DISSERTATION

# AGE-DEPENDENT DECLINE IN $K_V4$ CHANNELS, UNDERLYING MOLECULAR MECHANISMS, AND POTENTIAL CONSEQUENCES FOR COORDINATED MOTOR FUNCTION

Submitted by

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# ABSTRACT

## AGE-DEPENDENT DECLINE IN $K_v4$ CHANNELS, UNDERLYING MOLECULAR MECHANISMS, AND POTENTIAL CONSEQUENCES FOR COORDINATED MOTOR FUNCTION

The voltage-gated potassium channel, $K_v4$, is widely expressed in the central nervous system and it is responsible for a highly conserved rapidly inactivating A-type $K^+$ current. $K_v4$ channels play a role in the regulation of membrane excitability, contributing to learning/memory and coordinated motor function. Indeed, recent genetic and electrophysiological studies in *Drosophila* have linked $K_v4$ A-type currents to repetitive rhythmic behaviors. Because a deterioration in locomotor performance is a hallmark of aging in all organisms, we were interested in examining the effects of age on $K_v4$/Shal channel protein.

In this dissertation, I use *Drosophila* as a model organism to characterize an age-dependent decline in $K_v4$/Shal protein levels that contributes to the decline in coordinated motor performance in aging flies. Our findings suggest that accumulation of hydrogen peroxide ($H_2O_2$) is amongst the molecular mechanisms that contribute to the age-dependent decline of $K_v4$/Shal. We show that an acute *in vivo* $H_2O_2$ exposure to young flies leads to a decline of $K_v4$/Shal protein levels, and that expression of *Catalase* in older flies results in an increase in levels of $K_v4$/Shal and improved locomotor performance. We also found that the scaffolding protein SIDL plays a role in maintaining $K_v4$/Shal protein levels and that *SIDL* mRNA declines with age, suggesting that an age-dependent loss of SIDL may also lead to $K_v4$/Shal loss. In behavioral studies, we found that a knockdown of *SIDL* resulted in a lethal phenotype, leading to a large decline in *Drosophila*

eclosion rates, an event that requires coordinated peristaltic motions. Expression of *SIDL* or *Kv4/Shal* in this *SIDL* knockdown genetic background resulted in a partial rescue; these results are consistent with a model in which SIDL and $K_v$4/Shal play a role in coordinated peristaltic motions and are required for successful eclosion.

The results presented in this dissertation provide new insight into the possible molecular mechanisms that underlie an age-dependent decline in $K_v$4/Shal protein. We identify two contributing factors: 1) ROS accumulation, and 2) the interacting protein SIDL. Our data also suggests that this age-dependent decline in $K_v$4/Shal levels is likely to be conserved across species, at least in some brain regions. Because $K_v$4/Shal channels have been implicated in the regulation of long-term potentiation and in repetitive rhythmic behaviors, the loss of $K_v$4/Shal may contribute to the age-related decline in learning/memory and motor function.

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TABLE OF CONTENTS

# CHAPTER 1. INTRODUCTION

## 1.1 Overview

As a species, the average human lifespan is 85 years of age. Life expectancy, however, has risen from the mid-40s to the mid-70s just during this past century[1,2]. Along with a rising number of individuals surpassing 65 years, there has been an associated increase in chronic brain-related ailments affecting the population[3–5]. The topic of aging is quite broad. Research areas include understanding cognitive abilities, anatomy, physiology, cellular regulation, and molecular changes with the goal of understanding the effects of aging on an organism[6–10]. During aging, there are a series of physiological changes that lead to brain-related conditions including sarcopenia, age-related dementia, Parkinson's, and Alzheimer's diseases[7,9]. Together, these age-related ailments affect locomotion, learning, and memory performances at the organismal level. These diseases are typically attributed to changes in the brain, both structural and functional[2]. Ion channels and receptors choreograph brain synaptic activity through the movement of ions. In this chapter, I provide background on the effects of age on locomotor performance and on cellular function, and the consequences of age-related intracellular oxidation. I provide background on a series of ion channels that undergo age-related effects in protein levels and modulation. I also present a historical perspective on voltage-gated potassium currents, and I introduce $K_V4$ channels, which are the proteins I examined during aging in this dissertation.

## 1.2 Age-Effects on Locomotor Performance

The age-dependent decline in locomotor performance is a phenomenon that is well-established across species[11]. Age-driven changes in the neuromuscular system have been

described to be, in part, the culprit. In humans, the nervous system experiences age-related effects that result in a decline of neuronal and motor nerve fiber densities, which contributes to a decrease in the effectiveness of neurotransmitter signaling and slower nerve conduction velocities[12]. Rhesus monkeys have shown an age-dependent decline in walking and jumping[13]. In laboratory rodents, age-dependent decline in physical activity is well-known[11]. More specifically, 2-year old mice were found to have significantly less locomotor performance than 1-year old mice in wheel running experiments[14]. In rats, a 50% reduction in locomotor performance has been measured between 6 and 32 months of age while testing exploratory activities[13]. In other laboratory rodent aging studies, the Mongolian gerbil and the deer mouse have been found to also have decreased locomotor performances in wheel-running and home-cage activity experiments, respectively[15,16]. In the invertebrate world, an age-dependent decline in locomotor movement has been observed in nematodes, houseflies, and fruit flies[17–21]. More specifically, in a comparative *Drosophila* study, researchers found that adult flies from two different populations – Congo and France – had a decline in walking speed when measured between 2 and 13 days of age[22]. This decline in motor function is likely to be triggered by a decrease in nervous system function, which can contribute to a possible loss of muscle density. Indeed, in a recent study measuring motor activity in *C. elegans*, researchers found that a progressive decline in motor neuron function contributed to the age-dependent loss of motor function even before any loss of muscle mass could be measured[23].

### 1.3 Cellular Functions Impacted by Age

Cellular DNA mutations accumulate during aging. DNA mutations in germ cells are the basis for the evolutionary process and mutations in somatic cells can lead to detrimental effects on the function of the organism. Some mutations can occur during DNA replication where errors are

accidentally made by the molecular machinery[24]. Other mutations can arise from external sources such as UV radiation and chemical exposure[25]. In general, these endogenous and exogenous factors can lead to a wide variety of DNA damages including point mutations, single- and double-strand breaks, epigenetic alterations, genomic transpositions, chromosomal aberrations, and telomere shortening, all of which can affect cellular performance[26]. The accumulation of DNA damage to the point where repair is unsustainable is, in part, a hallmark of aging[25–27].

Gene expression is affected throughout development and aging. The variations in expression display themselves in the form of phenotypic changes that occur in organisms as they reach adulthood and as they reach old age. Particularly, gene expression can increase, decrease, or stay unchanged[28]. Researchers have performed comparative analyses from cDNA libraries derived from mRNA of young and old rats to identify genes that are upregulated or downregulated throughout aging[28]. Studies in rats have shown that the amount of mRNA molecules transported from the nucleus to the cytoplasm is negatively impacted by age, probably due to decreased mRNA synthesis, a lack of proper polyadenylation of mRNA, and errors in the trafficking of mRNA from the nucleus through the nuclear pore[29–31]. Furthermore, an increase in transcriptional noise – a process that yields a heterogeneous transcriptional response across cells of the same genetic composition to the same stimulus – increases with age leading to a wider variety of transcriptional responses which might be detrimental to an organism[26,32,33]. Two highly conserved signaling pathways play an important role in aging: Insulin/IFG-1 Signaling (IIS) and target of rapamycin (TOR). These pathways are responsible for regulating gene expression through aging in response to stress and nutrient availability[34,35].

Proteostasis is a term used to describe the maintenance of properly functioning protein. Protein turnover is critical for proper proteostasis[36]. A decline in proteostasis during aging is

characterized by the emergence of protein aggregates due to a disruption in the proteostasis network which includes the machinery for translation, chaperone proteins, and the principal protein degradation systems – proteasome and lyzosome[37]. Chaperone proteins, most of them from the heat-shock family of proteins (HSP), are at the vanguard of monitoring and ensuring proper protein folding. One important role of chaperone proteins is to aid other proteins to achieve a proper functional conformation, with the goal of counteracting aggregation of nascent protein[38,39]. There is also evidence of protein refolding by chaperones when misfolding occurs[40]. When a protein has been misfolded or incorrectly modified, and cannot be refolded, it is the role of chaperones to target the non-functional protein for degradation[41,42]. Unfortunately, both the ubiquitin-proteasome and autophagy-lysosome systems experience an age-dependent decline in activity[43–45].

Age-dependent mitochondrial dysfunction has also been described. The "mitochondrial damage-energy loss" hypothesis of aging was described by Medvedev in 1990[46]. In this hypothesis, age-dependent cellular injury by reactive oxygen species (ROS) occurs on the mitochondrial membrane and DNA, and it occurs especially in neurons[47]. This would lead to a decline in properly functioning mitochondria, and a decrease in readily available ATP molecules. With an increase in age, there is a reduction in mitochondria biogenesis as muscle studies have demonstrated in comparisons between young and 50-year plus men[47]. In a rat study, the enzymatic activity of heart mitochondrial oxidase was measured and found to be significantly diminished in older subjects[48]. Analyses of primate neocortex enzymatic activity of mitochondrial complex I and IV have shown that they also decline in activity with age[49]. These studies on the enzymatic activity of mitochondrial complexes were confirmed in mice, in which the activity of complex V – ATP synthase – was also found to decrease with age[47]. The age-dependent decrease in activity of

multiple critical mitochondrial enzymes is likely what leads to its dysfunction. Furthermore, in a recent study, Takihara and coworkers (2015) used mouse retinal ganglion cells (RGCs) as a model for measuring mitochondrial axonal transport in the central nervous system (CNS)[50]. Their findings reveal that mitochondrial axonal transport decreases with age; suggesting that loss of proper mitochondrial transport in CNS might be involved in the age-dependent dysfunction of mitochondria.

### 1.4 Oxidation During Aging

#### 1.4.1 ROS effects on nucleic acids

DNA base damage was first proposed as the root of aging in 1967[51]. As an organism ages, there is an accumulation of DNA damage that is both exogenous and endogenous in origin, and that this damage is likely to interfere with transcription[52]. Internal sources of this damage include genome reorganization[53], genomic instability[24], and improper DNA repair[25]. ROS have been largely considered a principal source of general DNA damage during aging. The primary site of ROS production is the mitochondria, where ROS are a byproduct of oxidative phosphorylation, even though other sources, such as from peroxisomes and cytochrome p450 enzymes, have also been described[54–56]. Because mitochondrial DNA (mtDNA) is closest to the source of ROS, it is thought that mtDNA damage occurs at faster rates than nuclear DNA damage, leading to the age-dependent mitochondrial dysfunction which causes a decline in ATP synthesis[47,55,57,58]. This is not to say, however, that nuclear genomic DNA does not undergo any oxidative damage, which was originally proposed in 1956 by Harman and experimentally confirmed in 2004 by Hartman *et al.*[56,59]. Indeed, 30 genes involved in synaptic plasticity, ranging from ion channels to calcium-binding proteins to vesicular/protein transport[60,61], were studied during human brain aging by Lu

and coworkers (2004). Their results show that, although there is some minor damage to exons, DNA damage had a higher occurrence in the promoter region of many of the genes expressed in the prefrontal cortex after 40 years of age, and was greatest in the promoter region of all measured brain genes after 70 years of age, leading to an age-dependent decrease in mRNA levels[58] . This age-dependent decline in mRNA levels of various genes is conserved across species. In a microarray analysis of over 6,000 mouse genes, Lee and coworkers (2000) found that 10-15% of those in neocortex and cerebellum have lower mRNA levels with age[62].

Moreover, results showing that some mRNAs increase with age have been published. In a study of over 11,000 genes focusing in the hypothalamus and cerebral cortex where researchers found that there is indeed an alteration in the expression levels of many genes with an increase in age; while mRNA coding for DNA-repair related proteins decreased with age, mRNA coding for protein degradation increased with age[63]. Their results also suggest that a measured increase in mRNA coding for mitochondrial enzymes involved in ATP production in the hypothalamus leads to an increase in ROS and, therefore, a greater effect of oxidative stress on the cells. In hippocampal studies, Blalock and coworkers (2003) found, in rat microarray analyses correlating gene expression to memory-related task performance, that almost half of the measured genes decreased with age, while the other half increased with age and negatively impacted memory performance[64].

### 1.4.2 ROS effects on protein

The age-dependent increase in ROS, likely caused by a deteriorating mitochondrial electron transfer, also leads to an increase in the probability of protein oxidation. This, along with the aforementioned decline in activity of the protein degradation machinery, results in an

accumulation of oxidized protein with age. Carney and coworkers (1991) were the first to observe an accumulation of oxidized protein in the cortex of gerbils. They administered a daily dosage of the spin-trapping chemical *N-tert*-butyl-a-phenylnitrone, a short-lived free radical interactor, with the goal of decreasing the amount of oxidized protein in the brain of aged gerbils. They observed a decline in the amounts of oxidized protein along with an increase in temporal and spatial memory performance in aged animals[65]. Today, it is well known that the age-dependent increase in ROS lead to oxidation of proteins which can cause them to become dysfunctional. Typical oxidative effects involve peptide bond cleavage catalyzed by free hydroxyl radical interaction with the carbonyl carbon, amino acid residue side chain oxidative modifications such as carbonylation, and disulfide bridge interactions via cysteine sulfhydryl group oxidation, leading to protein misinteraction and aggregation[66]. The accumulation of these aggregates leads to an increase in the cellular stress response and likely degradation of the toxic structures from protein aggregates. Unfortunately, as mentioned above, both proteolytic – proteasome and lysosome – systems become dysfunctional with age[43–45].

Indeed, work published by Friguet and colleagues (2000) has shown that the age-dependent decline in proteasome activity is, in part, due to oxidative modifications[67–69]. In neuronal studies, Keller *et al*. (2000) have described how chemically increasing oxidative damage on the spinal cord of young rats triggers a decline in proteasome activity similar to that measured in normal aging rats. Their results support the idea that the decline in proteolytic activity due to ROS exposure leads to its dysfunction, and possibly also contributes to neuronal cell death[70]. In regards to the lysosome, it was almost 50 years ago that it was first described that the lysosomal membrane is quite sensitive to oxidative damage[71]. Truly, these age-dependent oxidative effects are likely to cause changes in the pH within lysosomes affecting their stability and activity[72]. Kurz and

coworkers (2008) described how many of the proteins degraded by the lysosome are iron-containing. Iron, being highly susceptible to oxidation from the highly diffusible ROS, leads to the slow formation of lipofuscin – non-degradable pigment granules which increase in volume with age. This age-dependent lysosomal saturation with lipofuscin compromises the activity of the degradative activity of the machinery[73].

### 1.4.3 Regulation of intracellular ROS levels

There are a variety of enzymes that play a critical role in the regulation of reactive oxygen species (ROS) described in the literature. Superoxide dismutase 1 & 2, and catalase participate in ROS degradation while nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) generate ROS.

Superoxide dismutase (SOD) was first described in the late 1930s as a copper-containing protein. While attempting to gain an understanding of the role of copper in erythrocytes, Mann and Keilin (1939) found that a copper-containing protein was present in blood[74]. It was not until 20 years later that Markowitz and colleagues (1959) were able to isolate and purify this copper-containing protein[75]. A decade after, McCord and Fridovich (1968, 1969) published two reports characterizing this copper-containing protein. They described that the enzyme can catalyze the dismutation superoxide anion radicals into molecular oxygen and hydrogen peroxide ($H_2O_2$), and re-named this enzyme superoxide dismutase[76,77].

Today, we know of three members that comprise the family of SOD enzymes. SOD1 is a copper and zinc containing enzyme that is found in the cytosol of cells, SOD2 is a manganese containing enzyme that is found in the mitochondria of cells, and SOD3 is a copper and zinc containing enzyme that is exclusively targeted extracellularly and which is the least studied thus

far[78,79]. Studies in *Saccharomyces cerevisiae* have shown that a mutation in either or both *SOD1/SOD2* causes a significant decrease in cell viability[80]. In *Drosophila*, a *SOD1*-null mutant (*cSOD*$^{n108}$) was observed to have impaired performance in metabolizing superoxide anion, which correlated with reduced longevity[81]. In another report, Kirby *et al*. (2002) used the daughterless-GAL4 driver to express *SOD2-RNAi*, which resulted in undetectable levels of SOD2 in immunoblot experiments using whole adult males. It was described that this successful RNA interference caused high levels of mitochondrial oxidative stress and an enhanced onset of adult fly mortality[82]. A *SOD2* missense mutant, named *SOD2*$^{bewildered}$, has also been characterized in flies that results in detrimental effects during neurodevelopment and an anomalous brain morphology, and a reduced lifespan[83]. Overexpression of both *SOD1* and *SOD2* under the control of the constitutive *actin5C* promoter also resulted in a decreased life-span[84]. In contrasting studies, the overexpression of human *SOD1* in, specifically, motor neurons lead to an increase in fly life span by 40%[85]. The results of these studies reflect the importance of this family of enzymes organismal viability and suggests that an age-dependent increase in ROS levels may have detrimental effects.

Catalase was first recognized as a ubiquitous enzyme by Loew as early as 1900[86]. The role of catalase is to dismutate harmful $H_2O_2$ into water and molecular oxygen[87]. While these enzymes' role is to reduce the levels of intracellular ROS, there are other enzymes that produce ROS as a by-product or for purposes of intracellular signaling.

The role of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) family of enzymes is to produce ROS. NOX-dependent ROS production is quite varied and still under study; though originally thought to be only damaging, ROS are just beginning to be understood as a group of highly reactive molecules that are also involved in cellular signaling. The

NOX family is comprised of at least 7 different members identified so far: NOX1-5 and DUOX1-2. Much of what is currently known comes from studies using mouse knockout models of NOX protein function where different phenotypes such as loss of balance and hypothyroidism were described[88]. More specifically, NOX2 has been described to contribute to antimicrobial activity during phagocytosis[89], while NOX3 has been shown to be essential for proper development of components in the vestibular system[90–92]. DUOX has also been described to play a role in defense from microbial defenses, but in the airway epithelium; it has been characterized as necessary for the production of hydrogen peroxide which is used for iodination of the thyroid hormone as well[88].

The expression of the various oxidases from the NOX family in a variety of tissues, allows for them to play an important role in the upkeep of the vascular system from angiogenesis to tone[93]. In neuronal *in vitro* studies, researchers have made use of *N*-acetylcysteine, a ROS scavenger, to measure the effects of a decrease of ROS on a variety of neuronal development signaling pathways. Their results suggest that ROS generated by NADPH oxidases might play a role in neuronal cell differentiation[94–96]. In a study targeting NOX directly, Nitti and colleagues (2010) used the chemical DPI to block NOX activity to demonstrate that the product of the enzyme, possibly hydrogen peroxide, is involved in neuroblastoma cell differentiation[97]. *In vivo* mouse studies have reported that ROS produced by NADPH oxidases are present in the hippocampus and their localization responds to stimulation of hippocampal slices, suggesting that the ROS produced by these enzymes plays a role in LTP induction[98]. More specifically, NOX has been recently described to act as a regulator of the cytoskeletal organization in the hippocampus, by maintaining ROS physiological levels *in vivo*; this organization is critical for maintaining neuronal polarity and proper axonal length[99]. In *Drosophila*, a recent report published by the Landgraf laboratory (2017) uses the neuromuscular junction (NMJ) of larvae as a model to understand the role of $H_2O_2$ in

neuronal structural plasticity. Their results suggest a direct relationship between ROS levels and signaling involved in synaptic terminal growth at the NMJ[100].

### 1.4.4 ROS effects at the organismal level

Physiological ROS levels must be maintained as they play an important role in learning and memory, lifespan, and locomotor performance. An excess or a scarcity of these molecules can have a detrimental effect in any of the aforementioned behavioral functions. Indeed, in their recent paper, Haddadi *et al*. (2014) measured an increase in *Drosophila* ROS with age which correlated to an age-dependent loss of memory retention in adult flies. They suggested that this increase in ROS is possibly due to a decrease in the enzymatic activity of the antioxidant enzymes catalase and superoxide dismutase[101]. In addition, *in vitro* studies in mammalian hippocampus treated with superoxide scavenging molecules or with overexpression of SOD have shown impairments in LTP induction suggesting that it is the superoxide anion that is involved in processes related to learning and memory[102–105].

In mice, one study has been published where a *Catalase* knockout mouse was generated. The researchers reported that, though this mouse has no evident health problems up to 1 year of age and shows no apparent signs of clinical acatalasemia, they are highly susceptible to oxidative tissue injury, including deficiencies in oxidative phosphorylation after brain trauma[106]. Contrasting studies in which transgenic mice overexpressed *Catalase* showed increased lifespan; researchers also reported that the onset of cardiac and visual age-related diseases were also delayed[107,108].

Earlier studies on the effects of ROS on *Drosophila* lifespan have mostly focused on the detrimental oxidative effects of ROS at the organismal level and usually go in hand with the free-radical theory of aging[109–112]. In *Drosophila*, a study isolating and characterizing *Catalase*

mutant flies reported that, when catalase activity is decreased by 97%, lifespan is significantly reduced[113]. Interestingly, though overexpression of *Catalase* confers greater resistance to oxidative stress, and showed increased lifespan in mice, increased lifespan in *Drosophila* occurs only if catalase is co-expressed with *SOD*[114–116]. Research has suggested that, in tandem with SOD enzymes, the role of catalase is to catalyze the peroxide from SOD reactions into harmless products. Moreover, when both enzymes are co-expressed in motor neurons to above wildtype levels, there is typically an increase in life-span[117,118]. Some contradicting reports, however, have been recently published. Sanz and coworkers (2010) noticed that, though an increase in mitochondrial ROS (mtROS) correlates to a decreased lifespan, it does not necessarily affect fly longevity directly. In their report, they expressed the enzyme alternative oxidase from the urochordate *Ciona intestinalis* to decrease mtROS but they were unable to measure an increase in lifespan[119]. In a similar study, Scialò *et al*. (2016) determined that *Drosophila* expression of NDI1 dehydrogenase leads to an enhanced reduction of coenzyme-Q which causes a reverse electron transport in complex I of the mitochondria, culminating in increased levels of mtROS. Their results show that increased mtROS production via complex I causes an increase in *Drosophila* lifespan[120].

ROS have also been described to influence *Drosophila* locomotor performance. Long and colleagues (2009) fed an antioxidant containing grape extract to flies to determine how ROS play a role in a *Drosophila* model for Parkinson's disease. In their report, the extract improved the loss of locomotor performance of the model, suggesting that ROS are probably involved in this neurodegenerative disease[121]. In another study, Jimenez-del-Rio and coworkers (2010) restored *Drosophila* locomotor performance, in flies with high paraquat-induced ROS levels, by administering polyphenol antioxidants[122]. In a more recent study, a genetic screen was carried out to identify candidate genes linked to oxidative stress and locomotor phenotypes. Researchers used

the molecule menadione sodium bisulfite (MSB) which, unlike paraquat or $H_2O_2$, is a milder yet persistent inducer of chronic oxidative stress. Though their goal was to identify genes, their procedure led to the confirmation that oxidative stress in *Drosophila* leads to their loss of locomotor performance[123]. Taken altogether, the age-dependent increase in ROS has detrimental effects on *Drosophila* physiology and reports suggest that administration of neuroprotectant antioxidants ameliorate the reduced locomotor performance. I propose that ROS have an effect on $K_v4$ which, in turn, may have an effect on *Drosophila* locomotion. Chapter 4 of this dissertation describes the effects of ROS on $K_v4$ protein levels and describes the involvement of the enzyme catalase on $K_v4$ protein levels and an amelioration of locomotor performance in older flies.

### 1.5. Channel Proteins Affected by Age

#### 1.5.1. AMPA and NMDA ionotropic glutamate receptors are affected with age

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) and NMDA (*N*-methyl-D-aspartate) receptors respond to pre-synaptic vesicle release of the amino acid L-glutamate by opening and allowing post-synaptic cation permeability[124,125]. Functionally, AMPA receptors typically allow for the influx of the monovalent cation $Na^+$ into the cell which causes localized depolarization. In response to this depolarization, the $Mg^{2+}$ ion blocking the channel of neighboring NMDA receptors is dislodged, permitting influx of $Ca^{2+}$ [125–127]. These two channels control the majority of mammalian neuronal excitatory transmission and have been found to decline in levels with age[58,128–133].

Interestingly, scientists initially attempted to understand the effects of age on the levels of the amino acid L-glutamate rather than the levels of receptors themselves. Reports showed controversial results. Indeed, while some papers indicated an age-dependent decline of this amino

acid by ~12-17% in different regions of Fisher 344 rats[134,135], others reported that L-glutamate does not change in level with age as measured in different brain regions of both Wistar rats and post-mortem human brain tissue[136,137]. To test the effects of age on cerebral cortex mRNA, Carpenter and coworkers (1992) injected mRNA from either 24 or 3-month old rats into *Xenopus* oocytes and measured voltage-gated currents to determine if there were any changes associated with mRNA from different aged rats. They found decreased L-glutamate induced currents in those oocytes with older mRNA, suggesting that mRNA from older rats coding for these channels might lead to lower expression of neurotransmitter receptors in the cortex[128]. In human studies, Lu and others (2004) used DNA microarray analyses on the prefrontal cortex of 30 post-mortem individuals, aged between 26 and 106 years, to compare mRNA levels of 12,000 genes; they reported that mRNA coding for the GluR1 AMPA subunit and R2A NMDA subunit both show a 2-fold decrease in subjects 40 years of age or older[58].

Studies attempting to understand the effects of age on AMPA receptors in different mammalian model systems were later published. In a recent review, Henley and Wilkinson (2013) suggest that the age-related decline in AMPA receptors could be caused by an age associated defect in receptor trafficking, and that this may have an effect on long-term potentiation (LTP) and depression (LTD) in the hippocampus, which are involved in learning and memory[138]. In rat hippocampal studies, a report showed that, though *GluR-1* mRNA levels were not affected by age, the levels of the GluR-1 subunit of AMPA receptors decline with age as measured in 24-month old animals[130]. Also in the hippocampus, but using mice as a model organism, Magnusson and Cotman (1993) used autoradiographic density analyses of AMPA receptors ligand binding and reported that levels of AMPA receptors decrease with age in BALB/c and C57B1 mice strains[139]. Bahr *et al.* (1992) reported that in the brain telencephalon of BALB/c mice there is an

age-dependent decline in GluR AMPA subunit levels when compared to mice at 3 and 25 months of age[129].

Most of what is known about the effects of age on L-glutamate-dependent ionotropic channels, however, has been studied with NMDA receptors. In rodents, early reports described an age-dependent decline in NMDA receptors in different brain regions as a reason for age-related cognitive impairments[140,141]. Magnuson and coworkers (2002) later found that both *NR1* and *NR2B* transcripts showed decreased levels in aged mice. They expanded these results by also measuring protein levels of NMDA subunits NR1, NR2A and NR2B, and found that they all had lower levels in the cerebral cortex of older C57B1/6 mice, while only NR1 and NR2B subunits were decreased in the hippocampus[131]. Other studies have shown that age has a negative effect on the levels of the obligatory NR1 and NR2B subunits of the NMDA receptors in the hippocampus which results in a decline of spatial memory abilities in older rats[142–144]. Even in the brain of primates, a decline in NMDA has been measured; Wenk and coworkers (1991) described an age-dependent decrease in NMDA levels between young (~7-year old) and aged (~30-year old) monkeys[132]. Regarding motor function, Ossowska and coworkers reported that the age-dependent decline in NMDA receptors leads to a loss in muscle tone of rat leg muscles. Their data suggest that this loss in muscle tone is a reason for the poor performance of rats in their T-maze experiments[133].

### 1.5.2. Effects of age on $GABA_A$ ionotropic receptors

In the developed brain, γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter[145]. This neurotransmitter acts on GABA receptors, $GABA_A$ and $GABA_B$. Here, I describe the known effects of age on $GABA_A$ ionotropic receptor levels. Originally, measurements of $GABA_A$ receptor levels have been contradicting. While some studies reported a decline in

detectable $GABA_A$ in different brain regions of aged rats, others reported that $GABA_A$ remained unchanged in similar or other regions of the rat brain during aging[132,146–148]. Soon after, transcriptional studies in Fisher rats revealed that $GABA_A$ receptor subunit mRNA levels decreased by 70% in the cerebral cortex when comparing 6 month and 24-month old animals[149]. This report led Gutiérrez and coworkers (1994) to perform further testing on mRNA and protein levels of different subunits of the $GABA_A$ receptors. They reported that in the inferior colliculus of Sprague-Dawley and Fisher 344 rat brain both mRNA and protein levels of $\beta_2$, $\beta_3$, $\gamma_{2S}$, $\gamma_{2L}$, and $\alpha_1$ subunits declined with age[150]. Further testing in the same rats revealed no mRNA changes in the cerebral cortex [151], and that, though there are no changes in protein expression levels of $\alpha_1$, $\beta_2$, or $\beta_3$ subunits, mRNA levels coding for these three subunits decrease with age in the cerebral cortex[152].

### 1.5.3. Decline in $Ca^{2+}$-activated $K^+$ channels during aging in myocytes

In the heart, $Ca^{2+}$-activated $K^+$ channels play a critical role in regulating membrane potential and regulating muscle contractility by intracellular free $Ca^{2+}$ [153]. Although, not much has been done to understand the effects of age on these channels in myocytes, Marijic *et al*. (2001) reported that these channels, in both Fisher 344 rats and humans, decreases with age in coronary smooth muscle which could be an explanation for some heart problems in the aging population[154]. Another group, using 2 year old Fisher 344 rats, reported that low-intensity exercise training can partially reinstate the levels of $Ca^{2+}$-activated $K^+$ channels[155].

## 1.6. Voltage-Gated Potassium Channels Known To Be Affected By Age

### 1.6.1. Historical perspective on voltage-gated $K^+$ currents

Voltage-dependent $K^+$ currents were first described by Hodgkin and Huxley in 1952 in *Loligo* giant axons. The purpose of these delayed-rectifier $K^+$ currents is to restore the membrane to resting potential so the cell is able to propagate another action potential[156]. Almost a decade later, Hagiwara and coworkers (1961) were the first group to define a third conductance during action potentials; they described this conductance as very transient, hyperpolarizing, and not a part of delayed membrane rectification, an event that lasts much longer[157]. This third conductance was later characterized first by Connor and Stevens (1971) in marine gastropod *Anisodoris*' neuronal cell bodies and named the current A-type ($I_A$). They described the activation potential of this transient outward current to occur when the membrane potential changes from its resting state of -70mV to the range of -35 to -50 mV at lower temperatures[158]. This current was measured in cell bodies of snail neurons and recorded to lasts ~200-400 milliseconds[159]. Pharmacologically, a hallmark characteristic of transiently activating potassium channels is that they are sensitive to 4-aminopyridine (4-AP) and generally unresponsive to tetraethylammonium (TEA), unlike other potassium channels[160,161].

The first $K^+$ gene identified was from a mutant fly that exhibited a leg shaking phenotype under mild ether anesthesia, and was named *Drosophila* $K_v1$/Shaker[162–164]. Three research groups reported the cloning of this channel in *Drosophila*[165–167] and, soon after, Tempel *et al*. (1988) successfully cloned the first mammalian version[168]. These discoveries led to a series of other studies that resulted in the identification of three more *Drosophila Shaker*-like $K_v$ genes: *$K_v$2/Shab*, *$K_v$3/Shaw*, and *$K_v$4/Shal*[169–171]. The series of cloning studies in mammals and other systems revealed that, these genes, each represented a distinct family of ion channels conserved across

species[172–177]. Today there are 12 known subfamilies of $K_v$ channels: $K_v$1-12[178–189]. In this dissertation, I focus on $K_v$4 channels.

### 1.6.2. Structural characteristics of $K_v$ channels

Voltage-gated potassium channels ($K_v$) are the largest family of all ion channels and are coded by 40 genes in humans[182,189,190]. Phylogenetically, these families are subdivided into four major family groups: a) $K_v$1-$K_v$4, b) $K_v$5, $K_v$6, $K_v$8, $K_v$9, c) $K_v$7, d) $K_v$10-$K_v$12[189]. The functional $K_v$ channel is assembled in the ER membrane in which four nascent α-subunits of the same family form a tetramer by interacting via an N-terminal T1 tetramerization domain of ~130 amino acids[191–197]. Below, I focus on the structural characteristics of the channels $K_v$1-$K_v$4 family of channels.

