It's Justi*Flied*: The Endogenous Retrovirus K Integrase Induces Motor Disturbances in Transgenic Drosophila

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is an incurable neurodegenerative disease characterized by the loss of cortical and spinal motor neurons. Endogenous retrovirus K (ERVK) is a genomic viral symbiont that has been associated with motor neuron loss in ALS. The ERVK integrase (IN) is an enzyme with a role in driving neuropathology and motor deficit. The primary role of the viral IN enzyme is to insert viral DNA into the host cell genome. Accumulating evidence also points to ERVK IN activity causing DNA damage and genomic instability in the host. In Drosophila, retroelement activity contributes to deregulation of the ALS risk gene *TARDBP* (TDP-43, TBPH in Drosophila) via DNA damage-mediated cell toxicity. This suggests a dynamic interaction between TDP-43 biology, DNA damage and retroelements. I have determined that motor disability in ERVK IN expressing Drosophila correlates with neuropathological evidence of DNA damage, inflammation, and TDP-43 aggregation. Viability and behavioral assays and the Trikinetics DAM5H monitor were used to assess motor impairment in ERVK IN expressing flies. Two FDA approved HIV integrase inhibitors were administered to determine if the progression of motor impairments could be limited. Western blot analysis was used to monitor changes in ERVK IN, γH2AV (DNA damage marker), TDP-43, PARP1 and other related proteins over time. Pathological molecular markers were correlated with behavioural assays for motor function, to identify potential biomarkers. Establishment of this model allowed me to assess the association between ERVK IN-driven motor impairment and neuropathological outcomes. Determining the effect of integrase inhibitors in ERVK IN expressing Drosophila is a crucial step towards evaluating antivirals as a novel therapeutic strategy for the reversal of motor neuron damage and motor deficit in ALS.

1.0 Background

1.1 ERVs: The Silent Companions

Endogenous retroviruses are DNA remnants of ancient exogenous retroviruses that infected the germ-line cells of our human ancestors[1]. The oldest ERVs in humans are ERVF and ERVH which endogenated into primates approximately 60-70 million years ago[2]. ERVK is the youngest and most active ERV, endogenating into the primate lineage over the last 200,000 to 25 million years[2]. ERVs are passed from parent to offspring resulting in the presence of ERVs in both germline and somatic cells in the next generation. This vertical ERV transmission is inherited in a Mendelian fashion and accounts for approximately 8% of our genome[1]. The majority of ERVs have been rendered inactive due to the accumulation of mutations and post-insertional modifications that make most unable to produce viral protein and are thus non-infectious[3].

The structure of ERVK is analogous to exogenous retroviruses and consist of four main overlapping gene regions (*gag, pro, pol* and *env)* bracketed by viral promoters called Long Terminal Repeats (LTRs)[1]. The *gag* gene encodes the structural proteins consisting of the capsid, nucleocapsid and matrix. The *pro* gene encodes the protease enzyme, which is involved in viral polyprotein processing, resulting in the production of mature viral proteins. The *pol* gene encodes the reverse transcriptase (RT[4]) and integrase (IN[5]) enzymes, required for insertion of a viral DNA copy into the host genome. The *env* gene encodes the envelope which includes the surface unit, CTXLP[6] and transmembrane proteins. The *env* gene also encodes two spliced proteins, Rec and Np9[1].

Ancestral exogenous retroviruses that became ERVs had envelope proteins which targeted germline cells, enabling entry into the host cytoplasm through fusion with the plasma membrane [1]. Once inside, the retroviral RNA genome was converted into double-stranded DNA (dsDNA) by the reverse

transcriptase enzyme[1]. The viral linear dsDNA along with viral proteins IN and RT and additional cellular proteins formed a pre-integration complex (PIC). The PIC was transported into the nucleus where it associated with the host chromosome. IN catalysed the integration of the virus into the host genome. An integrated virus is termed a provirus; thousands of these insertion events populated our ancestral genome with ERVs. However, the ERVK lifecycle is not complete, as virus expression is still possible[7]. The LTRs formed during reverse transcription have a role in regulating proviral gene expression through the actions of enhancers, promoters and polyadenylation signals[1], which interact with host cell transcriptional machinery. Once transcribed, viral proteins can be produced and impact host cell function through interaction with a variety of cellular pathways.

1.2 ERVK: The Not So Silent Companion

ERVK is a member of the betaretrovirus-like supergroup[8]. Presently, the ERVK clade contains 10 groups HML-1 to HML-10 (HML meaning "human endogenous MMTV-like"). The K designation refers to these viruses using a lysine tRNA to prime reverse transcription[8]. In the human genome today, there are approximately 3,000 proviral remnants of HML-2, and of those about 200 are full-length proviruses[9]. However, only about 90 proviruses have been shown to produce viral protein[10]. In healthy adults, ERVK expression is silenced in most cell types or expression is low[8]. Reactivation has been linked to several types of cancer, inflammatory immune disorders and neurodegenerative disease, such as Amyotrophic Lateral Sclerosis (ALS)[8,11-13].

The transcriptional regulation of ERVK is controlled by the viral promoter within the LTRs, which interacts with viral and cellular transcription factors to modulate viral gene expression[14]. Tissue and cell-type specific reactivation of ERVK occurs in inflammatory diseases such as ALS, Rheumatoid Arthritis, Cancer and Systemic Lupus Erythematosus[12,14-16]. Neuroinflammation is a key factor in the reactivation of the

ERVK provirus in ALS[17]. While it is unclear how reactivation occurs, it is known that the ERVK promoter contains interferon-stimulated response elements (ISREs) that the proinflammatory transcription factors IRF1 and NF-κB can bind to elicit the upregulation of ERVK[17]. ERVK reactivation can lead to the formation of viral proteins and in limited circumstances intact virions[18].

1.3 Retroelements in Drosophila

As seen in humans, the Drosophila (fruit fly) genome is also populated with retroelements[19]. Among the order Ortervirales, insect retrotransposons are classified into the *Metavirdae* family, as opposed to mammalian ERVs which are members of the *Retroviridae* family[19]. Retrotransposons such as *gypsy, ZAM* and *nomad* among several others make up approximately 2% of the Drosophila genome[19]. Drosophila Errantiviruses are insect endogenous retrotransposons and are similar to ERVs in humans[20]. *Gypsy* is a Drosophila retrotransposon that possesses similarities to ERVK[21].

1.4 Viral Integrase: It's What's Inside That Counts

The most vital enzyme for retroelement integration into a host genome is integrase (IN). The structure of IN contains three domains: The N-terminal domain (NTD), the central catalytic domain (CCD), and the C-terminal domain (CTD)[22]. The NTD functions in IN multimerization and contains two conserved residues of histidine (H) and cysteine (C) forming a zinc-binding HHCC motif[5]. The zinc functions as a stabilizer for the folded three helical bundle shape of the NTD[23]. The CCD contains the IN active site and the catalytic triad called the DDE motif so named for the two aspartic acid (D) residues and the glutamic acid (E) residue[5]. The triad identifies and binds to Mg^{2+} which is necessary for IN function[5]. The CTD is the least conserved of the three domains and functions in the formation of the intasome, as well as interacts with DNA substrates[5,22].

The integrase has two main roles in facilitating the insertion of viral DNA into the host genome, the first is through 3' end processing of the viral DNA, the second role is the strand transfer reaction[5]. As a part

of the PIC, a tetramer of IN assembles on either side of the linear viral dsDNA forming the intasome[22]. In the presence of Mg^{2+}, IN can then remove 2-3 nucleotides from either of the 3' viral DNA ends producing 3' hydroxyl groups[5]. The binding of host DNA with the intasome forms the target capture complex[22]. Inside the complex, IN uses the viral 3' hydroxyl groups to cleave host DNA while at the same time joining the 3' viral ends to the host DNA[22]. The post catalytic strand transfer complex is then subject to disassembly by host machinery[22,24]. Unfortunately, the insertion of retroviral DNA is flawed. The integration process leaves ssDNA gaps at the host-proviral junction, and two 5' overhangs of viral DNA[5]. It is the job of host cell machinery to repair these DNA lesions and restore genomic integrity.

1.5 DNA Damage and Repair: What Doesn't Kill You…is Repaired?

DNA damage occurs to our cells everyday via genotoxic stressors[25]. It is the responsibility of the host DNA repair mechanisms to facilitate the DNA damage response (DDR)[26]. The ERVK IN enzyme is proposed to be a significant cause of DNA damage to the human genome[5]. IN enzymes damage DNA through the creation of single stranded breaks (SSBs) and double stranded breaks (DSBs) (**Figure 1**).

The most common type of DNA damage is SSBs, occurring more than 10,000 times per cell per day[27]. The induction of single nucleotide breaks occurs through several different mechanisms, including direct damage to the sugar or base of DNA, defective DNA repair, oxidative stress and through aborted cellular enzymatic activities[27]. If SSBs are unrepaired, it can result in DNA replicative stress, stalling of transcription and excessive PARP activity, which promotes genomic instability[27]. SSBs are repaired by excision repair mechanisms such as single-strand break repair (SSBR), base excision repair (BER) and nucleotide excision repair (NER)[28]. SSBs are formed during the process of retroviral DNA integration. It is the job of host DNA repair proteins to fix the break. PARP1 is an essential protein for the detection of SSBs and facilitates the recruitment of DNA repair proteins[28].

DSBs occur less frequently than SSBs, with approximately 10 breaks per cell per day, but can also be more problematic[29]. DSBs are repaired through one of two ways; homology directed repair (HDR) and non-homologous end-joining (NHEJ)[30]. During HRD, the damaged chromosome is fused with a completely or similarly homologous chromosome (sister chromatid) which is used as a guide to repair the break[30,31]. HR is mainly undergone during the S and G2 phases of the cell cycle when a sister chromatid is readily available[31]. As opposed to HR, NHEJ does not require a homologous sequence to be repaired; rather, it enables the direct re-ligation of the DNA[31]. NHEJ can occur during any stage of the cell cycle and involves the actions of several proteins including Ku70/80 which binds to DNA and acts as a docking sites for other proteins such as the nucleases DNA-PKcs and Artemis in humans[29]. DNA-PKcs and Artemis are not found in Drosophila and the polymerases/nucleases used in this process are not yet known[31]. Both the mammalian H2AX and homologous Drosophila H2AV histone proteins are phosphorylated in the presence of DSB[32]. Misrepaired DSBs can result in serious genetic mutations and even a single unrepaired DSB can be enough to trigger cellular apoptosis[31].

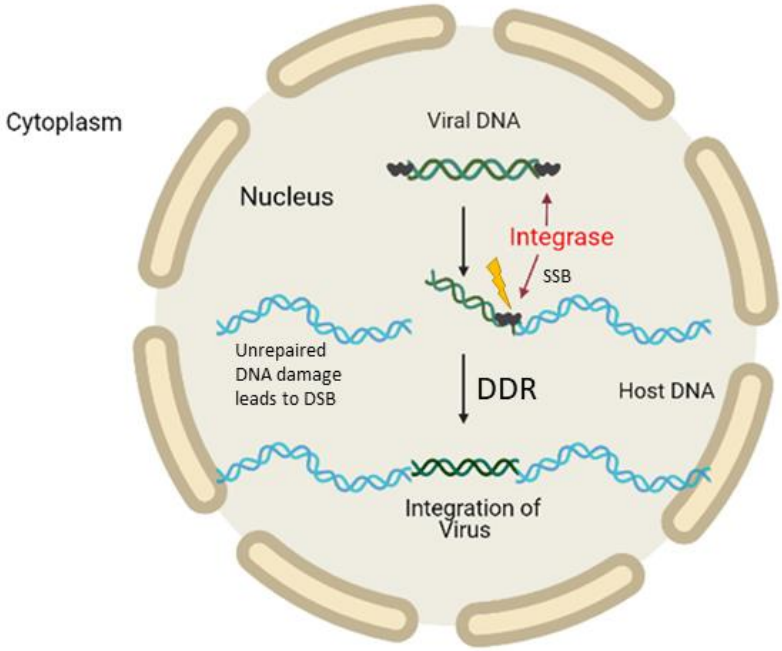


Figure 1: Viral integrase induces DNA damage. Viral DNA contains integrases on either end. The IN removes 2-3 nucleotides from its genome producing 3' ends containing hydroxyl groups (not shown). Using these hydroxyl groups, IN can nick the host genome creating single-stranded breaks (SSB) and joining viral and host DNA. The IN induced DNA damage initiates the DNA damage response (DDR) to facilitate genome repair. A successful DDR leads to proper proviral integration into the host genome. Unrepaired DNA damage can lead to double-stranded breaks (DSB) and genome instability.

1.6 DNA Damage Response Feeds into Innate Immune Pathways

How cells react to stress like DNA damage is an inordinately complex subject with many facets and even more unknowns. One pathway (of many) that is activated by DNA damage is innate immune signalling. Key drivers of inflammation include interferon response factors (IRFs)[33] and nuclear factor kappa B (NF-κB)[34]. These transcription factors are activated by several DNA damage signalling pathways including mediators PARP1, STING and DNA-PK[28,35].

PARP1 has been implicated in several DNA repair pathways and in the maintenance of genomic integrity[28]. DNA damage (SSBs, DSBs) results in the recruitment of PARP1 to DNA[28]. This activates PARP1 catalytic activity and the production of PAR chains[28]. Proteins involved in the DDR bind to the PAR chains located on PARP1 and are thereby recruited to sites of DNA damage to facilitate repair[28]. PARP1 also has roles in stabilizing replication forks, in chromatin remodeling[28] and links to innate immune signalling[36]. PARP1, ATM and TRAF6 form a signalling complex that directly interacts and activates STING[36]. STING activates the transcription factors IRF3 and NF-κB causing the expression of INFs and cytokines triggering an inflammatory response[35].

DNA-PK is also a protein activated during viral infection and involved in the repair of DSBs and triggering apoptosis in response to DNA damage[37]. At sites of DSBs, DNA-PK phosphorylates itself and repair

proteins involved in NHEJ. Moreover, DNA-PK activation during infection leads to IRF3 phosphorylation triggering transcription of other genes and the onset of an antiviral response[37]. While the main role of IRF3 is in inducing antiviral immunity, it also has immunomodulatory and neuroprotective functions[38]. Conversely, IRF3 facilitates virus and DNA damage-triggered apoptosis[39].

NF-κB transcription factors are involved in multiple cellular processes including inflammation, immunity, and apoptosis[40-42]. Upon activation, NF-κB translocates to the nucleus and increases pro-inflammatory gene expression[34]. NF-κB is a key player in regulating the DDR[43]. When DSBs occur, the kinase ATM is activated and phosphorylates NEMO (NF-κB essential modulator)[44]. This phosphorylation induces NEMO SUMOylation and ubiquitination. These modifications facilitate the transport of the ATM-NEMO complex into the cytoplasm where it associates with RIP1 and TAK1, which promotes IKKb degradation, ultimately allowing for nuclear translocation of NF-κB[44]. Together, IRF3 and NF-κB signalling drive inflammation in the context of DNA damage.

1.7 Innate Immune Response in Drosophila*:* Similar but not the Same

Drosophila orthologues have been identified in 75% of known human disease-associated genes[45]. However, Drosophila do not encode IRF genes and therefore have different mechanisms for initiating innate immune responses[46]. Nonetheless, similar to the NF-κB pathway in humans, Dorsal/Relish/Dif pathways drive inflammatory cascades in flies[45]. Analogous to the activity of IKKβ, the removal of Cactus frees NF-κB-like Dorsal and Dif, which translocate to the nucleus and induce the expression of many target genes important for immunity[45]. The NF-κB pathway can also be activated by TAK1 which activates the IKK complex leading to Relish phosphorylation[45]. Dredd cleaves Relish, which then translocates to the nucleus and activates downstream genes[45]. When cellular stress induced by pathogens or DNA damage becomes too great, there is only one other outcome: death.

1.8 Cell Death: The Final Frontier

Cell death is a normal part of the cell lifecycle; however, cell death in the brain and spinal cord contributes to neurodegenerative diseases, as neurons are post-mitotic and not easily replaced. The main cell death pathway triggered by DDR is p53-mediated apoptosis, whereas futile DNA repair leads to ATP depletion and necroptosis[47]. These modes of cell death are also observed in ALS, with either apoptosis or necroptosis occurring in degenerating motor neurons[48,49]. Moreover, these pathways are evolutionarily conserved in both humans and Drosophila, enabling translational research between model systems[50].

DNA damage and apoptosis

Apoptosis is an energy dependent form of programmed cell death characterized by cytoplasmic shrinkage, chromatin condensation, membrane blebbing and the formation of apoptotic bodies[49]. In humans, the apoptotic pathway is driven the executioner caspase-3 which initiates cell death[51]. In Drosophila, apoptotic stimuli can trigger a collection of four pro-apoptotic genes referred to as RHG (*Reaper, Hid, Grimm* and *Sickle)*[52]. RGH works by initiating a cascade leading to the activation of caspase-3-like proteins DriCE and Dcp-1, which drive apoptosis[52].

DNA damage and necroptosis

Necroptosis is a recently discovered form of programmed necrosis, which is characterized by pore formation and loss of plasma membrane integrity, allowing release of cell contents which perpetuate an inflammatory immune response[53,54]. Necroptosis has been implicated as a primary mode of motor neuron death in ALS[49,55]. Necroptosis is initiated upon activation of the kinase domain of the receptor interacting protein 1 (RIP1), which then activates RIP3. RIP3 phosphorylates and activates the mixed lineage kinase domain-like protein (MLKL) in order to form the necrosome. Once MLKL is induced, it translocates to the plasma membrane forming pores which disturb membrane integrity and initiates cell

death[49]. The Drosophila homolog to RIP1 is called IMD[56]. In flies, IMD signalling leads to Relish activation (discussed above) and apoptosis instead of necroptosis[57] (due to lack of RIP3 and MLKL in flies)[58].

1.9 ERVK and ALS: The Connection

ALS is an incurable and fatal neurodegenerative disease characterized by the progressive death of upper and lower motor neurons in the central nervous system[59]. In many cases, the increasing death of motor neurons eventually leads to paralysis-induced respiratory failure leading to death[60]. The prevalence of ALS is approximately 1-2 people for every 100,000, with a higher occurrence among Caucasian people, although it affects all ethnicities[59].

ALS causes atrophy of muscle fibers (amyotrophic) and degeneration of upper motor neuron axons in the lateral column of the spinal cord (lateral sclerosis) which are then replaced with reactive astrocytes[60]. Early-stage symptoms include muscle ailments such as cramps, stiffness, twitching, and weakness which eventually progresses to slurred speech and problems chewing and swallowing[60]. Later stage symptoms include further progression of muscle weakness and cramps and the development of muscle spasms and atrophy[60]. There are two types of ALS, sporadic (sALS) and familial (fALS), which make up 90-95% and 5-10% of cases, respectively. Select genes have been reported to be involved in ALS pathogenesis such as *TARDBP, C9ORF72*, *FUS* and *SOD1* [61]. The *TARDBP* gene is of particular interest as it is involved in both types of ALS[61].

ALS-like neuropathic symptoms have also been observed in viral infections such as HIV and human T-cell leukemia virus type 1 (HTLV-1)[62-64]. It has been shown that HIV-associated motor neuron disease is clinically indistinguishable from sALS[62]. Curiously, it has also been shown that in HIV patients there is a higher level of ERVK expression[62]. In three case studies, HIV patients had lower ERVK loads with variable effects on motor function after undergoing antiretroviral therapy[62]. ERVK expression is increased in ALS patient brain and spinal cord tissues[4,6]. The motor cortex had the highest amount of *pol* expression, with

cortical neurons exhibiting elevated levels of the RT protein[4]. The expression of ERVK is strongly associated with TDP-43, a neuropathological protein in ALS[4,65]. Moreover, the expression of ERVK proteins may contribute to the dysregulation of TDP-43, and conversely mutant TDP-43 forms can cause ERVK protein deposition in cells[65]. Further evidence for ERVK induced neuropathology is revealed in a ERVK *env* transgenic mouse study which has shown that ERVK envelope protein leads to neurotoxicity, as seen through neuronal death and neurite retraction[66]. In this model, ERVK *env* expression caused the death of upper and lower motor neurons, as well as a pattern of motor dysfunction which is typically observed in ALS patients[66].

1.10 TDP-43: A Pathological Player in Motor Neuron Disease in both Humans and Drosophila

TARDBP encodes TAR binding protein 43 (TDP-43), a highly conserved DNA/RNA binding protein[61]. TDP-43 has a N-terminal domain containing the nuclear localization signal, two RNA recognition motifs (RRM1/RRM2) and a C-terminal domain comprising both glutamate/asparagine rich and glycine rich domains[61]. Most of the TDP-43 protein pool is located in the nucleus with the rest continuously ferried between the nucleus and cytoplasm[67]. Nuclear TDP-43 targets a varied range of RNA transcripts including pre-mRNA, miRNA and long intronic sequences[68]. This allows TDP-43 to contribute to several RNA processing pathways such as alternative splicing, miRNA biosynthesis, transcriptional repression, and its own autoregulation[67,68]. Cytoplasmic TDP-43 functions under stress conditions to regulate mRNA stability, translation, and nucleocytoplasmic transport by forming and being recruited to complexes termed stress granules (SG)[67]. SGs form in response to cellular stress conditions and function in the storage of non-essential mRNAs, RNA binding proteins and translation factors[69]. TDP-43 is crucial for the proper development of the CNS from early embryogenic stages to adulthood; therefore, when a stressor results in the TDP-43 aggregation, cellular functions are greatly affected[67].

