A tale of two genes controlling behavior in Drosophila: role of DopEcR in ethanol-induced behavior and effects of epilepsy mutations on sleep

Emily Kay Petruccelli

University of Iowa

Copyright 2015 Emily Kay Petruccelli

This dissertation is available at Iowa Research Online: https://ir.uiowa.edu/etd/1998

Recommended Citation

https://doi.org/10.17077/etd.ubhjkg11

Follow this and additional works at: https://ir.uiowa.edu/etd

Part of the Genetics Commons
A TALE OF TWO GENES CONTROLLING BEHAVIOR IN DROSOPHILA:
ROLE OF DOPECR IN ETHANOL-INDUCED BEHAVIOR AND
EFFECTS OF EPILEPSY MUTATIONS ON SLEEP

by

Emily Kay Petruccelli

A thesis submitted in partial fulfillment
of the requirements for the Doctor of Philosophy
degree in Genetics in the
Graduate College of
The University of Iowa

December 2015

Thesis Supervisor:  Associate Professor Toshihiro Kitamoto
This is to certify that the Ph.D. thesis of

Emily Kay Petruccelli

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Genetics at the December 2015 graduation.

Thesis Committee:

Toshihiro Kitamoto, Thesis Supervisor

Donna Hammond

Wayne Johnson

Tina Tootle

Chun-Fang Wu
To my friends, family, and flies
Little Fly
Thy summer's play,
My thoughtless hand
Has brush'd away.

Am not I
A fly like thee?
Or art not thou
A man like me?

For I dance
And drink & sing;
Till some blind hand
Shall brush my wing.

If thought is life
And strength & breath;
And the want
Of thought is death;

Then am I
A happy fly,
If I live,
Or if I die.

William Blake, “The Fly,” 1794
ACKNOWLEDGEMENTS

I am forever grateful for the kindness, guidance, and motivation of my advisor Dr. Toshihiro Kitamoto. Throughout my graduate career, Toshi’s optimistic attitude and charismatic smile has seen me through all of my negative data, experimental errors, and foolish mistakes. He constantly shares my enthusiasm for science and encourages me to pursue any goal. Having such a wonderful mentor makes me both a better scientist and person.

To my thesis committee, I thank you for your patience and wisdom. Each of you has helped me realize my strengths and improve upon my weaknesses. I would also like to acknowledge current and past lab members of the Kitamoto lab, particularly Dr. Junko Kasuya, Dr. Hiroshi Ishimoto, Dr. Garrett Kaas, Hung-Lin Chen, Arianna Lark, Patrick Lansdon, and all of the undergraduate workers. All of you managed to somehow put up with me and make it fun to come to lab every day. Thank you also to the University of Iowa’s Drosophila community and Genetics department for providing an invaluable source of encouragement and advice. I am also very grateful for having received financial support from the University of Iowa’s Pain Research training grant and the National Institute on Alcohol Abuse and Alcoholism predoctoral fellowship.

And last, but by no means least, I am eternally indebted to my friends and family for keeping me sane during the rollercoaster ride that is graduate research.
ABSTRACT

Substance abuse and mental health disorders are a leading source of years lost to disability from medical causes worldwide. Unfortunately, for most neurological disorders it is unclear how underlying genetic predispositions govern behavioral response to environmental stressors. Owing to their convenience, genetic tractability, and small brains, the fruit fly, Drosophila melanogaster, has become an invaluable model in which to dissect the neurological basis of conserved complex behaviors. Here, I characterized the respective roles of two genes in alcohol response and sleep behavior.

Steroid hormones profoundly influence behavioral response to alcohol, yet the role of unconventional non-genomic steroid signaling in this process is unknown. I discovered that Drosophila DopEcR, a G-protein coupled receptor (GPCR) activated by dopamine or the major insect steroid hormone ecdysone, plays a critical role in ethanol-induced behaviors. DopEcR mutants took longer to sedate when exposed to ethanol vapor, and post-eclosion expression of DopEcR-RNAi phenocopied mutant resistance. DopEcR was necessary in particular neuronal subsets, including cholinergic and peptidergic neurons, and promoted ethanol sedation by suppressing epidermal growth factor/extracellular signal-regulated kinase signaling. In adult flies, ecdysone negatively regulated DopEcR-mediated ethanol-induced sedation. We also found that DopEcR inhibits ethanol-induced locomotion, a conserved dopaminergic behavior. Together, these findings provide novel insight into how an unconventional steroid GPCR interacts with multiple downstream signaling cascades to fine tune behavioral response to alcohol.
Despite an established link between epilepsy and sleep behavior, it remains unclear how epileptogenic mutations affect sleep and seizure susceptibility. To address this, I examined the rest/wake behavior of two fly models of epilepsy with \textit{paralytic} voltage-gated sodium channel mutations known to cause human generalized epilepsy with febrile seizures plus (GEFS+) and Dravet syndrome (DS). GEFS+ and DS flies display heat-induced seizure susceptibility, but at normal temperatures I found that both mutants had exaggerated nighttime sleep behavior. GEFS+ sleep was more resistant to pharmacologic and genetic reduction of GABA transmission as compared to control's response. This finding is consistent with augmented GABAergic suppression of wake-promoting pigment-dispersing factor (PDF) neurons in GEFS+ mutants. Contrastingly, DS sleep was almost completely resistant to pharmacologic GABA reduction, suggesting that PDF neurons are incapable of functioning despite disinhibition. The sleep of both GEFS+ and DS flies was largely suppressed, but not eliminated, by scotophase light, highlighting the importance of light stimulus and circadian signals in the manifestation of their phenotypes. Following sleep deprivation, GEFS+ and DS mutants failed show to homeostatic rebound. Sleep loss also unexpectedly reduced the seizure susceptibility of GEFS+ flies. This study revealed the sleep architecture of \textit{Drosophila} voltage-gated sodium channel mutants and provides a unique platform in which to further study the sleep/epilepsy relationship.
PUBLIC ABSTRACT

Genetic mutations are linked to nearly every human disease, including major health problems like alcoholism and epilepsy. But how can changes in our genes and environment affect these brain disorders? Fruit flies are an ideal genetic model in which to answer this question. They have simpler brains than humans, but still show complex behaviors. In this thesis, I used flies to study how specific genes influenced either drunk- or seizure-like behavior. It is still unclear which genes make people more likely to become alcoholics. I discovered that mutations in the *DopEcR* gene cause flies to become resistant to alcohol. Because this gene makes a unique type of protein in the brain, I looked at where, when, and how *DopEcR* works. My results suggest that similar human proteins are likely important in alcohol use disorders (AUDs). Next, I examined flies with human *SCN1A* gene mutations that cause epilepsy. Millions of people suffer from epilepsy, but why their seizures get worse without proper sleep is unknown. I found that epileptic flies not only had seizures, but also had many sleep problems. Also, after sleep loss, mutant flies were unable to recover their sleep and showed changes in seizure responses. These findings help us better understand how mutations that cause epilepsy affect the activity of brain cells in seizures and sleep. Altogether, my work highlights the value of fruit flies in scientific research and adds to our knowledge of how genes affect AUDs and epilepsy.
# TABLE OF CONTENTS

LIST OF FIGURES......................................................................................................................... ix
PREFACE........................................................................................................................................ xi

CHAPTER I: INTRODUCTION TO DROSOPHILA BEHAVIORAL NEUROGENETICS .......................................................... 1
  Evolution of the Field .................................................................................................................. 1
  Fly Behavioral Neurogenetics Today ....................................................................................... 3
  Future Directions ....................................................................................................................... 6

CHAPTER II: DROSOPHILA DOPECR, A UNIQUE GPCR FOR DOPAMINE AND ECDYSONE, MODULATES ETHANOL-INDUCED BEHAVIORS ................................................................. 9
  Introduction .............................................................................................................................. 9
  Materials and Methods ........................................................................................................... 11
  Results ..................................................................................................................................... 14
  Discussion ............................................................................................................................... 29

CHAPTER III: EXAGGERATED NIGHTTIME SLEEP AND DEFECTIVE SLEEP HOMEOSTASIS IN DROSOPHILA KNOCK-IN MODELS OF HUMAN EPILEPSY ......................................................... 35
  Introduction ............................................................................................................................. 35
  Materials and Methods .......................................................................................................... 37
  Results .................................................................................................................................... 40
  Discussion .............................................................................................................................. 55

CHAPTER IV: SUMMARY AND DISCUSSION ............................................................................ 62
  The Dopamine Ecdysone Receptor ....................................................................................... 62
  Epileptogenic Voltage-Gated Sodium Channels ................................................................. 64

REFERENCES .................................................................................................................................. 66
LIST OF FIGURES

Figure 1. \textit{DopEcR} mutants are resistant to ethanol sedation........................................ 15

Figure 2. \textit{DopEcR} virgin female mutants are resistant to ethanol sedation and \textit{DopEcR} mutants lack rapid sedation tolerance................................................................. 17

Figure 3. Adult \textit{DopEcR} expression is necessary for normal ethanol sedation......... 18

Figure 4. \textit{DopEcR-RNAi} expression in cholinergic or peptidergic neurons increases resistance to ethanol sedation................................................................. 20