Originally, in an attempt to describe the secondary and tertiary protein structure of the opening of voltage-dependent channels, Guy and Seetharamulu (1986) performed computational models to identify what today is known as the S5-S6 linker and the P-loop[198], Figure 1.1. With these modeling results, scientists focused on mutational analyses of the pore region in an attempt to characterize the P-loop of voltage-gated $K^+$ channels. MacKinnon and Miller (1989) published the first report, in which the pore-blocker peptide inhibitor charybdotoxin (CTX) from the scorpion *Leiurus quinquestriatus* was used to determine if the amino acid glutamate at position 422 was near the conduction pathway of $K_v$1[199]. Soon after, MacKinnon and Yellen (1990), by the use of CTX and the open-channel interactor chemical TEA, reported that residues 431 and 449 were likely involved in the ion permeation properties of $K_v$1, as previously proposed by the earlier computer model from Guy and Seetharamulu (1986)[198,200]. MacKinnon and coworkers (1990) then further characterized this region and identified other amino acid residues that altered toxin

interaction to determine that the region connecting the S5-S6 linker also matched the proposed computational model representing the pore region[201]. Yool and Schwarz (1991), performed additional site-directed mutagenesis and functional studies in this showing that, indeed, this region allows for passage of $K^+$ ions[202]. To confirm this, Hartmann and colleagues (1991) then created a chimera, in which the 21-amino acid pore region was transplanted into another potassium channel with different conductance and TEA sensitivity. Their results showed that this 21-amino acid replacement gave their channel the higher conductance and higher affinity to TEA, indicating that this 21-amino acid span controls the biophysical properties of the pore in these *Shaker*-like channels[203]. Further experiments by Yellen and colleagues (1991), using site-directed mutagenesis and internal TEA blocking, revealed that residues 431-449 are part of a reentrant loop between the S5-P-loop-S6 region[204,205]. Work by Heginbotham and coworkers (1992, 1994) later showed that the highly conserved 19-amino acid stretch is critical for the high $K^+$ selectivity of these channels[206,207]. All these discoveries were subsequently confirmed by crystallographic evidence (see below).

Today, we know that the general structure of a single α-subunit of the *Shaker*-like $K_v1$-$K_v4$ channels have both hydrophilic N- and C- termini located in the intracellular space. These termini flank the core region – an area of the α-subunit that has about 40% identity between $K_v1$-$K_v4$, which is composed of 6 α-helical transmembrane segments (S1-S6) with a potassium-selective pore (P) between S5 and S6[182,191,208–210], Figure 1.1. The tertiary structure of the selectivity filter has been confirmed through studies on KcsA, a prokaryotic potassium channel that has a similar amino acid sequence to the S5-P-loop-S6 region of *Shaker*-like subfamilies of channels[211,212]. These prokaryotic channels have been described to have a high structural similarity to the eukaryotic version, suggesting that the potassium selectivity pore structure and function are quite

conserved[213–215]. When closed, the structure of this pore forms a funnel-like structure with a diameter of 12 Å in the outer area constricting to 4 Å, the equivalent of a van der Waals interacting distance between two interacting atoms, at the most inner region of the pore. When open, allosteric interactions between the α-helices increase the inner region diameter to 12 Å[215]. The amino acid sequence of the selectivity filter is Gly-Tyr-Gly, a sequence highly conserved amongst $K_v$ channels[189,206,216–219]. Four, linearly arranged, coordinating interactions occur in the selectivity filter with the carbonyl oxygens of the pore side chain; here, $K^+$ enter in an alternating fashion with water molecules. More specifically, Zhou and coworkers (2001) were able to use X-ray crystallography to show that at lower concentrations of $K^+$, the conformation of the selectivity filter appears to be in a closed state with ions being absent at positions 2 and 3[218], Figure 1.2. The coordination chemistry of these carbonyl oxygens at the pore mimics that of the hydration shell of $K^+$ in solution, allowing for $K^+$ to diffuse freely through the pore. The coordination chemistry with $Na^+$ is not the same with the constrictions of the pore, making the selectivity for $K^+$ 1000-fold higher[220].

In $K_v$ channels, the pore opens in response to a membrane voltage change. The S4 transmembrane domain was first identified as the key voltage sensor of *Shaker*-like $K_v$ channels, and the movement of this domain led to conformational changes that opened the pore. The original reasoning was that this domain contained basic residues such as lysine and arginine at every third position, with non-polar amino acids in-between, that provide it with an overall positive charge allowing it to detect changes in membrane potential that are below the action potential threshold[208,219,221–224]. The structure of S4 was found to be highly conserved across all voltage-gated channels in different species and it was a collection of studies in different types of voltage-gated ion channels that led to the understanding of the membrane potential detecting

capabilities of this domain. Stühmer and coworkers (1989) were the first to investigate the role of these positive charges in membrane potential sensing by experimenting with rat voltage-gated sodium channels in *Xenopus* oocytes; by replacing the positively charged amino acids in S4 by neutral or negatively charged ones, they were able to modify the activation potential of a voltage-gated sodium channel. Their results showed that the removal of positive charges results in a decrease of the slope for voltage-dependent activation[225]. Surprisingly, a similar study by Auld *et al*. (1990) demonstrated that the positively charged amino acids were not the only ones responsible for the selective gating of the channel. Indeed, by mutating the non-polar amino acid leucine, at position 860, they measured a shift in activation of the channel to more positive potentials[226]. Soon after, analogous experiments were performed on *$K_v$1/Shaker* channels in *Drosophila*[227,228], and mammalian $K_v$1.1 channels[229]. These results confirmed that the highly conserved S4 domain has voltage sensing capabilities and that modifications to polar or non-polar amino acids lead to shifts in the voltage-sensing and gating of voltage-gated ion channels.

Later, it was found that the transmembrane domain S2 was also involved in the voltage-sensing capabilities through an acidic residue, in contrast to the basic amino acids from S4; this presented the idea that negatively charged residues interacting with S4 could also play a role in voltage sensing[230–232]. Indeed, the work of Li-Smerin and coworkers (1998) showed that a tarantula toxin consistently interacts with S2-S4 regions of different voltage-gated ion channels which resulted in changes on their voltage-gating properties. Moreover, Lu and colleagues (2001) created a chimera by fusing *Shaker* S1-S4 to KcsA which resulted in KcsA gaining voltage-sensing capabilities. Altogether, these studies suggested that S1-S4 might be involved in the voltage-sensing. Although S4 performs most of the voltage-sensing, S1-S4 seems to be a voltage-sensing module[224,231–235]. Campos and coworkers (2007) used a mutagenesis approach to uncover a

relationship between the S1-S4 and the S2-S4 domains. They replaced three isoleucine residues with cysteine (I241C, I287C and R362C) to allow the formation of disulfide bridges between I241C or I287C with the S4 residue R362C which constrained the closed-state position of the S4 segment; their data suggests that there are stabilizing hydrophobic interactions given by I241 and I287 in the channel's closed state[236]. When the module detects a change of +10 mM, the voltage-sensor undergoes conformational changes which results in the movement of the S6 domain, that possesses the conserved Pro-Val-Pro sequence allowing for segment mobility, leading to the opening of the pore[231,235,237–242].

*Shaker*-like $K_v$ channels are responsible for a wide variety of currents which include currents that are rapidly inactivating, non-inactivating, and slowly inactivating[171]. These A-type currents have since been described to play a physiological role in various types both non-excitable, such as epithelia, and excitable cells[182,219,243–251].

Fast inactivation of A-type $K_v$ channels is critical for modulating action potential firing in neurons. There are multiple of inactivation that have been described for *Shaker*-like channels[252–255]. The N-type, or ball-and-chain, inactivation occurs in the millisecond scale via a domain that interacts with the intracellular portion of the open pore[256–258]. This amino acid sequence may be present in the N-terminal domain of the channel or in an interacting β-subunit[259–261]. Both positive electrostatic charges and van der Waals contacts have been described to play a direct role in the interaction of this blocking amino acid sequence and the open pore blocking $K^+$ conduction[262,263]. C-type inactivation is generally a slower process that likely involves a structural change culminating in the pinching of the pore and is independent from N-type inactivation; the exact mechanisms that lead to C-type inactivation are still not fully understood[252,253,255,264,265]. The overall mode of inactivation has been proposed to involve allosteric mechanisms that couple both

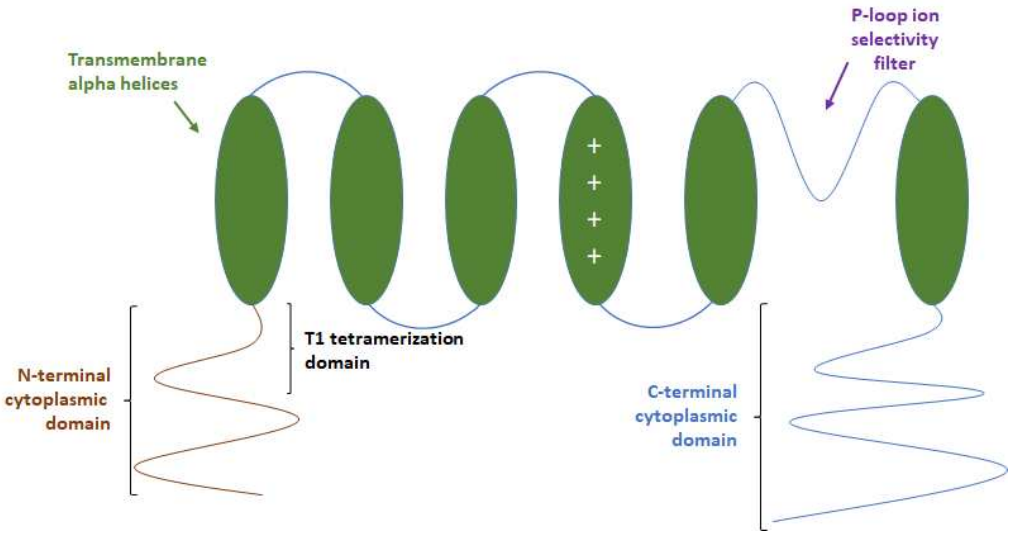


**Figure 1.1. Structure of $K_v$ channels.** There are six transmembrane domains (S1-6, green)) area flanked by both cytosolic N- and C-termini (red and blue, respectively). The P-loop ion selectivity filter is located between S5 and S6 (purple). The T1 tetramerization domain is situated in the N-terminal end of the polypeptide. S4 contains positively charged residues giving it the voltage-sensing capabilities.

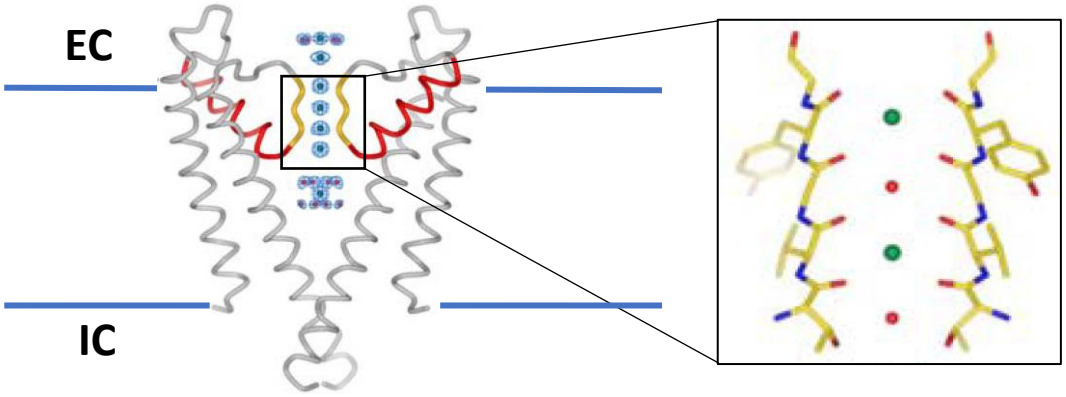


**Figure 1.2. $K^+$ pore and selectivity filter.** *Left*, homologous domain of KcsA representing the equivalent S5-P-S6 motif from two of the four interacting α-subunits is shown. Pore helices are in red and selectivity filter in yellow. Circles in blue mesh represent the electron density of ions entering through the filter. EC and IC are extracellular and intracellular domains, respectively. *Right*, enlarged section of the selectivity filter shows alternating $K^+$ (green, positions 1 and 3) and $H_2O$ (red, positions 2 and 4) ions as they enter the cell when the channel is open (adapted from MacKinnon 2003 with permission from John Wiley and Sons, License 4490440979830)[220].

N- and C-type inactivation processes. This was first noticed when Hoshi and coworkers found that a domain in the N-terminal of *Shaker* interacted directly with the open channel[260]. Along with this idea, Baukrowitz and Yellen have also described how N-type inactivation positively modulates C-type inactivation[266].

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### 1.6.3. Age-related decline in $K_v$1/Shaker levels

$K_v$1 is a voltage-gated potassium channel that is highly conserved across species with 70% identity between *Drosophila* and mice[267]. While in mammals there are at least twelve variants of $K_v$1 genes identified, there is only 1 representative of the $K_v$1 family in *Drosophila*[178,267] and it is found in both muscles and neurons[268,269]. In mammals, variants $K_v$1.1, $K_v$1.2, and $K_v$1.5 have been reported to play a role in myogenic control[270,271], while variants $K_v$1.4 and also $K_v$1.2 have been described to specifically localize to axons and nerve terminals[221]. The axonal localization of $K_v$1 in CNS is important for its function, where it likely plays a role in regulating neurotransmitter release[221].

The first publication reporting any effects of age on $K_v$1 channels surfaced in 2001. In this report, age was described to affect expression $K_v$1.1 and $K_v$1.2 in rat cerebellum. Levels of these two proteins were found to be increased in the cell bodies of cerebellar output neurons in ~20-months old rats when compared to ~5-month old rats[272], suggesting that changes in cerebellum function occur with age. These results were interesting as age-dependent effects on the functional activation and morphology have been reported, more recently in humans, to lead to a decline in locomotor performance[273–275]. Hearing loss is another ailment that is prevalent in the aging population and $K_v$1 has been described to undergo changes in the cochlear nuclei of rats. Specifically, $K_v$1.1 protein was found to be enriched with age, as it was previously described when studies were carried in the cerebellum[272,276]. In cardiac studies, mRNA and protein experiments

show that changes in $K_v1$ levels are not constant. Indeed, while transcriptional studies show that *$K_v1.4$* increases significantly with age[277], immunoblot analyses of ventricular tissue revealed that the levels of $K_v1.2$ decrease significantly in 2-year old mice when compared to 3-month old mice[278]. More recently, a study in rats reported that *$K_v1.3$* mRNA levels undergo an age-dependent increase. This transcriptional increase led to an upregulation of $K_v1.3$ protein levels which correlated to the measured age-dependent spontaneous increase in rat hypertension[279].

### 1.6.4. Age-related decline in $K_v2$/Shab levels

While there is only one representative of the $K_v2$/Shab family of channels in *Drosophila*, there are two known members in mammals, $K_v2.1$ and $K_v2.2$[267,280,281]. In mouse cardiomyocytes, $K_v2.1$ localization and mobility has been reported to be responsible for the slow potassium currents responsible for regulating the QT interval[282,283]. In neurons, though $K_v2$ channels were originally thought to only localize to the soma and proximal dendrites[284–286], reports have described $K_v2$ to also localize to the axon initial segment of cortical and hippocampal pyramidal neurons[287]. These channels are responsible for the slowly inactivating current in *Drosophila* embryonic neurons[171] and the delayed-rectifier voltage-gated potassium currents in mammalian cortical and hippocampal pyramidal neurons[281]. $K_v2$/Shab also plays a role in regulating myogenic response in cerebral arteries[288]. $K_v2.1$ has been reported to be present at high levels in mammalian central neurons and a major contributor to the delayed-rectifier potassium currents responsible for regulating action potential firing frequency and backpropagation of action potentials[287,289–292]. Because of this, it has been proposed that, at least in CA1 hippocampal neurons, the $K_v2.1$ localization in proximal dendrites works as a resistor and has the function of depressing neuronal intrinsic excitability[281]

Mammalian $K_v2.1$ channels have been described to be modified by the age-related increase in oxidant species. Indeed, Sesti and coworkers have published a series of papers over the past six years describing how the age-dependent increase in ROS lead to oxidation of the $K_v2.1$ channel which results in neurodegenerative diseases[293–296]. In transgenic mouse models of Alzheimer's disease, a neuropathy condition partially characterized by the age-dependent increased levels of oxidative stress, $K_v2.1$ was found to have high levels of oxidative damage which resulted in its aggregation, initiating apoptosis[296]. Interestingly, they also described that mutating a highly conserved oxidation-prone cysteine to alanine lead to neuroprotection in a mouse Alzheimer's model and also in *C. elegans* expressing the same mutation in their $K_v2.1$ homologue in presence of $A\beta_{1\text{-}42}$ [295]. When studying the oxidation-dependent importance of this highly conserved cysteine residue, Sesti and colleagues found that its oxidation caused oligomerization of $K_v2.1$ subunits via disulfide bridges formed by these cysteines. They suggested that this sulfhydryl-dependent $K_v2.1$ subunit interaction caused a decline in $K_v2.1$ internalization resulting in $K_v2.1$ membrane accumulation; these aggregates disrupted the neuronal lipid raft membrane structure which led to cell apoptosis and a decline in brain neuronal density[296]. Traumatic brain injuries lead to a significant increase in ROS levels in the affected area. Sesti and Coworkers (2016) showed that mice expressing the oxidation-resistant $K_v2.1$ cysteine-to-alanine mutation resulted in mice with better locomotor performance than those expressing wildtype $K_v2.1$[294]. Altogether, their reports suggest that there is an age-dependent oxidative disruption of $K_v2.1$ channel homeostasis which can contribute to neuropathies such as Alzheimer's disease.

### 1.6.5. Age-related decline in $K_v$3/Shaw levels

Four members make up the mammalian voltage-gated potassium family of $K_v$3/Shaw channels: $K_v$3.1, $K_v$3.2, $K_v$3.3, and $K_v$3.4[267,297,298]. In *Drosophila*, there is one representative of the $K_v$3/Shaw family of channels which has 55% identity with its mammalian counterpart[267]. In *Drosophila*, $K_v$3/Shaw channels have been described to have low voltage sensitivity and single channels open for a relatively short periods of time in embryonic neurons[171]. In mammals, $K_v$3/Shaw subunits localize mostly to the central nervous system, though its presence in skeletal muscle was also described, and it is responsible for enabling neurons to fire action potentials at high frequency[298,299].

Some reports have described opposing effects of age on $K_v$3 channel levels in different areas relating to the auditory system. *In situ* hybridization studies have revealed that *$K_v$3.1* channel mRNA levels increase during the development granule cells in the cerebellum[300], an area of the brain which receives input from the auditory system[301]. On the other hand, Jung and colleagues (2005) reported that $K_v$3.1 channel levels underwent an age-dependent decline in the posterior ventral cochlear nucleus of the rat auditory nuclei; they suggested that, because $K_v$3.1 levels decrease in the cochlea, this could be a reason to the age-dependent deterioration of hearing[276]. Moreover, the b-subtype $K_v$3.1 ($K_v$3.1b) was described to undergo an age dependent decline in the mouse medial olivocochlear feedback system, a component of the auditory system[302].

In an Alzheimer's related study, Boda and colleagues (2012) reported that, during aging, the levels of *$K_v$3* transcript were unchanged in several different brain regions of mice – olfactory bulb, septum, neocortex, hippocampus, brainstem and cerebellum – with the exception of $K_v$3.1 and $K_v$3.4. They reported that, while $K_v$3.1 increased in the olfactory bulb, $K_v$3.4 decreased in the septum and neocortex. Interestingly, when they measured levels of *$K_v$3* mRNA in their

Alzheimer's mouse model, their results showed that transcript and protein levels for *$K_v$3.1* decreased significantly by 12 months of age in the neocortex and the hippocampus, suggesting that this age-related disease leads to a decline in $K_v$3.1 levels which causes a neuronal impairment in repetitive action potential firing[303].

### 1.6.6. Age-related effects on KCNQ/$K_v$7-type Channels

The KCNQ/$K_v$7 family of voltage-gated potassium channels is composed of five members – $K_v$7.1, $K_v$7.2, $K_v$7.3, $K_v$7.4, and $K_v$7.5. While $K_v$7.1 is mostly present in cardiac myocytes, $K_v$7.2-$K_v$7.5 are primarily expressed in neuronal cells. $K_v$7 channels have been described to co-localize with sodium channels in the axon initial segment and at nodes of Ranvier to regulate action potential threshold, hence dampening neuronal excitability[304,305]. Its dysfunction has been reported to generate a variety of diseases including short and long QT syndrome, familial atrial fibrillation, benign familial neonatal seizures and autosomal dominant type 2 deafness[305].

Age-related dysfunctions of $K_v$7 channels have been described to be associated with some of the diseases mentioned above. Okada and coworkers (2003) investigated the connection between hippocampal $K_v$7 channels and benign familial neonatal convulsions in a rat model. They found that, within the first seven days of life, $K_v$7 channels strongly regulate action potential firing in rat hippocampal CA1 regions, while in mature neurons $K_v$7 channels were excluded from this role, likely through a decline in $K_v$7 current density[306]. However, in other studies, researchers reported an increase in *$K_v$7* mRNA levels during the first week of life[307,308]. No reports, however, on protein levels of $K_v$7 in this system are available to help in determining how age impacts this channel. Ocorr and colleagues (2007) performed an analysis of the *Drosophila* $K_v$7 channel in flies with the goal of understanding its role in the age-dependent arrhythmias of fly hearts. Their results

show that heart $K_v7$ mRNA levels decrease with age which correlated to the age-related increase in *Drosophila* arrhythmias. When they studied a $K_v7$ mutant fly, young flies displayed an early onset of arrhythmia, suggesting that the $K_v7$ mediated $K^+$ current influences fly heart repolarization[309]. In auditory studies, Lv *et al*. (2010) performed electrophysiological studies in 2-week and 17-month old mice to determine the differences in $K_v7$-mediated currents in cochlear spiral ganglion neurons (SGN). They found that the $K^+$ current contribution by $K_v7$ was higher in 17-month old mice when compared to 2-week old ones in both apical and basal SGN[310]. Their results suggest that, with increasing age, $K_v7$ dependent currents required for proper function of the cochlear SGN play an increasingly more important role. In memory-related studies, Cavaliere *et al.* (2013) reported that *Drosophila* $K_v7$ mRNA levels undergo a progressive age-dependent decline which correlates to a decline in short term memory abilities. They also tested short term memory in $K_v7$-null flies and found that their short-term memory abilities were eradicated. Their data suggests that the age-dependent decline in $K_v7$ channels plays an important role in the age-dependent memory impairment measured in flies[311]. Altogether, these studies show various age-related effects on $K_v7$ mRNA levels. Though levels of $K_v7$ mRNA increase or decrease in different organ systems with age, these reports illustrate the importance of maintaining proper ion channel levels in different cell types.

### 1.7. $K_v4$/Shal Channels

#### 1.7.1. $K_v4$/Shal expression

$K_v4$/Shal is a voltage-gated potassium ion channel whose sequence and function is conserved from jellyfish to humans[312,313]. Soon after the first cloning of these channels in *Drosophila* in 1990[173], the mammalian versions were also cloned: $K_v4.1$[312], $K_v4.2$[174,177], and

*$K_v4.3$*[314]. Since then, general studies on $K_v4$ channels have focused on its role in cellular excitability in the heart[315–321], smooth muscle[322–325] and lungs[326,327], as well as in mammalian and *Drosophila* neurons[171,191,221,267,328–331]. The mammalian $K_v4$ family of channels shares about 80% identity with *Drosophila* *$K_v4$* (*Shal*), the only representative of $K_v4$ channels in fruit flies[173,174,191,267,312,332]. In the fly, the *Shal* gene contains two splice variants, *Shal1* and *Shal2*, where the latter has a shorter C-terminus[173].

Much of the work performed characterizing expression of $K_v4$ channels in neurons has been performed in mammalian systems, and most neuron-related expression studies have focused on $K_v4.2$ and $K_v4.3$ channels which show strong expression levels in the mammalian brain[300,330,333–340]. In 1998, Serôdio and Rudy (1998) reported a thorough examination of *$K_v4.1$, $K_v4.2$, $K_v4.3$* mRNA levels and localization in the rat brain. *$K_v4.1$* was found to have very low levels of transcript and protein expression in the central nervous system (CNS). Interestingly, when comparing expression of *$K_v4.2$* and *$K_v4.3$*, they seem to have differing expression patterns in separate areas of the brain. *$K_v4.2$* was found to be present at high levels in the granule cells of the olfactory bulb, in most of the basal ganglia, CA1 pyramidal and granule cells of the hippocampus, the paraventricular nucleus of the thalamus, the granular cell layer of the cerebellum, and in the pontine nucleus of the brain stem. *$K_v4.3$* was measured to have high levels of expression in the neocortex, in stratum interneurons and granule cells of the hippocampus, in the ventroposterior complex and laterodorsal nuclei of the thalamus, in Purkinje cells of the cerebellum, and in the substantia nigra and superior colliculi of the brain stem[330]. In *Drosophila*, Tsunoda and Salkoff (1995) determined that $K_v4$/Shal currents made up virtually all of the A-type currents present in the soma of embryonic neurons[171].

### 1.7.2. $K_v$4/Shal neuronal subcellular localization and trafficking

The somato-dendritic localization of $K_v$4 was first described over 25 years ago in the rat hippocampus[338]. Early immunohistochemical experiments showed that $K_v$4.2 localizes in the granule cell layer, specifically in the soma of these cells in the cerebellum, while being absent in the axons. In these same studies, $K_v$4.2 was found to have high immunoreactivity in the soma of CA1 and CA3 pyramidal cells of the hippocampus while absent in axonal regions. These results supported the idea that the $K_v$4 A-type currents play a role in the regulation of post-synaptic membrane excitability[338]. Maletic-Savatic and coworkers (1995) extended these results by determining that $K_v$4.2 channels localize, not only to the soma, but also to distal dendrites of CA1 and CA3 pyramidal neurons[341]. Hoffman *et al*. (1997) later reported that in the soma and apical dendrites of rat CA1 hippocampal pyramidal cells there is a high density of transient outward currents that have fast activation and inactivation components. Single channel data revealed that their current reversed near the equilibrium potential of $K^+$, supporting the theory that $K_v$4 channel somato-dendritic localization has a critical role in the regulation of dendritic excitability[342]. Alonso and Widmer (1997) used immunoelectron microscopy and immunohistochemistry to elucidate the exact localization of $K_v$4.2 channels in the CNS. They were the first to report that $K_v$4.2 localization involves the clustering of the channel on post-synaptic membranes[343]. In *Drosophila*, Tsunoda and Salkoff (1995) genetically identified $K_v$4/Shal A-type currents in embryonic neurons which suggested that somatic localization of this channel was conserved on the fly[171]. In later studies, Diao *et al.* (2010) drove expression of a GFP-tagged $K_v$4/Shal protein (GFP-$K_v$4/Shal) in projection neurons of adult fly antennal lobe to examine the compartmentalization of $K_v$4/Shal. GFP-$K_v$4/Shal was found, not only to consistently localize to somato-dendritic compartments, but also to be present in the proximal segment of the antennal lobe inner antennocerebral tract axons,

suggesting that $K_v$4/Shal compartmentalized localization likely includes the axon initial segment[344]. In a recent publication using the same GFP-$K_v$4/Shal expressing fly, Jegla and colleagues (2016) showed that GFP-$K_v$4/Shal signal was also enriched in the axon initial segment (AIS)[345], supporting the idea that the subcellular neuronal localization of $K_v$4/Shal includes the proximal axon near the soma.

Amongst different types of conserved amino acid motifs used by neurons to maintain a polarized distribution, diverse forms of acidic di-leucine motifs have been reported to play a role in the sorting of ion channels towards compartmentalized targeting within these cells[346]. These motifs have been described as sufficient for basolateral membrane localization in epithelial cells lining the intestine[347] , for neuronal axonal targeting[348], and for neuronal somato-dendritic targeting[349]. The somato-dendritic targeting of $K_v$4 channels is mediated by a C-terminal di-leucine motif that is highly conserved from *C. elegans* to humans[221,349,350]. A comparison between the sequences of rat $K_v$4.2 and lobster $K_v$4 exposed this C-terminal conserved di-leucine motif. In the rat, the 16 amino acid sequence corresponds to residues 474-489 of the $K_v$4.2 polypeptide. While 14 residues are conserved in lobster, 13 are conserved in all the known $K_v$4 proteins in both vertebrates and invertebrates[349,350], Figure 1.3. Rivera and coworkers (2003) used a series of chimeric proteins containing the C-terminal $K_v$4 to identify the 16 amino-acid di-leucine motif that results in dendritic targeting[349]. They transfected these chimeric proteins into rat cortical slices and measured content of their proteins in dendrites and axons. Their results showed that this 16 amino-acid di-leucine motif is necessary and sufficient for dendritic targeting of $K_v$4.2. They extended their results by testing chimeras of axonal channels containing the C-terminal of $K_v$4.2 and found that these channels now localized to dendrites. Even adding the C-terminal of $K_v$4.2 to

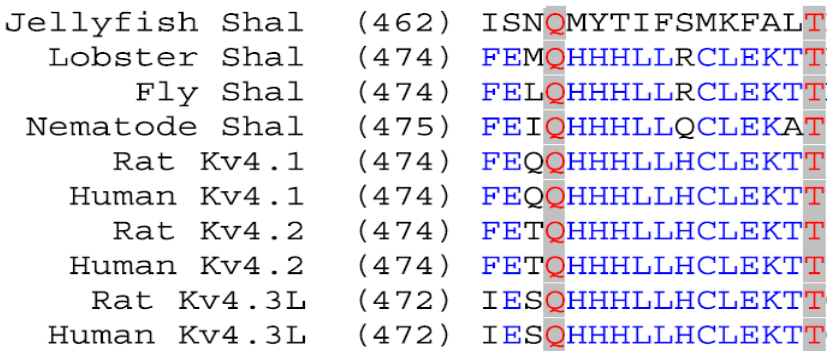


**Figure 1.3. The 16 amino acid di-leucine motif is conserved across species.** Protein sequence alignment of the C-terminal tail of $K_v4$ from various species uncovers a conserved 16 amino acid sequence centered between 2 leucine residues. In parentheses, the residue number of the polypeptide representing the first amino acid of the beginning of the 16 amino acid di-leucine motif. In red with grey background, amino acids that are identical. In blue, partial amino acid similarity across the examined species (adapted from Jerng *et al*. 2004 with permission from Elsevier, License 4490441153653)[221].

the transmembrane protein CD8 lead to its dendritic localization while deletion of the motif, or mutating the leucine to the smaller alanine amino acids, lead to a ubiquitous localization[349].

While looking for an interacting protein that may interact with this highly conserved di-leucine motif of $K_v4$, Chu *et al*. (2006) observed that the kinesin isoform Kif17 motor protein interacts with the C-terminus of $K_v4$ and contributes to the dendritic localization of the ion channel[351]. They used a system in which they replaced the motor domain of several kinesin isoforms with GFP or YFP to create a dominant negative version of the motor protein isoforms. Specifically, they looked at dominant negatives of Kif17 and Kif5B since these had been previously described as motor proteins responsible for the trafficking of dendritic ion channels NMDA receptor subunit NR2B and AMPA receptor subunit GluR2[352,353]. Chu and coworkers found that $K_v4.2$ and Kif17 co-immunoprecipitated and that they co-localized in dissociated cortical cultures. Their results indicate that Kif17 interacts either directly or indirectly with the outermost region of the C-terminus, but not with the di-leucine motif[351].

