Investigating the PECR-Mediated Mechanisms of Acamprosate in Ethanol Tolerance and Stress Resistance

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Magdalena Gompert

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Abstract

Alcohol use disorder (AUD) is a complex and chronic condition influenced by both environmental and genetic factors. Despite available treatments, relapse rates remain high, highlighting the need for more targeted therapeutic approaches. Acamprosate is a commonly used medication for maintaining abstinence in AUD, yet its exact mode of action and variable efficacy across individuals are not fully understood. Emerging evidence suggests a neuroprotective role of Acamprosate also outside of AUD. This thesis explores the interplay between a genetic factor contributing to AUD and its effect on the efficacy of Acamprosate to investigate the benefits of more personalized treatment strategies. To do this, this thesis aimed to elucidate the role of the peroxisomal trans-2-enoyl-CoA reductase (PECR), an enzyme with single nucleotide polymorphisms found to correlate with early onset of AUD, in EtOH-induced sedation and tolerance in *Drosophila*. Furthermore, it was investigated whether Acamprosate function is indeed able to reduce oxidative stress in the central nervous system (CNS) and if that function is reliant on PECR. PECR proved to be an important regulator for ethanol-induced functional tolerance. The findings identify PECR as a key regulator of ethanol-induced functional tolerance and reveal its broader role in enhancing resistance to oxidative and environmental stress. The effectiveness of Acamprosate in promoting both tolerance development and stress resilience was found to be dependent on sufficient levels of PECR in the CNS, suggesting a PECR-mediated mechanism of action. Furthermore, Acamprosate itself upregulates PECR expression, indicating a potential feedback loop that may enhance its therapeutic effects. This connection between PECR and Acamprosate not only advances our understanding of the drug's mode of action but also highlights the importance of individual molecular profiles in influencing treatment outcomes. These insights support the potential for more personalized strategies in the management of AUD.

Zusammenfassung

Alkoholabhängigkeit ist eine komplexe und chronische Erkrankung, die sowohl von Umweltfaktoren als auch von genetischen Faktoren beeinflusst wird. Trotz der verfügbaren Behandlungen sind die Rückfallquoten nach wie vor hoch, was den Bedarf an gezielteren therapeutischen Ansätzen unterstreicht. Acamprosat ist ein häufig verwendetes Medikament zur Aufrechterhaltung der Abstinenz bei Alkoholabhängigkeit, doch sind die genaue Wirkungsweise und die unterschiedliche Wirksamkeit bei verschiedenen Individuen noch nicht vollständig geklärt. Neue Erkenntnisse deuten auf eine neuroprotektive Rolle von Acamprosat auch außerhalb des Alkoholismus hin. In dieser Arbeit wird das Zusammenspiel zwischen genetischen Faktoren, die zu Alkoholabhängigkeit beitragen, und der Wirksamkeit von Acamprosat untersucht, um personalisierte Behandlungsstrategien zu entwickeln. Zu diesem Zweck wurde die Rolle der peroxisomalen trans-2-Enoyl-CoA-Reduktase (PECR), eines Enzyms, dessen Einzelnukleotid-Polymorphismus mit einem frühen Erkranken an Alkoholabhängigkeit korreliert, für die Ethanol-induzierte Sedation und Toleranz in *Drosophila* untersucht. Darüber hinaus wurde untersucht, ob Acamprosat tatsächlich in der Lage ist, oxidativen Stress im zentralen Nervensystem (ZNS) zu reduzieren, und ob diese Funktion von PECR abhängig ist. Die Ergebnisse weisen PECR als einen wichtigen Regulator der durch Ethanol induzierten funktionellen Toleranz aus und zeigen seine umfassende Rolle bei der Verbesserung der Widerstandsfähigkeit gegenüber oxidativem und umweltbedingtem Stress auf. Es wurde festgestellt, dass die Wirksamkeit von Acamprosat bei der Förderung der Toleranzentwicklung und der Stressresistenz vom PECR-Spiegel im ZNS abhängt, was auf einen PECR-vermittelten Wirkmechanismus hindeutet. Darüber hinaus erhöht Acamprosat selbst die PECR-Expression, was auf eine potenzielle Rückkopplungsschleife hinweist, die seine therapeutische Wirkung verstärken könnte. Diese Verbindung zwischen PECR und Acamprosat erweitert nicht nur unser Verständnis für die Wirkungsweise des Medikaments, sondern unterstreicht auch die Bedeutung individueller molekularer Profile für die Behandlungsergebnisse. Diese Erkenntnisse unterstützen das Potenzial für stärker personalisierte Strategien bei der Behandlung von Patienten mit Alkoholkrankheit.

1. Introduction

1.1 Alcohol Use Disorder

Alcohol is one of the most widely consumed psychoactive substances in the world, deeply embedded in social, cultural, and economic structures. While moderate consumption is often perceived as harmless, chronic and excessive drinking can lead to a complex condition called alcohol use disorder (AUD). AUD is a condition characterized by recurrent relapses and compulsive alcohol drinking, the loss of control over alcohol intake, and the emergence of a negative emotional state when alcohol is no longer available (Koob et al., 2020). While it is a worldwide problem, Germany specifically has one of the highest alcohol consumption per capita rates and has increasing alcohol-dependent hospitalization rates (Kraus et al., 2015; Peacock et al., 2018). In a German study, 3.1% of a randomly selected sample of citizens were found to be suffering from AUD, and the resultant economic expenses of the country were estimated at around 26.7 billion euros per year (Atzendorf et al., 2019). These expenses are a direct result of alcohol-related diseases and injuries. The Global Burden of Disease Study demonstrated that chronic use of alcohol enhanced the risk of cardiovascular diseases and cancer while even occasional use heightens the risk of injuries and irreversible brain damage (Griswold et al., 2018; Järvenpää et al., 2005; Mundt et al., 2009). In addition to the physical consequences of AUD, the condition has significant social impacts such as failed relationships, loss of employment and psychiatric symptoms. Considering the substantial societal impact of AUD, research in this field seeks to understand how alcohol alters brain function across a range of concentrations and time frames. The molecular networks of the underlying effects of stress and stress responses are important as stress in AUD patients often invokes craving which results in relapse. The stress response can be coordinated through the activity of the hormonal system called the hypothalamic-pituitary-adrenocortical (HPA) axis. Changes in the activity of the HPA axis can result in pain relief, but it can also enhance the organism's responsiveness to the rewarding effects of alcohol (Brady and Sonne, 1999). A dysregulation of the HPA axis can influence the motivation for alcohol-related behaviours including self-administered alcohol exposure and withdrawal (Koob and Le Moal, 1997). Alcohol abuse induces significant alterations in the nervous system, with protracted exposure to ethanol resulting in an imbalance between the inhibitory and excitatory neurotransmitters (Koob and Volkow, 2016). The brain undergoes

neuroadaptive changes in an attempt to compensate for these effects, resulting in the development of alcohol tolerance (Tsai and Coyle, 1998). Alcohol tolerance is characterized by a decrease in the effectiveness of a constant amount of alcohol, or an increase in the amount of alcohol required to achieve a similar effect. While it is defined as an acquired resistance to the inebriating effects of alcohol, tolerance can develop against the pleasurable as well as the aversive effects of alcohol intoxication (Tabakoff et al., 1986). In allosteric models of addiction, tolerance to the substance is a key motivational construct: individuals seek to regain positive reinforcement by consuming drugs, but fail due to their pre-existing tolerance, thereby leading to further tolerance development (Koob and Le Moal, 1997). While tolerance is considered a necessary factor for the development of AUD, it not sufficient on its own (Elvig et al., 2021). However, a high resistance to the effects of alcohol is associated with faster progression and greater severity of AUD (Schuckit, 1994). The hypothesis that the build-up tolerance in AUD patients might be inheritable has been postulated by many researchers (Enoch and Goldman, 2001; Heath et al., 1997; Slutske et al., 1999). Children of alcoholics have been shown to exhibit a lower increase in postural sway after alcohol consumption (Schuckit, 1985). Furthermore, social and environmental stressors as well as childhood trauma have been identified as risk factors for the development of AUD. However, a recent meta-analysis of twin and adoption studies concluded that AUD is approximately 50 % heritable (Verhulst et al., 2015), which calls for scientific research to identify genetic predispositions for developing AUD.

1.2 Ethanol Intoxication

A comprehensive investigation of the genetic and environmental risk factors associated with AUD necessitates a thorough analysis of the mechanisms underlying alcohol metabolism after exposure. Immediately following ingestion, the small two-carbon molecule ethanol (EtOH) crosses biological membranes by passive diffusion. The initial phase of “first pass” EtOH metabolism occurs in the stomach via the primary enzymes responsible for its oxidation: alcohol dehydrogenase (ADH) and the cytochrome P450 dependent enzyme (CYP2P1). These enzymes are present throughout the body, with the highest concentrations found in the liver where most of the EtOH is oxidized (Fagerberg

et al., 2014). Inside the cells, EtOH is oxidized to acetaldehyde, a cytotoxic substance that can impair protein secretion, enzyme activities and induces lipid peroxidation (Lieber, 1988). Acetaldehyde is oxidized to acetate and subsequently to acetyl-CoA, which, depending on nutritional, hormonal or energetic status can be used as a precursor for fatty acids, ketone bodies and cholesterol (Hardie, 1989). The Nicotinamide adenine dinucleotide (NAD^+/NADH) redox ratio is decreased due to the NAD^+-dependent nature of these reactions, which in turn results in the inhibition of important reactions such as glycolysis, the citric acid cycle and fatty acid oxidation (Dawson, 1979; Williamson and Cooper, 1980). This process leads to a significant reduction in the cytoplasmic and mitochondrial NAD^+ pools, resulting in the inhibition of gluconeogenesis and fatty acid oxidation. The rate of NADH production from EtOH oxidation to acetate has been shown to influence the citric acid cycle, consequently reducing CO_2 production in the liver (Krebs et al., 1969). Additionally, the concentration of malonyl-CoA is increased, which suppresses the uptake and oxidation of long-chain fatty acids in mitochondria (McGarry et al., 1977). Furthermore, the process of fatty acid synthesis is activated, and in combination with the excess fatty acids, it results in a fat accumulation in hepatocytes, leading to the manifestation of the characteristic fatty liver disease of alcoholics (Sozio et al., 2010; Wilson and Matschinsky, 2020). EtOH can induce alterations in the epigenetic regulation of cells which are characterized by the modulation of DNA and histones resulting in the condensation or relaxation of chromatin. Acute and chronic alcohol exposure can have opposite epigenetic effects highlighting the complexity of alcohol induced epigenetic dysregulation. For instance, in macaques, DNA methylation was found to be decreased in genomic loci involved in synaptic functions, such as neurotransmitter release or receptor trafficking, following alcohol self-administration (Cervera-Juanes et al., 2017). The body possesses several mechanisms that can lead to the development of tolerance against the intoxicating effects of EtOH. One such mechanism, a metabolic or pharmacokinetic tolerance is characterized by a reduced absorption of EtOH or a more efficient removal of EtOH from the body (Elvig et al., 2021; Tabakoff et al., 1986). Another, functional or pharmacodynamic tolerance, is achieved by adaptations in neuronal functions, consequently reducing the potency of EtOH (Fadda and Rossetti, 1998; Tabakoff et al., 1986). These include the upregulation of gene expression or transcription

factors that contribute to the termination of alcohol intake after chronic exposure to EtOH (Erickson et al., 2018; Warden et al., 2020; Ziv et al., 2019).

EtOH as well as acetate have been shown to cross the blood-brain barrier, enabling them to exert a direct influence on brain cells. However, EtOH is an unspecific drug, capable of interacting with other biomolecules via hydrogen bonding or weak hydrophobic interactions. The numerous molecular targets of EtOH contribute to the difficulty of inventing specific medications (Abrahao et al., 2017). Furthermore, the effects of EtOH are dependent on the age of exposure of the organism. In foetal development, for instance, EtOH consumption by the mother can result in severe neurodegenerative damage, craniofacial abnormalities, growth deficiencies and nervous system dysfunction (Halliday et al., 1982).

The acute reinforcing effects of EtOH are mediated by multiple neurotransmitter systems, including the GABAergic, dopaminergic, serotonergic and glutamate systems (Morato and Khanna, 1996). EtOH facilitates the formation of action potentials of dopaminergic neurons in the midbrain and increases extracellular dopamine levels in the ventral tegmental area (Deehan et al., 2016). The development of rapid tolerance, defined as a reduction in the effect of EtOH during a second exposure shortly after a first exposure, is dependent on GABA and NMDA receptors (Barbosa and Morato, 2001; Khanna et al., 1991, 1994). EtOH has been observed to potentiate Cys-loop ligand-gated ion channels, like $GABA_A$ and glycine receptors, while inhibiting glutamate receptors (Aguayo, 1990; Dildy and Leslie, 1989; Ye et al., 2001). Furthermore, it has been demonstrated that EtOH can directly interact with targets other than ion channels, including intracellular signalling molecules such as protein kinase C and adenylate cyclase (Pany and Das, 2015; Yoshimura et al., 2006). Indirect targets of EtOH include ion-channel subunits, intracellular signalling pathways, growth factors, transcription factors, proteins involved in the epigenetic regulation of gene expression, and membrane lipids (Abrahao et al., 2017). Additionally, EtOH has been shown to affect the splicing of mRNA at synapses which may disrupt synaptic function (O'Brien et al., 2018).

The impact of EtOH on the brains neuronal structure can be severe. Even the consumption of amounts of alcohol which do not disrupt sensory and motor skills or associative learning abilities, have been shown to reduce the production of cells in the dentate gyrus or hippocampus of adult rats (Anderson et al., 2012; Hansson et al., 2010).

As a result, the potential for structural plasticity of the brain is reduced. The nervous system is enriched in lipids and maintains a diverse lipid composition compared to other tissues. The diversity of the lipids is influenced by factors such as age, gender, neuronal activity, stress, and trauma. Lipids are characterized by their polar head and their fatty acid chain, which can vary in length and saturation state, thereby determining the size, shape and flexibility of the lipid (Lauwers et al., 2016). The double-layered lipid membranes of cells are susceptible to alterations following the administration of EtOH and are potentially involved in the compensatory mechanisms that protect against EtOH-induced damage (Hernández et al., 2016). EtOH has been shown to reduce the transition temperatures of membrane-bound enzyme activities and to decrease the order parameter. Therefore, EtOH enhances the cell membranes fluidity and disorders the membrane which may result in altered enzyme function for membrane-bound enzymes (Lenaz, 1987; Los et al., 2013). Following chronic EtOH administration, both values increase, indicating that chronic EtOH exposure leads to a resistance against the disordering properties of EtOH administration. For instance, an increase in neuron membrane cholesterol provides rigidity to the membranes and reduces EtOH binding while other membrane functions are simultaneously impaired (Barceló-Coblijn et al., 2013; Gustavsson, 1995; Rubin and Rottenberg, 1983). In addition to the direct effects of EtOH on lipids and membranes, the reactive oxygen species (ROS) derived from EtOH metabolism and its products further damages the cells. For example, lipid peroxidation affects polyunsaturated fatty acids in membrane phospholipids producing bioactive aldehydes such as 4-hydroxyalkenals and malondialdehyde, which in turn results in a decreased neuronal viability (Pizzimenti et al., 2013). In the brain, catalase and CYP2E1 are the main enzymes for the oxidation of EtOH during which both simultaneously increase ROS (Zimatkin et al., 2006). The induction of CYP2E1 has been shown to generate ROS and nitric oxide, whilst EtOH competes with H_2O_2 at the catalase binding side, thereby inhibiting ROS reduction (Haorah et al., 2008; Koop, 2006). The acetaldehyde derived from the catalase-dependent oxidation of EtOH contributes to tissue damage and the typical hangover symptoms after EtOH intoxication. Acetaldehyde induces an oxidative signalling cascade through phosphorylation of the extracellular-signal regulated kinase (ERK) and special MAP kinases (Li et al., 2004; Zhang et al., 2004). Additionally, oxidative stress itself activates ERK via irregular phosphorylation

(Perry et al., 1999). The activation of ERK can contribute to inflammatory pain and glutamate-induced oxidative toxicity, although it generally facilitates the transduction of extracellular signals (Stanciu et al., 2000; Xing et al., 2023). Acetaldehyde reacts with other substances to form biologically active compounds, such as salsolinol, which derives from a bond between acetaldehyde and dopamine and is involved in neurotoxicity (Xie et al., 2013). The consequences of these processes are increased ROS, a reduction of glutathione and intracellular ATP (Wanpen et al., 2004). Increased ROS in neurons results in damages or complete degradation of essential complex molecules, such as lipids, proteins and DNA (Hernández et al., 2016). In different conditions, ROS can either initiate or execute various molecular cell death mechanisms (Valencia and Morán, 2004). Therefore, obstruction of an accumulation of ROS is of utmost importance for the cell.

After chronic EtOH ingestion, enzymatic degradation processes are affected. The amounts of superoxide-dismutase (SOD), an enzyme which catalyses the dismutation of oxide radicals to hydrogen peroxide, as well as catalase and glutathione, both proteins which catalyse the decomposition of hydrogen peroxide to water and oxygen, are decreased (Hernández et al., 2016; Schrader and Fahimi, 2006; Wu and Cederbaum, 2003). To summarize, AUD patients experience high oxidative stress because the EtOH metabolism increases ROS while the capacity to compensate for ROS is simultaneously lowered.

1.3 Role of Peroxisomes in Oxidative Stress

The regulation of the cellular stress response, including metabolic and environmental stress, is mostly done via the peroxisomes. Peroxisomes are single membrane-enclosed organelles which exist in almost all eukaryotic cells. They are highly dynamic and can adapt their shape and size, the location and abundance in response to environmental or nutritional cues (Lodhi and Semenkovich, 2014). In general, peroxisomes carry out metabolic functions such as the catabolism of very long chain fatty acids (VLCFAs) to long chain fatty acids (LCFAs), branched fatty acids, amino acids and polyamines. The completion of the beta-oxidation of LCFAs takes place in the mitochondria (Sassa and Kihara, 2014; Verhoeven et al., 1998). Peroxisomal processes are calibrated in a tissue-specific manner based on the nutritional status of the organism (Palou et al., 2008;

Zechner et al., 2017). Excess energy is stored as triglycerides in lipid droplets and can be mobilized during nutrient deprivation via lipolysis. The activities of lipolytic enzymes are regulated by the nutrient status (Zechner et al., 2017). Fasting stimulates the physical interaction between peroxisomes and lipid droplets presumably to access the energy storage (Kong et al., 2020). In the brain adequate homeostasis of lipids is crucial, since lipid dysregulation can lead to energy loss, synaptic disfunction and neurodegenerative diseases (Vallés and Barrantes, 2022).

Peroxisomes are responsible for the production and catabolism of ROS and the biosynthesis of ether lipids. Ether lipids are completed in the endoplasmic reticulum and stabilise lipid raft microdomains while improving cellular fitness and facilitating membrane fusion processes (Dean and Lodhi, 2018; Lodhi and Semenkovich, 2014). Peroxisomes include multiple flavin adenine dinucleotide-dependent oxidoreductases which can accept and donate electrons in addition to catalases which reduce hydrogen peroxide to water. A disruption of the ROS balance in peroxisomes can lead to age-dependent diseases like diabetes, cancer and neurological disorders (Cipolla and Lodhi, 2017). There are several peroxisomal import pathway enzymes which can also modulate the cellular sensitivity to oxidative stress (Dubreuil et al., 2020). Pex5, an import receptor for peroxisomal matrix enzymes with a PTS1 motif, is a redox-sensitive protein which exhibits a reduced ability to import catalases when the oxidative stress in the cytosol is high (Apanasets et al., 2014). Inactivation of Pex5 increases the cell resistance against oxidative stress caused by exogenous H_2O_2 treatment as it confines the catalase in the cytosol to counteract H_2O_2 (Okumoto et al., 2020). Defects in peroxisomes increases the ROS production resulting in protein carbonylation, protein lipoxidation and glycoxidation, as well as lipid metabolism dysregulation. They can activate the NF-κB transcription factor inducing inflammation (Di Cara et al., 2019).

1.4 The Peroxisomal Trans-2-enoyl-CoA Reductase

In order to address the possible genetic risk variants for AUD, several genome-wide association studies (GWAS) were conducted. GWAS constitute an unbiased approach by analysing the whole genome without prior knowledge, and they analyse which single nucleotide polymorphisms (SNPs) occur at a higher frequency in people affected with the

disorder of interest compared a control group. As expected, given the association of the contained genes with EtOH metabolism, the ADH cluster was identified as a risk locus (Zuo et al., 2014). However, alterations in the enzymatic cascade of ethanol breakdown appear to be insufficient in explaining the multifaceted nature of AUD. Consequently, certain GWAS have concentrated on SNPs in genes with brain-specific expression. Chronic alcohol abuse has been linked to permanent neuronal loss in multiple brain regions, as well as to the deterioration of memory, cognition and motor function. Several promising genes were related to protein functions in neuronal development and membrane function (Zuo et al., 2014).

The chromosome region 2q35 is a region with linkage to alcoholic phenotypes (Nurnberger et al., 2001). Especially a component of the human event related brain potential (ERP), P300, a positive-going voltage change in response to an infrequent task-relevant event is used as a marker in a variety of psychiatric disorders and alcoholism (Porjesz et al., 1980). Significantly lower P300 amplitudes were observed in sons of AUD fathers and in alcoholism patients even after a sufficient period of abstinence which may be a vulnerability marker of alcoholism (Begleiter et al., 1984; Benegal et al., 1995). Variances at the area of chromosome 2q35 reduced the amplitude of P300 further indicating that genetic variation in this area may increase chances of alcohol dependence (Porjesz et al., 2002). Additionally, a linkage between low level response to alcohol and a microsatellite marker close to markers used for chromosome 2q35 was found (Schuckit et al., 2001). Low level response to alcohol is an endophenotype related to heavy drinking and alcoholism (Schuckit et al., 2008). A GWAS by Treutlein and colleagues specifically examined males with an early onset of AUD with the intention to exclude environmental and age-related factors as best as possible (Treutlein et al., 2009). They found 16 significant SNPs, nine of which were located within genes. These genes were associated with DNA repair and transcription, protein processing and translation as well as cell contact, cell growth and cell metabolism. Among them was *pecr,* a gene located on chromosome region 2q35, which encodes for the human peroxisomal trans-2-enoyl CoA reductase. This enzyme is involved in the fatty acid chain elongation cycle and is located in the peroxisomes of the cell (Das et al., 2000). This NADPH-dependent key enzyme of the fatty acid synthesis catalyses the reduction of medium-chained, unsaturated enoyl-CoAs to saturated acyl-CoAs which can undergo oxidation in the mitochondria as a mean

to produce energy (Das et al., 2000; Faust et al., 2012). When the energy supply is switched from glucose to fatty acids, typically induced by starving conditions, the transcript levels of PECR are increased (Zhang and Underwood, 1999). The fatty acid elongation system comprises enzymes which are responsible for the addition of two carbon units to the carboxyl end of a fatty chain. It is a very conserved process with four main enzymes: the condensing enzyme (β-ketoacyl-CoA synthase) followed by the beta-ketoacyl-CoA reductase, the β-hydroxyacyl-CoA dehydrase and finally the trans-2-enoyl-CoA reductase (Lee et al., 2006, Fig. 1). The fatty acid elongation cycle is initiated by the condensation of malonyl-CoA. There are multiple microsomal elongation systems with different chain length specificity. PECR functions best with chain lengths of 10:1 CoA but generally works between 6:1 and 16:1, however longer chained enoyl-CoAs (14:1, 16:1) are the best substrates for peroxisomal enzymes (Das et al., 2000). The conversion from unsaturated fatty acids to saturated acyl-CoAs which can be used in carnitine derivatives and exported to mitochondria for energy production, is likely an important metabolic pathway when the organism switches from using glucose to fatty acids during starvation conditions (Das et al., 2000).

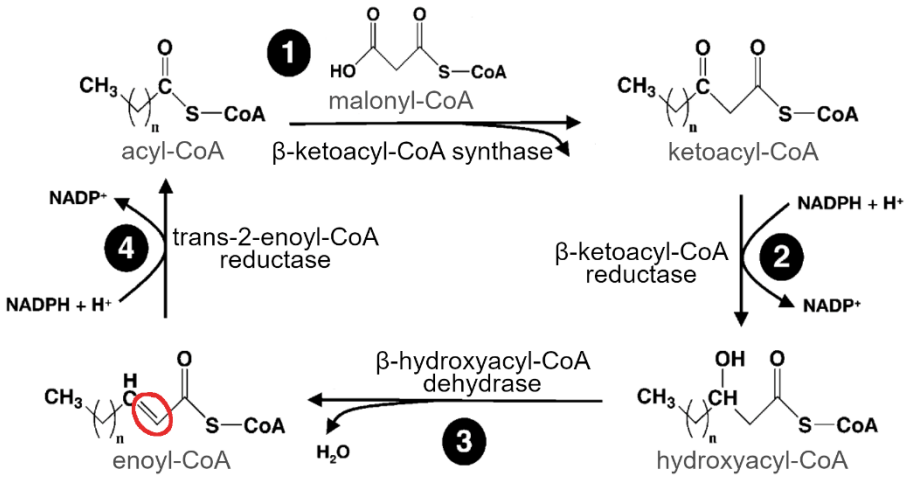

Figure 1: Involvement of PECR in lipid synthesis.
Acyl-CoA$_{(n)}$ is synthesised to Acyl-CoA$_{(n+2)}$. The red circle depicts the double bond which is then reduced by PECR. Modified after Lee et al., 2006.

Additionally, the elongated fatty acids can be used to generate VLCFAs. Saturated and monounsaturated VLCFAs are major fatty acid constituents of sphingolipids (Kihara, 2012). The trans-enoyl-CoA reductase was identified as the enzyme in the S1P metabolic

pathway responsible for the degradation of sphingosine in sphingolipids (Wakashima et al., 2014). In summary, the consequences of SNPs found in the *pecr* gene region might affect the adequate homeostasis of lipids in neurons, which would be crucial for synaptic function and tolerance development (Vallés and Barrantes, 2022), thus making *pecr* a plausible candidate for gene variants that predispose alcohol addiction.

1.5 Acamprosate as a Medication for AUD

Treatment for drug addiction can include pharmacotherapies: in the case of AUD, several medications were approved by the US Food and Drug Administration and the European Medicines Agency. Three main medications are prescribed and still studied: First, naltrexone which works via opioid receptor blockage and should reduce cravings, and the feeling of euphoria associated with substance abuse. Second, disulfiram, an inhibitor of ALDH, inducing immediate hangover due to the accumulation of acetaldehyde and its toxic effects. Lastly, acamprosate or Calcium-N-Acetylhomotaurine (Campral®), a synthetic taurine analogue which helps to increase the probability of maintenance of abstinence although its exact mechanisms are still unknown (Burnette et al., 2022). These medications should provide aid to maintain abstinence alongside the psychological support in drug facilities. However, only an approximated number of 1.6 % of adult AUD patients in 2019 used medication-based treatment in the US (Han et al., 2021). An individual patient data meta study showed a significant effect of acamprosate in improving the rates of abstinence and the reduction of heavy drinking in AUD patients. Additionally, they could associate acamprosate with higher rates of treatment completion (Mason, 2001; Mason and Lehert, 2012).

Acamprosate consists of a dimer of acetylhomotaurinate linked through a calcium salt and has structural similarities to both taurine and GABA (Durbin et al., 1996). While Acamprosate can cross the blood-brain barrier, its exact mechanism in the brain is still unknown, although there is evidence for its effects on neurotransmitter systems. Acamprosate reduces voluntary EtOH intake through GABA receptors (Boismare et al., 1984), specifically by blocking the inhibiting $GABA_B$ receptors (Berton et al., 1998). It can bind to taurine receptors with high affinity, since at the same concentration it is unable to bind to $GABA_A$ or glutamatergic receptors (Wu et al., 2001). Acamprosate functions as an

agonist of glutamate through NMDA receptors which are dysregulated in AUD patients (Iorio et al., 1992; Lovinger et al., 1989; Naassila et al., 1998). Treatment with Acamprosate decreases the expression of the excitatory amino acid transporter 2 (EAAT2), a solute transporter which clears glutamate from the extracellular space, implicating that Acamprosate stabilizes the hyper-glutamatergic condition in AUD mice. These mice showed reduced EtOH drinking and preference after Acamprosate treatment (Germany et al., 2018). Additionally, Acamprosate may rescue glutamate toxicity through restoring reticulon4 (RTN4) and NF-κB-mediated neuroimmune signalling. RTN4 regulates neurite fasciculation, branching and extension making neuronal restoration a potential efficacy mechanism in Acamprosate treatment (Germany et al., 2018). NF-κB is a transcription factor involved in immune signalling which can be downregulated by taurine (Sun et al., 2012).

