used in the liver to produce SAM [43,44]. SAM is produced by MAT (SAM synthase) from methionine in an ATP-dependent mechanism [43]. The adenosyl moiety of ATP is combined with methionine during this process to change it into a high energy reagent that can carry a sulphonium ion. SAM can then transfer a methyl group to a variety of substrates, including proteins, DNA, RNA, and lipids [45]. The cellular level of SAM can be affected by impaired dietary intake, absorption, transport, metabolism, or enzymatic processing of methionine [6,46-48]. For instance, dietary methionine limitation lowers SAM levels and increases the longevity of certain species [49-51]. Because cancer is frequently

characterized by abnormal methylation states and methionine or SAM dependency, SAM has been explored as a therapeutic target in the treatment of cancer [52,53]. For example, rats have been used in tests to determine how SAM treatment affected the growth of neoplastic liver lesions. The percentage of the liver that was occupied by GST-P-positive lesions significantly decreased when SAM was administered to rats during the clonal expansion of initiated cells (promotion), primarily as a result of a reduction in the size of the lesions [54-60]. The number and size of liver nodules decreased after receiving the same SAM doses for 11 weeks [54,55]. A consistent decrease in incidence and multiplicity of neoplastic nodules could be observed when SAM medication was continued for up to six months [61]. On a cellular level, SAM's chemopreventive action is linked to an increase in remodeling and a dose-related reduction in DNA synthesis in preneoplastic and neoplastic lesions [54,58,59]. Additionally, rats given SAM showed an increase in apoptosis in neoplastic nodules and hepatocellular carcinoma [55,58]. SAM therapy decreased carcinogenesis and metastasis in vivo while increasing apoptosis and decreasing the proliferation and invasiveness of breast cancer cells in vitro [62]. SAM treatment has been shown to be effective in inhibiting the proliferative and invasive potential of many cancer cell lines [63,64]. SAM selectively inhibits the proliferation and invasiveness of liver cancer cells by changing the transcriptome and methylome [65]. Although SAM has positive impacts on the treatment of cancer, more research is needed to establish SAM as a cancer therapy, as in many cases, the specific metabolic changes responsible for the observed anti-cancer effects are unclear.

2.4.4. Methionine Dependency in Cancer

Methionine metabolism and cancer have been linked on several levels. Even though they easily convert homocysteine into methionine, the majority of cancer cells are unable to proliferate if methionine in the media is replaced by homocysteine. Surprisingly, intracellular methionine levels in breast cancer cells remained substantially stable when they were transferred to homocysteine media and analyzed; however, in this situation, SAM levels were strongly depleted [66]. Homocysteine substitution for methionine has no effect on non-cancerous cells, suggesting they have less need for SAM. Cancer and normal cells are different in their growth rates with different metabolic needs, so it is frequently challenging to interpret the differences between the metabolic dependencies of normal and cancer cells. Perhaps unsurprisingly, there are some methionine-independent tumor cell lines, and in these cases, SAM levels are relatively normal [67,68]. According to the Hoffman effect, methionine is metabolized differently by cancerous and non-tumorigenic cells. Using 11C-methionine positron emission tomography, human cancers may be easily seen and

distinguished from normal tissue, demonstrating this higher need (Met-PET). Met-PET imaging often outperforms 18F-deoxyglucose PET (FDG-PET) imaging, particularly in glioma, as the increased brain glucose metabolism interferes with tumor-specific FDG signals. However, multiple myeloma and other malignancies have also been studied with Met-PET [69].

2.4.5. Homocysteine Metabolism and Cancer

Hyperhomocystinuria and cancer have been shown to be closely related by recent scientific developments. Homocystinuria is defined by a rise in the level of homocysteine (Hcy) in the serum and can come from an inborn mistake in the metabolic pathways of sulfur-containing amino acids [70]. Cancer patients have also been found to have increased plasma homocysteine concentrations. There are strong clinical correlations between a number of polymorphisms in the enzymes implicated in the Hcy detoxifying pathways and various cancer types [71-81]. Many cancer types exhibit high plasma Hcy levels in the advanced stages, although there may be little to no change in plasma Hcy levels in the earlier stages of the disease [73,82-90]. Why the Hcy levels differ between the early and late stages of cancer is unclear. However, since Hcy promotes the growth of cancer cells [91], increased generation and secretion of Hcy seems likely to be an adaptive metabolic mutation acquired during cancer progression. Caco-2 cell lines with higher homocysteine levels exhibit greater cellular proliferation. By including more folate in the culture media or by supplementing it with its metabolites, such as 5-MTHF [92], this increased proliferation can be reduced. However, because a very high Hcy concentration may potentially be lethal to the cancer cells, advanced-stage cancer cells may release Hcy. Clinically, the situation is less clear—in some studies, there is no evidence of a correlation between Hcy levels and cancer risk [93]. Further investigations are required to reveal the precise mechanism of how cancer patients deal with Hcy metabolism.

2.4.6. The Role of One-Carbon Metabolism in Nucleotide Synthesis in Cancer

The synthesis of purine and pyrimidine nucleotides, which are required for the synthesis of DNA and RNA, depends on the one-carbon cycle [94]. A single carbon, typically from serine, is transferred during one-carbon metabolism to create the pyrimidine and purine bases [52], hence the significance of serine in the production of nucleotides. During glycolysis, serine is produced from 3-phosphoglycerate (3-PG) [95]. Serine-derived one carbon transfer to tetrahydrofolate results in 5,10-methylenetetrahydrofolate (CH_2-THF), a substance essential for the synthesis of

pyrimidines [96]. CH2-THF is also the methyl donor used to regenerate methionine from homocysteine, so there is a balance between its use in pyrimidine synthesis versus providing the methyl group to SAM for use in DNA or protein methylation, polyamine synthesis, or the generation of glutathione. The subsequent transformation of CH2-THF into 10-formyltetrahydrofolate (CHOTHF) is an essential component of purine synthesis [96]. Therefore, the synthesis of both pyrimidines and purines depends on a carbon donor such as serine and the tetrahydrofolate carrier. Due to the need for a large quantity of DNA nucleotides, one-carbon metabolism is crucial for cancer cells to proliferate quickly. As a result, one-carbon metabolism is a prospective target for reducing cell growth. It was shown that lowering serine levels or blocking particular mitochondrial folate metabolic enzymes decreased the number of purine nucleotides, which in turn prevented proliferation [41,97,98]. As a result, researchers are actively looking at anticancer medications that target one-carbon metabolism [99,100].

2.4.7. Polyamine Metabolism in Cancer

Prostate cancer cell proliferation and differentiation, often controlled by androgen hormones, are correlated with levels of polyamines, particularly spermine [101] which is plentiful in the human prostate. Spermine may serve as a biomarker to distinguish between low-grade and high-grade prostate cancers because its content is lower in the latter [102]. In prostate cancer, the most significant metabolic disturbance observed was increases in polyamine metabolites and in dcSAM [103]. The PTEN-PI3K-mTOR complex 1 (mTORC1) pathway was shown to regulate the stability of SAMDC (AMD1), which controls the use of SAM for polyamine synthesis in prostate cancer [103,104]. Inhibitors of mTORC1 or SAMDC were able to significantly impede growth in prostate cancer cell lines, and this could be partly rescued by supplementing with spermidine. In this case, the role of ODC1 in polyamine regulation downstream of mTORC signaling was excluded—it was just via SAMDC regulation. However, in c-MYC transgenic mice, c-MYC has been shown to promote prostate cancer carcinogenesis by boosting polyamine production through the transcriptional control of ODC [105]. This is significant because ODC1 has been identified as a c-Myc-responsive rate-limiting step in polyamine synthesis [106]. Notably, PGC-1 inhibits c-MYC and hence ODC, which reduces polyamine production and lowers the aggressiveness and spread of prostate cancer [105]. By contrast, the androgen receptor typically acts in prostate cancer to upregulate ODC1 expression [107], and indeed ODC1 overexpression alone may be enough to drive prostate tumorigenesis [108]. Similar to the observation in prostate cancer, human breast cancer tissue has a lot more acetylated polyamines than healthy breast tissue [109]. In breast cancer patients,

estrogen signaling is linked to the creation of polyamines and purines. Estrogen directly contributes to the progression of breast cancer by activating the estrogen receptor (ER), which binds to estradiol (E2) [110]. Through the mitochondrial folate route, this binding activates ER and causes the activation of genes that boost polyamine and purine production [110,111]. Additionally, due to their effects on the activity of the insulin receptor, polyamines may control the mitogenic action of insulin in breast cancer [112]. ODC mRNA and protein levels are markedly increased in breast cancer patients, and they positively correlate with the tumor, node, and metastases (TNM) stages of the disease. Increased ODC activity is linked to higher cancer cell proliferation rates and a worse prognosis for breast cancer patients [113]. Arginase, which changes arginine into ornithine [114,115], is more prevalent in breast cancer, making it a potential market for breast cancer in its latter stages [116]. In addition to ODC, breast cancer also exhibits increased levels of ADC and agmatinase, enzymes involved in the synthesis of putrescine from arginine [117]. Early in metastasis, arginase and polyamine production are increased [118]. These considerations are relevant to this review because, in each of these cases in which polyamines are elevated in cancer, SAM and the one-carbon cycle are required for their synthesis. Patients with pancreatic cancer have polyamines found in their urine, serum, and saliva, which makes them potential biomarkers [119-122]. In human pancreatic ductal adenocarcinoma (PDAC), KRAS mutations are the most prevalent (representing around 95% of all mutations) [123]. In addition, the copy number of c-MYC has increased in more than 50% of human PDAC cell lines [124]. Similar to other cancers, KRAS and MYC are upstream activators of polyamine production in PDAC [123,125]. ODC levels rise in pancreatic cancer and aid in the development and spread of advanced pancreatic cancer [126-128]. Employing an ODC inhibitor (DMFO) and a polyamine transport inhibitor (Trimer44NMe) together greatly decreased the survival of PDAC cells by inducing apoptosis [125]. Immune privilege must be established in order for the PDAC tumor to survive, and spermine is critical for this process [129]. Poor prognosis is linked to the dysregulation of polyamines in neuroblastoma, and various polyamine homeostasis-related genes are transcriptional targets of cMYC/MYCN [130-132]. The modulation of the SLC3A2 polyamine exporter and other essential elements of the polyamine pathway in vitro is directly induced by MYCN, leading to increased polyamine production and accelerated neuroblastoma cell proliferation [133]. ODC has been recognized as a potent oncogenic transforming factor, and in neuroblastoma, it is the most well-studied target of the transcription factor c-MYC/MYCN [132,134,135]. In vivo neuroblastoma cell proliferation and MYCN-mediated oncogenesis are both reduced in animal models when ODC is disabled [136]. Along with ODC, SAMDC is a target of MYCN and plays a significant role in the growth of neuroblastomas [137,138]. In murine neuroblastoma,

S-adenosylmethionine synthetase overexpression is linked to the development of treatment resistance [137]. Transgenic mice used in a preclinical study that used polyamine antagonist regimens targeting ODC1 and SAMDC had their neuroblastoma initiation reduced [139,140]. Metabolic enzymes and polyamine levels affect both treatment and prognosis in leukemia [141]. High levels of polyamines are linked to a bad prognosis in leukemia cells. However, polyamine depletion in healthy cells also results in cell cycle arrest, highlighting the need to preserve polyamine homeostasis. Patients with acute lymphoblastic leukemia (ALL) have increased ODC activity and putrescine levels, and their recurrence can be detected by increased spermine levels in erythrocytes [141]. Polyamine depletion is a plausible approach to decreasing polyamine levels in cancer. Overexpression of the polyamine acetyltransferase SSAT drives the first step of polyamine breakdown and can result in diminished cell growth, migration, and invasion by blocking AKT and GSK3b signaling [142]. These findings were made using a variety of colon carcinoma cell models and human hepatocellular malignancies. It is not new to use polyamines and their metabolites as cancer biomarkers [143]. In lung and liver malignancies, polyamines and their metabolites in the urine and plasma can be helpful both for diagnosis and as indicators of tumor development [144,145]. Diacetylspermine has been linked to lung and ovarian cancers as a reliable urine biomarker [146-148]. Right-side colon tumors associated with biofilms have also been shown to contain significant quantities of diacetylspermine [149,150]. Urinary or serum measurements of polyamines and polyamine metabolites have demonstrated potential as biomarkers for colon, pancreatic, and prostate malignancies [119,151-154]. The development of more individualized methods for cancer diagnosis and therapy based on the use of polyamines as biomarkers may be aided by such analyses in conjunction with increasingly accurate genetic signatures.

2.5. Mechanisms Relating One-Carbon and Polyamine Metabolism to Cancer

2.5.1. The Function of One-Carbon Metabolism in Methylation Reactions

SAM is a common methyl donor used in the methylation of RNA, DNA, and histones [65]. The methyl group typically comes from serine via CH2-THF and is then transferred to methionine, then SAM before transfer to the final target molecule [155]. DNA methylation primarily takes place at the 5’ carbon of the pyrimidine base cytosine (5mC) in CpG islands. DNA methyltransferases (DNMTs) like DNMT3a, DNMT1, and DNMT3b catalyze DNA methylation using SAM as the methyl donor [156]. Numerous tumor cells, including colon, cervical, and breast cancer cells, have been found to exhibit hypermethylation in the DNA [157]. Reduced gene expression of tumor

suppressor genes is caused by the hyper-methylation of their promoters. Additionally, it has been noted that DNA hypermethylation and chemoresistance are associated [158]. A number of clinical kits are already being produced for detecting DNA methylation in cancer patients [159-167], demonstrating how this correlation has been incorporated into clinical practice. RNA methylation also occurs, primarily taking place at the N6 position of an adenine base (m6A) near a stop codon [168,169]. RNA methyltransferases like METTL3, METTL14, and WTAP catalyze the methylation of RNA using a SAM donor [170]. N6-Methyl Adenosine (m6A) in RNA has a variety of roles in the development and spread of cancer. By encouraging the translation of these mRNAs, METTL3 activity boosts and augments MYC, BCL2, and PTEN in human acute myeloid leukemia (AML) [171]. Similar findings suggest that RNA methylation fosters the development of tumors in other cancer types, including pancreatic, colorectal, hepatic, and breast cancer [172-175]. In addition, it has been noted that RNA methylation is a reliable diagnostic indicator for gastrointestinal malignancies [176]. However, RNA methylation can equally serve to increase the translation of tumor suppressors, and in these cases, overexpression of RNA methylation machinery is protective [177]. RNA methylation has also been linked to tumor immunity, so clearly, there is more work to be done to understand the full implications of RNA methylation in cancer. In cancer cells, histone methylation and demethylation are both crucial processes. Histone methylation has received a lot of attention as a protein modification, particularly for its function in regulating gene expression. Increased methylation of H3K4, H3K36, and H3K79 frequently promotes transcription, while increased methylation of H3K9, H3K20, and H3K27 typically represses transcription [178]. AKT1, MYC, and MAPK are just a few of the cancer-related genes that are impacted by H3K4 methylation [5]. Additionally, aberrant histone methylation and altered gene expression may be caused by mutations in the histone methyltransferases MLL2, EZH2, and UTX [179,180]. In addition, cancer stem cells (CSCs) in a variety of cancer types benefit from histone demethylation via the LSD1 or Jumonji C domain families [181-183]. SAM depletion alters the kinetics and development of histone methylation in vivo as well as in stem cells and cancer cells [5,46,184-186], but it is not yet clear whether this represents a viable therapeutic opportunity.

2.5.2. Oxidative Stress and One-Carbon Metabolism in Cancer

Reactive oxygen species (ROS) levels affect the development of cancer: initiating or promoting carcinogenesis at lower levels or at higher levels leading to cell death [187]. Tumor cells typically generate relatively high levels of ROS by their aberrant metabolism and tolerate oxidative stress through several adaptations, including the generation

of antioxidants such as glutathione. Glutathione can be regenerated following oxidative stress by glutathione reductase, but it requires NADPH. NADPH is generated in a number of ways, such as by activating AMPK, the Pentose phosphate pathway from glycolysis, and reductive glutamine and folate metabolism [187]. Redox-sensitive pathways are maintained in normal working order in physiological circumstances by a harmony between the creation and removal of reactive oxygen species (ROS). Oxidative stress can cause abnormal cell death and/or disease development when redox equilibrium is disrupted [188]. By restoring the activity of antioxidant defense enzymes like superoxide dismutase (SOD) and catalase and by raising levels of the anti-oxidant glutathione, cofactors of one carbon metabolism, in particular folate and B12, have been shown to be useful in lowering oxidative damage [189]. At least in rats, a long-term reduction in the intake of folate alters the activity of Mn-SOD, catalase, and glutathione peroxidase, as well as causing irreversible oxidative DNA damage [190]. Conversely, adding dietary folate may protect against oxidative stress [191,192]. The mechanisms have not always been identified in these cases, but SAM is known to boost SOD and glutathione-S-transferase (GST) activity and replenish glutathione levels [193], so it seems likely that a significant role of folate is to allow effective regeneration of SAM and hence glutathione when under oxidative stress. A potent antioxidant molecule, GSH is a tripeptide made of glycine, glutamate, and cysteine [194]. Cysteine catabolism via the trans-sulphuration pathway raises glutathione levels and speeds up the process of ROS detoxification [42]. Lack of dietary folate, and hence lack of methylene-THF, leads to hyperhomocysteinemia, as there is no methyl donor to use up homocysteine and regenerate methionine and SAM. Perhaps surprisingly, elevated homocysteine is not associated with elevated glutathione levels but rather with ER stress and DNA damage [195], as well as atherosclerosis and dementia. Homocysteine has some direct detrimental effects, including upregulating superoxide production by NADPH oxidase, leading to increased redox stress [196]. These deleterious outcomes underline the importance of one-carbon homeostasis, as folate is required to maintain SAM levels as well as to prevent elevated homocysteine [197]. One-carbon metabolism has come to be recognized as a significant cellular regulator of NADPH levels through the activity of MTHFD, which uses methylene-THF to make NADPH in the first step toward purine synthesis [28]. Cellular NADPH/NADP+ was lowered, and oxidative stress sensitivity was raised when either the mitochondrial or cytosolic MTHFD enzymes were depleted. In response to oxidative stress, Nrf2 activity promotes serine transit through the folate cycle, so cells produce more NADPH and the reducing equivalents required to detoxify ROS [42]. Methylene-THF is thus used both to produce glutathione via the methionine cycle as well as to maintain antioxidants in their reduced state by generating NADPH [198]. In summary, it is established that

there is a strong correlation between antioxidant defense mechanisms and one-carbon metabolism. It has also been established that one carbon metabolism has an impact on cancer progression. Consequently, it seems likely that at least one of the mechanisms by which one-carbon metabolism affects cancer outcomes will be its role in maintaining antioxidant defenses. The other principal defense against oxidative damage is the recycling of damaged molecules by autophagy, and this is also regulated by one-carbon metabolism.

2.5.3. The Linkage of Autophagy to the One-Carbon and Polyamine Metabolism in Cancer

Autophagy is induced in response to various stresses to maintain metabolic homeostasis and prevent the build-up of unnecessary or damaged cellular components [199-201]. The aberrant regulation of autophagy is linked to many diseases, especially in neurodegenerative disease and cancer [200,202,203], as well as in cells in which aneuploidy has been induced [204-207]. Autophagy can function as a pro-survival protective pathway in cancer cells to tolerate the effects of their increased metabolic demands for rapid cell proliferation and to respond to cellular stresses that may include genomic instability and metabolic stress [208-211]. Reduced autophagy may promote tumorigenesis by increasing DNA damage rates. Autophagy is thought to be mainly regulated by Target of Rapamycin Complex 1 (TORC1) in a nutrient-sensitive condition [212]. There are now ongoing clinical trials evaluating the combination of different modulators of autophagy with other chemotherapeutics [213,214].

Studies have shown that one-carbon metabolism is involved in the regulation of autophagy and antioxidant levels. S-adenosylmethionine (SAM) functions as a conserved metabolic switch that regulates autophagy by controlling methylation [186,215,216], sulphuration [217-219], and synthesis of polyamines [220,221]. Furthermore, SAM also controls the availability of natural antioxidant GSH and other sulfur-containing metabolites like cysteine [222]. GSH and cysteine are essential to reduce cancer-related oxidative damage [223]. GSH depletion and increased cellular oxidative stress can trigger the autophagic response [218,224,225]. Increased methionine levels in yeast result in the inhibition of starvation-induced autophagy through increased SAM levels and methylation of PP2A. Methylated PP2A dephosphorylates the negative regulators (Npr2, Npr3, and Iml1) of TORC1 [226]. In mammals, increased SAM levels enhanced its binding to SAMTOR, which disrupts the inhibitory complex (SAMTOR-GATOR1) of mTORC1 [226,227]. SAMTOR acts as a nutrient sensor via SAM; it links one-carbon metabolism to cellular growth and autophagy via mTORC1. Spermidine has also been demonstrated to trigger autophagy in flies, yeast, worms, and mammalian cells [220,228]. Spermidine controls autophagy by altering the expression of the autophagy-related gene

(Atg) via controlling the expression of the transcription factor elF5A and TFEB [229,230]. Spermidine also suppresses acetylation by regulating the expression of acetyltransferase E1A-associated protein p300 (EP300), which promotes the deacetylation of autophagy-related proteins [231]. In addition, spermidine also reduces the availability of acetyl-CoA, which decreases acetylation and promotes autophagy [228]. Cancer cells have altered metabolism to meet the high demands for energy which results in increased cellular stress and damage. Therefore, cancer cells have a higher dependency on autophagy and other repair mechanisms compared with normal cells. Maintaining cellular levels of autophagy prevents healthy cells from tumorigenesis by limiting tissue damage, inflammation, and genome instability, but cancer cells also utilize autophagy for tumor progression and drug resistance [232-235]. Therefore, inhibiting autophagy in cancer cells is a potential target, and clinical trials are ongoing on autophagy modulators to treat cancer, though clearly, more work needs to be done in this area.

2.6. Metabolic Cancer Therapy

2.6.1. Metabolic Therapy Targeting One-Carbon and Folate Metabolism

The relevance of FOCM has been unequivocally established, and clinics have been using related medications for many years. Numerous cancers have been treated with dihydrofolate reductase (DHFR) and thymidylate synthase (TYMS) inhibitors [22,236,237], such as methotrexate and pemetrexed. Similar to other chemotherapeutic treatments, these drugs are not ideal because cells develop resistance by, for instance, impairing drug absorption, decreasing the drug's retention inside the cell, and decreasing drug affinity. There is a need to develop further therapies that specifically target FOCM. Since many cancer cells appear to be somewhat dependent on the presence of exogenous serine, limiting the supply of serine may have medicinal advantages. Depletion of exogenous serine will obviously have less of an impact on tumors with increased serine synthesis enzymes, but p53 loss may increase their dependency. More than half of all malignancies have p53 mutations [238], which could lead to a tumor-specific dependency on serine availability. It is a well-known therapeutic technique to reduce phenylalanine intake in individuals with phenylketonuria [239], and it would appear that a similar strategy could be used to eliminate serine from a cancer patient's diet. Serum levels of serine and glycine can be selectively reduced by 50% in animals fed a diet missing serine and glycine [41], in mouse studies, despite the fact that serine synthesis by organs such as the liver and kidneys [240] might have been expected to maintain circulating serine levels. Mice fed on this diet showed delayed tumor formation in xenograft experiments [41]. Combining a serine-free diet with oxidative phosphorylation

inhibitors, such as the biguanides metformin and phenformin, which are used to treat type 2 diabetes, enhanced the therapeutic effectiveness of this method for treating cancer in an allograft mouse model [241]. According to experimental findings, the switch to de novo serine synthesis is followed by an increase in ROS levels. This raises the prospect that suppressing antioxidant defenses or encouraging the production of more ROS could work in conjunction with serine restriction to kill tumor cells. Limiting de novo serine synthesis is an alternative strategy for therapeutically addressing serine metabolism, particularly in tumors that exhibit serine synthesis enzyme amplification. For instance, the availability of PHGDH inhibitors [242,243] that block serine synthesis allows for preclinical and clinical examinations in patients chosen for having tumors with amplified PHGDH. However, a study employing xenograft mice models demonstrated that PHGDH depletion alone could not suppress tumor growth, casting doubt on the efficacy of this method for treating existing tumors [244]. Another problem with this strategy will be any negative consequences that may result from preventing de novo serine production. Exploiting serine metabolism clinically for the treatment of cancer is still in its infancy. A more specific method, or combination of approaches, is expected to emerge as we gain a deeper comprehension of the regulation and activity of these pathways. However, several approaches are currently in the initial phases of preclinical examination. Therefore, we are hopeful that this area of metabolism may lead to novel therapeutic possibilities. Dietary methionine restriction considerably slows down the growth of tumors in a number of preclinical models, including both solid tumors and blood malignancies [245-249]. Overall, Yoshida sarcoma survival improved as a result of their reaction to a methionine restricted diet. Regular diet mice all died by day 12, whereas Yoshida tumor-bearing mice all lived for 30 days, with the last one passing away on day 38. These tumor-bearing mice's body weights were unaffected by the methionine-off diet [250]. Although the results of clinical investigations utilizing diets low in methionine have been inconsistent, the endpoint data were primarily concerned with the effectiveness of plasma methionine reduction [251]. The amount of plasma methionine decreased by about 50%, and patients shed an average of 0.5 kg per week. When tumors were studied after surgery, the combination of 5-fluorouracil and methionine limitation in preoperative high-stage stomach cancer patients had a remarkable impact on tumor pathology [252]. A recombinant enzyme that breaks down methionine has been created [253,254]. The gene, methioninase (METase), was obtained from Pseudomonas putida and encoded an L-methionine-deamino-mercaptomethane lyase. Both patient-derived xenograft (PDX) and cell-based models of several malignancies demonstrated the efficacy of METase injection [249,255-259]. The most promising route to practical use involves methionine restriction along with chemotherapy or radiation.