It was not until 2010 that, in *Drosophila*, a protein that interacts with the highly conserved 16 amino-acid di-leucine motif was discovered. Diao *et al.* (2010) used the entire 166 amino acid C-terminal tail of *$K_v$4/Shal*, which includes the highly conserved di-leucine motif as "bait", and a cDNA library to screen the interacting "prey". From this experiment, SIDL (Shal/$K_v$4 Interactor of Di-Leucine) was identified. *SIDL* is a 4.1 kb gene that is located on the 3rd chromosome of *Drosophila* and its expression produces a ~130KDa protein. This same yeast-two-hybrid system was also employed to expand on their findings by demonstrating that SIDL can interact with both C-termini of $K_v$4/Shal1 and $K_v$4/Shal2, with the di-leucine motif alone, and even with the mouse version $K_v$4.2. When the di-leucine motif was removed from the C-terminal tail, SIDL did not interact. They also performed GST-pull-down assays on various constructs, expressed and purified from BL21 cells, to validate this interaction. Through this approach, they confirmed that the C-terminal tail of SIDL interacts with the C-terminal tail of $K_v$4/Shal1 and $K_v$4/Shal2, but not with a C-terminal tail of $K_v$4/Shal2 containing a partial deletion of the di-leucine motif. They also performed the reverse experiment, this time using *Drosophila* embryo membrane extract, and showed that the C-terminal of SIDL pulls-down $K_v$4/Shal protein. Tagged-SIDL was also found to co-localize with GFP-$K_v$4/Shal in *Drosophila* primary neuronal cultures. In their report, they confirmed that removal of the di-leucine motif from $K_v$4/Shal lead to mislocalization of the dendritic channel in a subset of neurons[344]. A recent paper describes the mechanism for the axon initial segment localization of $K_v$4/Shal. Jegla and coworkers (2016) showed that the AIS localization of GFP-$K_v$4/Shal is mediated by the N-terminal Ankyrin repeat region and dependent on Ank2 expression[345]. The polarized neuronal localization of $K_v$4 channels is important since they have been shown to participate in regulating the backpropagation of action potentials and synaptic integration by regulating membrane excitability in dendrites[191,342,354–356].

### 1.7.3. $K_v4$/Shal channel function

*In vitro* expression studies have shown that $K_v4$ is responsible for a highly conserved fast recovering and inactivating A-type transient current that can be measured at subthreshold membrane potentials[174,312,314,330,357,358]. The first study to genetically identify $K_v4$ $K^+$ currents in neurons was performed by Tsunoda and Salkoff (1995) in *Drosophila* embryonic neurons. Since there is only one representative of the $K_v4$ family of channels in *Drosophila*, they used a genetic deletion of *$K_v4$/Shal* to show that this channel encodes almost all fast inactivating A-type $K^+$ currents in the cell body; they also reported that, in this mutant fly, slowly or noninactivating $K^+$ currents remained. Because wildtype currents that were eliminated by this *Kv4/Shal* deficiency had showed variable time constants of inactivation, Tsunoda and Salkoff (1995) also explored $K_v4$/Shal single-channel currents. They found that the *Kv4/Shal* gene encodes a 4 pS channel, and that this channel exhibits two modes of gating, likely underlying the variation in whole-cell inactivation rates observed[171].

A dominant negative $K_v4$ ($DNK_v4$) subunit, in which a conserved tryptophan in the selectivity pore is substituted by phenylalanine[317], was used to examine the role of $K_v4$ *in vivo*. Ping and coworkers (2011) reported that expression of *$DNK_v4$* completely removed A-type currents in *Drosophila* neurons. Loss of this current resulted in the eradication of the latency for the first action potential firing, broadening of action potentials, a deficit in afterhyperpolarizations, and a lower threshold for inducing repetitive action potential firing. Their results also showed that $K_v4$ repolarizes the membrane during prolonged stimuli making this channel required for maintaining neuronal cell excitability during repetitive action potentials firing. When $DNK_v4$ was expressed in motor neurons, similar defects in repetitive firing were measured, suggesting that repetitive rhythmic behaviors that require proper motor function might be affected. In behavioral

analyses, loss of $K_v4$ A-type currents in motor neurons resulted in defects in larval and adult locomotion and fly grooming[359].

Mammalian studies to understand the role of $K_v4$ in neurons have been more extensive than in *Drosophila*, and a variety of neuron types have been used to perform them. Voltage-clamp studies in rat sympathetic neurons using a dominant negative form of $K_v4.2$ showed that knocking out function of the channel leads to a loss of the fast outward $K^+$ current component, indicating that $K_v4$ encodes the A-type current in these neurons[360]. Similar experiments in developing granule cells demonstrated that transient $K_v4$-specific $K^+$ currents are involved in their first action potential spike, an event required for granule cell maturation initiation[300,361]. Tests in rat primary visual cortex neurons also revealed that $K_v4$ encodes the same A-type current. When function is eliminated using $DNK_v4.2$, there is a hyperpolarization of the action potential threshold, a broadening of action potentials, and a detrimental effect on repetitive firing[362]. Regardless of species and neuron type, $K_v4$ channels encode A-type $K^+$ currents suggesting that these channels have a conserved neuronal function, Figure 1.4.

Hoffman *et al.* (1997) investigated the role of $K_v4$ A-type currents in dendrites during action potential firing. In their experimental procedures, they examine how this regulation occurs in CA1 hippocampal neurons. A-type current density was described to be directly related to an increase in the distance from the some through dendrites. Their findings that there is a higher density of A-type potassium channels in dendrites were the first to support three key ideas: 1) backpropagation of action potentials into distal dendrites is controlled by A-type currents, 2) A-type currents inhibit action potentials from initiating in dendrites, 3) A-type currents are strongly involved in the regulation of excitatory post-synaptic current (EPSC) amplitude[342].

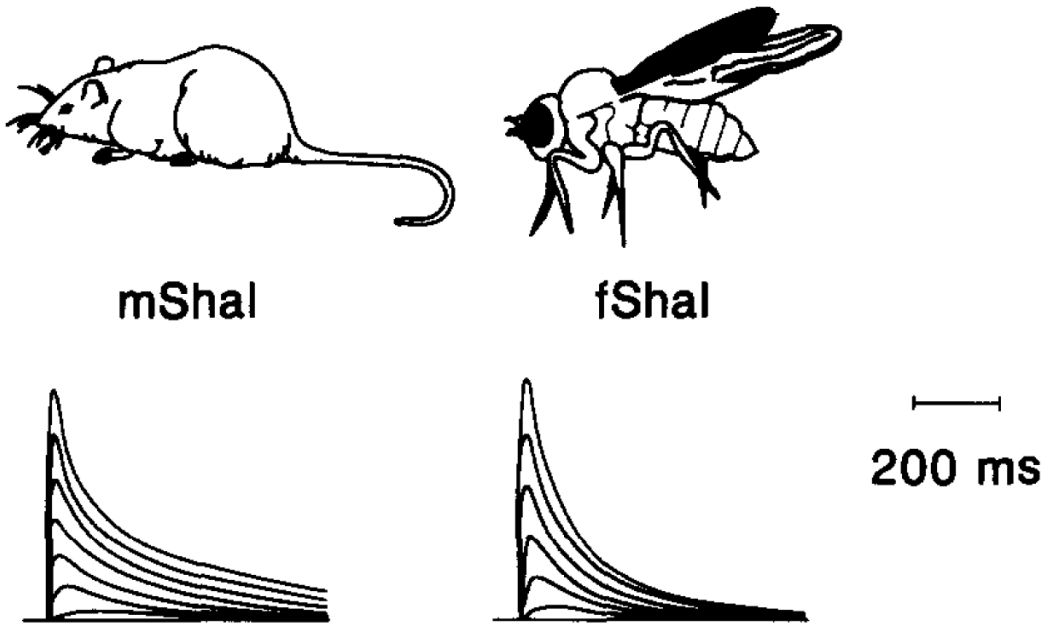


**Figure 1.4. The function of $K_v4$ channels is conserved across species.** Voltage-clamp experiments where currents were elicited from holding potential at -100 mV, with 10 mV step-wise increases from -80 to 20mV[312]. Left, voltage-sensitive response in mouse $K_v4$ (mShal). Right, voltage-sensitive response in *Drosophila* $K_v4$ (fShal). In both species, the A-type current shows a rapid rise and fast inactivation (adapted from Salkoff *et al.* 1992 with permission from Elsevier, License 4490441300619)[267].

The backpropagation of action potentials was first described in the late 1950s as an action potential initiated in the soma and propagated in a retrograde manner towards the dendritic tree[363], and confirmed by others and tested in different neurons[364–366]. Additional experiments on hippocampal CA1 pyramidal neurons demonstrated that this backpropagation of action potentials is maintained by axonal voltage-gated $Na^+$ channels and allows for voltage regulation at dendritic locations to control the permissive voltage for $Ca^{2+}$ influx[367]. Computational models have been used to help establish that a transient $K^+$ dendritic conductance could play a role in modulating the interaction between a synaptic input and a backpropagating action potential[368]. Through the use of genetic knockouts, $K_v4.2$ A-type currents were identified as important modulators of the backpropagation of action potentials which also play an important role in synaptic plasticity[356,369].

$K_v4$ has also been shown to be involved in the regulation of EPSCs. Indeed, the work by Ping and others (2011) showed that a loss of $K_v4$ channel current resulted in broadening of action potentials, a loss of afterhyperpolarization, and defects in repetitive firing; their results suggest that $K_v4$ plays a critical role in modulation of EPSCs and integration of post-synaptic potentials[359].

### 1.7.4. Physiological roles of $K_v4$/Shal channels

$K_v4$/Shal channels have been described to play a role in learning and memory formation, and to be required for proper neuronal function during rhythmic behaviors such as locomotion. Most of our current knowledge on how the brain stores information by using both long-term potentiation (LTP) and long-term depression (LTD) has been from decades of experiments studying AMPA/NMDA receptors[60,370–374]. The modulation of action potential backpropagation is critical in LTP and LTD, events that also depends on pre-synaptic stimulation and lead to synaptic strengthening or weakening and plasticity which is the paradigm of learning and memory[375–379]. Frick *et al*. (2004) described how induction of LTP produced an increase in dendritic excitability by allowing an increase of backpropagation of action potentials to this neuronal region. Their tests showed that a downregulation in A-type currents results in increased dendritic excitability[380]. Indeed, more recent studies using $K_v4.2$ knockouts in hippocampal CA1 pyramidal cells have shown that the lack of this channel allows for increased excitability in their dendrites, in turn leading to a lower threshold for induction of LTP[356,381–383]. These results suggested that functional levels of membrane-expressed $K_v4.2$ channels play a role in LTP. It was also found that internalization of $K_v4.2$ is involved in response to LTP in presence of high intracellular calcium concentrations in CA1 neurons[384]. Behavioral studies have expanded the *in vitro* findings and included behavioral responses to the knockout of $K_v4.2$ in mice which have been described to have

deficits in hippocampal-dependent learning and memory[381,385,386]. $K_v$4.3 is a form of the channel found in high levels in pyramidal neurons. In cortical pyramidal neurons, $K_v$4.3 has been found to regulate membrane attributes that play a role in the repetitive firing of action potentials but not on the threshold for firing[387]. Nevertheless, a recent study finding a mutation on the voltage sensor of $K_v$4.3 leading to a shift in its voltage-dependence shows that this mutant causes intellectual disability which in turn can affect memory performance[388,389].

$K_v$4 channels are implicated in homeostatic synaptic plasticity. In their report demonstrating that synaptic homeostasis is conserved across species in the central nervous system, Ping and Tsunoda (2012) showed that $K_v$4/Shal channel expression is increased in response to prolonged inhibition of nicotinic acetylcholine receptors (nAChRs). They reported that blocking synaptic activity for 24 hours, in different types of *Drosophila* motor neurons and projection neurons, resulted in increased mini excitatory post-synaptic current (mEPSC) amplitude and frequency, suggesting a likely increase in nAChRs in the post-synaptic cell. Further examination yielded that an increase in, specifically, Dα7 subunit levels was responsible for the rise in mEPSC amplitude and frequency. While investigating cellular changes resulting from synaptic homeostasis, they found $K_v$4/Shal A-type $K^+$ currents ($I_A$) to considerably increase following inhibition of nAChRs; this surge in $I_A$ was found to be the result of an increase in $K_v$4/Shal channels in the fly brain, suggesting that $K_v$4/Shal may be playing a role in downregulating the response. Altogether, they reported a molecular mechanism, in which synaptic inactivity by nAChR blockage caused Dα7 protein synthesis which resulted in an increase in transcription and translation of $K_v$4/Shal channels[390].

$K_v$4/Shal channels have also been described to play a role in movement. Repetitive rhythmic behaviors (i.e. feeding, eye blinking, locomotion, etc.) require the repetitive rhythmic

firing of neurons. As early as the 1970s, Connor and Stevens (1971) had described that potassium currents were likely involved in repetitive firing[391]. During the 1990s, reports were published, in which A-type currents were found to play a role in rhythmic patterns. An analysis using 4-aminopyridine, a potassium channel blocker, in the lobster stomatogastric ganglion showed decreased A-type currents that lead to a decline in the pyloric motor rhythm[392]. Studies in *Xenopus* embryos reported that a decrease in potassium currents has a detrimental effect on the motor activity of the swimming circuitry, similar to the effect seen when $K^+$ channels were blocked pharmacologically[393–396]. Pharmacological examination of the lamprey locomotor network neurons revealed that the transient A-type potassium current is involved in locomotor burst frequency[397]. Altogether, these results suggested that A-type currents, like those encoded by $K_v4$, play a role in repetitive movements and proper locomotor function.

Two decades later, the first report indicating that $K_v4$ channels are responsible for these A-type currents in rhythmic behaviors in *Drosophila* was published. Ping and coworkers (2011) generated a transgenic fly expressing DN$K_v4$ to knockout function of the channel without affecting other potassium currents. They found that the initiation of action potential firing is regulated by $K_v4$/Shal, and that this regulation is critical for continuous firing during sustained neuronal stimulus[359]. In nematodes, a recent report using a knockout of their $K_v4$ channel orthologue, SHL-1, showed a disturbed effect in turning behaviors[398]. In mice, a study using RNA-interference to acutely knockdown $K_v4.1$ levels *in vivo* showed that $K_v4.1$ contributes to the A-type currents found in the suprachiasmatic nucleus (SCN), the circadian pacemaker. The knockdown of these $K_v4.1$-dependent A-type currents led to shortened daily periods of wheel-running[399]. Moreover, $K_v4.2$ and $K_v4.3$ knockouts have also been used to test if their transient A-type currents have a role in modulating excitability of neurons in the SCN. The researchers found that the lack of $K_v4.2$, but

not $K_v$4.3, subunits caused mice to have a decreased period of locomotor activity caused by shorter circadian rhythm firing patterns on SCN neurons[400]. In other studies, however, $K_v$4.2 knockout mice did not display changes in locomotor behaviors, such as swimming or entries into the arms of an elevated plus maze[386,401], suggesting that some behaviors are not affected by shorter circadian rhythm firing patterns.

### 1.7.5. $K_v$4/Shal accessory proteins

$K_v$4 channels have been shown to be modulated by many different accessory proteins. Amongst the most prominent ones are $K_v\beta$, KChIP, DPP, SKIP3, PSD-95, Na$\beta$1, and SIDL. Below I provide a general summary on the relevance of these accessory proteins and then I introduce the scaffolding protein SIDL which is of significant interest in this dissertation.

The first discovered and the most characterized ancillary subunits are the family of $K_v\beta$-subunits. $K_v\beta$ are soluble proteins that lack a transmembrane domain and, at least in heterologous systems, modulate the voltage by which $K_v$4 is activated[191,402]. $K_v\beta$-subunits playing a role in $K_v$4 expression and inactivation were first observed by electrophysiology in experiments injecting low molecular weight fractions of rodent brain poly-(A) transcripts (2-4 kb) and cRNA encoding mouse $K_v$4. These experiments shed light, for the first time, on the idea that there are low molecular weight regulatory polypeptides that modulate gating of A-type potassium channels[403]. Experiments by Serôdio and colleagues (1994, 1996) confirmed this idea using rat mRNA. They injected rat $K_v$4.2 or $K_v$4.3 mRNA into *Xenopus* oocytes and measured currents that have slower inactivation kinetics and recover more slowly from inactivation than when measured in the brain. They also co-injected $K_v$4 channels along with the low molecular weight fractions of poly-(A) brain transcripts (2-4 kb), which code for polypeptides that do not exhibit $K^+$

conductance. They reported that this co-injection resulted a fast inactivating A-type current, suggesting that a polypeptide coded by the low molecular weight fraction was modulating $K_v4$ channels[314,358].

$K_v\beta$–subunits are ~39 kDa in size, have high hydrophilicity, and lack transmembrane domains, suggesting that these $K_v$ accessory subunits are likely located in the cytoplasmic side of the cell membrane[404]. To date, there are three known $K_v\beta$ genes in mammals – *KCNAB1*, *KCNAB2*, and *KCNAB3* – that can be alternatively spliced to produce variants within three different families of auxiliary subunits – $K_v\beta1$, $K_v\beta2$, $K_v3$[259,405–409]. A high resolution 2.1 Å crystal structure of the T1-tetramerization containing N-terminal domain of $K_v$ interacting with the cytoplasmic β-subunit revealed that a symmetric complex is formed with these α- and β- subunits[410]. Scott and colleagues (1994) were the first to suggest that this family of β-subunits was likely to extend the diversity of $K^+$ channel function[404]. This was confirmed by Rettig and coworkers (1994) when they co-expressed rat β-subunit with the α-subunit of $K_v1.1$ or $K_v1.4$, giving $K_v1$ rapid A-type inactivation properties[259]. $K_v\beta$ also exhibits a strong biochemical interaction with $K_v4$ α-subunits[411]. Molecular interactions between $K_v4$ and distinct splice-forms of different $K_v\beta$ families lead to $K_v$ functional diversity. For example, $K_v\beta1$ alone can provide oxidative sensing to $K_v4.2$, whereas either $K_v\beta1$ and $K_v\beta2$ can increase $K_v4.4$ current density and expression, while $K_v\beta3$ slows both $K_v4.3$ activation and inactivation kinetics leading to a slower recovery from inactivation[412–414]. In *Drosophila*, the homolog gene of $K_v\beta$ is called *Hyperkinetic* and has been shown to interact with $K_v1$/Shaker and $K_v4$/Shal channels[162,411,415].

$K^+$ channel interacting proteins (KChIP) are a family of calcium-binding proteins found to interact with the N-terminus of $K_v4$ channels. KChIP1-3 were first identified in yeast-to-hybrid experiments using the $K_v4.3$ N-terminus as bait[416], and KChIP4 was later discovered in co-

immunoprecipitation experiments when researchers identified it to interact with $K_v4.2$[417]. KChIP1, KChIP2, and KChIP3 have been described to be involved in increasing $K_v4.2$ surface expression and modulating recovery from fast inactivation in mammalian systems[416,418,419]. These accessory subunits also play a role in $K_v4.2$ trafficking from the endoplasmic reticulum to the cell membrane, as well as in stabilizing membrane forms of the channel, as measured by increases in $K_v4$ current density studies[191,402]. While characterizing *Ciona intestinalis* KChIP and its role in $K_v4$ function modulation, Salvador-Recatalà and colleagues (2006) reported that in their bioinformatics analysis, they had found a KChIP-like gene in *Drosophila*[420]. In a recent study, Chen and coworkers (2015) found that, in *C. elegans*, KChIP-like subunits regulate the expression of the $K_v4$ channel orthologue SHL-1[398].

Dipeptidyl peptidase-like proteins (DPPX) are another family of $K_v4$ channel related accessory proteins. DPP4, also known as CD26, was the first of the family to be originally identified in mammalian brain tissue and it is thought to participate in the modulation of synaptic plasticity[421–423]. While DPP6 has been described to be involved in the trafficking and membrane targeting of $K_v4$ channels, DPP10 has been reported to modulate inactivation of $K_v4$ channels[402,424,425]. Some other DPPs have a histidine-rich extracellular domain which, possibly, provides $K_v4$ channels with better stability in presence of variable extracellular pH levels[402]. In a recent report, Shiina and coworkers (2016) identified the fly DPP10 ortholog that interacts with rat $K_v4.3$ and can modulate $K_v4$ currents[426].

Shal/$K_v4$ $K^+$ channel interacting protein-3 (SKIP3) was identified using yeast-two-hybrid with the C-terminus of *Drosophila* $K_v4$/Shal by Diao and colleagues (2009). SKIP3 was found to be specific for $K_v4$/Shal and as it did not interact with the C-terminal tail of *Drosophila* $K_v1$/Shaker, $K_v2$/Shab or $K_v3$/Shaw in yeast-two-hybrid experiments. SKIP3 has been described

to play a role in $K_v4$ activity, in *Drosophila*, by modulating $K_v4$ current inactivation[427]. While looking for SKIP3 isoforms in various databases, Diao and colleagues (2009) identified *SKIP* which codes for two potential proteins, SKIP1 and SKIP2. Through the use of reverse-transcriptase experiments, they confirmed that SKIP3 is a true *Drosophila* isoform expressed in the fly embryo[427].

Post-synaptic density protein 95 (PSD-95) is a scaffolding protein that interacts with the C-terminal Val-Ser-Ala-Leu sequence of $K_v4.2$ as reported by Wong *et al.* (2002). In their report, they show that co-transfection of PSD-95 with $K_v4.2$ resulted in increased expression and clustering of $K_v4.2$ protein in CHO cells[428]. In later studies, Wong and coworkers (2004) found that PSD-95 can recruit a portion of $K_v4.2$ containing vesicles into lipid rafts[429]. In *Drosophila*, the *PSD-95* homolog is known as *Discs Large-1 (dlg*), and dlg protein has been reported to be required for normal morphology of synaptic buttons in *Drosophila* larva neuromuscular junctions[430,431].

$Na_v\beta1$ has been recently described as a $K_v4$ protein stabilizer in HEK-293 cells[432]. Through chimeric and mutational studies, Nguyen and colleagues (2012) identified $K_v4$ segments S1 and S5 as the molecular interaction sites of $Na_v\beta1$[433]. This voltage-gated sodium channel accessory subunit stabilizes cytoplasmic vesicle-bound $K_v4$ which, in turn, increases pools of the channel readily available for cell-surface expression[432,434]. In *Drosophila*, there are no genes with homology to mammalian *$Na_v\beta1$*, although *tipE* has been described to encode an auxiliary subunit that controls expression and function of the *Drosophila* $Na_v$ channel, Para[435,436].

The scaffolding protein SIDL (Shal/$K_v4$-interactor of di-leucine) has been found to directly interact with the highly conserved LL-motif of $K_v4$ (also see page 35), which is necessary and sufficient for the channel somato-dendritic localization, *in vitro*[344,349]. SIDL is expressed

throughout the life of the fly and it is present in neurons. A transgene containing a GFP-tagged $K_v4$ protein was found to mislocalize from cell bodies to axons in larval and adult CNS studies when the LL-motif was deleted in the GFP-tagged transgene[344]. Altogether, this leads to the possibility that SIDL may play a role in the localization of $K_v4$ proteins via interaction with its C-terminal LL-motif. Because mislocalized proteins are typically targeted for degradation[437], it is of interest to test if age has any impact on SIDL protein or mRNA. If SIDL protein or transcript declines with age, this may lead to mislocalization of $K_v4$ and targeting of this channel for degradation.

### 1.7.6. Age-related pathophysiology of $K_v4$/Shal

There are a wide variety of disorders attributed to a dysfunction of $K_v4$/Shal channels. Indeed, as described by Hille (2001), voltage-gated potassium channels are involved in the proper regulation of membrane excitability[224]; any changes to its balance may have detrimental changes to the organism. The studies reviewed below report pathophysiological effects, similar to those that occur during aging, from truncation, knockdown, knockout, mutation, or loss of function in $K_v4$ channels. Though most of the work published on $K_v4$ has been performed in excitable cells, $K_v4$ has also been reported to play roles in cell-cycle control in non-excitable cells.

Cancer is a disease that substantially affects people over 65 years of age[438]. During cancer, there is a dysregulation of cell division, and $K_v$ channels have been described to be partially involved in the mechanisms that regulate proper cell-cycle control in non-excitable cells[439]. Jang and colleagues (2009) described how transfection of siRNA targeting $K_v4.1$ in tumorigenic mammalian epithelial cells resulted in a suppression of cell proliferation. They also measured levels of *$K_v4.1$* mRNA in human breast cancer tissue and found that $K_v4.1$ mRNA levels were

increased in certain cell developmental stages when compared to wildtype[243]. Kim and coworkers (2010) reported that, in cancerous human gastric cells, transfection of siRNA targeting $K_v$4.1 resulted in an inhibition of cancer cell proliferation[251]. Though the molecular mechanisms by which $K_v$ channels regulate tumor progression remains uncertain[440], these publications suggest that $K_v$4 channels play a role in the progression and proliferation of cancer.

In the heart, the QT interval has been described to be prolonged with aging[441], and an increase in the QT interval is known to be coupled to arrhythmias, disease, and sudden cardiac death[442]. Barry and colleagues (1998) created a $K_v$4.2 functional knockout by expressing a dominant negative $K_v$4 $\alpha$-subunit with a pore mutation (tryptophan to phenylalanine) which, when interacting with three wildtype $K_v$4 $\alpha$-subunits to make a tetramer, it produces a non-conducting channel in mouse ventricular myocytes. Their mice experiments showed that $K_v$4 channels underlie $I_{to}$ currents in the heart, and that this functional knockout lead to longer durations of action potentials and increased QT intervals[317]. It is important to note, however, that though mice and rats have been shown to express mRNA coding for both $K_v$4.2 and $K_v$4.3 channels in heart ventricles[315], in human ventricles only mRNA coding for $K_v$4.3 is found[443].

In aging studies, Hauser (1997) reported that ~50% of elderly patients with no previous history of seizures suddenly began exhibiting them[444], suggesting that epilepsy may be considered an age-related disease. The onset of age-related epilepsy is typically attributed to stroke and neuronal degenerative diseases[445]. In their 2006 review, Leppik and colleagues (2006) brought attention to the lack of publications that correlate aging and epilepsy[445]. Indeed, one clinical study has described a patient having temporal lobe epilepsy at a young age, a medical effect that has been defined to be more prevalent in older populations. The cause was found to be a 44-amino-acid C-terminal truncation of Kv4.2[446,447]. Although clinical studies are lacking, in their review,

D'Adamo and colleagues (2013) report a wide variety of studies on $K^+$ channel dysfunction that have been associated to epileptic phenotypes[448].

Memory is a cognitive ability that has been extensively studied at many different levels, from molecule to behavior. While short-term memory remains typically unaffected by age, long-term memory is where most differences between young and old are observed[449]. Indeed, the elderly have been shown to have a decline in recalling information from long-term memory[450]. This age-related decline in cognitive function is, in part, attributed to molecular modifications that are directly involved with neuronal plasticity[451]. $K_v4$ channels have been described to play a role in neuronal synaptic plasticity. Simkin and coworkers (2015) have described an increase in membrane-bound $K_v4.2$/$K_v4.3$ in the hippocampus during aging, suggesting that $K_v4$ influences the hyperexcitability of CA3 neurons at an older age[339]. A publication by Smets and colleagues (2015) reported that a *de novo* mutation of $K_v4.3$ was the source of an early onset of cerebellar ataxia, a condition that is predominantly caused by age-dependent cerebrovascular disease, in a 3-year old patient[388,452,453].

In Alzheimer's disease studies, Ping *et al*. (2015) used a *Drosophila* Alzheimer's model to uncover the effects of an accumulation of human Aβ42 on the channel $K_v4$/Shal. This channel protein was found to be degraded while mRNA levels remained intact during the time of testing. With this decline in $K_v4$/Shal, many of the Alzheimer's ailments developed in the fly, including learning and locomotor defects, and neurodegeneration in the mushroom body. The loss of $K_v4$/Shal also lead to shortened lifespan in *Drosophila*. They demonstrated that overexpression of $K_v4$/Shal to near-wildtype levels restored all the dysfunctions mentioned above, slowed Aβ42-induced neurodegeneration, and partially restored lifespan[454]. In recent report, Feng and colleagues (2018) showed that accumulation of Aβ42 contributed to an age-dependent decline in

short-term memory using a courtship memory assay. Their studies highlight the importance of $K_v4$/Shal channels located in the fly mushroom body and projection neurons for courtship short-term memory[455]. In mammalian studies, overexpression of Aβ protein in hippocampal CA1 neurons resulted in a large decrease of $K_v4.2$ protein levels, which led to increased dendritic hyperexcitability[456]. In similar studies where Aβ was, instead, delivered intracellularly, Scala and coworkers (2015) measured a decline in A-type $K^+$ currents[457]. They also found that mechanisms regulating the decrease in these currents involve the activation of caspases and glycogen synthase kinase 3 (GSK-3). GSK-3 was found to phosphorylate $K_v4.2$ at residue 616 which was suggested to play a role in the reduction of A-type $K^+$ currents.

In Parkinson's disease, signaling from the substantia nigra to the stratum, the brain structure directly involved in decision-making, significantly decreases. Aidi-knani and colleagues (2015) report that, at the onset of this disease, $K_v4$ dependent A-type currents decline in medium spiny neurons (MSN) of the striatum as a homeostatic response to the ensuing nigrostriatal dopamine depletion[458]. They used the selective $K_v4$ toxin AmmTX3 to test the hypothesis that a further decline in $K_v4$ A-type currents would enhance the response of MSN, and assuage the symptoms attributed to this disease. They found that this drug treatment reduced Parkinsonian emotional, cognitive, and motor symptoms. It is important to note that these results are contradictory to other reports where a loss of $K_v4$ function resulted in no anxiety- or depression-related phenotypes in mice[401], a decline in mouse freezing behaviors during Pavlovian fear conditioning[386], a decline in cognition while traveling the Lashley maze[385], and, in *Drosophila*, a decline in rhythmic behaviors such as locomotor performance and grooming[359].

## 1.8. Overview of this dissertation

With the growing number of individuals surpassing 60 years of age, the incidence of age-related problems will also increase. Indeed, it is known that aging results in detrimental effects to several aspects of life, including a decline in cognitive and motor abilities. In a study involving over 2,000 people of similar health and education, Salthouse and colleagues (2009) reported an age-related decline in cognition; they found that reasoning and memory both decreased with age in humans[459]. Lee and co-workers (2017) later reported a decline in functional performance, such as gait speed, during aging in humans[460]. These hallmarks of aging have also been reported to occur in *Drosophila*. Indeed, in their 2014 report, Haddadi *et al.* measured the effects of age on *Drosophila* short, medium, and long-term memory[101]. They trained flies to associate a smell with an electric shock and found that all measured types of memory decreased by ~50% when comparing young (5 days) and old flies (50 days). *Drosophila* motor abilities also decrease during aging. Indeed, I present below that locomotor performance declines as a function of age in *Drosophila*.

Ion channels have been described to play a role in cognition and coordinated motor movement. From our lab, Ping and others (2011) used a $K_v4$ dominant negative expressing fly to study the effects of loss of functional $K_v4$ channels[359]. A transgene expressing point mutation (W362) in the $K_v4$ α-subunit was expressed in flies. Flies expressing this dominant negative $K_v4$ subunit ($DNK_v4$) form tetramers with endogenous α-subunits resulting in non-functional $K_v4$ channels. Neuronal expression of $DNK_v4$ resulted in a loss of $K_v4$ current, loss of the delay to the first action potential firing, and loss of repetitive rhythmic firing. When examining behavioral effects, they found that a loss of $K_v4$ function resulted in decreased larval crawling, and in deficits of adult fly grooming, locomotor performance, and learning and memory. Their results showed

that $K_v4$ is involved in functions that decline with age. *In this dissertation, my aim was to understand what happens to $K_v4$ during*.

**Chapter 3:** In this chapter, I characterized the age-dependent decline in *Drosophila* locomotor performance and the age-dependent decline in $K_v4$ protein levels. I found that the age-related decline in $K_v4$ protein levels is specific to $K_v4$ and not a general phenomenon that occurs to all potassium channels. I also tested the hypothesis that the decrease in $K_v4$ proteins is conserved across species.