The primary way that TDP-43 contributes to the neurodegenerative affects of ALS is a debated topic. One idea is that the removal of nuclear TDP-43 and subsequent cytoplasmic aggregation prevents TDP-43's normal function[70]. Another concept is that TDP-43 gains toxic properties which contributes to cell dysfunction[70]. Pathological TDP-43 is phosphorylated, ubiquitinated and found in truncated forms (TDP-25, TDP-35)[70], and its presence in SG is a hallmark of ALS[69-71]. Cleaved TDP-43 lacks the nuclear localization signal preventing its translocation into the nucleus and thus preventing it from carrying out its normal functions[68]. The CTD glycine-rich region of TDP-43 is involved in protein-protein interactions and has been proposed to resemble a prion like domain[68,72], potentially contributing to aggregate formation. Under normal physiological conditions the cell can degrade these small aggregates. If deposition persists, physiological conditions and cellular functioning can be disturbed and lead to neural degeneration[68]. TDP-43 positive inclusions have been found in affected neurons in approximately 97% of fALS and sALS cases[68]. Moreover, Drosophila containing mutated TDP-43 have displayed neuromuscular defects like those seen in ALS[72]. A study done on TDP-43 (TBPH) deficient Drosophila shows altered gene expression related to synaptic transmission and the release of neurotransmitters[73]. A notable function of TDP-43 that is lost in disease states results in de-repression of retroelements[42]. A Drosophila model expressing toxic human TDP-43 in glial cells shows increased expression of the Drosophila retroelement *Gypsy*[74]. Additionally, TDP-43 has a role in response to DNA damage[42]. With TDP-43 sequestered in cytoplasmic aggregates, the nuclear localization of TDP-43-associating DSB repair proteins is decreased, and DNA damage can accumulate leading to cell death[75]. Both factors contribute to a positive loop of DNA damage. TDP-43 also induces the expression of the proapoptotic genes *Bbc3* and *Bax*[76] and can initiate apoptosis in adjacent neurons leading to motor neuron disease phenotypes[74]. Genetically or pharmaceutically inhibiting the RT enzyme of *Gypsy* had the effect of reversing aggregated TDP-43 induced toxicity and extending lifespan[74]. It is possible that inhibiting ERVK activity via drug

intervention would have the same affect as inhibiting *Gypsy;* preventing further neuron death in motor neuron disease.

1.11 ALS Drug Treatments: A Sad Tale

ALS is a devastating disease that affects both diagnosed individuals as well as their support system; it is therefore surprising that so few treatments options are available, and no cure is in sight. There are currently only four FDA approved drugs used to treat ALS: Riluzole, Tiglutik, Edaravone and Nuedexta and one only Health Canada approved drug, Albrioza. Riluzole was first approved in 1995 and has proved to delay the onset of respiratory disfunction and increase patient lifespan by 2-3 months[59,77]. This drug works by decreasing glutamate levels by interfering with glutamatergic transmission which protects motor neurons from excitotoxicity induced deterioration[59]. Nuedexta was the next drug approved in 2010 and is a treatment for sudden emotional outbursts associated with pseudobulbar effects that can transpire concurrent with ALS as well as other neurological diseases such as Multiple Sclerosis and Parkinsons disease[78]. Edaravone was approved by the FDA to treat ALS in 2017[77], and has proven to slow the deterioration in physical ability in patients by over 30%[79]. The specific action of this drug in ALS is unknown, though it is thought to act as a neuroprotective agent against oxidative stress due to its known function as an antioxidant[60]. Tiglutik was approved in 2018 and is a thickened, liquid form of Riluzole for patients who have difficulties swallowing an oral tablet due to paralysis of throat and facial muscles[77]. Albrioza is the latest drug to be developed and thought to reduce mitochondrial and endoplasmic reticulum stress and while not yet FDA approved, it has been approved by Health Canada in June of 2022 as the newest ALS treatment option[80].

1.12 Raltegravir: A New Solution?

Drug development typically take decades from start to finish. ALS patients unfortunately don't have that kind of time. By looking at this disease from a virologic perspective instead of a canonical viewpoint we can hopefully repurpose already FDA approved drugs as potential treatments for ALS. Raltegravir is an integrase inhibitor used in the treatment of human immunodeficiency virus (HIV-1)[81]. It targets and removes the Mg^{2+} from the DDE motif of the IN CCD domain. Without Mg^{2+} IN is unable to perform strand transfer reactions and viral DNA insertion which damage host DNA[81]. Since ERVK and HIV integrases are so similar[5], Raltegravir may also limit ERVK IN activity in this fashion. With less ERVK IN-mediated DNA damage there may be reduced inflammation and cell death, particularly in motor neurons wherein lies the root of ALS neuropathology. Instead of maintaining and/or alleviating symptoms like the current drugs, Raltegravir targets a potential cause of the disease (ERVK IN-mediated DNA damage), potentially decreasing neuropathology, motor symptoms and increasing both quantity and quality of life.

2.0 Research Proposal

2.1 Background

ERVK is a viral genomic symbiont associated with the death of motor neurons in ALS. The ERVK integrase enzyme cleaves host DNA to facilitate the insertion of viral DNA, thus causing genomic instability and cell death. Establishment of a Drosophila model expressing ERVK IN provides the opportunity to assess the progression of motor deficit, identify pathological molecular markers of disease and compare with neurodegeneration patterns seen in ALS. The potential of HIV integrase inhibitors to halt and even reverse this neurodegeneration provides new hope for novel drug candidates to be moved into clinical trials for ALS.

2.2 Hypothesis

Motor dysfunction in ERVK IN expressing Drosophila will correlate with neuropathological evidence of DNA damage, inflammation, and TDP-43 aggregation.

2.3 Objective

To develop an *in vivo* model to study the effect of ERVK IN on ALS-like neuropathology.

2.4 Aims

Aim #1: Assessment of motor impairment and neuropathology in ERVK IN expressing *Drosophila* lines.

Aim #2: Identification of ERVK IN inhibitors which limit motor neuron disease. This strategy will identify candidate anti-retroviral drugs to bring forward into ALS clinical trials.

3.0 Methods

Drosophila husbandry

3.1 Ethics Statement *and Drosophila* Colony

Ethics statement

This project is part of Dr. Douville's ALS Association grant (HE #13781). Animal ethics approval is not required for the use of Drosophila in research[82].

Drosophila Stock Information

GenScript (Piscataway, USA) produced custom pUAST vectors with ERVK viral protein inserts. BestGene Inc. (Chino Hills, USA) injected Drosophila stocks with a vector containing a yeast upstream activator sequence (UAS) followed by the ERVK IN (UAS-ERVK_IN). The balancer chromosome, *CyO,* carries the curly wing mutation on the second chromosome and functions to prevent recombinant products and maintain desired inserts in the next generation. The presence of the ERVK IN insert was confirmed through PCR for each new line developed. ERVK IN was randomly inserted. Embryonic Lethal Abnormal Vision (ELAV BDSC 8760 (pan-neuronal)) and D42 BDSC 8816 (motor neuron) GAL4 driver stocks were ordered from the Bloomington Drosophila Stock Centre (Indiana, USA).

3.2 Drosophila Maintenance

ERVK transgenic and stock flies were fed a cornmeal-based diet (see **Table S1** in appendix) and were transferred into vials with new food every 10-11 days. Vials, bottles, and stoppers were cleaned and autoclaved before use to prevent infection. Flies were kept at 22°C, with 12-hour day and night light cycles including a 1.5-hour gradual light increase and decrease at dusk and dawn.

3.3 Mating Crosses

To generate ERVK IN expressing flies and littermate controls, crosses were performed with 10 male UAS-ERVK_IN Drosophila and 10 virgin female GAL4 driver Drosophila per 177ml bottle. For larger

experiments requiring an increased number of flies, 1L bottles containing 30 UAS-ERVK_IN males and 30 GAL4 virgin females were used. The tissue-specific GAL4 driver strains used were motor neuron (D42) and pan-neuronal (ELAV). Ten days after the initial cross the parental flies were discarded. In the following days, the progeny flies were collected and sorted into 1 of 4 genotypes: littermate control (curly wings) male or female, ERVK IN expressing (straight wings) male or female and used for experimentation.

3.4 Drosophila Drug Dosing and Administration

The FDA approved HIV integrase inhibitors Raltegravir and Cabotegravir were diluted with Reagent alcohol to a stock concentration of 5mM. ERVK IN and littermate control flies were dosed with 250µM of drug or vehicle control (reagent alcohol) mixed into the top layer of fly food. The flies were transferred into new drug dosed food every 10-11 days.

Drosophila viability and behavioural monitoring

3.5 Pupal Eclosion

A 10x10 cross was initiated (see mating crosses) and flies were permitted to eclose for 10 days. The cross bottle was then placed into a -20°C freezer to prevent further eclosion. Eclosion occurs when the adult fruit fly emerges from the pupae. The cross bottle was removed from the freezer and both the eclosed (light coloured) pupae casings and uneclosed (dark coloured) fly containing pupae present on the side of the bottle were counted.

3.6 Viability

To assess lifespan, 10 flies of each type were collected at the day of eclosion and placed into food vials. The flies were monitored every 5 days until day 15, and then every second day thereafter, for any deaths. Food was changed every 10-11 days. Trials (n=3) proceed until all flies had died.

3.7 Climbing

Flies were knocked unconscious using CO_2 and transferred into a climbing tube (clear tube with attached 8cm ruler). Once flies were awake (≈2-3 minutes) they were tapped to the bottom of the tube and timed for 30 seconds. The number of flies at each position on the side of the tube was recorded in centimeters (0-8). For each trial (n=3), data is presented as a graph of climbing ability over time, stratified by genotype and sex.

3.8 Walking

Ten flies per phenotype were knocked unconscious using CO_2 and transferred onto walking plates (petri plate with 1.5 x 1.5 cm grid). Once flies were awake (≈2-3 minutes) one fly was selected at a time. The top of the plate was gently tapped to initiate movement and the number of grid lines the fly crossed in 30 seconds was recorded. If the fly stopped moving, the top of the plate was gently tapped in an attempt to reinitiate movement. Any qualitative observations on decreasing motor function were recorded.

3.9 Trikinetics Drosophila Monitor System

The Trikinetics DAM5H monitor enables the analysis of 32 individual flies/monitor. One fly was placed in a glass tube containing a food cap on one end and a vented cap on the other and inserted into the monitor. 5 flies per phenotype were used for both control (no drug/ethanol spiked food) and drug (integrase inhibitor treated; Raltegravir or Cabotegravir spiked food) groups for each trial. Nine lasers spaced 3mm apart were used to collect data (parameters: moves, counts, dwell, latency, position, and rest) for each individual fly every minute for 24 hours. Only the movement parameter was analysed for this project. Flies were analysed at eclosion (Day 0), Day 20, Day 40, and Day 60. DAMSystem3 software records output from the activity monitors and saves the data as a .csv file. Data analysis and plots were performed using GraphPad Prism software.

Drosophila Specimens and Molecular Assays

3.10 Drosophila Specimens and Preservation

After all behavioural testing had been completed, the flies were placed in tubes and stored at -20°C until further processing (head removal) had been completed. After head removal, the samples were stored at -80°C to prevent proteolytic tissue degradation.

3.11 Fly Head Dissection and Processing

The flies from freezer storage were placed on a petri-plate located over ice. Using microdissection tools, the fly heads were removed from their body and placed in a labeled microcentrifuge tube (max 10 heads/vial). As there is approximately an equal amount of protein contained in each head, the need for BCA protein analysis is not needed. Fly head protein lysates were prepared using RIPA buffer, with samples homogenized using the Retch (MM 301) machine and clarified by centrifugation. Samples were boiled at 95°C for 10 minutes and prepared for western blot with Laemmli buffer containing bromophenol blue.

3.12 Western Blot

Western blot analysis was used to determine the presence and relative amounts of target proteins between ERVK IN expressing and littermate control Drosophila. Western blots were performed as previously described in Douville lab publications[17,65,83]. Briefly, from each sample (fly head lysate), 15µl was loaded into a polyacrylamide gel and run at 160V until proteins migrated through the gel. A 12% gel was used for lower molecular weight proteins (ERVK IN, γH2AV, TBPH) and thinner 10% gels were used for larger proteins (Relish, PARP1). The gel was transferred onto a prepared low fluorescence PVDF membrane using the GenScript eBlot L1 transfer system (GenScript L00686). The membranes were blocked for 5 minutes using BioRad Everyblot blocking buffer. The membranes were loaded into cassettes and placed into the EZ West Lite automated western device (GenScript L00816). The EZ West

lite machine performed the wash, primary antibody, and secondary antibody steps. For a list of antibodies used see **Table S2** in the appendix. The membranes were imaged using the FluroChem M chemiluminescent imager for the identification of target protein banding.

3.13 Data Analysis

Pupal eclosion analysis was done as percent eclosion (# eclosed /total pupae) and was graphed for each cross and averaged across trials. For each viability trial, data is presented as a graph of survival over time. For motility, lines crossed while walking, and distance climbed over time, stratified by genotype and sex, were recorded. Statistical differences between treatments (i.e., littermate control vs ERVK IN expressing and male vs female) were evaluated using two-way ANOVA for all assays. Trikinetics DAM5H data was analysed using two-way ANOVA and t-tests. All graphs and statistics were performed using GraphPad Prism software.

4.0 Results

4.1 ERVK IN induces DNA damage, Inflammation and Protein cleavage

Western blot analysis is a widely used technique to determine differences in protein concentration. This technique was used to determine which proteins were most effected by ERVK IN expression. Knowledge on which protein targets to search for is required for this assay. DNA damage, inflammatory immune signalling, and TDP-43 proteinopathy are known consequences of ERVK IN activity and the chosen antibody targets reflect these processes and are described below.

ERVK IN induces DNA Damage

The first questions were undoubtedly the most important for this project; is ERVK IN present in the transgenic flies and does ERVK IN induce DNA damage? This was answered using antibodies for ERVK IN and the DNA damage marker γH2AV. Integrase activity induces DNA double stranded breaks[84] and as γH2AV is phosphorylated at these breaks[85], it is an appropriate marker to indirectly measure ERVK IN activity. ERVK IN x D42 transgenic females were used for this experiment. Increased γH2AV was identified at Days 0 and 10 compared to littermate control females. What is interesting is this increase in γH2AV overlaps with the timepoints containing the highest concentration of ERVK IN (**Figure 2, Panel A**), pointing to ERVK IN as the cause of the increased DNA damage. ERVK IN is clearly present throughout all timepoints (0-80) but decreases over time most likely due to cell death. Bands are present in the littermate controls due to cross-reactivity with native fly ERVs which contain the same or similar epitopes as ERVK IN. Expression of native fly ERVs is relatively consistent throughout the littermate control timepoints.

ERVK IN induces TBPH cleavage

TDP-43/TBPH was the next target analyzed. ERVK IN x D42 expressing females have increased full length TBPH (43 kDa) at most timepoints, outside of Days 20 and 60 where the littermate control females have increased TBPH. The cell may not be able to keep up with the demand for TBPH leading to a lull in expression. The compelling nature of this experiment is the clear presence of ~35 kDa and ~25 kDa cleavage bands in the transgenic females (**Figure 2, Panel B**) with the highest concentration occurring at Day 0, the same as one of the higher concentrations of ERVK IN. Cleavage bands of this size are commonly found in ALS patients with TDP-43 proteinopathy[70]. What is unexplained is why the amount of TBPH cleavage decreases after this timepoint when ERVK IN expression is still high.

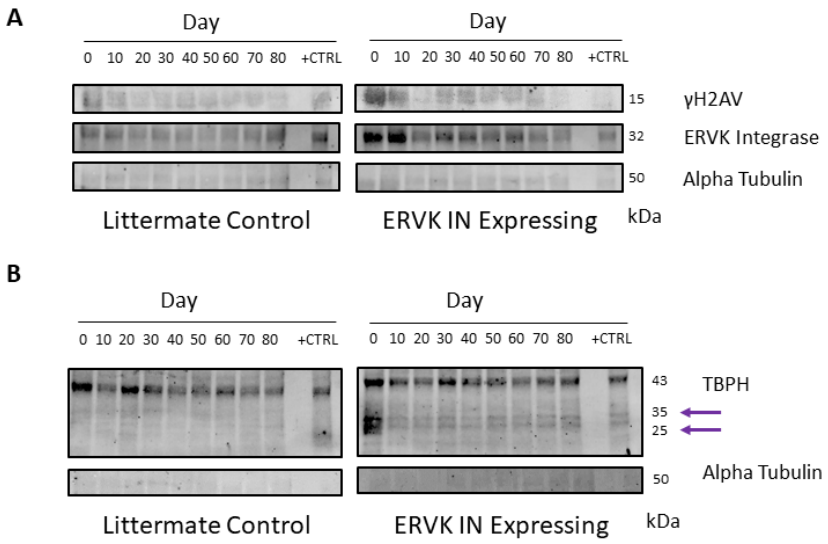


Figure 2: ERVK IN induces DNA damage and TBPH cleavage. Western blot of ERVK IN x D42 expressing and littermate control females. Flies were aged and the heads frozen and collected in 10-day intervals from Day 0 to Day 80; n=10 heads/sample. Alpha tubulin is the loading control. Positive control = 20 day old females expressing ERVK IN in motor neurons. **A)** ERVK IN expressing females show increased γH2AV at Days 0 and 10 that coordinates with the highest expression of ERVK IN. ERVK IN continues to be

present at all time points though decreases with time. **B)** Full length TBPH (43 kDa) is present in both ERVK IN expressing transgenic and littermate control females at all time points though appears higher in the ERVK IN expressing at later time points (Days 70 and 80). Cleavage of TBPH into 35 and 25 kDa bands is present in the ERVK IN expressing only (purple arrows).

To try to identify the potential cause of TBPH cleavage, bioinformatic analysis was performed. The Drosophila TBPH amino acid sequence was found in the NCBI protein database (https://www.ncbi.nlm.nih.gov/) under the accession number NP_477400.1. The amino acid sequence was analyzed using PeptideCutter (https://web.expasy.org/peptide_cutter/) which determines the size of TBPH cleavage products when cut with different proteases. The output of the search identified 24 proteases capable of cleaving Drosophila TBPH. This was narrowed down by removing proteases that generate multiple cleavage products, such as trypsin, as these are unlikely to be the source of the bands. After narrowing down the search, there were three potential proteases left: Factor Xa, Proline-endopeptidase and Thrombin (**Table 1**). The PeptideCutter software also provides the size of the cleavage products generated by each protease making it easy to determine their kDa size by using an amino acid to kDa calculator (https://www.bioinformatics.org/sms/prot_mw.html). After identifying the size of the cleavage bands potentially produced by the identified proteases the one most likely to have cleaved TBPH was found to be the Proline-endopeptidase, which has two potential cleavage sites in TBPH producing bands at 32.36 kDa and 25.12 kDa. However, Thrombin is also capable of producing a 32 kDa band that matches those found during Western blot and thus could also be responsible for TPBH cleavage.

Table 1: Proteases Capable of Cleaving Drosophila TBPH. TBPH (NCBI accession number: NP_477400.1) amino acid sequence was submitted to PeptideCutter software to generate a list of proteases capable of cleaving Drosophila TBPH. 24 proteases were initially identified, and the list narrowed to three.

Proline-endopeptidase produces 32.36 kDa and 25.12 kDa cleavage bands that match those found through Western blot analysis and may be the cause of TBPH cleavage seen with ERVK IN expression.

Protease	# Of Cleavage Sites	Amino Acid Cleavage Site	Kilodalton Size
Factor Xa	1	174	38.3
Proline-endopeptidase	2	226; 291	32.36; 25.12
Thrombin	1	225	32.46

Western blot of the ERVK IN x D42 expressing females shows increased γH2AV early in life. Closely associated with the activity of γH2AV is PARP1[86], a protein involved in the DDR and immune signalling.

ERVK IN induces Relish activation and PARP1 cleavage

The above experiments using ERVK IN x D42 females confirmed ERVK IN expression in the transgenic flies and that this expression correlates with the highest concentration of γH2AV and TBPH cleavage products. As the highest concentration of ERVK IN occurred at Days 0 and 10, these timepoints were used to screen the ERVK IN x ELAV males to assess the presence of the DNA damage and immune signalling protein, PARP1, the neuronal marker ELAV and the inflammatory marker Relish (NF-κB).

Relish was used to determine if immune signalling occurred with ERVK IN expression. ERVK IN x ELAV males show no apparent differences in full length Relish (110 kDa) but a much higher concentration of Rel 68 and Rel 49 was seen in the transgenic males at Day 10 (**Figure 3**). Relish is cleaved by Dredd to separate the inhibitory IκB-like Rel 49 from the NF-κB-like Rel 68. This allows for the translocation of Rel 68 into the nucleus and the expression of antimicrobial genes[87] Cleaved Relish is indicative of the activation of IMD pathway and the activation of a proinflammatory antiviral immune response[88]. One of the targets of Rel 68 are promotors inducing the expression of PARP1[89].