As mentioned above, there is no consistent research about the exact mechanism of Acamprosate in AUD, however the influence of the medication on the neurotransmitter systems was also proven in several non-AUD related studies. Acamprosate can reduce tinnitus perception or awareness when given in the same concentrations as prescribed to AUD patients (Farhadi et al., 2020; Sharma et al., 2012). Tinnitus is a phantom auditory perception, thus a perception of sound without the corresponding acoustic or mechanical correlates in the cochlea (Jastreboff, 1990). A GABA – glutamate imbalance is thought to be the cause for tinnitus (Zhang et al., 2021). Glutamate is the major neurotransmitter in the cochlea and the central auditory pathway and may cause “excitotoxicity”, thereby generating and maintaining sensory neural tinnitus (Puel et al., 2002). Similarly, a combination of Acamprosate and baclofen, another AUD medication, protected neuron-muscle co-cultures against glutamate toxicity (Boussicault et al., 2020).

Another example is the reduction of neuropathic pain in rats. Neuropathic pain is a chronic pain owing to nerve damage or disease of the CNS (Colloca et al., 2017). Abbasi and colleagues showed that treatment with Acamprosate reduced neuropathic pain and could even increase the thermal pain threshold of the animals (Abbasi et al., 2023). Further, Acamprosate may have anti-inflammatory effects because it decreases extracellular-signal-regulated kinases which lead to an inhibition of inflammatory cytokines (Germany et al., 2018; Tekari et al., 2022; Wang et al., 2021).

Some studies find no significant effect of the medication or hypothesize that the bound calcium is the main reason for its effectiveness (Spanagel et al., 2014). Calcium is associated with convulsive behaviour during withdrawal and EtOH withdrawal is accompanied by upregulated and hyper-excitable calcium-channels (Littleton et al., 1990; Morton et al., 1992; Whittington et al., 1995). Acamprosate reduces calcium entry mediated by excitatory amino acids, potassium-depolarization and membrane depolarization (Allgaier et al., 2000; Zeise et al., 1993). The interaction of Acamprosate with glutamate and its receptors and calcium channels may explain the ability of Acamprosate to reduce the hypermotility related to ethanol withdrawal (Dahchour and De Witte, 2000). In conclusion, there is a need for proper investigation of the function of Acamprosate, since its effects not only reduce the probability of relapse in AUD patients but also mitigate the effects of other diseases that target the equilibrium of neurotransmitters in the CNS.

1.6 *Drosophila* as a Model Organism for AUD

The fruit fly *Drosophila melanogaster* has proven to be a useful tool for measuring ethanol response behaviour. It has a general preference to consume ethanol over non-ethanol containing food (Devineni and Heberlein, 2009) and shows behavioural alterations after ethanol exposure similar to humans. Alcohol has intoxicating effects, it leads to hyperactivity, uncoordinated movement and, at last, sedation (Wolf et al., 2002). In *Drosophila*, EtOH exposure leads to an increase in ROS which negatively affects the organism's survival (Logan-Garbisch et al., 2015). Flies are able to develop a rapid tolerance, defined as increase in resistance after a single dose of EtOH, after its complete metabolic degradation (Crabbe et al., 1979; Scholz et al., 2000). A measurable rapid functional tolerance was described for the effects of ethanol on motor coordination and sedation (Scholz et al., 2000). Upon long-term, low-level continuous EtOH exposures, flies can develop a chronic tolerance, which persists significantly longer than rapid tolerance (Berger et al., 2004).

In humans, EtOH administration has various effects on different receptors in the CNS, mainly on the GABA- and glutamatergic system (Krystal et al., 2006, 2003). An impaired GABA clearance within brain structures like the amygdala contributes to alcohol addiction

(Augier et al., 2018). It was also determined that dysregulation of GABA and glutamate leads to withdrawal syndromes (Koob and Volkow, 2016; Tsai and Coyle, 1998). In flies, studies suggest a putative role for the $GABA_B$ receptor in the mechanisms of action of EtOH (Dzitoyeva et al., 2003). Additionally, NMDA receptors regulate synaptic plasticity and modulate a variety of behaviours. Alcohol functions as an NMDA receptor antagonist (Hoffman and Tabakoff, 1994; Lovinger et al., 1989), which enhances the number of NMDA receptors over the time of chronic alcohol abuse (Freund and Anderson, 1996; Tsai and Coyle, 1998). During withdrawal, a hyper-glutamic state was observed, and the resulting overactivity of the neurons elicits the symptoms of alcohol withdrawal (Dahchour and De Witte, 2000; Rossetti and Carboni, 1995). Mutations in NMDA receptors effect ethanol induced behaviour in flies as well: Flies with mutations in the NMDA receptor 1 have a reduced ethanol sensitivity and a faster recovery after sedation compared to their wild type controls (Troutwine et al., 2019).

AUD is a complex disease because of the multiplicity of factors which drive its development (Cummins-Beebee et al., 2023). Using the model organism *Drosophila* it is possible to exclude or adjust environmental factors like early life trauma or social standing and focus on the genetic predispositions which increase the probability for the development of AUD. GWAS findings can be reviewed in *Drosophila* as fly orthologues for GWAS AUD genes from studies in humans have shown an affected EtOH response in flies (Velo Escarcena et al., 2021). For example, genes involved in lipid metabolism have transcript alterations after EtOH exposure which leads to an accumulation of lipids (Logan-Garbisch et al., 2015; Velo Escarcena et al., 2021). One of the genes found by the GWAS by Treutlein and colleagues was *pecr*. The *Drosophila* orthologue CG10672 performs the same functions in lipid metabolism (Faust et al., 2012). The gene is located on chromosome 3L and spans a length of 1.2 kb. Its gene transcript is sectioned in three exons (Fig. 2). There are several genetic tools targeting the expression of *pecr*, one of which is the RNAi line HMS00753, which targets the second exon (Perkins et al., 2015). An RNAi mediated knockdown via $PECR^{HMS00753RNAi}$ led a reduction in transcript levels of about 54 % and to changes in the resistance against oxidative stress (Velo Escarcena et al., 2021). Further, a *PECR* mutant was created with a P-element insertion in the third exon, produced for the gene disruption project (Bellen et al., 2004). $PECR^{KG07864}$ mutant

flies showed a lower preference for EtOH-containing food after a withdrawal period (Velo Escarcena et al., 2021).

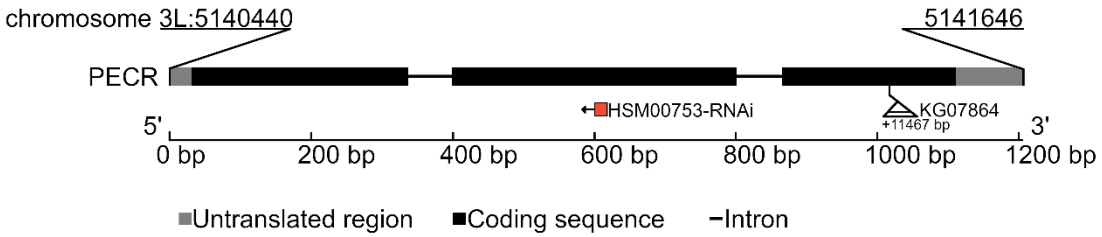

Figure 2: Genomic organisation of the *Drosophila pecr* gene.
The 1207 bp long gene is located on chromosome 3L between base pairs 5,140,440 and 5,141,646. Gene structure includes untranslated regions (grey), coding sequences (black) and introns (lines). Functional genetic tools targeting *pecr* include the RNAi construct $PECR^{HSM00753RNAi}$ (red) which spans parts of the second exon, and the P-element insertion mutant with an insertion located at 1016 bp in the third exon of *pecr.*

The medication Acamprosate can decrease the probability of relapse in wild type flies. Flies which were fed with EtOH-containing food for five consecutive days and afterwards went through withdrawal for one day showed a similar preference for EtOH as before the withdrawal. Flies fed with Acamprosate had a reduced preference after withdrawal (Velo Escarcena et al., 2021) similar to the findings in rodents (Boismare et al., 1984). Additionally, Acamprosate can rescue neuronal defects in flies with Fragile X Syndrome (Hutson et al., 2018). Fragile X Syndrome is an X-linked disability with defects in neuronal development and maturation which includes phenotypes like hyperactivity, attention deficit, hypersensitivity to sensorial stimuli, anxiety, aggression and epileptic seizures (Drozd et al., 2018).

In conclusion, *Drosophila* is an appropriate model organism for studies of alcohol use disorder because of its behavioural similarities to human intoxicated behaviour, its genetic availability and its susceptibility to medications like Acamprosate.

1.7 Scientific Aim

Alcohol use disorder is a prevalent and serious public health issue, contributing to psychological, and social problems worldwide. Understanding and addressing AUD is essential due to high relapse rates and the substantial burden it places on individuals and healthcare systems. The medication Acamprosate, licensed to support abstinence and reduce cravings, is still under investigation, as its mode of action remains unclear. Exploring how Acamprosate interacts with neurobiological systems is important, as the results may improve therapeutic strategies for patients struggling with AUD. Genetic predispositions can influence the efficacy of medications (Minikel et al., 2024; Plenge et al., 2013). It was shown that the anti-craving drug Naltrexone is more effective in patients with a specific polymorphism in an opioid receptor gene (Chamorro et al., 2012).

Acamprosate significantly improves the rates of abstinence in the withdrawal phase of patients recovering from AUD (Mason and Lehert, 2012). Furthermore, treatment with Acamprosate has been shown to increase the rates of treatment completion and medical compliance (Mason and Lehert, 2012). These results were reconfirmed in rats and *Drosophila*, since Acamprosate reduced withdrawal-elicited alcohol drinking in both model organisms (Dahchour et al., 1998; Velo Escarcena et al., 2021). In this study, possible effects of Acamprosate treatment on EtOH-induced behavioural changes, like tolerance to the sedative effects of EtOH, were analysed. The aim of this thesis was to analyse the efficiency of Acamprosate in relation to the AUD candidate gene *pecr* presented in the GWAS for AUD by Treutlein and colleagues, 2009. It was demonstrated that PECR expression is reduced in *Drosophila* heads following an exposure to EtOH (Velo Escarcena et al., 2021), indicating a function for PECR in EtOH-induced neuroadaptive changes. As these changes might affect EtOH-induced behaviour, like hyperactivity or sedation, $PECR^{KG07864}$ mutants were tested for their EtOH sensitivity and tolerance development. Furthermore, an RNAi-mediated PECR knockdown was induced in the fly CNS, muscles and fat body to explore whether PECR expression in the respective body part is needed to develop EtOH tolerance and to further discriminate between pharmacokinetic and pharmacodynamic tolerance. To exclude any metabolic changes due to PECRs role in fatty acid synthesis (Faust et al., 2012), body weight and food intake as well as glycogen, triglycerides and protein levels were measured and compared to

controls. Additionally, it was investigated whether any behavioural phenotypes might derive from a malfunctioning degradation of EtOH in the fly mutant body by measuring the degradation rate of EtOH after exposure. Since the aim was to investigate whether a degraded function of PECR might also affect Acamprosates efficacy, first, Acamprosate-treated flies were assessed for EtOH sensitivity and tolerance. The medication to prevent relapse in human patients has previously been shown to decrease preference for EtOH after withdrawal in rodents and flies (Dahchour et al., 1998; Velo Escarcena et al., 2021), however, its effect on EtOH-induced intoxication was not investigated. Since Acamprosate was described to have neuroprotective and anti-oxidant effects (Dahchour et al., 2005; Engelhard et al., 2000; Hutson et al., 2018), various stress-inducing assays were conducted to investigate the specificity of Acamprosate treatment. Additionally, two ROS-sensors were expressed in a small subset of neurons to confirm the proclaimed ROS-reducing effects of Acamprosate. Following the confirmation of Acamprosate as a neuroprotective agent, Acamprosate treatment was reviewed in flies with a low abundance of PECR. Since Acamprosate treatment was not effective to increase resistance to stress with low concentrations of PECR in the CNS, an RNAi-mediated knockdown in drivers targeting the GABA- and glutamatergic system, as well as taurine-affected neurons, was conducted and treatment efficiency was analysed.

The thesis provides further insights into the mechanism and the mode of action of Acamprosate and how treatment with Acamprosate could be personalized and improved, not only for AUD patients.

2. Material and Methods

2.1 Resource Availability

2.1.1 Material Availability

All materials produced will be made available upon request.

2.1.2 Data Availability

All raw data produced for this thesis was given to Prof. Dr. Henrike Scholz for storage and safekeeping. When necessary, data can be accessed through Prof. Dr. Henrike Scholz.

2.2 Material

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit Anti-GFP	Invitrogen	#A6455
Mouse Anti-Synapsin (3C11)	DSHB	#AB528479
Anti-Mouse-HRP	GE Healthcare UK Limited	#NA931V
Anti-Rabbit-Peroxidase	Sigma	#A0545
Primers		
PECR sense	Sigma	5'-AGTGCGATGAGAAGGTGTGG-3'
PECR antisense	Sigma	5'-TTGCTGACGGAATAGGCTCC-3'
Tubulin sense	Sigma	5'-TGTCGCGTGTGAAACACTTC-3'
Tubulin antisense	Sigma	5'-AGCAGTAGAGCTCCCAGCAG-3'
Chemicals		
Ca-Acamprosate	USP Reference Standard	#1000554
N-Ethylmaleimide (NEM)	Merck	E3876-5G
Key Reagents		
TRIzol	Invitrogen	#12044977
DNase	Roche	#04716728001
RNase	Roche	**#11119915001**
RNAse Inhibitor	Roche	**#**RNAINH-RO
Proto Script II RT	NewEnglandBiolabs	#M0368

MESA Blue qPCR Mastermix	Eurogentec	#UF-NSMT-B0701
Critical equipment		
Confocal microscope	Leica	SP8
iCycler iQ5	BioRad	IQ™5
Multiskan Spectrum	Thermo Scientific	1500
Critical commercial assays		
Glucose (HK) Assay Kit	Sigma-Aldrich	#GAHK20-1KT
Triglyceride (TG) Colorimetric Assay Kit	Thermo Fisher Scientific	#EEA028
NAD-ADH Reagent Multiple Test Vial	Sigma-Aldrich	#N7160
Bradford-Reagent	Sigma-Aldrich	#B6916
CNMCS Protein Extraction Kit	BioChain	#K3013010
Experimental models: Organisms/strains		
D. melanogaster. Strain background: *w^{1118}*	Scholz lab/ Lindsley &Zimm	N/A
D. melanogaster. w^{1118}; ;$PECR^{KG07864}$ *y[1] w[67c23]; P{y[+mDint2] w[BR.E.BR]=SUPor-P}Dhrs4[KG07864] ry[506]*	Bloomington Drosophila Stock Center	BDSC#14560
D. melanogaster. w^{1118}; appl-Gal4	Torroja et al., 1999	N/A
D. melanogaster. w^{1118}; FB-Gal4	Grönke et al., 2003	Linda Partridge
D. melanogaster. w^{1118};MB247-Gal80; mef-Gal4	Berger et al., 2024	N/A
D.melanogaster. w^{1118};GABA-Gal4 *w[1118];P{w[+mC]=Gad1-GAL4.3.098}2/CyO*	Bloomington Drosophila Stock Center	BDSC#51630
D. melanogaster. w^{1118}; EAAT2-Gal4	Besson et al., 2011	N/A
D.melanogaster w^{1118};dv-Glut-Gal4	Henrike Scholz	N/A
D.melanogaster. w^{1118}; UAS-$PECR^{RNAi}$ *y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00753}attP2*	Bloomington Drosophila Stock Center	BDSC#32958
D.melanogaster. w^{1118}; UAS-$SOD1^{RNAi}$ *y[1] sc[*] v[1] sev[21]; P{y[+t7.7] v[+t1.8]=TRiP.GL01016}attP40*	Bloomington Drosophila Stock Center	BDSC#36804
D.melanogaster. w^{1118}; UAS-$SOD2^{RNAi}$ *w[1]; P{w[+mC]=UAS-Sod2.RNAi.K}15/SM5*	Bloomington Drosophila Stock Center	BDSC#24489
D.melanogaster. UAS-mcD8::GFP;UAS-mcD8::GFP;UAS-mcD8::GFP	Lee and Luo, 1999	N/A

D.melanogaster UAS-mito-roGFP2 *w[1118]; P{w[+mC]=UAS-mito-roGFP2-Grx1}9*	Albrecht et al., 2011	BDSC#67664
D.melanogaster. UAS-Cyto-roGFP *w[1118]; P{w[+mC]=UAS-cyto-Grx1-roGFP2}13*	Albrecht et al., 2011	BDSC#67662
D.melanogaster: UAS-syt-GFP *w[*]; P{w[+mC]=UAS-syt.eGFP}2*	Bloomington Drosophila Stock Center	BDSC#6925
Software and algorithms		
Fiji ImageJ	(Schindelin et al., 2012)	imagej.net/software/fiji/
Python environment Spyder	Raybaut, P. (2009). Spyder-documentation.	Pythonhosted.org
SkanIt RE for MSS 2.4.4	Thermo Fisher Scientific	#5187139
FlyDetector	(Bodenstein et al., 2017)	https://github.com/cbodenst/FlyDetector
Bio-Rad iQ5	BioRad	v2.1.94.617

2.3 Methods

2.3.1 Fly Husbandry

The model organism employed in all experiments was *Drosophila melanogaster*. The transgenic lines are listed in the key resources table. The flies were raised and maintained on a standard agar cornmeal-based food at 25 °C and 60 % relative humidity on a 12/12 h dark/light cycle unless otherwise stated. The experimental flies were cultivated in a density-controlled manner. Adult male flies, ranging in age from 1 to 3 days, were collected followed by a two-day CO_2-recovery phase at 25 °C, unless stated otherwise.

2.3.2 Fly Weight

In order to ascertain the weight of a single fly of a specific genotype, 100 flies were weighed on a fine scale in an Eppendorf tube. At least five different sets of 100 flies were used for each measurement.

2.3.3 Acamprosate Treatment

The flies were subjected to treatment consisting of 25 mg/ml Ca-Acamprosate (USP #1000554) in 5 % sucrose or mixed with standard diet stained with red food dye (Ruth #1.7205.0100) for a period of seven consecutive days prior to the execution of any experimental procedures. The control group was fed a 5 % sucrose solution or standard diet stained with red food dye for seven days.

2.3.4 Food Intake

Food intake was quantified using the Capillay Feeder assay (Ja et al., 2007), as previously described in (Diegelmann et al., 2017). Briefly, 8 male flies were provided *ad libitum* access to food from four 5 µl capillaries over a 24-hour period. In each vial, a moist Whatman filter was employed to prevent dehydration. The capillaries (Blaubrand®) were filled with a 2 % red-dyed food solution (Ruth #1.7205.0100) and marked before and after the assay. To control evaporation, three set-ups without flies were treated in an identical manner. The amount of food consumed was measured with a digital calliper (GARANT). The food solutions comprised either 5 % sucrose (Sigma-Aldrich #S9378), 5 % yeast (AppliChem #A1552), or mixture of both. The amount of food consumed was calculated to reflect the intake per mg fly.

2.3.5 Starvation Resistance

Sets of 20 male flies were transferred into a vial containing a moist Whatman filter. The vial was sealed with a transparent lid with a single sponge plug. The vials were placed into the set-up of the FLORIDA assay (Bodenstein et al., 2017). Briefly, a light plate was positioned above the set-up and white light was emitted through the transparent vials. The vials were set in a grid holding them in place. The grid was connected to an electric engine that was controlled via a USB control switch. A camera (Canon EOS-5D Mark I SLR) was positioned beneath the vials and automatically focused on the bottom of the vials. The camera and the motor were connected to the FlyDetector (Bodenstein et al., 2017), a program designed to count immobile flies at the bottom of the vials. The camera was programmed to take a photo at 10,800 s (3 h) intervals, with the motor shaking for 2 s and 15 s prior the camera flashes. All images captured were stored for subsequent analysis, and the number of deceased flies was counted.

2.3.6 Ethanol Sedation

The sensitivity and tolerance development to EtOH were assayed using the eRING assay (Bhandari et al., 2009). Briefly, 20 male flies were transferred into empty vials which were then sealed with a plug wrapped in tissue. 2 ml of 75 % EtOH (VWR #20821.365) were added to the plug. Flies were then knocked down to induce a startle response. The flies that remained on the bottom were then counted as sedated. This process was repeated in one-minute intervals for at least 20 min or until all flies had been sedated. Thereafter, all flies were transferred to food vials for a recovery period of 4 h at 25 °C. After the 4 h resting period, flies were re-exposed to EtOH to determine their tolerance development. Tolerance was defined by the difference in sedation rates between the first and second EtOH exposure. As a simplified measurement, the sedation time at which 50 % of the tested flies were unresponsive to the startle reflex was determined as the SD50 value. The tolerance was defined as relative increase in resistance in the second exposure compared to the first exposure (Scholz et al., 2000). It was calculated as follows: $\text{Fold change} = \frac{SD50\ 2nd\ exposure}{SD50\ 1st\ exposure}$.

2.3.7 Heat Shock Resistance

The sensitivity to heat treatment of 38 °C was assessed by analysing the survival rate after an extended duration of heat treatment. 20 male flies were collected in medium food vials and submerged into a water bath at 38 °C. Every 30 minutes, the number of sedated flies was assessed. This process was repeated for at least 3 h or until all flies were sedated.

2.3.8 Immunohistochemistry

Male adult fly brains were dissected in ice-cold *Drosophila* Ringer and collected in ice-cold phosphate-buffered saline (PBS, pH 7.4) for a maximum period of 1.5 h. Brains were fixed with 3.7 % formaldehyde (Merck #F8775) for 15 min. Then, the tissue was washed with 0.5 % PBT (PBS with 0.5 % Triton) for 1 h and the tissue was treated with 50 % Glycerol/PBS for 30 min at room temperature and embedded in VectaShield©. Confocal images were obtained using the Leica SP8 laser scanning microscope and analyzed via Fiji ImageJ v1.54p.

2.3.9 Detection of Free Oxygen Radicals

An adapted protocol as described in Albrecht et al., 2011 was utilized to visualise ROS in neurons. Flies expressing *UAS-roGFP* (Albrecht et al., 2011) were collected and separated into two groups. For 7 days, flies were maintained in vials containing either a diet supplemented with Acamprosate or a control diet. Brains were dissected in 20 mM N-ethylmaleimide (NEM) in PBS, fixed with 3.7 % formaldehyde for 10 min and washed with 0.3 % PBT. Thereafter, the tissue was incubated in special mounting medium (85 % glycerol, 5 % ddH_2O, 10 % 1x PBS) overnight and was embedded the following day. Confocal images were obtained directly after mounting, with no change in laser intensity between the scans and the laser set to 488 nm with spectral detection between 490 – 530 nm. For image analysis Fiji ImageJ was used. Initially, a Z-Stack with “Sum Slices” was generated, converted to an 8-bit image, and adjusted to a specified threshold “MaxEntropy”. A region of interest (ROI) was generated from this image and overlaid as a mask onto the original image. The “Multi-Measure” tool of Fiji was utilized to quantitatively analyse the intensity and area of all slices within the ROI.

2.3.10 Protein Concentrations

Protein concentrations were determined using the Bradford method (Bradford, 1976). Briefly, 10 µl of samples were transferred into a 96 well plate. 250 µl Bradford reagent was added to all samples and left for incubation at RT for 15 min. Next, the samples were analysed using a Multiskan Spektrum plate reader at 595 nm.

2.3.11 Glycogen Levels

Glycogen concentrations were determined using the Glucose Assay Kit (GAH20-1KT), after Tennessen et al., 2014. Briefly, the weight of 10 flies (sated or 18 h starved) was determined. Next, 200 µl of ice-cold PBS was added and the samples were homogenised using a Biovortexer (Biospec). Following this, a brief centrifugation was performed to extract the supernatant for protein and DNA measurements. To reduce the risk of enzymatic degradation, the proteins and enzymes were heat inactivated at 70 °C for 10 min. The samples were then subjected to centrifugation at 4 °C and 20.000 rpm for 3 min. Subsequently, 60 µl of supernatant was added to 120 µl PBS and the contents of the tubes were vortexed. Then, 60 µl of the solution is added to 60 µl PBS and to 60 µl

Amyloglucosidase (Sigma #A9913) in PBS (1.5 µl AGS in 1 ml PBS). Standard solutions were prepared with a glucose standard in PBS for 0.16, 0.08, 0.04, 0.02 and 0.01 mg/ml. 30 µl of all solutions and standards were dispensed into a 96-well plate in triplicate, which was then sealed with a lid. Following a 1 h incubation at 37 °C, 100 µl of glucose reagent was added to each well and left for incubation at room temperature for 15 min. Then, photometric examination at 340 nm was conducted using a Multiskan Spektrum plate reader. The amount of glycogen was calculated by subtracting the total glucose concentration of the respective samples without AGS from the samples with AGS treatment.

2.3.12 Triglyceride Levels

The Triglyceride Colorimetric Assay Kit (Thermo Fisher Scientific #EEA028) was utilized to ascertain the triglyceride content of the fly. A total of 8 male flies were weighed in a 1.5 ml Eppendorf tube. 100 µl PBS was added and the samples were then homogenized using a Biovortexer (Biospec). Subsequently the samples were subjected to centrifugation, after which the resulting supernatant was collected for Brandford and DNA measurements. Standards were prepared from 2.26 mM Glycerinum in PBS to concentrations of 1.356, 0.678 and 0.226 mM. Then, samples were subjected to incubation at 70 °C for 5 min. Following centrifugation for 3 min at 4 °C and 4000 rpm, 2.5 µl of the resulting supernatant and all standards were dispensed in triplicate into a 96-well plate. Then, 250 µl of an enzyme working solution was added to each well, and the plate was then incubated at 37 °C for 10 min. The photometric read-out was conducted using a Multiskan Spektrum plate reader at 510 nm.

2.3.13 Ethanol Concentration

The ethanol concentration in *Drosophila* was determined using NAD-ADH Reagent N760, as previously described by Moore et al., 1998. Briefly, 50 male flies were frozen in liquid nitrogen and then stored in an Eppendorf tube at -80 °C. To each sample, 500 µl Tris/HCl (50 mM, pH 7.5) was added prior to homogenization via a Biovortexer (Biospec). The samples were spun at 4 °C and 20.000 rpm for 20 min before 400 µl of supernatant was extracted and transferred into a fresh Eppendorf tube. The following steps were executed under a hood: 500 µl of reagent in Glycine buffer and 5 µl of the sample were pipetted

together in an Eppendorf tube and gently mixed. Following a 10 min period of incubation, the solution was transferred into a cuvette and the measurements were made using a photometer at a wavelength of 340 nm. The EtOH concentration was then calculated as specified by the manufacturer.

2.3.14 Western Blot

Protein extraction was done following the CNMCS Protein Extraction (BioChain) protocol. Briefly, 200-400 male flies were collected and stored in liquid nitrogen. The heads of the flies were collected and 150 µl of buffer C with protease inhibitors was added. The tissue was then homogenized with a Biovortexer (Biospec) and incubated for 20 min on a shaker at 4°C. The homogenate was then subjected to centrifugation at 18000 rpm at 4 °C for 20 min, in order to yield the cytoplasmic protein fraction. The pellet was measured and resuspended in four times the volume of buffer W. Following a brief incubation, the probes were subjected to a second centrifugation, as previously described. The resulting pellet was resuspended with an equal amount of buffer N and the probes were incubated for another 20 min. The samples were then subjected to a third centrifugation step, in order to access the supernatant containing nuclear proteins. The pellet was resuspended once more in buffer M, and the incubation and centrifugation were conducted as before to collect the membrane protein-containing supernatant. The quantity of the respective proteins in the above extracts was measured using a NanoDrop. Per probe, 5 µl 4xLämmli (with 5 % β-mercaptoethanol), x µl extracted protein and (20-x) µl 1M Tris/HCl (pH 6.8) were added to a volume of 20 µl. Protein concentrations were adjusted to 50 µg/probe. Right before loading, the probe was heated for 10 min at 95 °C.

The Western blot was performed as in Mahmood and Yang, 2012. For development, a mixture of two home-made detection solutions was used. Solution 1 (100 mM Tris/HCl, 0.4 mM PCA, 5mM Luminol, pH 8.5) and Solution 2 (100 mM Tris/HCl, 6.85 mM H_2O_2) were put together directly on the membrane and left for incubation for 10 min. Afterwards the membrane was put into cassette and a film was laid on top. The film was taken after 15 min and developed using a developer (AGFA-153A& AGFA-153B) and rapid fixer (AGFA-G354). For data quantification images of the film loaded in Fiji ImageJ and the strength of bands was measured.