**Chapter 4:** In this chapter, I tested the hypothesis that reactive oxygen species (ROS) play a role in the age-dependent decline of $K_v4$ protein levels. I tested the hypothesis that exposure of hydrogen peroxide ($H_2O_2$) to *Drosophila in vivo* and to *Drosophila* primary culture neurons *in vitro* results in changes to $K_v4$ protein levels or qualitative changes in tagged $K_v4$, respectively. I also tested the hypothesis that overexpression of enzymes that regulate intracellular ROS have an effect on levels of $K_v4$ protein and fly locomotor performance at different ages.

**Chapter 5:** In this chapter, I tested the hypothesis that an age-dependent decline of the scaffolding protein SIDL (Shal interactor of di-leucine) contributes to the age-dependent decrease in $K_v4$ protein levels. We used a *Drosophila* line expressing *SIDL-RNAi* in neurons to test the hypothesis that a decline in *SIDL* mRNA results in a decline in $K_v4$ protein levels. We then tested the hypothesis that $K_v4$ and SIDL are implicated in *Drosophila* eclosion.

# CHAPTER 2. MATERIALS AND METHODS

## 2.1. *Drosophila* strains

The flies used involve wildtype, transgenics, and genetic deficiencies (*Df*): Canton-S (Bloomington *Drosophila* Stock Center, Stock 64349), $SK^{-/-}$ (obtained from Dr. Patrick Dolph, Dartmouth College, NH)[461], *Df(Shaker)* (obtained from Dr. Kyunghee Koh, Thomas Jefferson University, Philadelphia, PA)[462], *UAS-DN$K_v$4*[359], *UAS-$K_v$4/Shal*[454], *UAS-$K_v$1/Shaker* (obtained from Dr. William Joiner, University of California, San Diego), *UAS-SOD1*[463], *UAS-SOD2*[464], *UAS-Catalase*[463], *UAS-NOX-RNAi, and UAS-DUOX-RNAi* (these last five stocks obtained from Dr. Matthias Landgraf, University of Cambridge, UK)[465], *UAS-SIDL-RNAi* (Vienna *Drosophila* Resource Center, Stock 40390), *UAS-* SIDL[344], *UAS-CD8-GFP*[466], *UAS-GFP-$K_v$4/Shal*[344], *and UAS-mCherry-pHluorin-$K_v$4/Shal*[467–469]. We used the following lines that drive expression of UAS-genes in neurons: *elav-gal4*, *elav-gal4;gal80$^{TS}$*, and *elav-gal4,UAS-Dcr$^2$* (all obtained from the Bloomington *Drosophila* Stock Center). *elav-gal4* was used as pan-neuronal driver for constitutive expression. *elav-gal80$^{TS}$* was used for conditional temperature-sensitive expression. *elav-gal4,UAS-Dcr$^2$* was used for constitutive pan-neuronal expression with a double-stranded RNA-specific endonuclease.

## 2.2. Immunoblotting

All immunoblotting experimental samples were separated by size using electrophoresis in 10% acrylamide bisphosphate SDS-PAGE gel. Each gel containing separated proteins was then transferred, by wet electroblotting, into a 0.45 μm nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were then blocked in a solution containing 5% dry milk in 1X PBS (phosphate

buffered saline) with 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO) for at least 20 minutes on a table-top rocker. Blots were then incubated in 1° antibody diluted in block solution with 0.02% sodium azide (J.T. Baker Chemical Co., Phillipsburg, NJ) overnight at room temperature. Blots were then washed 4 times in 1X PBS and 0.05% Tween-20. After wash, blots were incubated for 60 minutes in 1:2500 2° antibody (Goat α-rabbit or Goat α-mouse) conjugated to horseradish peroxidase, HRP (Jackson ImmunoResearch Inc., West Grove, PA). Blots were then washed 4 times as described above. Then, each blot was incubated for 30-60 seconds in 5 mL 1:500 SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, Waltham, MA). Chemiluminescence signal was detected using an Epichemi$^3$ Darkroom and the Labworks Imaging Software (UVP BioImaging, Upland, CA). Each chemiluminescent band was quantified using densitometric analysis with the software Fiji, an image processing distribution of ImageJ[470] and each value transferred to Microsoft Excel for data analysis. All data obtained from each antibody in each lane was normalized to its corresponding loading control (Actin or Syntaxin). Dixon's Q-test was used to identify experimental outliers which were excluded from statistical analyses. Unless otherwise indicated, statistical evaluations were carried using the Student's t-test. All immunoblotting representative graphs in this dissertation were made in Sigma Plot and edited in Adobe Photoshop.

For *Drosophila* immunoblot analyses, each sample was prepared by sonicating 5 *Drosophila* heads in 20 μL 2X SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) sample buffer (100 mM Tris-HCL, pH 6.8, 200 mM dithiothreitol or 10% β-mercaptoethanol, 4% sodium dodecyl sulfate, 25% glycerol, and 0.2% bromophenol blue) in a sonifier (Branson Ultrasonics, Danbury, CT). Once in blots, proteins were probed with different 1˚ antibodies. α-$K_V$4/Shal antibody was affinity purified from rabbit serum which was bled from

rabbits previously injected with the peptide CGIELDDNYRD that is present on the C-terminus of both dShal1 and dShal2[427]. α-$K_v$4/Shal was used at 1:50-1:100 dilution depending on the purification stock. α-dSK antibody (kindly provided by Dr. Patrick Dolph, Dartmouth College, NH)[461] was verified with the use of a $dSK^{-/-}$ mutant (also provided by Dr. Patrick Dolph) using 1:100 dilution. $K_v$1/Shaker antibody (Abcam, Cambridge, MA) was verified with the use of a $K_v$1/Shaker *Drosophila* deficiency (kindly provided by Dr. Kyunghee Koh, Thomas Jefferson University, Philadelphia, PA)[462] using a 1:500 dilution. As controls, I used α-Actin (Clone C4, MilliporeSigma, MA) and α-Syntaxin (DHSB, University of Iowa, IA) antibodies at 1:2500 and 1:50, respectively.

### 2.3. Mouse Brain Experiments

#### 2.3.1. Mouse caring and sample storage

Mouse caring and brain dissections were carried by Dr. Mario Oyola, a post-doctoral associate in Dr. Bob Handa's laboratory at Colorado State University. He ordered 10 young (6-wk old) and 10 old (8-month-old) mice, strain C57BL/6 from Charles River Laboratories. Young mice, once they arrived, were kept in their new cages for 14 days so they could acclimate to their new environment (2-3 mice per cage). Old mice arrived at 8 months of age. They were housed independently until the age of 13 months. Dr. Oyola anesthetized mice with isoflurane prior to decapitation and brain extraction. Sections of the brain were separated with razor blades: 1) Cerebellum, 2) Hippocampus, 3) Motor Cortex, and 4) Olfactory Bulb. Material was submerged in 2 mL buffer (30 mM NaCl, 20 mM HEPES, 5 mM EDTA, pH 7.59, filtered sterile) and flash frozen in $N_{2(l)}$, then stored at -80°C. Note: not all mice survived; extractions were from 8-10 mice depending on age.

### 2.3.2. Sample preparation and immunoblot

I received all frozen samples from Dr. Oyola. Prior to each experiment, samples were thawed on ice. Protease Inhibitors (100X HALT, EDTA free, ThermoFisher Scientific, Waltham, MA) were then added to 1X concentration. Tissue was homogenized using a tissue mincer electric homogenizer. Homogenate was then transferred to a 15mL conical vial and spun at 1000x g for 10 minutes at 4°C – to pellet connective tissue and nuclear material. Supernatant was transferred to a Falcon tube, then spun at 20,000x g for 15 minutes at 4°C to pellet membrane fraction. Supernatant was removed and pellet re-suspended in 150-300 μL buffer + 1% Triton-X100. Quantification of the protein sample was performed using the BCA system from Pierce and a UV/Visible spectrophotometer (Model DU730, Beckman Coulter, Brea, CA).

Each sample was prepared with 15 μg protein in 2X SDS-PAGE buffer, and each gel was loaded such that one sample from each young and old brain would be present (7 young and 7 old). Sample protein separation was performed using electrophoresis as described above. Different 1° antibodies used: α-$K_v$4.1 (Alomone) at 1:400, α-$K_v$4.2 (gift from Dr. Michael Tamkun, Colorado State University) at 1:500, α-$K_v$4.3 (Neuromab) at 1:500, and α-mActin (Clone AC-40, Sigma-Aldrich, St. Louis, MO) at 1:1000.

### 2.3.3. Data collection and statistical analysis

Each experiment was carried at least 5 times. Seven young and seven old (total 14) brain extracts were loaded into a single SDS-PAGE gel each time. Once immunoblot results were obtained, we used densitometric analysis as described above to quantify the data representing $K_v$4.1, $K_v$4.2, $K_v$4.3 and the control mActin. For each lane, I normalized the $K_v$4 data to that of its corresponding mActin. I identified and removed outliers using Dixon's Q-test, as described above.

Then, I divided all values by the average of all 6-week (young mice) results – effective normalization to 1.

A Linear Mixed Effects Model was then used to analyze the effects of age on protein levels of $K_v4$ proteins. Because I wanted to determine the effects of age across many blots and multiple mice, in this model I used "age" as a fixed effect, and added "Experimental-Immunoblot" and "Mouse-Brain-Section" as random effects. In the mathematical expression, "age" (syntax: Age) represents the statistical test answering the question "do levels of the measured $K_v4$ protein ($K_v4.1$, $K_v4.2$, or $K_v4.3$) change with age?" "Experimental-Immunoblot" (syntax: Experiment) was defined as a variable effect which represents the error across experimental procedures, while "Mouse-Brain-Section" (syntax: Brain) is another variable that represents the measurable differences of the same $K_v4$ across different mouse brains. I fitted this model (syntax: model1) using the Maximum Likelihood of the "lmer" function in lme4 package[471] of the R statistical software using RStudio[472], setting REML to FALSE (this option is used when comparing different fixed effects which in this case was Age – 6 week and 1 year were fixed). The p-values were calculated from the fixed effects t-values obtained by the "lmer" function using my data (syntax: datavalues) as a function of age.

Software syntax for the mathematical expression:

model1 <- lmer(Data ~ Age + (1|Experiment) + (1|Brain), data = "datavalues", REML = FALSE)

## 2.4 RT-qPCR

### 2.4.1. RNA extraction

TRIzol reagent (300 μL) was used for homogenization of 10 fly heads for RNA extraction and storage at -80˚C. Chloroform (60 μL) was added to precipitate protein with vigorous vortexing

for 60 seconds before centrifuging samples at maximum speed (15,000 RPM) on a table top centrifuge at 4˚C. The supernatant was transferred into a new RNAse free microfuge tube containing 150 µL of 2-propanol and 10 µL glycogen and then incubated for 60 minutes at -20˚C to allow RNA to precipitate. After cold incubation, the sample was spun at maximum speed at 4˚C for 15 minutes. The supernatant was discarded, and the pellet washed at least 3 times with 500 µL of 70% ethanol/DEPC-treated water and spun at maximum speed at 4˚C to remake pellet each time. After washes, all ethanol/DEPC was removed by pipetting and the pellet was allowed to dry on the bench for 15-20 minutes prior to pellet resuspension in 15 µL DEPS-treated water. 0.5 µL of sample was used for gel electrophoresis to determine RNA integrity. Remaining sample was used for reverse transcriptase polymerase chain reaction (RT-PCR).

### 2.4.2. Reverse Transcription

Sample RNA concentration and purity was measured using a NanoDrop spectrophotometer. RNA purity limit for young and old fly samples were kept at ≥1.88 and ≥1.75, respectively. Samples that had proper RNA integrity and purity were used for RT reaction. 700 µg RNA for each sample was added to RNA-free PCR tube with 1 Unit DNAse I (ThermoFisher Scientific, Waltham, MA) and incubated at 37˚C for 15 minutes. DNAse inactivation was carried with addition of 1 µL 50 mM EDTA and incubation at 65˚C for 10 minutes, and then immediately placed on ice. 1 µL 0.5 µg/µL Oligo$(dT)_{12\text{-}18}$ primer (ThermoFisher Scientific, Waltham, MA) was added to each sample along with 1 µL 10 mM dNTP (ThermoFisher Scientific, Waltham, MA). The RT reaction was prepared with Superscript II Rnase H (ThermoFisher Scientific, Waltham, MA) and RNAse OUT (ThermoFisher Scientific, Waltham, MA). Sample was incubated at 42˚C to allow for primer binding to mRNA template before adding the reverse transcriptase. RT reaction

was carried for 50 minutes at 42˚C for elongation, and at 70˚C for 15 minutes for enzyme inactivation. cDNA was used immediately for qPCR.

### 2.4.3. qPCR

Samples were diluted 1:5 with autoclaved nanopure water. Each sample was tested in triplicates, 5 µL of diluted samples were added to 3 different wells of 96-well white qPCR plates (Roche, Switzerland) for each qPCR reaction. Each well also contained 3.8 µL of PCR grade $H_2O$, 0.4 µL of 20 µM forward primer, 0.4 µL of 20 µM reverse primer, 0.4 µL of 10 µM Probe from Universal Probe Library (UPL), and 10 µL LightCycler® 480 Probes Master Mix (Roche, Switzerland). Once all samples were loaded, a transparent plastic sheet (Roche, Switzerland) was used to seal the plates. Plates were spun for 2 minutes in a plate-centrifuge and loaded into a LightCycler® 480 Instrument II (Roche, Switzerland) for measurements using the UPL 96-well predefined program option.

Pimers for *$K_v$4/Shal* – which recognizes all 3 transcripts – using UPL Probe 66: GCTAACGAAAGGAGGAACG (forward) and TGAACTTATTGCTGTCATTTTGC (reverse). Primers for *SIDL* using UPL Probe 125: GTACAAGCAGGGTGACTGGAG (forward) and GATCGTGGCTTTGTAGGTGTC (reverse). For reference genes, we were unable to find genes that would be stable at all ages that we tested. For this reason, we selected *RpS20* and *eIF1A*; we used *RpS20* as a reference gene for measurements between 3 and 10 days, and we used *eIF1A* as a reference gene for measurements between 3 and 40 days, and 10 and 40 days. Primers for *RpS20* using UPL Probe 66: CGACCAGGGAAATTGCTAAA (forward) and CGACATGGGGCTTCTCAATA (reverse). Primers for *eIF1A* using UPL Probe 147: TCGTCTGGAGGCAATGTG (forward) and GCCCTGGTTAATCCACACC (reverse).

To verify primer amplification efficiency, we prepared enough cDNA samples using an equal combination of 3 and 40 day old fly heads so that we would have a large enough volume to perform test dilutions: undiluted, 1:5, 1:10, 1:15, 1:20, 1:25, and 1:35. Samples were loaded, in triplicates, into a 96-well white qPCR plates and loaded into the LightCycler® 480. The measured $C_t$ values for each dilution were graphed as a function of the Log dilution factor in Microsoft Excel and fitted with a linear regression curve ($R^2 \geq 0.95$). The efficiency was dictated by the slope of the regression line (m = -3.32 for 100% primer efficiency). Because a perfect amplification is not always possible, we allowed for a slope range, $-2.95 \leq m \leq -3.91$ (80 to 120% primer efficiency, an acceptable range[473]). We measured primer efficiency for $K_v4$/ShaI (m = -3.68), SIDL (m = -3.62), eIF1A (m = -3.60), and RpS20 (m = -3.33). Although our results show $C_t$ values that fall within the range of our dilutions, if future testing results in $C_t$ values outside of this range, the tests for primer efficiency should be expanded to include dilutions to that range.

To verify product amplification, we loaded amplified samples from the 96-well qPCR plates into an agarose gel and separated them by electrophoresis. Every amplification from each primer pair yielded only 1 band at the correct molecular weight for each pair of primers tested which was then gel-extracted (GeneJET™, ThermoFisher Scientific, Waltham, MA) and sequenced (Proteomics, Metabolomics Facility, Colorado State University, CO) to confirm that the amplicon matches the predicted PCR amplification.

For data analysis, all $C_t$ values were obtained using the Roche software on default options and exported into MS Excel. To be considered usable data, all triplicates needed to have a standard deviation of ≤0.15. If triplicates were ≥0.16, the Dixon's Q-test was applied to remove an outlier. If no outlier was found, the triplicate was discarded. The average of triplicates was used for the next data analysis step. $C_t$ averages were pulled together, and the standard deviation was measured.

For reference genes, this had to be true for data across experiments (e.g. together, 3d and 40d fly $C_t$ values needed to show a standard deviation of $\leq 1.0$ which would indicate the reference gene is stable for these experiments). In addition, a student's t-test was performed on experimental $C_t$ value groups to establish that there is no difference in the distributions (e.g. there should not be a difference between 3d and 40d data distributions). $C_t$ values were then further analyzed using the $2^{-\Delta Ct}$ method[473] and a Student's t-test was performed to determine differences across experimental tests.

### 2.5. ROS Fluorescence Detection

A fresh stock of 2',7'-dichlorodihydrofluorescein diacetate, $H_2$DCFDA (Invitrogen, Walthman, MA) was made each time at 1 mM concentration in dimethyl sulfoxide, DMSO (Sigma-Aldrich, St. Louis, MO). To calibrate fluorescence gain of each experiment, 1 μL of the $H_2$DCFDA stock was mixed with 1 pM, 1 nM, 1 μM, or 1 mM $H_2O_2$ (Sigma-Aldrich, St. Louis, MO) in separate wells. Samples were prepared by homogenizing 25 heads into 500 μL of 0.4 M Tris-HCL, pH 7.4, filtered sterile buffer by the use of a clean pestle in a microfuge tube and centrifuged at 5,000 RPM in top table centrifuge to pellet chitin. 100 μL samples were loaded into single wells (4 samples per each 500 μL extraction, with at least 5 extractions were performed per experiment) and 1 μL of 1 mM DCFDA was added to each well making sure it was done at random each time since the fluorophore begins reacting immediately and this could add a bias to the experiment. All data from 3 experiments was pulled for analysis and treated as an individual reading to include experimental variations. The Dixon's Q-test was performed on both control and experimental results to remove outliers. Both data groups were normalized to the control

experiment and a student's t-test was carried to determine if there is a significant difference in ROS levels between control and test.

### 2.6. *Drosophila* locomotor activity assay

35-40 adult males were collected at the bottom of a 12.4 cm test tube (VWR, Radnor, PA) and were allowed to climb for 30 seconds into a second inverted tube on top. The process was repeated and flies that arrived to the second tube were allowed to climb for another 30 seconds into an inverted third tube. This was performed on a total of 10 tubes and the population was fractioned by countercurrent distribution[474]. Each fly was given a score of 0.5 per each tube that they fully climbed to enter the next tube (e.g. score of 0.5 on first tube, 1.0 on second tube, 1.5 on third tube, etc.), as similarly performed by Xu *et al*[475]. and by Ping *et al*[454]., and this was taken into a total score which is defined as the "locomotor performance". This experiment was tested on at least 10 different groups of male flies for each genotype.

### 2.7. *Drosophila* longevity testing

To measure lifespan, 10 newly-eclosed adult males were collected into each of a total of 20 tubes which were kept at 25˚C and 60% humidity. Flies were transferred into new fresh food vials every 2-3 days and the number of living and dead were counted each time. We used GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) to graph survival curves and a log-rank analysis to determine differences, if any, in life span.

## 2.8. Immunocytochemistry

### 2.8.1. Embryonic Neuronal Culture Preparation

Single *Drosophila* embryos aged 1-2 hours were collected incubated at 25°C for 5-6 hours. Double-sided scotch tape was used to dechorionated single embryos which were then covered with halocarbon oil. Embryos were staged and stage 9-10 were used. A microelectrode with a broken tip was used to perforate the embryo and remove all the contents which were dissociated in a 20 μL drop of cultured medium of 18% fetal bovine serum, FBS (Invitrogen, Waltham, MA), 1% penicillin-streptomycin and Schneider's Medium (Gibco, Scotland, UK) on a 22x22 mm glass coverslip (VWR, Radnor, PA) placed inside a 35x10 mm culture dish (ThermoFisher, Waltham, MA). Cultures were then allowed to grow in a humidified chamber at room temperature overnight. After growth, coverslips were transferred into modified culture dishes for microscopy experiments.

Because we were working with an upright microscope, we needed to place the coverslip with neurons underneath the dishes. To modify these dishes, we took culture dishes of size 35x10 mm (Cellstar, Bioexpress) and drilled in the middle at a diameter twice the size of the 20 μL culture medium. Coverslips with cultures were adhered underneath these dishes with SYLGARD (World Precision Instruments, Sarasota, FL) so the culture-containing drop is situated in the middle of the drilled hole. Once cured, dish was flooded with 2 mL culture media and allowed to sit for 5 minutes to test for leaks. If no leaks, plate was placed into the inverted microscope for visualization.

### 2.8.2. Testing Different Experimental Conditions

Neuronal cultures mounted in floodable culture dishes filled with 2 mL culture media were visualized with a Carl Zeiss inverted microscope equipped with an ApoTome unit, for optical sectioning, and an EC Plan-Neofluar 40x/0.75 objective lens. All images of optical sections

through clusters of neuronal cell bodies were recorded with an Axiocam 503 and the Zen Blue Edition software (Carl Zeiss, Oberkochen, Germany). *Drosophila* embryos expressing CD8-GFP and GFP-$K_v$4/Shal were first used to determine if photobleaching was going to be a factor in the long exposure times needed for ApoTome microscopy. Depending on fluorescence signal intensity, we acquired images using exposure times between 700 milliseconds and 5 seconds. For GFP and mCherry signals, we used an eGFP filter block with excitation wavelength at 488 nm and emission wavelength at 509 nm. For mCherry signal, we used an AF568 filter block with excitation wavelength at 577 nm and emission wavelength at 603 nm. For testing the effects of exposure of ROS to neuronal cultures, $H_2O_2$ (Sigma-Aldrich, St. Louis, MO) diluted with nanopure water was directly added to the 2 mL of media in the embryonic culture dish to final concentrations of 100 μM, 1 mM, or 5 mM making sure the volume increase would not be more than 1% of the 2 mL.

# CHAPTER 3. AGE-DEPENDENT CHANGES IN $K_V4$ CHANNEL LEVELS AND THEIR CONTRIBUTION TO LOCOMOTOR PERFORMANCE

## 3.1. Overview

It is well-established that aging results in a decline in functional performance[12]. This decline is likely due to age-related modifications in the neuronal networks of the brain. Ion channels play a critical role in brain function and have been reported to be affected by age. Due to its relatively short life-span, wide genetic tool kit, general understanding of ion channel function, and the ability to perform population studies, *Drosophila* presents us with an ideal model system. We use *Drosophila* to characterize age-dependent decline in locomotor function and to uncover age-related molecular mechanisms that affect ion channels that may lead to this decline in locomotor behaviors. In this chapter, we characterized the decrease in *Drosophila* locomotor performance with age and found that there is an age-dependent decline in locomotor performance that begins at 30 days of age. We found that the ion channel $K_v4$, which has been reported to play a role in *Drosophila* rhythmic behaviors including locomotion[359], also undergoes an age-dependent decline in protein levels. Our data suggest that this progressive loss of $K_v4$ channels seems to be specific for $K_v4$, and not a general phenomenon occurring to all voltage-gated potassium ion channels. We also found that overexpression of $K_v4$ increased locomotor performance in flies at each tested age. To determine if the age-dependent loss of $K_v4$ channels is also seen in a mammalian system, we measured $K_v4.1$, $K_v4.2$, and $K_v4.3$ protein levels in different regions of the mouse brain. Though some levels of channels increase with age in different brain regions, we found that $K_v4.2$ decreases with age in the hippocampus of the mouse brain.

### 3.2. Age-Dependent Decline in Locomotor Performance In *Drosophila*

The age-dependent decline in locomotor ability is a well-known phenomenon affecting all species, from nematodes to humans[13,23,476–478]. In *Drosophila*, the half-life of the organism has been characterized extensively and has been defined at ~75 days of age[117,479–487]. To determine the onset of this age-dependent decline, we measured locomotor performance in *Drosophila* at 3, 10, 20, 30, 40, 50, and 60 days of age. We found that, between 3 and 20 days of age, there were no significant effects on performance (Figure 3.1). By 30 days, however, there was a 20% decline in locomotor performance compared to 20 days. Every subsequent measurement, after 30 days through 60 days of age, showed significant decreases in locomotor performance. When compared to 3 days, locomotion decreased by 30%, 60%, 70%, and 75% at 30, 40, 50, and 60 days, respectively, Figure 3.1. These results show that the onset of the age-dependent decline in locomotor performance occurs at 30 days of age in wildtype *Drosophila*.

In a previous study, expression of the dominant negative $K_V4$ subunit ($DNK_V4$) led to a loss of $K_V4$ function and reduced locomotor abilities in *Drosophila*[359]. We used the UAS/GAL4 system to express *UAS-$K_V4$* with the goal of testing if an overexpression of this channel would lead to a gain in performance and, if so, whether this gain in performance could delay the onset of the age-dependent decline in locomotor abilities. We first verified that *UAS-$K_V4$* could be overexpressed in *Drosophila* at different ages. We overexpressed *UAS-$K_V4$* pan-neuronally and measured $K_V4$ levels at 3 and 10 days post-eclosion and found that there is a significant increase in $K_V4$ protein levels at both time-points, Figure 3.2. Next, we performed locomotor performance measurements on these flies. We collected 35-40 adult males at the bottom of test tube 1 and allowed them to climb for 30 seconds into test tube 2. We performed this for a total of 10 tubes and the population of each tube was given a weighed score.

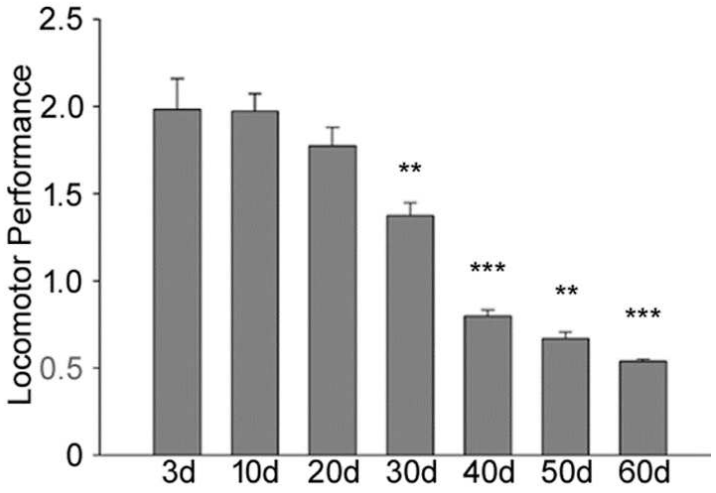


**Figure 3.1. There is an age-dependent decline in *Drosophila* locomotor performance.** Behavioral experiments measuring locomotor performance of adult flies during aging. Locomotor performance exhibits an age-dependent decline that begins to show a significant difference at 30 days of age. Each marked significance is compared to the previous day measurement (35-40 flies per sample, n=10 per day measured, ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test).

Though the onset of the decline in locomotion was not delayed, we detected a significant increase in locomotor performance at all ages, except at 60 days, Figure 3.2. To test the effects of overexpression of another A-type potassium channel on *Drosophila* locomotor performance, we expressed *UAS-$K_v1$*. The age-dependent decline in locomotor performance was exacerbated with *UAS-$K_v1$* overexpression at every age, Figure 3.2. These results suggest that the $K_v4$ A-type channel specifically plays a role in locomotor performance, and that loss of $K_v4$ cannot be compensated for by another $K^+$ channel.

Because a previous study reported that neuronal expression of $DNK_v4$ also led to a shortened lifespan in *Drosophila*[359], we tested the effects of constitutive expression of *UAS-$K_v4$* on longevity. We performed this experiment twice and in neither case were we able to detect any change in *Drosophila* lifespan, Figure 3.2, top-right panel. These results show that overexpression of $K_v4$

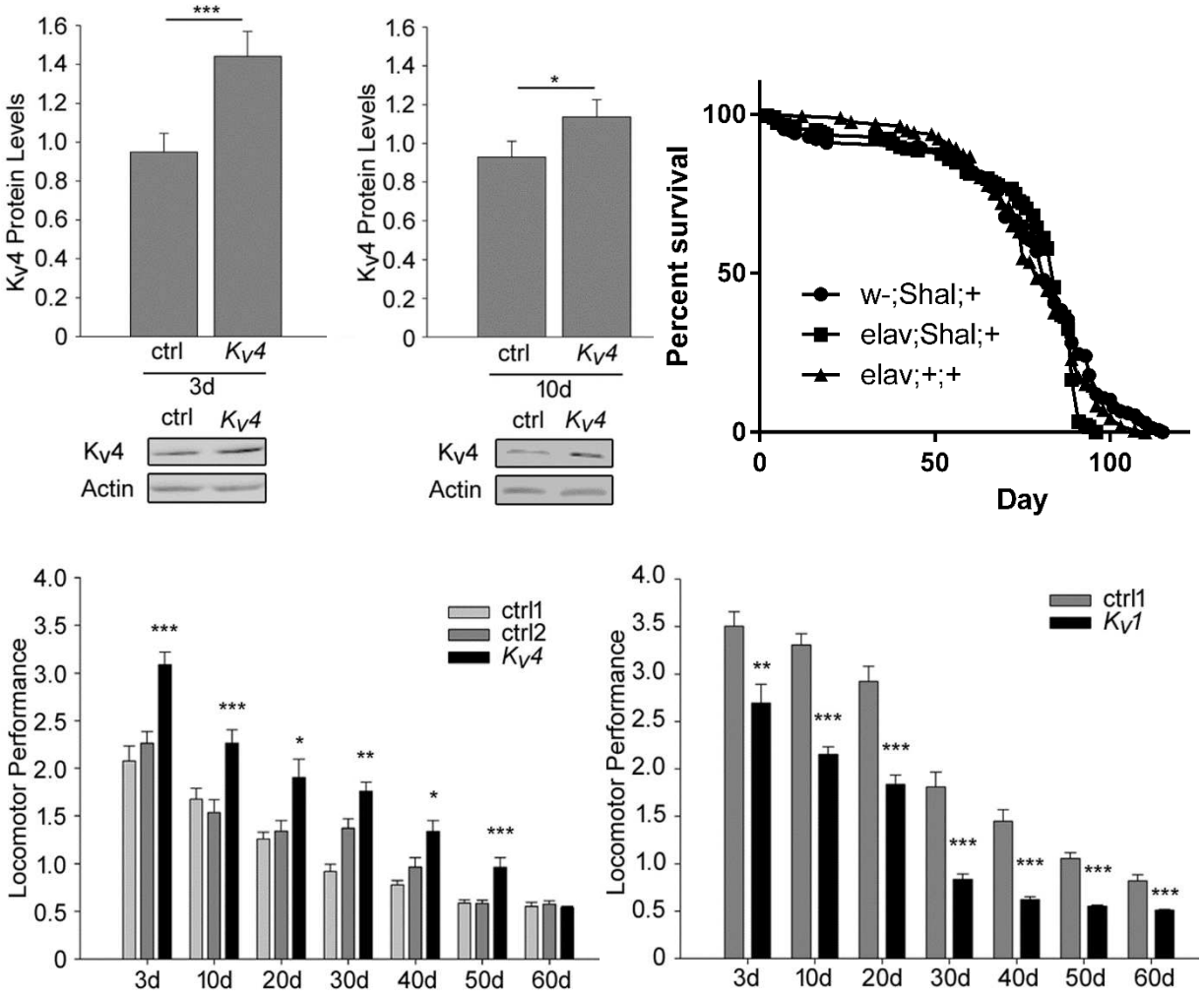


**Figure 3.2. Constitutive overexpression of $K_v4$ increases locomotor performance at every age tested but has no effect on longevity.** Top-Left, immunoblot experiments show that $K_v4$ channel levels have a significant increase with pan-neuronal overexpression of $K_v4$ at 3d and 10d of age, using Actin as a control (5 heads per sample, n=20, 6 blots, * $p \leq 0.05$, *** $p \leq 0.001$, Student's t-test). Top-Right, this constitutive expression of $K_v4$ does not increase lifespan in the fly (samples were 10 flies/vial, 20 vials per genotype, Log-rank statistical analysis). Bottom-Left, constitutive $K_v4$ overexpression significantly increases locomotor performance at each tested age up to 50 days of age – "ctrl1" represent wildtype control Canton-S flies, "ctrl2" represents *UAS-$K_v4$* genetic background flies, and "*$K_v4$*" represents flies constitutively expressing $K_v4$ channels (35-40 flies per sample, n=10 per genotype per day tested, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test between *$K_v4$* and ctrl2). Bottom-Right, constitutive expression of $K_v1$ channels significantly decreases locomotor performance at all tested ages – "ctrl1" represents *UAS-$K_v1$* genetic background flies, "*$K_v1$*" represents flies overexpressing $K_v1$ channels (35-40 flies per sample, n=10 per genotype per day tested, ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test).

does not increase the lifespan of the fly. These data suggest that, while increasing $K_v4$ does not increase longevity, it does enhance locomotor performance.