Figure 5. \textit{dunce} \textsuperscript{1}, but not \textit{rutabaga} \textsuperscript{1}, suppresses the \textit{DopEcR} mutant resistance to ethanol-induced sedation................................................................. 21

Figure 6. Inhibition of downstream EGFR/ERK signaling by DopEcR modulates sedation in response to ethanol................................................................. 23

Figure 7. Dopaminergic \textit{DAT} \textsuperscript{fmn} and \textit{DopR} \textsuperscript{PL00420} mutants display normal ethanol sedation and do not genetically interact with \textit{DopEcR} \textsuperscript{GAL4} ................................................................. 24

Figure 8. Ethanol-induced sedation sensitivity in ecdysone-defective DTS-3 mutants is \textit{DopEcR}-dependent and 20E feeding increases sedation resistance in flies overexpressing DopEcR................................................................. 26

Figure 9. DopEcR is required to inhibit ethanol-induced hyperactivity. .......................... 28

Figure 10. Working model of DopEcR in ethanol-induced behaviors............................ 29

Figure 11. GEFS+ mutation affects sleep/wake behavior.................................................. 41

Figure 12. Sleep abnormalities of GEFS+ mutants scrutinized using video tracking.... 43

Figure 13. Nighttime sleep parameters of control and heterozygous GEFS+ mated females from an outcross with wild-type genetic backgrounds (\textit{Canton-S} and \textit{w} \textsuperscript{1118}) ....................................................................................................................... 44

Figure 14. Percent survival of control and GEFS+ virgin females.................................. 44

Figure 15. Pharmacologic suppression of GABA\textsubscript{A} receptor function differentially affects sleep in control and GEFS+ flies..................................................... 46

Figure 16. Rdl GABA\textsubscript{A} knockdown in PDF-positive neurons differentially influences sleep latency behavior in GEFS+ mutants. ..................................................... 47
Figure 17. Effect of constant and acute light during the scotophase on GEFS+ and control sleep. .................................................................49

Figure 18. Circadian regulation is intact in GEFS+ mutants..................................................50

Figure 19. GEFS+ mutants lack homeostatic sleep regulation. ........................................51

Figure 20. Sleep deprivation reduces heat-induced seizure susceptibility of GEFS+ mutants. ..................................................................................................................52

Figure 21. Dravet mutants have sleep problems similar to GEFS+ mutants, but seizure susceptibility does not change after sleep deprivation..............................................54

Figure 22. Working model of how GEFS+ and DS mutations affect sleep behavior. .....55
PREFACE

This dissertation consists of both unpublished and published data describing two distinct projects. In Chapter I, I offer a brief review of *Drosophila* behavioral neurogenetics as a primer for subsequent chapters. In Chapter II, I provide evidence that *Drosophila* DopEcR mediates ethanol-induced sedation and locomotion. Knock-in *DopEcR* mutants were generated and shared by Dr. Yi Rao from the Peking University School of Life Sciences, Beijing, China. In Chapter III, with help from graduate student Patrick Lansdon on seizure assays, I characterize the sleep abnormalities of flies harboring human seizure-associated mutations (GEFS+ or DS) in their voltage-gated sodium channel gene (*paralytic*). Knock-in mutants were kindly shared by Dr. Diane O’Dowd at the University of California – Irvine, California, USA. Finally, in Chapter IV I summarize and provide future perspective for each research project.
CHAPTER I: INTRODUCTION TO DROSOPHILA BEHAVIORAL NEUROGENETICS

All living things rely on genetic instruction, but how do genes influence the behavior of an organism? The scientific field of behavioral neurogenetics seeks the answer to this question. To provide background and perspective for subsequent research chapters, I briefly highlight major historical events, current genetic tools, and future directions of behavioral neurogenetics in Drosophila.

Evolution of the Field

By the beginning of the 20th century, scholars and laypersons around the world were familiar with Charles Darwin's theory of natural selection and “indelible stamp of man's lowly origins.” In his works Darwin often emphasized the importance of generational inheritance, but struggled to precisely explain “genetics,” a term coined long after his death in 1882. It was not until the year 1900 with the rediscovery of Gregor Mendel’s 40-year old findings on heritable factors – now known as genes – that the foundations of genetics would be laid. To study genetics, a Harvard entomologist named C. W. Woodworth proposed that Drosophila fruit flies (hereinafter flies) would be a suitable model organism. There were, and still are, many advantages to using flies for biological research. Flies are much easier than mammals to manage in a laboratory; they are smaller, cheaper, and more fecund. Additionally, flies are exemplary genetic systems since they are readily amenable to genetic perturbation, large unbiased screening, and evolutionary analysis. Taking Woodworth's advice, the now celebrated geneticist Thomas Hunt Morgan turned his focus from embryology to “Mendelism,” the study of heredity and production of traits (see Nobel Biographical (Morgan, 1965)). With a mind to use flies for mutagenesis, Morgan erected the famous “fly-room” at Columbia University in 1910. Two years later, amongst his normally red-eyed flies Morgan found, and selectively propagated, a mutant white-eyed fly. Starting a Drosophila nomenclature tradition, where the affected gene is named after the mutant fly phenotype, he named the gene white. Interestingly, white mutants are still commonly used in
research labs worldwide since the proportionately large compound eyes of flies make mutants easy to identify (see the centennial commemorative review (Green, 2010)). Morgan and his students (jokingly called “Raiders”) continued creating and characterizing fly mutants of all kinds. By crossing different mutants to each other and analyzing the progeny, they eventually demonstrated that genes reside on hereditary units called chromosomes. They also discovered that recombination frequency, or the likelihood that mutant traits would be observed together or independent of one another could be used to map the distances between genes. The genetic unit of linkage would later be named a “morgan” (or centimorgan) by one of Morgan’s talented Raiders Alfred H. Sturtevant. In 1933, Morgan went on to receive the Nobel Prize for his genetical works, and forever immortalize the exploitation of flies for genetic research.  

Shortly after Morgan’s death, arguably one of the most important scientific discoveries was published in a short Nature paper (Watson and Crick, 1953). Based on work done by many pioneering scientists, biologist James Watson and physicist Francis Crick described the double-helical structure of deoxyribonucleic acid (DNA). Despite the enormity of this finding, the landmark science paper modestly states that, “This structure has novel features which are of considerable biological interest.”  

With this knowledge in hand, scientists quickly pieced together more of the DNA puzzle, exposing the nucleotide base-pairing and triplet codon nature of genomes. Darwin, himself, would likely have been delighted to learn that DNA’s semi-conservative replication and encryption system explains the biological inheritance of all living organisms. Scientists began translating and comparing DNA sequences, but were soon confounded by the fact that the majority of the human genome was considered to be “junk” – it

---

1 For more on T. H. Morgan’s family, education, and honors see the National Academy of Sciences biographical memoir written by A.H. Sturtevant (1959).

2 For an autobiographical, and controversial, tale on deciphering DNA’s structure see “The Double Helix: A Personal Account of the Discovery of the Structure of DNA” by James Watson (1968). Interestingly, much later in his life, Watson would become the first person to have their genome sequenced for less than $1 million (Wadman, 2008). He would also become interested in neuroscience, proclaiming that “The brain is the last and grandest biological frontier, the most complex thing we have yet discovered in our universe” (Discovering the Brain, 1992).
didn’t encode for proteins. It was logical that protein-encoding sequences would be conserved throughout the animal kingdom. So to find and characterize genes, many scientists looked to the fruitful nature of Morgan’s earlier fruit fly research. One of these scientists would even go on to become known as a “father of behavioral neurogenetics.”

In 1967, Seymour Benzer, a physicist turned geneticist, dropped his cutting-edge bacteriophage research and decided to open a fly lab at Caltech. His radical idea was that individual genes could underlie even the most complex of behaviors. Benzer reasoned that, “Behavior is the way the genome interacts with the outside world.” To test his theory he used DNA mutagens and carefully designed behavioral assays to detect, and creatively name genes that when mutated would cause behavioral defects. For instance, using an ingenious device known as the countercurrent apparatus, which tests the direction and magnitude of a fly’s innate phototactic or negative geotactic behavior, mutants with vision or locomotor defects, like *eyes absent* or *sluggish*, were quickly and unbiased-ly identified. And by recording a fly’s activity over long periods of time – flies break an invisible infrared light beam spanning their vials – the very first gene associated with circadian behavior, *period*, was discovered (Konopka and Benzer, 1971). Thus in spite of heavy criticism and skepticism, Benzer’s powerful forward genetics approaches and subsequent gene mapping, ultimately yielded the first fruits of behavioral neurogenetics. Following Benzer’s lead, many researchers have continued to identify and characterize genes linked to various complex behaviors such as sleep, stress response and learning and memory.3

**Fly Behavioral Neurogenetics Today**

The field of *Drosophila* behavioral neurogenetics now spans a wide range of reductionist to systems approaches. Today’s researchers are spoiled with the complete genome sequence

---

3 For more on Benzer and his academic progeny see the wonderful commemorative book by Jonathon Weiner’s called “Time, Love, Memory: A Great Biologist and His Quest for the Origins of Behavior” (1999).
of *Drosophila melanogaster* (Adams et al., 2000) – four chromosomes housing roughly 15,000 genes, 50% of which have mammalian homologs. Advances in microscopy have also produced a wealth of fly neurocircuitry information, a.k.a. the “connectome”. Scholars have created a surfeit of genetic tools, stock collections, and behavioral assays to help unlock the mysteries of the nervous system. Here, I touch on a few landmark tools, which have not only benefited the whole scientific community, but also directly impacted my own graduate research.