2.6.2. Therapy Targeting Polyamine Metabolism

Targeting polyamine metabolism, which is dysregulated in several types of malignancies, has been the focus of therapeutic treatments for some time. In the 1960s, methylglyoxal bis(guanylhydrazone) (MGBG) was utilized as an anticancer medication, for example, against leukemia [260,261], but its effectiveness was severely hindered by its toxicities. Later research revealed MGBG to be a SAMDC inhibitor [262], suggesting SAMDC as a possible therapy target. This effort resulted in the creation of many SAMDC inhibitors, such as 4-amidinoindan-1-one 20-amidinohydrazone (SAM486A). As an analog of spermidine and a competitive SAMDC inhibitor, MGBG lowers spermidine and spermine levels and raises putrescine levels [261]. MGBG inhibits the development of cancer cells by triggering the mitochondrial apoptosis cascade [263]. Even though these substances exhibited antitumor activity, they were nonetheless extremely hazardous. These analogs' antitumor activity and/or toxicity were caused by off-target effects such as antimitochondrial activities in addition to interference with polyamine metabolism. SAMDC can be rendered inactive by more potent inhibitors, such as 50-(((Z)-4-amino-2-butenyl)methylamino)-50-deoxyadenosine (AbeAdo) and its 8-methyl derivative (Genz-644131) [262,264]. These inhibitors have not yet been proven to be effective antitumor medicines though they are promising for treating trypanosomiasis. Similarly, inhibitors of the next step (aminopropyl transferases) have been demonstrated to lower polyamine content [265], but effective inhibitors have not yet been identified for clinical applications. The most well-known polyamine inhibitor, difluoromethylornithine (DFMO), was found in the 1970s and inhibited ODC irreversibly [266-268]. ODC is permanently rendered inactive once DFMO attaches to it, creating an extremely reactive intermediate that is then decarboxylated and covalently bound to ODC [268]. DFMO reactions result in polyamine depletion and are typically cytostatic in mammalian cells [269]. Its rapid clinical trial evaluation as a separate treatment agent was prompted by early observations of the impacts of DFMO in colon cancer, melanoma, small-cell lung cancer, and neuroblastoma [270-274]. Despite the fact that DFMO was well tolerated, the outcomes did not include notable clinical responses, which may have been the result of its ineffective distribution to cells [127,270]. Therefore, research centered on DFMO in combination with other medicinal drugs. In prostate, melanoma, breast, and neuroblastoma cell lines, the effective transport inhibitor AMXT 1501 synergizes with DFMO [275,276]. Patients with glioma have received DFMO in combination with the cytotoxic drugs procarbazine, nitrosourea, and vincristine, while those with neuroblastoma have received DFMO in combination with either bortezomib or etoposide, a proteasome inhibitor [277-279]. In vitro and in vivo, gemcitabine-resistant pancreatic cancer is efficiently inhibited by DFMO in

conjunction with the polyamine transport inhibitor Trimer44NMe [125]. A promising method to treat colorectal cancer in an in vivo model has been demonstrated to be preventing ODC expression by DMFO as a separate agent or together with other medications, which is yet to be evaluated in clinical trials [280-282]. Additionally, the discovery of inhibitors has focused on the spermine and spermidine synthases S-adenosyl-1,12-diamino-3-thio-9-azadodecane (AdoDATAD) and S-adenosyl-3-thio-1,8-diamino-3-octane (AdoDATO) [283,284]. However, although these substances effectively and selectively block aminopropyl transferase, they both have primary amines in their structures that act as SSAT and amine oxidase substrates. Their clinical usage is therefore constrained because cellular metabolism breaks them down. These inhibitors only marginally reduce the growth of cancer cell lines [283,284]. An alternative spermine homolog, None-carbonyclopropyl-methyl-N11-ethylnorspermine (CPENSpm) [285], is significantly cytotoxic to breast cancer and human lung carcinoma cells [286-288]. It results in the induction of elevated SSAT levels and the activation of apoptosis [288,289]. However, no clinical trials have yet been completed, largely due to the drug's poor cell type-specific cytotoxicity. In experimental animal models, drugs, or polyamine analogs that target polyamines and important enzymes connected to polyamine metabolism have been found to be beneficial against cancer. Some of these drugs have also been tested in human clinical trials. However, as far as we can tell, these inhibitors' adverse effects and toxicity have prevented them from producing adequate clinical results to date. Despite significant advancements in creative polyamine analogs and other polyamine-targeting drugs, the production of effective and secure therapeutic agents still needs further investigation.

2.7. Conclusions

Researchers' interest in cancer metabolism has increased over the past decade, which has resulted in a greater understanding of the metabolic pathways involved in cancer biology. Numerous pathways that are known to or are anticipated to increase the survival of cancer cells rely on one-carbon and polyamine metabolism. A more thorough comprehension of these could enable more focused targeting of the particular pathways that are most crucial for cancer cell survival. There are already several therapies that target one-carbon and polyamine metabolism. However, due to the significance of one-carbon and polyamine metabolites in healthily proliferating cells, it has been challenging to avoid harmful side effects. Nonetheless, there are encouraging prospects for therapies that deplete serine and methionine, particularly in combination with redox or autophagy intervention. Altering methionine or SAM levels has significant effects on cancers, but currently, the mechanisms responsible are unclear, so further work

is needed to develop specific and effective interventions. Polyamine-targeting drugs have been in clinical use for decades, and there are ongoing trials to optimize their use in combinations such as with NSAIDs in colorectal cancer. By more specifically blocking individual one-carbon and polyamines pathway enzymes, future treatments may be able to target one-carbon and polyamine metabolism more effectively in cancer cells. Therefore, this review strongly suggests the need for further investigations to explore a better understanding of one-carbon and polyamine metabolic pathways, particularly methionine and polyamine metabolism in cancer growth, and to discover novel inhibitors in these pathways.

2.8. References

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CHAPTER 3: CHROMOSOMAL INSTABILITY CAUSES SENSITIVITY TO POLYAMINES AND ONE-CARBON METABOLISM

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3.1. Abstract

Aneuploidy, or having a disrupted genome, is an aberration commonly found in tumours but rare in normal tissues. It gives rise to proteotoxic stress as well as a stereotypical oxidative shift, which makes these cells sensitive to internal and environmental stresses. Using *Drosophila* as a model, we investigated the changes in transcription in response to ongoing changes to ploidy (chromosomal instability, CIN). We noticed changes in genes affecting one-carbon metabolism, specifically those affecting the production and use of s-adenosyl methionine (SAM). The depletion of several of these genes has led to cell death by apoptosis in CIN cells but not in normal proliferating cells. We found that CIN cells are particularly sensitive to SAM metabolism at least partly because of its role in generating polyamines. Feeding animals spermine was seen to rescue the cell death caused by the loss of SAM synthase in CIN tissues. The loss of polyamines led to decreased rates of autophagy and sensitivity to reactive oxygen species (ROS), which we have shown to contribute significantly to cell death in CIN cells. These findings suggest that a well-tolerated metabolic intervention such as polyamine inhibition has the potential to target CIN tumours via a relatively well-characterised mechanism.

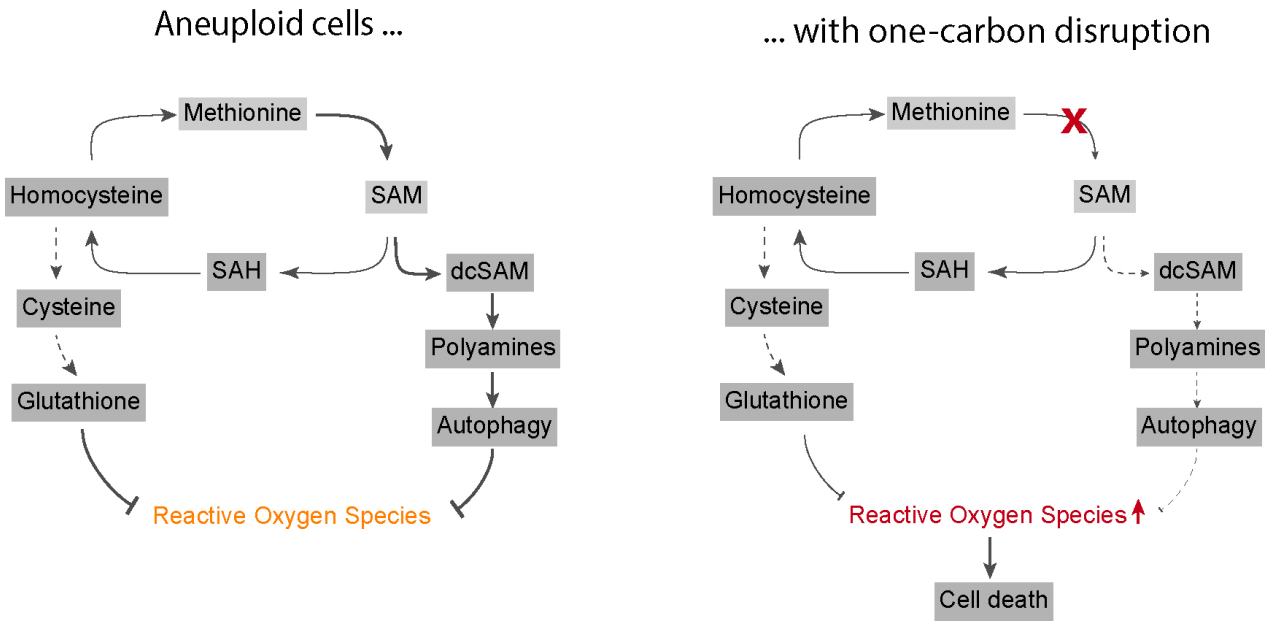

Figure 3-1-Graphical abstract

3.2. Introduction

Chromosomal instability (CIN) refers to karyotype variation that occurs over time, mainly due to a lack of mitotic fidelity [1]. It causes aneuploidy, which is characterized by a deviation from a normal chromosomal number, with either a gain or loss of DNA. CIN is a frequent characteristic of human tumours that causes increasing genetic variation, which has been linked to tumour evolution, medication resistance, and poor prognosis in CIN cancer patients [2]. Aneuploidy is a common hallmark of advanced malignancies, with chromosomal abnormalities detectable in more than 80% of solid tumours [3]. We and others have suggested that because CIN is a cancer-specific trait, it could be a good target for chemotherapy [4-7]. The process by which CIN is acquired by cancer cells, as well as how it impacts the tolerance of and response to cellular and environmental stressors, are therefore important research goals. Because CIN cells are genetically varied if not unique, identifying a conserved feature of CIN cells as a prospective target is challenging. However, CIN, and aneuploidy itself, is a common tumour specific phenotype that allows the prospect of tumour specific therapies. To achieve this, it will be necessary to identify the characteristics of aneuploid cells that remain constant regardless of which DNA has been gained or lost. Specifically, we aim to identify what gene expression profiles are essential for the survival of CIN cells, but not normal cells, so that the expression of these genes may become future therapeutic targets. To find genes that can be depleted to kill CIN cells without affecting normal proliferating cells, we used *Drosophila* to produce CIN in a genetically homogeneous population of cells in vivo [7-9]. By weakening the spindle checkpoint using mad2-RNAi, we were able to induce CIN. This shortens mitosis and leaves less time for any chromosome misorientation to be corrected during metaphase [10], leading to a considerably higher rate of chromosome segregation errors or CIN [7]. In this flat proliferating epithelium, we typically obtain an average of 25% of mitoses showing an anaphase error without inducing excessive cell death [2]. Using this CIN model system, we have identified several signalling pathways that show promise for therapeutic intervention, particularly metabolic pathways [8,9,11,12]. CIN is sensitive to several processes including proteostasis, redox balance, autophagy, and metabolism [5,8,9,11,13]. CIN induction results in metabolic stress sensitivity [8]. CIN cells are already dealing with high levels of stress, so a small metabolic change that does not harm normal cells can produce large levels of oxidative stress and, ultimately, cell death [8]. For

example, metabolic changes that cause decreased NAD+ and more reactive oxygen species (ROS) in aneuploid cells strongly impact their survival [11]. Oxidative stress occurs in response to aneuploidy in all eukaryotes that have been tested, including yeast, plants, *Drosophila*, mice, and humans [11,14]. Previous research has shown an increase in repair mechanisms [9], autophagy [5], and antioxidant levels [8] in response to aneuploidy, which are required to tolerate the deleterious effects of aneuploidy. The induction of aneuploidy increases cells' metabolic rates, making them vulnerable to oxidative stress so they show DNA damage and apoptosis in response to metabolic challenges that do not damage normal cells [8]. Moreover, aneuploid cells are metabolically different, with overactive mitochondria giving elevated ROS levels [8], which causes damage to macromolecules, resulting in protein-folding defects and ER stress [15]. It has been observed that increased chaperone levels and ER stress markers in aneuploid cells are dependent on ROS levels.

Currently, the mechanisms that regulate the change in metabolism in response to aneuploidy are unclear. Moreover, several aneuploidy-responsive processes, such as autophagy, redox stress, and proteostasis, are too broad to be good therapeutic targets. Therefore, our purpose was to find specific genes/metabolic targets that can be used to kill CIN cells without affecting the normal cells. We carried out RNA sequencing to find the genes or metabolic pathways that were altered in normal cells when we induced CIN. We found that the expression of one-carbon and polyamine metabolic genes is significantly upregulated in chromosomal instability (CIN) cells. We aimed to unravel the causal links between aneuploidy and these failures of homoeostasis caused by CIN and its consequences. We investigated the role of autophagy and ROS in mediating one-carbon sensitivity in CIN cells. Moreover, we saw an unexpectedly significant rescue of CIN cells with disruption to their one-carbon metabolism simply by feeding additional polyamines in the diet. Overall, our findings suggest that CIN cells are sensitive to one-carbon and polyamine metabolism and that targeting these pathways may represent a promising approach for the development of new strategies for treating and preventing diseases associated with chromosomal instability.

3.3. Materials and Methods

3.3.1. RNA-Seq

Total RNA was extracted from third instar larval tissue using RNEasy Plus minipreps (Qiagen). Six preps using approximately fifteen animals each were generated for each genotype, CIN (mad2 EY/Df(3L)BSC438) vs. non-CIN (mad2 EY/+), and sent for sequencing (150 bp paired end on NovaSeq using Illumina rRNA-depleted TrueSeq libraries). Analysis was carried out using CLC Genomics Workbench 20 (Qiagen). Approximately 40 M sequences per sample were trimmed for adapters, and reads below 15 bp were discarded before mapping to *Drosophila* genome BDGP6.22.98. The sequences have all been deposited in GEO (accession number GSE231601). The parameters used were default conditions: both strands; no global alignment; max. 10 hits per read; paired reads count as one. An average of 91.4% of reads were mapped in pairs, and 76.2% of reads were in annotated genes. Gene expression changes between CIN and non-CIN samples were quantified in Workbench both for whole genes (GE) and for individual splice variants (TE) and ranked by fold change following an FDR p value cutoff of 0.05. One sample from each genotype was discarded during quality control. The heat map was generated using the average Manhattan distances between clusters, which were filtered using a minimum FDR p value of 0.05 and a minimum fold change of 1.5. Gene ontologies of groups significantly enriched or depleted in the CIN genotype were compared using modEnrichr [16].

3.3.2. *Drosophila* Stocks

Stocks were obtained from the Bloomington Stock Center unless otherwise indicated. For the cell death assays in Figure 3-4 and Figure 3-6, the following stocks were used: engrailed-Gal4 (30564), UAS-*mad2*^{RNAi} (VDRC 47918), UAS-*Sam-s*^{RNAi} (29415), UAS-*spds*^{RNAi} (56011), UAS-*sms*^{RNAi} (52924), UAS-*Gnmt*^{RNAi} (53282), UAS-*Ahcy*^{RNAi} (51477), UAS-*Odc1*^{RNAi} (64498), UAS-*PAOX*^{RNAi} (36904), UAS-*Met-s*^{RNAi} (43986), UAS-*Cbs*^{RNAi} (43986), UAS-*Eip55E*^{RNAi} (36766), UAS-*SAMdc*^{RNAi} (VDRC 101753), and Df(3L)BSC438 (24942). For the autophagy assays, the following stocks were used: UAS-*Atg1*^{RNAi} (26731), UAS-*mTor*^{RNAi} (33951-scute removed), and UAS-*Atg18a* ^{RNAi} (34714).

3.3.3. Acridine Orange Staining

The extent of cell death in the engrailed induced third instar larval wing discs was determined using acridine orange (Invitrogen). Wing discs were dissected in PBS from third instar larvae and then stained for 2 min in a 1 µM acridine orange solution, rinsed briefly, mounted, and imaged in PBS. The stain was normalised using ImageJ software by subtracting the average acridine orange signal in the wild type anterior compartment from the average acridine orange signal in the *engrailed* Gal4-induced mutant posterior compartment (marked with mCD8-GFP). Subtracting the background, a rolling ball radius of 10 pixels or 50 pixels was used [17]. Multiple experiments were carried out, and reproducibility was confirmed by t-tests before grouping data.

3.3.4. Immunostaining

In this work, we used our lab's usual immunostaining procedure [13]. Wing discs were dissected in PBS, fixed in 3.7 percent formaldehyde for 20 min, blocked in PBS plus 0.2 percent Tween-20, and then incubated with primary antibodies overnight at 4°C. Before and after secondary antibodies were applied, discs were rinsed in PBSTw for 2 h at room temperature. Then, discs were mounted in 80 percent glycerol. All of the images are of larval wing discs in the third instar. The antibodies utilised in this research are listed below: Rabbit anti-DCP-1 (D175, 1:100) is the main antibody (cell signalling). The secondary antibody is rabbit-specific CY3 (1:200). In order to normalise the DCP-1 labelling, the average signal in the wild-type anterior compartment was subtracted from the average signal in the *engrailed* Gal4-driven mutant posterior compartment (marked with mCD8-GFP).

3.3.5. Image Data Analysis and Statistics

More information on background subtraction for DCP-1, acridine orange staining, and normalizing the signal from half wing discs to account for variations in staining intensity can be found in [8,12]. GraphPad Prism was utilised for statistical analysis, and ImageJ was used for quantification. The difference in means was calculated using two-tailed t tests with Welsh's correction, and all error bars indicated 95 percent confidence intervals for the mean. In cases in which multiple comparisons were needed, Dunnett's T3 method was used, comparing samples to the negative control

3.3.6. Drug Treatments

Unless otherwise specified, drugs were purchased from Sigma. Drugs were mixed with common fly food for larvae (water, molasses, yeast, glucose, acid mix, agar, semolina, and Tegosept), and when the mixture solidified, they were administered to the host fly. The medications utilised were N-acetylcysteine amide (NACA) 80 µg/mL, spermine 0.1 mM, and spermidine 0.1 mM and 1 mM.

3.3.7. ROS detection and Quantification

As stated, ROS detection was carried out [18]. Briefly, 1 mg of DHE was dissolved in 100 µL DMSO and diluted in Schneider's medium (SM) before use. The wing discs of dissected larvae were treated with 30 µM DHE in the dark for 15 min after being placed in SM at room temperature. Wing discs were promptly mounted on glass slides with VECTASHIELD Antifade Mounting Medium after being cleaned with PBS four times (Vector Laboratories). Imaging was completed within 4 h.

3.4. Results

3.4.1. The Expression of One-Carbon Metabolism Genes Was Affected by Chromosomal Instability (CIN) and Affected CIN Tolerance

To find new metabolic pathways altered in response to CIN, we carried out RNA-Seq in animals in which CIN had been induced by *mad2* mutations. A heat map shows consistent changes in gene expression in independent groups of animals of either genotype (Figure 3-2A). Gene enrichment analysis by modEnrichr shows the top 10 gene ontology hits for biological processes in the GeneRIF library ranked by combined score. Only the top hit, methionine metabolic process (GO:0006555), gave a statistically significant score when corrected for multiple comparisons (Figure 3-2B). Several genes involved in the methionine or one-carbon cycle showed an elevated transcription in CIN animals (bold in Figure 3-2C), the strongest hit being S-adenosyl methionine synthase (*SAM-s*). These genes regulate the production of S-adenosyl methionine (*SAM*), which is critical for several cellular processes, including methylation, antioxidant synthesis, and polyamine synthesis. Our next step was to test whether these genes were significant for the survival of CIN cells. We have previously generated models with inducible CIN expression using the RNA interference knockdown of the cohesin gene *Rad21* or the spindle assembly checkpoint gene *mad2* [3]. To test whether any of the one-carbon and polyamine regulatory genes affected CIN cell survival, we depleted them by RNAi in a proliferating epithelium (the developing wing disc) in which we induced chromosomal instability (Figure 3-2D). We observed elevated cell death in these CIN wing discs for several one-carbon regulatory genes, including SAM-s (Figure 3-2D), which is consistent with this pathway having a significant role in CIN cell survival.

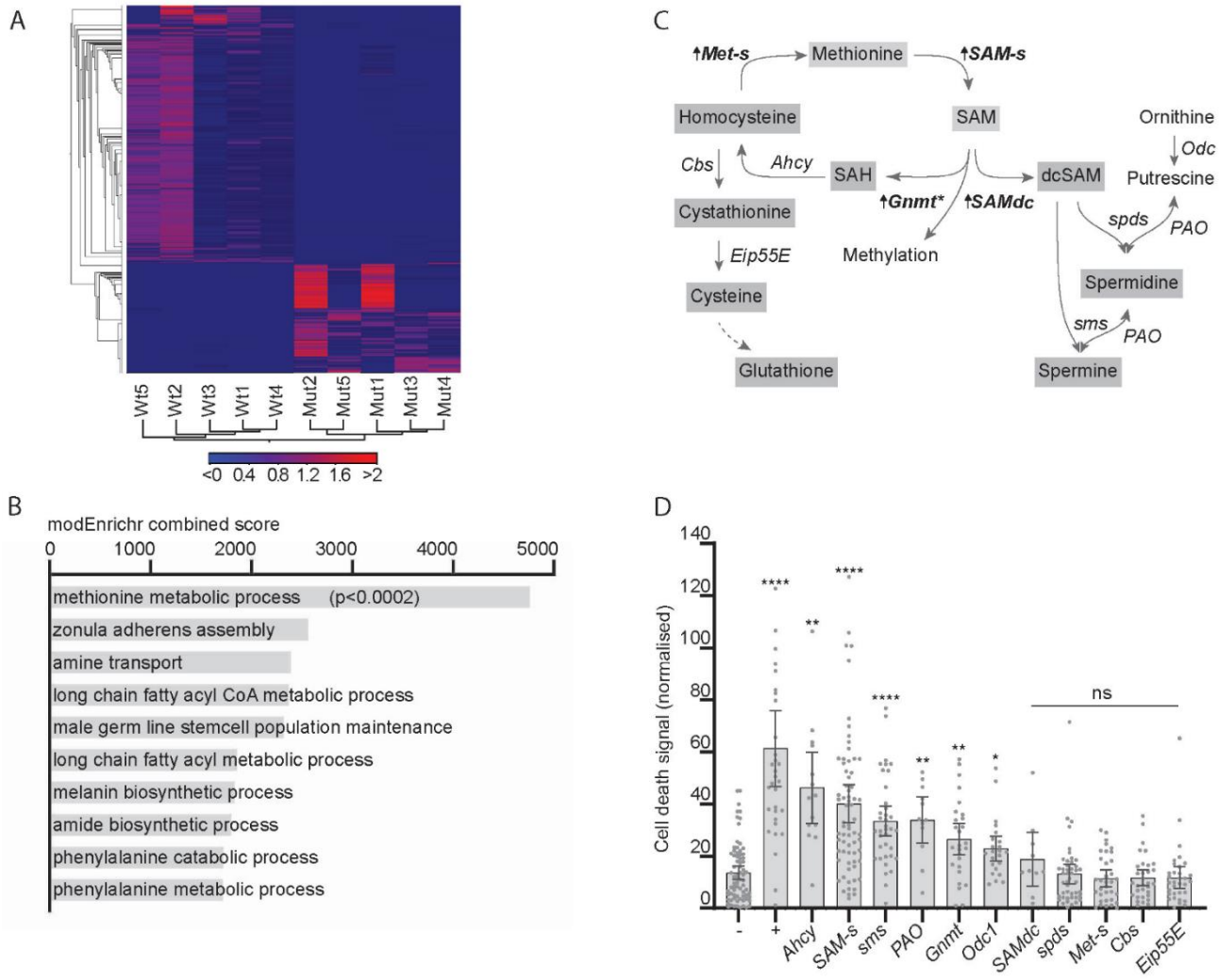

Figure 3-2. The expression of one-carbon metabolism genes is affected by chromosomal instability (CIN) and affects CIN tolerance. (A) Heat map showing changes in gene expression induced by loss of mad2. Mutant (mad2EY/Df(2R)BSC438) and wild type (mad2EY/TM6b) samples show distinct sets of transcriptional changes when clustering by genotype. The scale bar shows the shades of grey representing Z-scores for the significance of changes between genotypes in the heat map. (B) Gene enrichment analysis by modEnrichr shows the top 10 gene ontology hits for biological processes in the GeneRIF library ranked by combined score. Only the top hit, methionine metabolic process (GO:0006555), gave a statistically significant score when corrected for multiple comparisons. (C) One-carbon metabolism pathways, with arrows and bold type indicating changes in transcript expression in response to loss of *Mad2*. *SAM-s*, s-Adenosyl methionine synthase, FDR p < 0.0001; *Gnmt*, Glycine n-methyl transferase, FDR p < 0.01; *SAM*, s-Adenosyl methionine; *SAH*, s-Adenosyl homocysteine; Ahcy, Adenosyl homocysteinase; *Met-s*, methionine synthase, FDR p < 0.02; *Eip55E*, Cystathionine lyase; *SAMdc*, S-Adenosyl methionine decarboxylase, FDR p < 0.07; *dcSAM*, decarboxy-s-Adenosyl methionine. * There are over one hundred *Drosophila* genes with this predicted enzymatic activity (methyl transferases), of which only *Gnmt* expression is significantly elevated in *mad2* mutants. (D)

Cell death in third instar wing discs in response to depletion of *mad2* in combination with one-carbon metabolic gene depletion. Cell death is measured by acridine orange signal normalised to adjacent wild type tissue. Four data points lie outside the graph area. Negative control (−) shows little cell death in response to CIN alone (*w*1118 x *en* > *mad2*RNAi). Positive control (+) shows significant cell death in CIN cells (*JNK* is depleted: UAS-*bsk*RNAi x *en* > *mad2*RNAi; $p < 0.001$). Depletion of expression of some genes related to one-carbon metabolism showed significant cell death in CIN cells compared to the negative control (**** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns > 0.05 using Dunnett's T3 multiple comparisons test).

3.4.2. Knockdown of Candidates of One-Carbon and Polyamine Pathways Caused CIN Cell Death via Apoptosis

To test whether normal cells are similarly sensitive to one-carbon metabolic disruption, we depleted several candidate genes with and without CIN induction (Figure 3-3A). We observed a significantly elevated level of cell death in CIN cells compared to adjacent normally proliferating cells ($p < 0.0001$ in each case). This suggests that this pathway can be targeted to give death specifically in CIN cells. However, the assay used (acridine orange) did not clearly indicate the mechanism of cell death (apoptosis vs. necrosis, etc.). To test whether one-carbon and polyamine metabolic disruption induced apoptosis in CIN cells, we used DCP-1 antibody staining (Figure 3-3B). We observed considerable induction of apoptosis in proliferating CIN tissues when one-carbon genes were depleted. No induction of apoptosis was observed when CIN was induced alone or when one-carbon genes were depleted in normal proliferating cells. (Figure 3-3B). The quantification of these data confirmed the induction of CIN-specific apoptosis in candidate gene knockdown cells (Figure 3-3C). The depletion of some genes in related pathways, such as the folate cycle gene *Shmt*, showed cell death even in the absence of CIN, but this was not observed in the one-carbon or polyamine synthesis pathways. These experiments provided good evidence that one-carbon and polyamine-related candidate gene knockdowns cause CIN-specific cell death via apoptosis.