### 3.3. Age-Dependent Decrease In *Drosophila* $K_v4$ Channels

Because wildtype locomotor performance declined with age and an overexpression of $K_v4$ led to increased locomotor performance with age, we performed immunoblot experiments measuring $K_v4$ protein levels from wildtype *Drosophila* heads during aging. We first prepared adult fly head homogenates using β-mercaptoethanol as the reducing agent. We detected a signal with our α-$K_v4$ antibody in the high-molecular weight region of the resolving portion of the gel, Figure 3.3. When we used the stronger reducing compound dithiothreitol, this high-molecular weight signal was eliminated, Figure 3.3. This allowed us to more confidently quantify total $K_v4$ protein, eliminating possible high-molecular weight aggregations of the channel. We measured levels of $K_v4$ protein at 3, 10, 14, 20, 30, and 40 days of age. We found that $K_v4$ protein levels

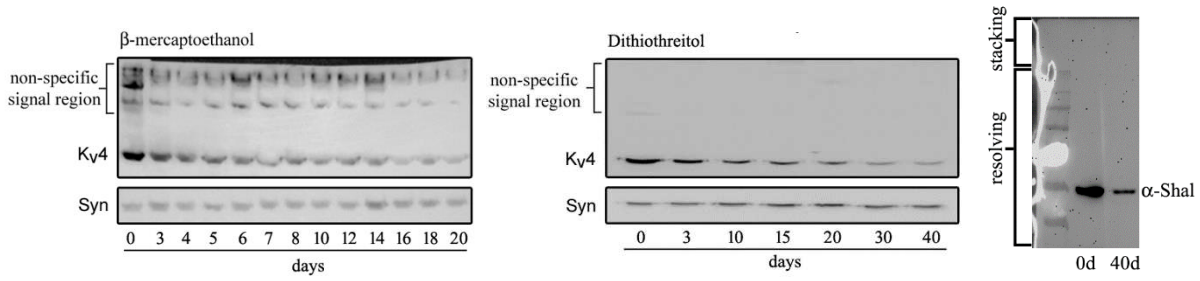


**Figure 3.3. Choosing the right reducing agent for sample preparation.** Immunoblot experiments labeling with α-$K_v4$ and α-Syntaxin as control (5 heads per sample, 4 blots per experiment). Left, resolving SDS-PAGE gel showing protein extract treated with the reducing agent β-mercaptoethanol shows an age-dependent decline in $K_v4$ signal between fly eclosion and 20 days post-eclosion. There is also immunoreactivity of high molecular weight which could, potentially, represent protein aggregation of $K_v4$. Middle, resolving SDS-PAGE showing protein extract treated with the stronger reducing agent dithiothreitol also shows an age-dependent decline in $K_v4$ protein levels but without the high-molecular weight species between newly eclosed and 40-day old flies. Right, immunoblot showing both resolving and stacking gels to confirm that no high-molecular weight signal is present even in the stacking portion of the gel.

undergo a decline characterized by an initial decrease of 50% in $K_v4$ protein levels between 3 and 10 days of age, followed by a more gradual yet progressive decline from 10 to 40 days of age, Figure 3.4. This protein decline, especially the more gradual decline after 10 days, might contribute to the age-dependent decline in locomotor performance observed.

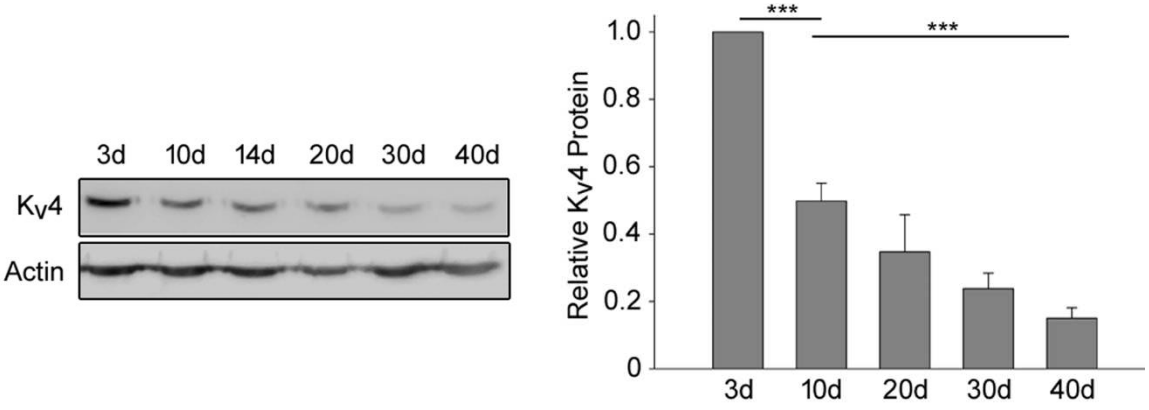


**Figure 3.4. $K_v4$ protein levels undergo a progressive age-dependent decline.** Immunoblot experiments labeling with α-$K_v4$ and α-Actin as control (5 heads per sample, n=7, 7 blots, ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test). Protein levels undergo an age-dependent progressive decline with age. There is ~50% decline in $K_v4$ proteins levels between 3d and 10d of age in adult flies. There is a 70% decrease in levels between 10d and the much older 40d flies.

### 3.4. The Decline In $K_v4$ protein Is Likely Specific For $K_v4$

To test if this progressive age-dependent decline in $K_v4$ channel protein levels is specific to $K_v4$, and not a consequence for all potassium channels, we measured protein levels in two other structurally-similar potassium channels in young and aged flies – dSK and $K_v1$, Figure 3.5. We first measured the levels of the *Drosophila* calcium-activated potassium channel dSK immediately post-eclosion (0d) and at 40 days of age. We detected no significant differences in dSK protein levels between newly eclosed and 40-day old flies, suggesting that dSK channels do not undergo an age-dependent decline in protein level. We found, however, a 30% decline in $K_v1$ protein from

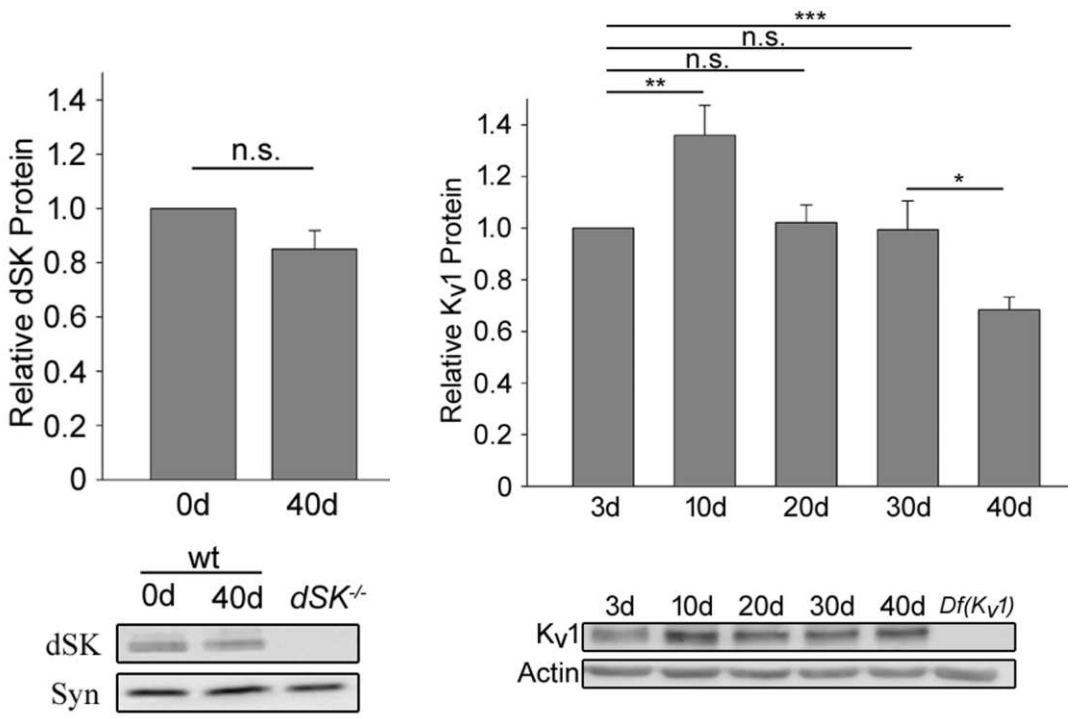


**Figure 3.5. The age-dependent decline in $K_v4$ channel protein levels is likely not a general characteristic of all potassium channels.** Left, immunoblot experiments measuring dSK and Syntaxin levels, using a *dSK*$^{-/-}$ as control. There is no significant difference in dSK protein levels between young newly eclosed flies and those at 40 days of age (5 heads per sample, n=10, 4 blots, n.s. denotes no significant difference, Student's t-test). Right, immunoblot experiments measuring $K_v1$ and Actin levels, using *Df($K_v1$)* as control. There is a transient significant increase in $K_v1$ protein levels between by 10d of age which reverts to original levels by 20 days of age, and a significant decline in $K_v1$ levels by 40 days of age (5 heads per sample, n=11, 11 blots, n.s. indicates no significant difference, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$Student's t-test).

3 to 40 days of age. When we examined additional ages, we found that this protein decrease mostly occurs between 30 and 40 days of age; $K_v1$ protein levels remain unchanged, for example, between 3 and 30 days of age. Interestingly, we also found that there is a transient increase in $K_v1$ levels between 3 and 10 days of age, which returns to baseline levels by 20 days of age. Bergquist *et al.* (2010) had described a mutual relationship between $K_v1$ and $K_v4$, and our data showed both consistent and inconsistent results to those reported by them. In their report, their *$K_v4$/Shal* knockout fly, *Shal495*, resulted in increased *$K_v1$/Shaker* mRNA levels. They also reported that

either on a $K_v1$ functional mutant (*Shaker*$^{14}$) or with expression of neuronal *Kv1/Shaker-RNAi*, $K_v4$*/Shal* mRNA levels increased. Because their report indicated that that $K_v4$ and $K_v1$ were reciprocally transcriptionally coupled[488], one possibility is that the decline in $K_v4$ protein resulted in an increase in $K_v1$ protein levels at 10 days of age. Consistent with their report, we found that the functional knockout, $DNK_v4$, led to increased $K_v1$ protein levels by 40% and 30% in 3 and 14-day old flies, respectively, suggesting that $K_v4$ function likely plays a role in expression of $K_v1$ and that this increase is not necessarily dependent on age, Figure 3.6. We also tested levels of $K_v4$ channels in a $K_v1$-deficient fly – *Df(*$K_v1$*)* – and the levels of $K_v1$ channels in a $K_v4$-functional knockout. We found that total $K_v4$ protein levels still undergo an age-dependent decrease in a fly that does not express $K_v1$ – *Df(*$K_v1$*)* – suggesting that the progressive decline in $K_v4$ protein does not depend on expression of $K_v1$ channels, Figure 3.6.

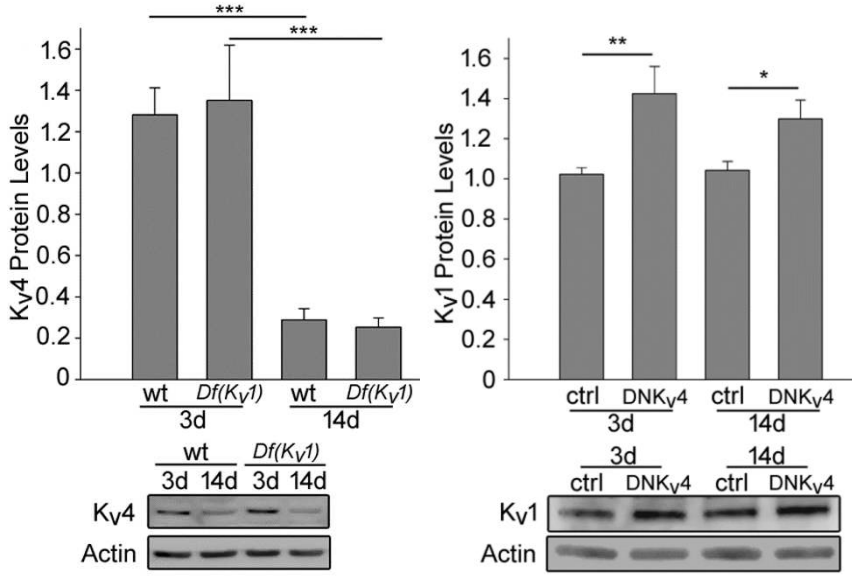


**Figure 3.6. $K_v4$ protein levels do not depend on $K_v1$ protein expression, instead $K_v1$ levels are, in part, controlled by $K_v4$ function.** Left, immunoblot experiments measuring $K_v4$ and Actin, as control, protein levels in 3d and 14d-old wildtype or *Df(*$K_v1$*)* flies. There is a significant decrease in $K_v4$ protein levels with age regardless of $K_v1$ channel presence (5 heads per sample, n=12, 6 blots, *** $p \leq 0.001$, Student's t-test). Right, immunoblot experiments measuring $K_v1$ protein levels in 3d and 14d-old wildtype or *DNK*$_v$*4*-expressing flies. The loss of $K_v4$ function leads to an increase in $K_v1$ levels at 3d and 14d post-eclosion (5 heads per sample, n=18, 8 blots, * $p \leq 0.05$, ** $p \leq 0.01$, Student's t-test)

### 3.5. Age-Effects On Mouse $K_v4$ Protein Levels

To determine if the effects of age on $K_v4$ channels is a phenomenon that is conserved across species, we measured the levels of $K_v4.1$, $K_v4.2$, and $K_v4.3$ in different regions of the mouse brain where $K_v4$ channels are required for proper function: hippocampus, olfactory bulb, cerebellum, and motor cortex[300,330,338,361,386,387,489]. Dr. Mario Oyola, a post-doctoral associate in Dr. Bob Handa's laboratory at Colorado State University extracted tissue from a total of 14 mice – seven at 6 weeks of age and seven at 13 months of age – and provided frozen samples to me for immunoblot analyses.

In the hippocampus, we were not able to reliably detect $K_v4.1$. It is possible that $K_v4.1$ is not expressed at high enough levels in this brain region. Because we collected data from many different blots and multiple mice, we performed a Linear Mixed Model (LMM) statistical analysis to compare $K_v4.2$ or $K_v4.3$ protein levels between 6 week and 1-year old mouse groups. Unlike the Student's t-test which compares two normal distributions, the LMM takes into account more variables and is compatible with distributions that have no normality. In our case, the comparison was protein levels between young and old mice, and with this mathematical function we were able to include the variability of protein concentrations across different mice and the variability across experiments into the equation, Figures 3.7-3.10. We were able to measure a ~20% age-dependent decline in $K_v4.2$ protein level between 6-week and 1-year old mice in the hippocampus, Figure 3.7. The levels of $K_v4.3$ protein, however, increased by ~50% with age, possibly as a compensatory mechanism because of the age-dependent loss of $K_v4.2$ channels. Alternatively, the levels of $K_v4.2$ might have decreased as a compensatory response to the large increase in $K_v4.3$ proteins.

Next, we looked at the concentration of $K_v4$ channels in the olfactory bulb. Here, we were able to measure the protein levels of all three $K_v4$ channel types, Figure 3.8. We were not able to

detect any significant differences in $K_v4.1$, $K_v4.2$, or $K_v4.3$ channel levels between 6-week and 1-year old mice. The protein across the different brain extractions is also quite varied.

In the cerebellum, the levels of $K_v4.1$ were very low; however, we were able to measure protein levels of $K_v4.2$ and $K_v4.3$ successfully, Figure 3.9. There was no change in $K_v4.2$ protein levels with age. Levels of $K_v4.3$, however, were significantly increased by ~40% in 1-year old mice when compared to 6-week old mice.

In the motor cortex, we did not measure significant differences in $K_v4.1$ or $K_v4.2$ protein levels, Figure 3.10. For $K_v4.3$ measurements, however, we noticed a ~30% increase in channel levels. Altogether, these results indicate that the effects of age on $K_v4$ protein are more complex in mammals. Indeed, though $K_v4.3$ was found to increase with age in the hippocampus, cerebellum, and motor cortex, levels of $K_v4.2$ decreased in the hippocampus, an area of the brain involved in learning and memory which has been described to decline with age[490].

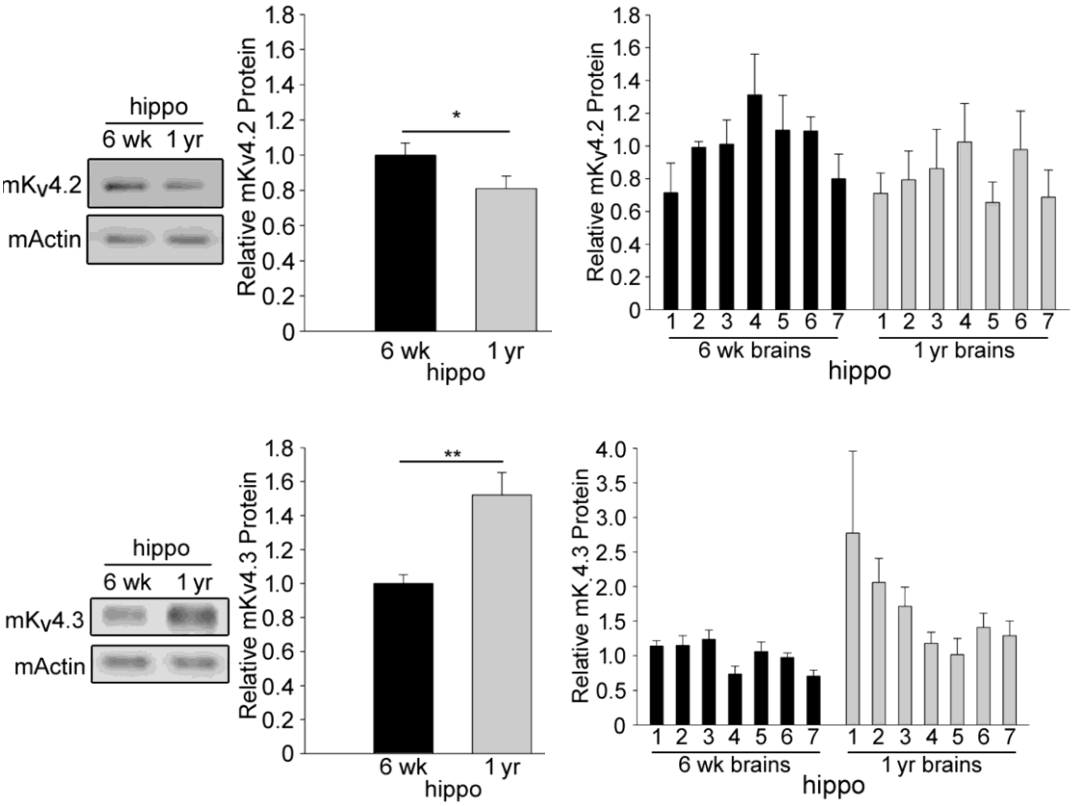


**Figure 3.7. $K_v$4.2 and $K_v$4.3 protein levels decrease and increase with age, respectively, in the hippocampus of mouse brain.** Black bars represent 6-week (6wk) old mice and grey bars represent 13-month (1yr) old mice (samples were obtained from individual hippocampus extractions from 7-6wk and 7-1yr brains, 15µg total protein per sample from homogenate loaded into each SDS-PAGE well, n=35, 5 blots, * $p \leq 0.05$, ** $p \leq 0.01$, Linear Mixed Model statistical analysis). Top, immunoblot experiments show that there is a significant decline in $K_v$4.2 protein levels in the hippocampus between 6-week old and 1- year old mice. The levels of $K_v$4.2 across different mice brains is not constant, as results show that they vary across each brain tested. Bottom, immunoblot experiments show that there is a significant increase in $K_v$4.3 protein levels in the hippocampus between 6wk and 1yr old mice. $K_v$4.1 signal was too low to perform any reliable data analysis.

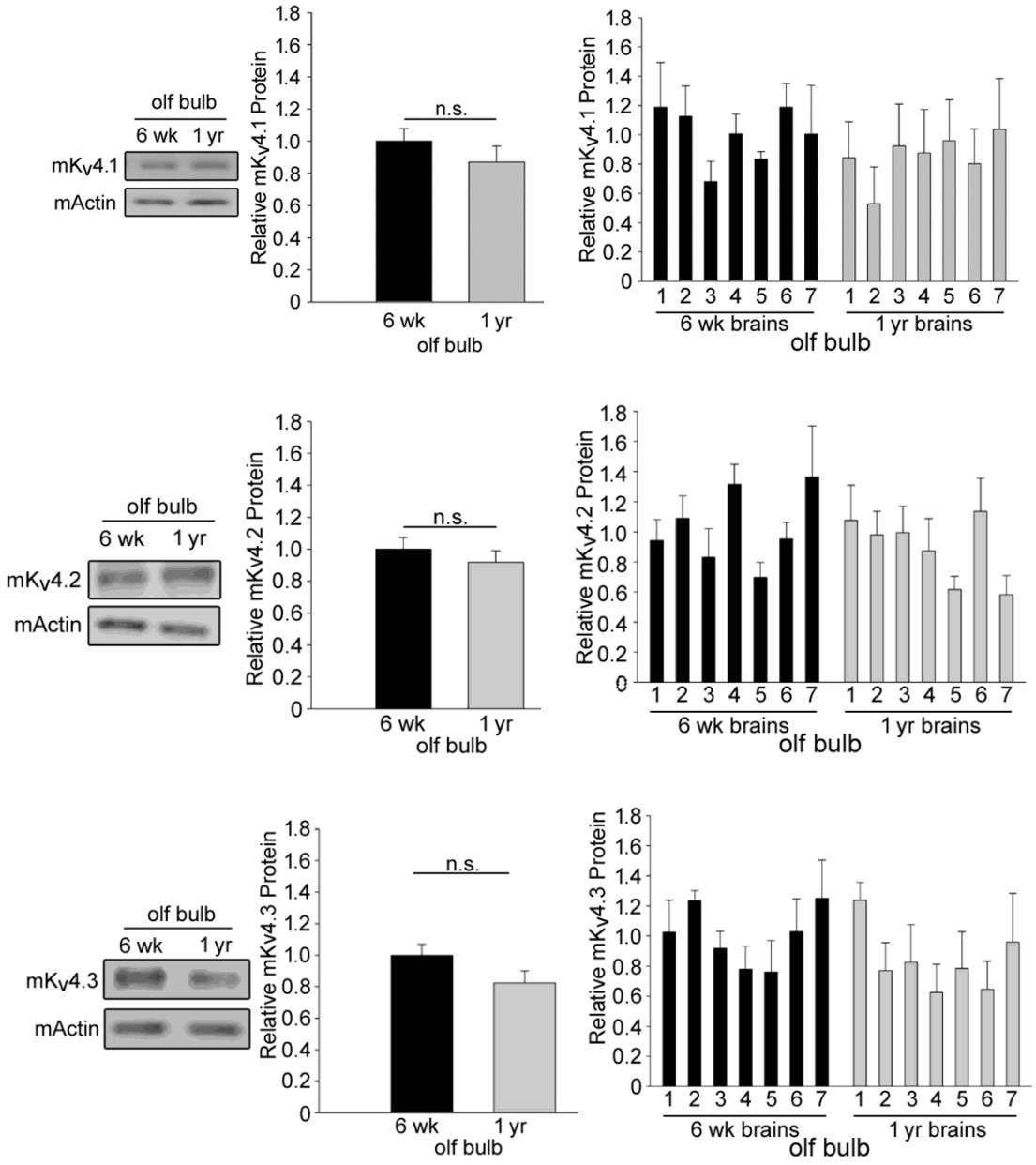


**Figure 3.8. $K_v$4.2 and $K_v$4.3 protein levels remain unchanged with age in the olfactory bulb of mouse brain.** Black bars represent 6-week (6wk) old mice and grey bars represent 13 month (1yr) old mice (samples were obtained from individual hippocampus extractions from 7-6wk and 7-1yr brains, 15µg total protein per sample from homogenate loaded into each SDS-PAGE well, n=35, 5 blots, n.s. indicates no significant difference, Linear Mixed Model statistical analysis). Top, immunoblot experiments show that there is no significant difference in $K_v$4.1 levels between 6wk and 1yr mice. Middle, immunoblot experiments show that there are no significant changes in $K_v$4.2 protein levels in the olfactory bulb between 6 week-old and 1 year-old mice. Bottom, immunoblot experiments show that there is no change in $K_v$4.3 protein levels in the olfactory bulb between 6wk and 1yr old mice.

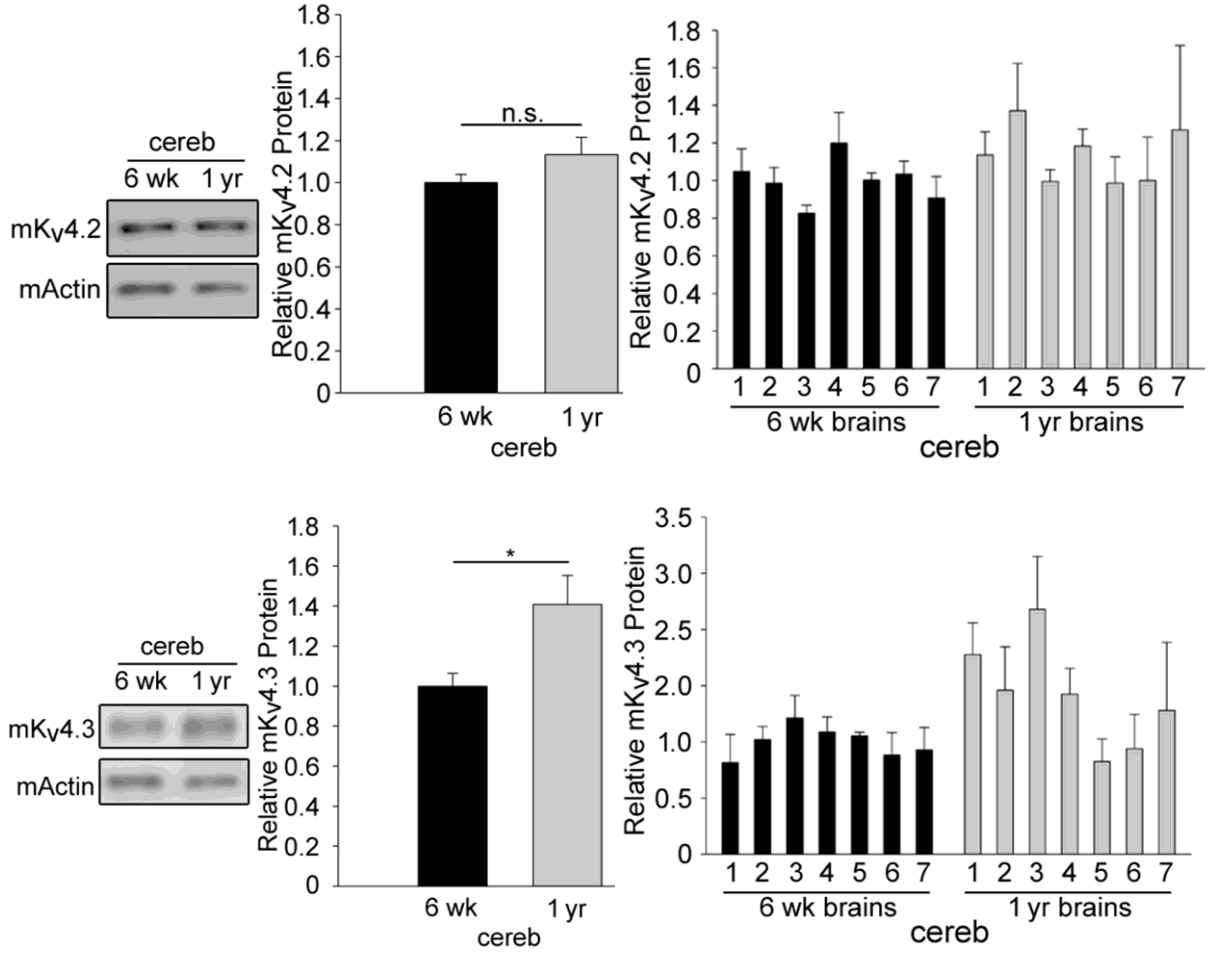


**Figure 3.9. Levels of $K_v$4.3, but not $K_v$4.2, protein increase with age in the cerebellum of mouse brain.** Black bars represent 6-week (6wk) old mice and grey bars represent 13 month (1yr) old mice (samples were obtained from individual hippocampus extractions from 7-6wk and 7-1yr brains, 15μg total protein per sample from homogenate loaded into each SDS-PAGE well, n=35, 5 blots, n.s. indicates no significant difference, * $p \leq 0.05$, Linear Mixed Model statistical analysis). Top, immunoblot experiments show that there is no significant difference in $K_v$4.2 protein levels between 6wk and 1yr mice. Bottom, immunoblot experiments reveal that there is a significant increase in $K_v$4.3 protein levels between 6wk and 1yr mice. $K_v$4.1 signal was too dim to perform any honest data analysis.

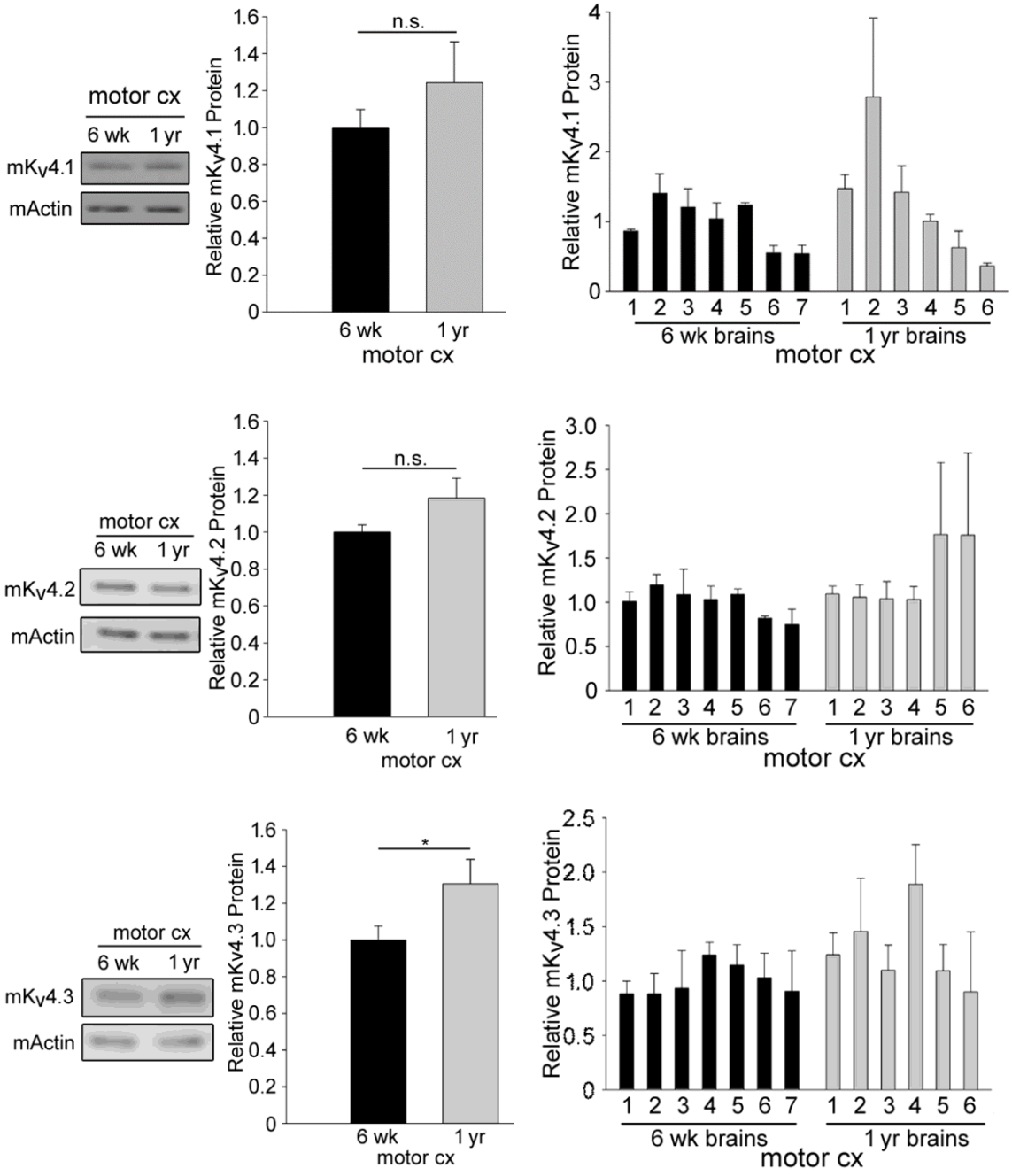


**Figure 3.10. Levels of $K_v$4.3, but not $K_v$4.1 or $K_v$4.2, protein increase with age in the motor cortex of mouse brain.** Black bars represent 6-week (6wk) old mice and grey bars represent 13 month (1yr) old mice (samples were obtained from individual hippocampus extractions from 7-6wk and 6-1yr brains, 15μg total protein per sample from homogenate loaded into each SDS-PAGE well, n=16-35, 3-5 blots, n.s. indicates no significant difference, * $p \leq 0.05$, Linear Mixed Model statistical analysis). Top, immunoblot experiments show that there is no significant difference in $K_v$4.2 levels between both young and old mice. Bottom, there is a significant increase in $K_v$4.3 levels in 1yr mice when compare to 6wk ones.