The jellyfish green fluorescent protein (GFP) has literally and figuratively illuminated science (Chalfie et al., 1994). Transgenic expression of this non-invasive, stable, and genetically encoded protein allows researchers to label, track, and sort living cells. GFP has also now been engineered to give rise to a diversity of colors, which has been extremely useful in biology. One remarkable example is the use of random genetic combinations of multiple GFP spectral variants to produce breathtaking mosaic neuron images of the brain (“Brainbow” (Livet et al., 2007) and “Flybow” (Hadjieconomou et al., 2011)). You should Google it. This technique and others have been crucial for delineating cell lineages and nervous system circuitry (Shimosako et al., 2014). Synthetic chemistry has also taken florescent techniques to staggering new heights. Many fluorescent sensors have been creatively co-opted to report various cellular features such as voltage (Han et al., 2013; Knopfel et al., 2015) or intracellular calcium (Nakai and Ohkura, 2003). These tools have provided crucial real-time visualization of electrophysiologial correlates in excitable cells. No matter the application, it is clear that without GFP the future of science would not be as bright.⁴

Most scientists seek omniscient power over where, when, how, and in which genetic background they want to express their favorite genes. Fly researchers have arguably the best tools for this job. Today, large stock collections of various genetic mutants and transgenic insertions are just a click away, and do not break the bank. Repositories like the Bloomington

---

*Drosophila* Stock Center (http://flybase.org) and Vienna *Drosophila* Resource Center (http://stockcenter.vdrc.at/control/main) are an invaluable resource for all Drosophilists. A particularly important collection of fly lines are those for the binary GAL4/UAS system (Brand et al., 1994). This system, borrowed from yeast, allows researcher to expression whatever (UAS-effector), wherever (enhancer-GAL4). For example, an effector gene, say UAS-*reaper* (a programmed cell death protein), can be ectopically expressed in the eye with *GMR*-GAL4 (an eye-specific enhancer), to subsequently produce flies without eyes. This method is also commonly used to reduce target gene expression by RNA-interference (RNAi) in specific tissues to identify the necessity of its protein product. So using the eye *GMR*-GAL4 with a UAS-*white*-RNAi produces white-eyed flies, while still maintaining the endogenous expression of *white* throughout the rest of the fly. Other tricks have subsequently been added to the fly’s genetic engineering toolbox including the following: a temperature-sensitive inhibitor of GAL4 called GAL80, the QF/QUAS and LexA/LexAop systems, an inducible FLP/FRT excision, multi-system intersectional strategies like FINGR, and more. With these genetic tools researchers can more easily ask, answer, and test hypotheses that help decipher complex biological processes.

Recent technological advancements have also been a boon for behavioral science. Specifically, video tracking and unbiased computational analysis has become an affordable and effortless means in which to assess behavior. Paradigms that once required subjective scoring by hand can now be digitally evaluated, allowing for better accuracy, scalability, and retrospective investigation. In flies, locomotor activity can be captured with a basic web camera and analyzed with available tracking software. Video analysis even allows researchers to examine behaviors over extended periods of time, including nighttime sleep behavior and circadian rhythm patterns. High resolution tracking can also be used to assess particular fly

---

activity in greater detail, like flight (Bath et al., 2014), fly-fly interactions (Branson et al., 2009), and feeding behavior (Itskov et al., 2014; Ja et al., 2007). Software is continually being refined to detect stereotypical ethograms – a fly’s behavioral repertoire – including grooming, singing, and walking behavior. Video tracking and analysis is also helpful for shedding light on behavioral responses to environmental stressors like alcohol and other drugs of abuse. And soon computer learning software and unbiased artificial intelligence will redefine organismal behavior, as we know it, thus propelling the field of behavioral neurogenetics into a fascinating new scientific generation.

**Future Directions**

There will always be a need to solve the unsolvable or comprehend the incomprehensible. In the future scientists will continue to face many ethical, technological, and political obstacles in the pursuit of knowledge. Here, I discuss three areas that I believe will dramatically shape the future of behavioral neurogenetics.

Nearly any genomic locus can now easily be modified, thanks to the CRISPR/Cas9 system (Ran et al., 2013). Comprised of clustered regularly interspaced short palindromic repeats and nuclease enzymes like Cas9, this prokaryotic system is a bacterial immune mechanism akin to RNA-interference in eukaryotic cells. Using chimeric CRISPR-derived biotechnologies, researchers can guide Cas9 to their favorite genomic sites and cause addition, removal or modification of endogenous loci with high efficiency. Though still in its infancy, CRISPR/Cas9 technology has quickly infiltrated academic, industrial, and medical communities, offering both great curative promises but also potential eugenics-like abuse (Ledford, 2015). In neurobiology research, getting CRISPR tools past the blood brain barrier provides researchers an alternative to embryonic stem cell technology for more effectively modeling neurodegenerative diseases (Agustin-Pavon and Isalan, 2014; Senis et al., 2014). In fly research, CRISPR targeting has proven to be much more efficient than other previously applied
genetic engineering methods and is quickly becoming the “go-to” method for manipulating
genes of interest (Port et al., 2015). With expertly designed genetic tools and thoughtful analysis
of genetic manipulation results, researchers now have the tools to help address some of the
biggest mysteries of behavioral neurogenetics.

Many outstanding questions in behavioral neuroscience are on the verge of being
resolved. For example, neuroscientists constantly grapple with how neural circuits are wired.
Though not very similar to mammalian brains, the spatiotemporal mapping of the ~135,000
neuron fly brain will likely be key to understanding how neuronal networks electrochemically
communicate (Kaiser, 2015). Researchers are also tantalizing close to understanding how
neurons acquire, store and retrieve memories. In flies, some short- and long-term memories
have remarkably been mapped to few – even single! –neurons (Aso et al., 2014; Haynes et al.,
2015). Recent research is also honing in on how and why brains sleep. Pioneering work in flies
has provided great insight into the genetic and circuitry control of circadian rhythms and sleep
homeostasis (Gilestro, 2012; Gilestro and Cirelli, 2009; Konopka and Benzer, 1971; Shaw et al.,
2000). These, and other, laboratory efforts hold great translational importance. But can
modeling human health and disease in rodents or flies ultimately improve health care?

The translation of scientific findings from “lower” organisms to humans has long been a
necessary and advantageous process for the advancement of medicine. Fruit flies, which
evolutionarily diverged from us nearly one billion years ago, have served many roles in
translational research. Human genes can easily be integrated into fly genomes, with flies
serving as proverbial petri dishes. The fly genome also has nearly 75% of known human
disease-causing homologs (Reiter et al., 2001), which lends them to valuable molecular
analysis and high throughput gene or drug screening. Even the punctuated development and
short lifespans of fruit flies are attractive for neurogenetic study since many clinicians embrace a
developmental perspective of neurological health. Moreover, since research using flies, unlike
rodents, does not require ethical regulation or oversight, it is easy to test chronic environmental
stressors that are known to affect the onset, progression, and prognosis of neurobiological
diseases. Finally, the burgeoning fields of molecular psychiatry and psychiatric genetics, which
hope to dissect complex higher order mental faculties like consciousness and mental disorders,
demand a clear understanding of basic synapto-physiology, signaling mechanisms and circuit
dynamics. Fly and other animal research provide this crucial infrastructure for the
neurobiological sciences.

In conclusion, it is my hope that this foray into the past, present, and future of behavioral
neurogenetics, particularly in Drosophila, has provided sufficient background and perspective
for grasping the significance and methodology of the following research chapters.
CHAPTER II: *DROSOPHILA DOPECR, A UNIQUE GPCR FOR DOPAMINE AND ECDYSONE, MODULATES ETHANOL-INDUCED BEHAVIORS*

Introduction

Alcohol abuse and addiction are personally and societally devastating. The Center for Disease Control and World Health organization have estimated that every year roughly 6% of deaths worldwide and over $200 billion in United States can be attributed to alcohol-related events. In May 2013 the newest *Diagnostic and Statistical Manual* (DSM-5) merged alcohol abuse and alcohol dependence into a single disorder called Alcohol Use Disorders (AUDs). Demonstrating alcohol’s wide-ranging effects, the AUD criterion includes overconsumption, neglecting obligations, persistent cravings, acquired tolerance, withdrawal manifestation, and other metrics that evaluate quality of life. Those suffering from AUDs often fail to seek treatment, or relapse when medication and behavioral therapies cease ([www.niaaa.nih.gov](http://www.niaaa.nih.gov)). Researchers had hoped that whole genome sequencing would reveal the heritable components of being at risk for alcoholism, but most studies have found genes with small effect sizes and unclear biological implications. Unlike other drugs of abuse, ethyl alcohol (ethanol) does not have a specific biological target, which further adds to the complex and multifactorial nature of developing and treating AUDs.