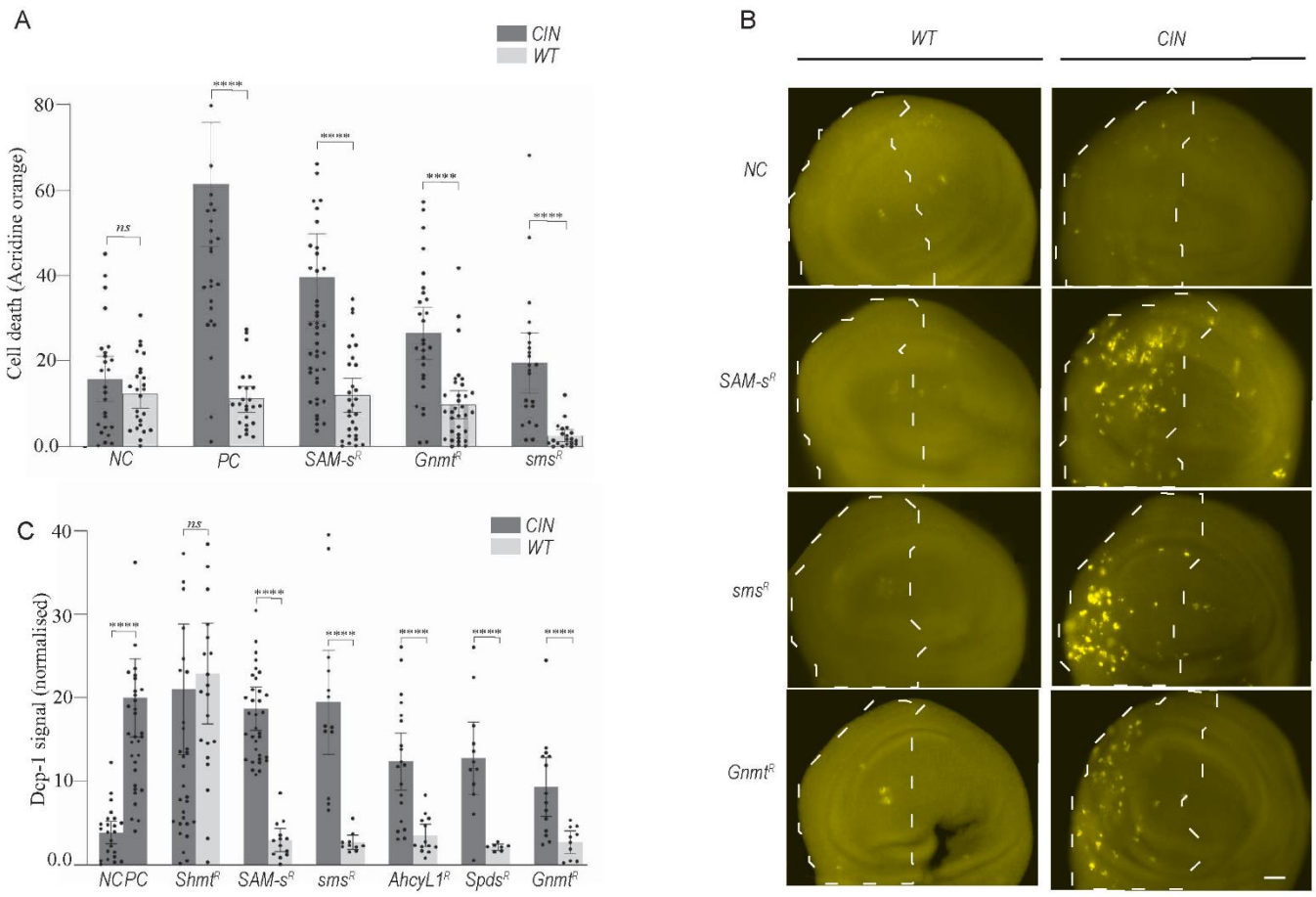

Figure 3-3. Depletion of one-carbon and polyamine metabolic genes causes CIN cell death but not in normally proliferating cells. (A) A quantitative investigation of acridine orange (AO) staining in third instar larvae wing discs with one-carbon metabolic and polyamine pathway genes knocked down with and without CIN (Figure 3-4). For each wing disc, the y axis depicts the normalised AO signals produced by subtracting the mean value of the control region from the affected region. The error bars show 95% confidence intervals (CIs). (B) DCP-1 staining in one-carbon and polyamine candidate RNAi imaginal wing discs with and without CIN. Dashed regions show cells depleted for the candidate gene (and *Mad2* in right panels). Original objective magnification, 20×; scale bar = 20 μm. (C) The normalised DCP-1 signals for each wing disc are shown on the y axis and are obtained by subtracting the mean value of the control region from the affected region. Shaded bars show one-carbon and polyamine candidate knockdowns in CIN cells, and white bars show the candidate knockdowns in wild type cells. In all graphs, the error bars show 95 percent confidence intervals (CIs). Two-tailed t-tests with Welch's correction were used to generate p-values: **** $p < 0.0001$. NC: Negative control; PC: positive control; *SAM-s*: S-adenosyl methionine synthase; *Gnmt*: Glycine n-methyl transferase; *Sms:* Spermine synthase; *Spds:* spermidine synthase; *Shmt:* Serine hydroxymethyl transferase; *Ahcy:* Adenosyl-homocysteinase.

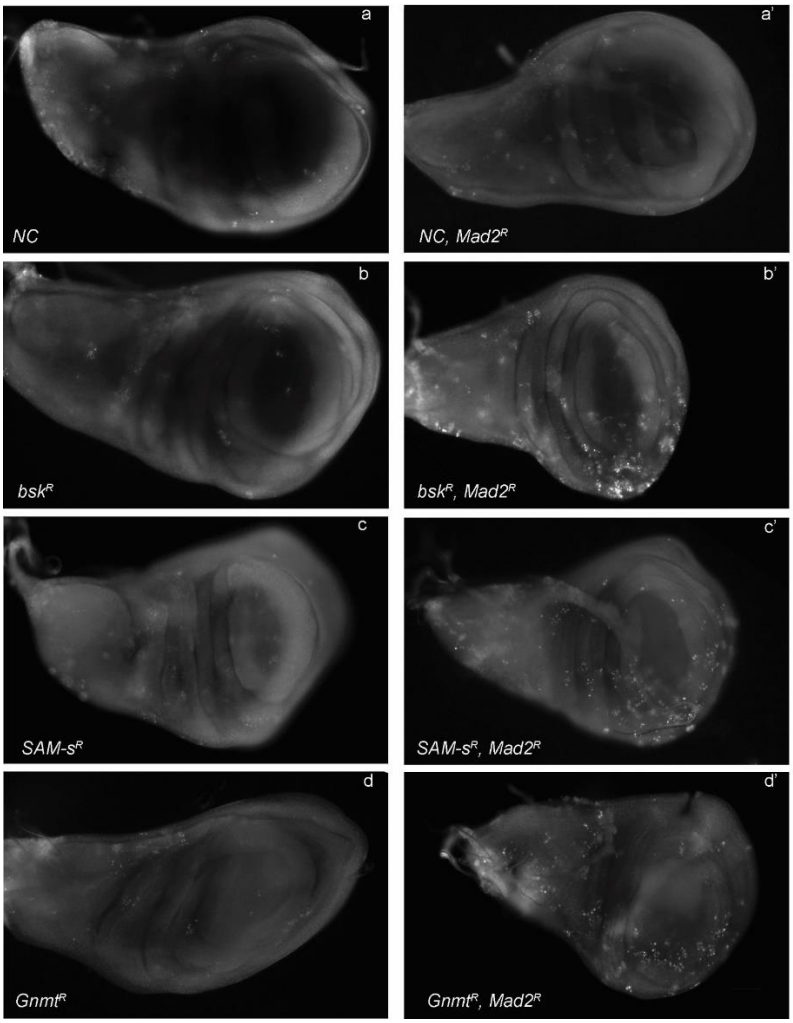

Figure 3-4. Cell death by acridine orange staining in third instar wing discs to examine the effects of *Mad2* in combination with one-carbon metabolic gene depletion. Depletion of *SAM*-s, *Gnmt* did not show much cell death (c, d) in normal proliferating cells, however, in the response to aneuploidy cell death (c', d') markedly increased. Negative control with (a') and without (a) CIN. Dashed area of the wing disc indicates affected GFP positive area. Scale bar 20 μm and original magnification 10x . Positive control with (b') and without (b) CIN. *SAM-s*: S-adenosyl methionine synthase, *Gnmt:* Glycine n-methyl transferase, *bsk:JNK*, Lac-Z: Negative control.

3.4.3. Feeding of Polyamines Rescued CIN Cell Death Caused by *SAM-s* or *Sms* Depletion

The depletion of *SAM-s* or *Spds* should decrease the level of spermidine, and the knockdown of *Sms* will similarly decrease the synthesis of spermine (see Figure 3-2C). Because the depletion of any of these enzymes showed death in CIN cells, it seemed plausible that some of the effect of *SAM-s* loss was being mediated by its effect on polyamine synthesis. To test this, we fed spermine to larvae knocked

down for *SAM-s* and *sms* in cells with CIN induced by *mad2* depletion. We observed that feeding spermine significantly rescued the AO cell death phenotype caused by the depletion of *SAM-s* in CIN cells. (Figure 3-5 A,B). As expected, the feeding of spermine was able to strongly rescue the depletion of spermine synthase in CIN cells (Figure 3-5 C,D). A similar rescue of the CIN cell death phenotype caused by *SAM-s* knockdown was seen when larvae were fed with spermidine (Figure 3-6).

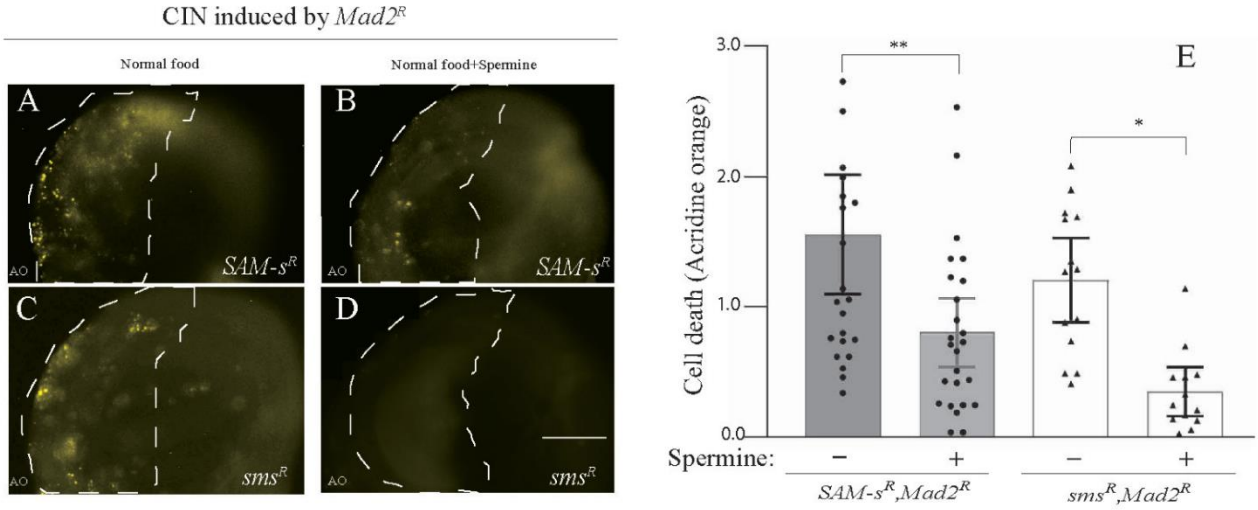

Figure 3-5. The CIN cell death phenotype caused by the depletion of SAM-s and sms was rescued by feeding polyamines. The depletion of *SAM-s* (A,B) and *Sms* (C,D) in CIN cells (induced by *mad2* RNAi) showed high AO staining, which was rescued by supplementing the larval diet with spermine (0.1 mM). (E) *SAM-s* knockdowns in CIN cells are represented by shaded bars with and without spermine, whereas the white bars show *Sms* knockdown in CIN cells with and without spermine feeding. In all cases, $n \geq 13$, and the error bars represent 95% Cis. The p-values were calculated by a multiple comparison test: ** $p < 0.0053$, * $p < 0.0245$. The scale bar is 50 μm, and the original magnification is 20×. Spermine − represents normal food; spermine + represents normal food + 0.1 mM spermine.

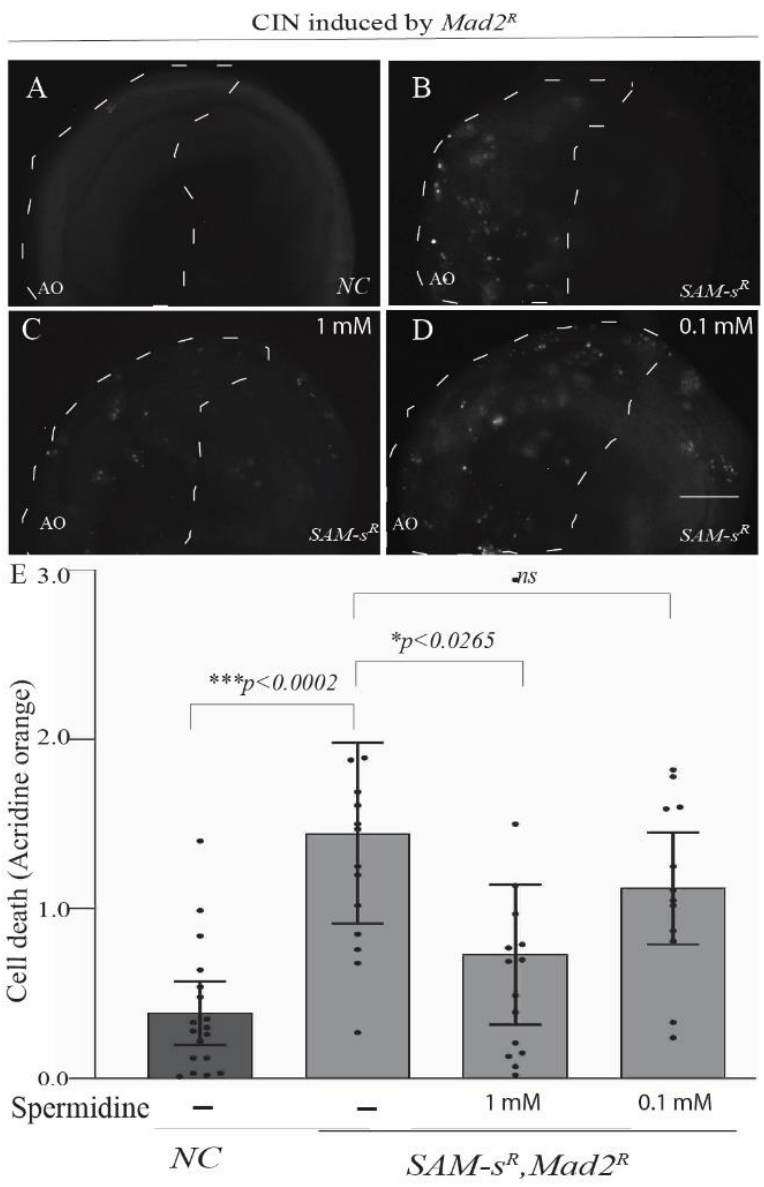

Figure 3-6. The CIN cell death phenotype caused by depletion of *SAM-S* was rescued by feeding spermidine. The depletion of *SAM-s* (B) in CIN cells (induced by *Mad2* RNAi) showed high AO staining compared to the negative control (A), which was rescued by supplementing the larval diet with 1 mM spermidine (C, E). In all cases n≥12 and the error bars represent 95% CIs. The P-values were calculated by multiple comparison test ***P*<0.0002, **P*<0.0265. The scale bar=50 μm and original magnification 20x. Spermidine **−**: normal food + no drug (A,B), Spermidine 1mM = Normal food + 1 mM spermidine (C); and Spermidine 0.1mM=Normal food+0.1 mM spermidine (D). NC: Negative control, *SAM-s*: S-adenosyl methionine synthase. 0.1 mM spermidine (D, E) does not have significant effect to rescue the cell death due to *SAM-s* (B) knockdown in CIN cells. NC:negative control, *SAM-s*: S-adenosyl methionine synthase.

3.4.4. Antioxidant Feeding Rescued CIN Cell Death Phenotypes Caused by One-Carbon or Polyamine Metabolic Gene Depletion

One of the roles of S-Adenosyl methionine is to provide homocysteine for antioxidant synthesis (for glutathione, see Figure 3-2C). Knowing that redox stress is a significant vulnerability of CIN cells [4], it was plausible that the loss of antioxidant response might be contributing to CIN cells' vulnerability to *SAM-s* depletion. We carried out ROS assays to examine whether the depletion of *SAM-s* may elevate oxidative stress in CIN cells. We found that the knockdown of *SAM-s* showed an increase in ROS in CIN cells (Figure 4A,B). Having observed elevated cell death in this genotype (Figure 3-3), we tested whether ROS were contributing to this cell death by feeding the animals an antioxidant (N-acetyl cysteinamide, NACA). We found that NACA feeding significantly reduced the CIN cell death phenotype compared to no drug feeding in the wing disc of third instar larvae (Figure 3-7C,D). This was consistent with S-adenosyl methionine being a vital antioxidant precursor molecule in CIN cells. However, we had seen a strong rescue of the Sam-s phenotype by feeding spermine (Figure 3-5), so we postulated whether the redox stress might be resulting from the disruption of the polyamine pathway rather than from the lack of homocysteine. To test this, we checked ROS levels in *sms*-depleted CIN cells and found that they were elevated (Figure 3-7F) compared to in control CIN cells (Figure 3-7A). We did the same antioxidant feeding experiment with the *sms* mutant animals and found that NACA significantly rescued the cell death seen in *sms*-depleted CIN cells (Figure 4G–I). From these data, we conclude that the depletion of either *SAM-s* or *Sms* leads to redox stress in CIN cells, which significantly contributes to the cell death seen in these genotypes.

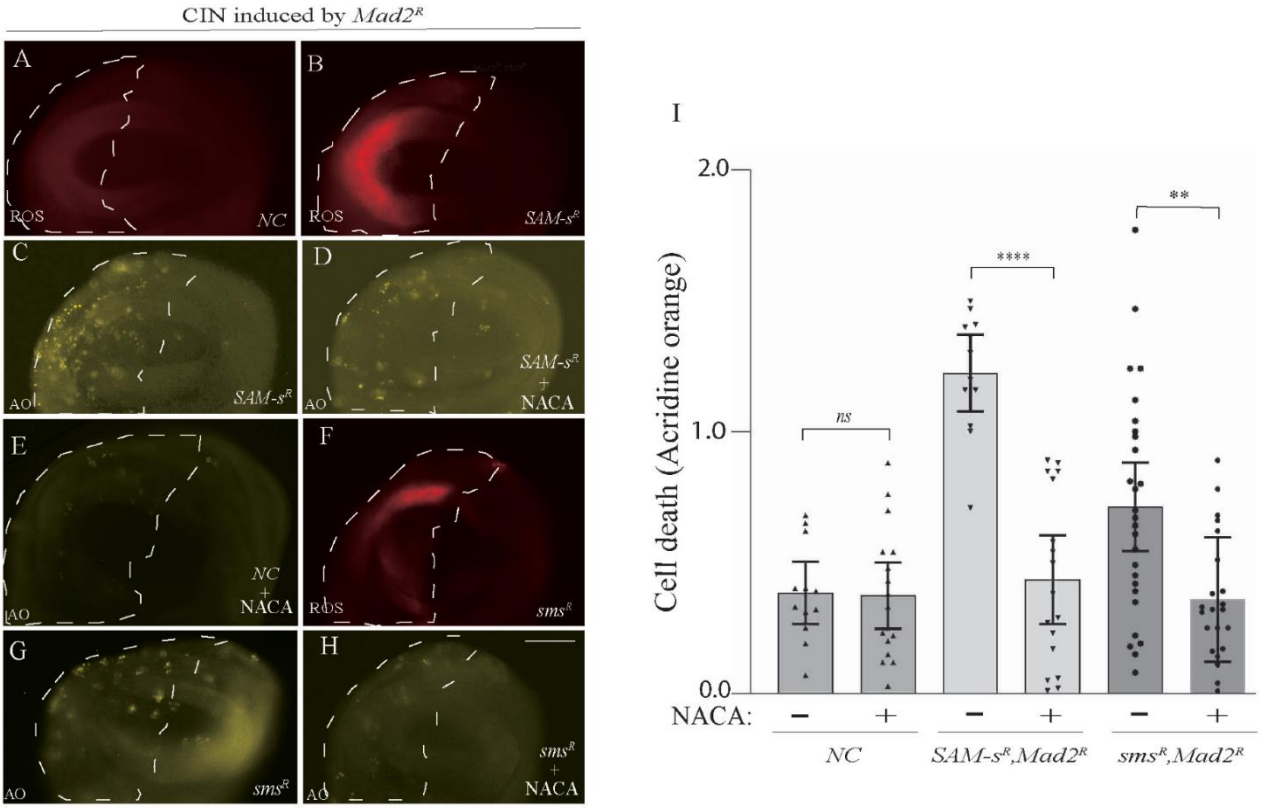

Figure 3-7. CIN cells were sensitive to polyamine levels because of their effect on the production of reactive oxygen species. ROS levels (DHE staining) were elevated in *Sam-s*-depleted CIN cells (B) compared to control CIN cells (A). Cell death (acridine orange staining) was decreased by feeding SAM-s-depleted CIN cells with the antioxidant N-acetyl Cysteinamide (NACA; compare (C) with (D) and negative control (E)). ROS levels are also elevated in Sms-depleted CIN cells (F). NACA feeding is also able to rescue the cell death seen in *Sms*-depleted CIN cells (compare (G) and (H)). Quantification of CIN cell death with and without NACA feeding in *SAM-s*- and *Sms*-depleted cells is shown in (I). The indicated genes were knocked down in the posterior half of each wing disc as indicated by the dotted line, while the rest of each disc was wild type. The p-values were calculated by multiple comparison tests: **** $p < 0.0001$, ** $p < 0.0020$. The scale bar is 50 μm, ns = not significant, and the original magnification is 20×. The different shading of bars indicates different genotypes (wild type, *SAM-s*, and *Sms*) with or without NACA drug feeding. In all cases, $n > 12$, and error bars indicate 95% confidence intervals.

3.4.5. CIN Cell Death from SAM-s and Sms Knockdown Responded to Autophagy

We previously found that autophagy is crucial for CIN cells' survival and that increasing autophagy could prevent CIN cell death in response to metabolic disruptions [5]. To test whether autophagy also affected *SAM-s*-depleted CIN cells, we checked cell death levels when we increased or decreased autophagy genetically (Figure 3-8). We found that decreasing autophagy by depleting Atg1 caused an increase in the AO staining seen in *SAM-s*-depleted CIN cells (Figure 3-8C,D). We also found that depleting SAM-s with *Atg18* was synthetically lethal in CIN animals. Although this change is in the direction we would predict, it could have been caused by an accumulation of unrelated cellular disruptions. To demonstrate that the effect of *SAM-s* knockdown depends on autophagy, we increased autophagy by *mTor* RNAi knockdown. Releasing autophagy from *mTOR* inhibition strongly rescued the CIN cell death phenotype caused by *SAM-s* RNAi (Figure 3-8E vs. Figure 3-8C).

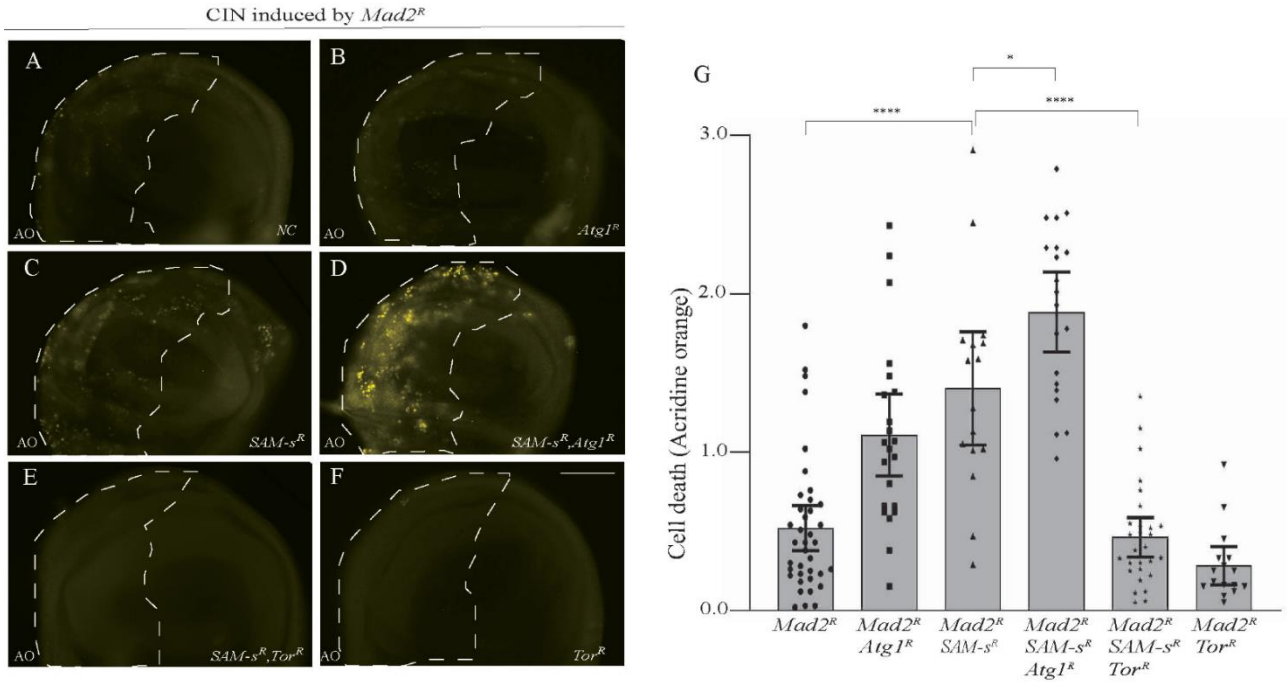

Figure 3-8. Blocking of autophagy by *Atg1* knockdown increased cell death in *SAM-s*-depleted CIN cells (**A**–**D**). Depletion of *SAM-s* with *Atg1* in CIN cells (induced by *Mad*2 RNAi) showed high AO staining (**D**) compared to only *SAM-s* knockdown (**C**), only *Atg*1 knockdown (**B**), or control CIN cells (**A**). Knockdown of *mTor* in *SAM-s*-depleted CIN cells rescued the cell death phenotype (**E**) to a level resembling the negative control of *mTor* alone (**F**). Quantification and statistical comparison of AO signals is shown in (**G**). In all cases, n ≥ 16, and the error bars represent 95% CIs. The *p*-values were calculated by multiple comparison test: **** $p < 0.0001$, * $p < 0.0418$. The scale bar is 50 μm and the original magnification is 20×.