2.3.15 Quantitative Real-Time PCR

Transcripts were measured by quantitative real-time PCR (qPCR). Previous to fly head acquisition, flies were treated with 25 mg/ml Ca-Acamprosate for 7 days. Briefly, RNA was isolated from a total of 200 fly heads, with 800 µl of Trizol® being added to each sample. The RNA pellet was resuspended in 50 µl of DEPC H_2O and digested with RNAse-free DNAse for 30 min at 37 °C followed by heat inactivation for 10 min at 95 °C. cDNA was synthesized with SuperScript II Reverse Transcriptase (New England Biolabs) and Random Primers (Invitrogen#74896). 100 ng of cDNA were used as a template for qPCR analysis with SYBR® master mix (Eurogentec). The experiments were carried out using an iCycler iQ5 Multicolor Real-Time PCR Detection System and its corresponding iQ5 Optical System Software from Bio-Rad. The obtained data was analysed using the Pfaffl method (Pfaffl, 2001). An ANOVA with post-hoc Tukey HSD test was used to analyse whether the differences between the groups were significant. cDNA was isolated from 3 different sets of flies and the qPCR was performed with three technical replicates. The data is presented as relative fold changes of gene of interest (GOI) transcripts relative to the house-keeping gene (HKG) transcripts.

2.3.16 Statistics

The obtained data was tested for normal distribution using the Shapiro-Wilk test before conducting further statistical analyses. The significant differences of normally distributed data between 2 groups were analysed using a Students t-test, when more than 2 groups were compared an ANOVA with post-hoc Tukey HSD test was performed. The survival probability and mean lifespan of flies were analysed and compared using the Kaplan-Meier estimator and a Log-Rank Test with Bonferroni correction using the Online Application for Survival Analysis 2 (OASIS2, Han et al., 2016). Data shown as boxplots show a box ranging from first to third quartile of the data, with a line as the median and an asterisk for the mean. Whiskers extend to the farthest data point within 1.5x the inter-quartile range from the box. Flier points represent data points past the ends of the whiskers. Data shown as scatterplots represent every data point obtained and the mean of the set as line.

3. Results

Genetic predispositions have been demonstrated to contribute to changes in the likelihood of developing AUD. A SNP in *pecr* is associated with an early onset of AUD (Treutlein et al., 2009). In *Drosophila* PECR levels in the heads of flies have been observed to decrease following exposure to EtOH, and $PECR^{KG07864}$ mutants have been shown to reduce the expression of PECR by 83 % and the flies exhibited a reduced EtOH preference after withdrawal (Velo Escarcena et al., 2021). The present study investigates the role of the peroxisomal enzyme PECR in alcohol-related behaviours and its potential impact on the efficacy of Acamprosate, a medication that has been shown to reduce cravings following periods of abstinence.

3.1 PECR is Associated with Decreased Sensitivity to and Increased Development of Functional Tolerance to EtOH

Addiction to EtOH results in individuals needing to consume a higher dose to access their positive reward due to their tolerance development (Koob and Le Moal, 1997). Low EtOH sensitivity and a high tolerance correlate with the severity of AUD (Schuckit, 1994). Since the build-up tolerance can be inheritable and genetic factors can influence the initial sensitivity of an organism (Enoch and Goldman, 2001; Heath et al., 1997; Slutske et al., 1999), this study aims to investigate the function of PECR in sensitivity and tolerance development to EtOH.

The eRING assay was used to test the response of flies to the sedating effects of EtOH (Bhandari et al., 2009). This assay utilises the negative geotaxis reflex of flies to ascertain the sedation rate during exposure to EtOH. The $PECR^{KG07864}$ mutant and its respective control w^{1118} were exposed to 75 % EtOH for 20 min and tested by inducing a startle response every minute. Flies that were startled and did not climb the wall reflexively were considered sedated and counted as such. This assessment was conducted through two experiment runs: an initial test and a subsequent test following a 4-hour resting period (Fig. 3, A,B). The initial sensitivity was analysed using the number of flies that sedated over time and by determining the point in time at which 50 % of the flies were sedated. This measure was termed SD50 (Fig. 3, C). The control flies succumbed to the sedative effects of EtOH significantly faster than the mutant flies (Fig. 3, C). The $PECR^{KG07864}$

mutant was significantly more resistant to the initial sedation indicated by a 15 % higher SD50 in the first EtOH exposure. However, the *w*^{1118} control exhibited a significantly higher level of resistance to EtOH in the second exposure (Fig. 3, D). Rapid tolerance is defined as an increase in resistance after a single exposure to EtOH and it was quantified by measuring the difference between the SD50 values of the first and second exposures (Lange and Wolf, 2023; Larnerd et al., 2023), via the fold change in SD50 values (Crabbe et al., 1979; Scholz et al., 2000), and the total difference of the curves, represented as the area between them. Both control and mutant flies showed a capacity to develop tolerance, as evidenced by the increase in latency to sedation between the first and second exposure (Fig. 3, A,B). The control flies showed a prolonged resistance by 8.2 ± 0.6 min, resulting in a relative increase in resistance of 104 % (Fold change 2.04), whereas the mutant flies demonstrated a significantly lower increase in resistance with 4.8 ± 0.6 min, resulting in a relative increase of 56 % (Fold change 1.56) (Fig. 3, E). Furthermore, the total difference in the sedation rate graphs between the first and second exposure is significantly reduced in *PECR*^{KG07864} mutants, indicating a reduced tolerance development (Fig. 3, F). Since the *PECR*^{KG07864} mutant has been shown to have significantly reduced PECR levels, these findings indicate that PECR is a negative regulator for ethanol sensitivity and a positive regulator for tolerance to the sedative effects of EtOH.

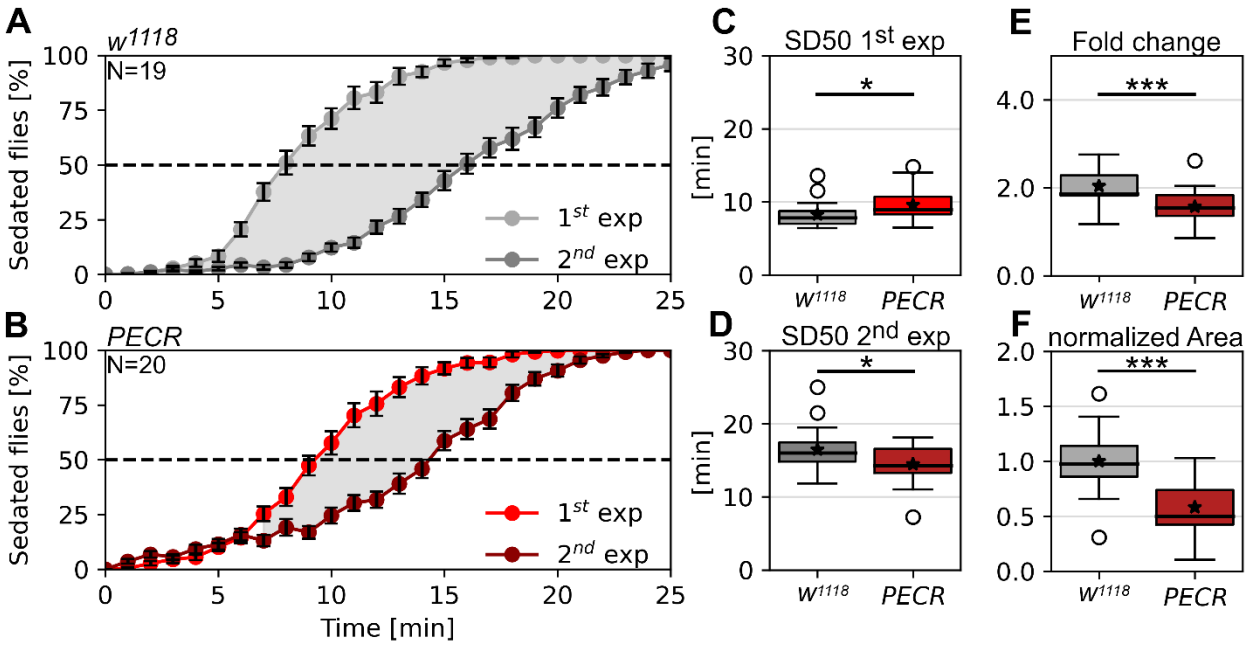

Figure 3: ***PECR*^{KG07864} mutant exhibited lower EtOH sensitivity and reduced EtOH tolerance development.**
A,**B**, Control flies and mutants showed a development of tolerance against EtOH after a 4 h recovery period, Students-t test to compare first and second SD50, $p < 0.001$; **C**, *PECR*^{KG07864} flies showed a reduced rate of sedation upon a first exposure to EtOH, mean ± SEM: w^{1118}: 8.3 ± 0.4 min, *PECR*^{KG07864}: 9.6 ± 0.5 min; Students-t-test F: 2.06, df: 39, p-value: 0.046; **D**, *PECR*^{KG07864} sedated faster than wild type after the second exposure to EtOH, mean ± SEM: w^{1118}: 16.4 ± 0.7 min, *PECR*^{KG07864}: 14.4 ± 0.5 min; Students-t-test F: 2.25, df: 39, p-value: 0.03; **E**, Controls exhibited a 2.04 ± 0.08 fold change in SD50 compared to the first exposure, while mutants had a significantly lower fold change of 1.56 ± 0.08; Students-t-test F: 3.81, df: 39, p-value: 0.00; **F**, The total difference between the graphs of the first and second exposure is significantly smaller in *PECR*^{KG07864} mutants, normalized to control, mean ± SEM: w^{1118}: 1 ± 0.1, *PECR*^{KG07864}: 0.6 ± 0.1; Students-t-test F: 4.92, df: 39, p-value: 0.00. Groups of 20 flies, N = 19/22; p-values derived from Students-t-test: * = $p > 0.05$, ** = $p > 0.01$, *** = $p > 0.001$.

3.2 The Role of PECR in Metabolic Homeostasis

3.2.1 Ethanol Metabolism Proceeds Independently of PECR Function

The preceding results indicated that PECR is required for the complete development of EtOH tolerance (Fig. 3). There are two forms of tolerance: pharmacokinetic tolerance or metabolic tolerance, which is defined by a reduced absorption of EtOH or a more efficient removal of EtOH from the body, and pharmacodynamic tolerance or functional tolerance, which is achieved by neuronal adaptations (Elvig et al., 2021; Fadda and Rossetti, 1998). To exclude any metabolic defects responsible for changes in the initial EtOH sensitivity and indirectly affecting tolerance development, EtOH concentrations were measured in *PECR*^{KG07864} mutants and their genetic controls before and during several points after the exposure to EtOH. For this, groups of 50 flies were exposed to EtOH vapor for 20 minutes, and at their specific time points subjected to an NAD-ADH reagent (Sigma Aldrich #N7160). The EtOH in the samples is oxidized to acetaldehyde while simultaneously reducing NAD to NADH. Consequently, there is an increase in absorbance at 340 nm which is directly proportional to the EtOH concentration in the samples (Moore et al., 1998). The results revealed that there were no significant differences in EtOH concentrations between the two groups at any given time point, and the flies exhibited comparable metabolic speed to remove EtOH (Fig. 4). These results indicate that PECR has no major role in the metabolism of EtOH. Therefore, the changes in sensitivity to EtOH and most likely the deficits in tolerance development observed in the mutant flies are due to alterations in their capacity to form functional tolerance to EtOH.

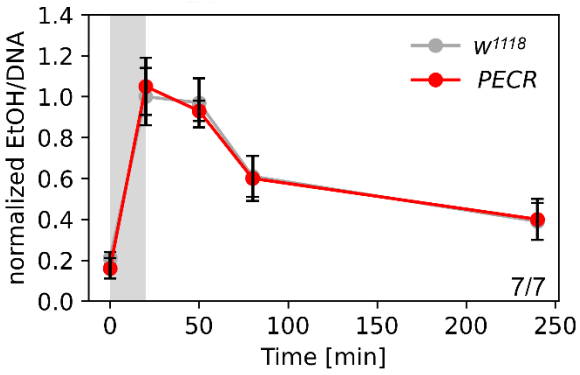

Figure 4: EtOH metabolism was not affected in *PECR*^{KG07864} mutants.
EtOH metabolism was similar in *PECR*^{KG07864} mutants and controls; before treatment: mean ± SEM: *w*^{1118}: 0.21 ± 0.03, *PECR*^{KG07864}: 0.16 ± 0.05; Students-t-test F: 0.89, df: 12, p-value: 0.39; immediately after treatment: mean ± SEM: *w*^{1118}: 1 ± 0.14, *PECR*^{KG07864}: 1.05 ± 0.14; Students-t-test F: 0.24, df: 12, p-value: 0.81; 50 min after treatment start: mean ± SEM: *w*^{1118}: 0.97 ± 0.12, *PECR*^{KG07864}: 0.93 ± 0.05; Students-t-test F: 0.27, df: 12, p-value: 0.79; 80 min after treatment start: mean ± SEM: *w*^{1118}: 0.61 ± 0.1, *PECR*^{KG07864}: 0.6 ± 0.09; Students-t-test F: 0.06, df: 12, p-value: 0.95; 4 h after treatment start: mean ± SEM: *w*^{1118}: 0.39 ± 0.1, *PECR*^{KG07864}: 0.4 ± 0.1; Students-t-test F: 0.09, df: 12, p-value: 0.92; groups of 20 flies, N = 7.

3.2.2 PECR Influences Nutrient Storage

Even though the EtOH metabolism of *PECR*^{KG07864} mutants was similar to that of controls, other metabolic defects which might affect functional tolerance were investigated. It was demonstrated that PECR is predominantly responsible for the elongation of fatty acids (Das et al., 2000; Faust et al., 2012). These fatty acids are often stored but can be broken down by β-oxidation, a process that provides energy to the organism (Schulz, 1991). Fatty acids are stored in organelles called lipid droplets (Walther and Farese, 2012). The accumulation of fat leads to an increased body mass (Birse et al., 2010). In order to investigate the hypothesis that reduced PECR leads to an accumulation of short-chain fatty acids or that the fatty acid stores in the fly body are reduced, the body weight of male and female flies was measured. No significant difference was found between controls and mutants (Fig. 5, A). However, there are metabolic pathways to produce the energy hypothetically missing from LCFA in PECR mutants from glycogen or protein stores (Gáliková and Klepsatel, 2023). The nutritional defects in other metabolites might contribute to the reduced tolerance. In larvae, studies demonstrated that ethanol induced a reduction in fat body metabolites, such as glycogen, lipids and proteins (Geer et al., 1989). The study suggests a metabolic defence mechanism in larvae to metabolize the

toxic ethanol into non-toxic storage products (Geer et al., 1989). To examine whether *PECR*^{KG07864} mutants exhibit any nutritional defects that contribute to their reduced ability to develop tolerance, male and female flies were subjected to a series of capillary feeder assays (CaFe assays, Ja et al., 2007). The assessment of appetite- and starvation-induced feeding provides insights into the nutritional requirements of the organism (Rion and Kawecki, 2007). Two distinct diets were utilised to determine whether the flies exhibited a deficiency in carbohydrate or protein metabolism. A mixture of both sucrose and yeast was used to determine whether there was any general effect on food consumption. Flies were fed *ad libitum* prior to the experiment to investigate their appetite-driven food intake. The appetite of the male *PECR*^{KG07864} mutants in respect to yeast intake was increased by 53 % compared to the genetic controls, indicating a protein deficit in the male mutants (Fig. 5, B).

Since hunger is elicited by a lack of nutrients and the energy demand of the organism, the flies were starved for 18 h prior to the experiment. When subjected to starvation, male mutants exhibited a heightened feeding response with an increase of 50 – 75 % compared to their respective controls, irrespective of the available food source (Fig. 5, C).

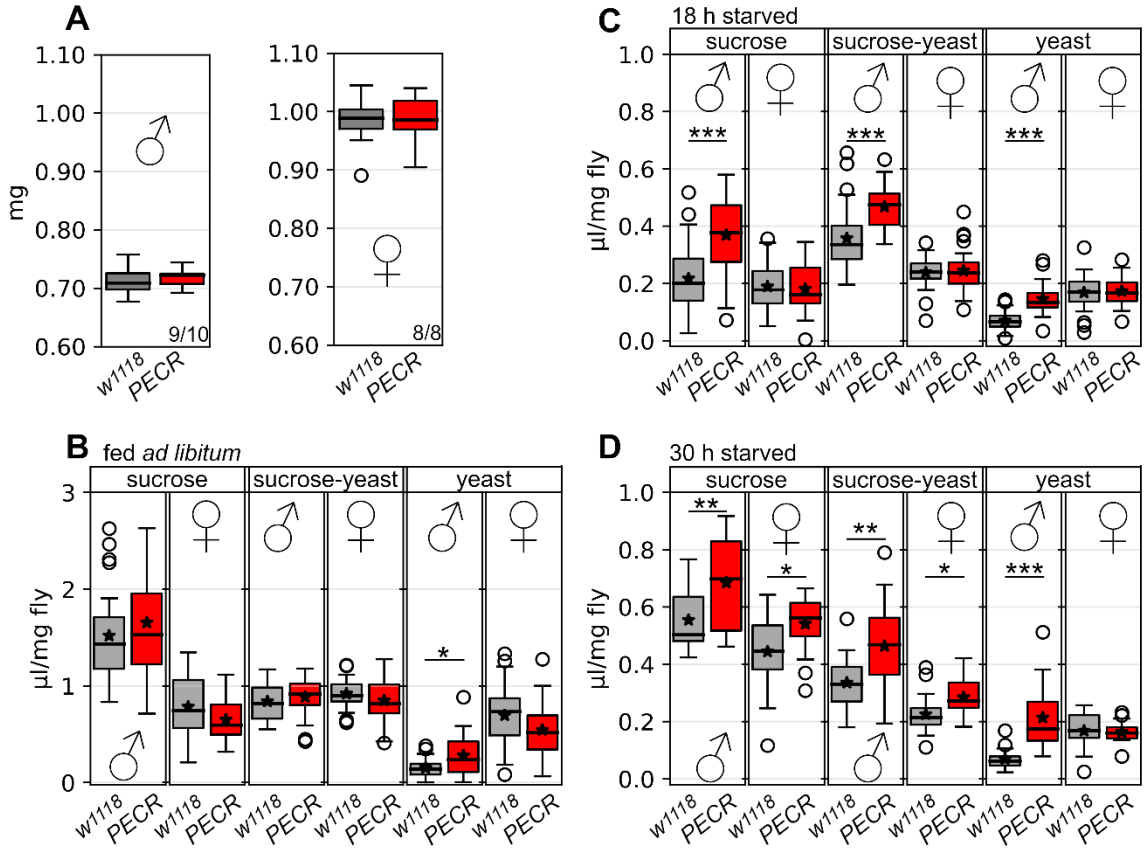

Figure 5: Starvation induced increased nutritional need in *PECR*^{KG07864} mutants.
A, Male and female body weight of *PECR*^{KG07864} mutants did not differ from controls, groups of 100 flies, N = 8-10; **B**, Appetite-driven protein intake was increased in male *PECR*^{KG07864} mutants; mean ± SEM: sucrose: males: 1.51 ± 0.1 µl/mg fly, 1.65 ± 0.11 µl/mg fly, females: 0.79 ± 0.06 µl/mg fly, 0.55 ± 0.06 µl/mg fly; sucrose-yeast: males: 0.84 ± 0.04 µl/mg fly, 0.88 ± 0.04 µl/mg fly, females: 0.92 ± 0.03 µl/mg fly, 0.85 ± 0.05 µl/mg fly; yeast: males: 0.15 ± 0.02 µl/mg fly, 0.28 ± 0.04 µl/mg fly; Students-t-test F: 2.88, df: 32, p-value: 0.006, females: 0.69 ± 0.06 µl/mg fly, 0.54 ± 0.05 µl/mg fly, group of 8 flies, N = 25-28. **C**, Hunger-driven food intake was increased in *PECR*^{KG07864} males after 18 h of starvation; mean ± SEM: sucrose: males: 0.21 ± 0.03 µl/mg fly, 0.37 ± 0.03 µl/mg fly, Students-t-test F: 4.38, df: 56, p-value: 0.00, females: 0.19 ± 0.01 µl/mg fly, 0.18 ± 0.02 µl/mg fly; sucrose-yeast: males: 0.36 ± 0.02 µl/mg fly, 0.47 ± 0.01 µl/mg fly, Students-t-test F:4.54, df: 58, p-value: 0.00, females: 0.24 ± 0.01 µl/mg fly, 0.24 ± 0.01 µl/mg fly; yeast: males: 0.07 ± 0.01 µl/mg fly, 0.14 ± 0.01 µl/mg fly, Students-t-test F: 5.98, df: 52, p-value: 0.00, females: 0.17 ± 0.01 µl/mg fly, 0.17 ± 0.01 µl/mg fly, groups of 20 flies, N=26-30. **F**, Hunger-driven food intake was increased in *PECR*^{KG07864} males and females after 30 h of starvation; mean ± SEM: sucrose: males: 0.55 ± 0.03 µl/mg fly, 0.69 ± 0.04 µl/mg fly, Students-t-test F: 2.81, df: 34, p-value: 0.008, females: 0.44 ± 0.03 µl/mg fly, 0.54 ± 0.02 µl/mg fly, Students-t-test F: 2.64, df: 38, p-value: 0.019; sucrose-yeast: males: 0.34 ± 0.02 µl/mg fly, 0.46 ± 0.03 µl/mg fly, Students-t-test F: 3.07, df: 34, p-value: 0.004, females: 0.23 ± 0.02 µl/mg fly, 0.28 ± 0.01 µl/mg fly, Students-t-test F: 2.68, df: 35, p-value: 0.011; yeast: males: 0.08 ± 0.01 µl/mg fly, 0.24 ± 0.04 µl/mg fly, Students-t-test F: 3.89, df: 28, p-value:0.008, females: 0.17 ± 0.01 µl/mg fly, 0.16 ± 0.01 µl/mg fly, groups of 20 flies, N = 18-25; Students-t-test * = $p<0.05$; ** = $p<0.01$, *** = $p<0.001$.

Only male mutants showed an increase in food intake compared to controls when starved for 18 h prior to the experiment. To investigate whether this phenotype is sex-specific, flies were subjected to a prolonged starvation period, as female flies have a higher body mass than males and may succumb to starvation later. The results of this experiment revealed a significant increase in sucrose and sucrose-yeast intake levels in both male and female mutants compared to their control groups. However, only male mutants exhibited an increase in protein intake (Fig. 5, C). These results demonstrate that PECR plays a pivotal role in the energy metabolism of flies and that its effects extend beyond those associated with a deficit in fatty acid metabolism.

Since the appetite of male mutants was elevated for protein-rich sustenance, the protein levels were measured with a Bradford reagent in both starved and fed flies (Bradford, 1976). It was observed that the protein levels remained constant even when the flies were starved, and no significant difference could be detected between controls and mutants (Fig. 6, A). Consequently, the increased appetite observed for protein-rich food is not due to protein deficiencies of the *PECR*^{KG07864} flies. Therefore, the previously observed defects in EtOH tolerance development are not due to metabolic defects in the *PECR*^{KG07864} mutant.

Starved *PECR*^{KG07864} mutants showed an increase in hunger-induced carbohydrate intake (Fig. 5, C, D). Since glycogen is the main carbohydrate storage in the fly (Wigglesworth, 1949), its levels were analysed in starved and fed flies. As anticipated, starved flies exhibited reduced glycogen levels compared to their fed controls. Furthermore, *PECR*^{KG07864} mutants exhibited reduced glycogen levels even under satiated conditions (Fig. 6, B). Similar to this result, the exposure of *Drosophila* larvae to EtOH-containing food reduces the internal glycogen stores in their fat body cells but simultaneously increased the lipid deposits (Geer et al., 1989). Therefore, the loss of PECR might already induce metabolic deficits which are further increased by EtOH exposure.

To address whether the fatty acid metabolism in *PECR*^{KG07864} mutants is changed, given PECR's role in fatty acid synthesis (Das et al., 2000; Faust et al., 2012) and the fact that endurance in EtOH-containing environments is reliant on the fatty acid content of *Drosophila* (McKechnie and Geer, 1993), triglyceride (TAG) levels of starved and fed wild type and mutant flies were examined. It was expected that *PECR*^{KG07864} mutants have reduced levels of TAG, as the initial fatty acid synthesis pathway through acyl-CoA is not available without PECR (Faust et al., 2012). In accordance with the aforementioned observations, starved flies exhibited reduced TAG levels in comparison to their fed controls. Contrary to the anticipated outcome, the TAG levels in the *PECR*^{KG07864} mutants did not differ from those observed in control groups (Fig. 6, C). Glucose can be converted to TAG in several steps, including glycolysis, pyruvate metabolism, acetyl-CoA and fatty acid synthesis, in a process which is not reversible for insects (Gáliková and Klepsatel, 2023). Therefore, the glycogen deficit might be a hint for a compensatory mechanism in the *PECR*^{KG07864} mutants to synthesise TAG through glucose, which results in decreased glycogen stores.

To analyse which parts of the body might be affected by the glycogen deficit observed in *PECR*^{KG07864} mutants, PECR was knocked down via RNAi (*UAS-PECR*^{HMS00753RNAi}, Velo Escarcena et al., 2021) utilizing different Gal4 drivers and glycogen levels were measured. Considering that the fat body is the main storage site for glycogen (Wigglesworth, 1949), a fat body-specific driver (*FB*-Gal4) was used (Grönke et al., 2003). Glycogen is also stored in the muscles of the fly for fast energy access (Wigglesworth, 1949), therefore a muscle-specific driver (*mef2*-Gal4) was selected as well (Crittenden et al., 2018). However, this driver also induces *UAS*-targeted gene expression in the mushroom bodies

(MBs), a brain region of the fly, so *MB247*-Gal80 was used in parallel to avoid side target effects. The Gal80 encodes a transcriptional repressor which inhibits the activity of the Gal4 transcriptional activator (Suster et al., 2004). Due to the target gene expression of Gal80 in the MB via the *MB247* promotor, any *mef-Gal4* side effects in the MB should be nullified (Berger et al., 2024).

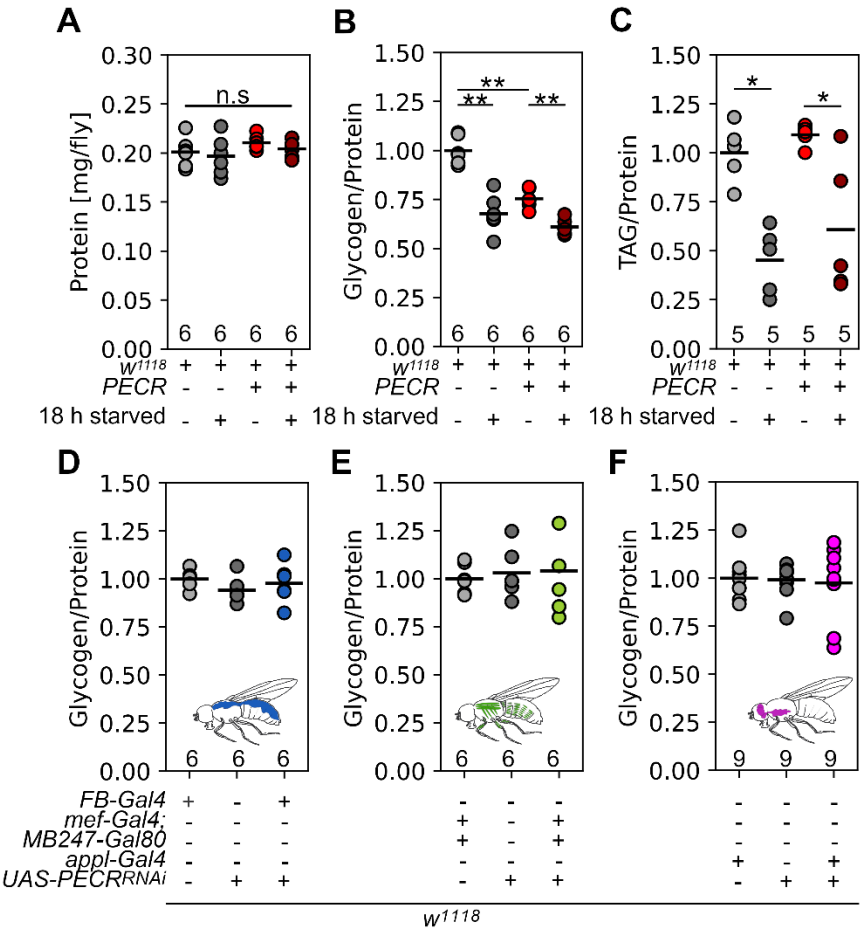

Figure 6: *PECR*^{KG07864} mutants had reduced glycogen levels.
A, Protein levels of fed and starved controls and mutants were similar; N=6; mean ± SEM: w^{1118}: 0.201 ± 0.006 mg/fly, w^{1118} starved: 0.197 ± 0.008 mg/fly, *PECR*^{KG07864}: 0.211 ± 0.003 mg/fly, *PECR*^{KG07864} starved: 0.204 ± 0.004 mg/fly. **B**, *PECR*^{KG07864} mutants had reduced glycogen levels, groups of 10 flies, N = 6; mean ± SEM: w^{1118}: 1.0 ± 0.03, w^{1118} starved: 0.68 ± 0.04, *PECR*^{KG07864}: 0.76 ± 0.02, *PECR*^{KG07864} starved: 0.61 ± 0.02, ANOVA F: 37.72, df: 3, p-value: 0.00, post-hoc TukeyHSD p-values: w^{1118}/w^{1118} starved: 0.001, w^{1118}/*PECR*^{KG07864}: 0.001, *PECR*^{KG07864}/*PECR*^{KG87064} starved: 0.007. **C**, TAG levels were reduced in starved flies; groups of 8 flies, N = 5; mean ± SEM: w^{1118}: 1.0 ± 0.06, w^{1118} starved: 0.45 ± 0.07, *PECR*^{KG07864}: 1.09 ± 0.02, *PECR*^{KG07864} starved: 0.61 ± 0.01, ANOVA F: 9.65, df: 3, p-value: 0.0002, post-hoc TukeyHSD p-values: w^{1118}/w^{1118} starved: 0.02, w^{1118}/*PECR*^{KG07864}: 0.36, *PECR*^{KG07864}/*PECR*^{KG87064} starved: 0.001. **D**, Knockdown of PECR in the fat body did not change the glycogen concentration; 10

abdomen per probe, N = 6, mean ± SEM: w^{1118}>*FB-Gal4*: 1.0 ± 0.02, w^{1118}>$PECR^{HMS00753RNAi}$: 0.929 ± 0.03, *FB-Gal4*>$PECR^{HMS00753RNAi}$: 0.94 ± 0.04. **E**, Knockdown of PECR in the fly muscles did not change glycogen concentrations; 10 abdomen per probe, N = 6; mean ± SEM: w^{1118}>*mef-Gal4;MB247-Gal80*: 1.0 ± 0.03, w^{1118}>$PECR^{HMS00753RNAi}$: 1.03 ± 0.05, *mef-Gal4;MB247-Gal80*>$PECR^{HMS00753RNAi}$: 1.04 ± 0.08. **F**, Knockdown of PECR in the CNS did not change glycogen concentration; 30 fly heads per probe, N = 9; mean ± SEM: w^{1118}>*appl-Gal4*: 1.0 ± 0.04, w^{1118}>$PECR^{HMS00753RNAi}$: 0.99 ± 0.03, *appl-Gal4*>$PECR^{HMS00753RNAi}$: 0.98 ± 0.06. ANOVA with post-hoc Tukey HSD * = $p < 0.05$, ** = $p < 0.01$.