The ERVK IN x ELAV males do not show any differences in the amount of full length PARP1 (113 kDa) at either Day 0 or Day 10 timepoints but the transgenic males did show an increase in a cleavage band just under the 75 kDa ladder and estimated to be around 70 kDa (**Figure 3**). This is not a typical cleavage product for human PARP1 (those being 24 and 89 kDa[90]) meaning PARP1 may be cleaved at noncanonical site by an undetermined protease. Bioinformatic analysis of Drosophila PARP1 was performed to determine the enzyme that could potentially produce a cleavage product that matches the identified band size. The Drosophila PARP1 amino acid sequence was found in the NCBI database (https://www.ncbi.nlm.nih.gov/) under accession number NP_001104452.1. The PeptideCutter search identified 23 proteases capable of cleaving Drosophila PARP1. After narrowing down the search, 3 proteases were left: Caspase 1, Hydroxylamine and Thrombin (**Table 2**). After analyzing the kDa size of each cleavage product, it was determined that the most likely protease that cleaved Drosophila PARP1 is Thrombin which could generate a 70.47 kDa cleavage product.

Table 2: Proteases Capable of Cleaving Drosophila PARP1. The Drosophila PARP1 (NCBI accession number: NP_001104452.1) amino acid sequence was submitted to PeptideCutter software to generate a list of proteases capable of cleaving this protein. 23 proteases were initially identified, and the list narrowed to three. Caspase 1 could generate a cleavage product of 12.93 kDa, Hydroxylamine a 43.51 kDa product and Thrombin two products at 70.47 kDa and 106.58 kDa.

Protease	# Of Cleavage Sites	Amino Acid Cleavage Site	Kilodalton Size
Caspase 1	1	881	12.93
Hydroxylamine	1	303	34.51
Thrombin	2	380; 933	70.47; 106.58

ELAV was then used to determine if ERVK IN caused the death of neurons. No apparent differences were seen at either Day 0 or Day 10, although there might be a slight reduction in ELAV at the Day 10 timepoint in the ERVK IN expressing males indicating neuron death may be starting (**Figure 3**). It is possible that as

ERVK IN and DNA damage are occurring at these timepoints (inferred from previous experiments; see above) that the cells expressing ERVK IN have not yet died and therefore significant changes in ELAV may not be seen until later timepoints.

ERVK IN was easily identified in the ERVK IN x ELAV expressing males at Day 0 and 10. Cross reactivity with native fly ERVs also results in bands being present in the littermate control males. Curiously, additional bands can be identified in the ERVK IN expressing males. This could be the ERVK IN antibody cross reacting with native fly ERV integrases. Alternatively, ERVK IN could become cleaved during an immune response rendering it inactive. These hypotheses are not mutually exclusive and could both be true. This could explain the decrease in ERVK IN after Day 10 but the continuing molecular pathology.

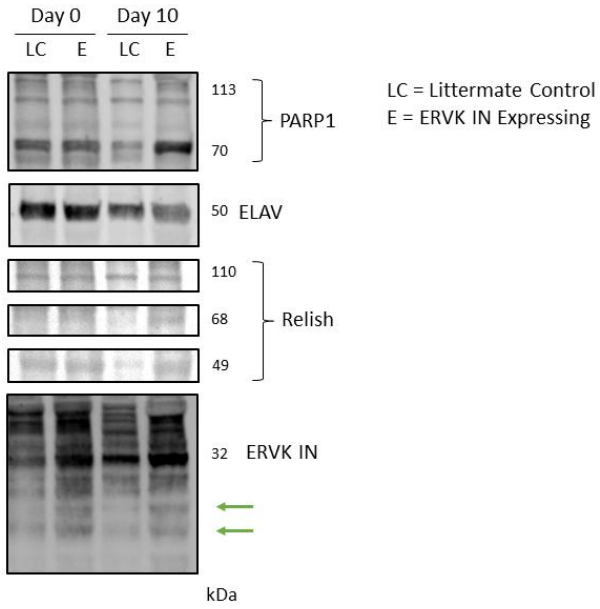


Figure 3: ERVK IN induces Relish activation and PARP1 cleavage. Western blot of ERVK IN x ELAV expressing and littermate control males. Flies were collected and frozen post eclosion and at Day 10; n=10 heads/sample. ERVK IN expressing males at Day 10 display an approximately 70 kDa PARP1 cleavage product, a slight decrease in ELAV, increased Rel 68 and Rel 49 and multiple bands present with ERVK IN (green arrows).

Confocal microscopy shows ERVK IN expression in transgenic flies

Lastly, confocal microscopy was also used to confirm the presence of ERVK IN in ERVK IN x ELAV larvae and adult flies. This method is more appropriate than Western blot to confirm the presence of ERVK IN as there is no cross reactivity with native fly ERVs. This is due to the 3D conformation of ERVK IN being different enough from native fly ERVs. Thus, the ERVK IN antibody is not expected to bind to anything other than the intended target. Western blot linearizes proteins for easier binding, which allows recognition of epitopes that can result in cross-reactivity. ERVK IN was only found in the ERVK IN expressing larvae and not the littermate controls (**Figure 4, Panel A**). ERVK IN was found to co-localize with ELAV (neuronal marker) in the larval brain along with what is yet an unidentified neural tract. A surprising find was the presence of neural puncta that were clearly apparent in the brains of the littermate control larvae but completely absent in the ERVK IN expressing larvae. This suggests that neuron death is occurring at the larval stage (and likely into adulthood). Once ERVK IN expression was confirmed in larvae, initial testing using a newly developed Fly Clear protocol (adapted from[91]) was used to remove the pigment from adult flies to enable full fly immunostaining. This method allows for *in situ* protein analysis of the whole fly and prevents tissue damage from occurring due to laborious fly brain microdissections. Preliminary experiments were successful as ERVK IN was identified in an ERVK IN x ELAV expressing female. ERVK IN co-localizes with DAPI indicating that ERVK IN is present in the nucleus (**Figure 4, Panel B**). ERVK IN is also found unassociated with DAPI showing that ERVK IN is also present in the cytoplasm.

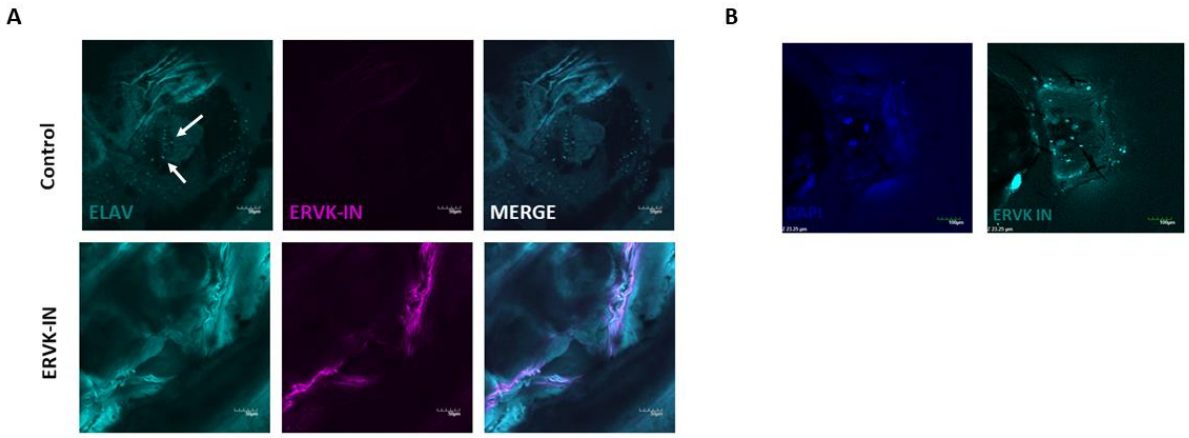


Figure 4: Confocal microscopy images of ERVK IN x ELAV expressing larvae and adult brain. A) Confocal image compilation of ERVK IN x ELAV third instar larvae highlighting the presence of ERVK IN in the ERVK IN expressing larvae. Neural puncta are present in the control larvae (white arrows) but not the ERVK IN expressing pointing to neuronal loss before adulthood. **B)** Adult ERVK IN x ELAV expressing female fly treated with a fly clear protocol. ERVK IN clusters are clearly seen in the fly brain and co-localize with DAPI (nuclear marker).

Molecular testing uncovered important information regarding ERVK IN expression however, it does not provide information on what ERVK IN is doing at the phenotypic level. To answer these questions, assays were developed to analyze survival and motor functioning with ERVK IN expression.

4.2 ERVK IN Expression Does Not Impact Eclosion

Eclosion was the first measurement of motor functioning that was analysed. Counting the number of dark (fly containing) pupae and light-coloured pupae casings, provided an estimate of the number of larvae produced and the speed of eclosion. The eclosion rate of the ERVK IN x D42 and the ERVK IN x ELAV crosses were 84.5% and 83.6% respectively, with no significant differences between them (X^2=0.14; *P*=0.71) (**Figure 5**). There was also a non-significant difference in the proportion of eclosed flies between

the ERVK IN x D42 (84.5%) and the control D42 x D42 cross (80.5%) (X^2= 3.11; *P*=0.08). In brief, ERVK IN expression does not affect pupal eclosion.

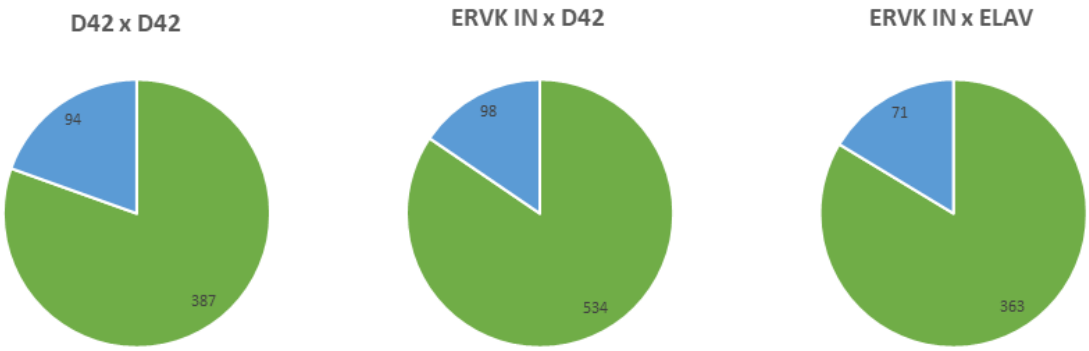


Figure 5: Total eclosion is not impacted by ERVK IN relative to the control. A 10x10 cross was initiated using ERVK IN males and D42 or ELAV virgin females. The parents were removed after 10 days, and progeny allowed to eclose for the next 10 days. On Day 20, adults were discarded and the number of dark fly containing pupae (uneclosed) and light pupae casings (eclosed) were counted. No significant differences were found between the control and experimental crosses. Blue slices in the pie-chart are proportion of uneclosed pupae; while green are proportion of eclosed pupae. The numbers within slices are the actual counts per group.

Pupae counts are indicative of the total number of flies without discerning their genotype (*i.e.*, littermate control or ERVK IN expressing). Moreover, our previous analysis of pupae counts ignored possible sex differences. To determine whether ERVK IN expression influenced the eclosion of transgenic flies, and to assess any possible sex effect, the experiment was repeated, and the adult flies were phenotyped (i.e. No ERVK IN expression = curly wings; ERVK IN expression = straight wings). One trial of this experiment was performed. No differences in the proportion of ERVK IN expressing and littermate control eclosed flies were observed (**Table 3**).

Table 3: ERVK IN expression does not impact pupal eclosion. A 10x10 cross was initiated using ERVK IN males and D42 or ELAV virgin females. The parents were removed after 10 days. Flies were allowed to eclose for 10 days. The flies were sorted based on sex and ERVK IN expressing or littermate control (based on wing phenotype) flies and their numbers recorded. A fly was considered unidentified if the wing phenotype could not be determined. No differences were observed in eclosion patterns between littermate control and ERVK IN expressing flies or between males and females.

	ERVK IN x D42			ERVK IN x ELAV		
	Fly #	%	P value	Fly #	%	P value
ERVK IN Females	129	22.3	**P=0.86**	90	27.7	**P=0.88**
Littermate Control Females	125	21.6		91	28	
ERVK IN Males	125	21.6	**P=1.00**	67	20.7	**P=0.77**
Littermate Control Males	125	21.6		73	22.5	
Unidentified	75	12.9		3	0.009	
TOTAL	**579**			**324**		

4.3 ERVK IN x D42 Expressing Males have an Increased Lifespan

An analysis of the effects of time and genetics on lifespan using two-way ANOVA shows no significant differences between ERVK IN expressing and littermate controls and no interaction effects: ERVK IN x D42 females ($F_{1,240}$ = 1.03, p = 0.31; $F_{60,240}$ = 0.30, p > 0.99), ERVK IN x ELAV females ($F_{1,240}$ = 0.16, p = 0.27; $F_{60,240}$ = 0.23, p > 0.99), ERVK IN x ELAV males ($F_{1,240}$ = 0.17, p = 0.69; $F_{60,240}$ = 1.16, p = 0.24). This result holds for the ERVK IN x D42 females and the ERVK IN x ELAV flies of both sexes. The ERVK IN x D42 expressing males did have a significantly increased lifespan compared to littermate controls ($F_{1,240}$ = 97.01, p < 0.0001; $F_{60,240}$ = 2.44, p < 0.0001), which was an unexpected find. Although only significant differences were found for the ERVK IN x D42 expressing males, an examination of survival dynamics overtime shows slight differences between sexes in each cross as described below.

ERVK IN x D42

No deaths occurred in either ERVK IN expressing or littermate control females during the first 80 Days (**Figure 6, Panel A**). At older ages (Days 90 to 120) the littermate control females had an approximately 10% increase in survival over the ERVK IN expressing females. The ERVK IN expressing flies were all dead at Day 120, while the littermate control females were all dead at Day 140. Regarding the males, both ERVK IN expressing and littermate controls both started dying at around Day 50. At Day 85, the ERVK IN expressing males had a 20% increase in survival whereas the littermate control males were all dead. All ERVK IN expressing males were dead at Day 105 (**Figure 6, Panel B**).

ERVK IN x ELAV

During the earlier timepoints (Days 0-60) the ERVK IN expressing and littermate control females display limited differences in survival. The ERVK IN expressing females then exhibit a brief period of increased survival ranging from approximately 10% to 40% from Days 70-80 respectively. After this point both littermate control and ERVK IN expressing females follow the same downward trajectory, until both were all dead at Day 130 (**Figure 6, Panel C**). The ERVK IN expressing males, on the other hand, consistently lived longer than the littermate control males (Days 10 to 80). Littermate and ERVK IN expressing males were all dead at approximately Day 90 (**Figure 6, Panel D**).

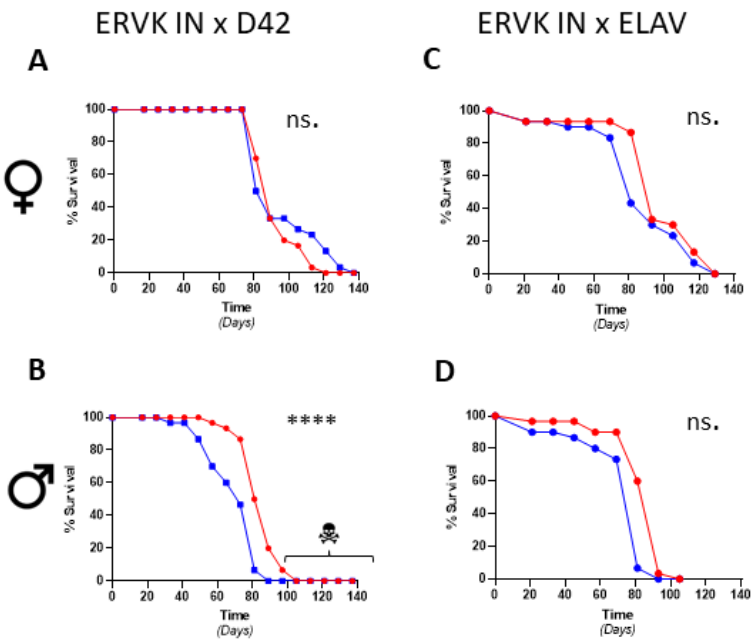


Figure 6: ERVK IN x D42 expressing males have an increased lifespan. Survival curve of ERVK IN expressing and littermate control flies. Flies were monitored for survival until all died. Every 5th time point is included on the graph for clarity. N=30 ERVK IN expressing or littermate control flies. Flies were kept at 22°C. **A)** ERVK IN x D42 females show no differences over the first 80 Days. The littermate controls show increased survival over the last 40 Days. **B)** ERVK IN x D42 males have a significantly increased lifespan over the duration of the experiment. **C)** ERVK IN x ELAV expressing females show a short period of increased survival from Days 70-80 but no other differences are seen. **D)** ERVK IN x ELAV expressing males have increased survival for the entirety of the experiment.

Overall, ERVK IN expression in motor neurons (D42) caused a significantly increase lifespan in the ERVK IN expressing males but not females (see above). Subtle alterations in lifespan were also present around midlife (approximately Days 60-80) as seen in a higher degree of survival in the ERVK IN x ELAV expressing

flies. While ERVK IN expression did not affect overall viability, it is possible for its activation to alter geotaxis and motor functioning.

4.4 ERVK IN Expressing Flies Display an Erratic Vertical Movement Pattern

Drosophila have a natural propensity to move against gravity when disturbed (negative geotaxis) and this behaviour can be used to analyze both motor functioning and behaviour[92]. ERVK IN expressing and littermate control flies were assessed both on their climbing ability and motor control. Analysis of the effect of genetics (i.e. ERVK IN expression vs. littermate control) and time (i.e. age) on climbing ability using two-way ANOVA showed no significant differences between ERVK IN expressing and littermate controls for both males and females (ERVK IN x D42: females $F_{1,32}$ = 2.71; P= 0.12, males $F_{1,32}$ = 0.06; P= 0.81, ERVK IN x ELAV: females $F_{1,32}$ = 0.38; P= 0.54; males $F_{1,32}$ = 3.08; P= 0.089) and no interaction effects (ERVK IN x D42: females $F_{8,32}$ = 0.54; P= 0.8, males $F_{8,32}$ = 0.71; P= 0.68, ERVK IN x ELAV: females $F_{8,32}$ = 1.18; P= 0.34, males $F_{8,32}$ = 0.96; P= 0.48) (**Figure 7**). A posteriori analysis of specific time-points shows an effect in the ERVK IN x ELAV females, where the ERVK IN expressing flies were able to climb approximately 2cm higher at Day 50 (Šídák's multiple comparisons test; P= 0.04). No evident differences in movement dynamics were distinguishable between ERVK IN expressing and littermate controls.

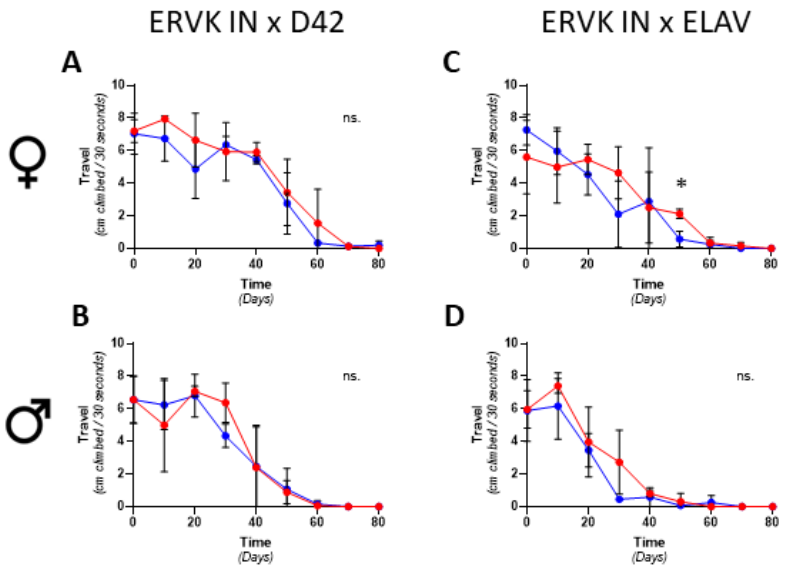


Figure 7: ERVK IN transgenic flies do not display differences in climbing ability. Climbing assay to assess vertical movement ability in ERVK IN expressing and littermate control flies. Two-way ANOVA shows no differences in climbing ability in the ERVK IN x D42 expressing or littermate control females or males and the ERVK IN x ELAV males. The ERVK IN x ELAV expressing females had a small difference at Day 50 (Šídák's multiple comparisons test; *P*= 0.04) with the ERVK IN expressing being able to climb about 2 cm higher than the controls. *P< 0.05; ns= non-significant.

There were some observed abnormalities in movement. Throughout most of the experiment, the ERVK IN expressing flies displayed a “popcorning” or erratic jumping phenotype especially apparent in the ERVK IN x D42 expressing flies. This movement abnormality made it quite difficult to collect data as the movement did not cease and progressively worsened over time, especially in the males. Moreover, at middle timepoints (Days 40-60) the ERVK IN expressing flies do not even climb the tube, rather, they jump to the sides and top of the vial. The ability to reach the top of the tube does not change between

the ERVK IN expressing and littermate control flies, but how they get there is drastically different. At later timepoints (Days 60-80) the littermate control flies can no longer climb the tube, but the attempt is still made. The ERVK IN expressing flies switched between the “popcorning” phenotype and no movement whatsoever. No limb movement and no grooming behaviour occurred in any tested ERVK IN expressing fly at these timepoints. Littermate controls still displayed grooming behaviour when not actively climbing.