Despite the complex pathophysiology underlying AUDs, it is well known that behavioral response to alcohol is greatly influenced by endocrine factors such as steroid hormones. Steroids dynamically encode for both external stressors and internal cues, and at the molecular level, produce their biological effects by binding their cognate nuclear receptors to eventually altering gene expression. Steroids are also known to elicit rapid cellular actions via G-protein coupled receptors (GPCRs) independent of transcriptional activity ([Losel and Wehling, 2003; Olde and Leeb-Lundberg, 2009](http://www.niaaa.nih.gov)). The role of this unconventional non-genomic steroid action in the nervous system, or in behavioral response to alcohol, remains unknown.
As a model organism, the fruit fly *Drosophila melanogaster* is uniquely suited to fill this gap of knowledge. As in mammals, exposing flies to constant ethanol vapor results in increased locomotion, loss of postural control and ultimately sedation (Wolf et al., 2002). *Drosophila*’s genetic tractability has facilitated the discovery and analysis of many evolutionarily conserved genes linked to AUDs (Devineni and Heberlein, 2013; Kaun et al., 2012). As for non-genomic steroid action, Srivastava et al. (2005) identified DopEcR, a novel fly GPCR that responds to both the major insect hormone ecdysone and the catecholamine dopamine. In heterologous cell culture DopEcR specifically induced rapid MAPK/ERK signaling in response to ecdysteroids and cAMP/PI3K signaling in response to dopamine. The *DopEcR* sequence is most similar to vertebrate β2-adrenergic receptors and its protein functionally resembles the mammalian GPCR for estrogen, GPER1 (Evans et al., 2014; Srivastava et al., 2005). Others, as well as our lab, have found that DopEcR can function as a dopaminergic receptor to control proboscis extension reflex behavior (Inagaki et al., 2012) and as a non-genomic ecdysone receptor to regulate experience-dependent courtship suppression (Ishimoto et al., 2013). In male *A. ipsilon* moths, DopEcR can act a dual receptor to modulate behavioral response to sex pheromones (Abrieux et al., 2013).

Intriguingly, the signaling components associated with DopEcR – steroids, dopamine, cAMP, ERK – are each known to influence evolutionarily conserved behavioral responses to alcohol (Corl et al., 2009; Kong et al., 2010; Koob, 2008; Moore et al., 1998). Here, we show that DopEcR is necessary for mediating ethanol-induced sedation and hyperactivity in flies. Our data indicate that DopEcR suppresses EGFR/ERK signaling to promote ethanol sedation, and that adult ecdysone negatively regulates this action. DopEcR also functions to suppress ethanol-induced locomotion, possibly by counteracting classical dopamine signaling. These findings contribute to our knowledge of GPCR-mediated non-genomic steroid actions and provide novel insight into the neurobiological mechanisms underlying AUDs.
Materials and Methods

Fly Stocks
Flies were raised on standard cornmeal/agar food at 25°C in 65% humidity under a 12 hr light/dark cycle. For all experiments newly eclosed males were collected over a two-day period, housed 10/vial, and aged 3-5 days. Two DopEcR mutant alleles were used in this study. A hypomorphic allele DopEcRPB1 (a.k.a. DopEcR<sup>c02142</sup>) (Ishimoto et al., 2013) that carries an intronic piggyBac transposon insertion was obtained from the Bloomington Stock Center. A presumptive null allele DopEcRGAL4 was generated by replacing the first 133 bp coding region of DopEcR (4370598…4370730) with an in-frame GAL4 and GMR-white. Gene targeting experiments were carried out as describe previously (Huang et al., 2008). DopEcRPB1 and DopEcRGAL4 mutants were backcrossed six generations to the w<sup>1118</sup> strain obtained from the Vienna Drosophila RNAi Center (VDRC). UAS-DopEcR-RNAi (KK111211) and UAS-Egfr-RNAi (KK107130) were obtained from the VDRC’s phiC31 RNAi library. UAS-DopEcR-cDNA was created previously in our lab (Ishimoto et al., 2013). The GAL4 lines, DTS-3 (a.k.a. mld<sup>DTS-3</sup>), and DopR<sup>PL00420</sup> flies were obtained from the Bloomington Stock Center and DATfmn flies were gifted by Dr. Kazuhiko Kume (Nagoya City University, Nagoya, Japan).

Ethanol Sensitivity Assay
Sensitivity to ethanol was assessed using the method similar to previous studies with minor modifications (Cavaliere et al., 2012; Chen et al., 2008). Approximately ten 3-5 day-old males were transferred from regular food vials to empty polystyrene vials (22.5 mm x 90 mm) with a cotton ball stopper. Following 5 min acclimation, 0.5ml of 50% ethanol was applied to the center of the cotton ball, quickly sealed with Parafilm and placed on a white background under constant light. Without stimulation, vials were scored every 5 min for loss of posture (on vial bottom) and sedation (on vial bottom absent normal posture and movement, except small twitches). The percentage of flies scored at each time point was calculated, and the time for
50% of flies to be scored – loss of posture (LT50) and sedation (ST50) – was determined by linear interpolation. Graphs represent the average and SEM across vials (n = the number of vials tested). All assays, including those using temperature shift paradigms, were performed at 25°C in 65% humidity between Zeitgeber Time (ZT) 0-6.

**Ethanol Absorption and Metabolism Assay**

Analysis of internal ethanol levels was performed similarly to others (Corl et al., 2009; Moore et al., 1998; Wolf et al., 2002). Approximately 30 flies were subjected to the ethanol sensitivity assay for 0, 15, 30 min (absorption) or 30 min followed by 30, 60, 120 min recovery on food (metabolism). Flies were then immediately transferred to empty polystyrene vials, frozen at -80°C, homogenized in 300 µl 50 mM Tris HCl pH 7.5, and centrifuged for 20 min at 4°C. Homogenate (2 µl) was added to alcohol reagent (200 µl) from an Ethanol Assay kit (Catalog #229-29 from Genzyme Diagnostics Sekisui). After a 20 min incubation at room temperature OD340 readings were determined using plate spectrophotometry. Readings were then averaged between technical replicates and ethanol concentrations were derived from standard curves. Experiments were performed at least four independent times and 0.6µl was used for individual fly volume (based on wet weight and comparable to (Devineni and Heberlein, 2012)).

**Western Blot Analysis**

Western blot analysis was performed essentially as described in (Harlow E., 1988). Approximately thirty 3-5 day-old flies were transferred to empty polystyrene vials, frozen at -80°C, and briefly vortexed to dissociate heads. Heads were then homogenized in 2x Laemelli’s loading buffer (5 µl/head), and the homogenates were centrifuged at 13,200 rpm for 10 min. Supernatant (12 µl for p-ERK and total ERK, 4 µl for tubulin) was run on 10% TGX polyacrylamide gels (Bio-Rad, Hercules, CA). Protein bands were semi-dry transferred to PVDF Immobilon membranes (Millipore, Billerica, MA) and probed with mouse anti-diphosphoMAPK
(1:2000) (Sigma M8159), rabbit anti-MAPK (1:2000) (Sigma M5670) or mouse anti-tubulin
(1:10,000) (Sigma T9026) antibodies (Sigma-Aldrich, St. Louis, MO). Antigen-antibody
complexes were then detected with appropriate goat anti-IgG antibodies conjugated with HRP
(1:10,000) (Santa Cruz Biotech, Dallas, TX). Membranes were exposed to Western Lightning
ECL-Plus reagent (PerkinElmer Life and Analytical Sciences, Waltham, MA) and signals were
developed on Kodak-Biomax X-ray film (Eastmen Kodak, Rochester, NY). ImageJ was used for
densitometric analysis (Schneider et al., 2012), and signals were normalized to tubulin.

Ethanol-Induced Locomotion Assay

We created an 8.5 cm x 14 cm x 2 cm plexiglass vapor chamber (Plexicraft Inc., LLC, Iowa City,
IA) modified from Wolf et al., 2002. Each side of our two-sided chamber has entry and exit
reservoirs to aide in vapor distribution, and are partitioned into four 58 mm x 12 mm stalls ~0.1
mm above the camber floor. Two bubble humidifiers with flowmeters (Concoa®, Virginia Beach,
VA) were used to produce humidified ethanol vapor – ~50% (3 L/min ethanol : 3 L/min dH2O)
and ~83% (5 L/min ethanol : 1 L/min dH2O). An aquarium air pump (Tetra® Whisper 30-60) was
used for constant air pressure. Tygon® tubing and Nalgene® connectors were used for delivery
to and removal from the chamber. Individual flies were gently mouth-pipetted into the chamber
stalls, placed on an infrared light box (140 LED Night Vision Illuminator Lamp) with a light
diffuser, and acclimated for 5 min under humidified air (6 L/min dH2O). A night vision web
camera (Agama V-132 1.3M Pixel) was mounted 15 cm above the vapor chamber and 640 X
480 resolution video was taken at 30 frames/sec. PySolo software (Gilestro and Cirelli 2009)
was used to analyze locomotion.