In a further experiment, a pan-neuronal driver (*appl*-Gal4) was employed to knock down PECR in the CNS (Torroja et al., 1999). This is relevant because glycogen serves as a key energy reserve in the brain, especially during periods of metabolic stress such as starvation (Zimmerman et al., 2004). Since PECR mutants exhibited increased compensatory feeding, this suggests a possible disruption in central energy sensing or storage mechanisms. PECR may influence lipid metabolism pathways that interact with carbohydrate metabolism; therefore, a knock down could affect how glycogen is synthesised, stored or mobilized in neural tissue. As a control for potential effects of the UAS and Gal4 constructs themselves, all experimental groups contained a UAS/+ and Gal4/+ control group. The glycogen levels in all experimental groups were found to be comparable to the controls, thereby indicating that the observed glycogen deficit in the $PECR^{KG07864}$ mutant is not reproduceable by targeting only specific organs of the fly (Fig. 6, D-F). As expected, the fatty acid synthesising enzyme PECR plays a pivotal role in the nutrient metabolism of the fly, particularly in starved conditions. It can be hypothesised that the absence of PECR leads to a reduction in glycogen stores because the flies are compelled to rely more heavily on their glycogen stores and utilise them more rapidly due to the deficiency in fatty acid synthesis and their resulting lack of additional energy sources. Since it was shown that high intracellular concentrations of EtOH reduce the capacities to synthesise protein, lipids and glycogen in larval fat body cells, already depleted stores in mutants might elevate the effect of EtOH on the organism (Geer et al., 1989).

3.3 Neuronal PECR Function is Essential for Tolerance to EtOH

3.3.1 Tolerance Development is Independent of Fat Body PECR Abundance

Since the role of PECR in tolerance development was evidenced by a reduced EtOH tolerance of *PECR*^{KG07864} mutants, an RNAi knockdown in specific body parts was used to confirm the role of PECR in tolerance development. The used construct *UAS-PECR*^{HMS00753RNAi} has been demonstrated to significantly reduce PECR expression in fly heads (Velo Escarcena et al., 2021). As PECR is involved in the synthesis of LCFA and the main store for fatty acids is the fat body (Canavoso et al., 2001; Faust et al., 2012), an experiment was conducted in which flies with RNAi knockdown of PECR in cells targeted by a fat body-specific Gal4 driver (*FB*-Gal4) were tested for their sensitivity and their ability to form functional tolerance to EtOH (Fig. 7).

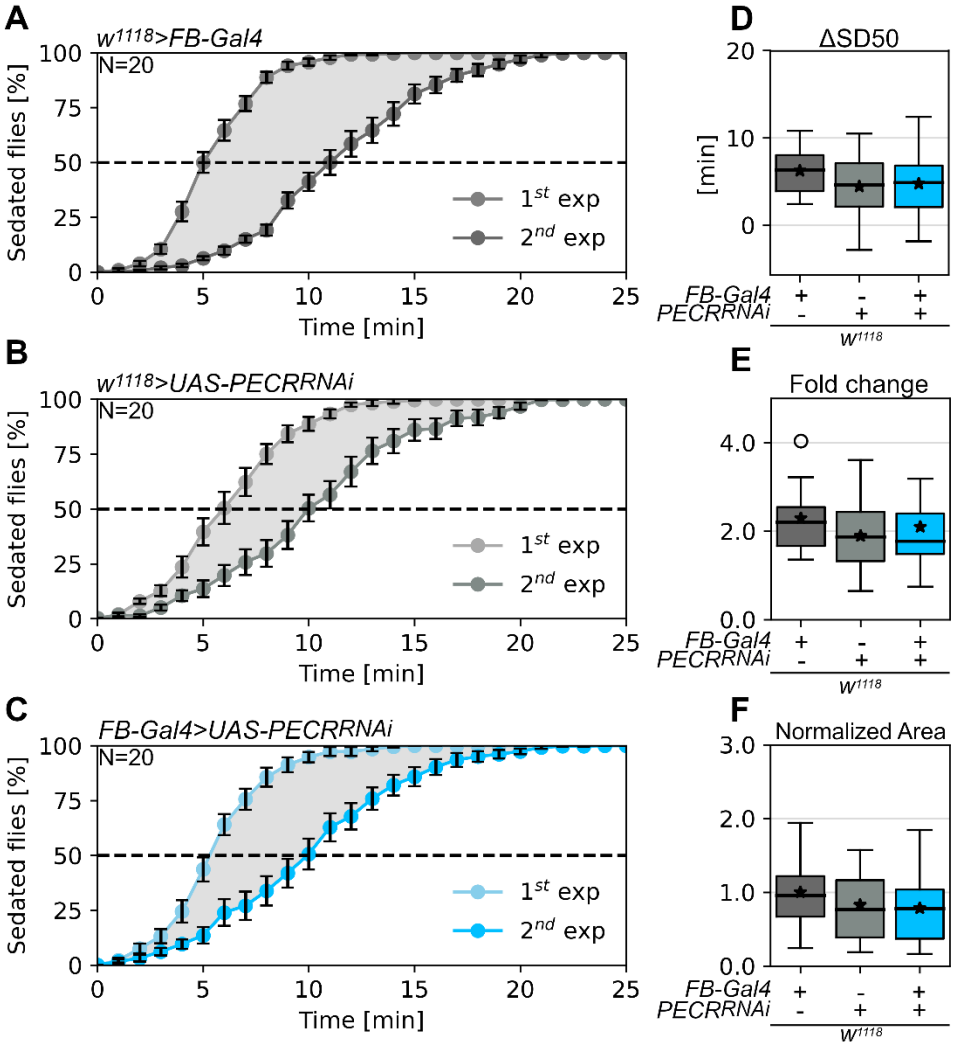

Figure 7: PECR within the fat body did not influence EtOH tolerance.
A-B, Control flies showed a tolerance to EtOH in the second exposure; **C**, Flies with a downregulation of *PECR* in the fat body showed tolerance development after previous EtOH exposure; **D**, All groups had similar differences in sedation rate during the second EtOH exposure, mean ± SEM: w^{1118};*FB-Gal4*: 6.22 ± 0.54 min; w^{1118};*UAS-PECR*^{HMS00753RNAi}: 4.43 ± 0.87 min; *FB-Gal4; UAS-PECR*^{HMS00753RNAi}: 4.75 ± 0.81 min; **E**, Controls and experimental group had a Fold change increase in the resistance to EtOH upon the second exposure; mean ± SEM: w^{1118};*FB-Gal4*: 2.29 ± 0.15; w^{1118};*UAS-PECR*^{HMS00753RNAi}: 1.89 ± 0.18 ; *FB-Gal4;UAS-PECR*^{HMS00753RNAi}: 2.09 ± 0.28; **F**, All groups showed comparable differences between first and second exposure rates; mean ± SEM: w^{1118};*FB-Gal4*: 1.0 ± 0.09; w^{1118};*UAS-PECR*^{HMS00753RNAi}: 0.8 ± 0.01; *FB-Gal4;UAS-PECR*^{HMS00753RNAi}: 0.78 ± 0.11; ANOVA Tukey HSD was not significant. Groups of 20 flies, N = 20.

The results demonstrated that the initial sedation times and the sedation rate during the second exposure to EtOH of the control flies were comparable to those of the experimental group (Fig. 7, A-C). Therefore, no significant differences were observed between the two groups in their ability to develop functional tolerance (Fig. 7, D-F). Consequently, these findings imply that even though the fatty acid content in the fat body can affect tolerance (McKechnie and Geer, 1993), a lower abundance of PECR in the fat body has no measurable effect on the development of functional tolerance against EtOH.

3.3.2 EtOH Tolerance Does Not Depend on PECR Expression in Muscle Tissue

Earlier results have demonstrated that PECR in the fat body does not influence the ability of flies to develop tolerance against EtOH. However, *PECR*^{KG07864} mutants showed an impaired energy storage in a food consumption assay, hence the second glycogen storage tissue, the fly muscles, were targeted next (Wigglesworth, 1949). In order to investigate whether PECR in muscles influences the fly's ability to develop functional tolerance to EtOH, PECR was knocked down via RNAi in *mef*-Gal4 driven cells. To circumvent any potential adverse effects that might be engendered by the supplementary expression of Gal4 in the mushroom body, the strategy of co-expressing *MB247*-Gal80 was employed (Berger et al., 2024). The EtOH sensitivity and tolerance of the flies was then assessed using the eRING assay (Bhandari et al., 2009). All tested flies developed a tolerance to EtOH following the initial sedation and showed no differences in their sedation curves during the first and second EtOH exposure (Fig. 8, A-C). There was no observed difference between the control and experimental groups in their development of

tolerance (Fig. 8, D-F). Consequently, it was determined that PECR abundance in muscles is not a prerequisite for flies to develop functional tolerance to EtOH.

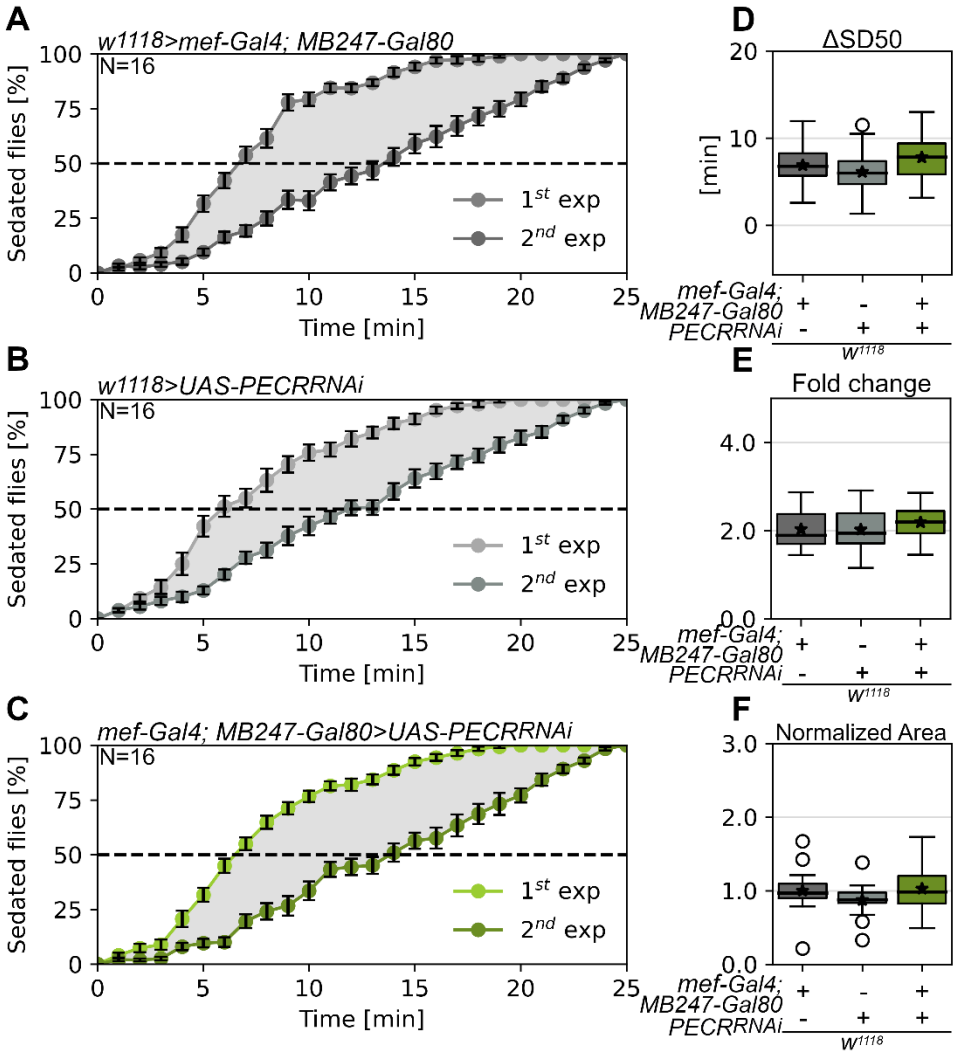

Figure 8: PECR in muscles was not required for the development of EtOH tolerance.
A-C, Control and experimental flies showed tolerance development after initial EtOH exposure. **D**, All flies showed similar differences in the sedation rates between the first and second exposure, mean ± SEM: *w*^{1118}*; mef-Gal4;MB247-Gal80*: 6.88 ± 0.64 min; *w*^{1118}*;UAS-PECR*^{HMS00753RNAi}: 6.12 ± 0.67 min; *mef-Gal4;MB247-Gal80;UAS-PECR*^{HMS00753RNAi}: 7.78 ± 0.72 min. **E**, Increase in resistance to EtOH did not differ between the groups as visible due to their fold changes: mean ± SEM: *w*^{1118}*;mef-Gal4;MB247-Gal80*: 2.03 ± 0.11; *w*^{1118}*;UAS-PECR*^{HMS00753RNAi}: 2.02 ± 0.13; *mef-Gal4;MB247-Gal80;UAS-PECR*^{HMS00753RNAi}: 2.18 ± 0.1; **F**, The normalized difference in the sedation graphs of the first and second exposure was similar for all genotypes mean ± SEM: *w*^{1118}*; mef-Gal4;MB247-Gal80*: 1.0 ± 0.08; *w*^{1118}*;UAS-PECR*^{HMS00753RNAi}: 0.87 ± 0.06; *mef-Gal4;MB247-Gal80;UAS-PECR*^{HMS00753RNAi}: 1.02 ± 0.08. Groups of 20 flies, N = 16, ANOVA Tukey HSD was not significant.

3.3.3 PECR in the CNS Mediates Functional Adaption to EtOH

PECR expression has been observed in the heads of *Drosophila* with alterations in expression levels resulting from exposure to EtOH (Velo Escarcena et al., 2021). The establishment of tolerance to EtOH is achieved through changes in the neuronal structure and functionality (Barceló-Coblijn et al., 2013). In order to ascertain whether flies with downregulated PECR expression in the CNS are able to establish functional tolerance, an RNAi-mediated knockdown driven by *appl*-Gal4 was conducted and the flies were tested using the eRING assay (Bhandari et al., 2009).

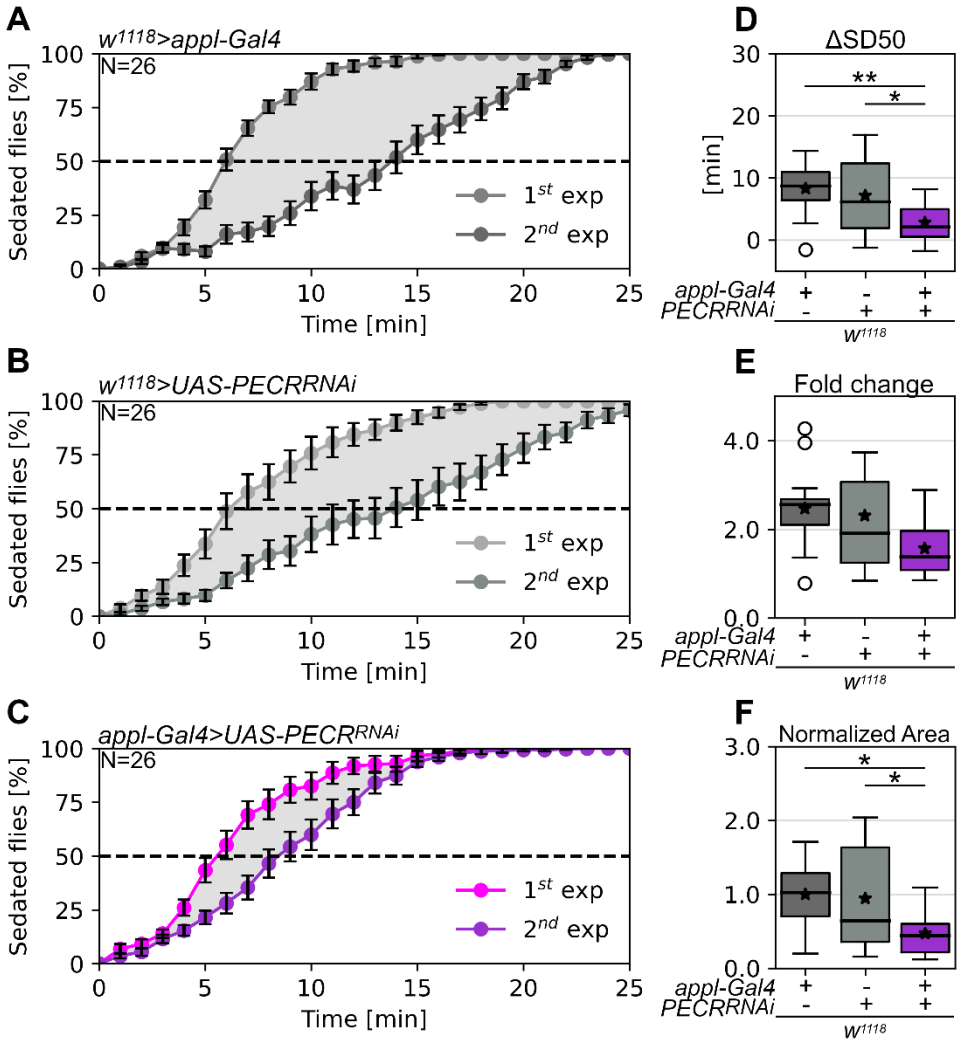

Figure 9: PECR was needed in the CNS for tolerance development to EtOH.
A-C, All tested genotypes were able to develop a tolerance; N = 26. **D**, Sedation times of second exposure did not differ significantly; mean ± SEM: *w*^{1118}*;appl -Gal4*: 14.4 ± 1.0 min; *w*^{1118}*;UAS-PECR*^{HMS00753RNAi}: 14.3 ± 1.5 min; *appl-Gal4;UAS-PECR*^{HMS00753RNAi}: 9.1 ± 0.7 min; One-way ANOVA F: 5.4, df: 41, p-value: 0.009, post-Hoc TukeyHSD: UAS/Gal4: 0.78, Gal4/Exp: 0.009, UAS/Exp: 0.047; **E**, The increase of resistance to EtOH did not differ between groups; Fold change

as mean ± SEM: w^{1118}; *appl-Gal4*: 2.47 ± 0.23; w^{1118};*UAS-PECR*^{HMS00753RNAi}: 2.31 ± 0.36 ; *appl-Gal4;UAS-PECR*^{HMS00753RNAi}: 1.57 ± 0.16; One-way ANOVA F: 3.05, df: 41, p-value: 0.058; **F**, The difference in sedation rate of first and second exposure to EtOH was significantly reduced in CNS knockdown PECR flies; mean ± SEM: w^{1118}; *appl-Gal4*: 1.0 ± 0.1; w^{1118};*UAS-PECR*^{HMS00753RNAi}: 0.95 ± 0.18; *appl-Gal4;UAS-PECR*^{HMS00753RNAi}: 0.47 ± 0.08; One-way ANOVA F: 4.58, df: 25, p-value: 0.016, post-hoc TukeyHSD: UAS/Gal4:0.957, UAS/Exp: 0.024, Gal4/Exp:0.047. ANOVA Tukey HSD * = p-value > 0.05, groups of 20 flies, N = 14.

The results showed a similar sedation rate during the initial EtOH exposure (Fig. 9, A-C). All flies exhibited the capacity to establish tolerance to EtOH (Fig. 9, A-C). The extent of tolerance of control flies tended to be stronger than the tolerance developed by the experimental group. The difference between the SD50s of the first and second exposure of the experimental group was reduced significantly by 27 % and the fold change was reduced by 23 – 27 % compared to the controls (Fig. 9, D,E). This finding is further substantiated by the significant difference in the sedation curves of the first and second exposures. Therefore, flies with a knockdown of PECR in the CNS demonstrated a significantly diminished tolerance development in comparison to their genetic controls (Fig. 9, F). These results demonstrate that PECR in the CNS is necessary to enhance the development of functional tolerance to EtOH.

3.4 PECR can Modulate Gene Expression

It is evident that the expression of PECR exerts a significant influence on the development of tolerance to EtOH in the CNS of flies. Consequently, further investigations on the potential involvement of fatty acid synthesis in the development of tolerance were analysed. As PECR is an important enzyme in the synthesis of LCFA and VLCFA, which are utilized in the construction of phospho- and sphingolipids (Faust et al., 2012; Wakashima et al., 2014), alterations in the capacity to generate membrane lipids may be discernible through confocal imaging. In order to visualise potential defects in neuron branching or protein transportation through vesicles, the membrane-bound GFP fusion protein *mouse*-cD8::GFP was expressed through a broad CNS driver (*appl*-Gal4) (Fig. 10, A). Concurrently, an RNAi-mediated PECR knockdown was conducted in the experimental group. A reduction in PECR resulted in a significant decrease in the fluorescence of the GFP, while no alterations in the expression pattern could be observed (Fig. 10, A'). However, given that the driver targets all neurons in the CNS, a smaller

subset of neurons (*PDF*-Gal4) was selected for observation to ascertain whether PECR knockdown might have an influence on neuron branching or mcD8::GFP expression. In this instance, no discernible alterations in neuronal branching or neuronal area were observed, however, a decrease in fluorescence was determined (Fig. 10, B-B'). The reduced mcD8::GFP fluorescence when co-expressed with $PECR^{HMS00753RNAi}$ can either result from lower mcD8::GFP expression or a reduced insertion into the membrane, since a change in cell lipid composition can influence the membrane dynamic (Bogdanov et al., 2014). To analyse the mcD8::GFP expression and localisation in the cell a Western blot was performed. To gain insight into the localization of the GFP a fractionation protocol was used on the proteins extracted from *Drosophila* heads. The extraction process of the proteins seperated cytoplasmic proteins, nuclear proteins and membrane proteins. The visualization of synapsin was utilized as an internal protein concentration control and demonstrated that the amount of protein used per probe was similar in all probes. Bands were expected at 70, 74, 80 and 143 kDa as denoted by the producer (DSHB Cat# 3C11, RRID:AB_528479). The results of the Western blot demonstrate that in the control group the highest abundance of mcD8::GFP (55 kDa) was found in the nucleus and the membrane. In the experimental group, the reduced PECR was found to decrease the overall abundance of mcD8::GFP. Furthermore, the relative abundances of mcD8::GFP were similar in the cytoplasm, nucleus and membrane (Fig. 10, C). Therefore, the knock down of PECR using $PECR^{HMS00753RNAi}$ reduces mcD8::GFP abundance in the neuron. However, it is unlikely that synapsin which is referred to as a vesicle regulating protein in the cytoplasm at the axon terminal (Hilfiker et al., 1999), and mcD8::GFP, which should be inserted into the membrane are both found in such a high abundance in the nucleus. Since no changes in the concentration of synapsin could be detected, the probability for a proper separation of the protein content is low. Therefore, the total amount of protein was calculated and the rate of GFP per synapsin was analysed (Fig. 10, D). Since the amount of synapsin did not differ between the probes, the amount of GFP with and without a PECR downregulation can give insights of possible effects of PECR on gene expression. When looking at the total mcD8::GFP content of the experimental group, the amount of detected mcD8::GFP is significantly lower than in the control, indicating that PECR indeed affects gene expression in neurons.

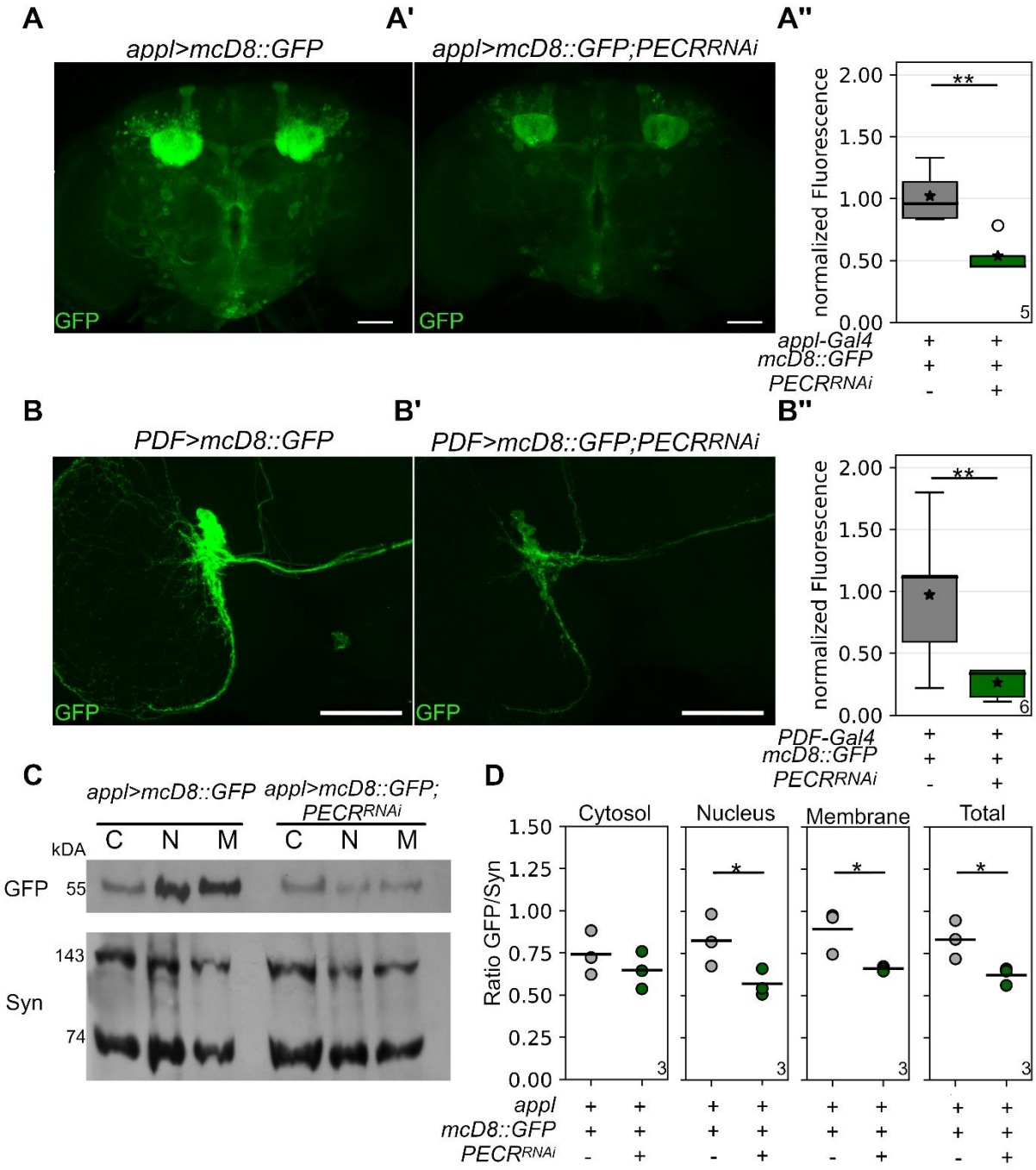

Figure 10: A knockdown of PECR reduced mcD8::GFP expression.
A-A'', Pan-neuronal downregulation of PECR via RNAi reduced GFP fluorescence, N = 5,5, mean ± SEM: 1 ± 0.09, 0.51 ± 0.06, Students-t-test F: 4.25, df: 8, p-value: 0.003; **B-B''**, RNAi-mediated knockdown of PECR in PDF neurons reduced GFP-induced fluorescence significantly, N = 6,6, mean ± SEM: 1 ± 0.22, 0.25 ± 0.05, Students-t-test F: 3.32, df: 10, p-value: 0.007; **C**, Western blot analysis of adult *Drosophila* heads. *appl;mcD8::GFP* showed strong bands for nuclear (N) and membrane (M) proteins and a weaker band for cytosolic proteins (C) at 55 kDA corresponding to mcD8::GFP. The expression of mcD8::GFP in *PECR*^{HSM00753RNAi} knockdown flies was significantly

weaker. Both genotypes showed similar expression of synapsin (Syn) at 143 kDa and around 74 kDa, indicating similar protein levels. **D**, The ratio of GFP to the corresponding synapsin control band in respective cell area an total showed a significant reduction of membrane mcD8::GFP in PECR knockdown flies; 3 Western blots were done, GFP and synapsin were detected and analysed on all 3 membranes, ratio of GFP/Syn are shown as mean ± SEM: cytosol: 0.74 ± 0.08, 0.65 ± 0.06, nucleus: 0.86 ± 0.06, 0.57 ± 0.05, Students-t-test F: 3.74, df: 4, p-value: 0.02, membrane: 0.90 ± 0.07, 0.66 ± 0.01, Students-t-test F: 3.12, df: 4, p-value: 0.03, total: 0.83 ± 0.07, 0.62 ± 0.03, Students-t-test F: 2.91, df: 4, p-value: 0.04 ; scale represents 50 µm; Students-t-test * = p-value < 0.05, ** = p < 0.01.