3.5. Discussion

Aneuploidy resulting from CIN is a common feature of solid tumours [6]. Abnormal cell development, proliferation, proteotoxic stress, and oxidative stress can all be caused by aneuploidy [7,8]. As the adaptability to these various stresses is necessary for survival during continual chromosomal gain or loss, cellular stress responses are a plausible candidate for being the target of cancer-specific apoptosis. The questions then becomes whether CIN cells inevitably approach a tolerance threshold as a result of the constraints of high energy utilisation, ROS formation, proliferation, and continuing genotoxic stress and whether we can leverage this vulnerability. The present study investigated the hypothesis that alterations in one-carbon and polyamine metabolism can contribute to surviving chromosomal instability (CIN), a hallmark of cancer. This study was based on our RNA sequencing results, which showed an increased transcription of one-carbon and polyamine genes in CIN cells compared to the wild type. Our results provide evidence that supports this hypothesis and suggests that targeting these metabolic pathways could represent a promising strategy for the development of novel cancer therapies. We report that the suppression of one-carbon and polyamine activity in wing imaginal disc cells exhibiting chromosomal instability (CIN) results in highly elevated levels of cell death not observed with either CIN or candidate gene knockdown alone. From these results, we conclude that one-carbon and polyamine metabolism are needed for the survival of CIN cells. Specifically, we found that SAM-s or sms knockdowns killed CIN cells but not normal proliferating cells and that the severity of these effects depended on oxidative stress and autophagy. We found that polyamine or *SAM-s*-depleted CIN cells showed oxidative stress and that their survival could be significantly enhanced by either adding antioxidants or increasing the level of autophagy.

The methionine cycle, which is connected to the folate cycle, polyamine synthesis, and the trans-sulphuration route, is a key component of one-carbon metabolism (Figure 3-2C) [9,10]. S-adenosyl methionine synthase (*SAM-s*) is the enzyme used to create S-adenosylmethionine (SAM) from methionine in the first phase of the methionine cycle. SAM is used for three main purposes: the methylation of protein and DNA substrates, the generation of antioxidants such as cysteine and glutathione, and the synthesis of polyamines. Any of these processes could have been the cause of the cell death seen when SAM was depleted in the CIN cells, so we used a genetic approach to identify

what was most important. SAM is needed to maintain glutathione and glutathione-S-transferase (GST) levels as well as SOD activity [11]. Hence, it appears likely that one important function of folate and methionine is to enable the efficient regeneration of SAM and, subsequently, glutathione when subjected to oxidative stress. Reactive oxygen species (ROS) levels influence how cancer develops by either supporting carcinogenesis at low levels or by causing cell death at high ones [12]. Due to their abnormal metabolism, tumour cells frequently produce many ROS and have evolved to withstand oxidative stress via a variety of mechanisms, including the production of antioxidants such as glutathione. Even cells that are otherwise normal become redox-stressed when CIN is induced due to a range of mechanisms including ER stress and mitochondrial disruption [2,4,13-18]. It seemed plausible that a key reason for needing SAM in CIN cells was to maintain glutathione levels. However, our data cast some doubt on this. We did not see the increased transcription of key enzymes in that pathway (such as cystathionine synthase, Cbs) in CIN cells nor were CIN cells sensitive to the depletion of Cbs or cystathionine lyase, Eip55E (Figure 3-2D). Nonetheless, we saw the rescue of the *SAM-s* phenotype in CIN cells when antioxidants were provided in the form of NACA feeding, and we show clear evidence for redox stress when SAM is depleted.

The second pathway that depends on SAM is methylation. There are over a hundred methylase enzymes that can use SAM to pass a methyl group onto a huge array of possible substrates [19]. Any of these could mediate the dependency on *SAM-s;* however, we saw a transcriptional response to CIN in only one enzyme, namely, glycine n-methyltransferase, *Gnmt* (Figure 3-2C), which is also needed for CIN cell survival (Figure 3-3). The metabolic consequences of *Gnmt* depletion are not obvious as its product, sarcosine, has no known metabolic roles [20]. Current models for the significance of *Gnmt* include its effect on autophagy and polyamine levels [21], which encouraged us to pursue these as possible explanations (see below).

The final metabolic pathway that depends on SAM is polyamine synthesis. SAM is decarboxylated by SAM decarboxylase (*SAMdc*), which produces s-adenosyl methioninamine or dcSAM, a crucial aminopropyl donor for the synthesis of spermidine. The other component of spermidine is produced via putrescine from ornithine via ornithine decarboxylase (ODC). Spermidine synthase produces spermidine by combining putrescine with dcSAM. The last step in this pathway route, spermine, is

produced by spermine synthase by combining spermidine with an additional aminopropyl group from dcSAM. We saw a modest increase in *SAMdc* transcription in CIN animals, and a strong, reproducible dependency on polyamine synthesis for CIN survival in multiple assays (Figure 3-2, Figure 3-3, Figure 3-5 and Figure 3-7). SAM is needed for polyamines (spermine and spermidine), and previous work has shown that these polyamines can affect rates of autophagy [22-24]. In flies, yeast, worms, and human cells, spermidine has been shown to trigger autophagy [23,25]. For example, spermidine regulates autophagy by modifying the expression of autophagy-related genes (*Atg*s) and the gene expression regulators elF5A and TFEB [26,27]. Spermidine also lowers acetyl-CoA availability, which diminishes acetylation and increases autophagy [25]. Polyamines interact with DNA and RNA and are strongly implicated in DNA condensation. This may be relevant as polyamine compaction can protect genomic integrity [28]. Polyamines have other functions, including as antioxidants and for protein synthesis, and CIN cells may also be dependent on these to some degree; however, we saw a strong effect by altering autophagy. Most strikingly, we were able to rescue the depletion of *SAM-s* either by adding spermine to the diet or, even more strongly, by increasing autophagy. These results strongly suggest that the primary dependency on SAM in CIN cells is not for methylation or for glutathione synthesis but rather to generate sufficient polyamines to support robust autophagy.

Our study also highlights the potential therapeutic implications of targeting one-carbon and polyamine metabolism in CIN-associated cancers. SAM has been investigated as a therapeutic target in the treatment of cancer because aberrant methylation states and methionine or SAM dependence are frequent characteristics of cancer [20,29-36]. Additionally, rats given SAM showed an increase in apoptosis in neoplastic nodules and hepatocellular carcinoma [31,34]. SAM treatment increased apoptosis and reduced proliferation and the invasiveness of breast cancer cells in vitro while lowering carcinogenesis and metastasis in vivo [37]. The ability of several cancer cell lines to proliferate and spread has been demonstrated to be effectively inhibited by *SAM* [38,39]. This evidence points to the need for homeostasis: on one hand, many cancers are methionine- and SAM-dependent, but, on the other, they may be inhibited by additional SAM. This may be partly explained by the effect of SAM levels on autophagy as elevated SAM can increase mTORC levels, which blocks autophagy [40]. The loss of SAM means the loss of polyamine synthesis and, hence, the loss of autophagy as described

above. Thus, the maintenance of SAM metabolism within normal ranges is important to avoid disruptions to autophagy.

Some phenotypic effects of CIN are reproducible, even though the genetic disruption is essentially random in each cell [2,41]. There is evidence that chromosomal instability causes sensitivity to metabolic stress [4], protein folding stress, and nucleotide depletion [13]. Proteotoxic stress, faulty mitochondria, and oxidative damage from ROS are three associated abnormalities reliably seen in aneuploid cells that autophagy has been shown to ameliorate [4,42]. To that list, we now add one-carbon and polyamine metabolism. From our results, it seems that cells with chromosomal instability upregulate genes that generate SAM and its polyamine metabolites primarily to increase autophagy, which is needed to survive the stressful metabolic environment of CIN cells.

In conclusion, our study provides new insights into the molecular mechanisms underlying CIN and cancer progression and highlights the importance of one-carbon and polyamine metabolism in regulating autophagy and oxidative stress in CIN cells. Our findings have potential clinical implications for the treatment of CIN-associated cancers, and further studies are warranted to explore the therapeutic potential of targeting one-carbon and polyamine metabolism in combination with other therapies.

3.6. References

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CHAPTER 4: ANEUPLOIDY IS LINKED TO NEUROLOGICAL PHENOTYPES THROUGH OXIDATIVE STRESS

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4.1. Abstract

Aneuploidy, having an aberrant genome, is gaining increasing attention in neurodegenerative diseases. It gives rise to proteotoxic stress as well as a stereotypical oxidative shift which makes these cells sensitive to internal and environmental stresses. A growing body of research from numerous laboratories suggests that many neurodegenerative disorders, especially Alzheimer's disease and frontotemporal dementia, are characterised by neuronal aneuploidy and the ensuing apoptosis, which may contribute to neuronal loss. Using *Drosophila* as a model, we investigated the effect of induced aneuploidy in GABAergic neurons. We found an increased proportion of aneuploidy due to *Mad2* depletion in the third-instar larval brain and increased cell death. Depletion of *Mad2* in GABAergic neurons also gave a defective climbing and seizure phenotype. Feeding animals an antioxidant rescued the climbing and seizure phenotype. These findings suggest that increased aneuploidy leads to higher oxidative stress in GABAergic neurons which causes cell death, climbing defects, and seizure phenotype. Antioxidant feeding represents a potential therapy to reduce the aneuploidy-driven neurological phenotype.

4.2. Introduction

Aneuploidy is the state in which cells carry an abnormal DNA complement [1]. Generally, the distribution of aneuploid chromosomes varies only by one or a small number of chromosomes from wild type. Numerous genetic and environmental causes can cause aneuploidy, which has long been recognized as a significant contributor to human diseases, particularly in the context of cancer. However, the potential role of aneuploidy in non-malignant disorders, such as neurological diseases, has gained increasing attention in recent years. Aneuploidy has been linked to an increased incidence of neurodegenerative diseases and with our aging population, these diseases are becoming more common [2,3]. Aneuploidy is well tolerated in the young brain, but aneuploid neural cells are sensitive to age related metabolic disorders and senescence that impact motor function and lifespan [4] as well as mental health [5]. Aneuploidy has been observed in the neural tissues of people who have neurodegenerative diseases, raising the question of whether it contributes to the onset and development of these disorders.

Owing to the in vivo inaccessibility of human brain tissue, several researchers used peripheral cells such as

lymphocytes and fibroblasts to examine the association between genomic damage and neurodegenerative diseases such as Alzheimer's disease (AD). Several studies have been published with a link between AD and enhanced peripheral aneuploidy [6-10]. Since then, a connection between aneuploidy and AD has been confirmed by various studies that show a role for aneuploidy, particularly hyperploidy, early in family and sporadic AD [11-13]. Alzheimer's brain disease has been shown to have elevated levels of spontaneous aneuploidy. [12,14-16]. Consistent with these findings, Alzheimer's disease genes are involved in molecular mechanisms that cause changes in chromosome missegregation and aneuploidy [17]. Even in the absence of disease, neurological aneuploidy is not rare. As many as a third of neuroblasts are aneuploid in developing mouse brains, a number which declines during development, but the remaining aneuploid neurons are incorporated into the circuitry [18]. Despite the mounting evidence suggesting a connection between aneuploidy and neurological diseases, the specific mechanisms by which aneuploidy might lead to neurological phenotypes remain poorly understood. Therefore, understanding the role of aneuploidy in the progression of neurodegeneration is important for successful therapeutic interventions.

To investigate the mechanisms by which aneuploidy changes neurological phenotypes, we used *Drosophila* as a model to knockdown *Mad2* specifically in GABAergic neurons. These cells are of particular interest as inhibitory neurons that are affected in epilepsy, Huntington's and other neurological diseases [19]. *Mad2* (Mitotic Arrest Deficient 2) is an essential Spindle Assembly Checkpoint (SAC) protein. The spindle assembly checkpoint, which guarantees normal chromosomal segregation during cell division, is a crucial regulatory checkpoint involved in preventing aneuploidy. Consequently, depletion of *Mad2* has been found to cause aneuploidy in a range of tissue types [20,21].

This study aims to address the gap in our understanding of how increased aneuploidy, following *Mad2* depletion, may contribute to the development of a neurological phenotype in a *Drosophila* model. We hypothesized that elevated aneuploidy induced by *Mad2* depletion could result in oxidative stress in neural tissues, ultimately leading to neurological dysfunction which might be rescued by feeding antioxidants. Antioxidants are endogenous or exogenous substances either prevent free radical formation or react with them to neutralise, potentially shielding the cell from oxidative damage. We can feed antioxidants that pass the blood brain barrier [22] and our previous published data revealed that antioxidant feeding can rescue cell death in other aneuploid cell types [23].

This study sheds light on the intricate molecular pathways that connect aneuploidy and neurobiology, with implications for future research in both model organisms and human neurodegenerative diseases.

4.3. Materials and Methods

4.3.1. Aneuploidy analysis by karyotyping

Third-instar larval brains were dissected in 1× PBS, and then the brain was soaked in 0.5% sodium citrate for 5 min. After that, it was transferred to the 45% acetic acid for 2 min. Then each brain was moved to a drop of 60% acetic acid on a coverslip for 1 min. The labelled glass slide was gently placed on the cover slip, inverted, and then squashed very hard with a thumb under filter paper. Then the slide was quickly placed in liquid nitrogen for 10 min, and after that, slides were removed from liquid nitrogen, and the coverslip flicked off with a scalpel. Then slides were placed in 100% ethanol for 10 min after that tissue was washed with 1×PBS and stained with Hoechst 33,342 solution (2 µg /ml) for 10 min. After staining, tissues were washed with 1×PBS and mounted with 80% glycerol. Finally, slides were ready for taking pictures for karyotyping using the 20×objective lens on an Olympus IX71 microscope.

4.3.2. Cell death Analysis by Hoechst stain

The slides for cell death analysis were prepared as described above for aneuploidy analysis. The pictures were taken under a 20×objective lens on an Olympus IX71 microscope. The bright, pyknotic, and round-shaped nuclei were considered to be undergoing cell death in our analysis, and we manually counted the numbers blinded for each genotype.

4.3.3. *Drosophila* Stocks

Drosophila were cultured in 12-h light/dark cycles at 25^0C on fortified medium (1% agar, 1% glucose, 6% fresh yeast, 9.3% molasses, 8.4% coarse semolina, 0.9% acid mix and 1.7% Tegosept). The following *Drosophila* lines were obtained from the Bloomington *Drosophila* Stock Centre (Indiana, USA): W^{1118} (BL3605), *GAD1-GAL4* (BL51630). The *GAD1-GAL4* line (BL51630, 3.089kb fragment of the GAD1 promoter) drives GAL4 expression in GABAergic neurons [24,25]. The UAS-$mad2^{RNAi}$ (VDRC 47918) obtained from Vienna *Drosophila* Resource Centre, Austria. The polyalanine repeat sequence stock (GCA90) was obtained from Dr Louise O'Keefe's laboratory at the University of Adelaide [26]. The expression pattern of *GAD1-GAL4* driver is shown Figure 4-2 [27]. In all cases where there is a negative control, it is generated from the cross between *Gad1-Gal4* with w^{1118}, while the experimental cross is *Gad1-Gal4* with UAS-$Mad2^{RNAi}$.

4.3.4. Climbing assays.

Climbing assays capitalize on the natural tendency of flies to climb, known as negative geotaxis. Climbing assays were performed according to the previous published methodology with little modification [26]. Climbing assays were performed on flies either wild type control, *Mad2* depleted flies and polyalanine repeat sequence stock (GCA90) under the *GAD1-GAL4* driver. For every genotype, three sets of 15 to 20 freshly enclosed male or female flies were gathered and maintained at 25°C on fortified medium until the test was carried out. When the flies were analysed, they were all 8–12 days old. Each batch of flies was moved to a 500 ml measuring cylinder with a 48 mm diameter and a parafilm-sealed lid and allowed to acclimatise for 3 minutes before the cylinder was tapped on the bench to give twenty strong mechanical shocks. A Dino-Lite digital microscope (Product# AD3713TB; AnMo Electronics Corporation, New Taipei City, Taiwan) was used to record and save the videos of all the experiments. Videos were replayed to count the number of flies remaining below the 1cm mark, between 1-5 cm mark and above the 5cm mark after 40 seconds. The polyalanine repeat sequence stock (GCA90) was obtained from Dr Louise O'Keefe's laboratory as a positive control known to be defective in climbing assays [26]. The Chi Squared test was used to analyse the proportion of climbing defects between each genotype compared to the wild-type control.

4.3.5. Bang-Sensitive Behavioural Assays to Investigate a Seizure-Like Phenotype

The bang-sensitivity behavioural assay (banging assay) was used to measure the recovery of *Drosophila* from seizure-like activities induced by mechanical shock and was performed as described previously [28]. Experiments were performed between 8am and 11am to minimise the potential effects of circadian oscillation on animal activity. Between ten and twenty *Drosophila* (females and males) aged between 8 to 12 days after eclosion were collected under CO2. The *Drosophila* were transferred to an empty clear 500 ml measuring cylinder and allowed to acclimatise for 3 minutes before the cylinder was tapped on the bench to give twenty strong mechanical shocks. We considered the flies for seizure like phenotype based on the following characteristics: unusual loss of posture, erratic flapping and buzzing of the wings, leg shaking, spinning, and uncontrollably flying, as well as total immobilisation and falling during clumsy attempts at rising and flying. A Dino-Lite digital microscope (Product# AD3713TB; AnMo Electronics Corporation, New Taipei City, Taiwan) was used to record and save the videos of all the experiments. Videos were replayed to score the *Drosophila* behaviour. *Drosophila* showing seizure-like behaviour were counted for 30 seconds. The Fisher's exact test was used to analyse the data between genotypes compared to the wild type

control. The polyalanine repeat sequence stock (GCA90) was obtained from Dr Louise O'Keefe's laboratory as a positive control for seizure assays [26].

4.3.6. Drug Treatments

Drugs were purchased from Sigma. Drugs were mixed with common fly food for larvae (water, molasses, yeast, glucose, acid-mix, agar, semolina, Tegosept), and when the mixture solidified, they were administered to the host flies. The antioxidant utilised was N-acetylcysteine amide (NACA) 0 µg /ml, 100 µg /ml, 200 µg/ml and 400 µg/ml.

4.3.7. Data Analysis

GraphPad Prism was utilised for statistical analysis. Comparisons of proportions were carried out using Fisher's exact test where possible; otherwise, chi-squared tests were used (where there were multiple outcomes such as Fig. 2A). Where samples had skewed distributions (e.g. Figure 4-1F), Mann–Whitney testing was used rather than a t test. All error bars indicate 95% confidence intervals for the mean.

4.4. Results

4.4.1. *Mad2* depletion in GABAergic neuron increased the aneuploidy rate in larval brain which enhance cell death.

To investigate the effect of *Mad2* depletion in GABAergic (inhibitory) neurons, we used *Drosophila* as a model, depleting *Mad2* by RNA interference using the Gad1-Gal4 driver. Knockdown of *Mad2* in GABAergic neurons gave a significant increase of the aneuploidy rate in 3rd instar larval brains compared to the wild type control (Fig-1C). We observed losses of a chromosome pair or one chromosome from the pair. (Fig-1A, 1B). This confirmed that we were able to generate the desired modest increase in aneuploidy in the central nervous system. Previous published results found that increased aneuploidy enhanced oxidative stress and cell death [29-31]. Different brain pathologies and clinical characteristics are present in age-associated neurodegenerative illnesses, all of which are linked to decreased neuronal numbers in particular brain regions [32]. Therefore, we wanted to see whether enhanced aneuploidy had any detrimental effects on neuronal cells. To measure cell death, we counted pyknotic nuclei in third instar larval brains. We found that cell death significantly increased in *Mad2* depleted larval brains compared to the wild type control (Figure 4-1D,1E,1F). Because increased aneuploidy enhanced brain cell death in early stages of development, we then looked for neurological defects, particularly locomotor defects, in adult *Drosophila.*

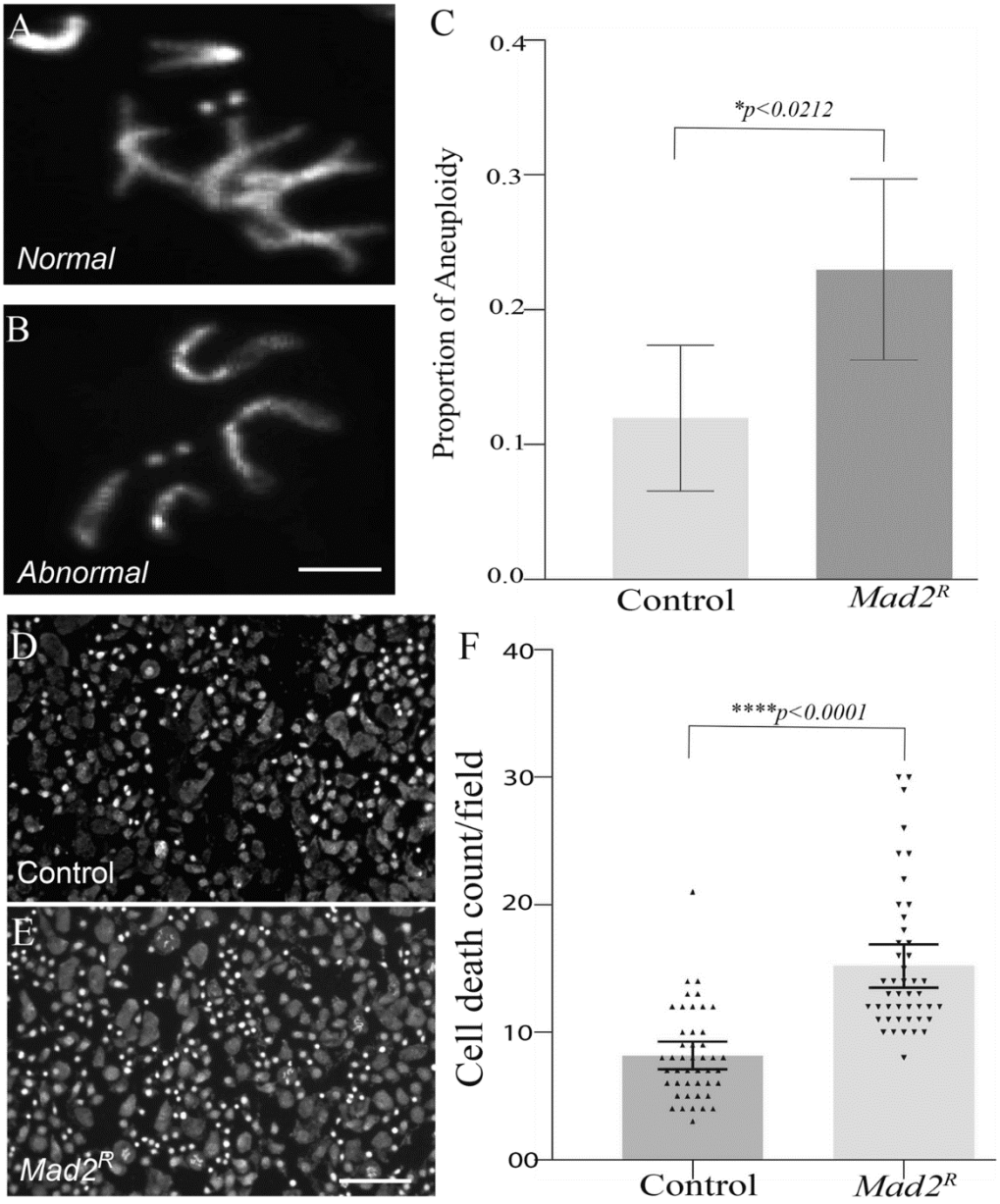

Figure 4-1: *Mad2* depletion in GABAergic neurons increased the proportion of aneuploidy and cell death. A Normal karyotype with four chromosome pairs; B abnormal karyotype missing a chromosome pair; and C analysis of aneuploidy proportion in wild-type controls and *Mad2*-depleted larval brains. There are aneuploid cells in wild-type brains, but the proportion of karyotypes that were aneuploid significantly increased in *Mad2*-depleted brain cells compared to the control (C). The p-values were calculated by Fisher's exact test *$p < 0.0212$, $n > 130$ karyotypes from ≥ 7 animals for each genotype. The scale bar = 50 μm. Hoechst stain was used to visualise DNA. D DNA staining in a wild-type larval brain; F DNA staining in a *Mad2*-depleted larval brain; and E showing the analysis of pyknotic nuclei in wild-type vs *Mad2*. The p-values were calculated by the Mann–Whitney test ****$p < 0.0001$. The scale bar = 50 μm, $n = 45$ from ≥ 8 animals for each genotype.

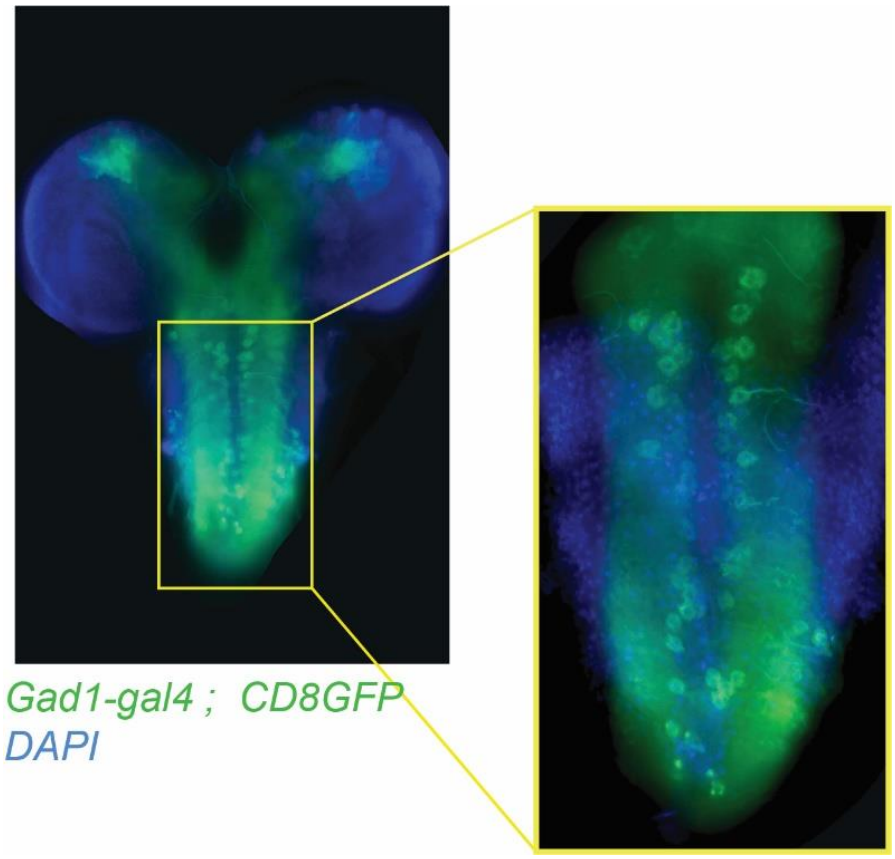

Figure 4-2: The expression pattern of Gad1-Gal4 driver visualised by CD8-GFP in a 3rd instar *Drosophila* larval central nervous system.