Statistics

SigmaPlot (Systat Software, San Jose, CA) was used to determine statistical significance for
ethanol-induced behaviors and Western blot results. Behavior over time was analyzed with
Two-Way Repeated-Measures ANOVA (One Factor Repetition) on Ranks followed by Holm-
Sidak multiple comparisons procedures. T50 data was assessed using Mann-Whitney Rank Sum tests. Chi-Square test was used to assess survival of sedated flies following 90-min ethanol exposure. All graphs display data averages with error bars representing standard error of the mean (SEM).

20-hydroxyecdysone (20E) Feeding
A 50 mM stock solution of 20E (Sigma-Aldrich) was prepared in 70% ethanol and added to re-heated food. 3-5 day-old adult male flies, raised 10/vial, were transferred to vials with food containing 0.01 mM 20E or vehicle for 24 hr prior to the ethanol sensitivity assay.

Results

DopEcR mutants are resistant to ethanol-induced sedation
To examine the potential role of DopEcR in behavioral response to alcohol we used hypomorphic \textit{DopEcR}\textsubscript{PBI} and null \textit{DopEcR}\textsubscript{GAL4} mutants. The \textit{DopEcR}\textsubscript{PBI} allele contains an intronic \textit{piggyBac} transposon insertion that reduced \textit{DopEcR} transcript levels (Ishimoto et al., 2013), whereas the newly generated \textit{DopEcR}\textsubscript{GAL4} knock-in mutant has an in-frame \textit{GAL4} construct replacing the first coding exon rendering it a null allele (Fig. 1A). Both \textit{DopEcR}\textsubscript{PBI} and \textit{DopEcR}\textsubscript{GAL4} homozygotes were viable, and displayed grossly normal development and morphology. Using \textit{DopEcR}\textsubscript{GAL4} and a fluorescent reporter (UAS-\textit{myrGFP-2A-RedStinger}) (Daniels et al., 2014), we observed diffuse nervous system expression with prominent signal in the mushroom bodies (MBs) and antennal mechanosensory motor center (AMMC) (Fig. 1B).

To assay ethanol sensitivity, control and \textit{DopEcR} mutant flies were subjected to ethanol vapor in an empty vial and their behavioral responses were observed every 5 min for 2 hr. Upon exposure flies gradually lost postural control and were increasingly seen on the vial bottom. Thus, the percentage of flies on the vial bottom was used as an indicator of ethanol-induced loss of postural control. \textit{DopEcR}\textsubscript{PBI}, \textit{DopEcR}\textsubscript{GAL4} and trans-heterozygotes (\textit{DopEcR}\textsubscript{PBI}/\textit{DopEcR}\textsubscript{GAL4}) lost their postures around the same time as controls (Fig. 1C). LT50
values (time for 50% of flies to lose posture) were around 20 min for all flies. Also around 20 min into exposure, control flies began to cease all motor activity, except for minor twitching, and were considered to be sedated (Fig. 1D). Nearly all control flies were sedated within 50 min of exposure. In contrast, DopEcR mutants continued moving in an uncoordinated manner typically while on their backs, as if trying to regain their posture, long after many control flies had
sedated. It took almost 2 hr for all DopEcR mutants to sedate. ST50 values (the time for 50% of flies to become sedated) were around 30 and 60 min for control flies and DopEcR mutants, respectively (Fig. 1D). We next addressed whether the absorption or metabolism of ethanol was abnormal in DopEcR mutants by measuring internal ethanol levels at various time points. Neither slow absorption nor fast metabolism of ethanol could explain the sedation resistance of the mutants (Fig. 1E), suggesting that DopEcR modifies behavioral response to alcohol though non-metabolic means. Since DopEcR is preferentially expressed in the nervous system (Graveley et al., 2011), we hypothesized that the receptor serves a neurobiological role in promoting sedation during naïve ethanol exposure.

We also found that heterozygous DopEcRPB1 or DopEcRGAL4 flies showed significant sedation resistance as compared to controls, albeit to a lesser extent than homozygous mutants (ST50 41±3 min or 43±4 min, respectively). In addition, virgin DopEcR mutant females exhibited normal loss of posture response, but resistance to ethanol-induced sedation (Fig. 2A-B). As other studies have shown, control flies develop rapid tolerance upon a second exposure to ethanol after being allowed to metabolically recover after an initial naïve exposure. We found that regardless of sex, DopEcR was required for rapid ethanol-induced sedation tolerance (Fig. 2C-D). From this point on we focused on male flies in order to avoid complications of ecdysone-regulated oogenesis and to better compare our results with the majority of previous literature using males.

**DopEcR mutants are more susceptible to death following ethanol sedation**

During the ethanol sensitivity assays we observed that unlike controls a large proportion of sedated DopEcR mutants displayed a “wings up” phenotype commonly seen upon death. To determine whether DopEcR mutants were more likely to die following sedation, control and mutant flies were exposed to ethanol for 90 min and then allowed to recover on food for 24 hr. As compared to sedated control flies, significantly fewer sedated DopEcR mutants survived
intoxication: 41% $w^{118}$, $n = 220$; 4% $DopEcR^{PB1}$, $n = 230$; 25% $DopEcR^{GAL4}$, $n = 177$; $X^2 = 87.079$, df = 2, $p < 0.001$. In summary, despite $DopEcR$ mutants being more resistant to sedate relative to control flies, sedated mutants were less likely to survive the ethanol exposure.

**DopEcR expression correlates with sensitivity to ethanol-induced sedation**

To further validate a role for DopEcR in modulating ethanol-induced sedation, we manipulated $DopEcR$ expression with the GAL4/UAS binary expression system (Brand and Perrimon, 1993). Ubiquitous knockdown of $DopEcR$ with $da$-GAL4 and $UAS-DopEcR-RNAi$ mimicked the
significant sedation resistance observed in $DopEcR$ mutants (Fig. 3A). Conversely, ubiquitous overexpression of a $DopEcR$ cDNA tended to increase sedation sensitivity, though values between experimental flies and UAS controls did not reach statistical significance (Fig. 3B). These results demonstrate an inverse relationship between $DopEcR$ expression levels and resistance to ethanol-induced sedation, further supporting a role for $DopEcR$ in promoting ethanol-induced sedation.

Figure 3. Adult $DopEcR$ expression is necessary for normal ethanol sedation. A) Ubiquitous expression of $DopEcR$-RNAi, da-RNAi (UAS-$DopEcR$-RNAi/+ ; da-GAL4/+, $n = 13$), significantly increased resistance to ethanol sedation as compared to RNAi (UAS-$DopEcR$-RNAi/+, $n = 14$) and GAL4 (da-GAL4/+, $n = 12$) controls. B) Ubiquitous overexpression of $DopEcR$-cDNA, da>cDNA (da-GAL4/UAS-$DopEcR$-cDNA, $n = 9$), showed a trend to increase sensitivity to sedate as compared to cDNA (UAS-$DopEcR$-cDNA/+, $n = 10$) and GAL4 (da-GAL4/+, $n = 12$) controls. C-F) Adult-specific knockdown of $DopEcR$ expression, UAS-$DopEcR$-RNAi/+ ; da-GAL4/tub-GAL80ts ($n = 11, 12, 11, 3$), resulted in increased resistance to ethanol-induced sedation as compared to RNAi (UAS-$DopEcR$-RNAi/+ ; tub-GAL80ts/+, $n = 9, 10, 12, 8$), and GAL4 (da-GAL4/+ ($n = 8, 7, 6, 6$) controls. Flies were either reared at constant temperatures (18°C or 29°C) or transferred to a different temperature after eclosion (18°C → 29°C or 29°C → 18°C). All figures display averages with SEM, Two-Way RM ANOVA on Ranks, Holm-Sidak, and Mann-Whitney Rank Sum tests; *p < 0.05, #p < 0.05 from both controls.
DopEcR is required in adulthood to promote ethanol-induced sedation

Next, we asked when during development DopEcR expression was necessary for modulating response to ethanol in adulthood. To this end, we employed the temporal and regional gene expression targeting (TARGET) method (McGuire et al., 2004), which allowed for temporal control of DopEcR-RNAi expression via the temperature-sensitive GAL4 inhibitor, GAL80\textsuperscript{ts}. When flies were raised at 18°C throughout development and adulthood, experimental flies (UAS-DopEcR-RNAi/+/tub-GAL80\textsuperscript{ts}/da-GAL4) did not exhibit resistance to ethanol sedation when compared to both control genotypes (UAS-DopEcR-RNAi/+/tub-GAL80\textsuperscript{ts}/+ and +/+ ; +/da-GAL4) (Fig. 3C). However, when raised continuously at 29°C, experimental flies showed greater ST50 values and were robustly more resistant than controls (Fig. 3D). The results of these constant temperature paradigms assured us that the TARGET system was working as expected. When flies were reared at 29°C during the embryonic, larval and pupal stages, and transferred to 18°C just after eclosion, experimental flies did not show ethanol sedation resistance (Fig. 3E). But when flies were reared at 18°C during development and then transferred to 29°C just after eclosion, experimental flies were significantly more resistant to ethanol than controls (Fig. 3F). These data suggest that post-eclosion expression of DopEcR is necessary for proper ethanol sedation behavior in adults.