4.5 ERVK IN x D42 Expressing Flies Display a Hyperactive Phenotype in Early Life

Multiple types of walking assays have been successfully developed to assess fly behaviour[93,94] and have been used in neurological disease modeling[95,96]. Techniques can range from hightech imaging to video tracking but low tech walking assays can be just as useful in assessing walking speed, coordination and muscle control as seen with the ERVK IN expressing flies. The above experiments using the ERVK IN expressing flies have revealed a distinct erratic vertical movement phenotype, but when movement was restricted to the horizontal plane the erratic behaviour continued. The initial hyperactive phenotype (Days 0-30) was characterized by a very fast walking speed and a sporadic jumping phenotype reminiscent of the “popcorning” seen in the vertical plane (**Figure 8**). The ERVK IN x D42 expressing and littermate control females had a significant genetics × time interaction ($F_{8,32}$ = 7.49; P < 0.0001) driven by effects at the Day 20 (Šídák's multiple comparisons test; P = 0.0005) and Day 30 (Šídák's multiple comparisons test; P = 0.018). While the ERVK IN x D42 males show no significant results, the activity over time shows a similar pattern to that of females. Later hyperactive phenotypes (Days 40-80) are seen in the spasming of leg muscles leading to jerky, uncoordinated movements and twitching of the wings. It is important to note that this horizontal phenotype is only observed in the flies with ERVK IN expression in motor neurons (D42). Although the ERVK IN x ELAV expressing flies do not display this hyperactive phenotype when compared to littermate controls (females: $F_{1,32}$ = 1.09; P = 0.30) (males: $F_{1,32}$ = 1.21; P =

0.33) they still exhibited motor deficits noticeable by a loss of limb coordination earlier than the ERVK IN x D42 expressing flies (starting at Day 20) that proceeds and worsens throughout the experiment (**Figure 8**).

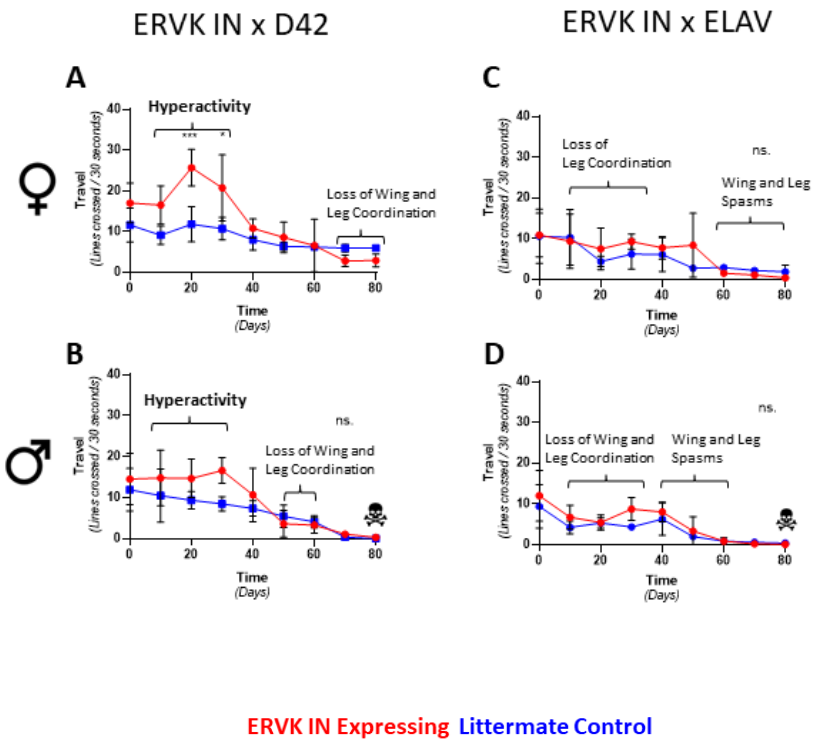


Figure 8: ERVK IN x D42 expressing flies display a hyperactive phenotype. Walking assays were performed to assess fly motor ability over time. Adult Drosophila were analyzed from eclosion and every 10 days thereafter until Day 80. N=30 control or transgenic Drosophila were analyzed at different time points. Statistical analysis was done using two-way ANOVA. **A) B)** Female and male ERVK IN expressing Drosophila exhibited a hyperactive phenotype early in life. This is followed by a progressive decline in motor function seen in a loss of wing and leg coordination. Significant effects were found for females but not males **C) D)** No hyperactive phenotype was observed in the IN x ELAV expressing females or males. The ERVK IN expressing flies were slower and had an earlier loss of limb coordination compared

to the D42 expressing flies. No significant differences were found in the IN x ELAV flies of either sex. *P< 0.05; ***P< 0.001; ns= non-significant.

After determining that the ERVK IN induces a hyperactive phenotype, the question became whether hyperactivity was continuous or a result of stimulation. To answer this the Trikinetics DAM5H Drosophila activity monitor was used to assess fly movement patterns over a 24-hour period.

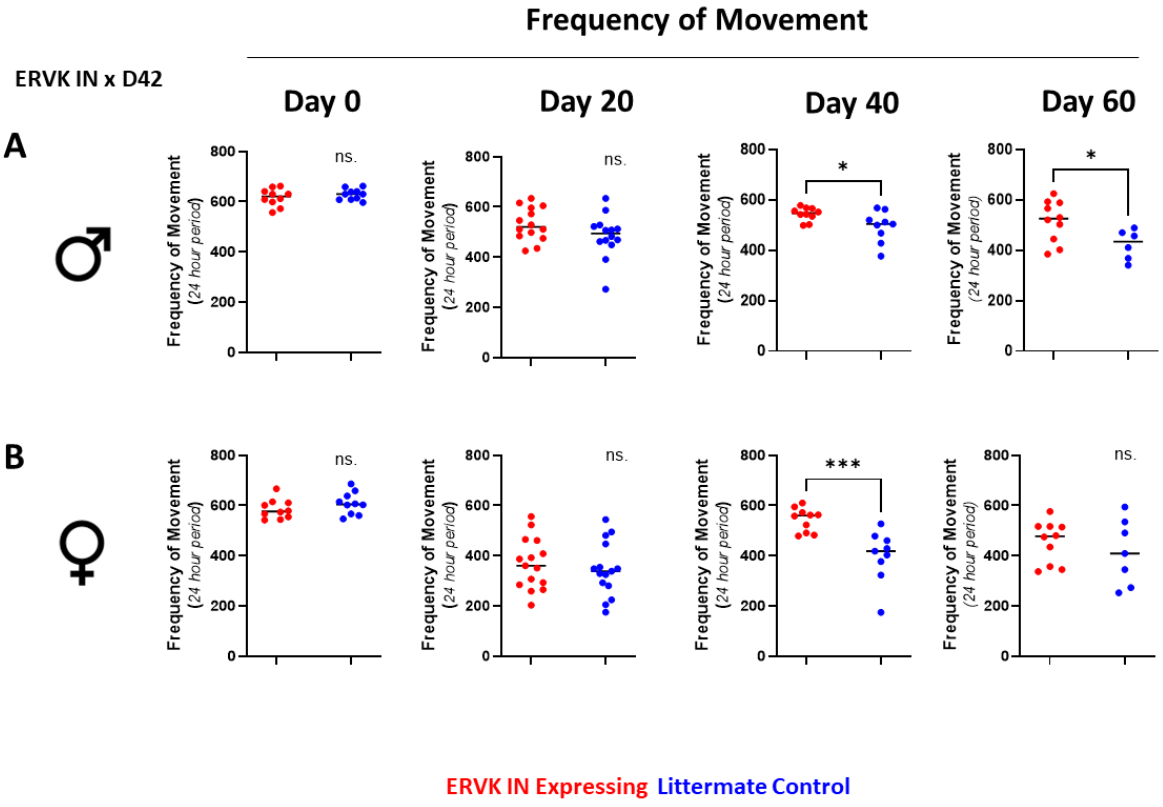
4.6 ERVK IN Induces Limited Motor Deficits Over a 24-Hour Period

To determine if the ERVK IN induced stimulated or continuous hyperactivity and/or other motor deficits the Trikenetics DAM5H Drosophila Activity Monitor was used to track movement patterns over a 24-hour period. The frequency of movement (movement peaks) and the total movement were calculated for each individual fly. ERVK IN expressing and littermate controls of both sexes and crosses were analyzed at Days 0, 20, 40 and 60 using unpaired T-tests and results are described below. For the results of all unpaired T-tests see Tables S3-S6 in the Appendix.

ERVK IN x D42

Newly eclosed flies (Day 0) did not show any significant differences in the frequency of movement or the total movement for either the males or females. The activity monitor showed no significant differences at Day 20 (**Figure 9**), a surprising result given that the walking assay (**Figure 8**) showed the highest hyperexcitability at Day 20. One possible explanation is that the tubes into which the flies are placed in the Trikinetics monitoring system limits movement to the horizontal plane, thus restricting the "popcorning" behaviour. The ERVK IN x D42 expressing males do display a small but significant decrease in total movement at Day 20 ($t(23) = 2.14$; $P = 0.04$) that may represent the beginning of motor decline. At Day 40 changes in motor control are more readily observable, particularly for the ERVK IN expressing females, which have a significantly higher frequency of movement ($t(17) = 4.02$; $P = 0.0009$). This is likely a result of muscle spasticity. The stop and start jerky movement phenotype of the ERVK IN expressing

females results in a higher frequency of movement. The littermate control females on the other hand have a more continuous movement pattern that translates into fewer movement peaks and a higher total movement ($t(17) = 2.84$; $P = 0.01$). The ERVK IN expressing males also have a higher frequency of movement ($t(17) = 2.39$; $P = 0.03$) but no difference in total movement is seen. At Day 60 the only significant difference is an increase in the frequency of movement observed in the ERVK IN expressing males ($t(14) = 2.41$; $P = 0.03$). This is expected, as this timepoint is when the littermate control and ERVK IN expressing lines intersect on the walking graphs for both sexes.



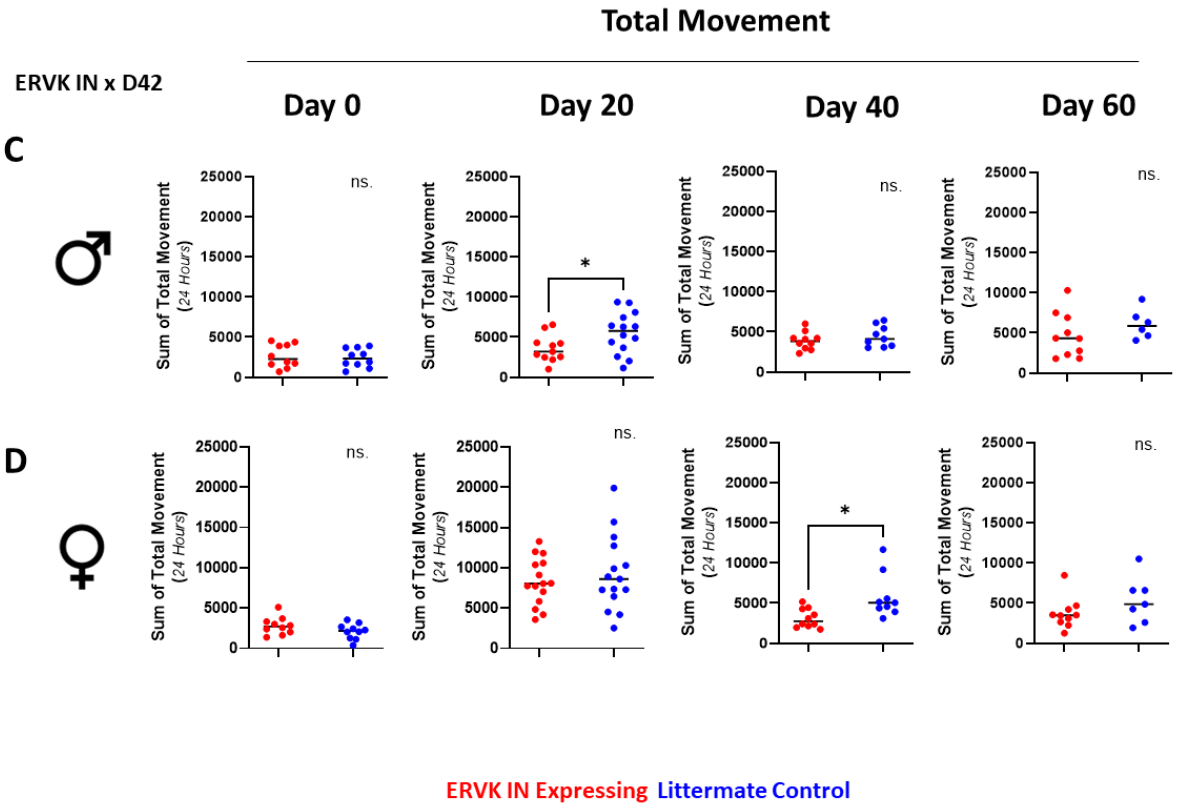
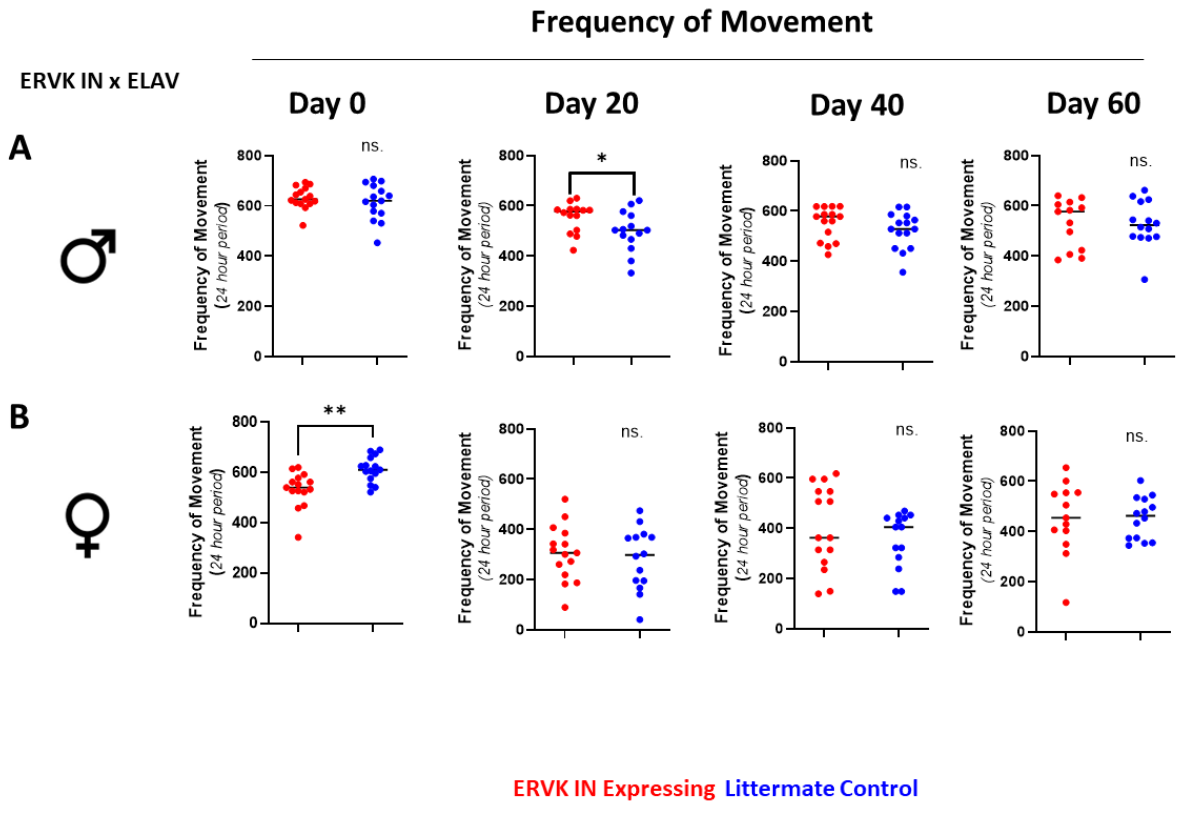


Figure 9: ERVK IN x D42 expressing flies display limited motor deficits when averaged over a 24-hour period. The Trikenetics DAM5H Drosophila Activity Monitor was used to analyze the frequency of movement and the sum of total movement of individual flies. Unpaired t-tests were performed to determine differences between the ERVK IN expressing and littermate control flies. **A)** ERVK IN x D42 expressing males show a higher frequency of movement at Days 40 and 60 (P = 0.03 for both days). **B)** ERVK IN x D42 expressing females show a significant increase in the frequency of movement at Day 40 (P = 0.0009). **C)** ERVK IN expressing males show a small decrease in total movement at Day 20 (P = 0.04). **D)** ERVK IN x D42 expressing females have a small decrease in total movement at Day 40 (P = 0.01).

ERVK IN x ELAV

The ERVK IN x ELAV expressing flies do not appear to display as noticeable movement deficits as the ERVK IN x D42 expressing flies. On the contrary, the ERVK IN x ELAV expressing females have a significantly lower frequency of movement ($t(28) = 3.55$; $P = 0.001$), which means a consistent movement pattern, and a significantly higher total movement ($t(23) = 3.37$; $P = 0.003$) at Day 0 than their littermate controls (**Figure 10**). ERVK IN may provide a benefit for females in early life when expressed in a certain neuron type (not motor neurons). However, at Day 20 the only significant difference observed is an increase in the frequency of movement in the transgenic males ($t(26) = 2.1$; $P = 0.05$), which is not paired with a decrease in total movement as previously seen. No significant differences are detected at Day 40 or 60.



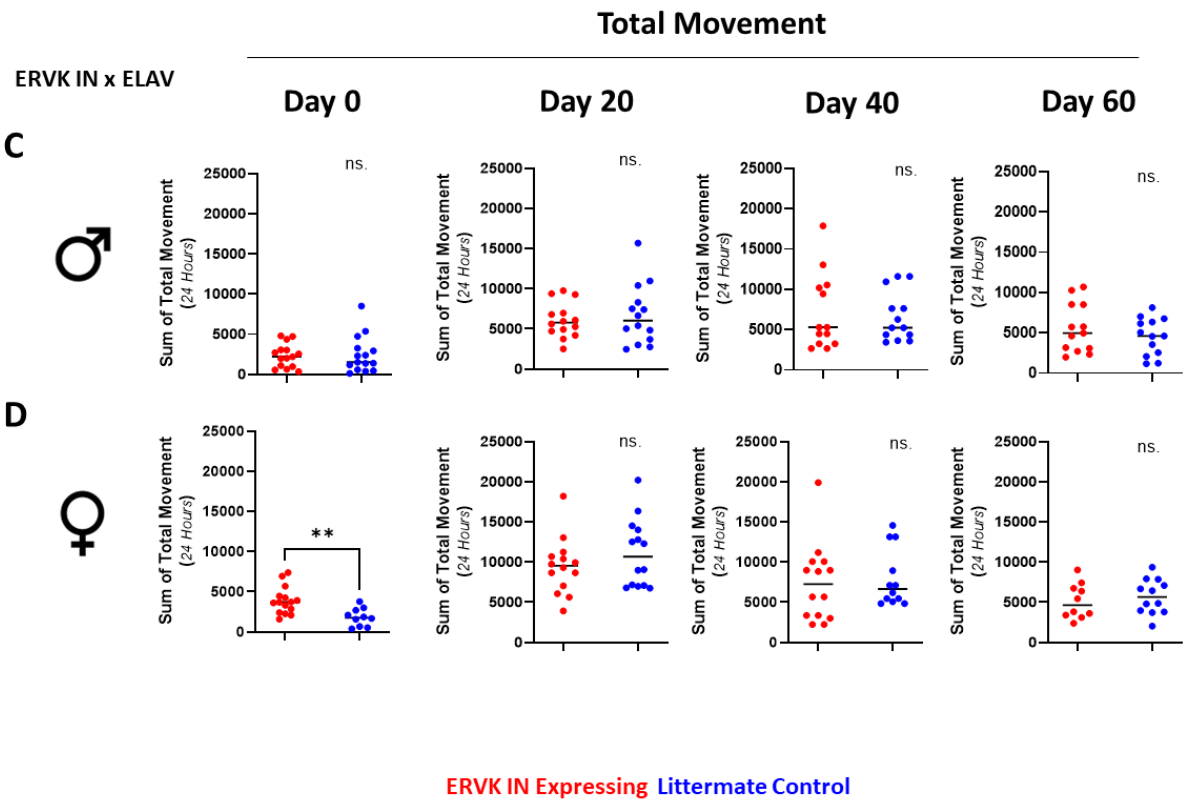


Figure 10: ERVK IN x ELAV expressing flies display limited motor deficits when averaged over a 24-hour period. The Trikenetics DAM5H Drosophila Activity Monitor was used to determine the frequency of movement and the sum of total movement of individual flies. Unpaired t-tests were performed to determine differences between the ERVK IN x ELAV expressing and littermate control flies. **A)** ERVK IN x ELAV expressing males have an increased frequency of movement ($t(26) = 2.1$; $P = 0.05$) at Day 20. **B)** ERVK IN x ELAV expressing females have a decreased frequency of movement ($t(28) = 3.55$; $P = 0.001$) at Day 0. **C)** ERVK IN x ELAV expressing and littermate control males display no significant differences at any timepoint. **D)** ERVK IN x ELAV expressing females have an increased total movement at Day 0 ($t(23) = 3.37$; $P = 0.003$).