4.4.2. *Mad2* depletion in GABAergic neurons caused neurological phenotypes.

We carried out neurological phenotype analysis based on previous experimental approaches known to detect neurodegeneration. The climbing assay has revealed neurodegenerative defects in the investigation of numerous conditions such as Alzheimer's disease [33]. We measured climbing performance in 8- to 12-day-old flies, recording the proportion that were unable to reach the 1cm mark, those between 1-5 cm mark and those above the 5cm mark (normal climbing ability). *Mad2* depletion in GABAergic neurons significantly degraded climbing ability compared to the wild type control (Figure 4-3A), close to the level of functional impairment seen when a well-characterized neurodegeneration was induced by overexpression of a polyalanine repeat sequence (+ve control) [26]. We also carried out seizure phenotype analysis in 8- to 12-day-old flies. In *Drosophila*, a seizure-like phenotype can be generated by a range of physical stressors. It is characterized by an unusual loss of posture, erratic flapping and buzzing of the wings, leg shaking, spinning, and uncontrolled flying, as well as total immobilisation and falling during clumsy attempts at rising and flying. The incidence of these phenotypes has been extensively used as a measure of neurological impairment in *Drosophila* models for Alzheimer's, Huntington's and Parkinson's disease [34-36]. *Mad2* depletion in GABAergic neurons significantly increased the incidence of seizure-like phenotypes compared to the wild type control (Figure 4-3B), reaching a level comparable with the repeat sequence positive control. Because male *Drosophila* are dosage compensated aneuploids relative to females, we tested whether there was any effect of sex on these *Mad2*-induced climbing and seizure phenotypes. We performed these analyses separately for males and females and found no significant difference between the sexes in climbing defects or in seizure phenotypes (Figure 4-5A, Figure 4-5B).

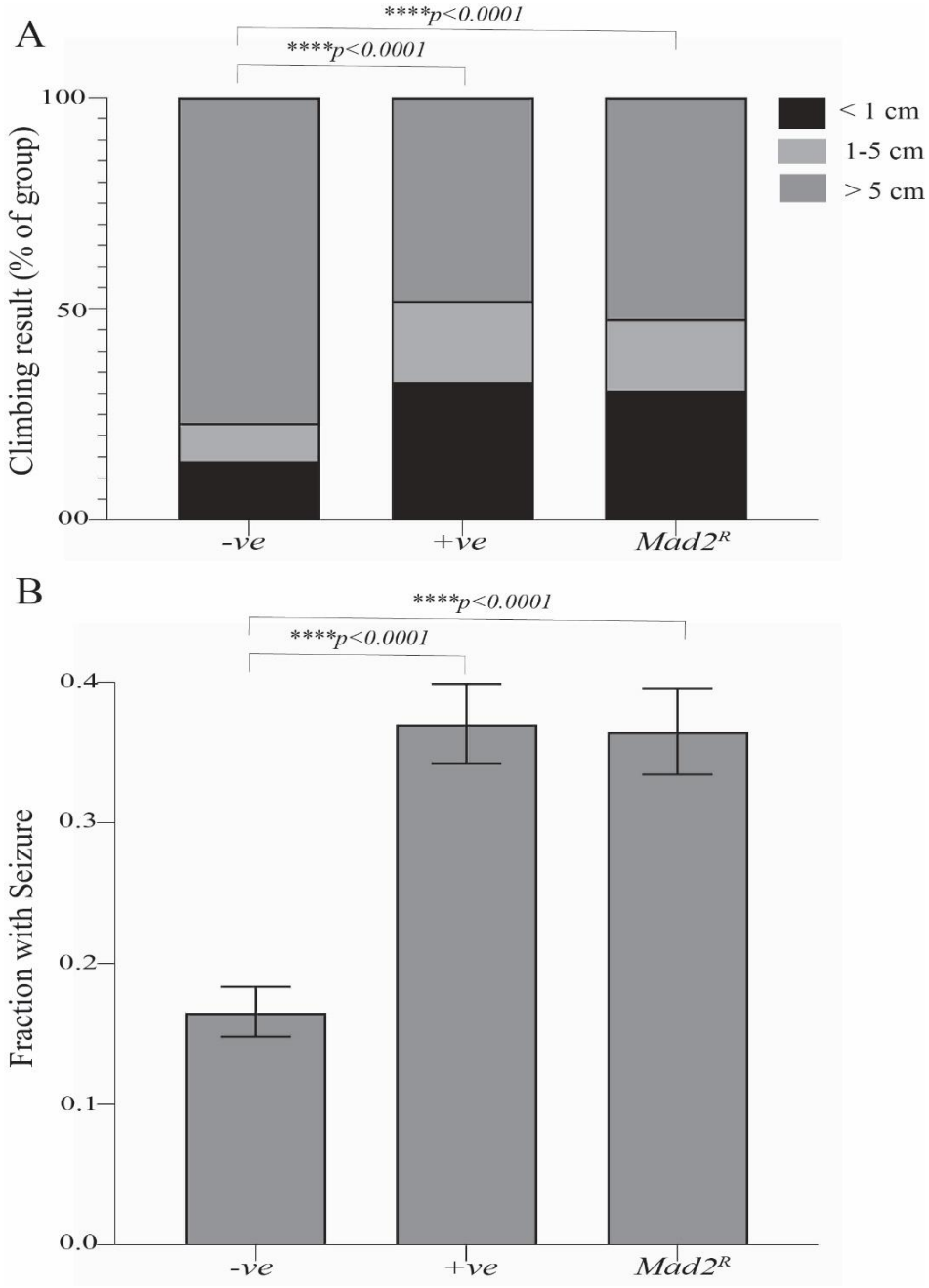

Figure 4-3: *Mad2* depletion in GABAergic neuron increased the frequency of climbing defects (A) and seizure phenotypes (B) in flies ages between 8 to 12 days. Flies with *Mad2* depletion in GABAergic neurons showed significant climbing defects compared to the wild type control (A). Positive control: +ve, wild type: -ve. We used overexpression in GABAergic neurons of GCA90, a polyalanine repeat sequence, as a positive control for neurodegenerative climbing defects. The p-values were calculated by Chi-Squared test ****P<0.0001. (B) The incidence of seizure-like phenotypes significantly increased in *Mad2* depleted flies compared to the wild type control flies. The GCA90 polyalanine repeat sequence was used as a positive control for seizure. The p-values were calculated by Fisher's exact test ****P<0.0001.

4.4.3. Antioxidant feeding rescued the climbing defect and seizure phenotype in adult *Drosophila.*

Oxidative stress is a typical cellular response to aneuploidy [29,30] and in some cases feeding antioxidants can rescue the cell death caused by aneuploidy [23,29]. NACA is an effective antioxidant in *Drosophila* and one of the benefits of NACA feeding is that it can pass the blood brain barrier [22]. NACA feeding showed significant reduction of climbing defects (Figure 4-4A) as well as significant reduction of seizure phenotypes (Figure 4-4B) in the flies. In our assays even 100ug/ml NACA feeding was sufficient to show rescue of the neurological phenotypes, with no advantage from higher doses (Figure 4-5C, Figure 4-5D). The rescue was not complete, suggesting that although oxidative stress is clearly a major part of the aneuploidy phenotype, there are likely to be other contributing factors (such as protein folding stress etc) [37,38]. The GCA90, polyalanine repeat sequence was used as a positive control for climbing defects and seizure phenotypes [26] and as expected, it was also significantly rescued by antioxidant feeding.

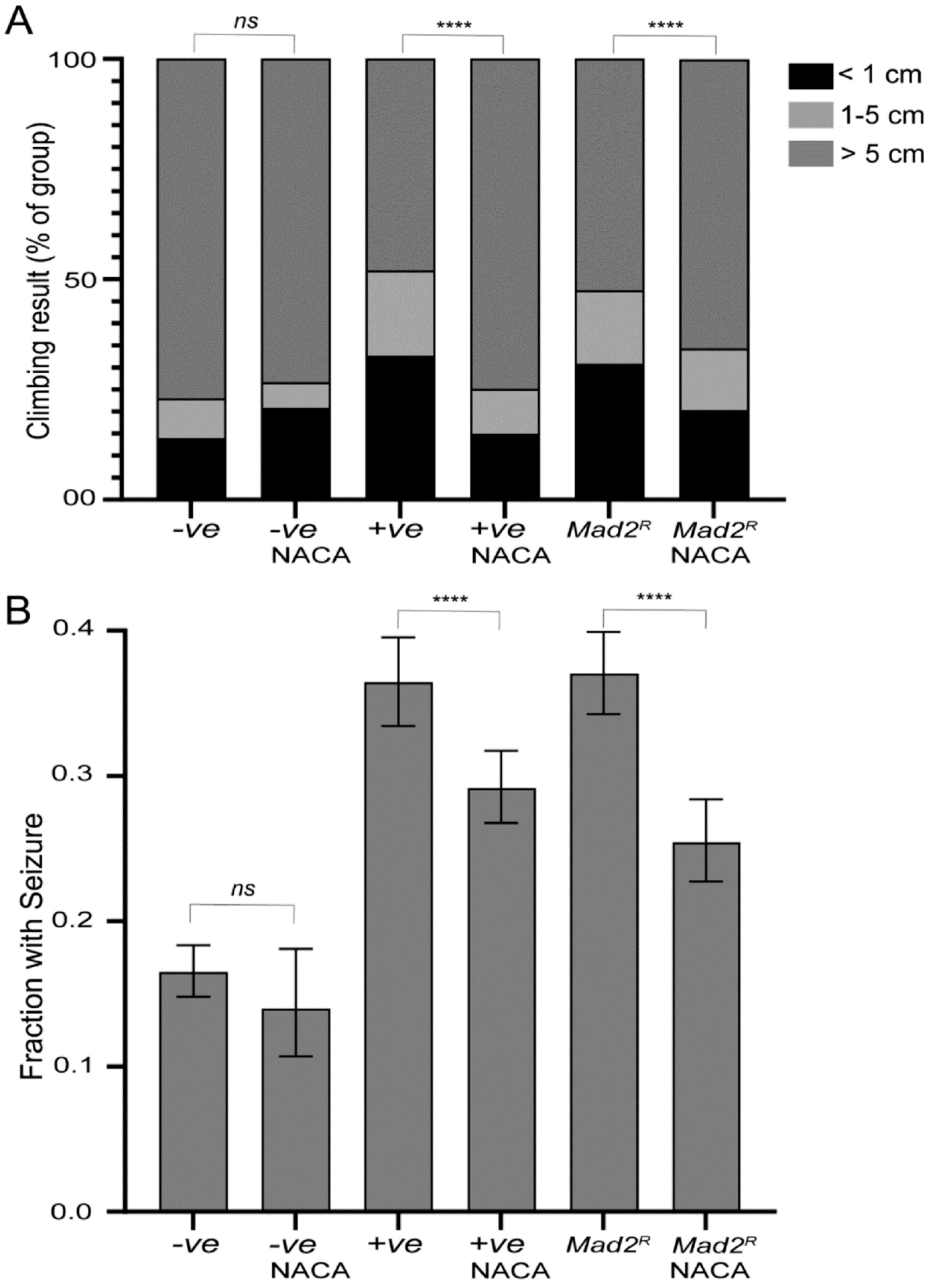

Figure 4-4: Antioxidant feeding rescues the climbing defects (A) and seizure phenotype (B) in aneuploid flies aged between 8 and 12 days. 100 µg/ml N-acetyl cysteine amide (NACA) feeding significantly decreased the climbing defects compared to the positive control (+ ve) and *Mad2*-depleted flies (A). The p-values were calculated by Fisher's exact test comparing proportions climbing less than 5 cm ****$p < 0.0001$, ns: $p > 0.05$, $n > 450$ for each genotype. The GCA90 polyalanine repeat sequence was used as a positive control for climbing defects and seizure phenotype. Seizure phenotype (B) significantly increased in *Mad2*-depleted flies compared to the wild type of control flies which were rescued by 100 µg/ml NACA feeding for both + ve (positive control) and *Mad2*-depleted flies. The p-values were calculated by Fisher's exact test ****$p < 0.0001$, ns: $p > 0.05$, $n > 300$ for each genotype.

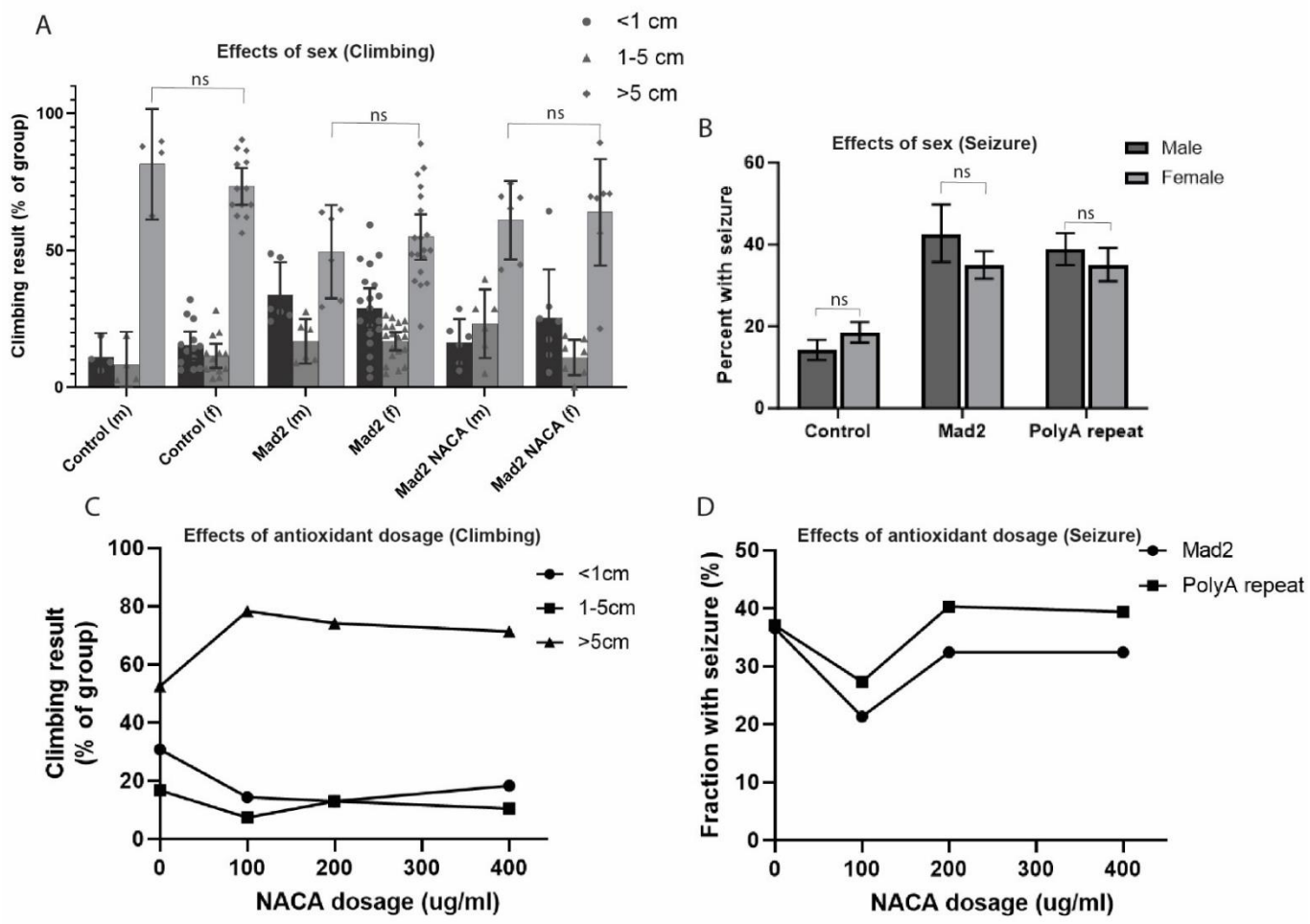

Figure 4-5: The climbing performance and seizure-like phenotype in male vs females are shown in A (NACA 100 ug/ml) and B. No significant difference between male vs females were detected regarding the climbing performance and seizure like phenotypes. The effect of various doses of NACA feeding on climbing defects and seizure like phenotypes have shown in C and D. There was no significant improvement above 100 ug/ml for rescuing the climbing defects and seizure like-phenotype (C, D).

4.5. Discussion

The role of aneuploidy in the pathogenesis of various diseases, particularly in neurological disorders, has been a subject of intense investigation. In this study, we explored the link between increased aneuploidy and the development of a neurological phenotype, focusing on the impact of *Mad2* depletion in the *Drosophila* model. Our findings provide compelling evidence that aneuploidy, induced by the depletion of *Mad2,* leads to oxidative stress and subsequently contributes to the manifestation of functional neurological deficits. We found that *Mad2* depletion in third-instar larval brains leads to increased aneuploidy, consistent with previous studies that have identified a role for *Mad2* in neurological disease progression [39,40]. Our findings align with studies in mammals, where aneuploidy has been implicated in neurodevelopmental disorders, including intellectual disabilities and autism spectrum disorders [41]. One of the most common causes of dementia among the aging population has implicated aneuploidy: Alzheimer's disease. Lymphocytes and splenocytes isolated from AD patients show mitosis and chromosomal segregation defects [42,43], demonstrating AD patients' vulnerability to aneuploidy and their predisposition to produce aneuploid cells. In some studies as much as 90% of the cell death observed in AD neurons has been attributed to aneuploid neurons [44].

In our current study, we present evidence suggesting that increased aneuploidy leads to more cell death in the brain. Consistent with this, there is evidence that microcephaly is caused by *KNL1* mutations. Like *Mad2, KNL1* mediates the spindle assembly checkpoint, which acts as a protective mechanism against aneuploidy. Segregation mistakes in mitotic neural progenitor cells subsequent to *KNL1* deletion result in DNA damage on the missegregated chromosomes. This leads to a large-scale eradication of cells with somatic genome damage by p53 activation and strong apoptotic and microglial phagocytic responses, ultimately resulting in microcephaly [39]. In several other experimental systems, aneuploidy has been shown to promote cell death like neurodegeneration [45-47] consistent with our current study. Previously we have shown that *Mad2* depletion causes chromosomal instability which generates aneuploid cells [48], resulting in mitochondrial stress, proteotoxic stress, DNA damage, alteration of

cellular signalling pathways, and cell death in proliferating *Drosophila* epithelia [23,29,30,38]. In these cases, cell death has been apoptotic, but we have not yet confirmed whether neuronal cell death in response to aneuploidy is also apoptotic. It would be desirable to validate our model of aneuploidy using alternative methods to deplete *Mad2*. We have previously used null mutants, but in this case, the effect of systemic aneuploidy would make the interpretation of behavioural phenotypes highly problematic. The advantage of the model we use here is that it is targeted to a very narrow subset of brain cells, so we can be confident that they are responsible for the behavioural phenotypes observed.

Since neurons have historically been believed to remain post-mitotic [49], how mosaic aneuploidy can occur in neurodegenerative or neurodevelopmental disorders has been unclear. Aneuploidy rates in the hippocampus, cortex and cerebellum of brain were studied by Iourov and co-workers, relating AD patients with a group of unaffected aged-matched controls [50,51]. They suggested that the increased levels of aneuploidy in AD were due to aberrant adult neurogenesis resulting from mitotic non-disjunction in neurons re-entering the cell cycle. Neurogenesis in the adult brain may be an indicator for initial identification of AD [52]. The main evidence for this hypothesis is that neurons of AD brain showed cyclin B1, cyclin D1, cdc2, and Ki67 phospho-proteins expression which are normally identified during mitosis [53-57]. Additionally, it has been demonstrated that Amyloid beta peptide (Aβ) triggers the expression of mitotic proteins and cell cycle entrance by mature neurones in culture [58-60]. This raises the possibility that re-entering into an aberrant cell cycle may result in aneuploidy. Previously, aneuploidy was presumed to result from proliferation during development, with defective clearance of these cells explaining their existence in the adult brain [61,62]. In recent times, it has become clear that neurogenesis is more common than had been assumed, and that the potential for neurogenesis persists into old age, even though it is not usually used [63-66]. There is good evidence that neurogenesis can arise in many areas of the brain during life. In general, genetic, and environmental stressors can produce and accumulate aneuploidy in the division or regeneration of cell populations at any time in life [46,67]. In addition, evidence from several studies indicate that neurogenesis in many brain regions can be induced in adult mice and rats in response to brain injury and attempted self-repair by the brain [68-70].

Recent evidence has shown that striatal astrocytes may transdifferentiate into new neurons able to form active neuronal circuits with pre-existing neurons following ischemic brain injury, which is the basis of an alternative potential mechanism for neuronal aneuploidy [69,70]. These results suggests that in AD and FTLD-MAPT brains, some of the aneuploid neurons may originate from glia. Increased total exposure to environmental stressors is related to aging, which can enhance missegregation of chromosomes and neuronal aneuploidy [67,71]. Age may be triggering all other mechanisms mentioned to form neuronal aneuploidy because evidence has shown that neuronal and non-neuronal aneuploidy increase with age [72-76]. It appears, then, that there are several possible mechanisms to account for the observed aneuploidy in adult neurons, which our experiments have been modeling with the objective of identifying potential interventions. Previous experiments to induce neuronal aneuploidy, such as by expressing mutant Tau protein have shown similar neuronal cell death [77], though in that case, because Tau has many effects, it was harder to confidently attribute the phenotype to aneuploidy.

We identified oxidative stress as a significant mediator of the neurological phenotype observed in *Mad2*-depleted *Drosophila*. Oxidative stress is a stereotypical cellular response to aneuploidy [78] and oxidative stress is also known to be a common detrimental observation in various neurodevelopmental disorders and neurodegenerative diseases [79]. Our data revealed that induced aneuploidy in GABAergic neurons increased oxidative stress and generated functional neurological phenotypes. Feeding antioxidants could rescue the neurological defects, showing that oxidative stress was a significant contributor to the neurological phenotype generated by aneuploidy. Reactive oxygen species (ROS) production typically leads to protein oxidation, lipid peroxidation, and DNA damage, all of which are implicated in the pathogenesis of neurodegenerative diseases [80]. The antioxidant we used, NACA, is known to significantly reduce the accumulation of lipid droplets (LD) and the production of peroxidised lipids in flies [22]. LD buildup in the brain is triggered by increased oxidative stress and the dysregulation of lipid droplets is known to play a role in the progression of neurological diseases [81]. The known associations between oxidative stress, neurodegeneration, and aging suggest a likely effect of neuronal aneuploidy on lifespan, which would be worth testing in the future.

In conclusion, our study provides important insights into the connection between increased aneuploidy

and the development of a neurological phenotype in a *Drosophila* model system. We highlight the role of oxidative stress as a mediator of this phenotype and suggest potential therapeutic avenues for neurodegenerative diseases. This research contributes to our understanding of the complex interplay between aneuploidy and neurobiology and opens new doors for further investigations into the pathogenesis and treatment of neurological disorders.

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CHAPTER 5: *DROSOPHILA* EXPRESSING MUTANT HUMAN *KCNT1* TRANSGENES MAKE AN EFFECTIVE TOOL FOR TARGETED DRUG SCREENING IN A WHOLE ANIMAL MODEL OF *KCNT1*-EPILEPSY

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5.1. Abstract

Mutations in the *KCNT1* potassium channel cause severe forms of epilepsy which are poorly controlled with current treatments. In vitro studies have shown that *KCNT1*-epilepsy mutations are gain of function, significantly increasing K+ current amplitudes. To investigate if *Drosophila* can be used to model human *KCNT1* epilepsy, we generated *Drosophila* melanogaster lines carrying human *KCNT1* with the patient mutation G288S, R398Q or R928C. Expression of each mutant channel in GABAergic neurons gave a seizure phenotype which responded either positively or negatively to 5 frontline epilepsy drugs most commonly administered to patients with *KCNT1*-epilepsy, often with little or no improvement of seizures. Cannabidiol showed the greatest reduction of the seizure phenotype while some drugs increased the seizure phenotype. Our study shows that *Drosophila* has the potential to model human *KCNT1*- epilepsy and can be used as a tool to assess new treatments for *KCNT1*- epilepsy.

5.2. Introduction

Mutations in *KCNT1* have been identified in a range of epilepsies with drug-resistant seizures [1]. *KCNT1* mutations are the major cause of epilepsy of infancy with migrating focal seizures (EIMFS) [1-3], where cognitive and developmental regression follow seizure onset. *KCNT1* mutations also cause other severe epilepsies beginning in infancy, including West Syndrome and Otahara Syndrome [1]. They also contribute to a range of focal epilepsies, which can have a later age of onset (in childhood or adolescence), including sleep-related hypermotor epilepsy [1,4,5]. *KCNT1*-epilepsy is often debilitating and while treatment with anti-epilepsy drugs can reduce seizures in some patients, seizure suppression is usually incomplete and the development of new treatments is needed [3]. *KCNT1* encodes an ion channel which is a major contributor of the sodium-activated potassium IkNa current which down regulates neuronal excitability. Following a rise in intracellular [Na+], *KCNT1* channels are thought to increase K+ current and prolong the slow after-hyperpolarisation phase following an action potential, thereby reducing the chance of repetitive neuronal firing [1,6,7]. Almost all *KCNT1* epilepsy mutations are heterozygous and missense, predicted to change a single amino acid in the protein [3] and de novo mutations most often account for severe cases. All mutations analysed in vitro to date (apart from T314A [8]) significantly increase K+ currents in comparison to normal *KCNT1* channels [8-12]. There is evidence suggesting that increased K+ currents in inhibitory neurons may be associated with *KCNT1*-related seizures [8,13,14]. Reducing the activity of inhibitory neurons may explain how neuronal hyperexcitability, the mechanism underlying seizures, occurs in *KCNT1*-epilepsy.

There are currently no highly effective treatments for *KCNT1*-epilepsy and no drugs available that specifically target the *KCNT1* channel. Demonstration that increased K+ current due to overactivity of the *KCNT1* channel is associated with seizures suggests that reducing or blocking *KCNT1* channel activity may inhibit seizures, and this has directed new drug screening efforts [15]. Currently, anti-epileptic drugs including carbamazepine, vigabatrin and valproic acid, as well as cannabidiol (CBD) are frontline medications commonly administered for *KCNT1*-epilepsy [3,16–18]. However, even combinations of multiple drugs (sometimes eight or more) usually fail to suppress seizures, with some

patients continuing to experience up to one hundred seizures per day [3,16-18]. In vitro studies have shown that the ion channel blocker quinidine reduces the increased current amplitude produced by *KCNT1*-epilepsy mutations [9,11,19]. However, it has shown variable results in patients and can have serious side effects [3,19-21].