Neuronal knockdown of DopEcR enhances resistance to ethanol-induced sedation

To determine the possible sites of DopEcR action for promoting ethanol-induced sedation, we examined the sedation response of flies expressing DopEcR-RNAi using a variety of cell type-specific GAL4 drivers. Significant increases in sedation resistance were observed when DopEcR-RNAi was expressed using a pan-neuronal driver, elav-GAL4, indicating that neuronal expression of DopEcR is required for normal sensitivity to ethanol (Fig. 4A). Among neuronal subset-specific GAL4 lines, cholinergic (Cha-GAL4) and pan-peptidergic (c929-GAL4) expression of DopEcR-RNAi also increased ethanol sedation resistance as compared to both
control genotypes (Fig. 4B-C). Knockdown of DopEcR with several other GAL4 lines, including a mushroom body (c772-GAL4), dopaminergic (TH-GAL4), and insulin-like peptide (dilp2-GAL4) drivers had little effect on ethanol sedation response (Fig. 4D, data not shown). Together, these data suggest that DopEcR is required in particular neuronal subsets for mediating ethanol sedation.
cAMP mutants modify the ethanol-induced sedation resistance of DopEcR mutants

Next, we sought to understand the downstream mechanisms by which DopEcR influences ethanol-induced sedation. Cyclic adenosine monophosphate, or cAMP, is an instrumental second messenger in intracellular signaling for a variety of biological processes. Amounts of cAMP depend on its production by adenylyl cyclase (AC) and degradation by phosphodiesterase (PDE). In Drosophila, AC mutants (rutabaga\(^1\)) and PDE mutants (dunce\(^1\)) have reduced and elevated cAMP levels, respectively, yet both mutants have been shown to be sensitive to ethanol-induced loss of posture (Moore et al., 1998). To determine whether DopEcR

![Figure 5](image)

**Figure 5.** dunce\(^1\), but not rutabaga\(^1\), suppresses the DopEcR mutant resistance to ethanol-induced sedation. A,B) rut (rutabaga\(^1\), \(n = 10\)) mutants exhibited normal ethanol-induced sedation. Double mutants rut\(;\)PB1 (rutabaga\(^1\) ;; DopEcR\(^{PB1}\), \(n = 12\)) and rut\(;\)GAL4 (rutabaga\(^1\) ;; DopEcR\(^{GAL4}\), \(n = 13\)) showed resistance nearly to the level of each DopEcR mutant. C,D) dnc (dunce\(^1\), \(n = 11\)) mutants were sensitive to ethanol-induced sedation, and double mutants dnc\(;\)PB1 (dunce\(^1\) ;; DopEcR\(^{PB1}\), \(n = 11\)) and dnc\(;\)GAL4 (dunce\(^1\) ;; DopEcR\(^{GAL4}\), \(n = 10\)) were significantly more sensitive than single DopEcR mutants. Ctrl (\(w^{118}\), \(n = 15\)) flies. PB1 (DopEcR\(^{PB1}\), \(n = 15\)) and GAL4 (DopEcR\(^{GAL4}\), \(n = 13\)). Two-Way RM ANOVA on Ranks, Holm-Sidak and ANOVA on Ranks, Dunn’s; \(a\), \(b\), and \(c\) represent \(p < 0.05\) from first, second, third genotype displayed.
facilitates cAMP signaling during ethanol exposure, we examined potential genetic interactions of *rutabaga*¹ and *dunce*¹ with *DopEcRPB1* and *DopEcRGAL4*. Reducing cAMP signaling with *rutabaga*¹ did not have a significant effect on sedation response relative to *w¹¹¹⁸* controls, but did slightly reduce the sedation-resistant phenotype of the *DopEcR* mutants (Fig. 5A,B). Increasing cAMP signaling with *dunce*¹ significantly reduced sedation timing as compared to controls, and almost completely restored normal ethanol-induced sedation timing in both *DopEcR* mutants (Fig. 5C,D). These findings are consistent with previous work highlighting both the importance of cAMP in conserved behavioral response to alcohol and that cAMP signaling can be activated downstream of *DopEcR* (Ishimoto et al., 2013; Moore et al., 1998; Srivastava et al., 2005).

**EGFR knockdown alleviates the sedation-resistant phenotype in *DopEcR* mutants**

Previous studies have demonstrated a bidirectional role of epidermal growth factor (EGF) signaling in ethanol-induced sedation, where EGFR/ERK activation increased resistance and suppression increased sensitivity to ethanol (Corl et al., 2009; Eddison et al., 2011). Since *DopEcR* has also been reported to activate the MAPK/ERK cascade in heterologous cell culture systems (Srivastava et al., 2005), we reasoned that in response to ethanol *DopEcR* might interact with the EGFR/ERK pathway to modulate sedation response. To test this hypothesis, we examined whether suppression of EGFR signaling in *DopEcR* mutant backgrounds could mitigate ethanol sedation resistance. Pan-neuronal expression of *Egfr-RNAi* increased ethanol sedation sensitivity in *DopEcRPB1* mutants (Fig. 6A). Similarly, expression of *Egfr-RNAi* in *DopEcRGAL4* mutants by its intrinsic GAL4 activity also increased sedation sensitivity, even beyond that of *Egfr-RNAi* controls (Fig. 6B). These behavioral observations for *DopEcR* mutants implicated that their resistance to ethanol-induced sedation is attributed to enhanced EGFR signaling. The strength of EGFR signaling can be estimated by the degree of phosphorylation of the downstream effector ERK. Western blot analyses revealed that although *DopEcR* mutants had lower total ERK levels in the heads, the basal levels of phosphorylated-ERK were
significantly increased relative to control flies (Fig. 6C). This finding suggests that in \( \text{DopEcR} \) mutants the EGFR/ERK pathway is aberrantly activated and likely contributes to their increased resistance to ethanol-induced sedation.

**Dopaminergic mutants \( \text{DAT}^{\text{fmn}} \) and \( \text{DopR}^{\text{PL00420}} \) display normal ethanol sedation**

Since both dopamine and ecdysone are known ligands for DopEcR, we examined if either was important for the role of DopEcR in ethanol-induced sedation. Dopamine mediates the locomotor activating effects of ethanol in \( \text{Drosophila} \) (Bainton et al., 2000; Kong et al., 2010) as well as in mammals (Boileau et al., 2003; Budygin et al., 2003; Shen et al., 1995). However,
dopaminergic manipulation, either by genetic or pharmacologic means, does not appear to influence ethanol-induced sedation in flies (Bainton et al., 2000; Kong et al., 2010). Consistently, we observed that in response to ethanol, neither increased synaptic dopamine due to defective dopamine transporters (DATfnn) nor reduced dopamine signaling by hypomorphic dopamine D1-like receptors (DopRPL00420) affected ethanol sedation responses (Fig. 7, red). Double mutants were then created and examined for potential genetic interactions between dopaminergic signaling and DopEcR. The sedation phenotype of DATfnn,DopEcRGAL4 was indistinguishable from that of DopEcRGAL4 mutants. DopRPL00420DopEcRGAL4 double mutants were similarly resistant, but slightly less resistant than DopEcRGAL4 mutants, to ethanol-induced sedation (Fig. 7, green). These results suggest that dopamine is not likely a major mediator for the DopEcR-dependent regulation of ethanol-induced sedation behavior.

Ecdysone negatively modulates DopEcR during ethanol exposure

To investigate the potential role of ecdysone in ethanol sedation, we first utilized the ecdysone-defective mutant Dominant temperature-sensitive-3 (DTS-3) (Holden and Suzuki, 1973). DTS-3
mutation is homozygous lethal irrespective of temperature, but heterozygous mutants survive at 22°C (permissive temperature) and only fail to molt at 29°C (restrictive temperature) due to reduced ecdysone levels (Holden and Suzuki, 1973). The ethanol sensitivity of control and DTS-3 (DTS-3/+) flies reared continuously at 18°C did not significantly differ (ST50 32±4 min and 25±2 min, respectively). However, when reared at 18°C during development and transferred to 29°C after eclosion, DTS-3 flies displayed increased sensitivity to ethanol-induced sedation as compared to controls (Fig. 8A). This suggests that reducing adult ecdysone levels increases sensitivity to ethanol-induced sedation, which is behaviorally opposite of the DopEcR loss-of-function phenotype. To examine the interaction between DTS-3 and DopEcR mutation, we generated flies heterozygous for DTS-3 and homozygous for DopEcR<sup>GAL4</sup>. When reared at 18°C and transferred to 29°C after eclosion, these double mutants showed significant ethanol sedation resistance comparable to that of DopEcR<sup>GAL4</sup> controls (Fig. 8A). This finding suggests that DopEcR function is required for the sensitizing effect of DTS-3 on ethanol-induced sedation. In further support of this, ubiquitous expression of DopEcR-RNAi in DTS-3/+ mutant flies also mitigated the sedation sensitivity of ecdysone-defective flies (Fig. 8B). Together these results propose that reducing post-eclosion ecdysone levels results in DopEcR-dependent ethanol-induced sedation sensitivity.