The previous experiments did not reveal many changes in the overall movement of ERVK IN transgenic flies. However, the analysis might have missed daily changes that could only be detected during more

continuous observations. Thus, I undertook a minute-by-minute behavioural tracking for an entire 24-hour period.

4.7 ERVK IN Induces Motor Deficits at Dusk

To understand the complete movement dynamics of the ERVK IN expressing and littermate control flies, actograms were created. The actograms show the movement of individual flies every minute over a 24-hour period. This method provides a full picture of the flies' activity throughout the day and night and better highlights differences between the littermate control and ERVK IN expressing flies that may be missed by averaging daily behaviours.

ERVK IN x D42

At Day 0 both the newely eclosed ERVK IN expressing and littermate control flies have exceedingly limited movement patterns that highly differ from later timepoints. Movement only begins during hours 21-24 of the assay, for both sexes (**Figure 11, Panel B**). It is important to note that the hours on the X axis of the figures represents the time since the start of the assay and not the actual time (*i.e.* if the assay started at 9:00am, hour 1 would be from 9-10am).

At Day 20 motor deficits start to become apparent in the ERVK IN expressing males. Interestingly, the time at which motor deficits occur is at dusk (hours 7 to 9; **Figure 11, Panel B**), with the ERVK IN expressing males moving apporximately 10 moves / minute less than the littermate controls. The littermate control males gradually increase to reach a peak dusk movement at hour 8 (15-17 moves / minute) and then gradually decrease their movement as the light dims. What originally appeared to be a clear movement deficit in the ERVK IN expressing males at dusk turned out to be a severe delay in dusk activity. The ERVK IN expressing males very slowly increase their activity, reaching a peak at hour 11 (around 10 moves / minute) followed by a gradual decrease. Nocturnal activity is also present at this

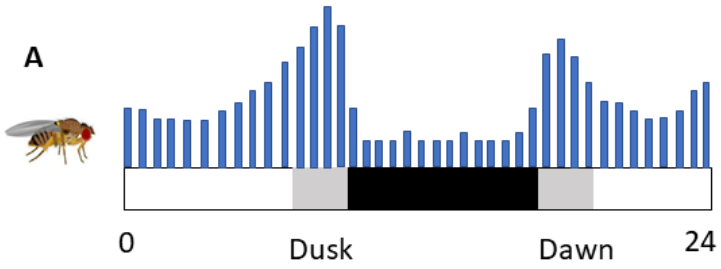
point and continues to worsen as time goes on. At Day 40 movement in the ERVK IN expressing males further decreases at dusk and a brief attempt at dusk movement is detected. At Day 60 several abnormalities are detected in the ERVK IN expressing males throughout the run. The first is a severe movement deficit at dusk, the second is a barely detectable dusk activity pattern and the third is an early dawn activity pattern. Early dawn activity is a new phenotype for the transgenic flies. The peak of dawn activity in the ERVK IN expressing flies occurs at hour 17 (around 10 moves / minute) which is during the night. The littermate controls have their peak dawn activity at hour 19 (around 10 moves / minute). The ERVK IN expressing males have a 2-hour shifted circadian pattern.

At Day 20 the ERVK IN expressing females do not display a dusk delay but do experience a movement deficit of about 5 moves / minute less from hours 0-8 (**Figure 11, Panel B**). The motor deficit continues to worsen at Day 40 with the ERVK IN expressing females moving 10 moves / minute less than the littermate controls from hours 0-10 that continues to Day 60.

ERVK IN x ELAV

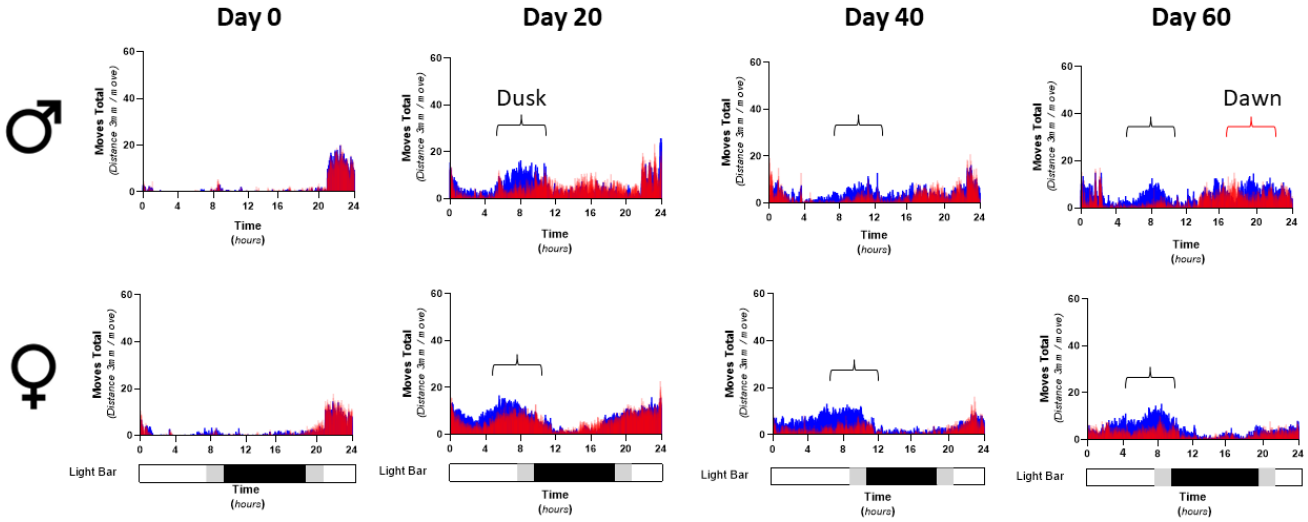
At Day 0 both ERVK IN expressing and littermate control flies have a reduced movement pattern compared to later timepoints. At Day 20 the ERVK IN expressing males have restricted movement (i.e. A sharp incline to and decline from the maximum peak) at dusk, resulting in a shortened dusk activity period compared to the littermate controls (gradual incline and decline) (**Figure 11, Panel C**). At Day 40 the restricted dusk activity disappears and the only abnormality is a slight 5 moves / minute motor deficit. At Day 60 motor deficits occur during hours 0-2 and at dawn (hours 19-20).

At Day 20 the ERVK IN expressing females consistantly show a slight decrease (2-3 moves / minute) in movement compared to the littermate controls for the duration of the 24-hour run. At Day 40 the ERVK IN expressing females motor decifit worsens to about 5 moves / minute less from hours 2-9.



B

ERVK IN x D42



ERVK IN Expressing **Littermate Control**

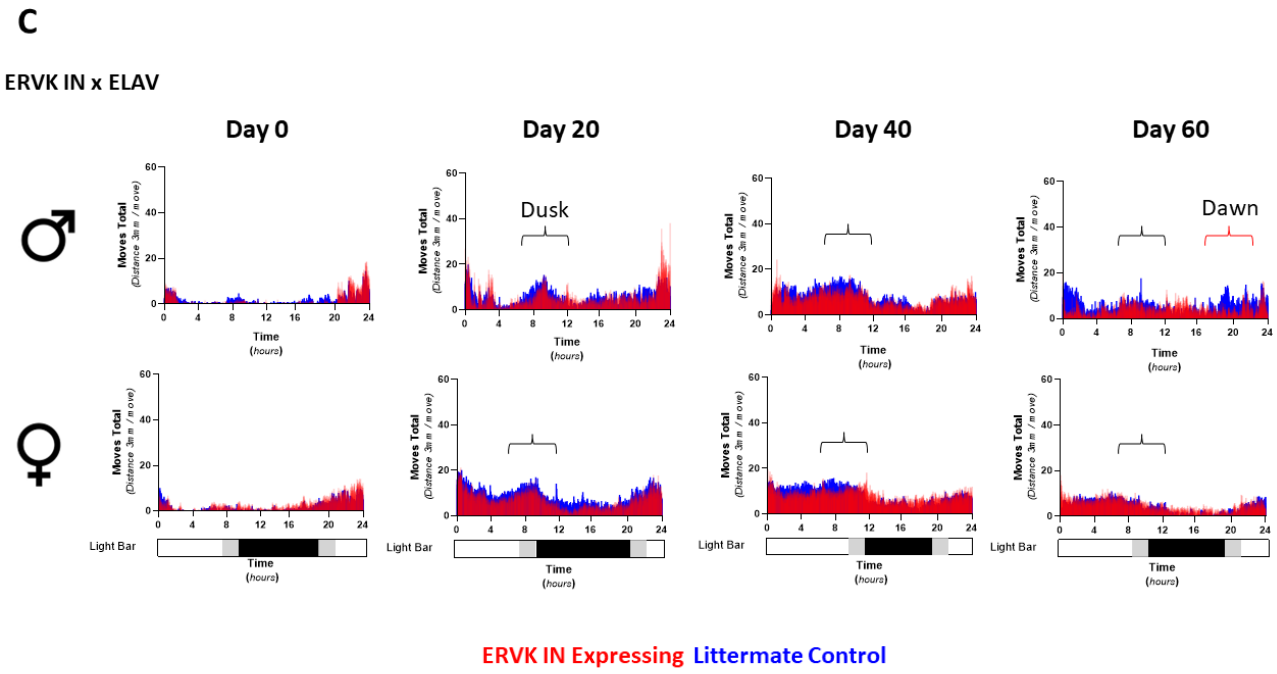
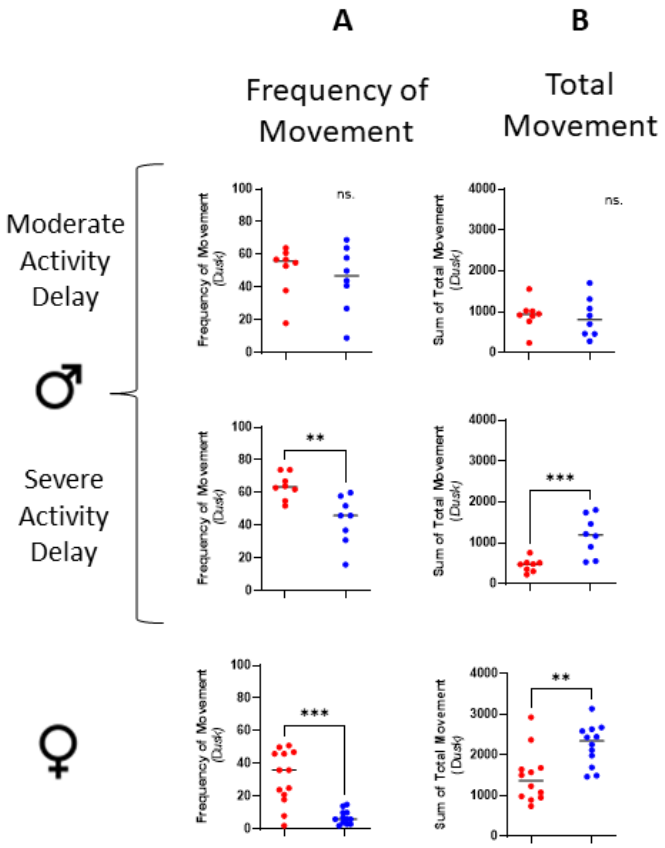


Figure 11: ERVK IN induces motor deficits at dusk in males. A) Actogram schematic of a healthy fly highlighting peak activity levels at dusk and dawn. **B)** ERVK IN x D42 expressing males display motor deficits at dusk and have increased activity at night at Day 20. Motor deficits continue to acrue at dusk at Day 40 and include deficits at dawn at Day 60. The ERVK IN x D42 expressing females have motor deficits from hours 0-7 that worsens at Days 40 and 60. **C)** The ERVK IN x ELAV expressing males have increased movement spikes and restricted dusk movement at Day 20 but no obvious motor abnomalities. A decrease in motor functioning (5 moves / minute) occurs at Day 40 and movement becomes more inconsistant at Day 60 with the ERVK IN x ELAV expressing males. ERVK IN x ELAV expressing females show a decrease (5 moves/minute less) in activity at Day 20. Activity decreases in both littermate control and ERVK IN expressing females at Day 60 but movement is more uncontrolled (more movement spikes) in the ERVK IN expressing females.

The 24-hour actograms uncovered previously missed motor deficits in the ERVK IN x D42 expressing flies and confirmed that the hyperactive phenotype observed in the climbing and walking experiments is not a continuous phenotype. The ERVK IN x D42 expressing males showed a delay in dusk activity that warranted further investigation.

4.8 ERVK IN x D42 Expressing Male Flies Display Different Phenotypes at Dusk

An extra trial using 16 littermate control and ERVK IN x D42 expressing flies of both sexes was performed at Day 20 (clearest delay in activity) to get a better understanding of the effect ERVK IN has on dusk activity. Side-by-side analysis of individual flies revealed two distinct phenotypes present in the ERVK IN x D42 expressing males at dusk. Actograms show that half of the ERVK IN x D42 expressing males display a moderate, though statistically insignificant, activity delay at dusk (Two-way ANOVA: $F_{1,10} = 0.99$; $P = 0.34$). While these ERVK IN x D42 expressing males showed no differences in their frequency of movement (t(14) = 0.47; $P = 0.58$) or in their total movement (t(14) = 0.47; $P = 0.27$), their peak dusk activity is shifted to occur approximately 45 minutes after the littermate controls (**Figure 12**). The other half of the ERVK IN x D42 expressing males had a significant increase in the frequency of movement (t(14) = 3.48; $P = 0.0036$) and a significant decrease in their total movement (t(14) = 3.95; $P = 0.0014$). Again, this is indicative of the jerky movement patterns and muscle spasms occurring in these ERVK IN x D42 expressing males. The actogram of these males also shows a significant activity delay (Two-way ANOVA: $F_{1,10} = 31.96$; $P = 0.0002$) and a much shorter dusk activity period (approximately 20 minutes vs 1.5 hours in the littermate controls). Interestingly, no dusk delay occurred in the ERVK IN x D42 expressing females indicating that this phenotype may be sex-specific. The frequency of movement was significantly increased (t(22) = 4.98; $P = <0.0001$) and the total movement was significantly decreased compared to the littermate controls (t(22) = 3.27; $P = 0.0035$. Two-way ANOVA: $F_{1,22} = 10.68$; $P = 0.0035$).


A
B
Frequency of Movement
Total Movement
Moderate Activity Delay
♂
Severe Activity Delay
♀
Frequency of Movement (Dusk)
Sum of Total Movement (Dusk)
ns.
**
***
ERVK IN Expressing
Littermate Control

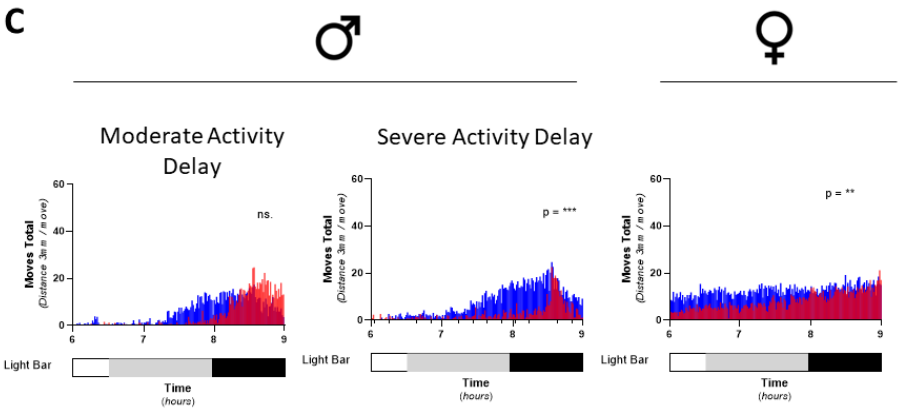

C
♂
♀
Moderate Activity Delay
Severe Activity Delay
Moves Total (Distance 3m / move)
ns.
p = ***
p = **
Light Bar
Time (hours)
ERVK IN Expressing
Littermate Control

Figure 12: ERVK IN x D42 expressing male flies display abnormal motor functioning at dusk. ERVK IN x D42 expressing and littermate controls of both sexes were aged to Day 20 and analyzed using the Trikinetics DAM5H Drosophila activity monitor. Hours 6-9 were extracted to determine differences at dusk **A) B)** Two phenotypes were observed in the ERVK IN x D42 expressing males at dusk. Half of the ERVK IN expressing male flies showed no statistical differences in either frequency of movement or total movement whereas the other half displayed an increase in the frequency of movement ($t(14) = 3.48$; $P = 0.0036$) and a decrease in total movement ($t(14) = 3.95$; $P = 0.0014$). The ERVK IN x D42 expressing females also displayed an increase in the frequency of movement ($t(22) = 4.98$; $P = <0.0001$) and a decrease in the total movement ($t(22) = 3.27$; $P = 0.0035$) compared to littermate controls. **C)** Actograms of the ERVK IN expressing males highlights the moderate and severe (Two-way ANOVA: $F_{1,10} = 31.96$; $P = 0.0002$) activity delays at dusk. The ERVK IN expressing females show a motor activity deficit (Two-way ANOVA: $F_{1,22} = 10.68$; $P = 0.0035$) but no observable delay at dusk.

The dichotomy in behaviour at dusk in the ERVK IN x D42 expressing males was an unexpected finding and suggests the possibility that ERVK IN may impact circadian activity dynamics. The previous experiments have highlighted the molecular and behavioural consequences of ERVK IN activity. Briefly, ERVK IN induces DNA damage and proteinopathy, erratic movements and hyperactivity along with motor deficits and dusk activity delays. The next step was to determine whether the severity of these abnormal disease-like phenotypes could be reduced with drug intervention.

4.9 Raltegravir Does not Improve Motor Functioning in ERVK IN Expressing Flies

Raltegravir is a first-generation HIV integrase inhibitor approved for use as an HIV therapeutic in 2007[97]. As the first drug in its class, Raltegravir is a logical choice to use as a first draft ERVK IN inhibitor. ERVK IN expressing flies typically experienced a high frequency of movement usually paired with a reduction in in the total movement. Raltegravir, if effective, will reverse the previously mentioned motor

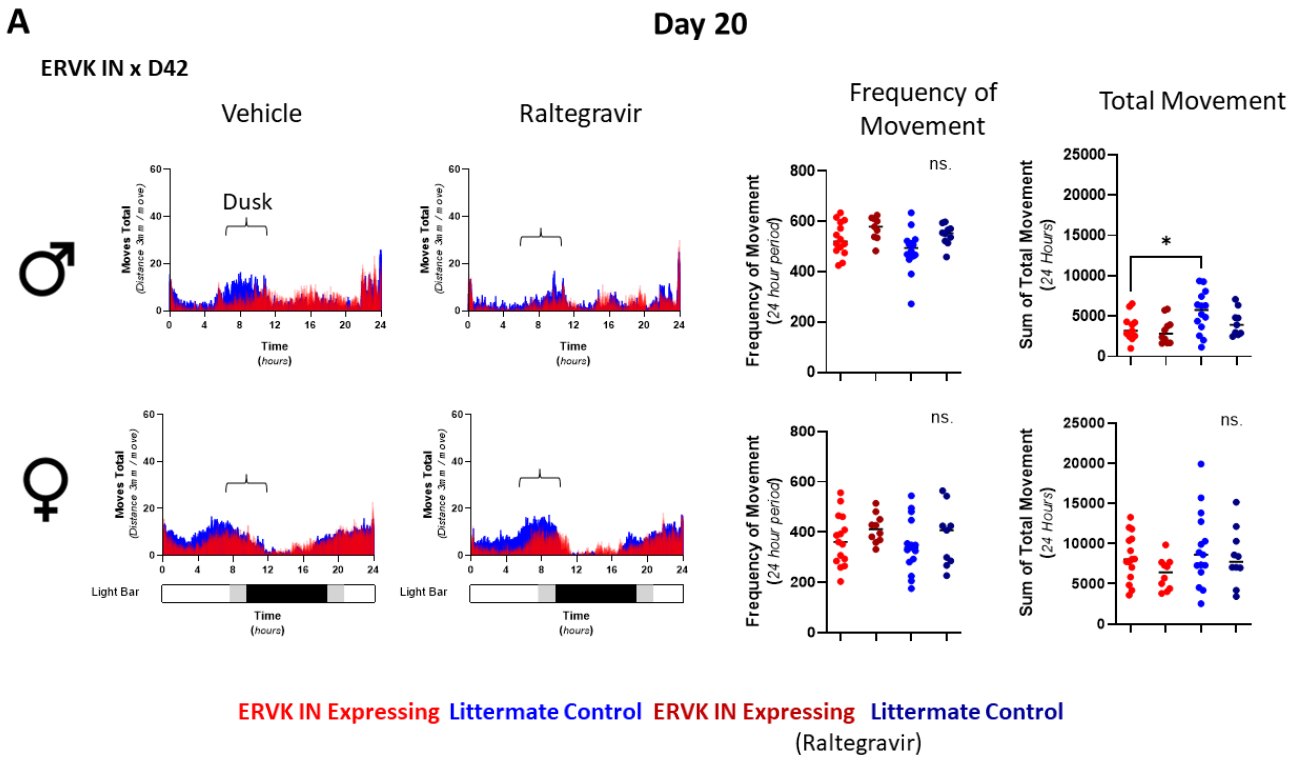
abnormalities (i.e., lower the frequency of movement and increase the total movement) without impacting the motor functioning of the littermate controls. From eclosion, ERVK IN expressing and littermate control flies were fed 250µM Raltegravir or vehicle (ethanol) spiked food and analysed using the Trikinetics DAM5H activity monitor at Days 20, 40 and 60. Result for each cross are described below. For all T-test results refer to Tables S3-S6 in the Appendix.