3.5 PECR in the CNS is Essential for Acamprosate Function

3.5.1 Acamprosate Treatment Promotes Tolerance Development

Exposure of the CNS to EtOH has been demonstrated to induce alterations in intrinsic excitability, synaptic transmission and plasticity (Abrahao et al., 2017). In individuals diagnosed with AUD, alterations in brain structures have been observed, resulting in a reduction in the functional connectivity of various brain regions. During the withdrawal phase, the brain is often in a hyperglutamatergic state (Rossetti and Carboni, 1995). The anti-relapse medication Acamprosate exerts a minimal effect on normal glutamate signalling, however, it has the capacity to reduce neuronal defects and even rescue neuronal defragmentation (Hutson et al., 2018). In order to investigate whether Acamprosate treatment might also change the susceptibility to EtOH, flies were treated with a 62 µM (25 µg/ml) Calcium-Acamprosate-containing diet for seven consecutive days and then tested for their sensitivity towards EtOH and their ability to develop tolerance. This amount of Acamprosate was chosen as it roughly reflects the daily dosage administered to humans (3x 666 mg per day, Saivin et al., 1998). The results demonstrated that Acamprosate treatment did not influence the initial sensitivity of the flies to EtOH. Both wild type control and treated flies exhibited a comparable response to the initial EtOH exposure (Fig.11, A,B). However, following a second exposure to EtOH, flies treated with Acamprosate exhibited a significantly increased resistance to the sedative effects of EtOH (Fig.11, C). The difference in SD50 of the first and second exposure was significantly increased by 83 % which was also reflected by a significant increase in fold change of 30 % (Fig. 11, D-E). Consequently, the present study demonstrates that Acamprosate has a positive effect on tolerance development yet does not affect the initial sedative effects of EtOH. This study previously demonstrated that

PECR^{KG07864} mutants exhibit impaired functional tolerance development. To investigate the possibility of Acamprosate decreasing the effects of the mutation or rescuing the tolerance deficit, *PECR*^{KG07864} mutant flies were treated with Acamprosate and tested for their ability to develop tolerance.

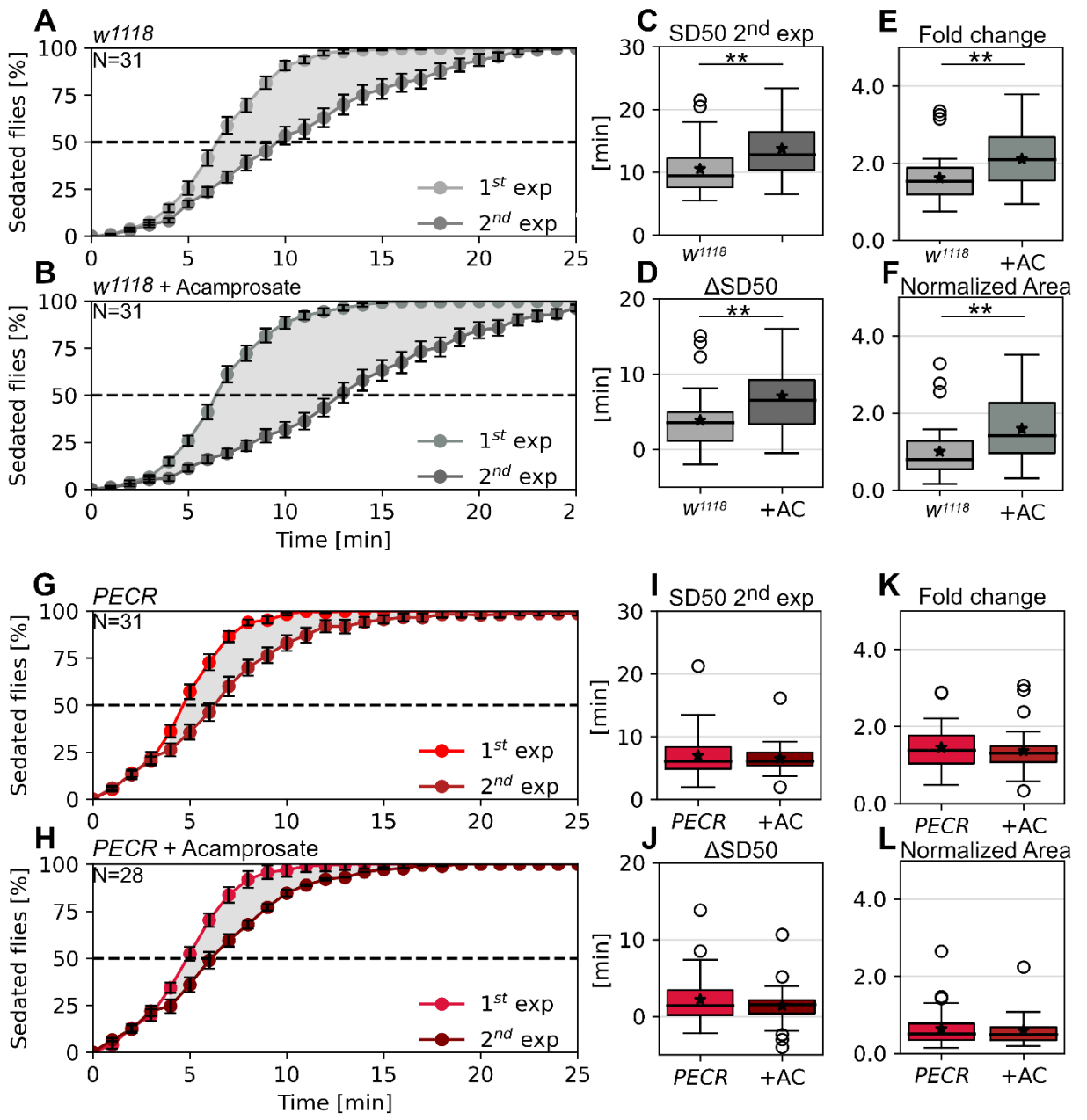

Figure 11: Acamprosate supports tolerance development in the presence of PECR.
A, *w*^{1118} flies treated with sucrose for 7 days developed a tolerance to EtOH. **B**, flies treated with sucrose and Acamprosate for 7 days developed a strong tolerance against EtOH-induced sedation. **C**, Treatment with Acamprosate prolonged time until 50% of flies were sedated in second EtOH exposure; mean ± SEM: *w*^{1118} 10.5 ± 0.8 min, with AC: 13.7 ± 0.7 min, Students-t-test F: 2.83, df: 60, p-value: 0.0062. **D**, Tolerance to EtOH was increased when flies were treated with

Acamprosate; mean ± SEM: w^{1118} 3.84 ± 0.75 min, with AC: 7.06 ± 0.8 min, Students-t-test F: 2.89, df: 60, p-value: 0.0052. **E**, Resistance to the sedating effects of EtOH was increased when flies were treated with Acamprosate; mean ± SEM: w^{1118}: 1.62 ± 0.12 min, with AC: 2.11 ± 0.13 min, Students-t-test F: 2.79, df: 60, p-value: 0.007. **F**, Difference between first and second exposure sedation rate was higher when flies were treated with Acamprosate, mean ± SEM: w^{1118} 1.0 ± 0.133, with AC: 1.588 ± 0.157, Students-t-test F: 2.8, df: 60, p-value: 0.0067.
G, $PECR^{KG07864}$ mutants developed a tolerance to EtOH when fed with sucrose for 7 days. **H**, Mutants treated with Acamprosate in sucrose solution were able to build a tolerance against EtOH. **I**, Acamprosate treatment did not affect the sedation rate in the second exposure in $PECR^{KG07864}$ mutants; mean ± SEM: $PECR^{KG07864}$ 7.0 ± 0.7 min, with AC: 6.5 ± 0.5 min. **J**, The development of tolerance was not affected by Acamprosate treatment in $PECR^{KG07864}$ flies; mean ± SEM: $PECR^{KG07864}$ 2.19 ± 0.59 min, with AC: 1.41 ± 0.51 min. **K**, The resistance against the sedative effects of EtOH was not affected by Acamprosate treatment in $PECR^{KG07864}$ flies as indicated by fold change; mean ± SEM: $PECR^{KG07864}$ 1.45 ± 0.11, with AC: 1.36 ±.0.11; **L**, Acamprosate treatment did not affect the difference in all time points between the first and second exposure in $PECR^{KG07864}$ mutants; mean ± SEM: $PECR^{KG07864}$ 1.0 ± 0.14, with AC: 0.93 ± 0.12. Students-t-test p-value: ** = p>0.01, groups of 20 flies, N = 31,31,31,28.

Similar to the observations in wild type, Acamprosate treatment did not affect the initial sedation to EtOH (Fig. 11, G-I). However, the treatment did not enhance the tolerance development of the mutants (Fig. 11, J-L). The sedation rates observed in both the control and treatment groups were comparable during the initial and secondary EtOH exposures, suggesting that the efficiency of Acamprosate is contingent on the presence of PECR. Additionally, there were no differences detectable in the fold change or the total difference of the curves (Fig. 11, K). Therefore, Acamprosate function in enhancing functional tolerance is reliant on PECR.

3.5.2 Acamprosate Promotes Tolerance Independently of Fat Body PECR

Since Acamprosate is ingested and may have a systematic effect and not only function in the brain, but it was also deemed necessary to ascertain whether the function of Acamprosate in the brain would enhance tolerance development or if the effect could be caused by side effects within other parts of the organism. This study established that PECR is required for Acamprosate to enhance the development of tolerance to EtOH (Fig. 11). Functional ethanol tolerance is connected to the metabolism of LCFAs (Geer et al., 1989; Swanson et al., 1995), which are primarily stored in the fat body of *Drosophila* (Canavoso et al., 2001). Therefore, flies with a knockdown of PECR in the fat body were treated with Acamprosate and subsequently analysed for their sensitivity to EtOH and their capacity to develop tolerance.

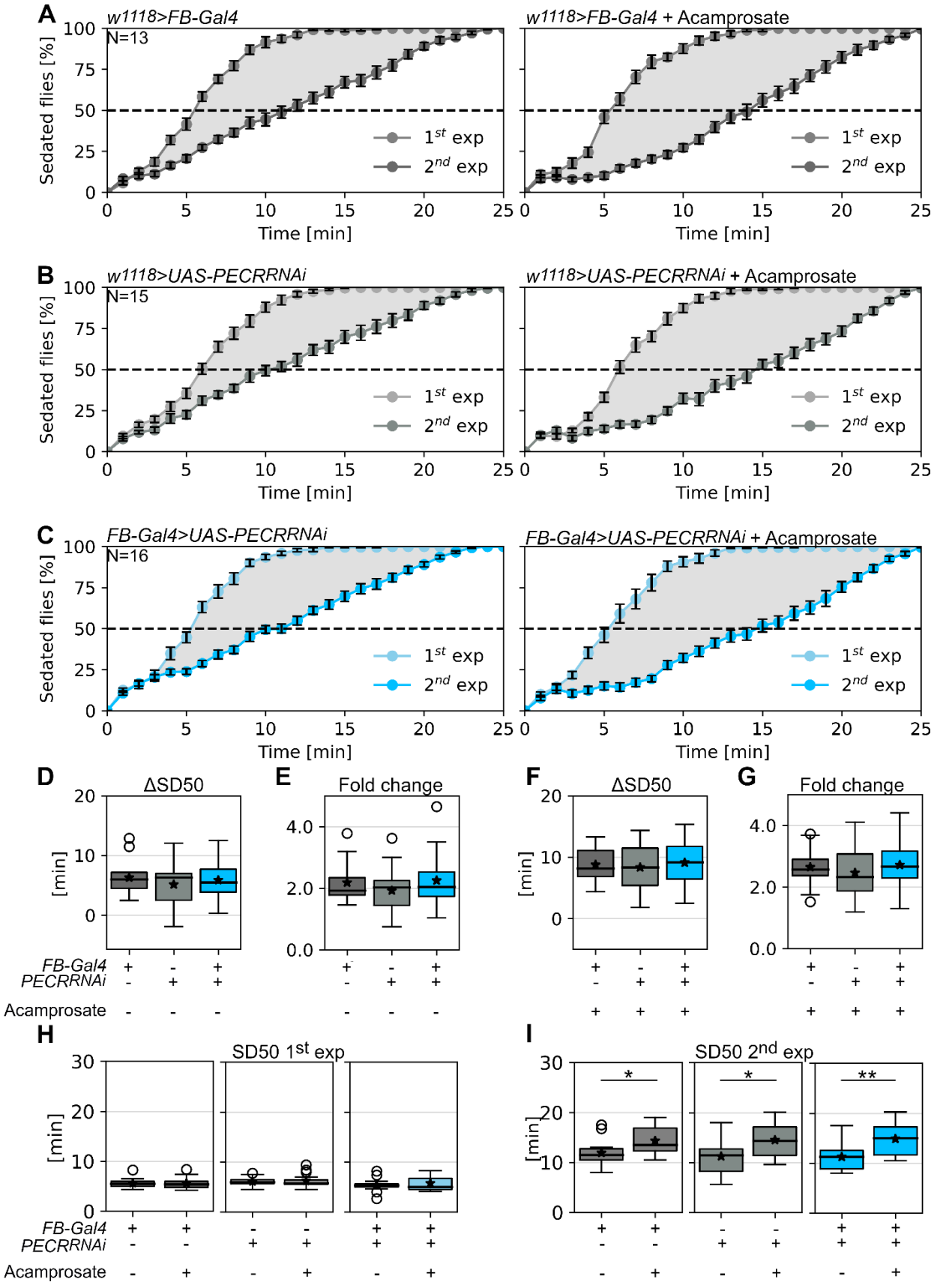
A
w1118>FB-Gal4
N=13
w1118>FB-Gal4 + Acamprosate
B
w1118>UAS-PECRRNAi
N=15
w1118>UAS-PECRRNAi + Acamprosate
C
FB-Gal4>UAS-PECRRNAi
N=16
FB-Gal4>UAS-PECRRNAi + Acamprosate
Sedated flies [%]
Time [min]
1st exp
2nd exp
D
ΔSD50
E
Fold change
F
ΔSD50
G
Fold change
[min]
FB-Gal4
PECRRNAi
Acamprosate
H
SD50 1st exp
I
SD50 2nd exp
*
*
**

Figure 12: Acamprosate induces stronger tolerance without PECR in fat body.
A - C, Flies treated with sucrose (left) or sucrose with Acamprosate (right) for 7 days developed a tolerance to EtOH. **D**, Untreated flies showed no differences in their second sedation time; mean ± SEM: w^{1118}>*FB-Gal4*: 11.9 ± 0.8 min, w^{1118}>$PECR^{RNAi}$: 11.2 ± 0.9 min, *FB*>$PECR^{RNAi}$: 11.3 ± 0.7 min. **E**, The fold changes in sedation times after the first and the second sedation were similar in all tested groups without Acamprosate treatment; mean ± SEM: w^{1118}>*FB-Gal4*: 1.928 ± 0.192, w^{1118}>$PECR^{RNAi}$: 2.18 ± 0.182, *FB*>$PECR^{RNAi}$: 2.25 ± 0.223. **F**, Treated flies showed no differences in their second sedation time; mean ± SEM: w^{1118}>*FB-Gal4*: 14.3 ± 0.7 min, w^{1118}>$PECR^{RNAi}$: 14.5 ± 0.8 min, *FB*>$PECR^{RNAi}$: 14.9 ± 0.9 min. **G**, The fold changes in sedation times after the first and the second sedation were similar in all tested groups with Acamprosate treatment; mean ± SEM: w^{1118}>*FB-Gal4*: 2.46 ± 0.2, w^{1118}>$PECR^{RNAi}$: 2.64 ± 0.17, *FB*>$PECR^{RNAi}$: 2.72 ± 0.21. **H**, Acamprosate did not affect the initial sensitivity to EtOH, mean ± SEM: w^{1118}>*FB-Gal4* 5.6 ± 0.25 min, +AC: 5.59 ± 0.3 min, w^{1118}>$PECR^{RNAi}$: 6.06 ± 0.24 min, +AC: 6.195 ± 0.33 min, *FB*>$PECR^{RNAi}$: 5.38 ± 0.3 min, +AC: 5.76 ± 0.38 min. **I**, Acamprosate treatment enhanced tolerance development in all tested genotypes, mean ± SEM: w^{1118}>*FB-Gal4* 11.89 ± 0.77 min, +AC: 14.33 ± 0.73 min, Students-t-test F: 2.22, df: 25, p-value: 0.0351, w^{1118}>$PECR^{RNAi}$: 11.24 ± 0.87 min, +AC: 14.52 ± 0.79 min, Students-t-test F: 2.7, df: 29, p-value: 0.0114., *FB*>$PECR^{RNAi}$: 11.257 ± 0.68 min, +AC: 14.87 ± 0.85 min, Students-t-test F: 3.21, df: 29, p-value: 0.0032. Students-t-test p-value: * = p<0.05, ** = p<0.01, groups of 20 flies, N = 13-16.

Concurrently, the experiment was conducted with mock-treated flies of the same genotype. It was observed that all groups, irrespective of treatment, exhibited the ability to develop tolerance. The untreated flies did not differ from each other in the time of sedation of the second EtOH exposure or the difference in the sedation rates between the first and second exposure (Fig.12, D,E). These results were consistent with previous findings for this genotype (Fig. 7). Furthermore, all flies treated with Acamprosate demonstrated comparable results, as there were no significant differences in the development of tolerance between the genotypes (Fig. 12, F,G). However, treatment with Acamprosate increased the SD50 of the second exposure by 20-32 %, which again proves its tolerance-enhancing effect (Fig. 12, I). Given that Acamprosate enhanced tolerance development in all treated groups and previous results have shown that Acamprosate requires the presence of PECR for its tolerance-promoting effect, it can be concluded that Acamprosate does not operate through the fat body of the flies.

3.5.3 Neuronal PECR is Required for the Tolerance-Enhancing Effects of Acamprosate

To further analyse whether the tolerance enhancing properties of Acamprosate are due to its efficacy in the brain, flies with a knockdown of PECR in the CNS underwent treatment with Acamprosate and were then tested for their sensitivity towards EtOH and their ability to develop tolerance.

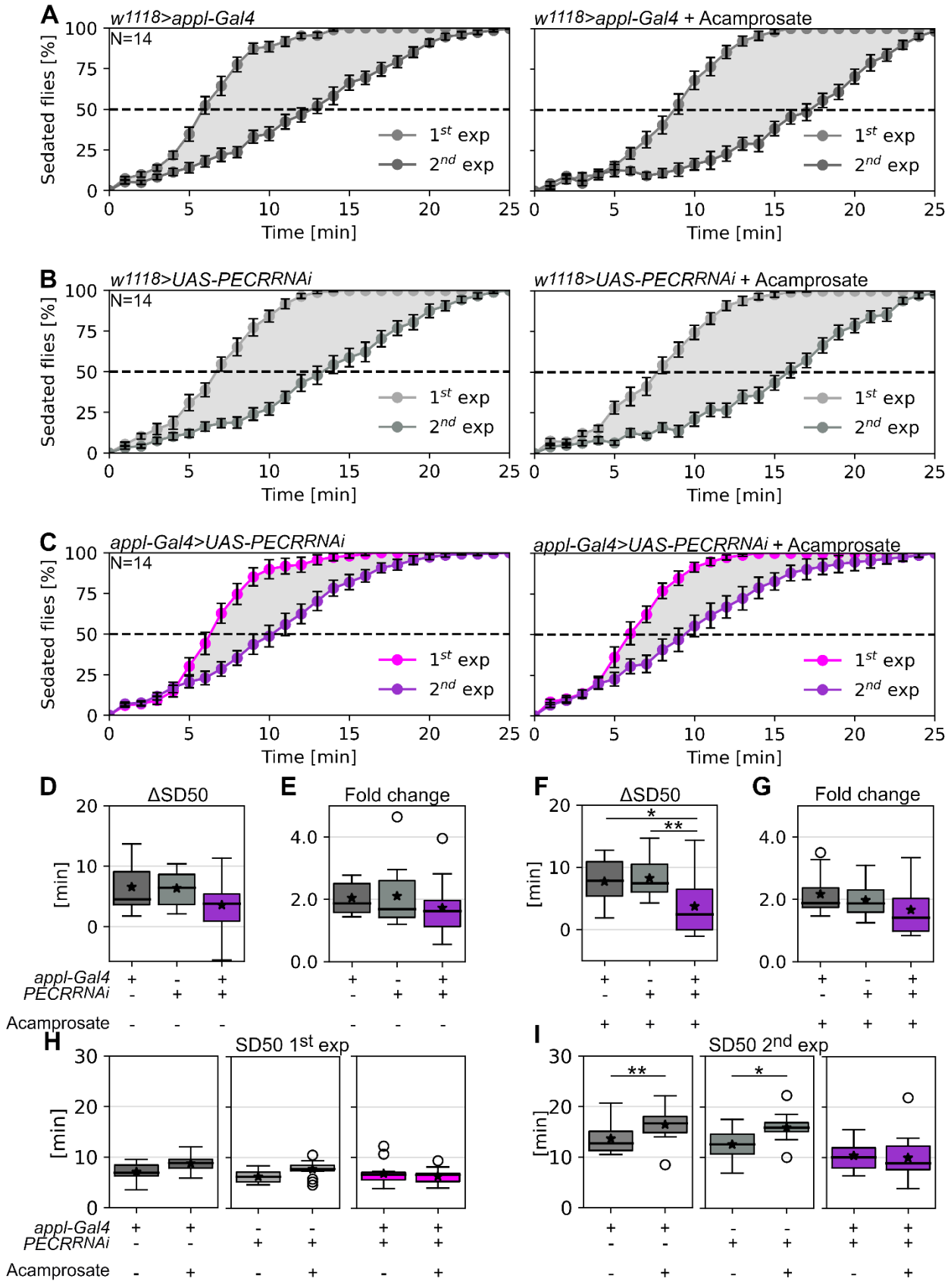
A
w1118>appl-Gal4
N=14
w1118>appl-Gal4 + Acamprosate
B
w1118>UAS-PECRRNAi
N=14
w1118>UAS-PECRRNAi + Acamprosate
C
appl-Gal4>UAS-PECRRNAi
N=14
appl-Gal4>UAS-PECRRNAi + Acamprosate
Sedated flies [%]
Time [min]
1st exp
2nd exp
D
ΔSD50
E
Fold change
F
ΔSD50
G
Fold change
[min]
appl-Gal4
PECRRNAi
Acamprosate
H
SD50 1st exp
I
SD50 2nd exp

Figure 13: Acamprosate function relies on PECR in the CNS.
A - C, Flies treated with sucrose (left) or sucrose with Acamprosate (right) for 7 days developed a tolerance to EtOH. **D**, Flies with a knockdown of PECR in the CNS tend to gain less tolerance: mean ± SEM: w^{1118}>*appl-Gal4*: 6.54 ± 1.01 min, w^{1118}>$PECR^{HMS00753RNAi}$: 6.32 ± 0.75 min, *appl*>$PECR^{HMS00753RNAi}$: 3.57 ± 1.11 min. **E**, The differences in sedation times after the first and the second sedation were similar in all tested groups without Acamprosate treatment, shown by fold changes: mean ± SEM w^{1118}>*appl-Gal4*: 2.1 ± 0.25, w^{1118}>$PECR^{HMS00753RNAi}$: 2.04 ± 0.13, *appl*>$PECR^{HMS00753RNAi}$: 1.72 ± 0.23. **F**, Treated control flies exhibited an increase in resistance to the sedative effects of EtOH compared to flies with reduced concentration of PECR in the CNS: mean ± SEM: w^{1118}>*appl-Gal4*: 7.72 ± 0.92 min, w^{1118}>$PECR^{HMS00753RNAi}$: 8.22 ± 0.79 min, *appl*>$PECR^{HMS00753RNAi}$: 3.7 ± 1.16 min, ANOVA F: 6.059, df: 41, p-value: 0.0051, post-hoc TukeyHSD: UAS/Gal4: 0.899, Gal4/Exp: 0.02, UAS/Exp: 0.007. **G**, The fold changes in sedation times at the second sedation were similar in flies compared to groups with Acamprosate treatment; mean ± SEM w^{1118}>*FB-Gal4*: 1.96 ± 0.15, w^{1118}>$PECR^{HMS00753RNAi}$: 2.17 ± 0.17, *appl*>$PECR^{HMS00753RNAi}$: 1.66 ± 0.2. **H**, Acamprosate did not affect the initial sensitivity to EtOH; mean ± SEM w^{1118}>*FB-Gal4*: 7.1 ± 0.4 min, +AC: 8.7 ± 0.5 min, w^{1118}>$PECR^{HMS00753RNAi}$: 6.2 ± 0.3 min, +AC: 7.7 ± 0.4 min; *appl*>$PECR^{HMS00753RNAi}$: 6.8 ± 0.6 min, +AC: 6.2 ± 0.4 min. **I**, Acamprosate treatment resulted in a delayed sedation rate in second exposure in controls but not in the experimental group, mean ± SEM w^{1118}>*appl-Gal4*: 13.6 ± 0.8 min, +AC: 16.4 ± 0.8 min, Students-t-test F: 2.95, df: 26, p-value: 0.0067, w^{1118}>$PECR^{HMS00753RNAi}$: 12.5 ± 0.9 min, +AC: 15.9 ± 0.7 min, Students-t-test F: 2.43, df: 26, p-value: 0.022, *appl*>$PECR^{HMS00753RNAi}$: 10.3 ± 0.8 min, +AC: 9.9 ± 1.2 min, Students-t-test F: 0.28, df: 26, p-value: 0.78; groups of 20 flies, N = 14; Students-t-test p-value: * = $p < 0.05$, ** = $p < 0.01$.

Previous results indicated a reduced tolerance development, although the flies were still able to gain tolerance (Fig. 8). Since Acamprosate treatment increased tolerance to EtOH in wild type flies (Fig. 11), the PECR knockdown flies in the CNS were used to discern if Acamprosate is reliant on PECR in the CNS to exert its tolerance-enhancing function. As anticipated, the untreated experimental group demonstrated a tendency for diminished tolerance in the second EtOH exposure compared to untreated controls (Fig. 13, D,E). Treatment of the control flies with Acamprosate resulted in a significantly increased tolerance compared to the PECR knockdown flies, even though the fold changes were not affected (Fig. 13, F,G). As previously demonstrated, Acamprosate treatment did not affect the initial sedation rate of the flies (Fig. 13, H), but it increased the development of tolerance in control flies, as evidenced by the increase of 20-29 % of SD50 of the second exposure (Fig. 13, I). The treatment with Acamprosate did not affect the tolerance development of PECR knockdown flies (-4%) (Fig. 13, I). Consequently, it can be concluded that Acamprosate is contingent upon the presence of PECR within the CNS to elicit its positive effects on tolerance development to EtOH.