# CHAPTER 4. EFFECTS OF ROS ON $K_v4$ ION CHANNEL LEVELS

## 4.1 Overview

The age-dependent accumulation of ROS is a well-established phenomenon that occurs in cells[491] and has been termed the "mitochondrial free radical theory of aging"[56]. Oxidative damage can cause proteins to become nonfunctional which typically triggers them to be targeted for degradation. We hypothesized that ROS may play a role in the age-dependent decline of $K_v4$ channels. In this chapter, I present the effects of acute exposure of $H_2O_2$ *in vivo* and *in vitro* on $K_v4$ channel levels. We found that, though $H_2O_2$ exposure did not cause any qualitative changes in tagged-$K_v4$ (GFP-$K_v4$ or mCherry-pHluorin-$K_v4$) in neuronal cultures, $K_v4$ protein levels were affected by $H_2O_2$ in whole flies. We then confirmed that ROS levels increase in *Drosophila* with age and examined the effects of expressing superoxide dismutase 1 and 2 (SOD1 and SOD2), and catalase, enzymes that regulate intracellular ROS, on levels of $K_v4$ protein at different ages of the adult fly. We found that *Catalase* expression in 40-day flies, but not *SOD1* or *SOD2*, ameliorates the decline in $K_v4$ levels. This *Catalase* expression in 4-day old flies resulted in lower ROS levels when compared to wildtype. I also make use of RNAi to knockdown expression of two ROS producing enzymes, *NOX* and *DUOX*, and test their effects on $K_v4$ channel levels. We found that a knockdown of *NOX*, but not *DUOX*, causes increased levels of $K_v4$. Because flies that expressed either *Catalase* or *NOX-RNAi* resulted in increased levels of $K_v4$, we tested their locomotor performance. We found that with expression of *Catalase*, but not *NOX*, *Drosophila* locomotor performance is improved.

### 4.2 Effects of exposing *Drosophila* to $H_2O_2$

To test if ROS have an effect at the organismal levels that leads to a decline in $K_v4$ channels, we exposed live *Drosophila* to $H_2O_2$ and tested the effects of this exposure on $K_v4$ protein levels. We placed 30-35 flies in scintillation vials and included a round paper filter with 100 μL of 30% (8.82 M) $H_2O_2$, Figure 4.1. After a 4-hour incubation, we transferred the flies into regular food vials for recovery at room temperature. We measured $K_v4$ levels immediately after $H_2O_2$ exposure (0 hours), and after 4, 24, and 48 hours recovery. Though there were no significant differences in $K_v4$ protein levels for at least 4 hours after $H_2O_2$ exposure (data not shown), we measured a ~20% decrease in $K_v4$ levels 24 hours after $H_2O_2$ exposure, Figure 4.1. These results show that a short 4-hour exposure to $H_2O_2$ has a detrimental effect on $K_v4$ channel protein levels

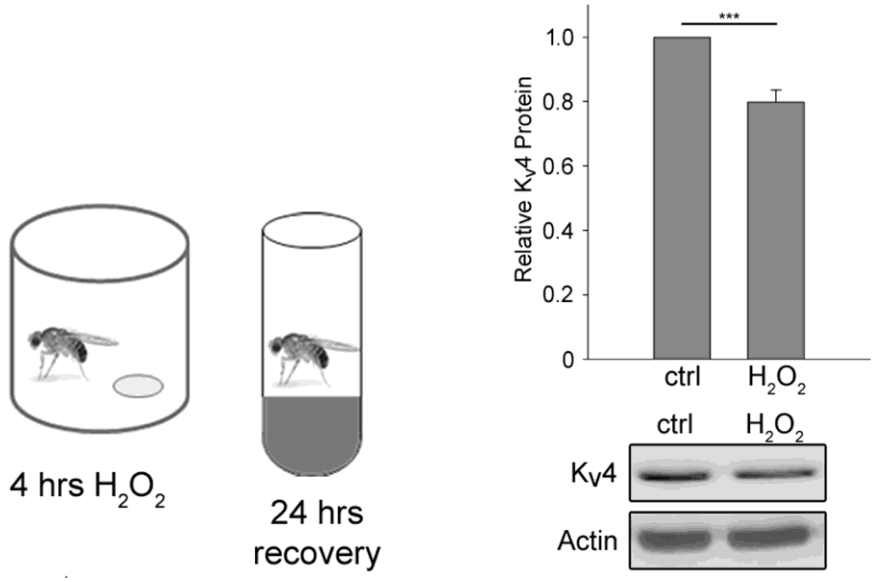


**Figure 4.1. Acute exposure to hydrogen peroxide leads to decreased $K_v4$ protein levels in *Drosophila*.** Left, cartoon depicting *Drosophila* in a closed scintillation vial with a paper filter containing 100 μL $H_2O_2$ for 4 hours and 24 hours recovery in a regular *Drosophila* food vial at room temperature. Right, immunoblot experiments showing a decrease in $K_v4$ protein levels, using Actin as a control, upon 4 hour $H_2O_2$ exposure with 24 hours recovery – "ctrl" represents the experiment where $H_2O$ was added to the paper filter, "$H_2O_2$" represents the experiment where $H_2O_2$ was added to the paper filter (5 heads per sample, n=15, 15 blots, *** $p \leq 0.001$, Student's t-test).

in living flies. One possibility is that it takes 4 hours for $H_2O_2$ to overwhelm cellular antioxidant defenses in *Drosophila* and cause enough oxidative damage to $K_v4$ which could then be targeted for degradation. Recovery of channel levels likely depends on the time required for $K_v4$ protein turnover. After 48 hours recovery from a 4 hours $H_2O_2$ exposure, $K_v4$ levels were restored, suggesting that damaged $K_v4$ protein were replaced by newly synthesized channels. We hypothesized that accumulation of ROS with age might contribute to the progressive age-dependent decline of $K_v4$ channels we measured previously, Chapter 3, Figure 3.4

### 4.3. Effects of Exposing Cultured Neurons To $H_2O_2$

We next tested the effects of $H_2O_2$ on $K_v4$ channels in primary *Drosophila* cultured neurons. We took advantage of the *UAS/GAL4* system[492] and used *elav-GAL4* to pan-neuronally express *UAS-GFP-$K_v$4* or *UAS-mCherry-pHluorin-$K_v$4* in embryonic cultures.

Neuronal clusters are loosely attached to the glass coverslips and obtaining still images over 30 minutes or more was difficult. To solve this problem, I took note of the location of the neuronal cluster of interest using 20X and 40X objective lenses, so I could remove the plate, place it in the humidifier for the allotted incubation time, and then return it in the microscope to identify the same cluster over time. Once this system was implemented, I developed a systematic approach for repeatedly re-focus to the same focal plane. To do this, I selected a cell that was on the edge of the cluster in the focal plane. I focused on the edge of this cell and scanned an image for reference.

Preliminarily, we tested increasing concentrations, from 1 pM to 5 mM, of $H_2O_2$ on neuronal cultures to determine the maximum amount of $H_2O_2$ that could be added without grossly affecting neuronal morphology (data not shown). We determined that at 1 mM $H_2O_2$, a value well beyond physiological concentrations, neuronal morphology appeared relatively normal. To test

whether the addition of $H_2O_2$ would have any effects on GFP itself, we expressed *CD8-GFP*, a GFP-tagged non-specific transmembrane protein, in neurons and exposed them to 1 mM $H_2O_2$ for 1 hour. We used Zeiss ApoTome microscopy to visualize the GFP signal. In comparison to standard fluorescent microscopy, the ApoTome delivers enhanced optical resolution with much better contrast while allowing to visualize images with depth discrimination[493]. We visualized samples in an optical section through 10 neuronal clusters from different culture preparations, Figure 4.2. We noticed no qualitative difference in GFP signal from CD8-GFP expressing neurons between pre- and post- treatment with $H_2O_2$ after 1-hour incubation, suggesting that exposure to $H_2O_2$ likely has no effects on GFP fluorescence, Figure 4.2.

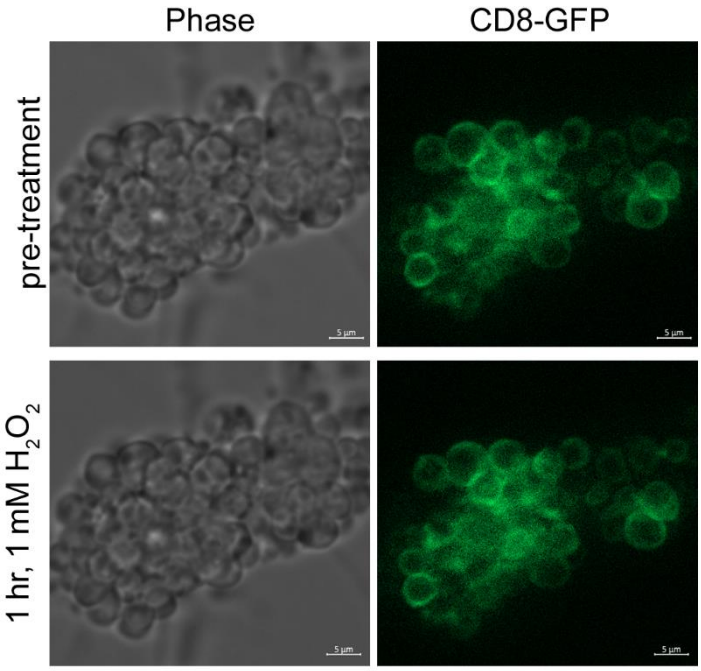


**Figure 4.2. Acute exposure of $H_2O_2$ to CD8-GFP shows no qualitative changes in GFP signal intensity.** Representative neuronal cluster section showing GFP signal from flies expressing *UAS-CD8-GFP* using ApoTome microscopy, using 40X objective lens. Sections correspond to a cross-section of the cluster pre-treatment or exposed to 1 mM $H_2O_2$ for 1 hour. Neuronal cluster morphology was unaffected with $H_2O_2$ exposure. CD8-GFP is non-specific and therefore expressed all throughout the cell membranes. There are no apparent changes in GFP signal after acute exposure to 1 mM $H_2O_2$ for 1 hour (10 clusters from different culture preparations).

We then tested the hypothesis than an acute 1-hour exposure to $H_2O_2$ would lead to a decrease or re-localization of the GFP signal in neurons expressing *GFP-$K_v$4*. We visualized GFP-$K_v$4 in an optical section through 7 neuronal clusters from different culture preparations. The GFP signal appears "moon-shaped", suggesting that these channels do not localize uniformly as did CD8-GFP. Upon addition of 1 mM $H_2O_2$ and incubation for 1 hour, morphology of the cells remained relatively unaffected, Figure 4.3. The GFP signal from GFP-$K_v$4 did not qualitatively decrease in intensity or show any gross re-distribution. Overall, our data shows that a 1-hour incubation with $H_2O_2$ had no effect on GFP signal in neurons expressing *GFP-$K_v$4*, Figure 4.3.

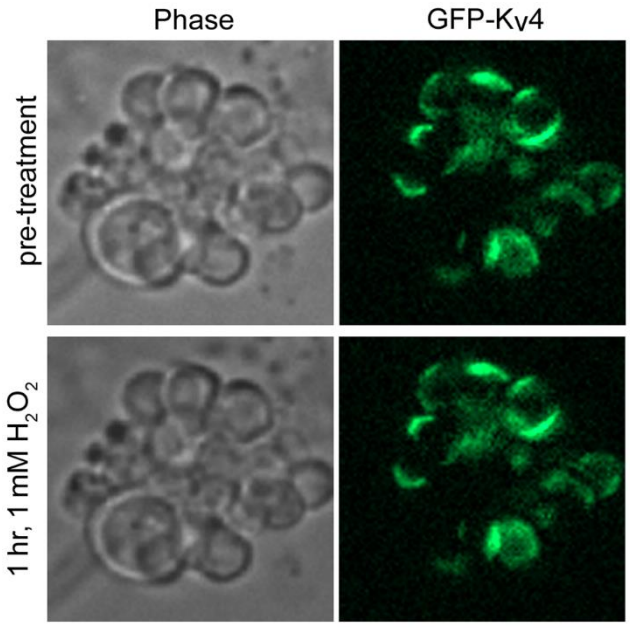


**Figure 4.3. Acute exposure of $H_2O_2$ shows no effects on GFP-$K_v$4 localization and qualitative signal intensity in neuronal cultures.** Representative neuronal cluster section using ApoTome microscopy. Sections correspond to a middle region of the cluster. These clusters were used to measure GFP-$K_v$4 signal with and without $H_2O_2$. Neuronal cluster morphology looks relatively normal between pre-treatment (control) and those exposed for 1 hour to $H_2O_2$. The GFP signal in GFP-$K_v$4 appears moon-shaped and seems to localize to or near the membrane of cells in cluster. There are no evident changes in GFP signal after acute exposure to 1 mM $H_2O_2$ (7 clusters from different culture preparations).

We then performed similar experiments using a *mCherry-pHluorin-$K_v$4* expressing *Drosophila* transgenic line to test the hypothesis that $K_v$4 might be internalized into low pH compartments such as lysosomes and multivesicular bodies, and possibly targeted for degradation. Developed by Miesenböck (1998) and colleagues, the pH-sensitive GFP protein tag (pHluorin) has been useful in fluorescent microscopy studies that aim to monitor internalization of proteins into low pH environments[468]. A mutant of GFP, pHluorin shows a strong fluorescence signal in neutral pH environments and the signal is quenched in lower pH environments such as the inside of a vesicle or lysosome. This tag has been successfully expressed and used to monitor vesicle exocytosis and recycling in mammalian and *Drosophila* cell systems[468,494]. In *Drosophila*, Pankiv and coworkers (2007) further developed this pH-sensitive tag by adding *mCherry* to its sequence. The tandem fusion of the acid-insensitive mCherry with the pH-sensitive typically detected in both red (mCherry) and green (pHluorin) channels when present in a non-acidic environment. Upon internalization, the only signal that can be detected is mCherry. Since then, the mCherry-pHluorin tag has been used in studies that aim to understand pHluorin allowed them to monitor proteins p62 and LC3 being targeted for lysosomal degradation[467]. They described that when the double tagged proteins are protein internalization and vesicular trafficking for degradation in *Drosophila* neurons[495,496].

The N-terminus of $K_v$4 localizes to the cytosol when the channel is in the plasma membrane, and the channel was N-terminally tagged with *mCherry-pHluorin*. Upon internalization of tagged $K_v$4 channels into vesicles, the *mCherry-pHluorin* tag will still be present in the cytosol and both fluorescent signals will be detected. When the vesicle is internalized into, likely, multivesicular bodies (MVB) and further into the lysosome, the pHluorin signal is expected to be quenched and, at this point, we would then only detect mCherry fluorescence. A collaborative

experiment with the laboratory of Dr. Andrew Bean at the University Of Texas Health Science Center in Houston provided us with some evidence that $K_v4$ channels are likely sorted into MVB during turnover. I prepared an expression construct of $K_v4$ with a C-terminal V5 tag. Monica Gireud, in Dr. Bean's laboratory, used this construct in their cell-free reconstitution of MVB cargo sorting system[497,498] and found that $K_v4$-V5 recycles through MVB, data not shown.

We cultured primary *Drosophila* neurons and allowed them to grow for 3 days at room temperature in a humidified chamber. We then visualized optical sections through clusters of neurons and were able to detect both mCherry and pHluorin signals. Both signals qualitatively co-localized to or near the cell membrane of neurons, Figure 4.4. We also noticed the absence of

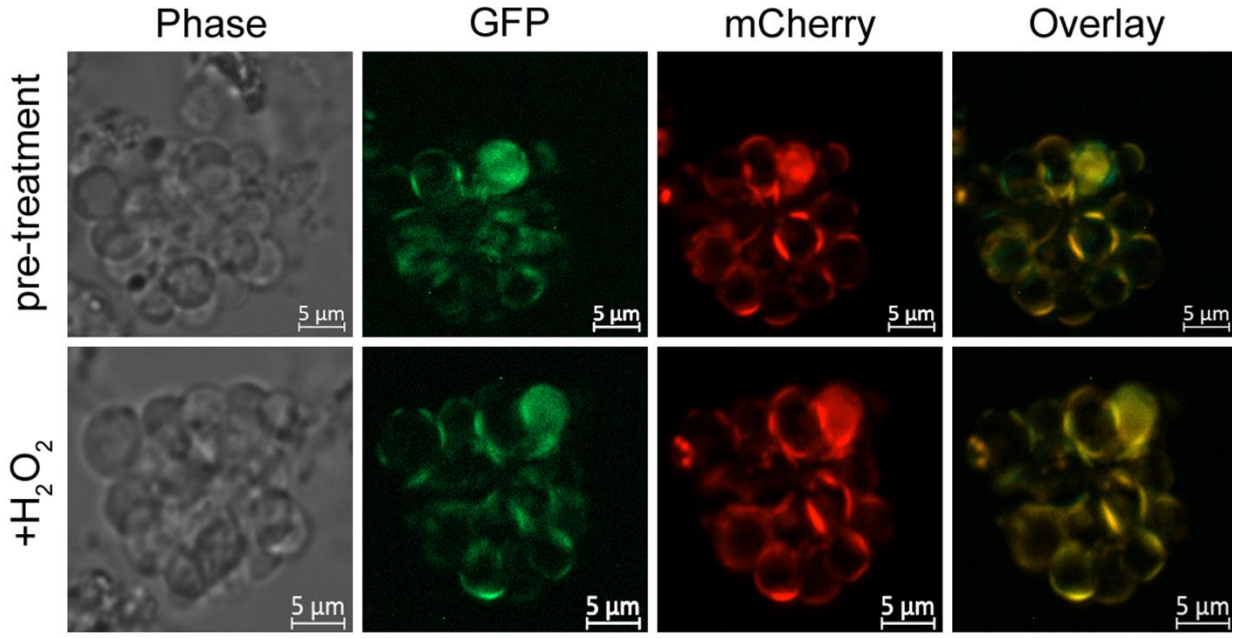


**Figure 4.4. There are no distinct differences in internalization of $K_v4$ into low pH compartments within neurons on cells acutely exposed to 1mM $H_2O_2$ for 24 hours.** Both pre- and post- treatment of $H_2O_2$ are shown. No substantial changes in morphology upon exposure of 3-day-old cultures to 1 mM $H_2O_2$ for 24 hours. pH-sensitive GFP signal showing localization of $K_v4$ not in a low pH environment. mCherry signal depicts the complete neuronal localization of $K_v4$. There is no evident decline in GFP signal, suggesting that exposure to $H_2O_2$ does not produce a relocalization of $K_v4$ into a lower pH environment such as a vesicle (5 clusters from different culture preparations).

any mCherry-positive puncta suggesting that 3-day old cultures do not exhibit detectable mCherry-pHluorin-$K_v4$ in acidic compartments. In 7 to 9-day old cultures, we were also able to detect co-localized mCherry and pHluorin signal. In these older cultures, however, we additionally observed mCherry-positive puncta that did not express pHluorin, Figure 4.5. This qualitative data suggests that after a week, neurons exhibit detectable $K_v4$ protein turnover.

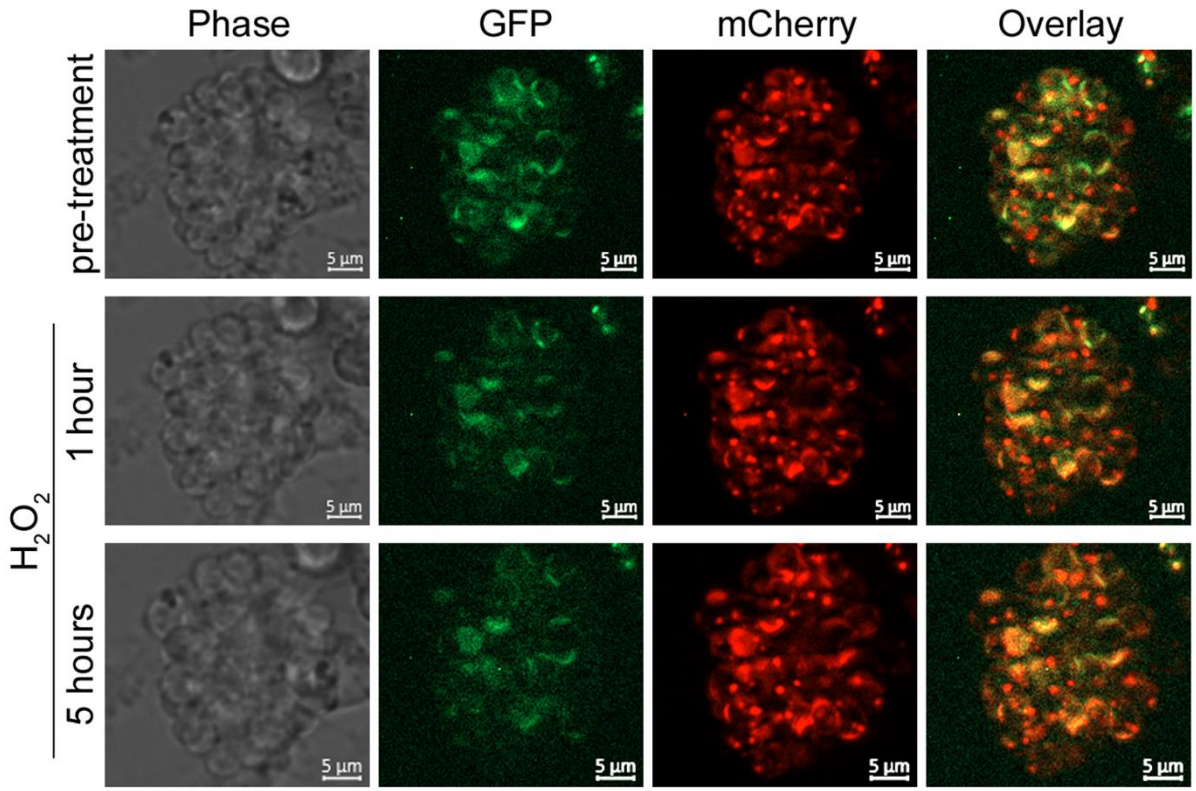


**Figure 4.5. There are no distinct changes on $K_v4$ internalization in 7-day old cultured neurons expressing *mCherry-pHluorin-$K_v4$*.** Pre-treatment, and 1 and 5 hours post-treatment with 1 mM $H_2O_2$ on *Drosophila* cultured neurons that are 7 to 9-days old. pH-sensitive GFP signal shows localization of $K_v4$ in the plasma membrane. mCherry corresponds to the pan-neuronal presence of $K_v4$. Red puncta likely represent internalization of $K_v4$ into low pH compartments. There is no apparent increase in the number of intracellular red puncta after 1 or even 5 hours incubation in 1 mM $H_2O_2$ (4 clusters from different culture preparations).

To test the hypothesis that $K_v4$ internalization would be detected with $H_2O_2$ exposure, we performed a 24-hour 1 mM $H_2O_2$ exposure on 3-day old neuronal cultures and checked for red puncta formation, Figure 4.4. We did not observe formation of any mCherry-labeled puncta in

these cells, suggesting that a 24-hour exposure to 1 mM $H_2O_2$ does not lead to targeting $K_v4$ for internalization into low pH compartments such as lysosomes or multivesicular bodies. Next, to test the hypothesis that $K_v4$ internalization is enhanced with $H_2O_2$ exposure in 7 to 9-day old neuronal cultures, we subjected these cells to 1 mM $H_2O_2$, Figure 4.5. We did not observe any qualitative increase in mCherry-labeled puncta at either time point. Altogether, these results suggest that direct exposure of 1 mM $H_2O_2$ to neuronal cell cultures do not lead to changes in $K_v4$ expression or subcellular localization.

### 4.4. ROS Levels Increase With Age In *Drosophila*

ROS levels have been previously described to increase by 10% in 50 -day old flies compared to 5-day old flies[101]. To verify this finding in our system, we measured ROS levels in 3 and 40-day old flies. We used the reduced form of the ROS reporter 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) to measure ROS levels in young (3d) and old (40d) flies. DCFDA is oxidized by ROS into 2',7'-dichlorofluorescein (DCF). DCF, now a fluorescent compound, was measured using fluorescence spectrophotometry. We found a 10% increase in ROS-related fluorescent signal in 40-day old flies when compared to 3-day old flies,

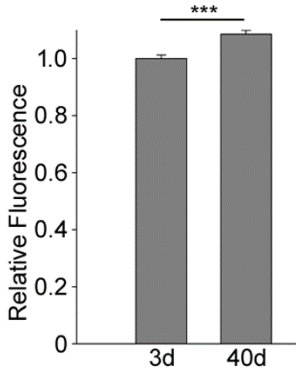


**Figure 4.6. ROS increase with age in wildtype flies.** Spectrofluorimetry experiments measuring fluorescence changes with the ROS sensitive DCFDA compound. There is a significant increase in ROS levels between 3 and 40 day old flies (25 heads per extraction, 5 extractions per experiment with 4 technical replicates each, a total of 3 experiments were performed, *** $p \leq 0.001$, Student's t-test).

Figure 4.6. Although we do not know the source of ROS, these results support the idea that ROS increase with age in the adult fly.

### 4.5. Overexpression And Knockdown Of Enzymes That Regulate ROS Levels

Superoxide dismutase (SOD) 1 and 2, and catalase are enzymes that actively participate in ROS damage control within cells. The role of SOD is to convert superoxide anions to $H_2O_2$, while catalase converts $H_2O_2$ to $H_2O$ and $O_2$[78]. Our previous results showed that half-life of *Drosophila* is 75-80 days and that population numbers do not begin to decrease exponentially until ~50 to 60-day old flies, Figure 3.2. A recent publication reported that *Drosophila* SOD and catalase show decreased enzymatic activity by 50 days of age[101] which is likely a reason for the higher concentrations of ROS measured in older flies. This and our previous results showing that live fly exposure to $H_2O_2$ causes a decline in $K_v4$ levels suggest a model in which increasing ROS levels play a role in the progressive decline in $K_v4$ channel levels.

We next tested whether overexpression of SOD or catalase, which should decrease ROS levels, would reduce the loss $K_v4$ protein. We used the *UAS/GAL4* system to constitutively overexpress *SOD1*, *SOD2*, or *Catalase* in neurons using the genetic driver *elav-GAL4* and measured the effects of expression of these genes on levels of $K_v4$ protein in 20-day old (mid-age) flies. We measured no significant changes in $K_v4$ protein levels with constitutive expression of any of these enzymes, Figure 4.7. One possible explanation is that there is not enough ROS accumulation at 20 days for SOD or catalase to make any difference in ROS-affected $K_v4$ levels.

Since we measured a significant increase in ROS at 40 days, Figure 4.6, we next performed the same experiment on 40-day old flies to allow for ROS accumulation. We did not detect a significant difference with overexpression of SOD1 or SOD2. Expression of catalase, however,

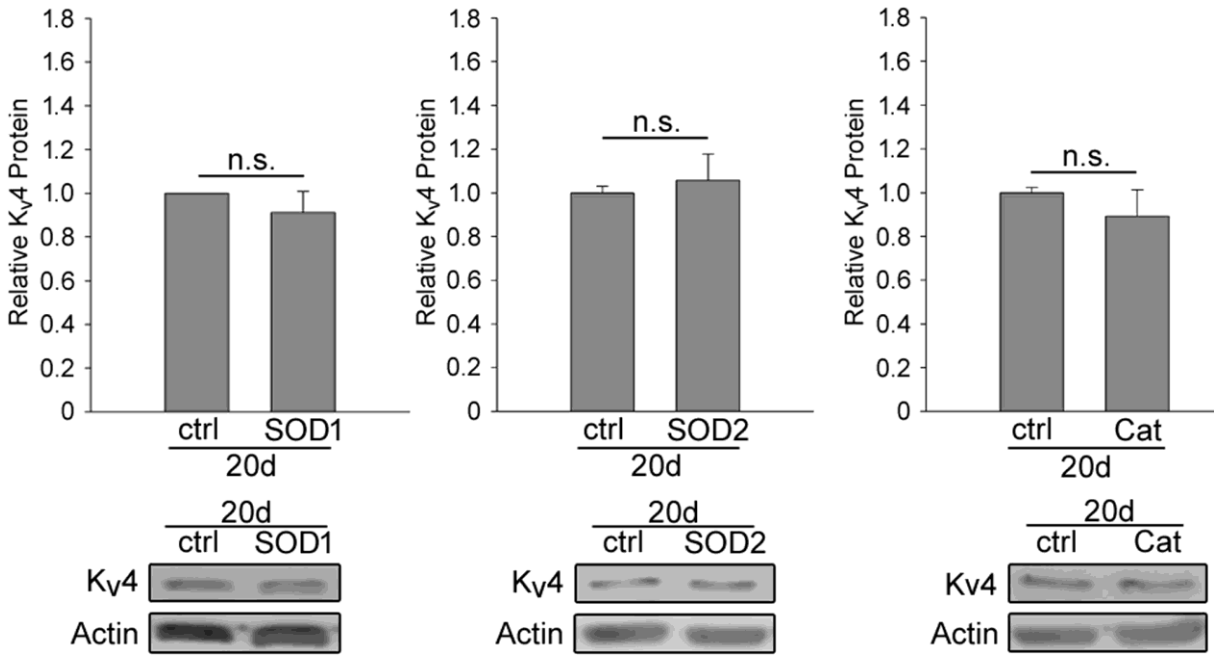


**Figure 4.7. Constitutive overexpression of SOD1, SOD2, or Catalase do not influence $K_v4$ protein levels in 20d old flies.** From left to right, there is no significant difference with overexpression of neither SOD1, SOD2, nor Catalase, respectively – "ctrl" represents the *UAS-SOD1*, *UAS-SOD2*, or *UAS-Catalase* genetic backgrounds, respectively (5 heads per sample, n=18, 6 blots, n.s. denotes no significant difference, Student's t-test).

significantly increased levels of $K_v4$ by ~30% when compared to *elav-GAL4* and by ~60% when compared to its genetic background *UAS-Catalase*, Figure 4.8.