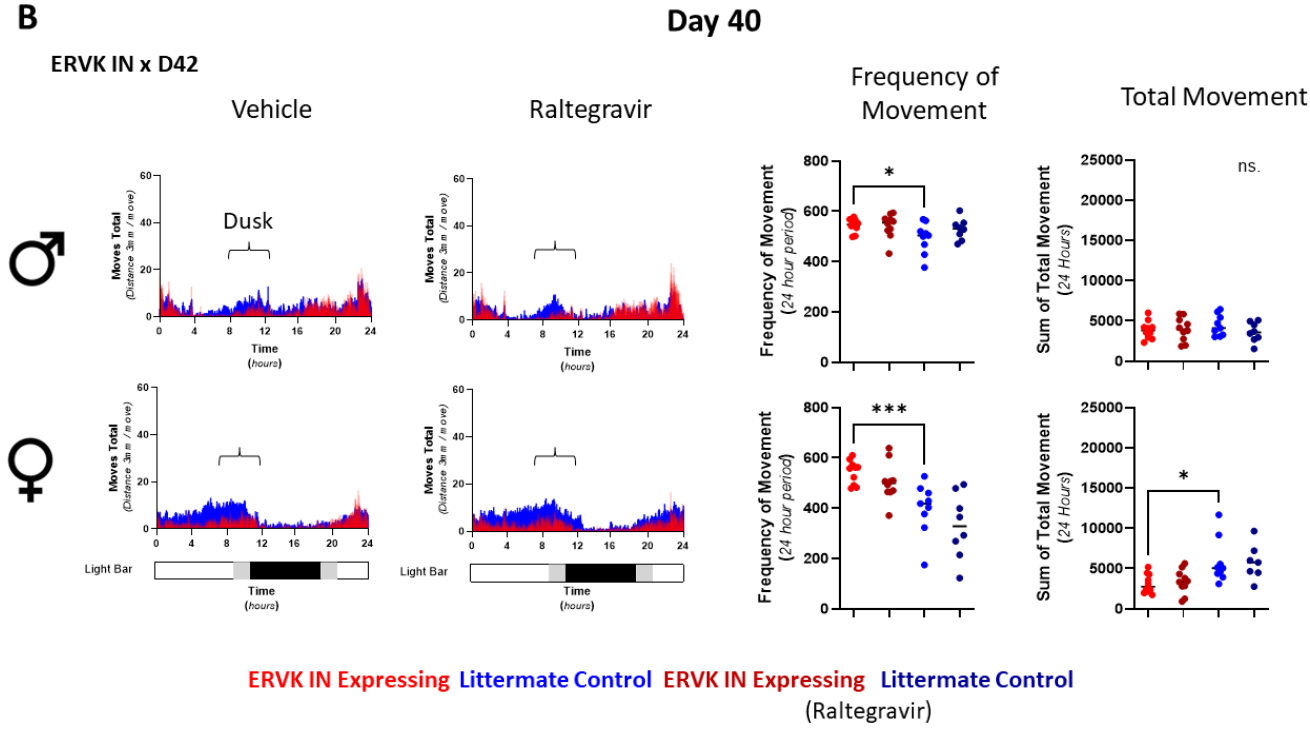
ERVK IN x D42

Instead of increasing the total movement and reducing the frequency of movement in the ERVK IN expressing males, the drug seems to worsen it. The ERVK IN expressing males had significantly decreased total movement ($t(23) = 2.14$; $P = 0.044$) at Day 20 and a higher frequency of movement at Day 40 ($t(17) = 2.39$; $P = 0.029$) and Day 60 ($t(14) = 2.41$; $P = 0.035$). No improvements in the previously mentioned impairments occurred with Raltegravir treatment. The actograms further highlight the reduced movement seen in both ERVK IN expressing and littermate control males with Raltegravir treatment at Days 20 and 40 (**Figure 13, Panels A and B**). At Day 60 the actograms also showed that the ERVK IN expressing males had increased night activity under Raltegravir treatment at this, but not other timepoints (**Figure 13, Panel C**). This is most likely a side effect of Raltegravir as insomnia has been reported in patients using this drug[98]**.**

No significant differences were found between treated and non-treated flies in the ERVK IN expressing or littermate control females at Day 20. At Day 40 motor deficits start to become apparent with a significantly higher frequency of movement ($t(17) = 4.02$; $P = 0.0009$) and a reduced total movement ($t(17) = 2.84$; $P = 0.011$) in the ERVK IN expressing females that is not improved with Raltegravir. Day 60 is the first timepoint when Raltegravir treatment had an impact on motor functioning in the ERVK IN expressing females. Unfortunately, it was not an improvement as the frequency of movement increased

with drug treatment (t(18) = 3.16; *P* = 0.005) and, as previously shown, this is most likely a consequence of the ERVK IN expressing females jerky movements. The actograms feature the progressing decline in motor functioning in the ERVK IN expressing females and the worsening with Raltegravir treatment.





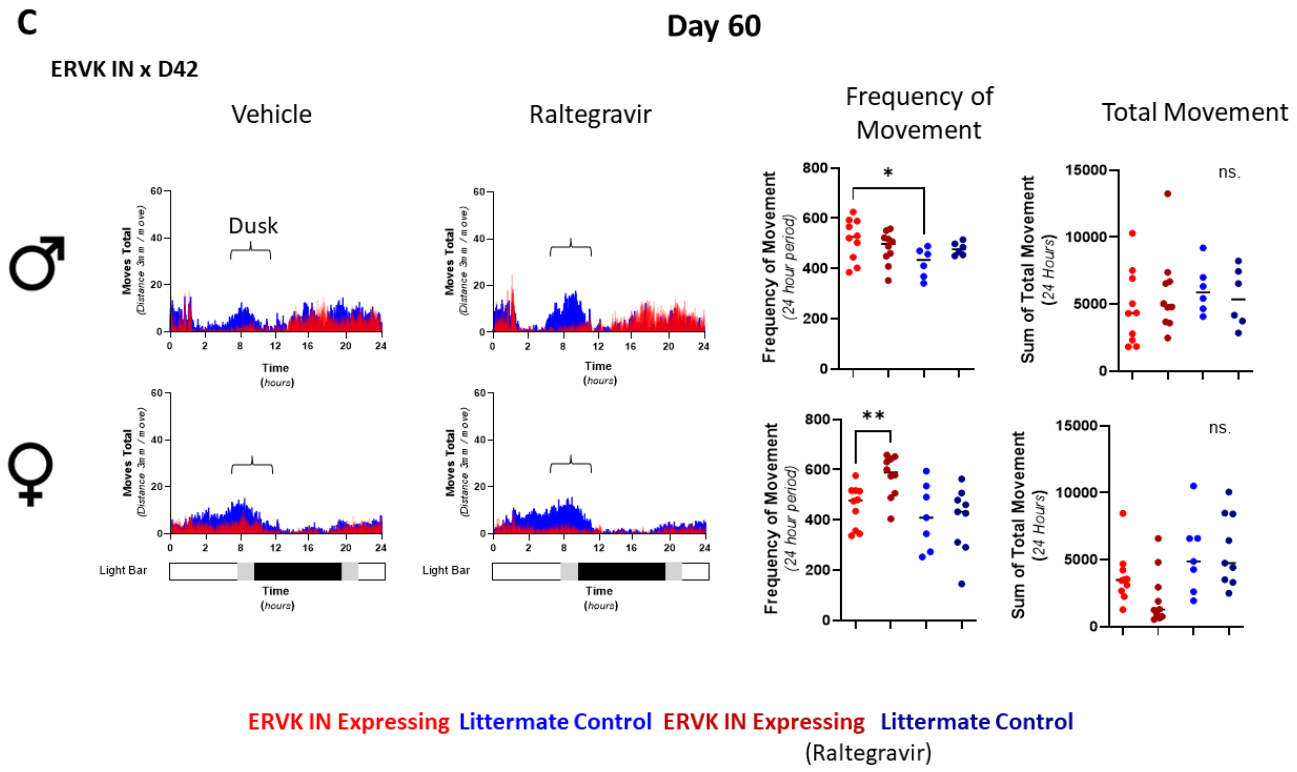


Figure 13: Raltegravir does not improve motor functioning in ERVK IN x D42 expressing flies. ERVK IN x D42 expressing and littermate controls were fed 250µM Raltegravir or Vehicle food. The food was changed every 10-11 days. 24-hour analysis was performed using the Trikinetics DAM5H Drosophila activity monitor at Days 20, 40 and 60. Actograms depicting 24-hour movement patterns and graphs showing the frequency of movement and total movement were generated. Unpaired T-tests were used to determine differences between ERVK IN expressing and littermate control flies with and without Raltegravir treatment **A)** Day 20 analysis shows worsening motor function in ERVK IN expressing males and females with Raltegravir treatment. **B)** Day 40 shows a significant increase in the frequency of movement in the ERVK IN expressing males and females that is not improved with Raltegravir treatment. The ERVK IN expressing females show a decrease in total movement that is again not improved with Raltegravir. **C)** Day 60 analysis shows an increase in the frequency of movement in the ERVK IN expressing males that is not changed with Raltegravir. Dusk activity increases in the littermate control males and

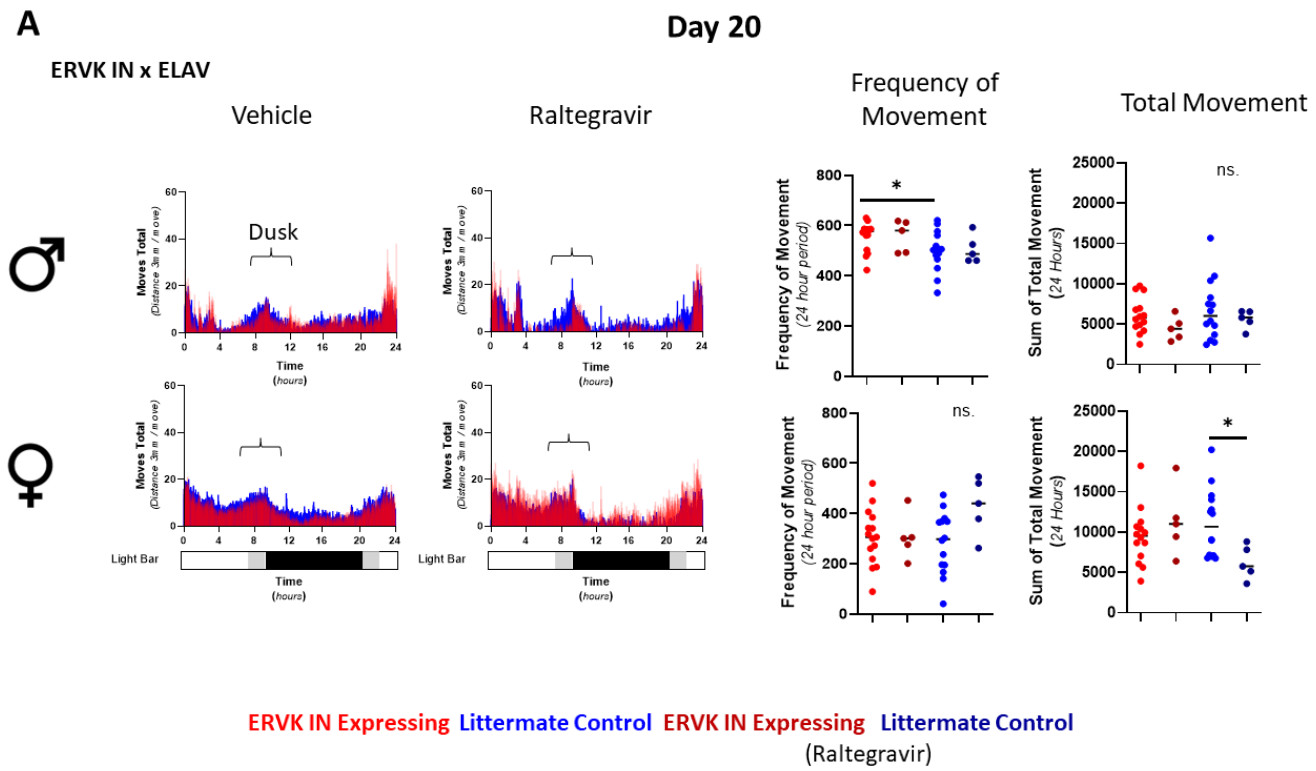
night activity increases in the ERVK IN expressing males with treatment. The frequency of movement also increased in the ERVK IN expressing females with Raltegravir and while not significant, movement decreases with treatment as well.

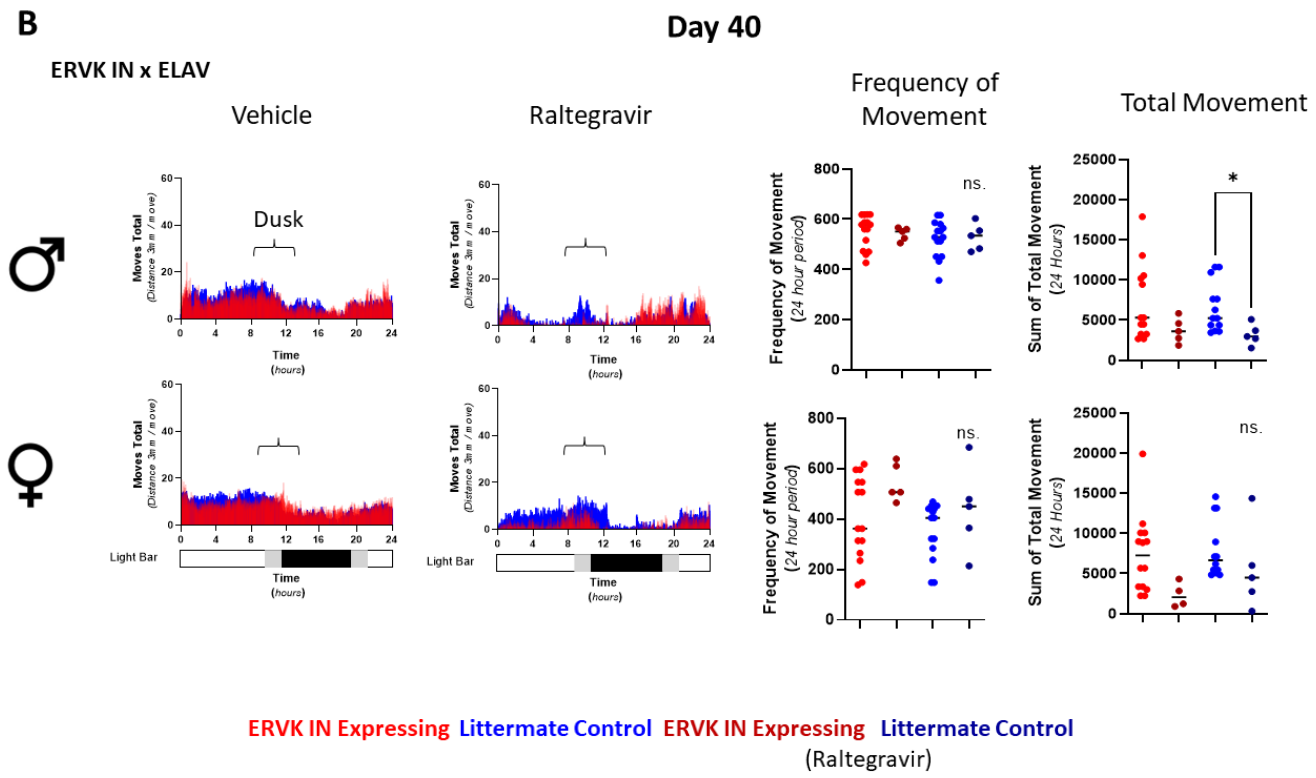
ERVK IN x ELAV

Raltegravir treatment in the ERVK IN x ELAV expressing males showed the same pattern as seen in the ERVK IN x D42 expressing males at Day 20, with an increased frequency of movement ($t(26) = 2.09$; $P = 0.047$) compared to littermate controls (**Figure 14, Panel A**). Additionally, the ERVK IN expressing males' actogram showed an overall reduction in motor activity along with a 50% shortening of the dusk activity period with Raltegravir treatment. Curiously, the littermate control males' activity did not decrease as it did with the ERVK IN x D42 littermate control males, highlighting differences in drug reactions that can occur with different genetic backgrounds. At Day 40 Raltegravir treatment continues to reduce activity and while not significant in the ERVK IN expressing males, it is significant in littermate control males ($t(16) = 2.36$; $P = 0.032$) showing that negative side-effects can occur in healthy flies with this drug. This reduction in movement can clearly be seen in the actograms along with a lack of dusk movement and increased nocturnal movement in the ERVK IN expressing males (**Figure 14, Panel B**). At Day 60, no significant differences were observed with Raltegravir treatment. However, the actograms showed elevated movement in ERVK IN expressing males which wase different from previous timepoints (**Figure 14, Panel C**). It may take a transition period for Raltegravir to become effective in the ERVK IN expressing males.

The ERVK IN expressing and littermate control females showed little differences at Days 20, 40 and 60. However, with Raltegravir treatment alterations in activity occurred in both ERVK IN expressing and littermate control females. At Day 20, Raltegravir treatment induced a significant reduction in the total

movement in the littermate controls (t(17) = 2.45; *P* = 0.026) further showcasing negative side-effects in the healthy flies. Fatigue is another common side effect found with Raltegravir treatment[99] and may cross over into Drosophila explaining this reduction. It was with the ERVK IN expressing females where we saw an alternate response to Raltegravir treatment than previously observed. While no significant differences were found in the frequency of or total movement, the actogram did show brief spikes of activity that resulted in a frantic motor pattern that resembled the ERVK IN x ELAV expressing males at Day 60 (with Raltegravir treatment). Although not significantly different, Raltegravir did increase the frequency of movement and decreased the total movement in the ERVK IN expressing females at Day 40 as seen in the actograms. This is the opposite of the expected outcome. Finally, at Day 60 the littermate control females showed increased movement whereas the ERVK IN expressing females showed a significant decrease in movement with Raltegravir treatment (t(14) = 2.16; *P* = 0.049).




B
Day 40
ERVK IN x ELAV
Vehicle
Raltegravir
Frequency of Movement
Total Movement
♂
♀
Dusk
Moves Total (Distance 3in / move)
Time (hours)
Light Bar
Frequency of Movement (24 hour period)
Sum of Total Movement (24 Hours)
ns.
*
ERVK IN Expressing Littermate Control ERVK IN Expressing Littermate Control
(Raltegravir)

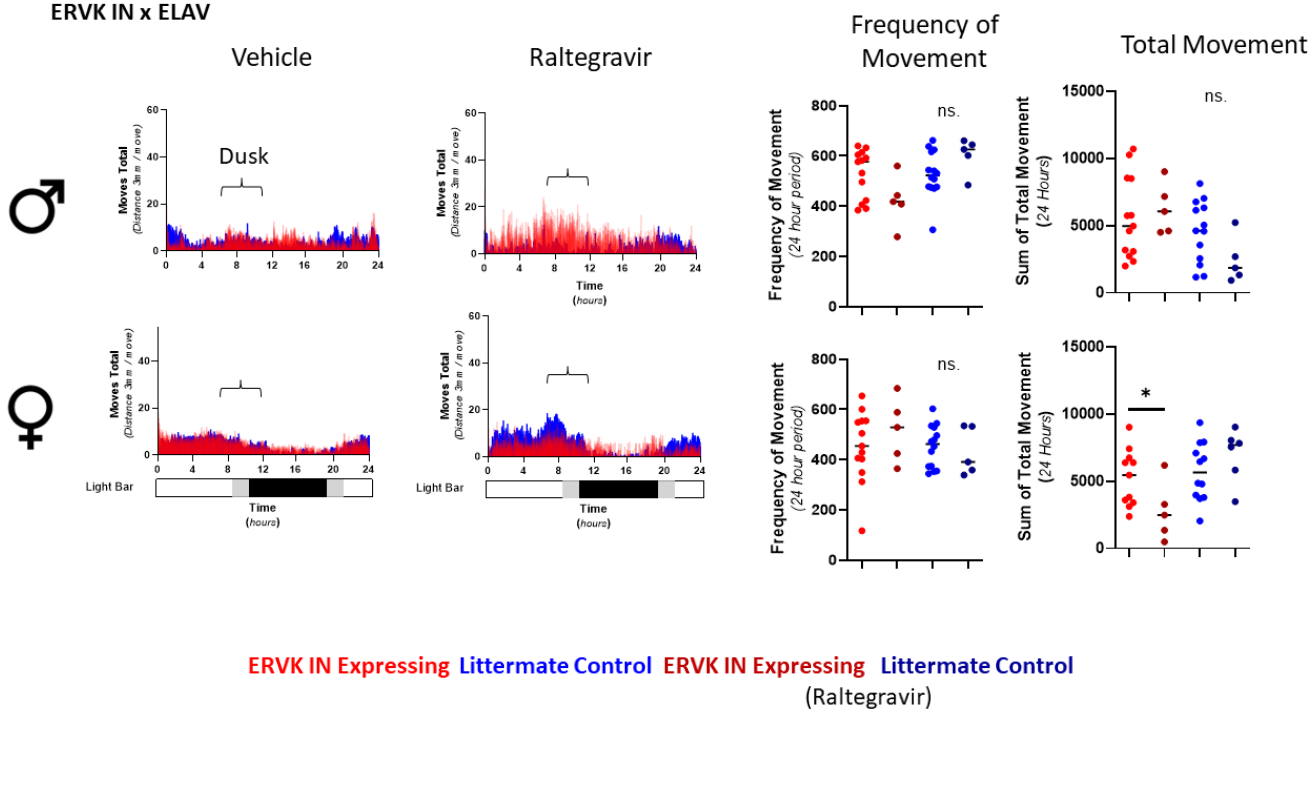

C
Day 60
ERVK IN x ELAV
Vehicle
Raltegravir
Frequency of Movement
Total Movement
♂
♀
Dusk
Moves Total (Distance 3in / move)
Time (hours)
Light Bar
Frequency of Movement (24 hour period)
Sum of Total Movement (24 Hours)
ns.
*
ERVK IN Expressing Littermate Control ERVK IN Expressing Littermate Control
(Raltegravir)

Figure 14: Raltegravir treatment does not improve motor functioning in ERVK IN x ELAV expressing flies. ERVK IN x ELAV expressing and littermate controls were fed 250µM Raltegravir or vehicle food. The food was changed every 10-11 days. 24-hour analysis was performed using the Trikinetics DAM5H Drosophila activity monitor at Days 20, 40 and 60. Actograms depicting 24-hour movement patterns and graphs showing the frequency of movement and total movement were generated. Unpaired T-test were used to determine differences between ERVK IN expressing and littermate control flies with and without Raltegravir treatment **A)** Day 20 analysis the ERVK IN expressing males showed an overall reduction in movement with Raltegravir treatment. The ERVK IN expressing females had an increased and more frantic activity pattern (not significant) whereas the littermate control females showed a significant reduction in movement with Raltegravir treatment **B)** At Day 40 there is an overall reduction in activity in both ERVK IN expressing males and females with Raltegravir treatment. The littermate control males also displayed a significant decrease in total movement with Raltegravir treatment **C)** ERVK IN expressing males' actogram at Day 60 showed increased, though insignificant, movement The ERVK IN expressing females have significantly decreased total movement with Raltegravir treatment whereas the littermate control females had increased daytime activity.