In this study we sought to investigate if *Drosophila* could be used to model *KCNT1*-epilepsy and if the seizure phenotype responded to some of the drugs currently used to treat patients. Many of the genes and pathways identified in human epilepsy are highly conserved in *Drosophila.* The animal has been used to model other forms of human epilepsy, including Dravet Syndrome and Generalised Epilepsy with Febrile Seizures Plus (GEFS+) due to mutations in the sodium channel gene *SCN1A* [22,23] and can make powerful tools for therapeutic screens [24]. The majority of *KCNT1*-epilepsy mutations cluster around three, highly conserved, functional domains in the *KCNT1* protein [1]. A mutation from each region was selected to be analysed: c.862G > A, p.G288S in the S5 segment of the pore domain, c.1193G > A, p. R398Q in the RCK1 (Regulator of K+ Conductance) domain and c.2782C > T, p.R928C adjacent to the NAD+ binding domain (Figure 5-1). G288S and R398Q mutations have been identified in patients with a range of epilepsy phenotypes, ranging from very severe infantile onset epilepsies to less severe and later onset focal epilepsies, whereas R928C mutations are only associated with the latter. The three mutations are recurrently found in patients, collectively accounting for approximately 20% of cases [1,3]. Thus, successful modelling of human *KCNT1*-epilepsy with these mutations would be relevant for a significant proportion of patients.

To generate the *Drosophila* models, human *KCNT1* with the patient mutation G288S, R398Q or R928C was introduced into *Drosophila* by transgenesis. The mutant *KCNT1* channels were expressed in different neural tissues, including pan-neural, glial, excitatory (cholinergic) neurons and inhibitory (GABAergic) neurons, and investigated for a seizure phenotype using bang sensitive behavioural assays [25,26]. To investigate the potential of the *Drosophila* models in assessing treatments for patients with *KCNT1* epilepsy, five of the frontline anti-epilepsy drugs currently administered to patients with *KCNT1* epilepsy which have been shown to reduce, but rarely stop, seizures in some patients were analysed for

their effects on the seizure phenotype in our *Drosophila* models.

5.3. Materials and Methods

5.3.1. Generation of constructs and germ line transformation

Transgenic *Drosophila* were generated using the attP2 locus and PhiC31 integration system [27]. Full-length cDNA for Human *KCNT1* Transcript 1 NM_020822.1 (Origene Technologies Inc, Rockville, MD, USA) was mutagenized to induce point mutations, G288S, R398Q and R928C using QuikChange lightning site-directed mutagenesis kit (Agilent Technologies Inc. Santa Clara, CA, USA). Primers used for mutagenesis are as follows: p.G288S (5′-ACGGGGACCTGCAGCATCCAGCACC-3′ and 5′-GGTGCTGGATGCTGCAGGTCCCCGT-3′), p.R398Q (5′-CGCCCACCCCCAGCTCCAGGACT-3′ and 5′-AGTCCTGGAGCTGGGGGTGGGCG-3′) and p.R928C (5′-CCACCCTTCCAACATGTGCTTCATGCAGTTCCG-3′ and 5′-CGGAACTGCATGAAGCACATGTTGGAAGGGTGG-3′). The resulting constructs were cloned into EcoRI/XhoI restriction sites of pUAST-attB. All introduced mutations were verified by Sanger sequencing prior to injection into embryos (BestGene Inc. Chino Hills, CA, USA).

5.3.2. *Drosophila* stocks and culture

Drosophila were cultured in 12-h light/dark cycles on a standard fortified medium containing 1% agar, 1% glucose, 6% fresh yeast, 9.3% molasses, 8.4% coarse semolina, 0.9% acid mix (4.6% orthophosphoric acid v/v, 43.9% propionic acid v/v Sigma-Aldrich (Gillingham, U.K.) and 1.7% Tegosept (Chem Supply, Australia). The following *Drosophila* lines were obtained from the Bloomington *Drosophila* Stock Center (Indiana, USA): W118 (BL3605), elavC155-GAL4 (BL458), GAD1-GAL4 (BL51630), Chat-GAL4 (BL56500) and Repo-GAL4 (BL7415). The GAD1-GAL4 line BL51630 (created by Gero Miesenbock and deposited into Bloomington *Drosophila* Stock Centre by Prof Hugo Bellen) drives GAL4 expression with a 3.089 kb fragment of the GAD1 promoter, immediately adjacent from the translation start site. GAD1-GAL4 has been used numerous times by researchers to drive expression of UAS transgenes in GABAergic neurons [28-33]. The vector control BL8622 (y[1] w[67c23]; P(y[+t7.7] = CaryP)attP2) was used to generate transgenic *Drosophila* with human *KCNT1*. Transgenic *Drosophila* harbouring human *KCNT1* generated in this work were: UAS-

KCNT1 WT, UAS-*KCNT1* G288S, UAS-*KCNT1* R398Q and UAS-KCNT1 R928C.

5.3.3. Bang-sensitive behavioural assays to investigate a seizure phenotype

Seizures were studied using the bang-sensitive behavioural assay which measures the occurrence of seizures induced by mechanical shocks [34]. All the experiments were performed between 8 and 11 am to minimize the effect of circadian rhythms on neuronal activity. Male and female flies aged between 4 to 8 days after eclosion were collected in batches of 10–20 flies under CO2 anaesthesia and transferred to a 100 ml (inner diameter: 29.5 mm) measuring cylinder (Cat# 612–3836, VWR International) where they were allowed to recover from anaesthesia and acclimatise for 5 min before they were assayed separately. Visibly unhealthy flies and those with damaged appendages were excluded from the assay. Seizures in *Drosophila* melanogaster are characterised by a repertoire of behavioural abnormalities which include: an abnormal loss of posture, random wing flapping, leg shaking, spinning and uncontrolled flight, complete immobilization and falling down during uncoordinated flight and climbing efforts [26,35,36]. For this study, animals were considered as normal if they were able to climb past a 5 cm mark on the cylinder after mechanical shock. Flies that did not climb past a 5 cm mark and exhibited immobilization but had a normal standing posture or were grooming themselves were also considered as normal. Flies were classified as displaying seizures if they were unable to climb past a 5 cm mark and showed one or more of the accepted seizure-like behaviours throughout 30 s post mechanical shock. The ‘seizure-like behaviours’ scored were: buzzing (fly was upside down at the bottom of the cylinder exhibiting continuous flapping of wings and legs), spinning (fly at the bottom erratically moving in circles), leg shaking (fly upside down continuously shaking its legs), uncontrolled flight attempts (attempted flight jump and then falling down to abnormal posture, recovery and then trying to fly), uncoordinated climbing effort (attempted climbing and then falling down and then again try to climb) and immobilized flies with an abnormal posture (bending sideways).

To look for the presence or absence of a seizure phenotype, flies were subjected to mechanical shocks by forcefully tapping the cylinder 20 times in “banging assays” which are a well-established method in investigating a seizure phenotype. A single researcher conducted the ‘banging’ and scoring for all experiments for intra-experimental consistency. To facilitate analysis and scoring the flies were

videotaped during the process for 3 min with a Dino-Lite digital microscope (Product#AD3713TB; AnMo Electronics Corporation, New Taipei City, Taiwan). To determine the proportion of animals displaying seizure like activity, the resulting videos were zoomed in to cover up to the 5 cm mark on the cylinder. Individual flies were observed for a minimum of 30 s at a slower video playback speed (0.30X) to score seizure activity. In the banging assays a minimum of 50 *Drosophila* were analysed for each genotype with each concentration of a drug. The ≥ 50 flies were collected from a minimum of 3 independent crosses (N) and were individually analysed in measuring cylinders in groups of 10–20 adult flies per cylinder. Brown-Forsythe and Welch's ANOVA tests, followed by Dunnett's multiple comparisons tests (GraphPad Prism 9) were used to determine the statistical difference of recovery from mechanical stimulation between different groups.

5.3.4. Analysis of the effects of selected drugs on seizure activity in *Drosophila.*

Based on clinical data, five of the frontline epilepsy drugs most commonly administered to patients with *KCNT1*-epilepsy. Drugs: carbamazepine (Sigma Aldrich: PHR1067), valproic acid (Sigma-Aldrich: P4543), vigabatrin (Sigma-Aldrich: V8261), quinidine (Sigma-Aldrich: R751839) and CBD (Gift from Professor Sanjay Garg) were analysed in vivo to determine their effects on the seizure phenotype of the three *Drosophila* lines expressing the mutant human *KCNT1* transgenes. We used feeding experiments with a range of drug concentrations, followed by the bang sensitive behavioural assay. Each drug was dissolved in absolute ethanol and a constant volume (1 µl) of 100% ethanol was dispensed per 1 ml of *Drosophila* food for all drug concentrations. *Drosophila* were raised on standard fortified medium (composition mentioned above). *Drosophila* crosses were performed in cages with apple juice agar plates and yeast to encourage egg laying. Embryos were collected and transferred to vials with food containing the drugs at different concentrations (0.001–100 µM) at 24 °C. Adult *Drosophila* from these vials were allowed to age between 4 to 8 days on food containing the respective concentrations of drug to be tested. A range of concentrations of each drug dissolved in *Drosophila* food, between 0.001 µM and 100 µM, were used in the experiments. Multiple replicates were performed for each dosage of the drugs and controls. Controls were Vehicle Control (VC) which was the normal food with just the solvent ethanol present (1 µl/1 ml) in the *Drosophila* food, and Normal Food control (NF) where no drug or

solvent (ethanol) was added in the *Drosophila* food. For Adult only feeding, R398Q mutant flies eclosed from normal food vials were allowed to age between 4–5 days on food vials containing CBD (0.1–10 μM) followed by the bang sensitive behavioural assay.

5.3.5. Electrophysiology and analysis of effects of drugs

Whole-cell and inside-out patch-clamp recordings of *KCNT1* mediated currents were performed using transiently transfected HEK293T cells 20–36 h post transfection. Whole-cell patch clamping was performed using a computer-based patch-clamp amplifier (EPC-9, HEKA Elektronik) and PULSE software (HEKA Elektronik) as previously described [8]. The bath solution contained 140 mM NaCl, 4 mM KCl, 2 mM CaCl2, 2 mM MgCl2 and 10 mM HEPES adjusted to pH 7.4 with NaOH. The pipette solution contained 80 mM K gluconate, 50 mM KCl, 10 mM NaCl, 1 mM MgATP, 10 mM EGTA and 10 mM HEPES adjusted to pH 7.3 with KOH. Patch pipettes were pulled from borosilicate glass and fire polished to give a pipette resistance between 1 and 2 MΩ. Series resistance ranged between 2.5 and 4 MΩ and was 80–90% compensated. For comparing amplitudes of *KCNT1* currents produced by different constructs, the plasmids containing WT or mutant KCNT1 cDNA were transfected into HEK293T cells at the same amounts (0.8 μg in 35 mm Petri dish) and cells were used for patch clamping within a short time window 24–28 h post transfection. For the analysis of the drugs' effects, the amounts of the plasmids and/or the time post transfection were adjusted to produce *KCNT1* currents of similar amplitudes and amenable to voltage clamp with the voltage error less than 10% due to a residual uncompensated series resistance. The holding potential was set to − 78 mV and cells with the membrane potential more positive than − 72 mV were excluded from the analysis. Cell and the pipette capacitance were compensated for by the EPC-9 amplifier automatically. Expression of mutant *KCNT1* channels had no effect on cell capacitance compared to cells expressing wild type *KCNT1* (20.7 ± 7.4 pF (WT; n = 15) vs 19.9 ± 4.8 pF (G288S; n = 13), 21.0 ± 6.4 pF (R398Q; n = 14), or 20.9 ± 7.9 pF (R928C; n = 12)). Leakage through the seal determined immediately before achieving whole-cell configuration was subtracted using "leak subtraction" function of the EPC9 amplifier. No P/N protocol have been used due to *KCNT1* channel non-zero open probability even at very negative potentials [8]. In the inside-out patch clamp experiments pipettes with a resistance between 2 and 4 MΩ were filled with a standard

bath solution (see above) and the membrane patch was perfused from the intracellular side with a solution containing 110 mM KCl, 35 mM NaCl, 0.2 mM EGTA and 10 mM HEPES adjusted to pH 7.3 with KOH. Single channel current traces used for the analysis were recorded at 0 mV for the duration of 15 s, using 5 kHz sampling rate and 2 kHz filtering. For the display purposes all traces were further filtered by the 8-pole Bessel filter at 1 kHz. All drugs were dissolved in DMSO, aliquoted, and stored at − 20 °C. The maximum DMSO concentration in the bath solution did not exceed 0.05%. Drugs were applied using a gravity-fed perfusion system with the outlet positioned within 1–2 mm of the patched cell and the perfusion rate of 0.5 ml/min.

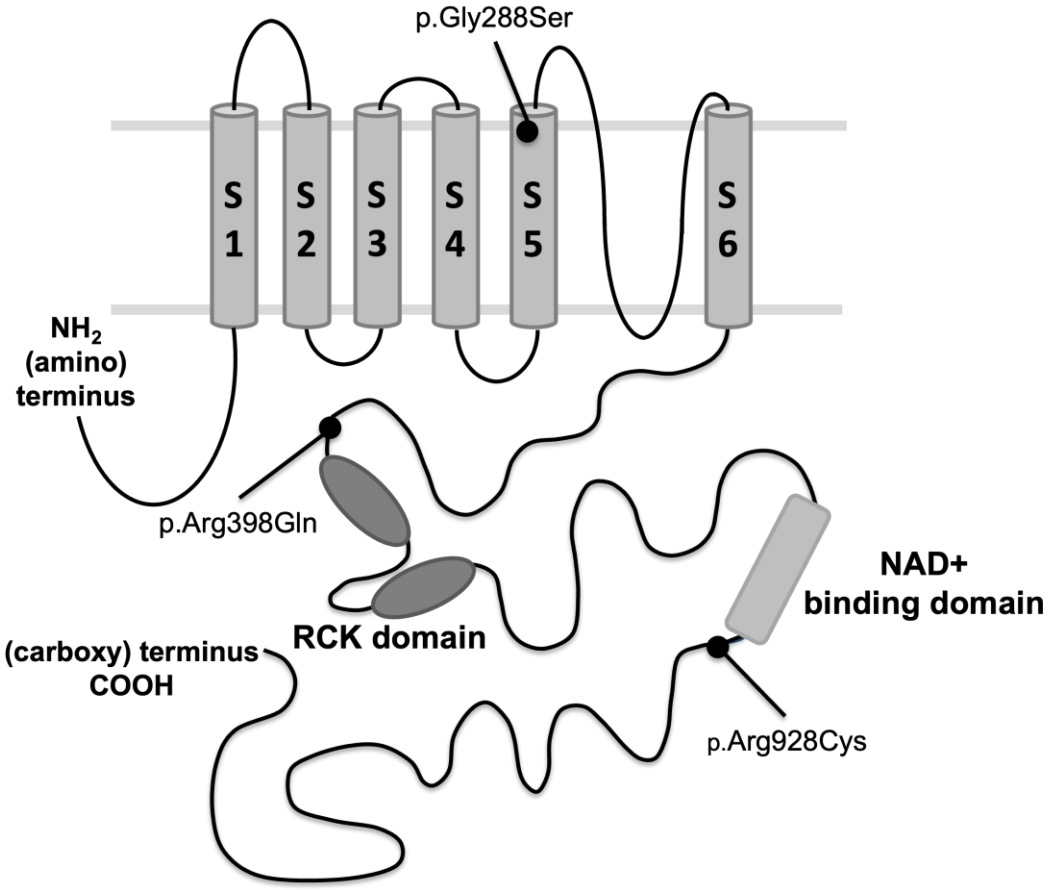
5.3.6. Data Analysis and statistics

Data were analysed using GraphPad Prism 9 software (San Diego, CA, USA). All values are reported as mean ± standard deviation (SD). All data passed normality and lognormality Shapiro–Wilk tests, determined by GraphPad Prism 9. In patch clamping data 'n' represents number of cells, and all experiments were repeated using cells from a minimum 2 separate transfections. To determine the IC50 of KCNT1 inhibition by CBD, the data were fitted with Hill equation of the form: Y=Bottom+(Top−Bottom)/(1+10^((LogIC50−X)∗HillSlope)) where Top was constrained to 100% and Hill Slope to − 1. Single channel data were analysed using Ana software developed by Dr Michael Pusch (Istituto di Biofisica, Genova, Italy) (http://users.ge.ibf.cnr.it/pusch/programs-mik.htm). The amplitude histograms generated by the Ana software were used to determine the changes in the open probability (PO) of KCNT1 channels. As most inside-out patches contained more than one active channel, typically 2–5, the PO was calculated as 1-PC, where PC is the probability of all channels in the patch being closed simultaneously. PC was determined by dividing the bin count (equivalent to the area under the curve) corresponding to the closed state in the amplitude histogram by the total bin count of the entire histogram. In statistical analysis of seizure-like behaviour of *Drosophila* (Table 2) 'N' represents independent trials using independent fly crosses, with the total number of flies for each condition shown in brackets. In all experiments, statistical significance of differences between groups was determined using Brown-Forsythe and Welch's ANOVA tests, assuming non-equal SDs, followed by Dunnett's multiple comparisons tests (GraphPad Prism 9).

5.4. Results

5.4.1. Investigation of a seizure phenotype in *Drosophila* with G288S, R398Q or R928C mutant *KCNT1*.

To investigate if *Drosophila* carrying KCNT1 mutations showed a seizure phenotype, three transgenic lines were generated with mutated human KCNT1 (Figure 5-1a,b). Each Drosophila line carried a human KCNT1 transgene with a heterozygous missense mutation that has been identified in patients, G288S, R398Q or R928C. Wild type (WT), normal, human KCNT1 (NM_020822.3) was used as a control. The UAS-GAL4 expression system was used to drive overexpression of the human KCNT1 transgenes [37]. Each *Drosophila* line contained an upstream activating sequence (UAS) positioned before the KCNT1 transgene. Genetic crosses were used to introduce the GAL4 transcription factor under the control of selected promoters, to drive expression of the WT or mutant KCNT1 channels in different tissues.



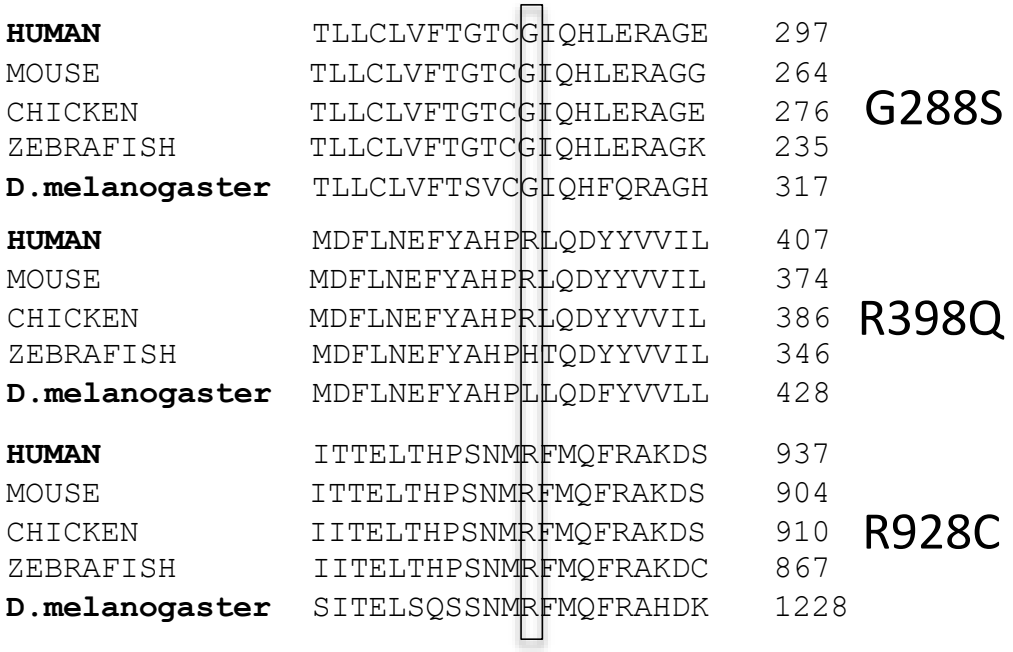


Figure 5-1-Position and evolutionary conservation of mutated amino acid residues. Position and evolutionary conservation of the amino acid residues altered by the three patient *KCNT1* missense mutations investigated in this study, G288S, R398Q and R928C. (a) Schematic diagram of the KCNT1 channel showing the positions of the three mutations investigated in this study. (b) Alignment of the orthologous KCNT1 proteins found in different species showing the high evolutionary conservation of the amino acids altered by the 3 *KCNT1* mutations investigated in this study.

The KCNT1 channel is found in a wide variety of neurons in mammals [38,39] and we expressed them in a range of neural tissue types in the *Drosophila* models. We first tried to see if driving expression of the mutant transgenes in all neurons using a pan-neural GAL4 driver could give a seizure phenotype. Using the pan-neural promoter *elav*^{C155}*-GAL4* to drive mutant *KCNT1* expression in all neurons did not give any progeny and was embryonic lethal, thus a seizure phenotype was not able to be investigated (Table 1). Expression of WT human *KCNT1* in all neurons with *elav*^{C155}*-GAL4* produced living progeny indicating overexpression of the WT human *KCNT1* channel was not lethal. We then tried to express mutant human KCNT1 transgenes in smaller subsets of neural tissues to see if they could give a seizure phenotype. First, the three mutant and one WT transgenes were expressed in excitatory neurons with the *CHAT-GAL4* driver containing the *Choline-Acetyltransferase* (Chat) gene promoter to drive expression in excitatory cholinergic neurons [40]. As was observed for pan-neural expression, only

expression of WT *KCNT1* in cholinergic neurons gave living adult flies and expression of the three mutant human KCNT1 lines was lethal (Table 1). The effects of expressing WT and mutant human KCNT1 transgenes in glia, were assessed using the reversed polarity *(Repo)-GAL4* driver [41]. None of the 3 mutants gave any surviving progeny, and as before, the WT human KCNT1 expressed in glia gave viable offspring (Table1). Interestingly, none of the surviving WT human *KCNT1* expressing flies with either *ELAV-GAL4*, *CHAT-GAL4* or *REPO-GAL4* displayed any seizure phenotype in bang-sensitive assays (Figure 5-3).

Table 1. Effect of WT and mutant human KCNT1 transgene expression in neuronal subsets.

Driver / **Mutant**	**Pan-neuronal** ***Elav-GAL4***	**Cholinergic** ***Chat-Gal4***	**GABAergic** ***GAD1-GAL4***	**Glial** ***Repo-GAL4***
UAS-*KCNT1*-WT	Viable no effect	Viable no effect	Viable no effect	Viable no effect
UAS-*KCNT1*-G288S	Embryonic Lethal	Embryonic Lethal	Viable- Bang Sensitive	Embryonic Lethal
UAS-*KCNT1*-R398Q	Embryonic lethal	Embryonic Lethal	Viable- Bang Sensitive	Embryonic Lethal
UAS-*KCNT1*-R928C	Embryonic lethal	Embryonic Lethal	Viable- Bang Sensitive	Embryonic Lethal

It has been postulated from experiments in cells and mice that one possible mechanism of seizure genesis may be due to the inhibition of inhibitory GABAergic interneurons [14]. As in humans, the major inhibitory neurotransmitter in the *Drosophila* central nervous system is γ-aminobutyric acid (GABA)26. Previous studies have suggested that reduced activity of inhibitory GABAergic neurons may be associated with seizures [8,13,14], so we next expressed the WT and mutant human *KCNT1* transgenes in GABAergic neurons using the *GAD1-GAL4* line which drives GAL4 expression from a 3.089 kb fragment of the GAD1 promoter in GABAergic neurons [28-33]. Unlike, glial, pan-neural and excitatory neuron expression, *GAD1-GAL4* driven expression of the WT and three mutant human *KCNT1* transgenes gave surviving adults which were healthy and lived long enough to be investigated for a seizure phenotype in bang-sensitive behavioural assays [25,26]. To quantify the effects on viability of driver and transgene combinations, the heterozygous (Driver/Balancer) *Gad1-Gal4/CyO* flies were crossed with WT human *KCNT1* and the three human KCNT1 mutants. The average viable count of progeny with a given driver expressing human KCNT1 (*Gad1-Gal4: UAS-human-KCNT1*) versus progeny with no-driver (*CyO: UAS-human-KCNT1*) was calculated and gave a viability ratio between 0.8 and 1. This suggested that the expression of WT human *KCNT1* or mutant human *KCNT1* using *GAD1-GAL4* at 24 °C showed minimal or no effect on the survival of offspring. In contrast, viability ratio analysis using the balanced drivers *CHAT-GAL4/CyO* and *REPO-GAL4/Tm3* for expressing any of the three mutant human *KCNT1* transgenes was less than 0.01 at 24 °C whereas expression of WT human KCNT1 transgenes was close to 1. As only the *GAD1-GAL4* driver gave any viable adult *Drosophila*, when expressing the mutant *KCNT1* transgenes, only this driver was able to be used for all future experiments on seizure analysis and drug rescue of seizures. We performed the 'bang sensitive behavioural assay' on *Drosophila* expressing the three human *KCNT1* patient mutations or WT human *KCNT1* in GABAergic neurons and calculated the percentage of animals showing a seizure phenotype for each genotype. The mutant *KCNT1* lines G288S, R398Q and R928C each showed a statistically significant seizure phenotype, while expression of WT human *KCNT1* in GABAergic neurons did not (Figure 5-2). R398Q gave the strongest seizure phenotype with 48% of animals showing seizure activity followed by G288S with 41% and R928C with 38%.