To test the potential negative relationship between ecdysone and DopEcR in controlling ethanol-induced sedation, we asked whether exogenous feeding of the active form of ecdysone, 20-hydroxyecdysone (20E), could influence ethanol sedation response. Acute 24 hr feeding of 0.1mM 20E was not able to significantly affect the timing of control flies (w<sup>1118</sup>) to sedate from ethanol (ST50 vehicle 34±5 min and 20E 37±4 min). However, the sedation sensitivity of flies ubiquitously overexpressing DopEcR was significantly lessened (Fig. 8C) with supplemental 20E feeding. This suggests that ecdysone may negatively regulate DopEcR activity during ethanol exposure and, thus, control ethanol-induced sedation timing.
DopEcR is required to inhibit ethanol-induced hyperactivity

Dopaminergic signaling in primates, rodents, insects, and nematodes significantly contributes to alcohol-induced disinhibition behaviors like increased locomotor activity. To determine whether DopEcR plays a role in ethanol-induced locomotion, individual control and DopEcR mutant flies were subjected to humidified ethanol vapor in a chamber modified from Wolf et al., 2002. In
response to 50% ethanol, control flies exhibited a stereotypical odor-induced startle response and then maintained a fairly constant 6 mm/sec speed during a 15 min exposure (Fig. 9A). Following the startle response, $DopEcR^{Pb1}$ mutants acutely increased in locomotion and $DopEcR^{GAL4}$ mutants displayed prolonged hyperactivity relative to control flies (Fig. 9A). Both $DopEcR$ mutants traveled significantly farther than controls throughout the course of the experiment. Exposure to 83% ethanol expectantly reduced the speed of control flies and eventually led to sedation (Fig. 9B). At this higher ethanol concentration $DopEcR^{Pb1}$ mutants showed a locomotor profile indistinguishable from control flies, though many mutants never visually reached a state of sedation. $DopEcR^{GAL4}$ mutants, however, still exhibited abnormal hyperactivity and rarely sedated during the 83% ethanol exposure (Fig. 9B).

To further support our findings with $DopEcR$ mutants, we examined the effect of $DopEcR$ knockdown in ethanol-induced locomotion. As compared to either GAL4 or UAS alone controls, flies ubiquitously expressing $DopEcR$-RNAi displayed increased ethanol-induced hyperactivity at high ethanol concentration exposure (Fig. 9C). Since either increasing or decreasing DopEcR bidirectionally influenced ethanol-induced sedation behavior, we examined whether this was also the case for ethanol-induced hyperactivity. Ubiquitous overexpression of DopEcR did not significantly affect ethanol-induced locomotion under our conditions (Fig. 9D). Using a lower ethanol concentration, though, might help to rule out possible floor effects, which limit the detection of hypo-locomotion.

Others have shown that classical D1-like DopR signaling promotes ethanol-induced hyperactivity, and that mutants for DopR have reduced ethanol-induced locomotion responses (Kong et al., 2010). We also observed this behavior in our assay with $DopR^{600420}$ mutants exposed to high ethanol concentration vapor (Fig. 9E). To determine the potential dopaminergic interaction between DopEcR and DopR signaling, we observed the response of double mutants to ethanol vapor. Surprisingly, double mutants had extremely high hyperactivity (Fig. 9E). Together, these data suggest that DopEcR inhibits ethanol-induced locomotion possibly by
Figure 9. DopEcR is required to inhibit ethanol-induced hyperactivity. Average speed and total distance traveled when exposed to A) 50% and B) 83% ethanol vapor; Ctrl (w^{1118}^{/}, \(n = 95, 80\), DopEcR^{PB1} \((n = 80, 64)\), and DopEcR^{GAL4} \((n = 80, 64)\). C) Ubiquitous DopEcR knockdown \(da > RNAi\) (UAS-DopEcR-RNAi\(^{+/+}\); da-GAL4\(^{+/+}\), \(n = 32\)), but not D) overexpression \(da > cDNA\) (UAS-DopEcR-cDNA/da-GAL4, \(n = 32\)), significantly affected ethanol-induced hyperactivity as compared to RNAi (UAS-DopEcR-RNAi\(^{+/+}\), \(n = 77\)), da (da-GAL4\(^{+/+}\), \(n = 32\)), and cDNA (UAS-DopEcR-cDNA\(^{+/+}\), \(n = 32\)) controls. E) In response to ethanol vapor, DopR (DopR\(^{P00420}\), \(n = 31\)) mutants showed hypo-locomotion, a phenotype opposite of DopEcR mutants. Interestingly, double mutants DopR GAL4 (DopR DopEcR^{GAL4}, \(n = 32\)) mutants exhibited extreme hyperactivity. Two-way RM ANOVA on Ranks, Holm-Sidak, ANOVA on Ranks, Dunn’s, and Mann-Whitney Rank Sum tests; *\(p < 0.05\), from both controls #\(p < 0.05\), \(a, b, c = p < 0.05\) from first, second, third genotype.
counteracting DopR-mediated ethanol-induced locomotion.

**Discussion**

**Brief summary**

In humans, naïve resistance to ethanol-induced sedation positively correlates to an individual’s risk for becoming an alcoholic (Schuckit et al., 2000). Therefore, a better understanding of the biological mechanisms for ethanol sedation is of great clinical importance. Fruit flies are a resourceful model in which to study evolutionarily conserved genes involved in drug addiction (Kaun et al., 2012). In this study we found that mutants for *Drosophila* DopEcR, a unique dual GPCR for dopamine and ecdysone, took longer to sedate despite having normal initial behavioral responses to ethanol vapor (i.e. loss of postural control) as well as proper ethanol absorption and metabolism (Fig. 1, 2). These findings indicate that DopEcR is necessary for normal ethanol-induced sedation and that it functions to promote sedation during ethanol exposure. RNAi-mediated knockdown of *DopEcR* after eclosion (Fig. 3) or in specific neuronal populations, including peptidergic and cholinergic neurons (Fig. 4) recapitulated the sedation-resistant phenotype of *DopEcR* mutants. We implicated cAMP (Fig. 5) and EGFR/ERK signaling downstream of DopEcR action (Fig. 6) and found that ecdysone may negatively regulate DopEcR to influence ethanol-induced sedation (Fig. 8). Additionally, we showed that DopEcR may not influence sedation using a dopaminergic signal (Fig. 7), but may serve a dopaminergic function in suppressing ethanol-induced hyperactivity (Fig. 9). Together these findings provide a working model for the role and mechanism by which DopEcR controls ethanol-induced behaviors in *Drosophila* (Fig. 10).
and future investigations into the actions of DopEcR offer important insight into the elusive function and mechanism of GPCR-mediated non-canonical steroid signaling, particularly in the context of physiological response to alcohol.

**Role of DopEcR ligands in ethanol-induced behaviors**

Dopamine and ecdysone are known ligands of DopEcR in flies (Inagaki et al., 2012; Ishimoto et al., 2013; Srivastava et al., 2005). As mentioned by others (Bainton et al., 2000; Kong et al., 2010) and shown in our study, dopaminergic signaling does not appear to affect naïve ethanol sedation response (Fig. 7). Thus, dopamine is not a relevant ligand for activating DopEcR to promote ethanol-induced sedation. Interestingly, however, DopEcR-mediated dopaminergic signaling may play a role in ethanol-induced hyperactivity, a behavior known to be dependent on conventional dopamine receptor, DopR, signaling (Kong et al., 2010). Our recent experiments show that this dopaminergic behavioral response to ethanol is exaggerated in DopEcR mutants (Fig. 9). This observation raises the possibility that, although dopamine is not involved in the DopEcR-mediated regulation of ethanol-induced sedation, it may act through DopEcR to counteract DopR-mediated ethanol-induced hyperactivity. Future studies will be needed to further elucidate this potential dopaminergic role of DopEcR in behavioral response to ethanol.

In the case of ecdysone, we found that acute 20E feeding had no effect on the ethanol-induced sedation of control flies. However, DTS-3 mutants that are deficient in adult ecdysone synthesis displayed increased sensitivity to ethanol-induced sedation in a DopEcR-dependent manner. In addition, acute 20E feeding significantly increased the sedation resistance of flies overexpressing DopEcR (Fig. 8). These data suggest that ecdysone can negatively affect DopEcR activity to promote ethanol-induced sedation.