Overall, the flies' activity is reduced under the influence of Raltegravir treatment in both ERVK IN expressing and in some cases the healthy littermate controls. The exceptions being the IN x ELAV expressing females at Day 20 and males at Day 60. Raltegravir treatment was expected to decrease the frequency of movement and increase the total movement in the ERVK IN expressing flies (aspects that typically differ from littermate controls). Unfortunately, Raltegravir did not match expected outcomes at the current dosage and delivery method.

4.10 Cabotegravir Improves Motor Functioning in ERVK IN Expressing Flies

Cabotegravir is the newest HIV integrase strand transfer inhibitor manufactured[97] and has benefits over Raltegravir. These include a longer elimination half-life allowing for treatment only once every 2 months, administration via intramuscular injection and perhaps most importantly, a higher resistance barrier to common HIV integrase mutations[100]. Due to the before mentioned benefits, Cabotegravir was a second attempt at finding an effective ERVK IN inhibitor. A preliminary screen was performed in ERVK IN x D42/ELAV expressing and littermate control flies at the Day 20 timepoint and the results are described below. For all T-test results see Table S3-S6 in the Appendix.

ERVK IN x D42

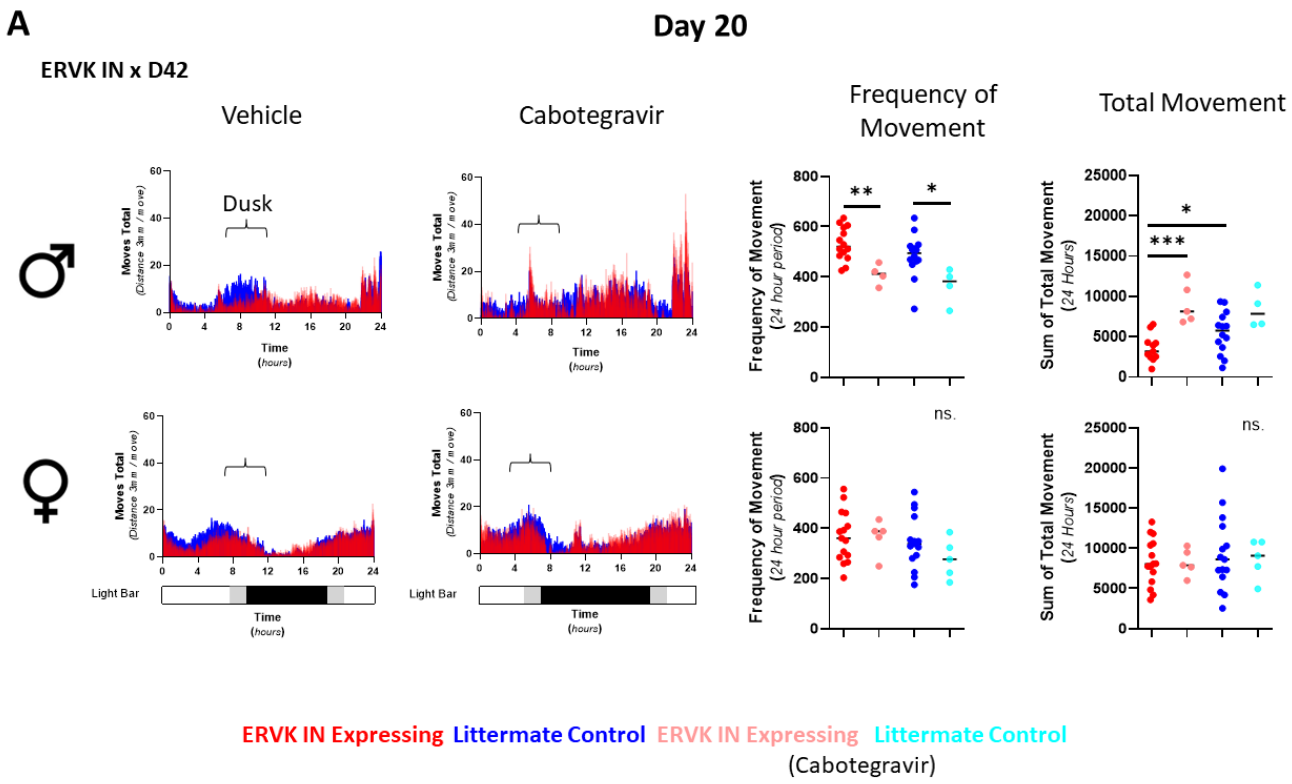
At the Day 20 timepoint positive results were found for the ERVK IN expressing flies under Cabotegravir treatment. The ERVK IN expressing and littermate control males both had a significant reduction in the frequency of movement ($t(16) = 3.44$; $P = 0.0033$) and ($t(16) = 2.57$; $P = 0.021$) respectively (**Figure 15, Panel A**). This means that both the ERVK IN expressing, and littermate control males had a more continuous movement pattern resulting in less movement peaks. The main benefit is observed in the total movement. Without treatment the ERVK IN expressing males had a significantly lower total movement ($t(23) = 2.14$; $P = 0.044$) compared to littermate controls. With Cabotegravir treatment this deficit was significantly improved, increasing the total movement ($t(14) = 5.28$; $P = 0.0001$) of the ERVK IN expressing flies compared to ERVK IN expressing flies without treatment. The actograms further reflect the increased movement of the ERVK IN expressing males. The most prominent Cabotegravir induced side-effect is the increase in nocturnal activity which occurred in both ERVK IN expressing and littermate control males. Insomnia is one of the most common side-effects associated with Cabotegravir usage[100]. Alterations in drug dosage may aleviate this side-effect.

The ERVK IN expressing females while not having any significant differnces in the frequency of movement or the total movement do show visible improvements. The actograms ehxibit a partial recovery of motor ability from hours 0-6 in the ERVK IN expressing females with Cabotegravir treatment compared to without treatment. Interestingly, the insomnia present in the treated males is not observed to as high of a degree in the treated females. This could be due to differences in drug metabolism or eating habits resulting in more or less drug incorporation or ingestion.

ERVK IN x ELAV

The promising results observed with Cabotegravir treatment in the ERVK IN x D42 expressers continues with the ERVK IN x ELAV expressers. Without treatment the ERVK IN expressing males had an increased frequency of movement ($t(26) = 2.09$; $P = 0.047$) (compared to littermate controls) that was effectively reduced with Cabotegravir treatment ($t(22) = 2.39$; $P = 0.026$) (compared to ERVK IN expressing males without Cabotegravir) (**Figure 15, Panel B**). Cabotegravir also increased the total movement of the ERVK IN expressing males ($t(22) = 2.66$; $P = 0.014$) that was seen in the partial recovery of dusk activity and in an increase in daytime activity spikes. Curiously, insominia was not observed in the ERVK IN x ELAV males like what was seen in the ERVK IN x D42 males. This could be due to the higher impact of ERVK IN activity in the ELAV transgenics (expression in all neurons rather than motor neurons exclusively) requiring a higher drug dosage and perhaps accruing less side-effects.

The ERVK IN expressing females experienced a reduction in the frequency of movement ($t(22) = 2.39$; $P = 0.026$) and increased total movement ($t(21) = 2.94$; $P = 0.008$) with Cabotegravir treatment. Importantly, the littermate control females activity was not impacted by Cabotegravir treatment indicating this drug was well tolerated in this group.



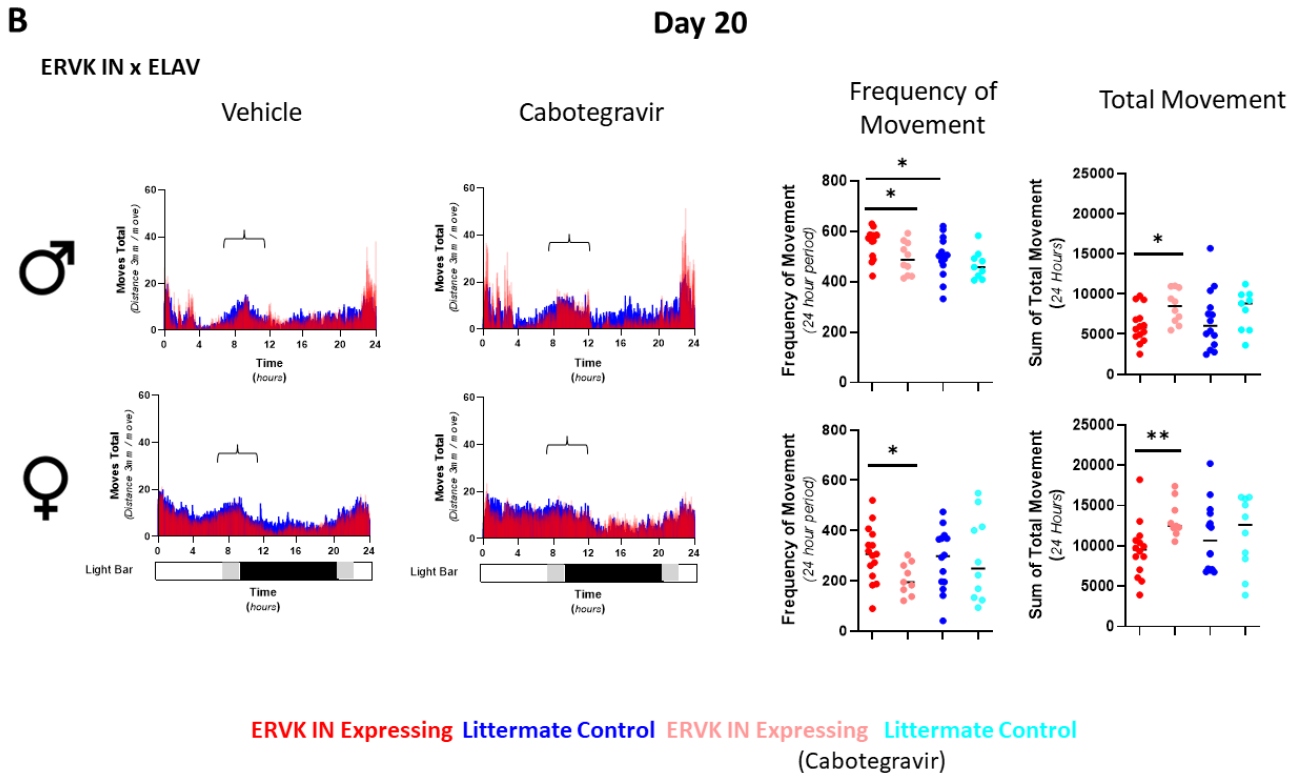


Figure 15: Cabotegravir improves ERVK IN induced motor deficits at Day 20. ERVK IN x D42 and ELAV expressing, and littermate control flies were fed 250µM Cabotegravir or Vehicle (ethanol) spiked food.

The food was changed every 10-11 days. 24-hour analysis was performed using the Trikinetics DAM5H Drosophila activity monitor at Day 20. Actograms depicting 24-hour movement patterns and graphs showing the frequency of movement and total movement were generated. Unpaired T-test were used to determine differences between ERVK IN expressing and littermate control flies with and without Cabotegravir treatment **A)** Cabotegravir treatment significantly reduced the frequency of movement in both ERVK IN x D42 expressing males and littermate control males. The total movement of the ERVK IN expressing males also significantly increased No significant differences were found in the ERVK IN x D42 expressing females **B)** Cabotegravir treatment was able to significantly decrease the movement frequency of ERVK IN x ELAV expressing males ($t(22) = 2.39$; $P = 0.026$) and increase the total movement Treatment was also able to significantly decrease the movement frequency of ERVK IN x ELAV expressing females and increased the total movement.

Comparing the effects of Raltegravir and Cabotegravir on their ability to alleviate ERVK IN induced motor pathology revealed a facinating pattern. Regarding the ERVK IN x D42 expressing males and females, Raltegravir reduced activity and Cabotegravir increased activity (**Figure 16, Panel A**). This patterns continued with the ERVK IN x ELAV expressing males. The ERVK IN x ELAV expressing females had a slightly different phenotype, showing increased activity with both Raltegravir and Cabotegravir treatments (**Figure 19, Panel B**). Moreover, the ERVK IN x ELAV expressing females under Raltegravir treatment displayed an erratic activity pattern.

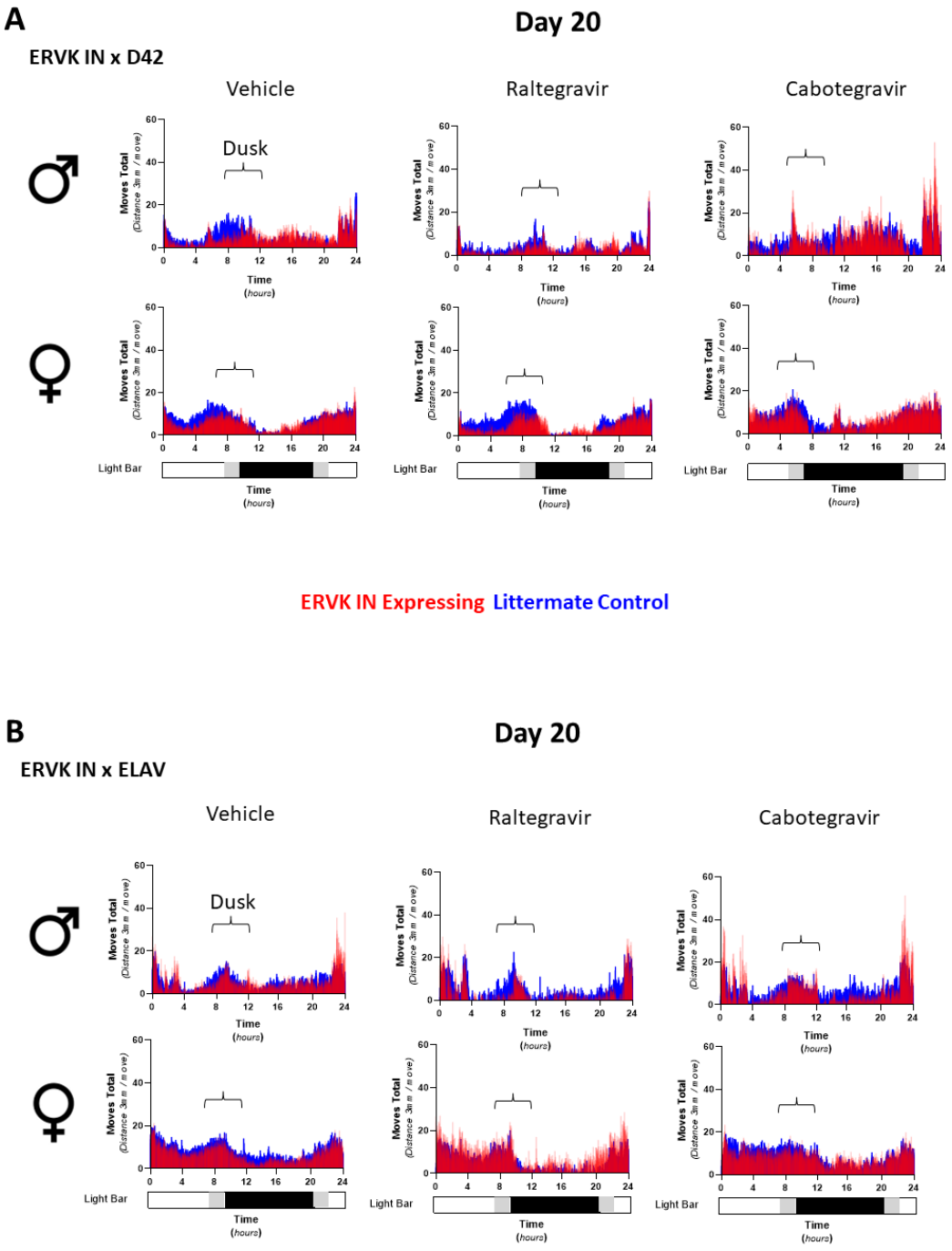


Figure 16: Raltegravir and Cabotegravir treatment induce opposite effects on motor functioning at Day 20. ERVK IN expressing and littermate control flies were fed 250µM Raltegravir or Cabotegravir or vehicle food and analysed at Day 20. **A)** ERVK IN x D42 expressing males showed a delay in activity at dusk and

increased nocturnal activity. Raltegravir treatment reduced motor activity whereas Cabotegravir treatment increased motor activity in both ERVK IN expressing and littermate control males. Cabotegravir treatment also improved ERVK IN expressing males activity at dusk but worsened night activity. The ERVK IN expressing females showed reduced activity at dusk but no activity delay. Raltegravir treatment decreased activity and Cabotegravir treatment increased activity in the ERVK IN expressing females but not the littermate controls. **B)** The ERVK IN x ELAV expressing males displayed spikes of increased activity throughout the 24-hours and condensed dusk activity but overall did not vary much from the littermate controls. Raltegravir treatment reduced and Cabotegravir treatment increased these activity spikes in the ERVK IN expressing males. ERVK IN x ELAV expressing females showed a slight decrease (5 moves/minute less) in activity. With Raltegravir treatment the ERVK IN expressing females activity increased but became more uncontrolled and with Cabotegravir treatment day activity decreased but night activity increased.

In summary, ERVK IN induced DNA damage, TBPH and PARP1 protein cleavage along with an inflammatory immune response. ERVK IN did not significantly impact eclosion or lifespan and caused a hyperactive phenotype in early life. Actograms displaying 24-hour activity patterns revealed that the hyperactive phenotype was not continuous and further characterized multiple abnormalities including motor deficits, dusk activity delays and nocturnal activity. The most commonly observed phenotypes in the ERVK IN expressing flies was an increased frequency of movement (i.e. increased movement peaks) indicative of jerky, stop and start actions that were usually but not always coupled with a decrease in total movement. Additionally, ERVK IN x D42 expressing males were found to display two phenotypes at dusk. The first, a moderate dusk delay with no significant differences between ERVK IN expressing and littermate controls. The second, a severe dusk delay with significant differences in the frequency of movement and the total movement. This discovery may implicate ERVK activity in the alteration of

circadian rhythms. First attempts at an ERVK IN inhibitor using repurposed HIV antivirals revealed opposing drug effects. Raltegravir mostly lowered activity and in some cases seemed to worsen ERVK IN induced phenotypes whereas Cabotegravir universally increased activity in the ERVK IN flies improving motor deficits and dusk activity levels.

5.0 Discussion

ERVK is the most recent ERV to enter the primate genome[2]. While normally inactive, ERVK can become reactivated by inflammatory signalling events[14]. Increased ERVK expression has been linked to several disease states including the neurodegenerative disease ALS[101]. ERVK IN is an understudied ERVK protein that has been implicated as a source of DNA damage[5]. *Drosophila melanogaster* is one of the most used model organisms to study human diseases including ALS[102-104]. Drosophila has been used throughout this project to gain a better understanding of the cellular and behavioural consequences of ERVK IN activity in neurons. Fruit flies share a high degree of similarity to the human innate immune system[105] making them ideal when studying viral infections in the nervous system.