However, it should be noted that these results were obtained with flies exposed to a carbohydrate-rich but protein-low diet for seven consecutive days, which rendered dietary effects on the flies' behaviour possible. Consequently, the experiment was repeated with flies fed on a standard diet with and without Acamprosate for a period of seven days prior to the experiment. The results demonstrated a significantly diminished capacity of the flies to develop tolerance to EtOH, and the absence of any impact of Acamprosate on enhancing tolerance development in the presence of a low abundance of PECR in the CNS (Fig. 14). Thus, the previously obtained results are independent of the diet prior to the experiment.

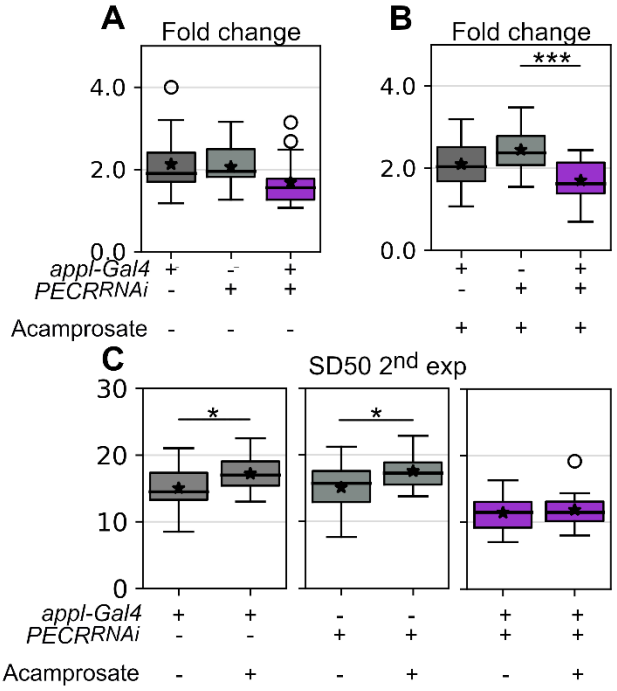

Figure 14: Dietary effects of treatment solution can be excluded as a cause of the tolerance development phenotype.
A, The fold change in SD50 of the first and second EtOH exposure was similar to controls in flies with a PECR knockdown in the CNS; mean ± SEM w^{1118}>*appl-Gal4*: 2.12 ± 0.155, w^{1118}>$PECR^{RNAi}$: 2.05 ± 0.111, *appl*>$PECR^{RNAi}$: 1.68 ± 0.123. **B**, The difference in SD50 of the first and second EtOH exposure in flies treated with AC was significantly reduced in CNS PECR knockdown flies seen by fold change; mean ± SEM w^{1118}>*appl-Gal4*: 2.09 ± 0.126, w^{1118}>$PECR^{RNAi}$: 2.44 ± 0.112, *appl*>$PECR^{RNAi}$: 1.69 ± 0.108. ANOVA F: 9.87, df: 59, p-value: 0.0002, post-hoc TukeyHSD: Gal4/UAS: 0.106, Gal4/Exp: 0.053, UAS/Exp: 0.001. **C**, Treatment with AC increased tolerance development except in flies with low PECR concentrations in the CNS; mean ± SEM w^{1118}>*appl-Gal4*: 15.0 ± 0.7 min, +AC: 17.2 ± 0.6 min, Students-t-test F: 2.3, df: 38, p-value: 0.02, w^{1118}>$PECR^{RNAi}$: 15.1 ± 0.8 min, +AC: 17.5 ± 0.5 min, Students-t-test F: 2.54, df: 38, p-value: 0.015, *appl*>$PECR^{RNAi}$: 11.4 ± 0.5 min, +AC: 11.8 ± 0.6 min, Students-t-test F: 0.5, df: 38, p-value: 0.615; groups of 20 flies, N = 20; ANOVA post-hoc TukeyHSD or Students-t-test *= $p < 0.05$.

3.6 Acamprosate Decreases Oxidative Stress Through Neuronal PECR

3.6.1 PECR Contributes to Lifespan Maintenance

It has been demonstrated that EtOH exposure can induce oxidative stress in cells (Logan-Garbisch et al., 2015; Wu and Cederbaum, 2003). Tolerance to EtOH partly relies on the cellular stress pathway (Scholz et al., 2005). Increased ROS results in lipid peroxidation which reduces neuronal viability. The cell is able to decrease ROS in the peroxisomes since the main anti-oxidant enzymes, like catalase and SOD, are located there (He et al., 2021). Further, it has been demonstrated that various lipids are involved in the compensatory mechanisms protecting against EtOH-induced damage (Hernández et al., 2016). PECR is involved in fatty acid synthesis (Das et al., 2000; Faust et al., 2012), and thereby induces the synthesis of lipids which might have anti-oxidant properties. Increased oxidative stress reduces the lifespan of flies (Fleming et al., 1992). It has been demonstrated that levels of oxidative stress increase with age in flies, and that accumulation of this stress can result in premature death (Fleming et al., 1992). To analyse whether *PECR*^{KG07864} mutants are less resistant to oxidative stress, similar to their reduced tolerance to the intoxicating effects of EtOH (Fig. 3), an analysis of their lifespan was conducted.

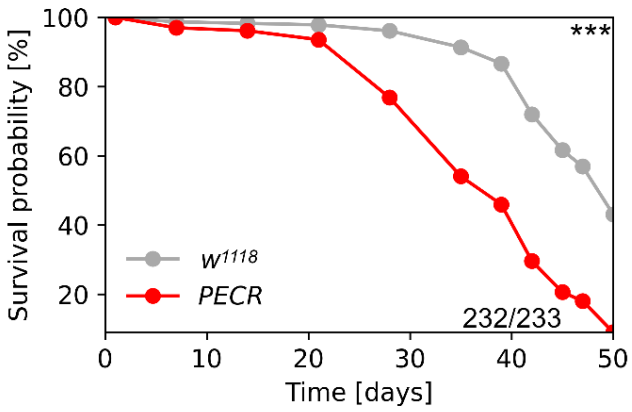

Figure 15*: PECR*^{KG07864} mutants have a reduced lifespan.
Longevity was impaired in *PECR*^{KG07864} mutants; N = 232/233; Log-Rank test with Bonferroni correction: ***= p<0.001

Wild type and mutant males were collected in groups of 20 and placed on a standard diet. The analysis on the lifespan under regulated conditions revealed that *PECR*^{KG07864} mutants had a significantly lower survival probability with time (Fig. 15). The observed outcome can be attributed to either a deficient metabolism or an impaired oxidative stress

resistance in the mutants. However, since the flies always had access to their standard diet, the significantly reduced lifespan can likely be attributed to a reduced ability to withstand oxidative stress.

3.6.2 Acamprosate Enhances Starvation-Induced Oxidative Stress Resistance

Acamprosate has been demonstrated to attenuate symptoms of stress, including anxiety and hyperactivity (Schaefer et al., 2017). In order to investigate a link between Acamprosate treatment and probable oxidative stress-reducing effects, oxidative stress was induced in the flies. The enzymes superoxide-dismutase 1 and 2 (SOD1/SOD2) have been demonstrated to reduce oxidative stress in form of superoxide in cells (McCord and Fridovich, 1969). To induce oxidative stress in flies particularly in the CNS, RNAi-mediated knockdown of SOD1 and SOD2 was conducted using the *appl*-Gal4 driver to target the CNS. Additionally, the flies were subjected to starvation, as starvation has been demonstrated to further induce oxidative stress (Morales et al., 2004).

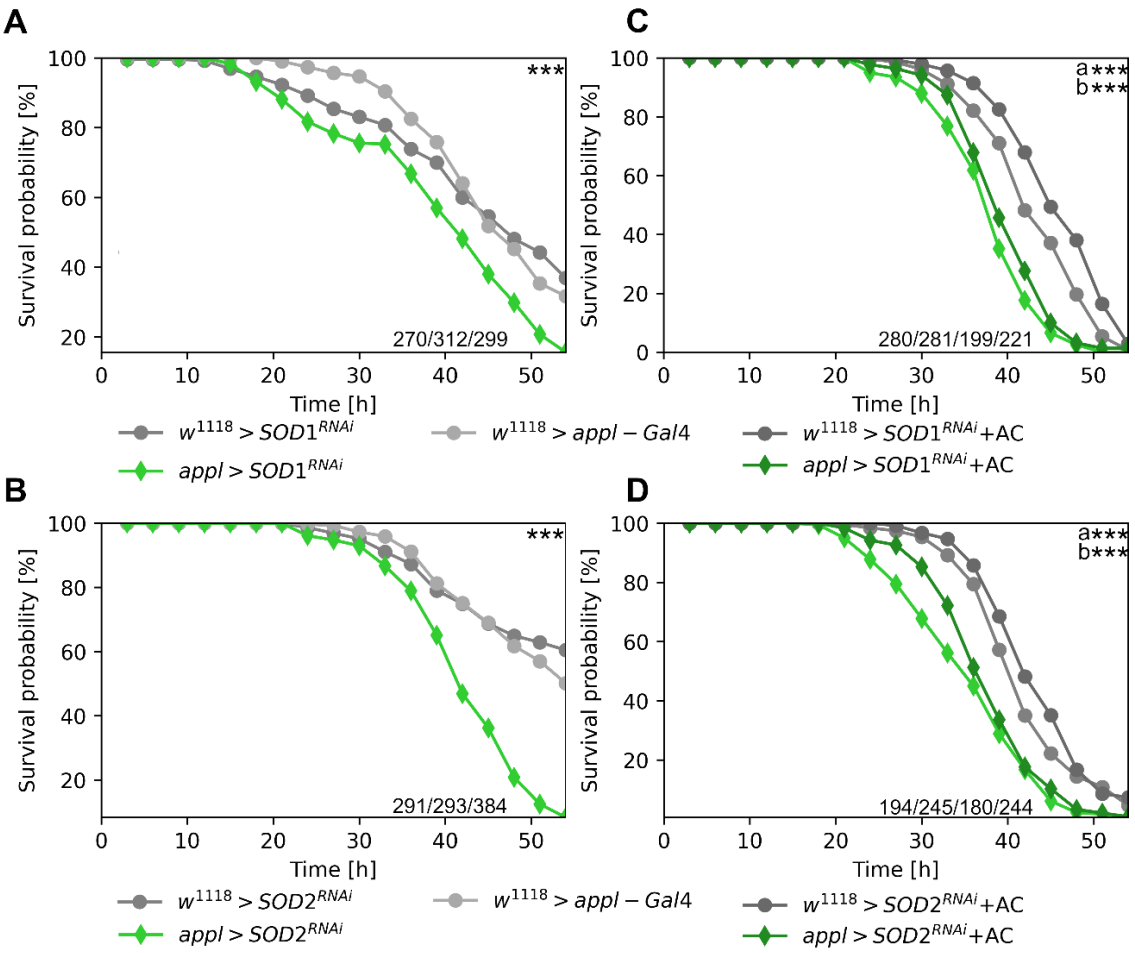

Figure 16: Acamprosate increases resistance against starvation-induced stress.
A, Knockdown of SOD1 in the CNS reduced survival probability of starved flies, N = 270/312/299; **B**, Knockdown of SOD2 in the CNS reduced survival probability of starved flies, N = 291/293/384. **C**, Acamprosate treatment enhanced survival probability in starved control flies and in starved SOD1 knockdown flies, N = 280/281/199/221. **D**, Treatment with Acamprosate promoted survival probability of starved control and SOD2 knockdown flies, N = 194/245/180/244. Log-Rank test Bonferroni controlled *** = $p < 0.001$, a: Comparison between treated and untreated controls, b: Comparison between treated and untreated experimental groups.

The survival of the flies was then surveyed every three hours for a period of at least 54 hours using the fly detector software (Bodenstein et al., 2017). The resulting data was analysed with a Kaplan-Meier estimator and a further Log-Rank test to obtain a survival probability for each genotype (Han et al., 2016). The obtained results demonstrated a clear correlation between increased oxidative stress during starvation and a decrease in survival probability (Fig. 16, A,B). In both SOD knockdown experiments, the experimental group exhibited a reduced resistance to starvation-induced stress. Treatment with Acamprosate was successful in significantly increasing the survival probability of both the control and experimental groups (Fig. 16, C,D). These results demonstrate that Acamprosate can reduce starvation-induced stress and is not dependent on the abundance of SOD1 or SOD2.

3.6.3 PECR Promotes Resistance to Starvation-Induced Stress

In a previous experiment, *PECR*^{KG07864} mutants exhibited a reduced lifespan in comparison to control flies (Fig. 15). It is well established that lifespan and ageing are very susceptible to oxidative stress (Warner, 1994). To ascertain whether *PECR*^{KG07864} mutants exhibit a diminished tolerance to starvation-induced stress, male and female flies were analysed for their starvation-induced stress resistance. The results demonstrated that *PECR*^{KG07864} mutants exhibited a diminished survival probability compared to the control group, irrespective of their sex (Fig. 17). These results indicate that PECR plays an important role in the resistance against stress. However, it is imperative to consider the alterations in the metabolism of *PECR*^{KG07864} mutants. *PECR*^{KG07864} mutants exhibited metabolic defects, likely arising from an impaired fatty acid synthesis (Faust et al., 2012). Still, the results showed that PECR can be associated with starvation-induced stress resistance and with resistance against EtOH-induced intoxication, therefore it is likely that PECR is involved in environmental stress resistance.

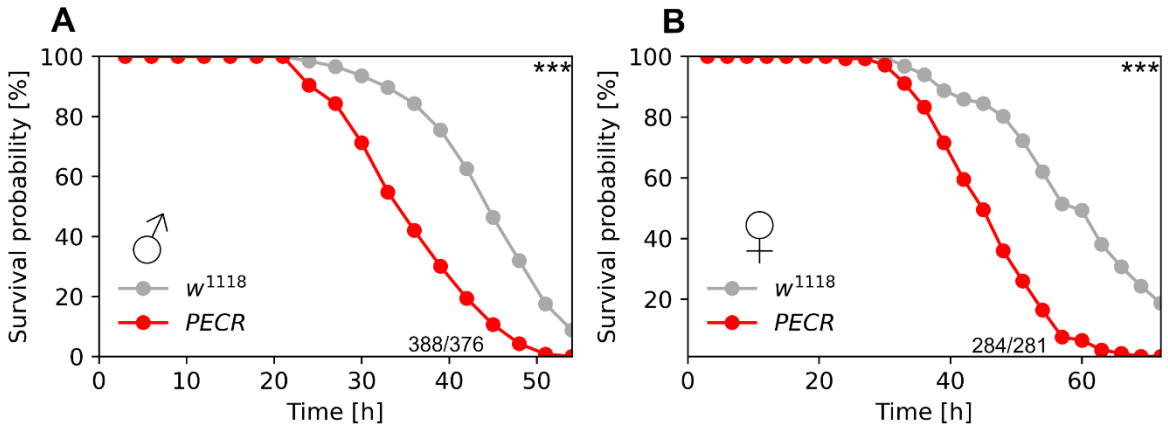

Figure 17: PECR is necessary to endure starvation-induced stress.
A, Starved male *PECR*^{KG07864} mutants had a lower survival probability than controls; N=388/376; Log-Rank test with Bonferroni correction: *** = p < 0.001. **B**, Starved female *PECR*^{KG07864} mutants had a lower survival probability than controls; N=284/281; Log-Rank test with Bonferroni correction: *** = p < 0.001.

3.6.4 Acamprosate Requires PECR to Reduce Starvation-Induced Stress

PECR^{KG07864} mutants exhibited a diminished survival probability in a starved state, however it was unclear if the decrease in survival is due to metabolic deficiencies or less oxidative stress resistance. Still, the mutants already showed a decreased lifespan, and a malfunctioning fatty acid synthesis can result in heightened oxidative stress (Sassa and Kihara, 2014). It was hypothesised that Acamprosate treatment should be able increase the survival probability of the mutants, as shown in previous experiments (Fig. 16). Therefore, control and mutant flies were treated with an Acamprosate-entailing diet or a mock diet for seven consecutive days. Subsequently, the flies were subjected to a starvation period of at least 60 h and their survival rate was analysed.

Acamprosate enhanced the survival probability of wild type control flies, however, it had no effect on *PECR*^{KG07864} mutants (Fig. 18). In a manner analogous to EtOH tolerance, Acamprosate was unable to execute its effect to enhance the survival probability in the absence of PECR. These results provide further confirmation of a connection between Acamprosate function and PECR abundance.

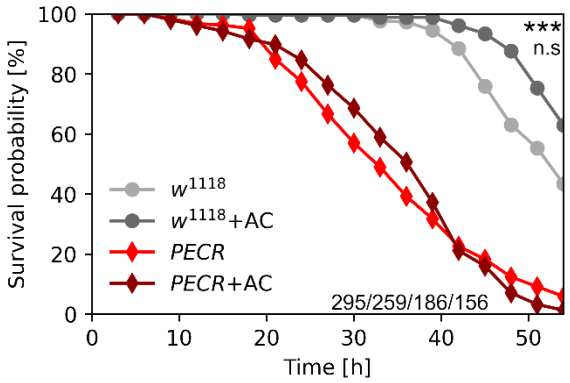

Figure 18: Acamprosate did not increase survival probability without PECR.
Acamprosate enhanced resistance against starvation-induced stress in controls but not in PECR mutants. Log-Rank test Bonferroni controlled: *** = $p < 0.001$; N = 295/259/186/156

3.6.5 Acamprosate Requires Neuronal PECR to Increase the Resistance Against Starvation-Induced Stress

To further examine the relationship between Acamprosate and PECR, a PECR knockdown was conducted in the fat body, muscles and CNS of flies. These flies were subjected to starvation to test their starvation resistance. Additionally, all genotypes were treated with Acamprosate to ascertain whether PECR is required in specific body parts of the fly for Acamprosate function. Since the fat body is the main storage for lipids (Canavoso et al., 2001), a knockdown of PECR in the fat body was conducted. Reduced PECR in the fat body led to no significant changes in their survival probability (Fig. 19, A). Treatment with Acamprosate led to a marked increase in survival probability for both the control and the experimental group (Fig. 19, D). Hence, these findings suggest that PECR in the fat body is not a critical factor for starvation resistance or for the impact of Acamprosate treatment. Conversely, the downregulation of PECR in the fly muscles led to a decrease survival probability. This is supported by the observation that glycogen stores are depleted in *PECR*^{KG07864} mutants and muscle tissue is the primary storage location for glycogen. Consequently, the downregulation of PECR in muscles may already lead to a depletion of the muscle glycogen levels, despite this not being detected in the previous glycogen assay (see Fig. 6, B). However, it should be noted that the glycogen measurements were conducted on the whole thorax of the fly which may have introduced variations in the results. Nevertheless, Acamprosate treatment was found to enhance survival probability in both the control and experimental groups (Fig. 19, E). These results

suggest that PECR in the muscles plays a role in general resistance against starvation, however, it is not directly relevant to the improved resistance against starvation-induced stress in the context of Acamprosate treatment. Finally, flies with a knockdown of PECR in the CNS were observed.

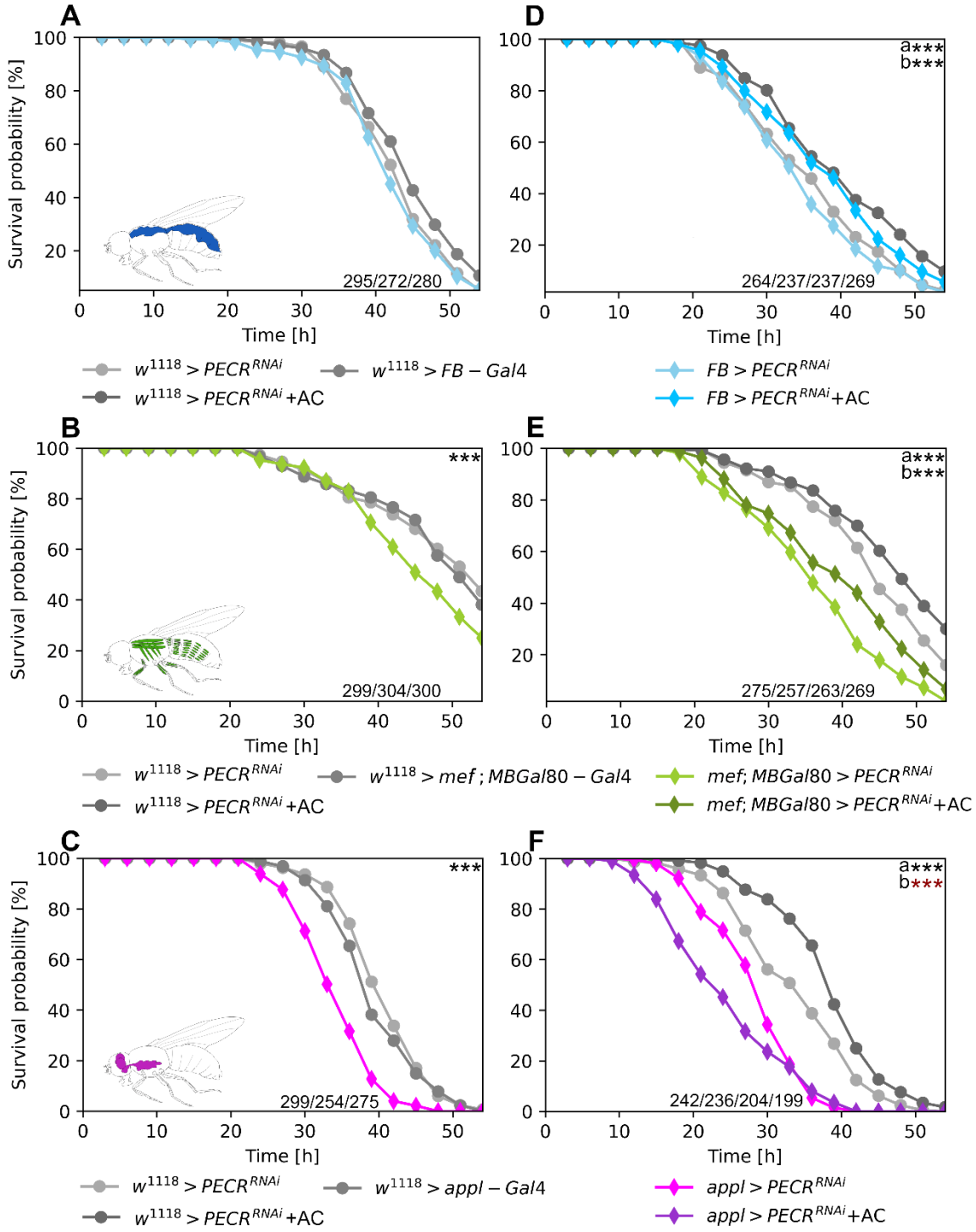

Figure 19: CNS PECR facilitates starvation-induced stress resistance.
A, Starvation-induced stress resistance was not dependent on PECR in the fat body; N = 295/272/280. **B**, PECR in the muscles promoted starvation-induced stress survival, N = 299/304/300. **C**, PECR in the CNS facilitated resistance against starvation-induced stress, N = 299/254/275. **D**, Acamprosate was not dependent on PECR in the fat body, N = 264/237/237/269. **E**, Acamprosate function did not depend on PECR in the muscles of the fly; N = 275/257/263/269. **F**, Function of Acamprosate was reliant on PECR in the CNS; N = 242/236/204/199. Log-Rank test Bonferroni controlled: *** = $p < 0.001$, a: difference between controls with and without Acamprosate, b: difference between treated and untreated experimental groups.

A lower CNS PECR abundance was found to result in a significantly diminished survival probability during starvation (Fig. 19, C). Treatment with Acamprosate reduced the survival probability even further, indicating that PECR in the CNS is important for Acamprosate function in starvation resistance and that Acamprosate exerts effects on neurons, which decrease their survival probability in the event of low PECR abundance.

3.6.6 Acamprosate Requires PECR in Neuronal Subtypes to Enhance Stress Survival

As Acamprosate requires PECR in the CNS to induce EtOH tolerance or increase the starvation-induced stress resistance, a range of neurotransmitter systems were evaluated. Acamprosate has been shown to work via the GABAergic system through inhibition of GABA receptors and via the glutamatergic system by exciting NMDA receptors (Berton et al., 1998). The glutamic acid decarboxylase (Gad1) is an essential, CNS-specific enzyme which synthesizes GABA, thus *Gad1*-Gal4 (GABA-Gal4) was used to target GABAergic neurons (Ng et al., 2002). The *Drosophila* vesicular glutamate transporter is expressed in glutamatergic moto- and interneurons, as well as in the synaptic terminals of the neuromuscular junctions (Daniels et al., 2004). Therefore, glutamatergic neurons were targeted by *dV-Glut*-Gal4.

Acamprosate is a homotaurine and binds taurine receptors with a high affinity (Wu et al., 2001). It was shown to reduce the expression of the EAAT2 in rodents (Germany et al., 2018). In *Drosophila*, EAAT2 is mainly expressed neuronally in sensory pathways, and it was demonstrated that taurine is co-expressed in those neurons (Besson et al., 2005). Therefore, a driver targeting EAAT2-expressing neurons (*EAAT2*-Gal4) was used to knock down PECR expression and investigate the starvation resistance of those flies.

The knockdown of PECR in GABAergic neurons and neurons expressing EAAT2 led to a significant reduction in survival probability (Fig. 20, A,C). Thus, PECR increases the

resistance to starvation-induced oxidative stress in GABAergic neurons and neurons expressing taurine. In glutamatergic neurons, however, RNAi-mediated PECR knockdown did not affect the survival (Fig. 20, B), therefore the glutamatergic neurons targeted by *dV-Glut*-Gal4 were not further investigated for their role in starvation induced-stress resistance and Acamprosate treatment.

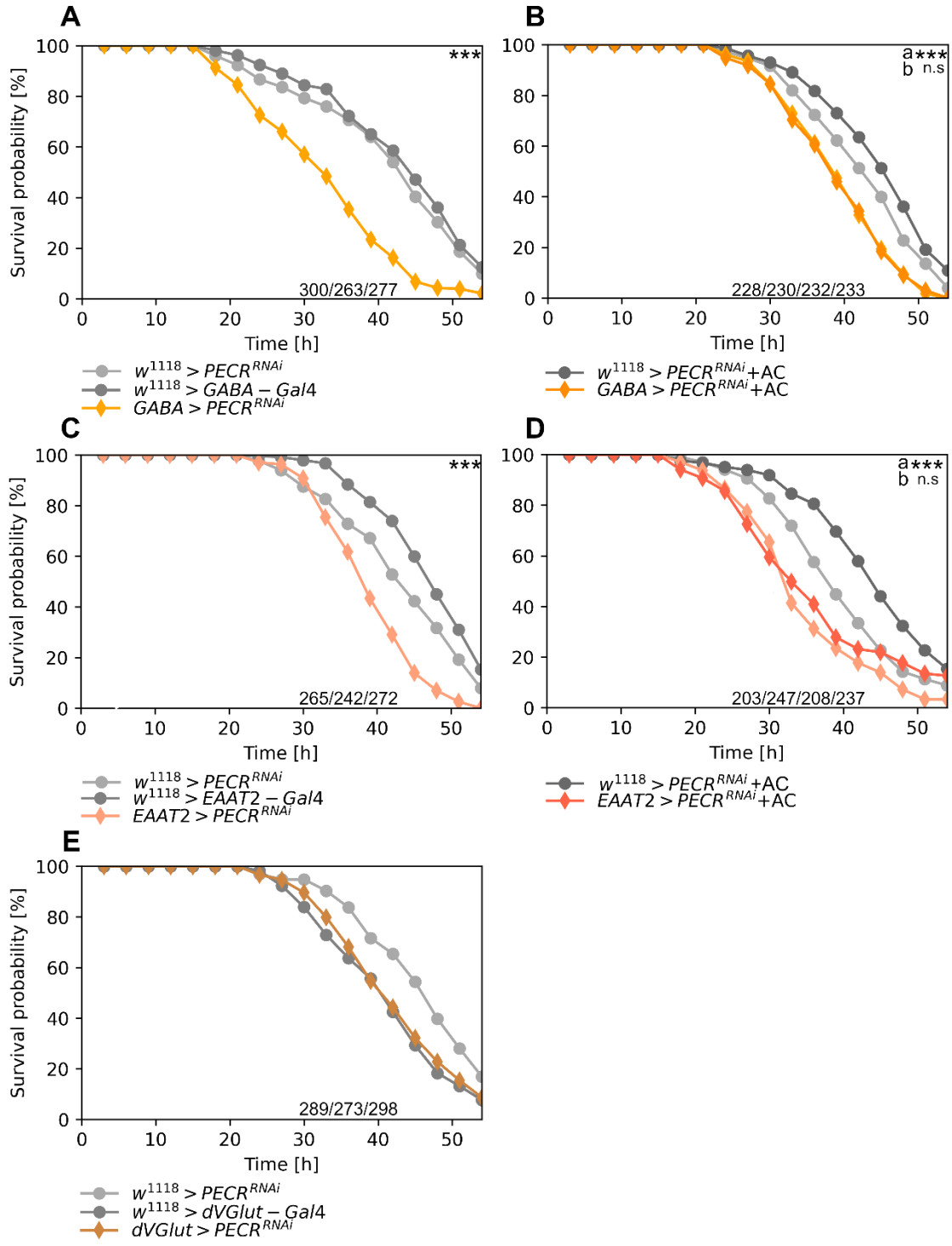

Figure 20: Acamprosate acts through GABAergic and EAAT2-expressing neurons.
A, Downregulation of PECR in GABAergic neurons decreased survival probability in starved flies; N = 300/263/277. **B**, Knockdown of PECR in glutamatergic neurons did not influence survival probability in starved flies; N = 289/273/298. **C**, PECR knockdown in cells expressing EAAT2 reduced survival probability in starved flies; N = 265/242/272. **D**, PECR is needed in cells expressing EAAT2 for Acamprosate to function; N = 203/247/208/237. Log-Rank test with Bonferroni correction: *** = $p < 0.001$; a: difference between controls with and without Acamprosate, b: difference between treated and untreated experimental groups.