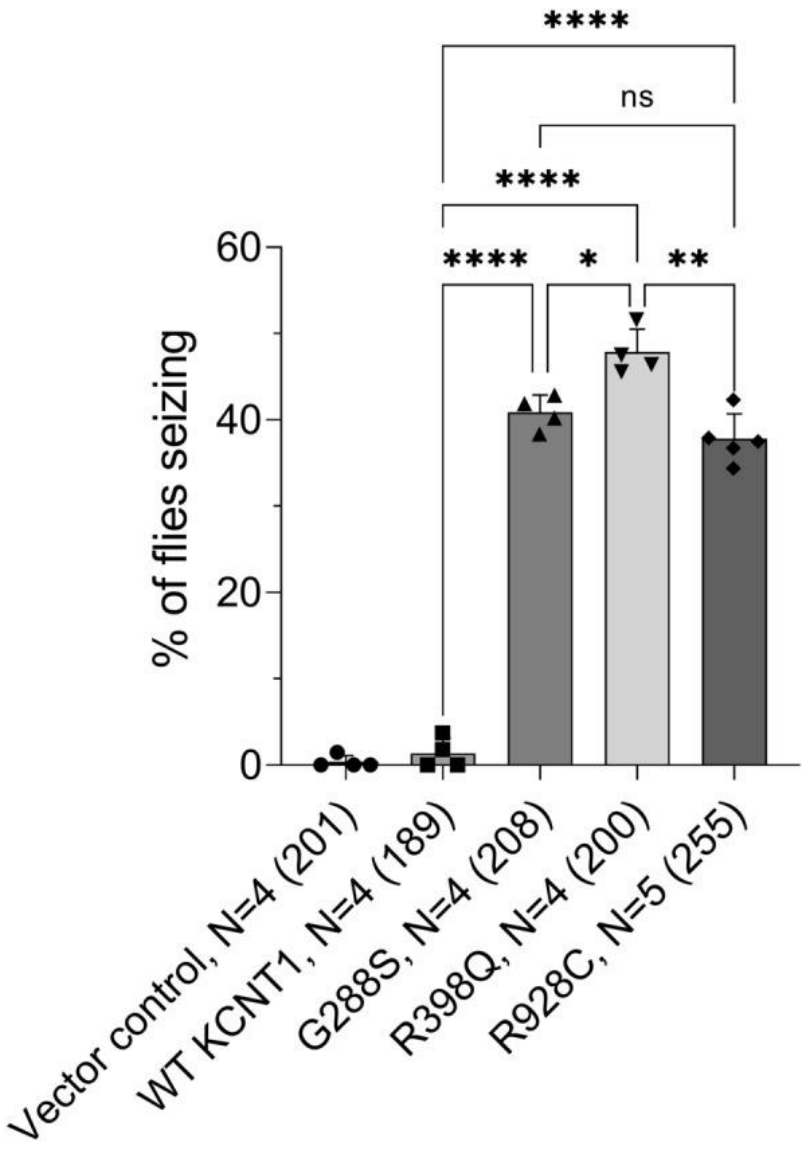


Figure 5-2- Expressing human *KCNT1* mutants in GABAergic neurons of *Drosophila* gives rise to seizures in a bang sensitive behavioural assay. *Drosophila* with the vector control and *Drosophila* expressing either WT or G288S, R398Q or R928C mutant human *KCNT1* in GABAergic neurons were analysed in the bang sensitive behavioural seizure assay. Percentage of *Drosophila* showing a seizure phenotype are shown for each line. N, is the number of independent crosses, with total number of flies in all crosses shown in brackets. The data were analysed using Brown-Forsythe and Welsh's one-way ANOVA followed by Dunnett's T3 multiple comparisons test; ****$P < 0.0001$, ***$P = 0.0003$, **$P = 0.0080$, *$P = 0.0411$, ns—no significant difference.

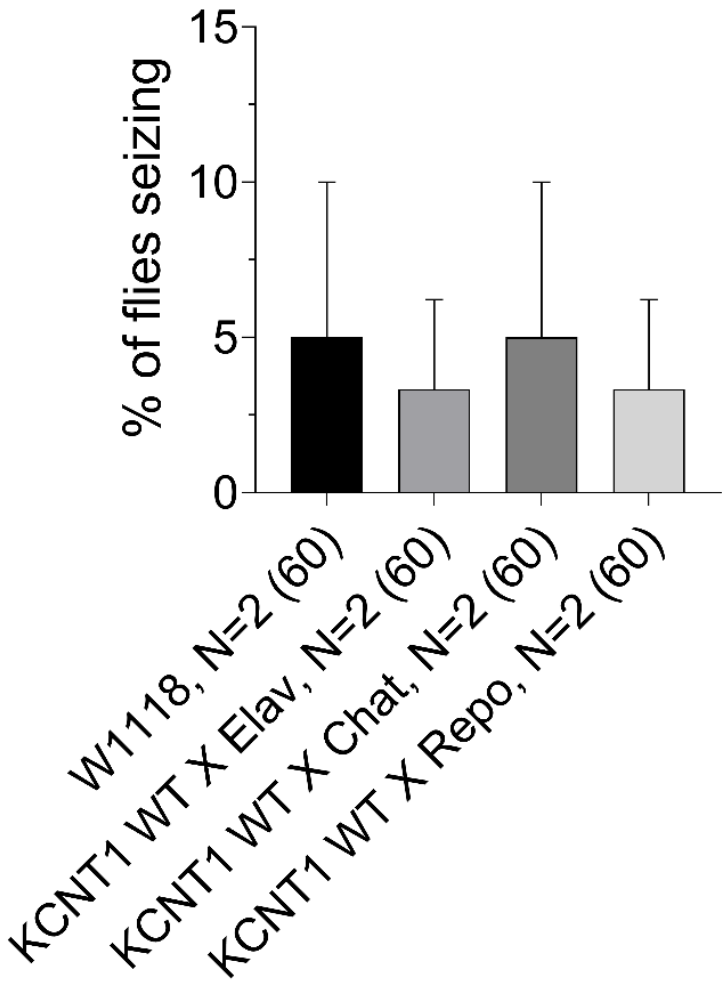


Figure 5-3. Expressing human *KCNT1* WT in different neurons of *Drosophila* gives no seizures in a bang sensitive behavioural assay. *Drosophila* with the vector control and WT human KCNT1 expressed pan-neuronal (elavC155-GAL4), in excitatory neurons (CHATGAL4) and in glia (*Repo-GAL4*) were analysed in the bang sensitive behavioural seizure assay. Percentage of *Drosophila* showing a seizure phenotype are shown for each line. N, is the number of independent fly crosses, with total number of flies in all trials shown in the brackets. Brown-Forsythe and Welsh's one-way ANOVA followed by Dunnett's T3 multiple comparisons test showed no significant difference between the experimental groups.

5.4.2. In vitro effects of currently used drugs on *KCNT1* channels.

Having shown that expression of G228S, R398Q and R928C mutant *KCNT1* in GABAergic neurons gives a seizure phenotype, we next investigated if the phenotype generated by each of the three mutations responded to some of the drugs most commonly used to reduce seizures in patients with *KCNT1*-epilepsy. In the initial experiments, we looked at the in vitro effects of the drugs on KCNT1 channels in human cells using a HEK293T cell expression system and patch clamping analysis. Consistent with previous findings, the amplitudes of *KCNT1* currents in cells expressing the G288S, R398Q and R928C mutants were significantly larger than those expressing WT KCNT1 (Figure 5-4) [8]. Furthermore, the kinetics of KCNT1 currents were affected by the mutations as shown and discussed in our previous publication [8].

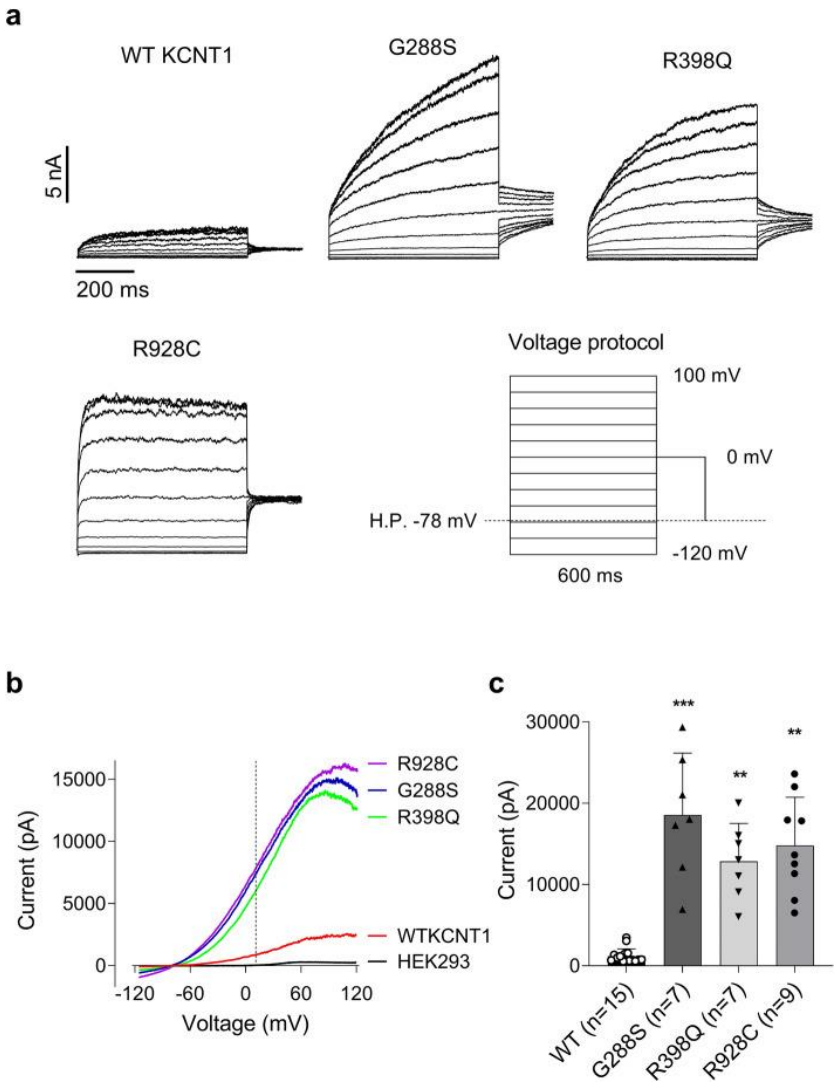


Figure 5-4-WT and mutant KCNT1 currents recorded in HEK293T cells. (a) KCNT1 currents recorded in HEK293T cells in response to the voltage steps ranging from − 120 mV to 80 mV in 20 mV increments followed by a voltage step to 0 mV. (b) Examples of the I-V plots of WT and mutant KCNT1 currents recorded in response to 100 ms voltage ramps between − 120 and 120 mV. For comparison, an example of the I-V plot recorded in non-transfected HEK293T cell is shown. The vertical dash line corresponds to 10 mV. (c) The average amplitudes of the WT and mutant KCNT1 currents measured at 10 mV using the I–V plots recorded in response to the voltage ramps between − 120 and 120 mV, similar to those shown in panel (b). Brown-Forsythe and Welsh's one-way ANOVA followed by Dunnett's multiple comparisons test indicated that currents produced by the mutant constructs were significantly larger, compared to WT KCNT1 ($P = 0.0003$ (G288S); $P = 0.0017$ (R398Q) and $P = 0.0026$ (R928C)).

Next, we analysed the effects of carbamazepine, valproic acid, vigabatrin and cannabidiol (CBD). The in vitro effects of quinidine were not investigated as part of this study as these have been published previously [9,11]. Each drug was first tested on WT *KCNT1* channels at a range of concentrations, including well above their currently accepted potential doses for therapeutic use. The drugs were applied to the bath through the perfusion system and the amplitude of WT *KCNT1* current was monitored by applying voltage ramps between − 120 and 120 mV every 2 s. Carbamazepine (100 μM), valproic acid (30 μM), and vigabatrin (50 μM) showed no effect on the current amplitude produced by WT *KCNT1* within 5–10 min of application (Figure 5-5a). Since the drugs showed no effects on the WT *KCNT1* channels we did not test their in vitro effects on the mutant channels. In contrast, CBD (25 μM) inhibited almost 90% of the WT *KCNT1* current in HEK293T cells (Figure 5-5a). Addition of CBD to HEK293T cells expressing each of the *KCNT1* mutant channels showed a significant reduction in K+ current amplitude (Figure 5-5b). CBD was seen to inhibit *KCNT1* mutant currents with higher potency compared to WT *KCNT1* (Figure 5-5b). The IC_{50} for WT *KCNT1* (5.6 ± 1.88 μM; ($n = 4$)) was significantly higher than IC_{50} for R398Q (0.41 ± 0.22 μM; ($n = 4$, $P = 0.0436$)) and R928C (0.47 ± 0.31 μM; ($n = 4$, $P = 0.0458$)) mutant channels (Figure 5-5b). The difference between the IC_{50} for WT *KCNT1* and G288S mutant channel (0.81 ± 0.34 μM), however, was not statistically different ($n = 4$, $P = 0.0554$). The time course of inhibition was similar between WT and mutant *KCNT1* channels (Figure 5-5c, data for WT and R928C *KCNT1* are shown), and CBD was fully washable.

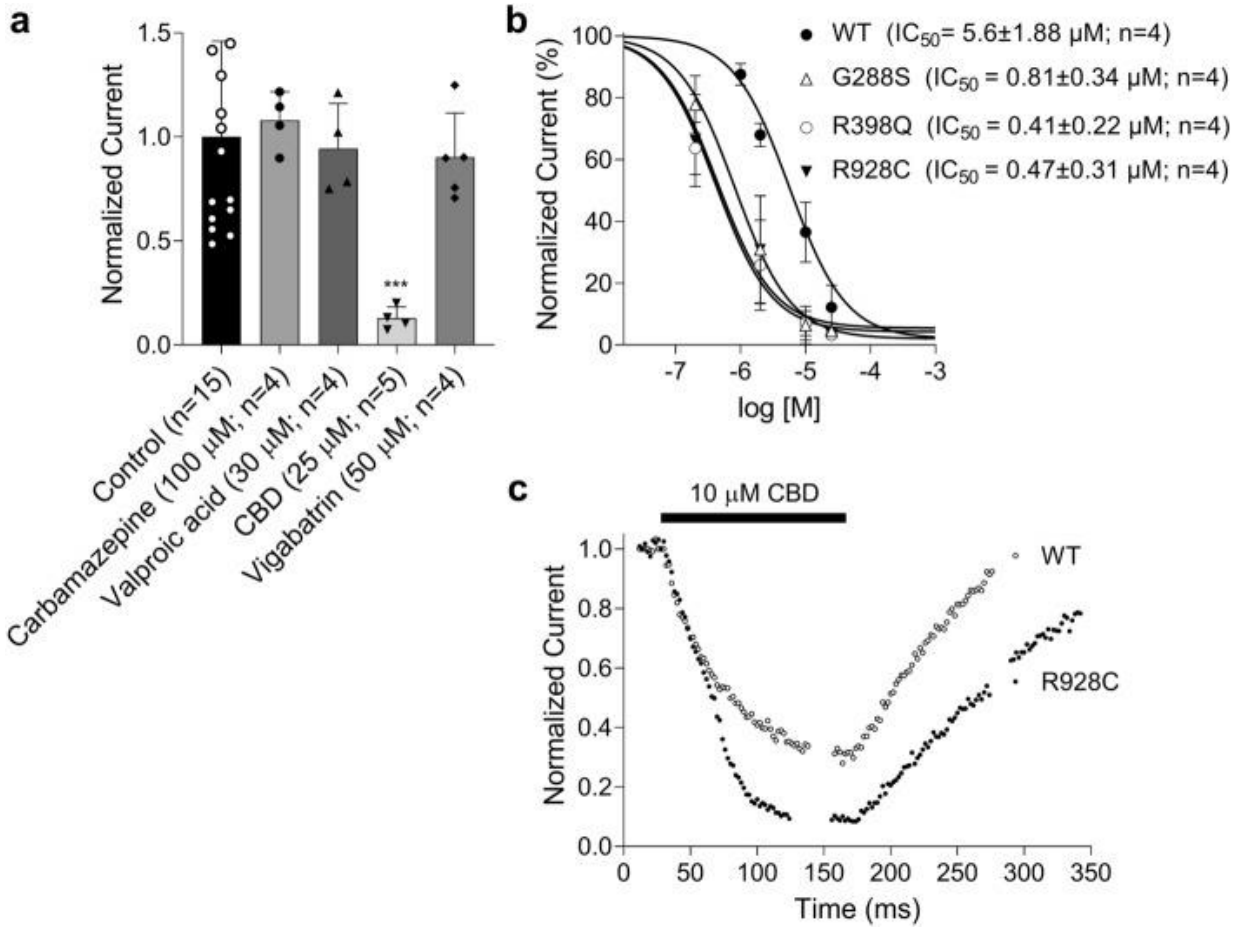


Figure 5-5-The in vitro effects of drugs on *KCNT1* channels. (a) The average normalised WT *KCNT1* current amplitude measured at 10 mV using I–V plots similar to those shown in Figure 5-4b in the presence of the drugs. (b) The dose dependent inhibition of the WT and mutant *KCNT1* channels by CBD. The curves represent the fit of the Hill equation (Eq. 1, Methods) to the experimental data. (c) The time course of *KCNT1* inhibition by 10 µM CBD followed by the washout. Each point represents current amplitude measured at 10 mV from the I–V plots (see Figure 5-4b) in response to the voltage ramps applied every 2 s.

Investigation of *KCNT1* single channel activity using inside-out patches showed that CBD applied to the intracellular side of the membrane inhibited WT *KCNT1* channels at lower concentrations, compared to the extracellular applications (Figure 5-6a–c). Application of 0.5 µM CBD reduced PO of WT *KCNT1* by 17 ± 5% (n = 3) and 1 µM by 84 ± 11% (n = 3) (c.f. IC_{50} for extracellular application 5.6 ± 1.9 µM) without any effect on the single channel current (i = 3.6 ± 0.23 pA (control, n = 9) vs i = 3.7 ± 0.26 pA (0.5 µM CBD, n = 5) and i = 3.6 ± 0.34 pA (1 µM CBD, n = 5)). Similar to the

extracellular applications, CBD inhibited R928C mutant more potently, compared to WT *KCNT1*, reducing PO of R928C *KCNT1* by 25 ± 5% (n = 3) at 0.25 μM and by 80 ± 7% (n = 3) at 0.5 μM (Figure 5-6d–f). However, the difference between the efficacy of CBD inhibition of WT and mutant *KCNT1* in inside-out patches was not as obvious as in the whole cell experiments.

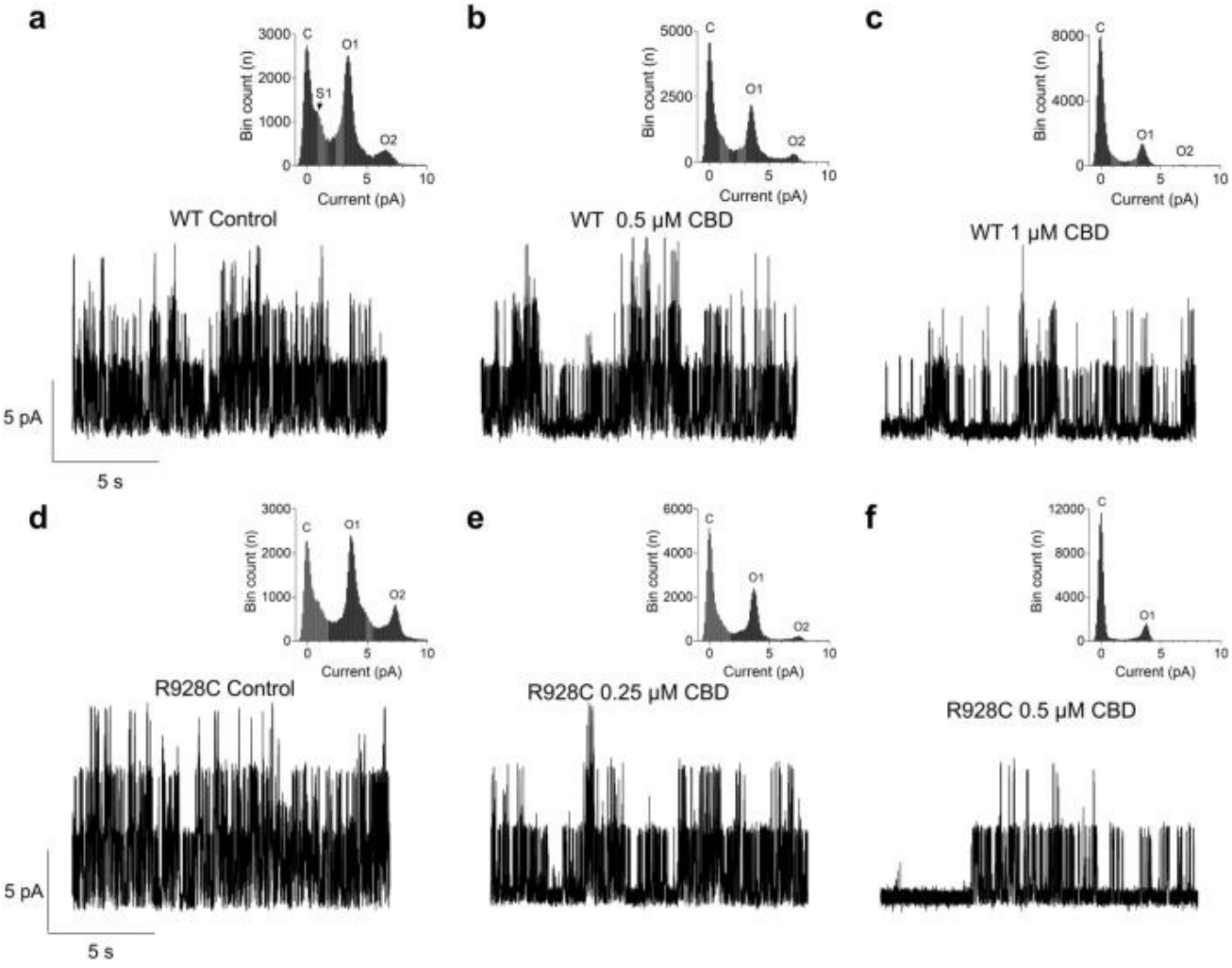


Figure 5-6. The effect of CBD on single WT and mutant (R928C) *KCNT1* channels. Single WT (a–c) and mutant channel currents (d–f) were recorded at 0 mV in inside-out patches under control conditions and in the presence of the indicated amounts of CBD in the bath. The insets show the all-point amplitude histograms of the corresponding traces. C denotes the closed state, O1 and O2 are open states corresponding to the opening of one or two *KCNT1* channels, whereas S1 is a substate.

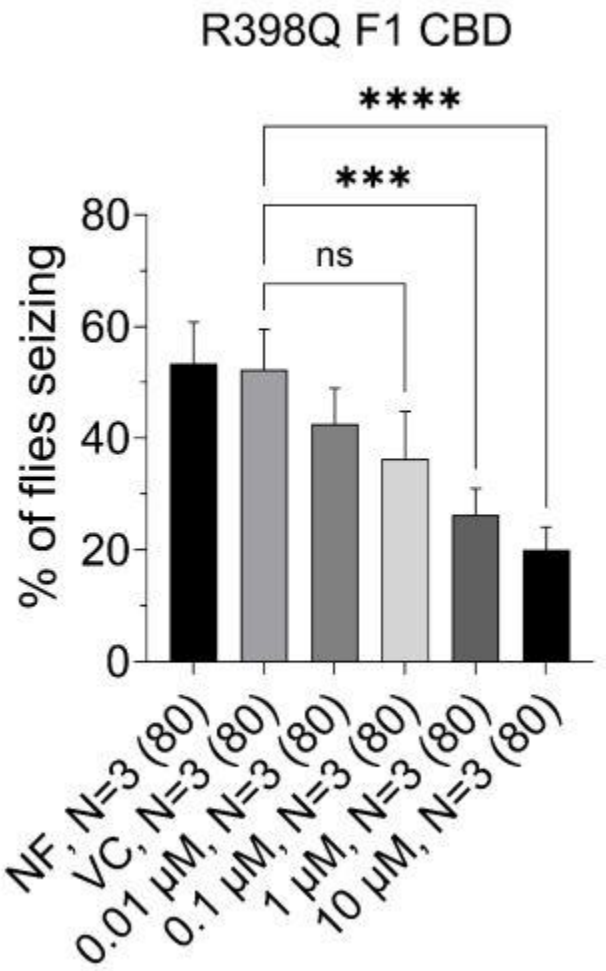


Figure 5-7. Feeding CBD to adult flies expressing human KCNT1 mutant (R398Q) in GABAergic neurons of *Drosophila* rescues seizures in the bang sensitive behavioural assay. *Drosophila* expressing mutant human KCNT1 with the R398Q mutation in GABAergic neurons were raised from embryos on normal food (NF) and the offspring were transferred on a food containing a range of concentrations of CBD (0.01-10 µM) and then analysed in the bang sensitive behavioural seizure assay. The percentage of *Drosophila* showing a seizure phenotype are shown for each dose of drug. N, is the number of independent fly crosses, with total number of flies in all trials shown in the brackets. All data points were compared to the vehicle control (VC) using Brown-Forsythe and Welsh's oneway ANOVA followed by Dunnett's multiple comparisons test. Compared to vehicle control, CBD significantly reduced seizures in R398Q mutants at 1 and 10 µM concentrations. ****P<0.0001, *** P=0.0006, and ns - no significant difference (P=0.0856 for 0.1uM).

5.4.3. Responses of *KCNT1* G228S, R938Q and R928C *Drosophila* seizure models to currently used drug treatments.

We next investigated the effects of the drugs carbamazepine, valproic acid, vigabatrin, quinidine and CBD on the seizure phenotype of the three *Drosophila* lines expressing the mutant human *KCNT1* transgenes. We used feeding experiments with a range of drug concentrations, followed by the bang sensitive behavioural assay. To most closely match the human disease model of onset in infancy we fed *Drosophila* immediately from shortly after birth (i.e., first instar larvae) through to just before assay for seizures 4–8 days post eclosion (hatching). None of the drugs were seen to have an effect on the viability or behaviour of *Drosophila* expressing WT *KCNT1* (data not shown). Carbamazepine, valproic acid and quinidine, were each seen to exacerbate the seizure phenotype in the three *KCNT1* mutant *Drosophila* lines (Figure 5-8). Vigabatrin was seen to reduce the seizure phenotype in the lines expressing G288S and R398C mutant *KCNT1*, while it increased the seizure phenotype in the R928C mutant. CBD showed significant reduction of the seizure phenotype in all three *KCNT1* mutant lines and showed a dose-dependent response (Fig. 6). The maximum reduction of the seizures was 52% in the lines expressing G288S mutant (at 0.01–10 µM CBD); 63% in the lines expressing R398Q mutant (at 10 µM CBD); and 53% in the lines expressing R928C mutant (at 10 µM CBD). We also conducted adults only feeding of flies expressing R398Q *KCNT1* to see if that was sufficient for rescue of seizures. Data for the rescue of *Drosophila* expressing R398Q human *KCNT1* mutant in GABAergic neurons only during adult stages (i.e., post eclosion) is shown in Figure 5-7. While adult feeding also reduced seizures it was not as efficient as feeding from early larval stages, as they required higher doses of CBD (1uM and 10uM) to get significant amounts of rescue (Figure 5-7).