Because catalase expression led to an increase in $K_v4$ levels at 40 days of age, we measured *in vivo* levels of ROS to test whether ROS were indeed decreased with overexpression of this enzyme. We performed these experiments using two different genetic controls, *elav-GAL4* and *UAS-Catalase*. We found that expression of *Catalase* led to a significant decline in ROS levels; we measured a 20% decline when compared to the control *elav-GAL4*, and a 50% decline when compared to the genetic background *UAS-Catalase*, Figure 4.9. Altogether, these results suggest that the age-related accumulation of $H_2O_2$ contributes to the age-dependent decrease of $K_v4$ protein levels in older flies.

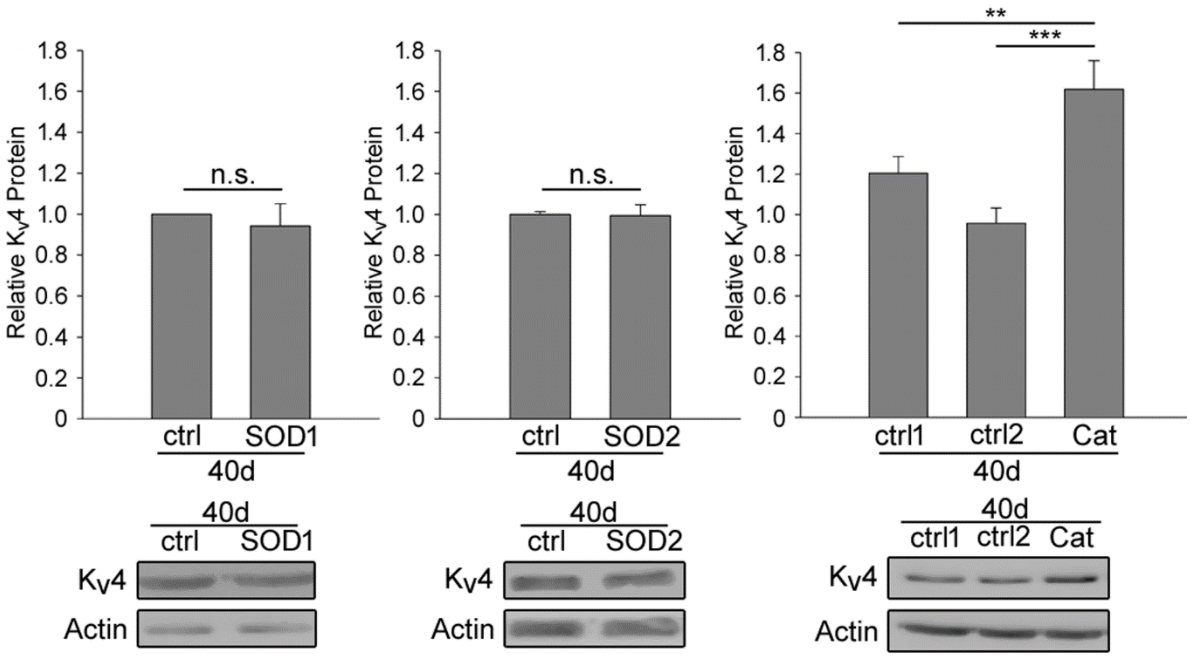


**Figure 4.8. Constitutive overexpression of Catalase, but not SOD1 or SOD2, leads to increased $K_V4$ levels in 40d old flies.** Left and middle, there is no significant difference with overexpression of neither SOD1 nor SOD2, respectively – "ctrl" represents the *UAS-SOD1* or *UAS-SOD2* genetic backgrounds, respectively. Right, overexpression of Catalase significantly increases levels of $K_V4$ protein in 40d old flies – "ctrl1" and "ctlr2" represent the genetic backgrounds *elav-gal4* and *UAS-Catalase*, respectively (5 heads per sample, n=18, 6 blots, n.s. indicates no significant difference, ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test).

To further test the idea that ROS play a role in $K_V4$ channel levels, we investigated the role of two ROS-generating enzymes in *Drosophila*, NOX and DUOX. While the structure of NOX is homologous to that of phagocyte oxidase (phox), DUOX enzymes are composed of a phox catalytic subunit that includes an N-terminal extracellular peroxidase-homology domain[499,500]. We made use of two *Drosophila* transgenic lines that knockdown these ROS-generating enzymes by ~60%, *DUOX-RNAi* and *NOX-RNAi*[501]. We measured levels of $K_V4$ in both transgenics in 40-day old flies. We found that, although a *DUOX* knockdown does not lead to a significant change in $K_V4$ expression, a *NOX* knockdown leads to a 40% increase in $K_V4$ protein levels, Figure 4.10.

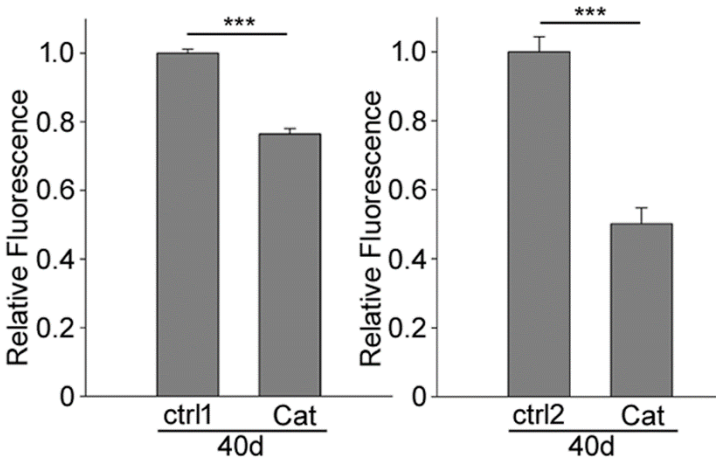


**Figure 4.9. The age-related accumulation of ROS levels can be reduced with catalase overexpression.** Catalase overexpression leads to a significant decrease in detectable ROS-dependent DFC fluorescence when compared to two different genetic controls – "ctrl1" and "ctrl2" represent *elav-GAL4* and *UAS-Catalase*, respectively (25 heads per extraction, 5 extractions per experiment with 4 technical replicates each, a total of 3 experiments were performed, *** $p \leq 0.001$, Student's t-test).

Because a transcriptional knockdown of NOX and an overexpression of *Catalase* both resulted in increased $K_v4$ channel levels at 40 days of age, we tested the hypothesis that expression of *UAS-NOX-RNAi* or *UAS-Catalase* would lead to increased locomotor performance in 40-day old flies. We found that the transcriptional NOX knockdown led to a decline in locomotor performance in *Drosophila*, Figure 4.11. Since NOX is an important enzyme that participates in many signaling pathways in the cell, effects on general health may have been a confounding factor. Conversely, expression of *Catalase* resulted in significantly increased levels of locomotor performance by 50%, Figure 4.11. Altogether, our results support the idea that there is an accumulation of ROS during aging, and that the accumulation of $H_2O_2$ contributes to a decrease in $K_v4$ protein levels in older flies (40 days) which, in turn, results in locomotor problems.

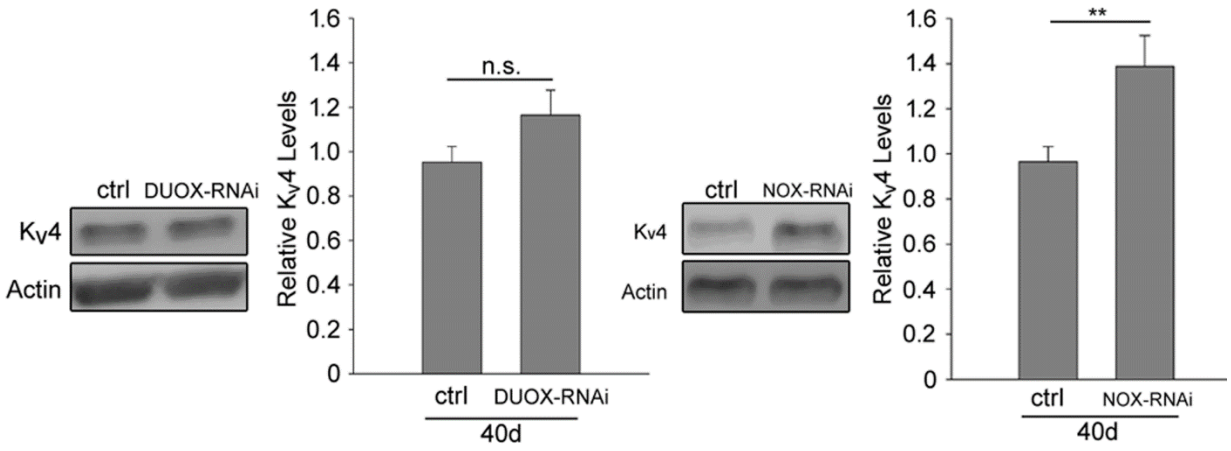


**Figure 4.10. Genetic knockdown of two ROS generating enzymes in *Drosophila*.** Left, immunoblot experiment shows that there are no significant changes in $K_v4$ protein levels in 40-day old flies with expression of RNAi targeting *DUOX* – "ctrl" represents the genetic background *UAS-DUOX-RNAi* (5 heads per sample, n=27, 10 blots, n.s. denotes no significant difference, Student's t-test). Right, transcriptional knockdown of the NOX enzyme leads to increased levels of $K_v4$ proteins in 40-day old flies – "ctrl" corresponds to the genetic background *UAS-NOX-RNAi* (5 heads per sample, n=26, 10 blots, ** $p \leq 0.01$, Student's t-test).

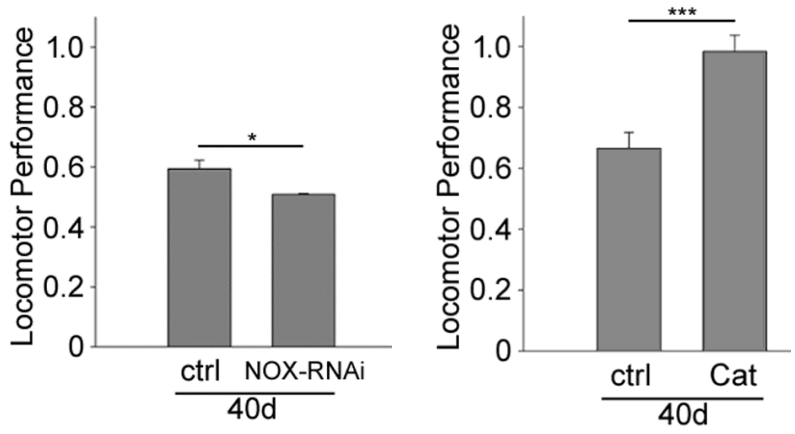


**Figure 4.11. Catalase expression, but not a transcriptional knockdown of NOX, increase locomotor performance in *Drosophila*.** Left, there is a significant decrease in locomotor performance of flies expressing NOX-RNAi at 40d of age (35-40 flies per sample, n=10 per day measured, * $p \leq 0.05$, Student's t-test). Right, expression of Catalase significantly increases locomotor performance in 40d flies – "ctrl" represents the genetic background *UAS-Catalase* (35-40 flies per sample, n=10 per day measured, *** $p \leq 0.001$, Student's t-test).

# CHAPTER 5. ROLE OF THE SCAFFOLDING PROTEIN SIDL ON $K_v4$ LEVELS DURING AGING, AND ITS CONTRIBUTION ON *DROSOPHILA* ECLOSION

## 5.1. Overview

The loss of a scaffolding protein may lead to destabilization and possible degradation of its protein associates[502]. The scaffolding protein SIDL has been previously described to interact with the highly conserved C-terminal di-leucine motif of $K_v4$ and to co-localize with $K_v4$ in *Drosophila* primary neuronal cultures[344]. This project aims to understand the importance of the scaffolding protein SIDL for $K_v4$ channels *in vivo*. We found that *SIDL* mRNA levels decline with age. To test the hypothesis that a decline in *SIDL* mRNA influences the stability of $K_v4$ protein, we conditionally expressed *SIDL-RNAi* in adult flies post-eclosion. We found that after 6 days of *SIDL* knockdown, levels of $K_v4$ were decreased. When we extended the knockdown period, $K_v4$ levels decreased even more, suggesting that the expression of *SIDL* plays a role in maintaining levels of $K_v4$ protein. Next, we examined the effects of a SIDL-dependent loss of $K_v4$ on *Drosophila* eclosion rates (exiting the pupal case to become an adult) which requires a rhythmic peristaltic movement. Because $K_v4$ channels have been reported to be required for rhythmic behaviors[359], we knocked-down *SIDL* during development and measured percent of successful eclosion. Flies expressing *SIDL-RNAi* had a 15% success of eclosion, which is significantly decreased from the 80% observed in wildtype flies. We were able to partially rescue this phenotype by overexpressing *SIDL* or *$K_v4$* in the *SIDL-RNAi* line. We also measured eclosion rates of flies that only overexpressed *SIDL*, *$K_v4$*, or *CD8-GFP*. Eclosion rates were enhanced in flies expressing *SIDL* or *$K_v4$*, but not *CD8-GFP*, consistent with the model that SIDL plays a role in stabilizing $K_v4$ protein which, in turn, enhances coordinated motor function.

### 5.2. *SIDL* mRNA declines with age

In the absence of a SIDL-specific antibody, we measured if levels of *SIDL* mRNA change with age. We used RT-qPCR to compare mRNA levels of *SIDL* at 3d, 10d, and 40d. We were not able to find one reference gene that would be stable at all ages tested; instead, we made use of two different reference genes, *RpS20* and *eIF1A*. We first measured *SIDL* mRNA levels at 3 and 40--days post-eclosion. We found that *SIDL* mRNA levels decrease by 20% between 3 and 40-day old flies, Figure 5.1. Next, to determine if *SIDL* mRNA levels might play some role in the 50% $K_v4$ protein decline we found between 3 and 10-days post-eclosion, we measured *SIDL* mRNA levels at these time points. We found no significant differences in *SIDL* mRNA levels between 3 and 10 days of age, Figure 5.1, suggesting that the decline in $K_v4$ protein levels between 3 and 10 days of age likely does not involve a $K_v4$ destabilization by a decline in SIDL protein. We next tested if SIDL might be involved in the gradual decline of $K_v4$ protein we measured between 10 and 40 days of age. We found that there is a 10% decline in *SIDL* mRNA levels, Figure 5.1. These

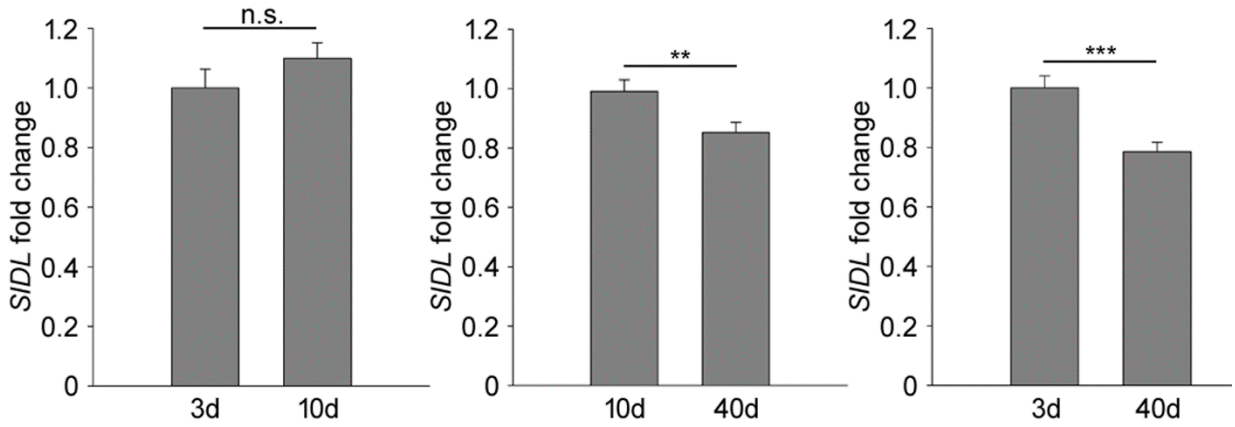


**Figure 5.1. *SIDL* mRNA levels undergo an age-dependent decline.** Three bar graphs with RT-qPCR measurements of *SIDL* mRNA, relative to RpS20 or eIF1A, show that there is a significant age-dependent decline in *SIDL* transcript between 10d and 40d, and between 3d and 40d flies, but not between 3d and 10d (each sample was prepared from 700 ng RNA extracted from 10 heads, n = 17-24, n.s. represents no significant difference, ** $p \leq 0.01$, *** $p \leq 0.001$, Student's t-test).

results show that *SIDL* transcript begins to decline after 10 days of age in *Drosophila* and suggest that a decline in SIDL protein after 10 days of age may play a role in the stability of $K_v4$ protein levels.

### 5.3. SIDL plays a role in $K_v4$ protein stabilization

Because SIDL protein interacts with $K_v4^{344}$, and we found that both $K_v4$ protein and *SIDL* mRNA levels decline with age, we tested the hypothesis that SIDL plays a role in maintaining steady-state levels of $K_v4$. We made use of a fly containing an RNAi construct that targets *SIDL*, *UAS-SIDL-RNAi*, and conditionally expressed it in *Drosophila* using the UAS/GAL4 system. To conditionally express *UAS-SIDL-RNAi* in adult *Drosophila* neurons, we used the temperature-sensitive GAL80 molecule to suppress the pan-neuronal promoter *elav-GAL4*. At 18˚C, GAL80 binds to the C-terminal 30 amino acids of GAL4, inhibiting GAL4 interaction with the upstream activating sequence of transgenes. At 30˚C, GAL80 decreases its GAL4-binding affinity allowing GAL4-mediated transcription activation[503]. We collected 1-day old adult *Drosophila* and placed them at the permissive expression temperature of 30°C for several days. After incubation for 6 days, we performed immunoblot experiments to measure levels of the channel $K_v4$. We detected a 35% reduction in $K_v4$ protein levels with *SIDL* knockdown, Figure 5.2. To test if a longer knockdown of SIDL would lead to further loss of $K_v4$ protein, we allowed conditional expression of *UAS-SIDL-RNAi* for 10 days and found a 55% decrease in $K_v4$ protein levels when compared to control, Figure 5.2. These results suggest that the scaffold protein SIDL might play a role in regulating steady-state levels of $K_v4$ protein. Altogether, these data suggest that after 10 days of age, the decline in *SIDL* mRNA levels might lead to a decrease in the scaffold protein SIDL which likely results in $K_v4$ protein instability and loss.

### 5.4. $K_v4$ and SIDL are implicated in *Drosophila* eclosion

The peristaltic process *Drosophila* performs to successfully eclose from the pupal case to become an adult fly is a process that requires maintained rhythmic contractions of the abdomen[504,505]. Because $K_v4$ channels are required for maintaining excitability during repetitive firing in

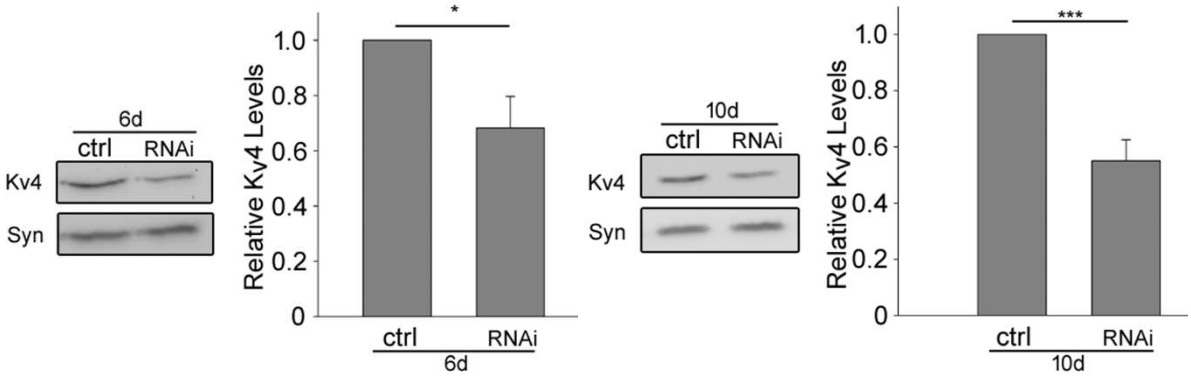


**Figure 5.2. Knockdown of *SIDL* leads to decreased levels of $K_v4$ channel *in vivo*.** Immunoblot experiments after conditional expression of *UAS-SIDL-RNAi* for 6d and 10d post-eclosion. Left, expression of RNAi targeting SIDL for 6 days shows a ~35% decline in $K_v4$ channels (5 heads per sample, n=5, 2 blots, * $p \leq 0.05$, Student's t-test). Right, extended expression of RNAi targeting SIDL for 10 days decreases $K_v4$ protein levels even more, to ~55% from control (5 heads per sample, n=8, 2 blots, *** $p \leq 0.001$, Student's t-test).

*Drosophila* neurons[359], and we found that a knockdown of *SIDL* resulted in decreased $K_v4$ levels, we tested the hypothesis that knockdown of *SIDL* during development might affect successful eclosion. We used the UAS/GAL4 system to constitutively express *UAS-SIDL-RNAi* pan-neuronally using the driver *elav-GAL4* to perform these studies. With the help of Timothy Vernier, while he was an undergraduate at Colorado State University, we collected ten *Drosophila* 3rd instar larva at a time and transferred them to new food vials. We followed these flies until pupation and counted the number of success or failure to eclose; we examined 15-17 total vials per genotype. We found that 80% of wildtype flies eclosed, Figure 5.3. We then measured eclosion events of flies expressing *UAS-SIDL-RNAi*. Successful eclosion rates significantly decreased to

~15%, Figure 5.3. We next tested for a rescue by co-expressing *UAS-SIDL-RNAi* with *UAS-SIDL*. *Drosophila* significantly increased successful eclosion rates to 60% supporting the idea that *SIDL* is required for successful the eclosion.

Because we previously measured decreased $K_v4$ protein levels with a knockdown of *SIDL*, we tested if unsuccessful eclosion in *SIDL-RNAi* flies is due to loss of $K_v4$. We co-expressed *UAS-SIDL-RNAi* with *UAS-$K_v4$* to increase $K_v4$ levels in the *SIDL* knockdown background.

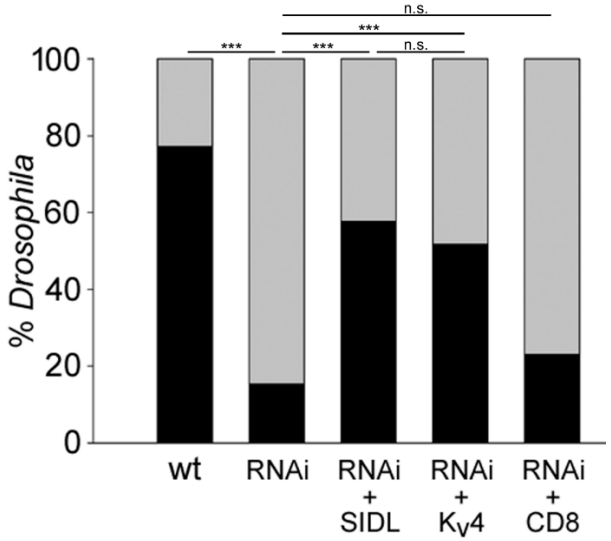


**Figure 5.3. The decline in successful eclosion rates by constitutive expression of *UAS-SIDL-RNAi* is partially rescued by co-expression with *SIDL* or *$K_v4$*, but not with *CD8-GFP*.** From 100% total tested flies, black bar represents larvae developing to pupal stage and successfully eclosing, grey bar represents larvae that arrived at the pupal stage and were unable to eclose. There is a ~80% successful eclosion rate in wildtype flies. Knockdown of *SIDL* throughout development is characterized by a significant decrease in successful eclosion rates to less than 20%. This partial lethal phenotype was ameliorated with co-expression of *UAS-SIDL* or *UAS-$K_v4$*, but not with *UAS-CD8-GFP* (10 flies per vial, 15-17 vials per genotype, n=135-167, n.s. denotes no significant difference, *** $p \leq 0.001$, Analysis of Proportions).

Increasing $K_v4$ expression indeed increased successful eclosion to 55%. As a control, we co-expressed *UAS-SIDL-RNAi* with the non-specific transmembrane protein CD8-GFP. We measured no significant difference between *UAS-SIDL-RNAi* and the co-expression with *UAS-CD8-GFP*. Because a *SIDL* knockdown led to a decline in $K_v4$ protein levels in adult flies,

Figure 5.2, and caused a decrease in successful eclosion rates from pupating flies, these results support the idea that SIDL and $K_v4$ interact *in vivo* and suggest that stabilization of $K_v4$ channels by SIDL is required for successful eclosion.

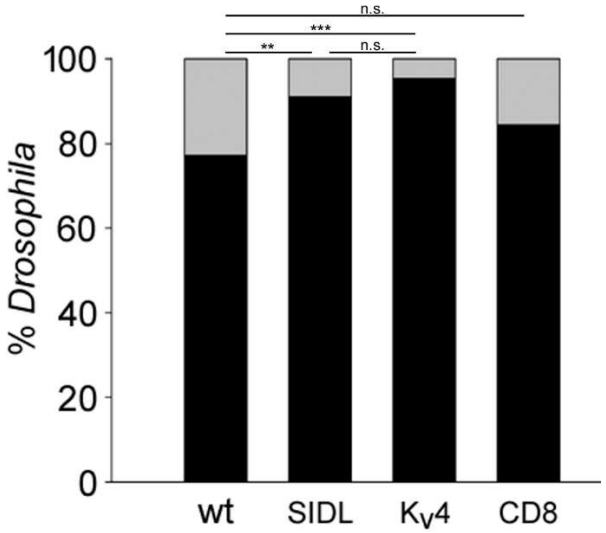


**Figure 5.4. Overexpression of *SIDL* or *$K_v4$*, but not *CD8-GFP*, increases successful eclosion rates.** From 100% total tested flies, black bar represents larvae developing to pupal stage and successfully eclosing, grey bar represents larvae that arrived at the pupal stage and were unable to eclose. Overexpression of *SIDL* or *$K_v4$* significantly increase eclosion rates to above 90% success when compared to wildtype. Overexpression of *CD8-GFP* has no significant effects when compared to wildtype (10 flies per vial, 15-17 vials per genotype, n=115-142, n.s. denotes no significant difference, ** $p \leq 0.01$, *** $p \leq 0.001$, Analysis of Proportions).

We also tested whether overexpression of SIDL or $K_v4$ alone would increase eclosion rates. We measured 90% successful eclosion with either *UAS-SIDL* or *UAS-$K_v4$* overexpression; this did not occur with *UAS-CD8-GFP*, Figure 5.4. Altogether, these data suggest that $K_v4$ channel levels are involved rhythmic behaviors, such as the peristaltic process required for pupal eclosion, and that the scaffolding protein SIDL is required for maintaining required levels of $K_v4$.

# CHAPTER 6. DISCUSSION

## 6.1. Overview

The age-dependent decline in motor abilities is a well characterized phenomenon[11–13]. This is partially due to an overall loss of neuronal density and age-related neuronal modifications of the dendritic arborization, a neuronal region implicated in signal integration[506]. Though age-dependent dendritic morphological changes have been described and comprehensively reviewed[506–509], little is known about the molecular mechanisms that may alter dendritic functional properties during aging. The somato-dendritic voltage-gated A-type $K_v4$ channel[191,344] has been previously described to play an important role in repetitive rhythmic behaviors[359,397,454,455,510]. In this study, we showed that $K_v4$ channels undergo an age-dependent decline in protein levels which contributes to the loss of *Drosophila* locomotor performance throughout aging. We measured an increase in ROS levels with age, and we also found that $K_v4$ channel levels in young *Drosophila* decline with *in vivo* exposure to $H_2O_2$. Overexpression of *Catalase*, not only decreased levels of ROS in older flies, but it also resulted in increased levels of $K_v4$ protein and ameliorated locomotor performance in older flies, suggesting that the age-related increase in levels of $H_2O_2$ contributes to the decline in $K_v4$ protein levels. Another mechanism that we uncovered was the age-dependent decline in mRNA levels of *SIDL*, a scaffolding protein that plays a role in regulating the levels of $K_v4$ *in vivo*. We also measured a decline in mouse hippocampal $K_v4.2$ protein levels suggesting that this age-dependent decline of $K_v4$ is likely to be conserved across species, at least in some brain regions. Since $K_v4$ channels have been implicated in regulation of long-term potentiation, age-dependent loss of $K_v4.2$ in the hippocampus may also contribute to aging effects on learning and memory, and susceptibility to neurodegenerative diseases.

### 6.2. $K_v4$ Channels Are Implicated In Locomotor Performance During Aging

The age-dependent decline in *Drosophila* locomotor performance is likely due, in part, to a decrease in $K_v4$ protein levels. We measured a decline in *Drosophila* locomotion that began by 30 days of age and became progressively reduced; by 60 days of age flies were quite inactive. Although we did not measure levels of functional $K_v4$ channels in neurons, we did detect a decrease in total $K_v4$ protein levels; this locomotor performance decline correlated with a loss of $K_v4$ protein levels which, at 30 days of age, is ~25% of the total we measured at 3 days of age. Remarkably, when we pan-neuronally expressed $K_v4$, locomotor performance was increased by ~50% at all tested ages, except at 60 days of age. Altogether these results suggest that total $K_v4$ protein levels in neurons participate in motor function and that the variability in $K_v4$ channel levels may have direct consequences on the density of A-type currents.

Because the levels of $K_v4$ channel protein decline during aging, one possibility for the decline in locomotor performance is that the density of $K_v4$ somato-dendritic A-type currents also decreases with age in neurons that participate in locomotor abilities, such as sensory neurons, central patter generators (CPG), and motorneurons[511]. Indeed, the role of somato-dendritic A-type currents in motor neuron function was described as early as 1979 when Byrne and coworkers were studying the control of ink release in *Aplysia*[512]. Pharmacological experiments by Choi and coworkers (2004) using the A-type current blocker 4-aminopyridine (4-AP)[513] later showed that somato-dendritic A-type currents play a role in firing of the first action potential and regulating repetitive firing in *Drosophila* 3$^{rd}$ instar larva motor neurons, suggesting that a somato-dendritic A-type channel, likely $K_v4$, is involved in the regulation of motor neuron action potential firing[514]. To test the hypothesis that $K_v4$ channel A-type currents in neuronal firing patterns and rhythmic behaviors, such as locomotion, Ping and colleagues (2011) introduced a functional knockout of

$K_v4$ ($DNK_v4$) to motor neurons in *Drosophila*. They measured a loss in the delay to the first action potential firing and a loss of neuronal excitability for repetitive action potential firing. These electrophysiological effects led to defects in locomotor performance in larvae and adult flies[359], suggesting that $K_v4$-regulated neuronal function is required for proper motor output.

To test the possibility that the age-dependent decline in $K_v4$ channel levels leads to a decrease in A-type current density in neurons, electrophysiological experiments on adult *Drosophila* during aging are needed. Ping and colleagues (2015) measured the current of mushroom body neurons in intact adult fly brains at 3 and 8 days of age and found these currents to be similar[454], suggesting that if there is a change in A-type current density it must occur after 8 days. Older brains, however, are more difficult to dissect and maintaining viability is more of a challenge (personal communication: Dr. Yong Ping).

With the goal of determining the effects of a loss of $K_v4$ function on *Drosophila*, Ping and colleagues (2011) used $DNK_v4$ to knockout $K_v4$ function in either the whole nervous system, motor neurons, or sensory neurons, and measured larval motor function. They found that loss of $K_v4$ function in all three resulted in decreased locomotor performance for larvae. When they knocked out $K_v4$ function in the whole nervous system or only motor neurons, adult flies showed decreased locomotor performance[359]. Although Ping and colleagues (2011) did not measure *Drosophila* grooming with aging, the expression of $DNK_v4$ resulted in defects in other repetitive behaviors, such as grooming. In our experiments, however, we measured total $K_v4$ protein levels from fly heads which do not contain motor or sensory neurons. Along with our results, this would suggest that the age-dependent decline in $K_v4$ channels results in an age-dependent decline in A-type currents, possibly in central pattern generator neurons, leading to a decrease in proper

motor coordination. Other coordinated behaviors, such as proboscis extension could also be examined as a model.