Acamprosate treatment was not able to increase the starvation resistance of flies with a low abundance of PECR in GABAergic and EAAT2-expressing neurons (Fig. 20, B,D). These results indicate that Acamprosate indeed targets GABA receptors and EAAT2 receptors and that the expression of PECR in these neurons is crucial for its function.

3.7 Neuronal PECR is Essential for Acamprosate to Alleviate Heat Stress

3.7.1 Acamprosate Treatment Reduces Heat Shock-Induced Stress

The previous assays targeted EtOH-induced and starvation-induced stress. In both cases, Acamprosate treatment was sufficient to either increase tolerance to EtOH or increase the starvation resistance. Another way to induce stress in flies is via heat shock. The heat shock response in flies buffers several stressors besides heat, including oxidative stress and DNA damage (Morrow and Tanguay, 2003). Heat shock proteins (HSPs) are ubiquitously expressed and highly conserved as they function on a wide range of cellular housekeeping processes (Hartl et al., 2011). High temperatures can disrupt neuronal membranes thus affecting ion channels which results in their inhibition and thereby a loss of motor control, and unresponsiveness of the entire organism (Robertson, 2004). In this experiment, the flies were exposed to a 38 °C heat shock and sedated flies were counted every half an hour. As done before, $PECR^{KG07864}$ mutants were used to test their resistance against heat shock-induced stress. Control flies showed a significantly higher survival rate than $PECR^{KG07864}$ mutants (Fig. 21, A,C). To investigate, whether Acamprosate treatment can also affect heat shock-induced stress, control flies and mutants were treated with Acamprosate for one week before the procedure. Acamprosate prolonged the survival of controls but, like in the previous experiments, it did not induce heat shock resistance effects in $PECR^{KG07864}$ mutants (Fig. 21, D,E,F). However, there was a strikingly steep decline in the survival rate of the untreated control flies. Unlike the

previous dataset with flies aged two days, the survival rate of the older, treated flies declined at a much faster rate. Hsp70 expression decreases in flies with age (Sørensen and Loeschcke, 2002). To ensure that the decline in survival is due to the age of the flies, the heat shock survival was repeated with untreated flies aged seven days. The aged control and mutant flies did not differ from each other, indicating that age is a relevant factor in heat shock-induced stress resistance (Fig. 21, B,C).

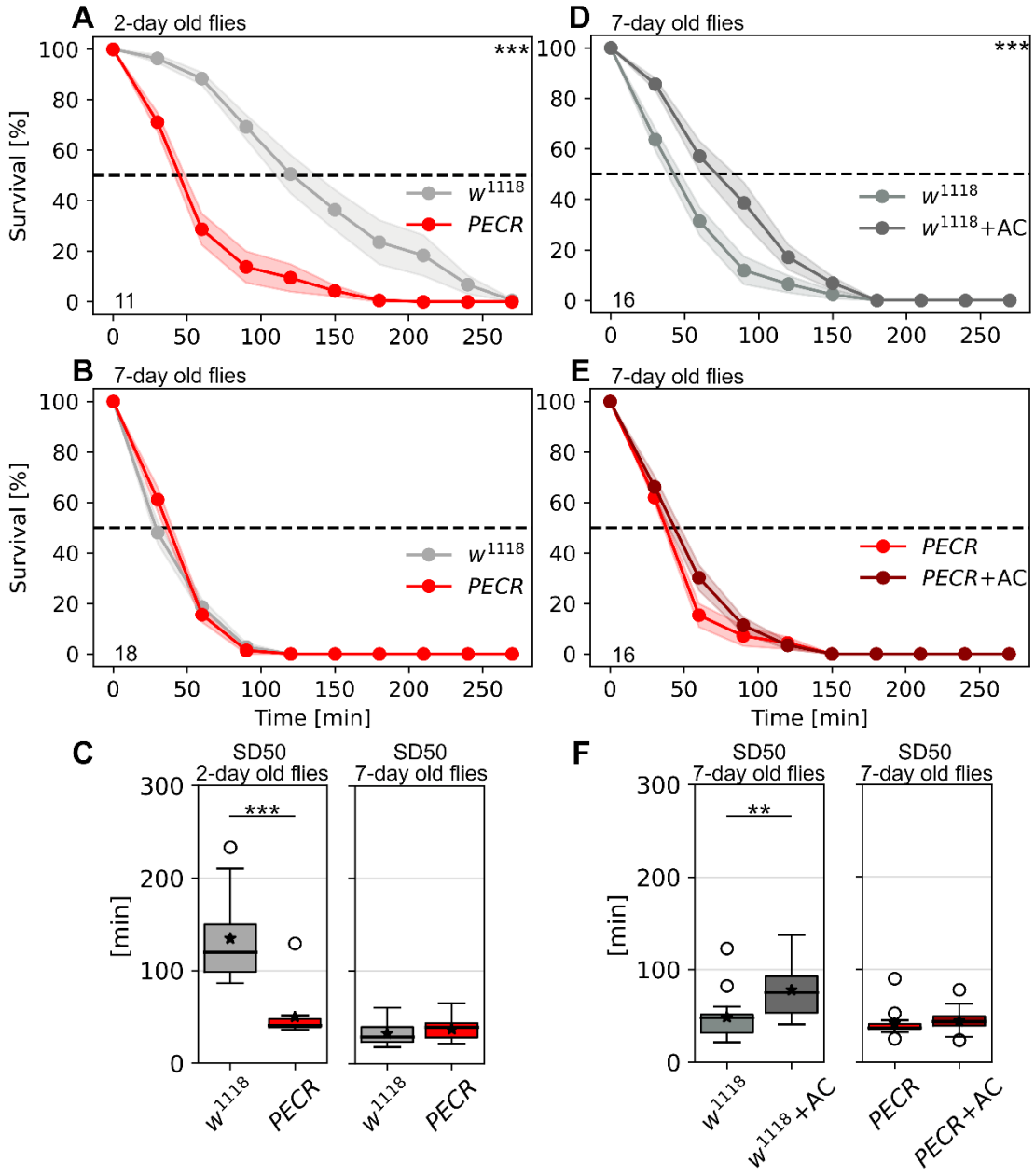

Figure 21: Acamprosate promotes heat shock-induced stress resistance.
A, *PECR*^{KG07864} mutants had less tolerance to heat shock-induced stress; Log-Rank test Bonferroni corrected: *** = p < 0.001. **B**, Aged mutants showed similar heat shock resistance as

controls. **C**, SD50 values of 2 and 7 day old flies showed the importance of age for heat shock resistance; mean ± SEM: 2-day old: w^{1118}: 134.7 ± 14.6 min, $PECR^{KG07864}$: 50.0 ± 8.1 min, Students-t-test F: 4.87, df: 19, p-value: 0.0001; 7-day old: w^{1118}: 32.2 ± 2.8 min, *PECR*: 37.0 ± 2.7 min. **D**, Acamprosate increasds resistance to heat shock in control flies; Log-Rank test Bonferroni corrected: *** = p < 0.001. **E**; Acamprosate was not able to increase heat shock resistance in $PECR^{KG07864}$ mutants. **F**, Acamprosate increased resistance to heat shock in w^{1118} flies but not in $PECR^{KG07864}$ mutants, mean ± SEM: w^{1118}: 48.5 ± 6.3 min, w^{1118}+AC: 77.8 ± 7.3 min, Students-t-test F: 3.02, df: 30, p-value: 0.0051, $PECR^{KG07864}$: 41.6 ± 3.7 min, $PECR^{KG07864}$ +AC: 44.1 ± 3.4 min. Groups of 20 flies, N = 11-18, Students-t-test: *** = p-value < 0.001, ** = p-value < 0.01.

3.7.2 Acamprosate Requires Neuronal PECR to Increase Resistance Against Heat Shock-Induced Stress

To ensure that the stress resistance-increasing effects of Acamprosate towards heat shock-induced stress are similar to the effects it has on starvation- or EtOH-induced stress, PECR knockdown flies were tested for their heat shock-induced stress resistance.

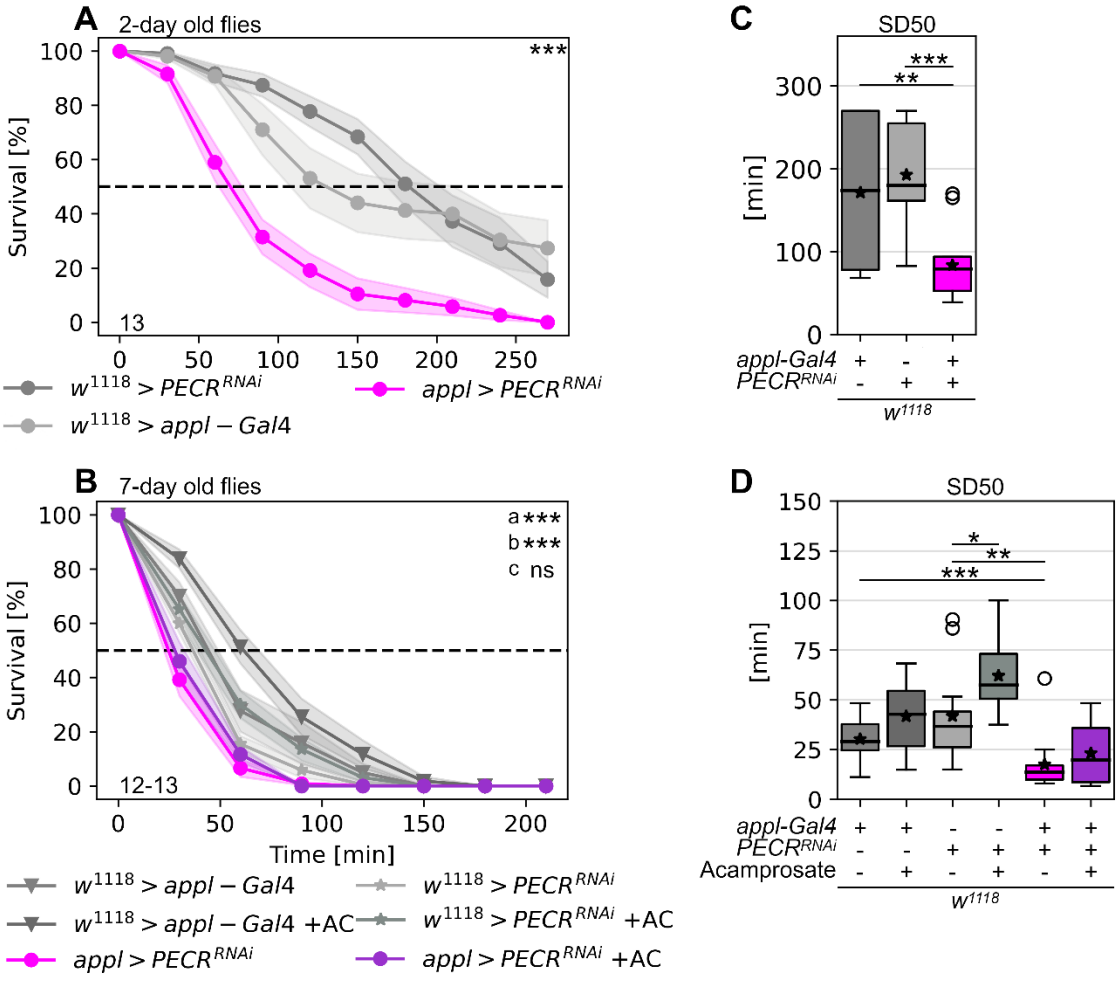

Figure 22: Heat shock-induced stress is reduced through Acamprosate and PECR.
A, PECR in the CNS was important for heat shock induced stress resistance, N = 13. **B**, Acamprosate increased stress resistance only in the presence of PECR in the CNS, N = 12-13; Log-Rank test Bonferroni controlled: *** = p < 0.001, a: difference between treated and untreated Gal4-controls, b: difference between treated and untreated UAS-controls, c: difference between treated and untreated experimental group; **C**, Heat shock-induced stress resistance was reduced in CNS PECR knockdown flies, mean ± SEM: w^{1118};*appl-Gal4*: 171.1 ± 22.9 min, w^{1118};$PECR^{HMS00753RNAi}$: 192.5 ± 16.7 min, *appl*;$PECR^{HMS00753RNAi}$: 83.5 ± 11.2 min, ANOVA F: 9.93, df: 38, p-value: 0.0004, N = 13. **D**, Acamprosate treatment did not delay the time point where 50 % of flies were sedated in *appl*>$PECR^{HMS00753RNAi}$ flies, mean ± SEM: w^{1118};*appl*-Gal4: 30.3 ± 3.0 min, + AC: 41.8 ± 4.8 min; w^{1118}; $PECR^{HMS00753RNAi}$: 41.8 ± 6.6 min, +AC: 62.1 ± 4.8 min; *appl*;$PECR^{HMS00753RNAi}$: 17.5 ± 4.0 min, +AC: 23.1 ± 4.1 min; ANOVA F: 10.65, df: 72, p-value: 0.00, post-hoc TukeyHSD: $Gal4^-/Gal4^+$: 0.052, UAS^-/UAS^+: 0.046, Exp^-/Exp^+: 0.082, $Gal4^-/Exp^-$: 0.092, UAS^-/Exp^-: 0.00; N = 12-13, ANOVA Tukey HSD * = p < 0.05; ** = p < 0.01, *** = p < 0.001

Like in the previous stress-related assays, a downregulation of PECR in the CNS significantly reduced the survival compared to controls (Fig. 22, A). This was quantified by inspecting the time when 50 % of the flies submitted to heat shock were sedated (Fig. 22, C). Treatment with Acamprosate increased the survival probability of both controls, however there was no significant difference between the treated and untreated experimental group. Therefore, Acamprosate can improve the resistance against heat shock induced stress only with high PECR abundance in the CNS.

3.8 Acamprosate Reduces Oxidative Stress

The previous results from behavioural analyses on stress resistance in Acamprosate-treated flies indicated that Acamprosate might contribute to the reduction of oxidative stress. To analyse whether Acamprosate increased the resistance to multiple stressors, a more direct approach was conducted. Two cellular ROS-sensors, designed by Albrecht and colleagues were used to investigate the concentration of ROS in neurons. The sensor consists of a redox-sensitive GFP (roGFP), which has an engineered dithiol/disulfide switch on the surface that is associated with redox-dependent ratiometric fluorescent changes. The roGFP is directly fused with glutaredoxin which facilitates the measurement of the glutathione redox potential representing the major protection system against oxidants. The signal from the ROS-sensor is directly proportional to the amount of oxidative stress in its vicinity (Albrecht et al., 2011). Higher oxidative stress therefore results in a stronger fluorescence. Flies expressing the cytoplasmic *UAS-cyto-Grx1-roGFP2* (ROS^{cyto}) or the mitochondrial matrix-targeting ROS-sensor *UAS-mito-roGFP2-*

Grx1 (ROS^{mito}) in neurons targeted by *PDF*-Gal4 were treated with Acamprosate for seven consecutive days.

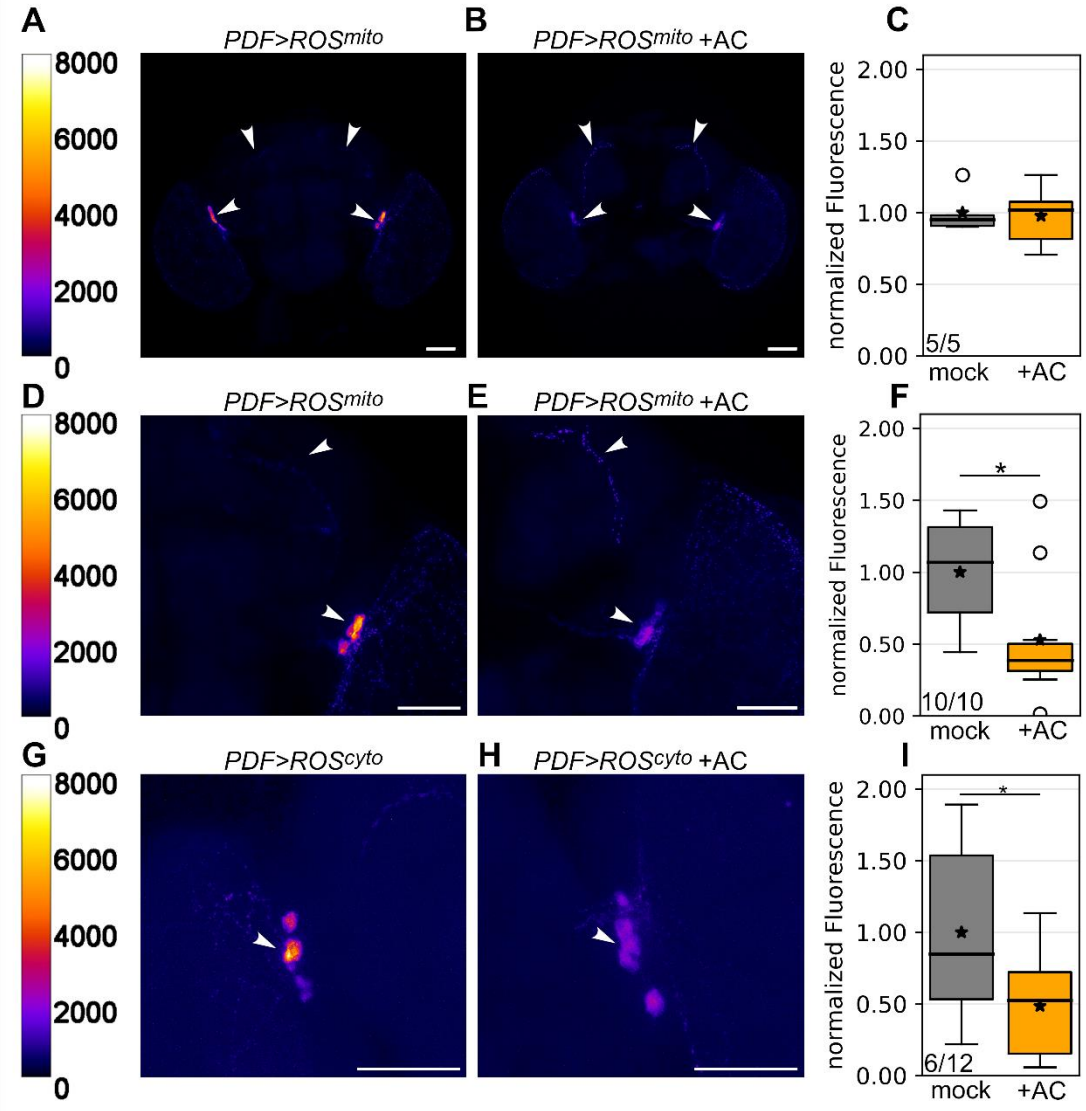

Figure 23: ROS is reduced in neuron somata through Acamprosate treatment.
A,**D**, ROS sensor at mitochondria strongly visible in PDF neuron somata. **B**,**E**, In treated flies, the fluorescence of the mitochondrial ROS sensor was reduced in the soma but increased in the axon. **C**, Fluorescence of the whole brain is not different in treated and untreated flies, Students-t-test F: 0.2, df: 8, p-value: 0.839, N = 5/5. **F**, Fluorescence of ROS^{mito} sensor in the soma was significantly decreased in treated flies, Students-t-test F: 2.62, df: 18, p-value: 0.0172, N = 10/10. **G**, Cytoplasmic ROS sensor expressed in PDF neurons; **H**, Flies treated with Acamprosate showed reduced intensity of fluorescence via cytoplasmic ROS sensor; **I**, Fluorescence signal on ROS^{cyto} sensor was reduced in flies treated with Acamprosate, Students-t-test F: 2.15, df: 16, p-value: 0.0467, N = 6/12. Arrows point to axonal branches and somatas of the PDF neurons, scale bar represents 50 µm. Comparison done via Students-t-test * = $p < 0.05$.

The dissection of the brain was conducted in parallel, one day prior to the imaging. Subsequently, confocal images were taken. Each with the same settings for the comparability of the intensity of the fluorescence. ROS^{mito}-Sensor signal was found in brains of the control group and the experimental group. Although the overall ROS^{mito} fluorescence showed no difference between control and treatment group, the distribution of the fluorescence seemed to be different (Fig. 23, A-C). When focussed only on the soma of the PDF neurons, the flies treated with Acamprosate showed significantly less ROS^{mito} fluorescence than their controls (Fig. 23, D-F). Additionally, flies expressing ROS^{cyto} treated with Acamprosate had a decreased fluorescence in the soma of PDF neurons compared to their controls, indicating less ROS (Fig. 23, G-I). In summary, these results indicate that Acamprosate treatment reduces oxidative stress in the soma of the neuron.

3.9 Acamprosate Treatment Upregulates PECR Expression

All previous experiments implied that Acamprosate function in relation to EtOH-, starvation- or heat-induced stress requires PECR in the CNS. To see whether there is a direct connection between Acamprosate treatment and PECR expression, a quantitative real-time PCR (qPCR) was conducted. Flies were treated with Acamprosate or a mock solution for seven consecutive days before the heads of the flies were removed for RNA isolation. cDNA was generated from RNA isolated out of fly heads to ascertain the involvement of Acamprosate in the transcription of CNS PECR. Primer efficiencies for the housekeeping gene (HKG) *tubulin* and the gene of interest (GOI) *pecr* were determined. Furthermore, the experiment was done with $PECR^{KG07864}$ mutants as negative controls which showed almost no *pecr* transcript, indicating that the chosen primers successfully recognized *pecr* (Fig. 24). qPCR results showed an increase in *pecr* RNA abundance of 176 % in w^{1118} flies after treatment with Acamprosate, further indicating that Acamprosate relies on PECR expression and its function in stress resistance.

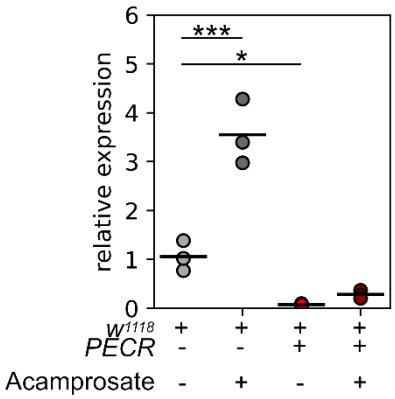

Figure 24: Relative *pecr* expression was increased after Acamprosate treatment.
Acamprosate treatment increased *pecr* expression relative to HKG *tubulin* in w^{1118} controls but not in $PECR^{KG07864}$ mutants, 3 independent cDNA extractions per group; experiment repeated 3 times, data shown as all datapoints and mean; mean ± SEM: w^{1118}: 1.06 ± 0.18, +AC: 3.55 ± 0.38, PECR: 0.08 ± 0.01, +AC: 0.28 ± 0.05. ANOVA F: 55.48, df: 11, p-value: 0.00, post-hoc Tukey HSD: w^{1118}/w^{1118} +AC: 0.001, w^{1118}/$PECR^{KG07864}$: 0.04, $PECR^{KG07864}$/ $PECR^{KG07864}$+AC: 0.89; * represents ANOVA with post-Hoc TukeyHSD $p<0.05$, *** = $p < 0.001$.

4. Discussion

4.1 PECR Increases Resistance Against Oxidative Stress

4.1.1 Neuronal PECR Induces Resistance Against EtOH-Induced Oxidative Stress

Acute EtOH exposure increases oxidative stress in the brain, as the EtOH metabolism produces NADH which enhances the activity of the respiratory chain at the mitochondria. This increases ROS production which in turn induces degradation of essential complex molecules like lipids, proteins and DNA (Hernández et al., 2016). Oxidized fatty acids lead to a decrease in neural viability and negatively affect mitochondrial function, decreasing energy production (Hernández et al., 2016). Lipid levels are elevated in the CNS and lipid dysregulation can lead to synaptic dysfunction while blocked lipid synthesis results in neuronal death (Ruiz-Pérez et al., 2021; Vallés and Barrantes, 2022).

Studies in the model organism *Drosophila* have demonstrated that the molecular targets of oxidative stress in form of H_2O_2 and of EtOH significantly overlap, resulting in similar phenotypes in development (Logan-Garbisch et al., 2015). Additionally, the study suggests that developmental EtOH exposure increases oxidative stress through abnormal fatty acid metabolism. They showed that mutations disrupting LCFA metabolism confer sensitivity to EtOH-induced oxidative stress (Logan-Garbisch et al., 2015). Developmental exposure to EtOH results in metabolic dysregulations with an increased expression of proteins involved in the fatty acid biogenesis and modification (Hwang et al., 2023).

PECR, an enzyme for fatty acid synthesis (Das et al., 2000), can potentially modify the lipid composition of the cell and consequently alter its capacity to adapt to EtOH and its oxidative effects following an EtOH exposure. In this thesis, $PECR^{KG07864}$ mutants exhibited a reduced sensitivity to the sedating effects of EtOH and a significantly diminished tolerance to EtOH, with a 47 % smaller increase in EtOH resistance compared to controls (Fig. 3). PECR is expressed in the CNS of *Drosophila* demonstrated via an RNAi-mediated knockdown in neurons which decreased PECR transcript levels in the head of the fly (Velo Escarcena et al., 2021). PECR abundance within the CNS of *Drosophila* is essential for normal progression of tolerance development to the sedating effects of EtOH, but it is not involved in the initial sensitivity to EtOH (Fig. 9).

PECR transcript levels in the heads of flies have been demonstrated to be reduced after a single exposure to EtOH (Velo Escarcena et al., 2021). Therefore, the concentration of

PECR in the CNS might be crucial for the development of a functional tolerance to EtOH. Flies mainly gain tolerance due to neural and genetic adaptations rather than increased metabolic breakdown of ethanol (Scholz et al., 2000). Therefore, it is improbable that PECR is directly implicated in the regulation of the EtOH metabolism and thus affects pharmacokinetic tolerance. In this thesis, the EtOH concentration in the *PECR*^{KG07864} mutants after exposure to EtOH vapour remains comparable to controls (Fig. 4). A decrease in PECR expression was measured four hours after the exposure (Velo Escarcena et al., 2021), and there were no differences in the degradation speed of EtOH between wild type and mutant flies during four hours after the exposure, indicating that there are no changes in the EtOH metabolism and indicating that the reduction in rapid tolerance is not due to metabolism deficiencies (Fig. 4).