ERVK IN activates the IMD immune signalling pathway

Western blot revealed that the Drosophila NF-κB homologue Relish was cleaved into Rel-49/RelC and Rel-68/RelN which is indicative of IMD pathway activation. As ERVK IN activity occurs inside the cell, this pathway is most likely not initiated by external influence. The IMD pathway can also be initiated intracellularly via dSTING. The mammalian STING is usually activated by cyclic dinucleotides, but this pathway can also be activated through a noncanonical pathway initiated by DNA damage[106]. DNA damage is detected by PARP1, ataxia telangiectasia mutated (ATM) and interferon-inducible protein 16 (IFI16) that results in the formation of a STING signalling complex leading to NF-κB nuclear translocation and expression of interferon stimulated genes[106]. It could be that this noncanonical pathway translates to Drosophila with the fly PARP1 activating STING initiating the IMD signalling cascade ending with Relish cleavage and subsequent activation of antimicrobial peptides. Interestingly, the inflammatory cytokine TNFα, produced by the human tumour necrosis factor signalling pathway (equivalent to Drosophila IMD) is upregulated in ALS[107,108]. TNFα can also induce an inflammatory mode of cell death called necroptosis[109] which is the mode of cell death implicated in ALS[49]. MLKL, RIP1 and RIP3 are mediators of

the mammalian necroptosis pathway[109]. While MLKL has no equivalent in flies, RIP1 and RIP3 contain a RHIM-motif that resembles one found in the Drosophila IMD protein[87]. This provides further evidence that ERVK IN may induce equivalent signalling cascades in both flies and humans.

ERVK IN may use PARP1 as a targeting system to locate damaged DNA

I found that activation of ERVK IN in the Drosophila model led to PARP1 cleavage. ERVK IN contains a high affinity BRCT interaction domain[110], meaning it can bind to proteins containing the BRCT domain which includes PARP1. PARP1 is also able to bind DNA without initiating its catalytic activity[111]. What is particularly intriguing is PARP1s' ability to traverse the genome using what is termed the monkey-bar mechanism[112]. By "swinging" through the genome, PARP1s ability to find sites of DNA damage increases threefold compared to random detection through diffusion[111].

The HIV integrase is known to bind the DNA repair and antiapoptotic protein Lens epithelium-derived growth factor (LEDGF) and use it as a co-factor for integration. LEDGF works to dock the HIV PIC to the chromatin and protect it from degradation[113]. Importantly, LEDGF recruits and tethers DNA repair proteins to DSBs located in sites of active transcription[114,115]. Here I present a proposed mechanism where ERVK IN binds PARP1 to detect DNA damage in sites of active transcription, as HIV IN does with LEDGF. This effectively increases ERVK INs' chance for a successful reintegration event.

PARP1 cleavage as a viral immune evasion strategy

Facilitating the cleavage of proteins involved in the antiviral immune response is a commonly employed viral immune evasion strategy. For example, the C3 protease of the human Enterovirus 68 binds and cleaves IRF7[116] and the Dengue virus NS2B3 protease binds and cleaves STING[117] preventing the induction of type 1 interferons and inflammatory cytokines in both cases. Drosophila are also victims of the same antiviral strategies. The Cricket paralysis virus 1A protein can hijack a host ubiquitin ligase

which cleaves AGO2 restricting the RNAi response[118]. PARP1 is also targeted by viral proteins to evade detection[119,120]**.** The detected PARP1 cleavage in ERVK IN expressing flies could be a viral immune evasion strategy used to prevent PARP1 from initiating an antiviral immune response. ERVK IN may recruit thrombin, the protease predicted to form the detected 70 kDa cleavage product (see **Table 2**). Furthermore, inhibition of thrombin has been found to improve motor functioning and decreases oxidative stress in a Drosophila model of Parkinsons Disease[121], another neurodegenerative disease associated with PARP1 pathology[122]. Alternatively, and perhaps more likely, cleavage of PARP1 by thrombin occurs independently of ERVK activity. Neurotrauma creates instability in the blood-brain-barrier which is known to allow thrombin to enter the CNS inducing neuroinflammation[123]. Trauma induced neuroinflammation could be a triggering event for ERVK INs' initial expression. Moreover, those who have experienced a neurotrauma are at a much higher risk of developing ALS and other neurodegenerative diseases[123].

ERVK IN may induce activation of other Endogenous Retroviruses

Western blot revealed multiple ERVK IN bands in the ERVK IN expressing male flies. The extra bands were both smaller and larger than ERVK IN (32 kDa). The simple explanation is cleavage of ERVK IN results in smaller sized bands and post-translational modifications results in larger sized bands. However, there is another explanation that not only fits with previous findings but is also quite disconcerting. ERVK IN activity may result in the derepression and subsequent reactivation of native fly ERVs. Multiple proteins are known to function in the repression of transposable elements (TEs). In Drosophila, PARP1 is a known repressor of TE activity[124-126]. Additionally, a mutation identified in the Parp locus removes the DNA binding ability of PARP1 and results in increased expression of TEs[125] further highlighting PARP1s role in the repression of TEs. PARP1 overactivity has also recently been reported to be required for TDP-43 translocation and aggregation in the cytosol of neurons[127]. ALS associated cleavage of TDP-43 is generally

thought to occur in the cytoplasm and caused by caspases such as caspase-3[68]**.** Bioinformatic analysis suggested that the only protein capable of producing the 25 kDa and 35 kDa TBPH cleavage products was proline-endopeptidase. Unfortunately, the software used does not contain information regarding cleavage by fly caspases; one of which could be responsible for the resulting cleavage. However, it has been found that the asparaginyl endopeptidase is capable of cleaving human TDP-43 into 35 kDa and 32 kDa immunoreactive fragments in the brain[128]. Therefore, it is not out of the realm of possibilities that slight differences between TDP-43 and TBPH result in cleavage sites for different endopeptidases. Normally, TDP-43 directly binds the transcripts of TEs, preventing transcription machinery from gaining access and facilitating their repression, but in situations where the proper functions of TDP-43 are compromised, increased expression of TEs occurs[129]. Additionally, ALS associated forms of TDP-43 increases ERVK protein accumulation[65]. This phenomenon is also observed in Drosophila. TBPH null flies display an upregulation in TE expression in glia, with the majority belonging to the LTR family of TEs including *accord* and *gypsy*[21]. Moreover, transgenic flies expressing pathological human TDP-43 display alterations in the siRNA signalling pathway inducing increased TE expression[74]. The continued expression of these TEs leads to increased levels of DNA damage and the degeneration of motoneurons[21]. ERVK IN could have a hit-and-run effect. A short initial burst of ERVK IN activity induces PARP1 and TBPH proteinopathy that potentiates longer lasting consequences in the form of TE derepression that continues the pattern of DNA damage and eventually neuron death.

Hyperexcitability is associated with ALS pathology

The clearest phenotype identified in the ERVK IN transgenic flies was hyperexcitability. Initially this phenotype was characterized by random, vertical, up, and down, “popcorning” type movements and an excessively fast walking speed (ERVK IN x D42 expressing flies only). Later, the hyperactivity was seen in muscle spasms in the legs and wings and was represented by a high frequency of movement usually

paired with a lower total movement. What is fascinating is a replica of this pattern has also been observed in ALS patients. The idea of physical exercise as a risk factor for the later development of ALS is controversial. Some studies have concluded that no link exists between the two[130] but other studies have uncovered causal links between physical exercise and ALS. For example, physical exercise is more likely to induce motor neuron injury in those with an ALS risk genotype[131] and both sports-related and work-related physical exercise are risk factors[132]. For instance, not only are Italian soccer players at an increased risk for developing ALS, but they also develop the disease over 20 years earlier than the general population[133]. The same pattern is found in the military. Veterans deployed during the gulf war were found to have an increased risk of developing ALS[134]. Likewise, the Danish found the same results with their military[135]. These occupations have a high occurrence of head trauma and as previously mentioned, trauma induced neuroinflammation may be a triggering event for ERVK IN reactivation.

Muscle spasms, like what was observed in aging ERVK IN expressing flies, is another form of hyperexcitability that is also seen in ALS. Alterations in excitability have been found in both sporadic and familial ALS cases and has been suggested to be a mechanism that predisposes neurons to degeneration[136]. Glutamate is the most abundant excitatory neurotransmitter in the CNS and is important in the regulation of most neuronal activities in both mammals and fruit flies[137,138]. Excitotoxicity occurs where there is prolonged activation of glutamate that leads to an influx of ions into neurons causing death[137]. Moreover, tumour necrosis factor signalling can potentiate glutamate mediated excitotoxicity in mammals[139]**.** In Drosophila*,* glutamate excitotoxicity impairs CPG circuit and muscle activity leading to overexcitation of motor neurons[138]. Hyperactivity may be the initial phenotype associated with ERVK IN activity and excitotoxicity the consequence of long term ERVK IN activity.

ERVK IN may alter the sleep cycle

Sleep disturbances are common early indicators of neurodegenerative diseases including Parkinsons disease, Alzheimer's disease[140] and ALS[141]. The sleep cycle is tightly intwined with the DNA damage response and impairments effecting one system, will to some degree, impact the other[142]. In multiple species, including fruit flies and humans, PARP1 promotes sleep after neuronal injury and inhibition of PARP1 impairs sleep-dependant DNA repair[143]. PARP1 cleavage may prevent the DNA damage induced drive for sleep. This both reduces the effectiveness of the DNA damage response and induces a delay in the onset of sleep. The rising amounts DNA damage and PARP1 cleavage induced by ERVK IN activity could reduce the drive to sleep in early stages preventing rest and efficient DNA repair. Interestingly, my monitoring of daily activity showed some significant changes in activity at dusk, but the effect was sex-specific.

The relevance of sex-specific effects in health and treatment

Multiple sex-specific effects were uncovered in the ERVK IN expressing flies including differences in lifespan and drug response. Increased lifespan in the ERVK IN x D42 (significant) and ELAV (non-significant) males paired with dusk activity delays were unexpected finds and highlighted the role of sex on the presentation of disease phenotypes.

It is known that there are robust sex differences in the aging process and in responses to genomic instability, but sex specific effects are not typically conserved between species[144]. Although, there is a sex bias in the diagnosis of ALS with males having a higher occurrence and an earlier age of diagnosis[145]. This could be due to sex differences found in the nervous system, DNA damage response or epigenetic modifications among many other factors[145] As you age your ability to repair DNA damage decreases leading to genomic instability, a hallmark of aging[144]. It could be that ERVK IN promotes the continuing expression of DNA repair genes thus extending lifespan. In fact, overexpression of PARP1 in the nervous

system has been associated with an extended lifespan in Drosophila[146]. However, this phenomenon was only observed in females and not males. As PARP1 was cleaved in ERVK IN expressing flies, PARP1 expression would not extend life in the females. Another protein involved in DNA repair, with a counterpart in Drosophila is GADD45. Overexpression of D-GADD45 in the nervous system has a significant lifespan lengthening effect in male flies[147]and may be responsible for the life extension in the ERVK IN expressing males. Overall, the underlying factor dictating lifespan is the effectiveness of the DNA damage response in preventing genomic instability. Unfortunately, there is a disproportionate amount of research performed in males over females[148] and assuming that responses in both are equal puts a significant part of the worlds population at a disadvantage.

The ERVK IN x ELAV females under Raltegravir treatment experienced adverse drug effects at Day 20. Pharmacokinetics and pharmacodynamics differ between sexes and influences the speed drugs are metabolised and the occurrence of side-effects[149]. Females generally have slower gastric emptying and renal clearance, making them more sensitive to certain medications and requiring a lower drug dosage compared to men[150]. Moreover, females are 50-75% more likely to experience adverse drug reactions[150]. This shows how crucial it is to move away from general treatments towards implementing sex-specific considerations based on drug effectiveness and dosage effects.

HIV strand transfer inhibitors induce opposite effects on ERVK IN expressing flies

Raltegravir and Cabotegravir are the first and latest FDA approved HIV integrase inhibitors, respectively. These drugs work by binding to the HIV intasome containing the integrase enzymes[151]. This prevents the strand transfer reaction and the viral protein from integrating into the genome and averting the associated DNA damage[151]. The most common reaction to Raltegravir treatment in the flies was a decrease in movement whereas an increase in movement was most common with Cabotegravir

treatment. If these two drugs work fundamentally the same, then why is there such a dichotomy in their effects? One reason drug responses may differ is due to their different protein binding capabilities. Cabotegravir and Raltegravir have protein binding capacities of over 99% and 83% respectively[152]. This means that Cabotegravir is more effective at binding to the integrase than Raltegravir. Cabotegravir also possesses a wider resistance barrier compared to Raltegravir and due to this, Cabotegravir can be used to treat patients with Raltegravir resistant HIV integrase mutants[153]. Another reason for the dichotomy could be the drug dosage. Several methods of drugging fruit flies have been developed, most of them relying on oral administration through food consumption[154]. Physiologically effective drug concentrations in Drosophila can vary greatly from 0.01 to 100 mM[154]. It could be that a higher or lower drug dosage would be more effective and limit negative side-effects. Lastly, the administration of the drugs could be altered for better absorption, particularly in the case of Cabotegravir. Oral administration of Cabotegravir is available, however this drug is primarily delivered via intramuscular injection[100]. Drug injections would permit the exact dosage received by each fly to be known rather than relying on the unlikely chance each fly consumes the same amount of drug. Cabotegravir's main benefit is its classification as a long-acting antiretroviral treatment, only needing to be injected once every 1-2 months[100]. If proven effective in clinical trials, this drug would be ideal for those living with ALS as swallowing medication is a major limitation of current ALS drug treatments.

The diagnosis of ALS is life changing. Over 80% of people don't live more than 2-5 years after diagnosis. The slow decline in motor ability including walking, talking, and eating eventually leads to death from respiratory failure. The best ALS drug currently on the market only extends life by 2-3 months[77]. This research using ERVK IN expressing Drosophila has the potential to fill a desperately needed void in the treatment of ALS. As ERVK is implicated in ALS neuropathology, determining the effect of integrase

inhibitors in an ERVK IN expressing Drosophila model is a critical step towards evaluating antivirals as a novel therapeutic strategy for the reversal of motor neuron damage and motor deficit in ALS.

6.0 Appendix

Table S1: Fly food recipe.

Ingredient	Amount
Boiled Water	760 ml
Cold Water	170 ml
Cornmeal	65 g
Yeast	13 g
Agar	6.6 g
Molasses	54.6 g
Tegosept	20 ml
Propionic Acid	5ml

Notes: As water boils, a slurry is created using cold water, cornmeal, yeast and agar. The slurry is added to the boiling water and mixed vigorously. Once a running boil is achieved, the food is removed from the heat and the molasses is added. When the temperature has cooled to 65°C the tegosept and propionic acid are added. The food is dispensed using a squeeze bottle into autoclaved vials and bottles and covered with cheesecloth to solidify.

Table S2: Primary and secondary antibodies used in Western Blot analysis.

Target	Concentration	Product Number
PRIMARY ANTIBODIES		
Mouse anti Relish	1:500	DSHB #21F3
Rat anti ELAV	1:500	DSHB #7E8A10
Rabbit anti γH2AV	1:500	NBP1 #78103
Rabbit anti PARP1	1:500	ab32138
Rabbit anti TDP-43	1:500	12892-1-AP
Rabbit anti ERVK IN-2	1:500	Custom
SECONDARY ANTIBODIES		
Goat anti Rabbit (594nm)	1:2000	ALEXA FLUOR #A11072
Goat anti Rabbit (488nm)	1:2000	ALEXA FLUOR #A11070
Goat anti Mouse (594nm)	1:2000	ALEXA FLUOR #A11020
Goat anti Rat (594nm)	1:2000	ALEXA FLUOR #A11007

Table S3: ERVK IN x D42 Transgenic Females unpaired T-tests

ERVK IN x D42		ERVK IN Expressing vs Littermate Control Females			
Treatment	**Day**	**Frequency of Movement**	**P Value**	**Total Movement**	**P Value**
Vehicle	**0**	t(18) = 1.24	**0.23**	t(18) = 1.51	**0.15**
	20	t(28) = 0.57	**0.57**	t(28) = 0.71	**0.48**
	40	t(17) = 4.02	**0.0009***	t(17) = 2.84	**0.01***
	60	t(15) = 0.79	**0.44**	t(15) = 1.39	**0.18**
		ERVK IN Expressing (Vehicle vs Drug)			
Raltegravir	**20**	t(23) = 1.28	**0.21**	t(23) = 1.92	**0.07**
	40	t(18) = 1.41	**0.18**	t(18) = 0.38	**0.71**
	60	t(18) = 3.16	**0.005***	t(18) = 1.75	**0.09**
Cabotegravir	**20**	t(18) = 0.05	**0.96**	t(18) = 0.02	**0.98**
		Littermate Control (Vehicle vs Drug)			
Raltegravir	**20**	t(22) = 0.77	**0.45**	t(22) = 0.56	**0.58**
	40	t(14) = 1.24	**0.24**	t(14) = 0.04	**0.97**
	60	t(14) = 0.19	**0.85**	t(14) = 0.30	**0.77**
Cabotegravir	**20**	t(18) = 1.29	**0.22**	t(18) = 0.29	**0.77**

*Significant result

Table S4: ERVK IN x D42 Transgenic Males unpaired T-tests

ERVK IN x D42		ERVK IN Expressing vs Littermate Control Males			
Treatment	**Day**	**Frequency of Movement**	**P Value**	**Total Movement**	**P Value**
Vehicle	**0**	t(18) = 0.91	**0.37**	t(18) = 0.43	**0.68**
	20	t(23) = 1.60	**0.12**	t(23) = 2.14	**0.04***
	40	t(17) = 2.39	**0.02***	t(17) = 1.01	**0.33**
	60	t(14) = 2.41	**0.03***	t(14) = 1.08	**0.3**
		ERVK IN Expressing (Vehicle vs Drug)			
Raltegravir	**20**	t(19) = 1.28	**0.21**	t(19) = 0.52	**0.61**
	40	t(18) = 0.11	**0.91**	t(18) = 0.13	**0.89**
	60	t(18) = 1.05	**0.31**	t(18) = 0.84	**0.41**
Cabotegravir	**20**	t(18) = 0.05	**0.96**	t(18) = 5.28	**0.0001***
		Littermate Control (Vehicle vs Drug)			
Raltegravir	**20**	t(22) = 2.11	**0.05***	t(22) = 1.32	**0.2**
	40	t(15) = 1.36	**0.19**	t(15) = 1.35	**0.19**
	60	t(10) = 2.10	**0.06**	t(10) = 0.53	**0.61**
Cabotegravir	**20**	t(16) = 2.57	**0.02***	t(16) = 2.02	**0.06**

*Significant result

Table S5: ERVK IN x ELAV Transgenic Females unpaired T-tests

ERVK IN x ELAV		ERVK IN Expressing vs Littermate Control Females			
Treatment	**Day**	**Frequency of Movement**	**P Value**	**Total Movement**	**P Value**
Vehicle	**0**	t(28) = 3.55	**0.001***	t(28) = 3.37	**0.003***
	20	t(26) = 0.52	**0.61**	t(26) = 1.12	**0.27**
	40	t(24) = 0.93	**0.36**	t(24) = 0.35	**0.73**
	60	t(21) = 0.005	**0.99**	t(21) = 0.52	**0.61**
		ERVK IN Expressing (Vehicle vs Drug)			
Raltegravir	**20**	t(17) = 0.03	**0.97**	t(17) = 0.96	**0.35**
	40	t(16) = 1.83	**0.08**	t(16) = 2.03	**0.06**
	60	t(14) = 0.89	**0.39**	t(14) = 2.16	**0.04***
Cabotegravir	**20**	t(21) = 2.39	**0.026***	t(21) = 2.94	**0.008***
		Littermate Control (Vehicle vs Drug)			
Raltegravir	**20**	t(17) = 2.31	**0.034***	t(17) = 2.45	**0.026***
	40	t(15) = 1.26	**0.23**	t(15) = 1.09	**0.29**
	60	t(16) = 0.81	**0.43**	t(16) = 1.18	**0.26**
Cabotegravir	**20**	t(22) = 0.12	**0.91**	t(22) = 0.21	**0.83**

*Significant result

Table S6: ERVK IN x ELAV Transgenic Males unpaired T-tests

ERVK IN x ELAV		ERVK IN Expressing vs Littermate Control Males			
Treatment	**Day**	**Frequency of Movement**	**P Value**	**Total Movement**	**P Value**
Vehicle	**0**	t(28) = 0.78	**0.44**	t(28) = 0.13	**0.9**
	20	t(26) = 2.09	**0.05***	t(26) = 0.55	**0.59**
	40	t(26) = 1.13	**0.27**	t(26) = 0.34	**0.74**
	60	t(24) = 0.03	**0.97**	t(24) = 0.96	**0.35**
		ERVK IN Expressing (Vehicle vs Drug)			
Raltegravir	**20**	t(17) = 0.14	**0.89**	t(17) = 1.52	**0.15**
	40	t(16) = 0.32	**0.75**	t(16) = 1.54	**0.14**
	60	t(16) = 2.09	**0.053**	t(16) = 0.48	**0.64**
Cabotegravir	**20**	t(22) = 2.39	**0.026***	t(22) = 2.66	**0.01***
		Littermate Control (Vehicle vs Drug)			
Raltegravir	**20**	t(17) = 0.18	**0.86**	t(17) = 0.64	**0.53**
	40	t(16) = 0.68	**0.5**	t(16) = 2.36	**0.03***
	60	t(16) = 1.68	**0.11**	t(16) 1.88	**0.08**
Cabotegravir	**20**	t(21) = 0.99	**0.33**	t(21) = 0.88	**0.39**

*Significant result

7.0 References

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