It would be interesting to test whether $K_v4$ protein also declines in the peripheral nervous system (PNS). Because CPG neurons in the central nervous system (CNS) receive input from the PNS[515], another possibility underlying the age-dependent decline in locomotion is that the density of the somato-dendritic A-type currents required for signal integration in these sensory neurons declines after 30 days of age. Indeed, in *Drosophila*, when multiple dendritic (MD) sensory neurons of the PNS were conditionally silenced, larvae exhibited peristaltic locomotion impairment[516]. When subsets of MD neurons were analyzed, two classes of CNS feedback proprioceptor neurons, bipolar dendrites and class I MDs, were identified as necessary for this peristaltic locomotor movement[517]. Larval locomotion speed also declined when the functional knockout of $K_v4$ was introduced and expressed in MD neurons[359]. In our *SIDL* knockdown experiments which resulted in lower $K_v4$ levels, we suggested that this decline in $K_v4$ might play a role in the possible loss of peristaltic movement required for eclosion. One possibility is that lower levels of $K_v4$ in this subset of MD neurons resulted in the loss of peristalsis required for *Drosophila* eclosion. To test which subsets of sensory or motor neurons where an age-dependent decline in $K_v4$ protein might affect peristalsis, experiments could be performed in which $DNK_v4$ is expressed in identified subsets of neurons.

Brigui and coworkers (1990) reported that *Drosophila* proboscis extension, in response to sensing sucrose, needs higher concentrations of sucrose with an increase in age[518], suggesting that sugar sensing capabilities undergo an age-related decline. Similar experiments testing proboscis extension could be performed with overexpression of either $DNK_v4$ or $K_v4$ in sensory or motor neurons. A decrease in *Drosophila* proboscis extension response in young flies expressing $DNK_v4$

would support the model that $K_v4$ protein levels decrease in these neurons during aging. In contrast, an improvement proboscis extension response in aged *Drosophila* overexpressing $K_v4$ would support the idea that a decline in $K_v4$ channels in these types of neurons is occurring with age. Indeed, $K_v4$ might play a role in either sensation or motor response, or both.

### 6.3. Age-Related $K_v4$ Protein Decline Effects On Learning And Memory

Aging is characterized by a decline in cognition which includes the ability to learn and remember[519]. In *Drosophila*, we found that $K_v4$ levels decrease with age in the CNS and that this age-related decrease in $K_v4$ protein may influence the age-related decline in learning and memory. Indeed, in transgenic flies expressing $DNK_v4$ in which $K_v4$ channel function has been knocked out in the entire CNS or selectively in mushroom body (MB) neurons[454], the center for learning and memory of the fly, larval olfactory associative learning was negatively affected. [520].

Alzheimer's disease is more prevalent in the aged population and ultimately leads to a loss of learning and memory[508]. Because $K_v4$ channels have been described to play a role in learning and memory, one possibility is that an accumulation of $A\beta_{42}$ in Alzheimer's disease may lead to a loss of $K_v4$ protein. Ping and colleagues (2015) neuronally expressed the human $A\beta_{42}$ protein which resulted in decreased $K_v4$ A-type currents in MB neurons of the brain. They found that the accumulation of Aβ42 induced a decline in $K_v4$ protein which resulted in a lower density of somato-dendritic A-type currents which, in turn, led to larvae associative learning and memory defects. Remarkably, expression of $K_v4$ in this *Drosophila* Alzheimer's model resulted in a rescue of the learning and memory defects[454].

In vertebrates, the hippocampus is the primary center for learning and memory, and changes in synaptic strength in neurons within this brain region has been described to be necessary

for memory formation[521,522]. Long-term potentiation (LTP) leads to synaptic strengthening and has been measured to occur in the hippocampus, and mouse $K_v$4.2 knockout studies have shown that $K_v$4 channel A-type currents have been shown alter synaptic properties thought to be involved in the regulation of LTP induction in the hippocampus[356,369,386,521]. Chen and coworkers (2006) used a $K_v$4.2 knockout mouse to demonstrate that deletion of the $K_v$4.2 gene results in an increase of backpropagation of action potentials to the dendrites resulting in an increase in $Ca^{2+}$ influx leading to a lower threshold for the induction of LTP in hippocampal neurons[356]. It is important to note that $K_v$4.2 is internalized as a normal response to LTP induction[384], and not that LTP is induced because of a decline in membrane-bound $K_v$4.2. Lugo and colleagues (2012) later subjected $K_v$4.2 knockout mice to a battery of behavioral tests[386]. They found that mice lacking $K_v$4.2 have deficits in learning and memory, behavioral events that are dependent on a proper functioning hippocampus. An age-dependent decrease in the levels of $K_v$4.2 protein would lead to an increase in backpropagation of action potentials and likely allow for easier dendritic depolarizations. Indeed, changes in levels of $K_v$4.2 have been described to directly impact synaptic remodeling by altering NMDA subunits composition which, in turn, lead to modifications in synaptic plasticity and memory[523,524]. Our results show that, in mice, total hippocampal $K_v$4.2 levels decrease with age which might contribute to the age-related decline in learning and memory abilities.

Other isoforms of $K_v$4 may also play some roles in the hippocampus. We were unable to measure any levels of $K_v$4.1 protein in this brain region. Our results are not surprising, however, as Serôdio and coworkers described *$K_v$4.1* mRNA levels to be present in very little abundance within this brain region[330]. $K_v$4.3, conversely, is present in abundant levels within certain regions of the hippocampus[330,525], and we measured increased levels of $K_v$4.3 with age. $K_v$4.3 is found predominantly in interneurons[526,527], and an increase in these neurons is likely to resulted in

increased hippocampal excitability. What's more, changes to hippocampal interneurons likely result in hippocampal dysfunction which has been reported to contribute to bipolar disorder[528].

Rizzo and colleagues (2014) proposed a connection between the loss of cognitive function in bipolar disorder and aging[529]. One possibility for increased incidence in bipolar disorder during aging is an age-related dysfunction of hippocampal interneurons. $K_v4.3$ A-type currents are responsible for the rhythmic activity in hippocampal interneurons involved in learning and memory[530,531], and we measured total $K_v4.3$ levels in the hippocampus to increase with age. It is probable that this increase in total $K_v4.3$ levels is a result of age-dependent effects on the channel. In another report, Simkin and coworkers (2015) measured $K_v4.2$ and $K_v4.3$ levels in hippocampal CA3 neurons and found that protein levels of these two $K_v4$ isoforms were increased in 30-month old rats. They reported that this increase in channel expression resulted in faster action potential repolarization in aged CA3 pyramidal neurons which resulted in increased hippocampal CA3 neuronal excitability[339,532]. Although we did measure an increase in hippocampal $K_v4.3$ protein levels with age in mice, $K_v4.2$ levels decreased. It is important to note that we measured total hippocampal $K_v4.2$ and $K_v4.3$ protein levels and we cannot discern $K_v4$ levels between different regions of the hippocampus. One possibility is that the decline in $K_v4.2$ protein levels in other regions of the hippocampus is large enough to offset the increase in CA3 pyramidal neurons. In contrasting results, Haberman and colleagues (2011) reported that 24 to 26-month old rats with preserved cognition had, instead, lower levels of $K_v4.2$ protein in CA3 neurons[533]. Altogether, these two reports – Simkin (2015) and Haberman (2011) – suggest that levels of $K_v4.2$ and $K_v4.3$ play a direct role in cognition, and that a decline in CA3 $K_v4.2$ channel levels might be a response to maintain cognition during aging.

We also measured an increase in only $K_v4.3$ levels in the cerebellum and the motor cortex. Our findings support previous publications in which high *$K_v4.3$* mRNA levels in these regions have been described[330,534]. Though most reports on $K_v4.3$ function have been reported in myocardial cells[318,335,535–538], at least in cortical pyramidal (CP) neurons, $K_v4.3$ has been described to regulate action potential duration and neuronal repetitive firing[387,539]. Using knockout mice studies, Carrasquillo *et al.* (2012) investigated the functional roles of $K_v4.2$ and $K_v4.3$ subunits in CP neurons. While $K_v4.2^{-/-}$ mice resulted in increased action potential firing in response to small depolarizing current injections, $K_v4.3^{-/-}$ mice resulted in increased action potential firing in response to large current injections, suggesting that $K_v4.3$ channels recover faster from inactivation[387]. Because $K_v4.3$ recovers faster it is possible that the age-related increase in $K_v4.3$ levels we measured might be a response to maintaining neuronal excitability in the pyramidal neurons of the cortex.

### 6.4. ROS Accumulation During Aging Leads To Lower $K_v4$ Channel Levels

Our results show that an accumulation of reactive oxygen species (ROS) contributes to the age-dependent loss of $K_v4$ channel protein levels. A 4-hour *in vivo* exposure of *Drosophila* to $H_2O_2$ after a 24-hour recovery led to decreased $K_v4$ levels, suggesting that an age-related accumulation of $H_2O_2$ might have an effect on the age-dependent decline of $K_v4$ channels and locomotor performance. Our results are contradictory to previously published data in which researchers measured an increase in *Drosophila* activity when they fed them $H_2O_2$, or injected $H_2O_2$ abdominally[540]. Because flies were fed $H_2O_2$, it is possible that the ingested molecule oxidized areas of the digestive system which lead to a noxious response in the fly. In a similar manner, abdominal injection of $H_2O_2$ could cause a sense of burning in the fly abdomen due to the oxidative

properties of $H_2O_2$, which could cause the fly to have a noxious response. Noxious response may produce escape/avoidance behavior[541], which could explain their measured increase in fly activity, which persisted for 6 hours[540].

Because we measured lower levels of $K_v4$ protein in whole heads, we tested the effects of $H_2O_2$ exposure on neuronal cultures expressing tagged-$K_v4$. Exposing *Drosophila* cultured neurons directly to $H_2O_2$, however, led to no qualitative changes in overall levels, subcellular localization, or vesicular internalization of tagged-$K_v4$ channels. In our neuronal culture experiments we used the pan-neuronal *elav-GAL4* driver. One possibility is that the neurons we analyzed, which were the ones that visually had the strongest tagged-$K_v4$ signal, were a subset of neurons with greater antioxidant properties. Future experiments could investigate identified cell types using many specific GAL4 lines available to label particular neurons to determine if changes can be seen in more specific neuronal cell types. Another possibility could be that treating neuronal cultures with such high levels of $H_2O_2$ could have quickly damaged the autophagy degradation machinery. Indeed, oxidation of the lysosomal membrane proteins leading to a dysfunctional system has been described, in general, to occur with aging[542], and oxidative damage has been reported to inhibit vesicular transport of both synaptophysin and synaptotagmin I in mouse microglial cells[543]. Experiments adding physiological levels of $H_2O_2$ to cultures for longer periods of time, possibly days, could be performed to minimize oxidative damage to other cell compartments and to examine effects on $K_v4$ channels. Since we were unable to detect any changes in cultured neurons, we performed follow up tests for $H_2O_2$ effects on $K_v4$ in the aging fly.

We found that neuronal overexpression of *Catalase*, an enzyme that degrades $H_2O_2$ [113,544,545], decreases detectable ROS levels, increases $K_v4$ protein levels, and ameliorates locomotor performance in 40-day old flies. Our results corroborate data published by Haddadi and coworkers

in which they measure a similar increase in ROS levels between 5 and 50-day old flies[101], and with the general concept that ROS increase with age[55,293,546]. In their report, they also described catalase activity to be reduced by ~25%. This decline in catalase activity alone could be a reason for the intracellular accumulation of, specifically the $H_2O_2$ species. Another study found that when *Catalase* was expressed within the mitochondrial matrix, however, there was a decrease in the walking speed of *Drosophila* during aging[547]. One possibility is that, in our experiments we expressed *Catalase* with a pan-neuronal driver, the levels of $H_2O_2$ are likely decreasing more in the cytosol rather than within mitochondria. A specific decrease in ROS levels within the mitochondria could cause its dysfunction which is detrimental to the cell, and mitochondrial ROS has been described to be important for other cellular signaling[548]. *Catalase* expression in leg muscles (soleus, gastrocnemius, tibialis anterior, and extensor digitorum longus) in a muscular dystrophy mouse model increased locomotor performance, although the study only measured muscle function[549], suggesting that catalase is also playing a role in muscle. Protein carbonyl content has been described as a method to determine the level of oxidation state of a protein[550]. To test whether $H_2O_2$ is directly affecting $K_v4$ protein, site-directed mutagenesis of cysteine residues could be performed.

In mice, overexpression of *SOD*, which metabolizes superoxide into $H_2O_2$, was reported to increase oxidative damage and result in a decline in locomotor performance[551]. Although we did not measure locomotor performance, we did not find any changes in *Drosophila* $K_v4$ protein levels with expression of either *SOD1* or *SOD2*. One possibility is that at the ages we tested, the levels of superoxide are not high enough to increase $H_2O_2$ intracellular concentration to detrimental levels. To test this hypothesis, we could measure ROS levels in 20 and 40-day old flies that overexpress *SOD1* and *SOD2*. Another possibility is that endogenous levels of catalase, at 20 and

40 days of age, are enough to breakdown all $H_2O_2$ produced by *SOD1* and *SOD2* overexpression. An experiment to test this idea would be to overexpress *SOD1* or *SOD2* in a fly with a *Catalase* mutation. Altogether, this raises our interest in understanding the role of SOD1 and SOD2 in the age-related production of $H_2O_2$. Perhaps co-expression of both SOD1 and SOD2 would lead to higher levels of $H_2O_2$ for us to strengthen the model that $H_2O_2$ causes a decline in $K_v4$ protein levels.

The two splice forms of *Drosophila* $K_v4$ channels share 25 cysteine residues in their polypeptide chain, and 19 residues are conserved from *Drosophila* to mice and rats, suggesting that oxidation of any of these conserved residues might have a similar effect across species. Selective oxidation by $H_2O_2$ has also been described to have effects on $K_v$ function. A study testing the effects of addition of $H_2O_2$ to CHO cells expressing the $K_v2$ homologue KVS-1 channel from *C. elegans* revealed that there are changes in the electrophysiological properties of KVS-1 channel due to oxidation of a cysteine amino acid[552]. Because the *Catalase* expressed in our experiments was intracellular, one possibility is that conserved cytosolic cysteine residues might be subject to oxidation. I used the secondary structure prediction software TMHMM[553] and found that there are 6 potential cysteine residues located in the cytosol (3 N-terminal and 3 C-terminal). Three of these residues are localized in the N-terminal tail, and three others are localized in the C-terminal tail, Figure 6.1. If GFP signal decreases in a fly conditionally expressing *GFP-Shal* that has been exposed to $H_2O_2$ *in vivo* as we described above, then, an experiment that could be performed would involve a fly conditionally expressing *GFP-Shal* as a control, and as tests, flies conditionally expressing *GFP-Shal* constructs in which each of the six cysteine residues have been modified to alanine, similar to that described by Sesti[293].

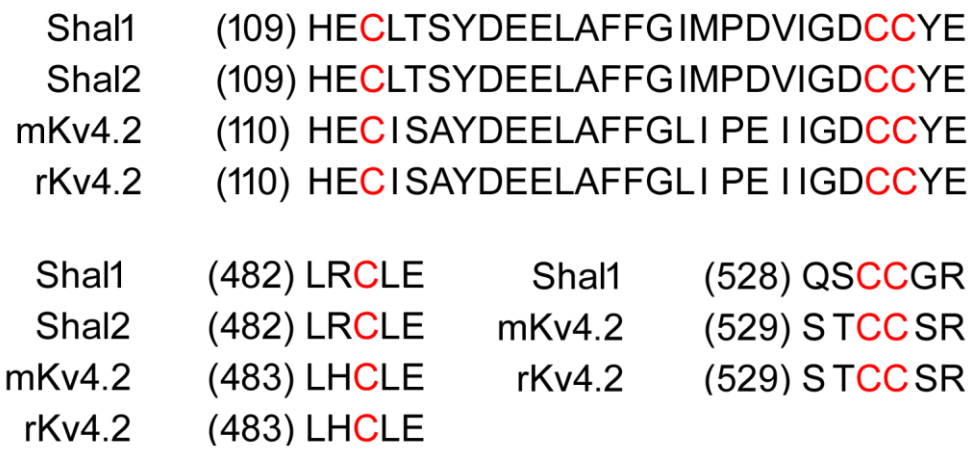


**Figure 6.1. Conserved cytosolic cysteine residues.** Shal cysteine residues 111, 132, and 133 are localized to the N-terminus. Residues 484, 530, and 531 are present in the C-terminus of Shal.

To test if the proteasome is involved in the decline of $K_v4$ levels with age, we could use transgenic flies that overexpress the dominant-negative 20S proteasome subunits *Pros26*[1] and *Pros*$\beta^2$ [554,555] and expose them to $H_2O_2$ for 4 hours. After 24 hours recovery, we would measure $K_v4$/Shal protein levels. While a decrease in $K_v4$ levels would suggest that the proteasome is not involved in $K_v4$ degradation, no change in $K_v4$ protein levels would suggest that the proteasome plays a role in the $H_2O_2$-dependent decline in $K_v4$ protein.

As described in Chapter 4, we exposed 3-day old flies to $H_2O_2$ for 4 hours and immediately placed them in food vials for 24 hours. After this recovery, we measured a decline in $K_v4$ protein levels which returned to baseline after 48 hours. To determine the rate of $K_v4$ protein turnover after exposing 3-day old flies to $H_2O_2$ *in vivo*, we could employ the use of the protein synthesis inhibitor cycloheximide (CHX). In our 2012 publication, we described a protocol in which we fed CHX to transgenic flies expressing *inaD* under the control of a heat-shock promoter. CHX successfully blocked inaD protein synthesis when flies were fed CHX for at least 30 minutes before induction by heat-shock, and protein synthesis inhibition could be maintained for up to 24 hours[556].

These experiments could be used to determine if protein synthesis plays a role in returning $K_v4$ levels back to baseline after 48 hours recovery from a $H_2O_2$ exposure.

### 6.5. Oxidation And Neurodegenerative Diseases

Potassium channel oxidation by ROS results in oxidative modifications of channel activity in normal and disease states; these oxidation-based modifications might be a factor underlying neurodegeneration[557]. Because $K_v4$ channels have been described to be implicated in neurodegenerative diseases, such as Alzheimer's[454] and Parkinson's[458], an $H_2O_2$-dependent $K_v4$ protein oxidative modification might considerably contribute to these diseases. Benzi and Moretti (1995) proposed the idea that ROS might be involved in Alzheimer's disease[558]. Indeed, some publications had reported that Aβ protein aggregates in presence of ROS in a variety of experimental conditions, and these aggregates are even stable in high concentrations of the denaturing agent urea[559–562]. Furthermore, $H_2O_2$ has been described to potentiate Aβ protein neurotoxicity, and the antioxidant Vitamin E has been reported to protect this toxicity in hippocampal cultures[558]. As it ensues with age, a mitochondrial imbalance occurs with the onset of Alzheimer's disease[563], and the mitochondria of mammals and insects is the principal site for the production of $H_2O_2$[564,565]. The levels of ROS, therefore, are expected to rise with Alzheimer's disease.

Protein oxidation contributes to changes in protein structure through oxidation-dependent amino acid modifications and cleavage of peptide bonds, and these structural changes may cause the damaged protein to be targeted for degradation[566–568]. To test levels of $K_v4$ protein oxidation, we could measure protein carbonyl content[550] in 3, 10, and 40-day old flies that were exposed to $H_2O_2$ *in vivo*. To test if oxidized $K_v4$ protein is targeted for degradation, we could perform

proteasome or lysosome inhibition and protein synthesis inhibition studies as previously described in Section 6.4. Neurodegenerative diseases such as Huntington, Parkinson's, and Alzheimer's have been attributed to oxidative damage which may lead to large accumulations of damaged protein aggregates[569–575]. The common variable for these neurodegenerative diseases is that they all display some level of mitochondrial dysfunction[576–578]. One possibility is that dysfunctional mitochondria results in increased cellular ROS, and that this increase in ROS may have detrimental oxidative effects on $K_v4$. Indeed, Manczak and coworkers suggested, in their transgenic mice studies, that both human amyloid precursor protein and human Aβ change metabolic properties of mouse mitochondria resulting in increased levels of $H_2O_2$ production[578]. In a recent publication, Ping *et al.* used a *Drosophila* model expressing the human Aβ42 to show that $K_v4$ channels are targeted for degradation[454]. It is possible that during Alzheimer's disease, the increase in $H_2O_2$ by the Aβ42-dependent mitochondrial dysfunction causes $K_v4$ to be targeted for degradation. The current method of ROS detection using $D_2$DCFDA has been described fairly reliable approach for detecting ROS[579], however, $D_2$DCFDA does not provide information regarding the identity of the ROS being detected. Alternatively, an older method for, specifically, measuring $H_2O_2$ was developed by Hyslop and Sklar (1984) in which *p*-hydroxyphenylacetate fluorescence is measured during the reaction of horseradish peroxidase and $H_2O_2$[580]. This method would allow one to confirm if concentrations of, specifically, $H_2O_2$ are increasing in the Aβ42 model.

Though oxidative damage to RNA has not been a research priority, RNA molecules are also vulnerable to oxidation by ROS and some studies have suggested that oxidation of RNA may contribute to neurodegenerative diseases. Guanosine is the predominant RNA base that becomes oxidized, in presence of highly reactive hydroxyl radicals, to form 8-hydroxyguanosine (8-OHG)[581]. Indeed, Abe *et al.* (2002) found that the levels of 8-OHG in the cerebrospinal fluid of

patients with early stages of Alzheimer's disease was five times higher than that of controls suggesting that RNA damage may play a role on the onset of this disease[582]. Furthermore, mRNA has also been reported to undergo age-dependent oxidation. Shan and coworkers (2003) reported that, in postmortem brain tissue of patients with Alzheimer's disease, there is a high level of oxidation on poly(A)-mRNA species that are related to Alzheimer's disease. They reported that many of these genes include some that play roles in synaptic plasticity and LTP, suggesting that RNA oxidation is very selective, and not an arbitrary consequence[583]. Though we did not test the effects of ROS on *$K_v4$* mRNA levels, it is possible that ROS is involved in the age-dependent decline of *$K_v4$* mRNA we measured, Figure A in Appendix, and that this damage by 40 days of age may contribute to the decline in protein levels at this age. It would be interesting to measure the 8-OHG content on older flies, and on flies that were previously used in an Alzheimer's model[454]. With a similar method as the one used by Shan *et al.* (2003), we could also determine the effects of ROS on $K_v4$ mRNA for both older flies and those used in the Alzheimer's model. It is possible that the accumulation of ROS during aging, or in Alzheimer's disease, not only leads to oxidation of the $K_v4$ protein but also to oxidation of $K_v4$ mRNA.

### 6.6. The Scaffolding Protein SIDL Contributes To Maintaining $K_v4$ Levels

A complementary mechanism that may contribute to the age-dependent decline in $K_v4$ protein levels is the expression of the scaffolding protein SIDL (Shal interactor of di-leucine). We found that 6 and 10 days of expression of *SIDL-RNAi* in young flies causes a decline by 30% and 50% in $K_v4$ protein levels, respectively, and that mRNA levels of *SIDL* decrease by ~20% at 40 days of age. These data suggest that the age-related decline in *SIDL* transcript is likely another mechanism involved in the age-dependent decrease of $K_v4$ protein levels. $K_v4$ contains a highly

conserved dileucine motif that has been described to be necessary and sufficient for proper somato-dendritic targeting of the channel in neurons[349]. SIDL was the first protein identified to interact with this motif, to co-localize with $K_v4$ in *Drosophila* neurons and when a GFP-tagged $K_v4$ carrying a deletion of the dileucine motif was expressed in *Drosophila* neurons, its somato-dendritic localization was altered[344].

Because the dileucine motif is important for proper subcellular targeting of $K_v4$, and SIDL interacts with this motif, one possibility is that the age-related decline in *SIDL* transcript may cause decreased expression of the SIDL protein which, in turn, may lead to mislocalization of $K_v4$. Mislocalization of membrane proteins has been shown in other cases to cause aggregation and disruption of proper cell function[437,584]. In future studies, we could conditionally express *SIDL-RNAi* in *Drosophila* neurons and use *GFP-$K_v4$* or *mCherry-pHluorin-$K_v4$* to follow the fate of $K_v4$ to test if knockdown of *SIDL* leads to a mislocalization or internalization of $K_v4$. Furthermore, protein scaffolds have been described to be required for stability of their associated proteins. In *Drosophila* photoreceptors, the deletion of the scaffolding protein InaD contributes to the instability and loss of signaling proteins that associate with it[502]. We could make use of CRISPR/Cas9[585] technology to create a *SIDL* mutation that would allow us to measure more robust detrimental effects on $K_v4$ protein levels. A report showed that the overexpression of the scaffold protein Galectin-3 in HEK cells led to increased levels of nanoclustering of the protein K-Ras GTP[586]. In similar experiments, we could overexpress the scaffolding protein SIDL which may stabilize and increase $K_v4$ protein levels. Altogether, the loss of SIDL likely results in $K_v4$ mislocalization and instability which may result in a decline of motor function.

Peristalsis is a synchronized rhythmic movement required for successful *Drosophila* eclosion[504,505]. The repetitive firing of *Drosophila* neurons requires proper function, and likely

localization, of $K_v4$ channels to maintain excitability[359]. We found that a *SIDL* knockdown resulted in a large decrease of successful eclosion, as only 15% of flies were able to exit the pupal case to become adults. These results suggest that expression of *SIDL-RNAi* led to mislocalization and instability of $K_v4$ which resulted in defects in peristalsis and successful eclosion. Indeed, Ping and colleagues showed that $K_v4$-dependent A-type currents are required for repetitive rhythmic movement[359].

We were able to partially rescue the lethal phenotype of the *SIDL* knockdown with either *SIDL* or *$K_v4$*, but not with the transmembrane protein *CD8-GFP*, supporting the idea that stability of $K_v4$ protein levels is required for proper motor coordination to ensue. Further testing is needed, however, to determine if $K_v4$ is being targeted for degradation in the absence or low levels of SIDL, and if SIDL protein is indeed lower in 40-day old flies. We were also able to enhance eclosion rates with expression of *SIDL* or *$K_v4$*, but not with *CD8-GFP*. It is possible that the increase in SIDL levels might increase the stability of $K_v4$, and with this an increase in the density of $K_v4$-dependent A-type currents. Experiments measuring $K_v4$ levels with overexpression of *SIDL* could help determine if SIDL leads to increased expression of $K_v4$.

Although there is no known protein with high homology in mammals, transmembrane protein 1 (TMEM1) has been identified as one potential homolog candidate of *SIDL*, with 29% amino acid identity[344]. *TMEM1*, originally named *EHOC-1* (Epilepsy, holoprosencephaly candidate-1), encodes a protein with two predicted transmembrane domains[587]. This gene has been mapped to the human chromosomal region 21q22.3 which has been linked to hereditary syndromes[587]. To date, only *Drosophila* SIDL[344] and *S. cerevisiae* Trs130[588] have been described as orthologs of the TMEM1 protein, and there are no present studies that report TMEM1 expression or function. In future directions, we could express human TMEM1 in *Drosophila* to

first determine if it co-localizes with $K_v4$ channels in neuronal cultures. It would be of interest to determine if TMEM1 interacts with the C-terminal tail of $K_v4$, and if so, if it interacts with the highly conserved di-leucine motif in the C-terminal of $K_v4$. Another study of interest would be to determine if expression of human TMEM1 rescues, at all, the phenotype we measured with expression of *SIDL-RNAi*.

### 6.7. Conclusion

In this dissertation my aim was to shed light on the molecular mechanisms underlying an age-dependent decrease in $K_v4$ channels which I found to correlate with a decline in coordinated motor function. Although there have been reports describing how age affects channel levels, localization, and function, little is known about the effects of age on $K_v4$ channel protein. I found this phenomenon to be specific to $K_v4$ and to be conserved in the hippocampus of mice where I measured an age-related decline in $K_v4.2$ levels.

I showed that ROS accumulate with age in *Drosophila* and that $H_2O_2$ contributes to the decline in $K_v4$ levels during aging, and even in young flies acutely exposed to $H_2O_2$. In my experiments, however, I measured steady-state levels of $K_v4$ protein, and much remains to be done to understand the effects of $H_2O_2$ accumulation during aging on $K_v4$ current. It will also be important to determine whether $K_v4$ A-type current is affected by age. In future experiments it will be of interest to determine the mode of oxidation of $H_2O_2$ and what happens to the channels once oxidized. Indeed, we do not know if $H_2O_2$ exposure is also modifying channel function, leading to aggregation by creating sulfhydryl bonds between channels, or signaling channels for internalization to degrade or to increase internal vesicle channel pool. Neither do we know the effects of $H_2O_2$ on mRNA coding for $K_v4$ protein.

Scaffolding proteins play a critical role in intracellular organization of subcellular components and SIDL has been described as a scaffolding protein that interacts with the highly conserved di-leucine motif of $K_v4$. Although SIDL has been previously described to likely play a role in $K_v4$ somato-dendritic localization in neurons, I found that SIDL is directly involved in the stability of $K_v4$ channels, as a knockdown of SIDL lead to a decline in steady-state levels of $K_v4$. The question of whether SIDL is required for proper localization of $K_v4$ still remains unanswered. It would be of interest, as well, to determine if SIDL is regulated by ROS. Experiments using the *SIDL-RNAi* transgenic line should be performed to determine if it leads to mislocalization of $K_v4$ channels. Interestingly, our behavioral experiments studying *Drosophila* eclosion showed that overexpression of the scaffolding protein SIDL increased successful eclosion rates, as did an overexpression of $K_v4$. It would be of interest to test if the overexpression of SIDL leads to increased steady-state levels of $K_v4$. If so, we would like to know if there are increased levels of functional $K_v4$. Because Ping and colleagues (2011) described that $K_v4$ channels enable neurons to repetitively fire during prolonged input/output, these results would suggest that an overexpression of SIDL may increase or stabilize levels of functional $K_v4$ channels which may result in enhancing rhythmic behaviors like the peristaltic movement involved in *Drosophila* eclosion.

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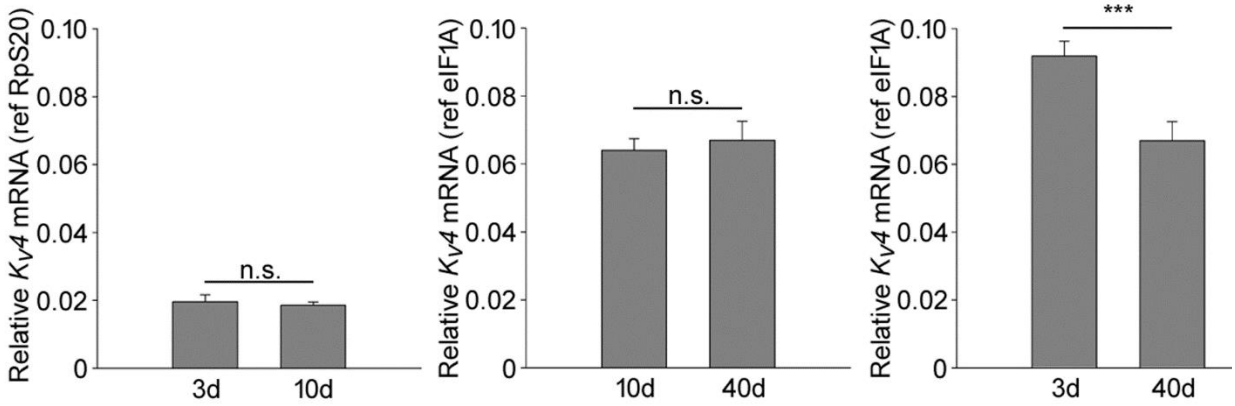


**Figure A. *$K_V4$* mRNA levels decline between 3d and 40d old adult wildtype flies.** Left and middle, *$K_V4$* relative mRNA levels do not undergo a significant change between 3d and 10d or between 10d and 40d of age, using RpS20 or eIF1A genes as reference (700 ng RNA from 10 heads per sample, n=17-24, n.s. represents no significant difference, Student's t-test). Right, *$K_V4$* mRNA levels show, however, a significant age-dependent decline between 3d and 40d of age, using eIF1A as a gene reference (each sample was prepared from 700 ng RNA extracted from 10 heads, n=16-23 amongst the three experiments above, n.s. denotes no significant difference, *** $p \leq 0.001$, Student's t-test).