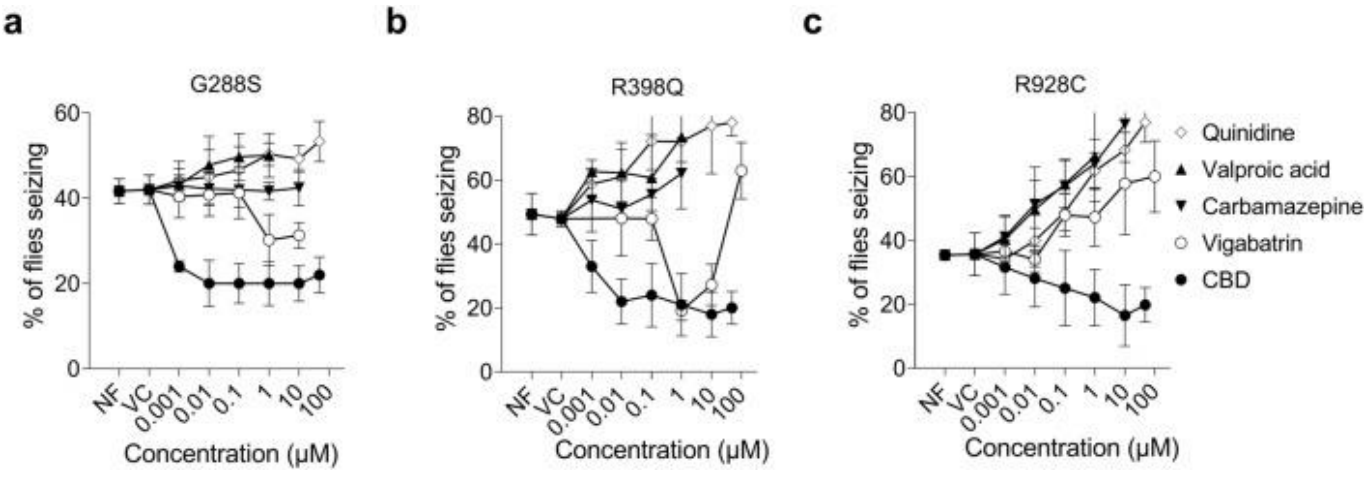


Figure 5-8. In vivo analysis of drug effects on seizure phenotype in *Drosophila* models. *Drosophila* expressing either G288S (a), R398Q (b) or R928C (c) mutant human *KCNT1* in GABAergic neurons were raised from embryos on normal food (NF) or on food containing a range of concentrations of CBD, vigabatrin, valproic acid, carbamazepine or quinidine and then analysed in the bang sensitive behavioural seizure assay. The percentage of *Drosophila* showing a seizure phenotype are shown for each dose of drug. All data points were compared to the vehicle control (VC) using Brown-Forsythe and Welsh's one-way ANOVA followed by Dunnett's multiple comparisons test. Compared to vehicle control, CBD significantly reduced seizures in G288S and R398Q mutants at all concentrations; and R928C at 10 and 50 µM. Vigabatrin significantly decreased seizures in G288S and R398Q mutants at 1 and 10 µM, but increased seizures at 100 µM in R398Q mutant and R928C mutant at 10 and 100 µM. Valproic acid had no effect on G288S mutant, but increased seizures in R398Q at 1 µM and R928C at 1 and 10 µM. Carbamazepine had no effect on G288S and R398Q mutants, but significantly increased seizures in R928C at 0.1, 1 and 10 µM. Quinidine exacerbated seizures in G288S mutant at 50 µM, and R398Q and R928C mutants at 0.1, 1, 10 and 50 µM. The total numbers of flies, the number of independent crosses and the exact P values for all data points are presented in Table 5-2.

Table 5-2. P values obtained using One way ANOVA with Dunnett's multiple comparisons test of the data presented on Figure 5-8. Blue asterisks denote the statistically significant decrease in seizures compared to vehicle control, whereas red asterisks denote significant increase. Ns – not significant.

	G288S					**R3928Q**					**R928C**				
[µM]	CBD	Vigabtr	Valpr	Carbam	Quin	CBD	Vigabtr	Valpr	Carbam	Quin	CBD	Vigabtr	Valpr	Carbam	Quin
0.001	<0.0001 ****	0.9604 ns	0.9999 ns	0.9996 ns	0.9979 ns	0.0136 *	0.1252 ns	0.0526 ns	0.6311 ns	0.3140 ns	0.9608 ns	0.9998 ns	0.8910 ns	0.8675 ns	0.9996 ns
0.01	<0.0001 ****	0.9819 ns	0.4985 ns	0.9977 ns	0.9758 ns	<0.0001 ****	>0.9999 ns	0.0613 ns	0.9479 ns	0.1634 ns	0.5964 ns	0.9996 ns	0.0904 ns	0.0550 ns	0.8641 ns
0.1	<0.0001 ****	0.9944 ns	0.2154 ns	0.9924 ns	0.7269 ns	<0.0001 ****	>0.9999 ns	0.1073 ns	0.4074 ns	0.0012 **	0.1644 ns	0.2033 ns	0.0037 **	0.0023 **	0.0125 *

1	<0.0001 ****	0.0124 *	0.1815 ns	0.9997 ns	0.1240 ns	<0.0001 ****	<0.0001 ****	0.0006 ***	0.0569 ns	0.0022 **	0.0583 ns	0.1963 **	<0.0001 ****	0.0003 ***	<0.0001 ****
10	<0.0001 ****	0.0240 *	0.9999 ns	0.9996 ns	0.2295 ns	<0.0001 ****	0.0010 **			0.0001 ***	0.0021 **	0.0014 ***		<0.0001 ****	<0.0001 ****
50	<0.0001 ****				0.0153 *	<0.0001 ****				<0.0001 ****	0.0096 **				<0.0001 ****
100							0.0153 *					0.0005 ***			

5.5. Discussion

In this study we found that expression of human KCNT1 channels containing three patient-specific mutations gives a seizure phenotype in *Drosophila.* The phenotype was observed when the mutant channels were ectopically expressed in inhibitory GABAergic neurons. These results indicate that overactivity of the KCNT1 channel and increased K+ currents in inhibitory neurons is sufficient to induce a seizure phenotype in *Drosophila*. This finding is consistent with previous studies, including our recent analysis on the electrophysiological properties of a large series of patient *KCNT1* mutations [8]. KCNT1 channels are integral membrane proteins thought to be important in hyperpolarising the cell membrane, acting to inhibit excitability by reducing the likelihood of repetitive firing of action potentials. Greater silencing of inhibitory neurons by increased KCNT1 activity may lead to neuronal hyperexcitability, the mechanism that underlies seizures. Studies in mice also suggest that decreased excitability of inhibitory neurons contribute to seizures [13,14]. Expression of the three *KCNT1* mutants in all neurons, excitatory neurons or in glia in *Drosophila* were damaging, resulting in lethality. The reasons for this are not yet understood. The seizure phenotype of *KCNT1* R398C was significantly stronger than that seen with R928C. This may be consistent with R928C being only identified in patients with the “milder” phenotype of sleep-related hyper motor epilepsy (SHE), while R398C (and G288S) are also found in patients with more severe phenotypes including epilepsy of

infancy with migrating focal seizures (EIMFS) [3]. To ascertain whether our *KCNT1 Drosophila* models may be useful as tools for assessing drug treatments for *KCNT1*-epilepsy, we looked at the effects on the seizure phenotype for some of the drugs currently used to treat patients and reduce seizures in some patients. We also looked at the in vitro effects of the drugs in cell models expressing KCNT1 channels to see if findings from the two systems are comparable. Feeding vigabatrin, valproic acid and carbamazepine to *Drosophila* expressing WT KCNT1 channels showed no evidence of toxicity or alteration of normal behaviour. In the *KCNT1* mutant lines, valproic acid and carbamazepine, which are both known to inhibit voltage and use-dependent sodium channels [39], were seen to exacerbate the seizure phenotype in a dose-dependent manner. Vigabatrin, which increases GABA levels by inhibiting GABA aminotransferase [42], was seen to reduce the seizure phenotype at some doses in the animals expressing *KCNT1* G288S, and R398Q, and exacerbated the seizure phenotype in those expressing R928C. The seizure phenotype in the *Drosophila* models showed responses to the anti-epileptic drugs, with varied effects on the different mutant channels. Variable responses to the drugs are also seen in patients, with none being highly effective in inhibiting seizures. Further studies are needed to investigate if the *Drosophila* models will be useful in preclinical pharmacogenetics for predicting the response of patients with particular *KCNT1* mutations to different drugs. Quinidine is a long-known blocker of ion channels and has previously been used as an anti-arrhythmic drug [43]. It has previously been shown in vitro to significantly reduce the K+ currents of WT [44] and epilepsy associated mutant KCNT1 channels [9,11,19]. However, it has shown variable results and serious side effects in some *KCNT1* epilepsy patients [3,16,19-21,45,46]. Given the conflicting in vitro and in vivo (patients) results, we analysed the effects of quinidine on *Drosophila* expressing the G288S, R398Q or R928C *KCNT1* mutants. The R928C and R398Q mutant models each showed a strong dose-dependent increase in the seizure phenotype, which was less pronounced in the G288S mutant, and no effect was seen in animals expressing WT *KCNT1*. The in vivo effects of quinidine on KCNT1 channels in this study therefore again differ from those seen in previous in vitro studies which show blocking of the channel activity. This is likely due to the presence, in vivo, of multiple interacting neural networks in the context of a functioning nervous system in a whole animal, and/or due to the inhibition of ion channels other than KCNT1 controlling the activity of the

excitatory neurons, due to the higher concentrations of quinidine required to see the blocking effects in the in vitro studies [46]. Ingestion of medicinal cannabis derivatives containing the compound cannabidiol (CBD) reduces the frequency of seizures in some *KCNT1*-epilepsy patients and has not been reported to exacerbate seizures [3,18]. Although the exact mechanism of action of CBD in epilepsy treatment is yet to be elucidated, it has been postulated that CBD modulates intracellular calcium and adenosine mediated signalling to inhibit neuronal activity [47,48]. In this work, CBD was seen to significantly reduce the K+ currents in HEK293T cells expressing WT and each of the G288S,R398Q, R298C mutant KCNT1 channels. The effect was rapid and at least similar or stronger in 'inside-out' patches suggesting CBD may be acting directly on the binding site in the channel, accessible from both sides of the membrane, rather than through cannabinoid receptor signalling. Significantly, CBD was approximately tenfold more efficient at blocking K+ currents of R398Q and R928C KCNT1 channels than WT KCNT1 channels. While this preferential action on mutant KCNT1 channels is a highly desirable property for a *KCNT1* therapeutic, it is unclear why this might be the case. It is possible that CBD binding to KCNT1 channel is state-dependent and the higher open probability of the mutant KCNT1 channels results in a higher efficacy of the drug [8]. This is supported by the results of the inside-out experiments where higher concentration of Na+ (35 mM) was employed to increase the PO of WT channels. The dependence of GoF KCNT1 mutant channels on the intracellular Na+ is significantly shifted towards lower concentrations compared to WT channels [12]. As a result, with higher intracellular Na+ the difference in the PO of WT and mutant KCNT1 channels is diminished, so is the difference in the CBD efficacy. While this is the first demonstration of direct inhibition of the KCNT1 K+ currents by CBD, further investigation into the mechanism of action on KCNT1 as well as the efficacy and dosing regimens for treatment of patients with CBD may also be warranted.

In summary, our study shows that the expression of patient-specific *KCNT1* mutations in *Drosophila* gives a seizure phenotype, modelling human *KCNT1*-epilepsy. The seizure phenotypes in the *Drosophila* models were affected by the addition of drugs currently used to treat people with *KCNT1* epilepsy, suggesting they may be useful as preclinical tools for screening new therapeutics for these epilepsies. Importantly, we were able to demonstrate differential effects, some positive and others

negative, as well as differential efficacies, of currently used drugs. Together, the three *KCNT1* mutations investigated in this study account for approximately one fifth of patients identified to date, thus dosing information and any candidate drugs identified using the models will potentially benefit a significant proportion of people with *KCNT1* epilepsy.

5.6. References

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CHAPTER 6: FUTURE DIRECTIONS

6.1. Future directions

Aneuploidy is a hallmark of cancer. In this thesis **(chapter-2),** I thoroughly reviewed the literature for one-carbon and polyamine metabolism as a therapy target for aneuploid cancer. I also investigated the mechanism of sensitivity of aneuploid cells to depletion of metabolic candidates of the one-carbon and polyamine pathways in *Drosophila* **(Chapter-3).** I found that disruption of polyamine and one carbon metabolism affects survival for aneuploid cells but not for normal proliferating cells. In aneuploid cells, the depletion of some of these genes resulted in apoptosis, but not in normally proliferating cells. More specifically, SAM metabolism has a role in producing polyamines, which contributes to the heightened sensitivity of CIN cells to SAM depletion. It was observed that feeding spermine to animals can prevent the cell death in CIN tissues usually induced by SAM synthase depletion. Methionine is the main amino acid that the liver uses to generate SAM [1]. It also makes up half of the body's daily demand for amino acids. Methionine is converted to SAM by MAT (SAM synthase) in an ATP-dependent process [2]. In this mechanism, methionine and ATP's adenosyl moiety combine to transform ATP into a high-energy reagent that is capable of carrying a sulphonium ion. SAM can then transfer a methyl group to a range of substrates, such as lipids, proteins, DNA, and RNA [3]. Reduced autophagy and susceptibility to reactive oxygen species (ROS) resulted from the loss of polyamines, and my research has demonstrated that these factors greatly contribute to CIN cell death. However, it also raised new questions that need to be addressed.

1) By producing centrosome amplification, microtubule defects, and altered microtubule dynamics, sodium arsenite (NaAsO3) impairs mitosis and results in aneuploidy and multipolar spindles. It has been demonstrated that SAM (S-adenosylmethionine) lessens these consequences by decreasing centrosome amplification, microtubule abnormalities [4], and the creation of micronuclei, especially from lagging chromosomes [5]. Additionally, in treated cells, SAM reduces aberrant microtubule dynamics, multipolar spindle formation, and the duration of mitotic progression [6]. According to my current research, SAM-S depletion promotes apoptosis in aneuploid cells but not in normally growing cells and feeding polyamines reduces the phenotype of aneuploid cell death caused by SAM-S depletion. However, the impact of SAM-S depletion on mitotic dynamics has not been examined in our current model.

If SAM depletion is discovered to worsen CIN via a specific mitotic mechanism, addressing SAM metabolism may become a viable therapeutic method for minimising CIN in cancer patients. Moreover, identifying a mitotic mechanism influenced by SAM depletion would provide better knowledge of how metabolic changes influence mitotic regulation, potentially revealing novel features of how cellular metabolism governs cell division. In addition, if SAM depletion alters microtubule behaviour and mitosis, it may open up new pathways for treatment. For example, medicines that alter SAM levels could be utilised to inhibit the mitotic cycle in rapidly proliferating cells, notably in cancer.

2) Methylation of DNA is a heritable, enzyme-induced modification of DNA structure that does not alter the specific sequence of base pairs required for genome encoding. The methyl groups required for DNA methylation, a crucial epigenetic alteration, are produced by one-carbon metabolism. Through the silencing or activation of particular genes, such as tumour suppressor genes and oncogenes, DNA methylation can control the expression of genes. Cancer and developmental aberrations are linked to abnormal DNA methylation patterns and gene mutations encoding enzymes or DNA methylation regulators [7-10], such as hypermethylation or hypomethylation. Additionally, polyamines have an effect on the one-carbon metabolic pathway. For example, SAM is consumed during their production, which lowers SAM's availability for DNA methylation. As a result, an increase in polyamine production may cause DNA hypomethylation, which is a characteristic of cancer cells. On the other hand, it has been demonstrated that blocking polyamine production slows the growth of cancer and restores normal DNA methylation patterns. In my current study, supplementation of polyamines (spermine and spermidine) reduces the aneuploid cell death, which could have been affected by DNA methylation. However, we have not studied DNA methylation aspects in relation to SAM and polyamines, which is one of the shortcomings of this study. We could extend the current study by investigating the expression of tumour suppressor genes (using RTPCR or RNASeq) **when we alter SAM and polyamines?.** The expression pattern of a common marker representing overall DNA methylation (5-methylcytosine (5mC)) levels across the genome could also provide insight that may suggest a therapeutic intervention options for CIN cancers.

3) The National Cancer Institute defines a biomarker as "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease", such as cancer. Tumour biomarkers are extremely important for tumour detection and treatment. They provide critical reference parameters for early tumour detection, tumour stage assessment, anticancer strategy selection, treatment response monitoring, and prognosis [11,12]. A biomarker can also be a series of changes, such as gene expression, proteomic, or metabolomic patterns. It is well known that aneuploidy is a common characteristic of solid tumour as it occurs in approximately 90% of solid human tumours and 75% of haematological malignancies [13]. In my current study, the survival of aneuploid cells requires one carbon and polyamine metabolic pathway candidates, and the depletion of these candidates causes aneuploid cell death. Upregulation of one carbon and polyamines could be a biomarker for aneuploid cancer. To find out the biomarkers of aneuploid cancer in response to one-carbon and polyamine metabolic candidates, we could do metabolomics analysis to detect levels of Cysteine, Homocysteine, Glutathione, S-adenosyl Methionine synthase, spermine, spermidine, Putrescine, dcSAM by using LCMS or GCMS. We would test one-carbon and polyamine pathways in wild type, *Mad2* RNAi, *Mad2+SAM-S*-RNAi, *Mad2+Sms*-RNAi, *Mad2+Spds*-RNAi, *Mad2+Gnmt*-RNAi flies. This could provide a more clear understanding about one-carbon and polyamine metabolism in cancer and their potential use as a biomarkers for later stage of cancer. Moreover, we could overexpress the *SAM-S, Sms, Spds, Gnmt* expression in CIN flies and do the metabolomics of the same candidates of one-carbon and polyamine pathways to identify the potential of polyamine metabolites as biomarkers.

4) Tumour cells contain higher amount of ROS when compared to normal cells. In our prior work, it is well established ROS level is elevated in aneuploid cells [14]. Our laboratory already has data that there are genetic candidates (PARP, CLAMP) whose knockdown can reduce the ROS production in aneuploid cells and consequently reduce aneuploid cell death. However, we do not know the detailed mechanism. Therefore, we could investigate the effects of PARP and CLAMP knockdown in combination with SAM-S in aneuploid cells. This may provide new insight about the effect of one-carbon metabolism on control of redox stress responses to CIN.

5) We do not know if there is any difference between the *Drosophila* model and other animal models in response to aneuploidy for one-carbon and polyamine metabolic signaling pathways. Therefore, it could be helpful to conduct the study in human cancer cell lines, mouse or rat models. A mouse model of aneuploidy and tumorigenesis [15] could be used to investigate the regulation of one-carbon and polyamine metabolic signaling pathways in response to aneuploidy in mouse embryonic fibroblasts cells to validate our findings. Moreover, a recent study showing the dependency of human aneuploid cells on the RAF/MEK/ERK pathway for overcoming DNA damage could be a useful tool to further study the regulation of one-carbon and polyamine metabolic signaling pathways in response to aneuploidy in human cell lines[16].

In another study **(Chapter 4),** I examined the role of aneuploidy in the development of neurological abnormalities in *Drosophila.* The ultimate target is to generate a *Drosophila* model to investigate the involvement of aneuploidy in the severity of *KCNT-1* epileptic neurological disease. Further advances in the treatment of associated serious seizure disorders are the main purpose of using *KCNT1* mutant which may be achieved by gaining a greater understanding of the mechanisms underlying *KCNT1*-related disease. In my current study, I observed increased aneuploidy due to *Mad2* depletion in third instar larval brains of *Drosophila*. The increased aneuploidy and higher cell death in GABAergic neurons is accompanied by a defective climbing and seizure phenotype. The defective climbing and seizure characteristics of the animals was rescued by feeding them an antioxidant (NACA). However, subsequent studies should focus on a number of important areas:

1) My current *Drosophila* model opens the door to examine the contribution of aneuploidy induced oxidative stress in the severity of neurodegenerative disease models. In future, there is scope to make a combined *Drosophila* model of aneuploidy in *KCNT1* epilepsy. However, in this approach, one challenge is to get viable progeny after crossing *KCNT1* mutant flies with *Mad2*-RNAi. So, we require to test several neuronal drivers (*Gad1, D42-Gal4, nsyb-Gal4, elav-Gal4*) to get viable progeny which may be challenging. If we can generate the necessary stocks, we have some novel genes whose knockdown may rescue the viability (CLAMP, PARP) which could be candidates as a therapeutic tool for

aneuploidy related neurological abnormalities. This approach is also relevant for other neurological diseases such as- Alzheimer's disease and Parkinson's disease.

We also can compare the oxidative stress profiles between *KCNT1* epilepsy to aneuploidy+*KCNT1* epilepsy to determine the effect of aneuploidy induced oxidative stress in neurological disease. The traditional antioxidant treatment against oxidative stress in neurological diseases is problematic. Because a certain level of ROS is also necessary for cell signaling and homeostasis [17]. If antioxidants are used excessively to decrease ROS, this could upset important cellular processes. Furthermore, it is important to consider the fact that many antioxidants are rapidly metabolized and eliminated from the body, have poor bioavailability, and do not easily pass the blood-brain barrier [18]. High dosage and frequent administration are required for antioxidant treatments which have detrimental side effects [19]. But we will test an alternative approach: to intervene by blocking the aneuploidy response, as we find this significantly lowers oxidative stress in aneuploid cells and should not impact normal cell redox signaling. Therefore, our current approach is beneficial if we could metabolically prevent the detrimental effects of ROS in neurodegenerative diseases and exploit as a therapeutic strategy in neurological diseases.

2) Based on our preliminary screening of male-specific lethal (MSL) related genes (Unr, Msl1, Mof, roX1, roX2, CLAMP, JIL-1, PARP). We have found that the loss of CLAMP and PARP rescued cell death in aneuploid cells by lowering the ROS levels. There is scope to extend the investigation into how the aneuploidy can be sensed by the cell and triggers the stress response which results in cell death, and how these genes mediate the effect. If we could identify the novel pathways involved by depleting these genes and screening for others with similar effects, the ability to rescue the lethality of aneuploid cells and lower ROS levels could be used as a therapeutic tool for neurodegenerative diseases. As we cannot replace neurons, if neuronal cells become aneuploid and die, the brain carries this effect for life. So, it would be a significant finding if we can prevent the death of aneuploid cells without needing to use damaging levels of systemic antioxidants.

3) In our previous publication, we demonstrated that aneuploidy induces protein folding stress [20], and it is well known that neurological abnormalities are linked to the misfolding and aggregation of proteins [21]. In our current model, we could extend this work by depleting *Mad2* in GABAergic neurons to investigate how aneuploidy contributes to neurological phenotypes through protein misfolding. In brain aneuploid cells, protein aggregation can be demonstrated using Myc-tagged CAG91 (a polyglutamine repeat). This approach could provide insights into interventions aimed at reducing the burden of neurological phenotypes.

4) Autophagy is crucial for aneuploid cells survival [22]. In my thesis, I found that decreasing autophagy by depleting Atg1 caused an increase cell death in a SAM-s-depleted aneuploidy background and depleting SAM-s along with Atg18 was synthetically lethal in CIN animals. Autophagy plays a critical role in neurological diseases and has potential as a therapeutic tool. We could see the changes in autophagy-related gene expression by using quantitative reverse transcription PCR (qRT-PCR) in *Mad2* depleted GABAergic neuron flies vs wild type flies in response to SAM and polyamines and see how these genes can affect the aneuploidy related neurological phenotypes. We could also use GFP-tagged Atg8a transgenic *Drosophila* to quantify GFP-Atg8a puncta in the Drosophila brain tissues by using fluorescence microscopy.

5) In the mammalian nervous system, DNA methylation controls the fate of neural stem cells, native brain function, neurodevelopmental abnormalities, and neurodegenerative illnesses [23,24]. In our current study, there is a possibility that *Mad2* depletion in GABAergic neuron can cause abnormal DNA methylation which may have effects on neurological phenotypes in *Drosophila.* The methodology from previous published papers [25] can be used to detect DNA methylation *Drosophila* brains.

6) Aneuploidy was genetically induced by *Mad2* depletion in *Drosophila* larval proliferative wing discs to test the sensitivity of various metabolic signaling pathways in response to aneuploidy in our current and prior published works. We found JNK, one-carbon and polyamine metabolic pathways are regulated in response to aneuploidy [26,27]. Moreover, autophagy and oxidative stress also regulates the survivability of aneuploid cells [22]. In addition, prior evidence also suggests that aneuploidy increases susceptibility to nucleotide depletion, protein folding stress, and metabolic stress [14,20]. As neuronal tissues are metabolically different from proliferative epithelium, therefore, there is scope to examine the regulation of these pathways in response to neuronal aneuploidy. Moreover, we could also investigate how neuronal tissues/cells tolerate aneuploidy and how the aneuploidy threshold makes them die which could be exploited as a therapeutic strategy for neurological diseases.
7) We do not know if there is any difference between the *Drosophila* model and other animal models in response to *Mad2* depletion in GABAergic neuron. Therefore, it could be helpful to conduct the study in *Mad2* depleted mouse GABAergic neuron and validate the *Drosophila* model findings.

In Chapter 5, we created *Drosophila melanogaster* lines carrying human *KCNT1* with the patient mutations G288S, R398Q, or R928C in order to test if *Drosophila* may be utilized to replicate human *KCNT1* epilepsy. Each mutant channel's expression in GABAergic neurons produced a seizure phenotype. While some medications increased the seizure phenotype, cannabidiol demonstrated the largest reduction in seizure phenotype. However, there are still some key points can be addressed in future research:

1) The lack of knowledge about the medications' bioavailability in the brains of *Drosophila* is one of the study's main shortcomings. The amounts of cannabidiol in the fly brains can be found using ultra-performance liquid chromatography quadrupole time-of-flight mass

spectrometry selective ion recording (UPLC-QTOF-MS SIR).

2) To validate the *Drosophila* model, it is important to carry out the same research in mouse or rat models before going to clinical trial. Moreover, pharmacokinetic and toxicokinetic profiling also needs to be addressed.

In conclusion, aneuploidy induces oxidative changes and proteotoxic stress, which makes cells more vulnerable to stress and is commonly seen in tumours. This thesis showed that chromosomal instability (CIN) modifies the expression of genes involved in one-carbon metabolism in a *Drosophila* model, specifically affecting the production of polyamines and s-adenosyl methionine (SAM). CIN cells also exhibited increased sensitivity to SAM disruption, which resulted in cell death that could be prevented by supplementing with spermine, indicating that polyamine pathway targeting could be a viable therapeutic approach for CIN tumours. I also found that induced aneuploidy in GABAergic neurons led to seizures-like phenotypes and increased cell death. Antioxidant therapy (NACA) restored these phenotypes, suggesting antioxidants as possible therapeutic agents and demonstrating the important role oxidative stress plays in aneuploidy-driven neurodegeneration. Furthermore, we used *Drosophila* human *KCNT1* mutations (G288S, R398Q, and R928C), which produced seizure symptoms resembling those of humans. Responses to a test of five leading epileptic medications varied, but cannabidiol significantly decreased seizure activity. This work emphasizes how useful *Drosophila* is for simulating *KCNT1* epilepsy and assessing novel therapies.

6.2. References

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