Pharmacodynamic tolerance to EtOH, for example via neuronal changes in the lipid composition of membranes, can serve to protect against EtOH-induced damage (Barceló-Coblijn et al., 2013; Saito et al., 2007). The role of PECR in the synthesis and elongation of fatty acids is crucial for the synthesis of membrane lipids, such as sphingo- and phospholipids (Honda et al., 2023; Wakashima et al., 2014), indicating a role for tolerance-inducing changes in the membrane fluidity. Despite the absence of alterations in protein or TAG concentration in either fed or starved *PECR*^{KG07864} mutants (Fig. 6, B,C), a deficiency in those metabolites can be implied through the analysis of food intake preferences (Fig. 5, B-D). Additionally, the measurements might be imprecise due to the use of the whole thorax of the fly instead of just the RNAi-targeted tissue. Results obtained with a colorimetric assay should be validated with thin layer chromatography, which allows for fractionation and detection of fatty acids, diacylglycerol and TAG (Tennessen et al., 2014). Also, several different enzymes are involved in TAG synthesis and transformations between the metabolites, for example by breakdown of TAG into fatty acids and glycerol, which can then enter the glucose metabolism, might even out moderate deficiencies (Heier and Kühnlein, 2018). Regardless, further studies on the metabolite composition of *PECR*^{KG07864} mutants, especially regarding cholesterol and ethanolamines, should be done. The concentration of cholesterol in neuronal membranes can be increased by chronic ingestion of EtOH, consequently resulting in an increased rigidity of the membrane and thereby its resistance against EtOH-induced damages (Barceló-Coblijn et al., 2013). More rapid changes are applicable to the fatty acid amines N-acylethanolamine (NAE)

and N-acylphosphatidylethanolamine (NAPE). Exposure to EtOH results in an increase in the amount of NAE and NAPE in the cell, resulting in neuroprotection. One NAE, palmitoylethanolamine, has been demonstrated to enhance neuronal protection against oxidative stress, thereby impeding apoptosis (Duncan et al., 2009; Garg et al., 2010; Lombardi et al., 2007). Additionally, an increase in NAPE, TAG, cholesterol ester and ceramide has been observed in the brain shortly after exposure to EtOH (Saito et al., 2007). NAPE is involved in neuroinflammatory processes and the oxidative stress response (Hansen et al., 2001). Hence, influence in the synthesis of fatty acids can increase neuroprotection. In conclusion, there are multiple mechanisms by which PECR, due to its function in fatty acid synthesis, can influence the functional tolerance to the sedative effects of EtOH or induce resistance against EtOH-induced oxidative stress.

4.1.2 PECR Improves Resistance Against Environment-Induced Stress

In this thesis, flies with a low abundance of PECR, due to either mutation or RNAi-mediated knockdown in certain tissues, demonstrated a lower resistance against environmental stressors, such as starvation and prolonged exposure to heat (Fig. 3,9,17,19,21,22). The ability of an organism to resist starvation is dependent on its capacity to withstand oxidative stress, as well as the internal energy stores (Rion and Kawecki, 2007). Prolonged starvation induces oxidative stress and lipid peroxidation whilst additionally activating antioxidant defences (Morales et al., 2004; Pascual et al., 2003). To counteract starvation, the organism requires an elevated respiration rate to satisfy its energy demands, which generates ROS (Yu et al., 2006). Further studies demonstrated that hyperactivity and sleep deprivation caused by starvation increased ROS in the *Drosophila* brain and gut (Kempf et al., 2019; Vaccaro et al., 2020). Consistently, in this thesis, flies with elevated ROS concentrations in the CNS due to an RNAi-mediated knockdown of SOD1 or SOD2 in neurons exhibited a reduced ability to withstand starvation (Fig. 16). Therefore, it can be hypothesised, that the elevated ROS level within the CNS, attributable to both starvation and diminished SOD levels, was apparent through the decreased starvation resistance. These findings suggest that, provided internal energy stores and developmental factors are comparable across compared groups, starvation resistance can serve as an indicator of oxidative stress

resistance. In rats, starvation increases the transcript levels of PECR, suggesting a potential role in starvation resistance (Zhang and Underwood, 1999). The reduced starvation resistance of *PECR*^{KG07864} mutants can be ascribed to metabolic defects in the mutant, evidenced by their increased food intake following a starvation period and their diminished glycogen levels in a sated state (Fig. 5, Fig. 6, B). Flies with a deficiency in glycogen production are starvation sensitive, as this nutrient becomes essential under nutritional stress (Rion and Kawecki, 2007). In the adult fly glycogen is abundant in the FB as well as in the flight muscles, body wall muscles and halteres (Wigglesworth, 1949). In this thesis, flies with reduced PECR abundance in the FB showed no alteration in their starvation resistance (Fig. 19, A). Further, downregulation of PECR in the muscles reduced the starvation resistance (Fig. 19, B), which aligns with research demonstrating that inhibition of fatty acid synthesis in muscle tissues inhibits life span extension during dietary restriction (Katewa et al., 2012). However, in both cases changes in the glycogen levels of those flies could not be detected (Fig. 6). This might be due to inaccuracies of the assay since the thorax and abdomen of the flies naturally contribute additional glycogen sources. Possibly, the changes in glycogen levels can only be observed in starved flies, because tissue-specific downregulation of PECR might be sufficiently compensated through feeding. To decrease the amount of glycogen in the fly significantly, a whole-body mutant is necessary as shown in Figure 6. The other main energy store, TAG, was comparable to controls in concentration of both *PECR*^{KG07864} mutants and flies with a knockdown of PECR in the FB and muscles. Together, the results indicate that the reduced starvation resistance displayed by *PECR*^{KG07864} mutants is independent of the possible defects in the lipid metabolism of the fly. Additionally, PECR knockdown in the CNS of the flies resulted in a severely decreased starvation resistance (Fig. 19, C). Given the capacity of these flies to maintain their metabolic rate due to the abundance of PECR in other tissues, these findings suggest an additional role for PECR in the CNS, most likely in the regulation of the resistance to starvation-induced oxidative stress.

A further method of inducing environmental stress is through the prolonged exposure of an organism to elevated temperatures. The heat shock response cascade is comprised of several heat shock proteins (HSPs), which act as chaperones. These proteins are responsible for the prevention of non-native protein aggregation during the processes of protein folding and refolding. Furthermore, they have been shown to facilitate the

proteolysis of damaged proteins, thereby reducing cell damage (Wickner et al., 1999). HSPs have been found to be associated with not only heat shock but also various cellular stress response pathways, including apoptosis, immune response and multidrug resistance (Barna et al., 2018). In the context of the fruit fly, studies have demonstrated that neuronal expression of HSPs confers benefits, including enhanced lifespan and increased resistance to oxidative stress (Liao et al., 2008). The expression rate of HSP70 is associated with lifespan expansion as its expression rate decreases with age, concurrently with the decline in survival rates (Sørensen and Loeschcke, 2002). In the present thesis, it is demonstrated that heat shock-induced stress resistance is contingent on PECR expression in the CNS of flies (Fig. 22). It is evident that the results of this study are unable to demonstrate a link between PECR and a reduction of heat shock- induced oxidative stress, since the heat shock response has been shown to reduce not only oxidative stress but also affects other cellular stress pathways. To further analyse the involvement of PECR in the heat shock response, HSP expression in the *PECR*^{KG07864} should be analysed. However, it can be deduced from the data that PECR, given its already documented role in enhancing resistance to oxidative stress induced by EtOH and starvation, may play a part in the general cell answer to oxidative stress. This hypothesis is supported by the observation that *PECR*^{KG07864} mutants exhibit a reduced lifespan (Fig. 15), which can also be attributed to a diminished resistance to oxidative stress. In summary, it can be concluded that PECR is likely to be involved in the reduction of oxidative stress or the induction of the cellular oxidative stress response.

4.1.3 Potential Mechanisms Linking PECR to Oxidative Stress Resistance

Contrary to the obtained results, PECR was expected to induce oxidative stress since it reduces the antioxidant cofactor NADPH which increases the redox potential (Das et al., 2000). The enzymatic activity of PECR should increase the number of oxidative molecules, therefore a loss of PECR was thought to increase the resistance against oxidative stress due to a higher abundance of reducing agents. Additionally, the redox enzyme catalase, which is also present in the peroxisome, is positively impacted by bound NADPH, but with PECR converting NADPH to $NADP^+$ in close proximity, catalase would be expected to have lower capacities for its antioxidant function (Cattani and Ferri, 1994).

Therefore it was surprising that a low PECR abundance decreased the resistance against oxidative stress, especially because a study demonstrated that reduced PECR levels in subsets of neurons increased the resistance to elevated oxidative stress elicited by H_2O_2 (Velo Escarcena et al., 2021). Similar to the results in this thesis, *PECR*^{KG07864} mutant flies have been shown to have a reduced ability to handle bacterial infection, which is a form of environmental stress (Von Ohlen et al., 2012). Oxidative stress can induce the carbonylation of PECR, which impairs its function (Méndez et al., 2014). Therefore, PECR may be directly affected by ROS and might act differently with increased ROS levels.

Since PECR is responsible for fatty acid synthesis in which educts and products are acyl-CoAs of various lengths, a further investigation into the effects of acyl-CoA on oxidative stress might be fruitful (Das et al., 2000; Faust et al., 2012). Acyl-CoA can elicit damage to the cells which is similar to ROS-mediated cell damage and especially the longer chained acyl-CoAs are very potent (Trub and Hirschey, 2018). Harmful derivatives of acyl-CoA drive inflammation and even apoptosis. When acyl-CoA is used for β-oxidation an incomplete process can be harmful for the cell, since partially oxidized fatty acid intermediates can accumulate. Those can disrupt membrane integrity, trigger stress and apoptosis pathways and contribute to lipotoxic stress (Schulz, 1991; Yadav et al., 2019). High levels of acyl-CoA can induce ROS through the electron transport chain at the mitochondria and drive the inhibition of key metabolic enzymes like the pyruvate dehydrogenase complex (Szrok-Jurga et al., 2023). However, the increased ROS production due to β-oxidation is anticipated by the cell and directly combatted via redox enzymes in close proximity at the mitochondria and the peroxisomes. Furthermore, the resulting energy from β-oxidation is crucial for the organism, especially in a nutrient deprived state (Cabodevilla et al., 2013). Additionally, Acyl-CoA is critical for the biosynthesis of phospho- and sphingolipids which are both important for membrane integrity and the protection against lipid peroxidation (Szrok-Jurga et al., 2023). Some derivates of acyl-CoAs can also regulate peroxisome-proliferator activated receptors (PPARS) which act as transcription factors and can control expression of genes involved in oxidative stress response and lipid metabolism (Elholm et al., 2001; Murakami et al., 2001). Therefore, the balance of the amounts of different medium- to long-chained acyl-CoAs in the cell may be crucial for the expression rate of PECR. In summary, PECR can act on multiple pathways through its function in fatty acid synthesis and might even

influence ROS-induced inflammation. The protective effects of PECR were analysed in recent research in human medicine. PECR acts protectively in cases of liver cancer and overexpression of PECR inhibits proliferation, migration and invasion of hepatocellular carcinoma while promoting apoptosis (Luo et al., 2023a; Qiu et al., 2022). Further, it was demonstrated that low levels of PECR promote liver cancer growth (Luo et al., 2023a). High abundance of PECR in a kidney transplant patient was correlated to an increased auto-immune response to the transplant and a higher risk of rejection (Dinavahi et al., 2011). Further, PECR was associated with the protection of development of sepsis-induced lung injury (Song et al., 2024). Therefore, PECR levels were discussed as a biomarker for inflammation responses. These findings further indicate a connection between PECR and cell responses to stressors such as ROS, toxic reagents or cancer. However, it is still unclear whether an increased immune response elevated PECR expression or an elevated PECR expression induces a stronger immune response.

4.2 Acamprosate Acts Through PECR to Mitigate Oxidative Stress

4.2.1 Acamprosate is a Negative Regulator of Oxidative Stress

In this thesis, Acamprosate treatment in wild type flies did not affect the initial sensitivity to the sedative effects of EtOH but increased the acquired tolerance to EtOH (Fig. 11). These results complement previous findings that Acamprosate induces mechanisms which are involved in modulating the capacity of functional tolerance development (Cole et al., 2000; De Witte et al., 2005). Functional tolerance is gained through neuroadaptive processes (Tabakoff et al., 1986). In an acute EtOH exposure, the excitability of the CNS is inhibited through the exciting effects of EtOH on GABA receptors and its inhibitory effect on glutamate signalling. In AUD patients, due to the chronic exposure to EtOH, neuroadaptations counteract the inhibition induced by EtOH to regain the balance in the CNS (De Witte et al., 2005). These neuroadaptations result in behavioural changes as a preparation for future exposures, similar to EtOH-cue learning. Administration of Acamprosate was demonstrated to reduce changes in behaviour evoked by EtOH exposure implying an effect on synapse remodelling (Cole et al., 2000). These results also hint towards an additional effect of Acamprosate besides its efficacy in acute withdrawal

situations. The balance of excitatory and inhibitory neurotransmitters in the CNS gained via neuroadaptive changes is lost in the withdrawal phase, since the EtOH-driven inhibition stops while the compensatory neuronal changes still induce excitation (De Witte et al., 2005). EtOH withdrawal is very strenuous for the organism. A study with alcohol-dependent rats undergoing withdrawal phases several times demonstrated that after four repeats of withdrawal and re-consumption phases, 80 % of the rats were dead (Dahchour et al., 1998). When treated with Acamprosate before the experiment, the behavioural manifestations of withdrawal were prevented and the mortality of abstinence was significantly reduced, allowing 90 % of the rats to survive the fourth cycle (Dahchour and De Witte, 1999). Withdrawal after chronic ethanol exposure induces excitotoxicity in cells which results in increased cell death (Prendergast et al., 2000). Treatment with Acamprosate reduces cell death during the withdrawal phase, implying neuroprotective effects of Acamprosate (al Qatari et al., 2001). In this thesis, wild type flies treated with Acamprosate were able to significantly increase their resistance against EtOH-, starvation-, or heat-induced stress (Fig. 11, 18, 21). These findings suggest that Acamprosate is capable of reducing EtOH-induced oxidative stress while it simultaneously facilitates EtOH abstinence by reducing preference for EtOH in *Drosophila* (Velo Escarcena et al., 2021). Since Acamprosate is a homotaurine it might mimic taurine and attenuate the influences of EtOH on the antioxidant system in liver and kidney in mammals (Goc et al., 2019), yet most of the literature is consistent on the fact that Acamprosate primarily acts in the CNS on the glutamatergic and GABAergic system (Durbin et al., 1996; Naassila et al., 1998). In the CNS, EtOH exposure increases ROS while treatment with Acamprosate during EtOH intoxication prevents the ROS increase (Dahchour et al., 2005). Using two different ROS-sensors, this thesis also demonstrated a ROS-reducing effect of Acamprosate in neurons (Fig. 23). Similar to those results, other studies demonstrated that Acamprosate has antioxidant effects in the CNS unrelated to EtOH. For example, the rat model for incomplete cerebral ischemia and reperfusion had significantly lower neurological deficits when treated with Acamprosate, indicating its role in neuroprotection (Engelhard et al., 2000). Furthermore, combined with Ribavirin, an antiviral medication, Acamprosate treatment positively modulated neuropathic pain through induced regenerative mechanisms in sensory axons (Romeo-Guitart and Casas, 2020). However, changes in the antioxidant defence system were demonstrated to be

dependent on the affected organism, as Acamprosate treatment enhanced oxidative stress only in non-alcoholic rats (Ewertowska et al., 2015). Additionally, Acamprosate treatment is dosage dependent. Research indicates that Acamprosate can improve clinical outcomes in young individuals with Fragile X Syndrome (FXS), an autism spectrum disorder caused by inactivation of the conserved FMR1 gene, which causes overgrowth of neuronal processes and hyperactive synaptic signalling (Santoro et al., 2012; Zhang et al., 2009). FXS is linked to elevated levels of ROS, increased lipid and protein oxidation and impaired antioxidant systems, which suggest a chronic state of oxidative stress in the brain and plasma of affected individuals (Bekay et al., 2007; Diego-Otero et al., 2009). The *Drosophila* model of FXS, *dfmr1* null mutant larvae exhibit defects in locomotion and overgrown NMJs with increased synaptic bouton formation and neuronal branching (Xu et al., 2004; Zhang et al., 2001). FXS larvae treated with 10 mM Acamprosate showed a significant rescue of travel distance while this concentration did not change control behaviour. Treatment with 100 mM reduced travel distance in control larvae significantly, while it tended to increase the travel distance in mutant larvae, although not significantly (Hutson et al., 2018). Thus, the effect of Acamprosates on the behaviour of the organism depends on the genetic characteristics of the organism and the administered dose of Acamprosate. In the mice model for multiple sclerosis, different concentrations of Acamprosate delayed the emergence of neurological deficits and reduced inflammation (Sternberg et al., 2012). Interestingly, Acamprosate treatment also reduced the weight loss correlated with the stress of the injections (Sternberg et al., 2012). To conclude, the results of this thesis concerning an antioxidant effect of Acamprosate are in line with the neuroprotective effects demonstrated through Acamprosate treatment in animal models of diseases unrelated to EtOH. Hence, Acamprosate might have a broad effect and its efficacy in EtOH withdrawal and tolerance could be a positive side effect of its neuroprotective characteristics.

4.2.2 Acamprosate Efficacy Depends on Neuronal PECR

Acamprosate treatment was able to increase the resistance against oxidative stress deriving from heat, nutritional deficiency or EtOH exposure only in flies with wild type levels of PECR in the CNS (Fig. 11, 12, 13, 18, 19, 22). Surprisingly, flies with low abundance of PECR in the CNS treated with Acamprosate exhibited an even lower

starvation resistance than untreated flies, which might be due to the fact that Acamprosate can have an enhancing effect on oxidative stress and induce neurotoxicity (Ewertowska et al., 2015; Wu et al., 2001). Still, in all other cases Acamprosate was able to increase the resistance to the stressor, as long as PECR was abundant in the CNS. In a further investigation, two specific subsets of neurons demonstrated a dependency on PECR for starvation resistance (Fig. 20). Treatment with Acamprosate was not able to increase the starvation resistance as it was shown to do in controls when PECR was reduced in these subsets of neurons. Thus, they are likely the site of action of Acamprosate. One of the two subsets was the GABAergic subset targeted by the *Gad*-Gal4 driver. GABA receptors have been shown to be inhibited in response to Acamprosate administration (Berton et al., 1998). Considering the results of this thesis Acamprosate might block GABA receptors to disable the neurotoxic effects of EtOH. Depending on the EtOH concentration, frequency and amplitude of the miniature inhibitory postsynaptic current of GABA are modulated (Sebe et al., 2003). Moreover, depending on the subset of neurons EtOH exposure can potentiate the $GABA_A$ receptor-induced chloride current into the neuron (Aguayo, 1990). Although the activation of $GABA_A$ receptors blocks the development of tolerance, the use of a negative allosteric modulators of $GABA_A$ receptors significantly increased the development of rapid tolerance to EtOH (Barbosa and Morato, 2001). Furthermore, the activation of $GABA_B$ receptors through EtOH or a $GABA_B$ agonist decreases locomotor activity in the fly and was shown to be needed to elicit rapid tolerance (Dzitoyeva et al., 2003). Thus, the binding of Acamprosate on GABA receptors can prevent the neuronal changes elicited by EtOH binding to the receptors and thereby influence the development of tolerance to its inhibitory influence on the CNS. In line with this hypothesis, Acamprosate treatment rescued the changes in the excitatory and inhibitory neurotransmitter balance in patients with neuronal tinnitus, which develops after an overexposure to sound changes the GABAergic terminal density (Sharma et al., 2012; Zhang et al., 2021). Neuronal tinnitus is elicited by similar changes to the neuronal system as those included by chronic EtOH intoxication and its rescue with Acamprosate further proves the efficacy of Acamprosate on the excitability of the glutamatergic and GABAergic neurotransmitter systems. However, the effect of Acamprosate on the tolerance development of flies with reduced PECR in GABAergic neurons should be analysed to see whether the GABAergic system is also responsible for functional tolerance to EtOH.

The second subset of neurons on which Acamprosate exerts its function are the EAAT2 expressing cells (Fig. 20). In *Drosophila*, *d*EAAT2 is the only identified taurine transporter (Besson et al., 2005). Taurine exhibits various functions in the CNS, most of which are related to neuroprotection, including antioxidant action, reducing glutamate excitotoxicity through GABA receptor-independent regulation of intracellular calcium homeostasis, activation of $GABA_A$ receptor-induced neurotransmission and inhibiting inflammatory mediators (El Idrissi, 2008; Jong et al., 2012; Paula-Lima et al., 2005; Wu et al., 2009). Further, taurine can decrease alcohol-induced inflammation in liver cells through decreasing NF-κB signalling (Lin et al., 2015). NF-κB is a transcription factor which influences gene expression for cell proliferation and apoptosis (Zinatizadeh et al., 2021). Acamprosate can work similarly to taurine and is very potent in antagonistic binding to taurine receptors (Wu et al., 2001). The expression of the taurine transporter EAAT2 is regulated through the NF-κB transcription factor. Acamprosate can decrease the NF-κB signalling, similar to taurine, fitting to the reduced EAAT2 expression after Acamprosate treatment (Germany et al., 2018). Thus, treatment with Acamprosate can reduce the immune response elicited by NF-κB to prevent neuronal death. In the mouse model of alcoholism, $ENT^{-/-}$ mice show a hyperglutamatergic state which is stabilized by Acamprosate treatment while RTN4 and ERK signalling are increased to induce a stronger immune response (Germany et al., 2018). However, other studies showed that Acamprosate treatment reduces inflammatory cytokines (Abbasi et al., 2023). In conclusion, Acamprosate affects GABAergic, glutamatergic and taurine signalling, possibly balancing chronic EtOH-induced synaptic changes and modulating cell immune responses. Additionally, Acamprosate has a variety of targets in the CNS and induces different responses in different situations. Since the effects of Acamprosate in functional tolerance or immediate resistance against stressors such as nutrient deficiency or excess heat were not visible in this thesis without PECR abundance in the CNS, the search for the intracellular mechanisms of Acamprosate continues. EtOH-preferring rats treated with Acamprosate showed changes in their lipid metabolism. Acamprosate influenced the triglyceride metabolism by modulating the balance between hydrolysis and transacylation of long chain fatty acids (Piorunska-Mikolajczak, et al., 2004). Further, certain metabolite concentrations were hypothesized to be important in the outcomes of relapse therapy in patients, since lower ethanolamine concentrations during Acamprosate treatment had a

negative influence on relapse (Ho et al., 2022). Thus, there might be a direct connection between the fatty acid synthesising protein PECR and Acamprosate.

Another way to connect Acamprosate treatment to PECR is through the ability of Acamprosates to influence gene expression. In this thesis, Acamprosate-treated flies had significantly enhanced levels of PECR compared to their untreated controls. Studies showed that Acamprosate treatment induces or reduces the expression of multiple genes (Germany et al., 2018; Ho et al., 2022; Hutson et al., 2018), supporting the observation of Acamprosate-induced increase in the PECR expression. Acamprosate induces PECR expression while EtOH exposure reduces PECR expression (Velo Escarcena et al., 2021). Recent studies proved that there are several genes with an “inverted” expression, similar to PECR, demonstrating that EtOH reduces the expression of certain genes while Acamprosate increases it (Ho et al., 2022). However, there are examples, like genes modulating plasma serotonin levels, where EtOH and Acamprosate both reduce the genes expression rates (Ho et al., 2021). Additionally, Acamprosate treatment showed different results on gene expression depending on the internal state of the animal. For example, treatment of the alcohol model mice $ENT^{-/-}$ reversed the changes of protein expression induced by the mutation and it induced protein levels similar to the mutant in wild type mice (Germany et al., 2018). Therefore, the obtained results of Acamprosate increasing PECR in wild type flies should be investigated via a repetition of the qPCR in alcoholic flies. The protein expression rates altered by treatment with Acamprosate were multifunctional, since they are involved in synaptic plasticity and immune response (Germany et al., 2018). One of the affected proteins is ERK as it was demonstrated that Acamprosate treatment reduces its levels in rodents (Abbasi et al., 2023; Germany et al., 2018). ERK is involved in several signalling cascades, neuronal plasticity and inflammatory responses (Tekari et al., 2022; Wang et al., 2021). In *Drosophila*, PECR interacts with CG6180, the orthologue to the human phosphatidylethanolamine binding protein 1 (PEBP1) (Guruharsha et al., 2011). This protein is predicted to be involved in the negative regulation of the ERK cascade, as well as the NF-κB signalling pathway (Yeung et al., 2001; Zuo et al., 2015). This link leads to the speculation that Acamprosate treatment through PECR might affect an immune response cascade. However, immune response pathways have several targets and can influence the cells health positively and

negatively (Morris et al., 2022). Therefore, further investigations into the connection between Acamprosate, PECR and their neuroprotective functions need to be made.

4.3 Conclusion

The present thesis has established novel correlations between a candidate gene involved in the genetic predispositions of AUD and the effectiveness of Acamprosate treatment which may have ramifications for the future prescription of Acamprosate to patients diagnosed with AUD.

In this study, I demonstrated that in the CNS of *Drosophila*, PECR is required to increase the development of rapid functional tolerance to the sedative effects of EtOH. It was proposed here that altered lipid composition in neuronal membrane of $PECR^{KG07864}$ mutants might limit their ability to adapt to the neurotoxic effects of EtOH. Nevertheless, the findings in this study demonstrate that PECR improves the resistance to oxidative stress caused by heat, starvation, and EtOH. Exposure to EtOH leads to an increase in ROS within the organism which can result in brain damage (Hernández et al., 2016). Therefore, PECR might induce its tolerance-facilitating function through its effect on ROS. The precise mechanisms by which this occurs require further investigation; however, it is established that ROS can directly affect PECR, as it induces PECR carbonylation (Méndez et al., 2014), and thus may start a ROS-reducing cell response. This agrees with the fact that PECR is a peroxisomal enzyme and the peroxisome is the main compartment of the cell in which the ROS response takes place (Lodhi and Semenkovich, 2014).

Concurrently, I determined that Acamprosate treatment can facilitate the development of tolerance to EtOH, contingent on PECR abundance in the CNS. It is imperative that further investigations be conducted in the cellular pathways connecting Acamprosate with PECR. As indicated by the findings of multiple preceding studies, there is a possibility that Acamprosate could play part in neuroprotective processes, with the effects potentially differing according to the condition of the organism in question (Germany et al., 2018; Ho et al., 2022). Through the investigations carried out in this thesis, the antioxidant effects of Acamprosate have been confirmed, and its role, especially in the GABAergic and taurinergic system, has been elucidated.

Future investigations should focus on the causal relationship between immune response and PECR expression (Dinavahi et al., 2011; Luo et al., 2023b; Song et al., 2024). It is not yet known whether PECR induces an immune response, and if so, how, or whether PECR expression is increased due to the immune response.

In conclusion, I demonstrated that Acamprosate is a medication with potential use in a variety of settings due to its neuroprotective effects and the capacity to reduce ROS in the CNS. Acamprosate treatment was only effective in cases where PECR expression was at wild type levels, while Acamprosate treatment itself was found to upregulate the PECR expression rate. This suggests that the effectiveness of Acamprosate treatment depends on PECR expression levels. This connection has the capacity to enhance the efficacy of Acamprosate in treating AUD in human subjects, since an analysis of SNPs in *pecr* or an analysis of PECR levels in a patient might reveal if they are eligible for Acamprosate treatment. In cases where patients are not eligible for Acamprosate treatment due to their genetic profile, alternative therapeutic interventions could be considered.

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6. Abbreviations

AC	Acamprosate	ENT	Equilibrative nucleoside transporter
ADH	Alcohol dehydrogenase	eRING	Ethanol Rapid Iterative Negative Geotaxis
AGS	Amyloglucosidase	ERK	Extracellular signal-regulated kinase
ALDH	Aldehyde dehydrogenase	ERP	Event-related brain potential
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	EtOH	Ethanol
ANOVA	Analysis of variance	FB	Fat body
ATP	Adenosine triphosphate	FXS	Fragile X Syndrome
AUD	Alcohol use disorder	GABA	Gamma-aminobutric acid
Ca	Calcium	Gad1	Glutamate decarbocylase 1
CNS	Central nervous system	GFP	Green fluorescent protein
CYP2P1	Cytochrome P450 dependent enzyme	GOI	Gene of interest
DEPC	Diethyl pyrocarbonate	GWAS	Genome-wide association study
DNA	Deoxyribonucleic acid	HKG	House-keeping gene
dV-glut	Drosophila vesicular glutamate transporter	HSP	Heat shock protein
EAAT2	Excitatory amino acid transporter 2	HPA	Hypothalamic-pituitary-adrenal

MAP kinase	Mitogen-activated protein kinase	RNA	Ribonucleic acid
MB	Mushroom body	RNAi	RNA interference
NAD	Nicotineamide adenine dinucleotide	roGFP	Redox-sensitive GFP
NADP	Nicotinamide adenine dinucleotide phosphate	ROI	Region of interest
NAE	N-acyletanolamine	ROS	Reactive oxygen species
NAPE	N-acylphophatetanolamine	RTN4	Reticulon 4
NEM	n-ethylmaleimide	SNP	Single nucleotide polymorphism
NMDA	N-Methyl-D-aspartic acid	SOD	Superoxide dismutase
PBS	Phoshate-buffered saline	Syn	Synapsin
PDF	Pigment dispersing factor	TAG	Triglyceride
PECR	Peroxisomal trans-2-enoyl-CoA reductase	VLCFA	Very long chain fatty acids
qPCR	Quantitative real-time polymerase chain reaction	ZNS	Zentrales Nervensystem