Despite the resistance of DopEcR mutants to ethanol-induced sedation, our results indicate that neither dopamine nor ecdysone is required for activation of DopEcR during ethanol
exposure. One potential explanation for this is that other agonistic ligands for DopEcR are responsible for promoting ethanol-induced sedation. Another conceivable possibility is that DopEcR is able to exert agonist-independent activity. GPCRs generally have two distinct conformation states — an active form and an inactive form, which are stabilized by agonists and inverse agonists, respectively. Considering that many GPCRs have agonist-independent basal activity (Bond and Ijzerman, 2006; Nakashima et al., 2013), our experimental findings raise the possibility that ecdysteroids act as inverse agonists to suppress agonist-independent activity of DopEcR. This hypothesis would explain how reduced ecdysone levels in DTS-3 mutants could lead to DopEcR disinhibition and thus increased sensitivity to ethanol-induced sedation — a result similar to that of overexpressing DopEcR. This scenario also predicts that increasing 20E levels should then increase ethanol-induced sedation resistance, yet an effect of acute 20E feeding was only observed in flies overexpressing DopEcR. There are potential reasons for this discrepancy. First, perhaps the majority of DopEcR receptors are already occupied by 20E, or other endogenous DopEcR steroidal ligands, such as makisterone A and the prohormone ecdysone. Secondly, ecdysteroids may be acting more like neuro-hormones with refined site-specific actions and thus 20E feeding fails to recapitulate any localized ecdysone signaling necessary for DopEcR-mediated processes. In addition, genomic and non-genomic actions of the canonical ecdysone receptor, EcR, in the control of alcohol response are unknown. Detailed analyses at the molecular and cellular levels are required to fully understand the mechanisms by which DopEcR activity is regulated through interactions with its ligands and other modulators during ethanol exposure.

**cAMP and EGFR/ERK signaling downstream of DopEcR-dependent behavioral response to ethanol**

We provide evidence to suggest that DopEcR requires cyclic adenosine monophosphate (cAMP) signaling to mediate ethanol sedation timing. Specifically, cAMP phosphodiesterase mutation (dunce¹), but not by adenylyl cyclase mutation (rutabaga¹), eliminated the sedation
resistance of \textit{DopEcR} mutants (Fig. 5). These findings are consistent with previous work highlighting the well-established importance of cAMP in conserved behavioral response to alcohol and that cAMP signaling can be activated downstream of DopEcR (Ishimoto et al., 2013; Moore et al., 1998; Srivastava et al., 2005).

We also found that DopEcR negatively affects EGFR/ERK signaling to promote ethanol sedation (Fig. 6). This finding is consistent with prior investigations demonstrating that the EGFR/ERK activity positively correlates with resistance to ethanol-induced sedation in both flies and rodents (Corl et al., 2009). Previous studies have also provided insight into the spatiotemporal necessity of EGFR/ERK signaling in ethanol-induced sedation. Specifically, reducing EGFR/ERK in either insulin-producing (\textit{dilp2}+) or dopaminergic (\textit{TH}+) neurons increases resistance to ethanol-induced sedation (Corl et al., 2009) and that the EGFR pathway is required during development for a normal ethanol-induced sedation response (Eddison et al., 2011). At first glance our data seem incompatible with these models since \textit{DopEcR-RNAi} in TH+ or dilp2+ neuronal subsets or restricted to development did not produce significant effects on the sedation response to ethanol (data not shown, Fig. 4). However, in mammals EGFR can be trans-activated or -inhibited by alcohol itself or GPCRs via intra- or inter-cellular mechanisms (Berasain et al., 2011; Mill et al., 2009). Therefore, DopEcR may directly or indirectly influence EGFR/ERK signaling to inevitably alter ethanol-induced sedation timing.

Unlike sedation-resistant \textit{happyhour} (Ste20 kinase) mutants, which showed increased p-ERK levels only after short exposure to ethanol vapor (Corl et al., 2009), \textit{DopEcR} mutants exhibited higher basal levels of p-ERK (Fig. 6). We suspect that this disparity is caused by distinct roles of DopEcR and Ste20 kinase in regulation of EGFR/ERK signaling; DopEcR is likely involved in maintenance of the steady state activity of EGFR/ERK signaling, while Ste20 kinase may play a role in modulating the acute response of the signaling pathway upon exposure to ethanol. We also found that EGFR/ERK signaling and DopEcR function have different temporal requirements. Experiments employing pan-neuronal knockdown of EGFR
indicate that the EGFR/ERK pathway is required both during development and in adulthood to impact ethanol-induced sedation sensitivity (Eddison et al., 2011). Our findings using DopEcR-RNAi in combination with the TARGET system suggests that DopEcR function is particularly required after eclosion for normal sensitivity to the sedative effect of ethanol (Fig. 3). Although we cannot rule out the possible role of DopEcR in post-eclosion development, our results support the idea that DopEcR modulates adult physiological processes critical for the proper sedative response to ethanol.

Accumulating evidence suggests that DopEcR mediates a variety of biological functions including appetitive gustatory reflex behavior (Inagaki et al., 2012), experience-dependent courtship conditioning (Ishimoto et al., 2013), olfactory plasticity during mating (Abrieux et al., 2013), and now modulation of ethanol-induced sedation. When considering the role of DopEcR in these various behaviors, its widespread neuronal expression, and dual ligand capacity, it is tempting to speculate that DopEcR could directly or indirectly interacts with various neurotransmitter systems – small molecules (ACh, OCT, DA, 5-HT, GABA) or peptides (NPF, dilps, Crz) – many of which are known to underlie specific drug-related behaviors. Further investigation will be required to determine direct and indirect interactions between DopEcR and these systems.

**Functional similarities of DopEcR to vertebrate GPER1**

DopEcR bears many functional similarities to GPER1, the vertebrate GPCR for 17β-estradiol, in terms of their cellular location, signaling properties and pharmacology (Evans et al., 2014). Although the in vivo functions of GPER1 largely remain unclear, recent studies have identified a neuroprotective role for GPER1 in a murine Parkinson’s model (Bourque et al., 2013; Bourque et al., 2014) and found that GPER1 knockout mice display reduced anxiety-like behaviors (Kastenberger and Schwarzer, 2014). We found that DopEcR affords a slight protection from dying after ethanol-induced sedation, and propose that DopEcR is uniquely suited to respond to
stressful conditions since both dopamine and ecdysone signaling have been previously implicated in stress responses (Bainton et al., 2000; Gruntenko et al., 2003; Ishimoto and Kitamoto, 2011; Neckameyer and Weinstein, 2005; Regan et al., 2013).

GPER1 is known to activate cAMP production and transactivate EGFR/ERK signaling (Olde and Leeb-Lundberg, 2009). GPER1 also responds to dopamine in a dose-dependent manner when expressed in Xenopus oocytes (Evans et al., 2014). Furthermore, both the DopEcR and GPER1 protein sequences contain putative cholesterol recognition/interaction amino acid consensus (CRAC) sequences that have been proposed as a sterol-binding domain in GPCRs (Jafurulla et al., 2011). In consideration of these intriguing parallels and our current findings, GPER1 warrants further investigation into its potential function and mechanism in mammalian responses to ethanol.
CHAPTER III: EXAGGERATED NIGHTTIME SLEEP AND DEFECTIVE SLEEP HOMEOSTASIS IN DROSOPHILA KNOCK-IN MODELS OF HUMAN EPILEPSY

Introduction

Substantial evidence supports an intimate reciprocal relationship between sleep and seizures. On one hand, the sleep state has a significant impact on seizure activity (Matos et al., 2011; Sinha, 2011). For example, seizures can be facilitated by the neuronal synchronization that typically occurs during non-rapid eye movement (NREM) sleep (Neiman et al., 2010). Also, sleep deprivation is generally considered to trigger or worsen seizures in patients with epilepsy (Ellingson et al., 1984; Malow, 2004). On the other hand, seizures often influence the quality and quantity of sleep, causing irregular sleep patterns or circadian disruption in epileptic patients (Derry and Duncan, 2013; Giorelli et al., 2013). Furthermore, antiepileptic drugs commonly affect sleep (Jain and Glauser, 2014), making the interactions between sleep and seizures even more complex. Altogether these interactions can set into motion a vicious cycle of seizures and sleep abnormalities. Despite this well-recognized interplay between seizures and sleep, the neurobiological underpinnings of this relationship remain largely elusive. A better understanding of the sleep-seizure relationship is thus expected to provide important insights into seizure pathophysiology and sleep mechanisms.

Although evolutionarily distant from mammals, the fruit fly Drosophila melanogaster has emerged as a powerful model to study fundamental molecular and cellular processes underlying sleep/wake behavior (Cirelli, 2009; Hendricks et al., 2000; Potdar and Sheeba, 2013; Shaw et al., 2000). Defined as 5 min or more of inactivity, fly sleep embodies many characteristics of mammalian sleep. Specifically, the sleep state of flies is: 1) subject to circadian and homeostatic regulation, 2) associated with increased arousal thresholds and altered brain activity, 3) susceptible to sleep-wake drugs, and 4) controlled by highly conserved molecular pathways such as inhibitory GABAergic signaling (Potdar and Sheeba, 2013; Shaw et al., 2000; Shaw et al., 2002; Vanin et al., 2012; Wu et al., 2009). In Drosophila, a number of mutants display
seizure-like neuronal activities, and their behaviors have been molecularly and physiologically characterized (Parker et al., 2011; Song and Tanouye, 2008). These mutants include loss-of-function forms of voltage-gated potassium channel subunit genes, *Shaker (Sh)* and *Hyperkinetic (Hk)*, and their modulator *quiver/sleepless (qvr/ssss)*. Interestingly, these seizure-prone mutants have significantly reduced sleep (Bushey et al., 2007; Cirelli et al., 2005; Koh et al., 2008). In addition, Lucey *et al.* recently found that sleep deprivation increases the seizure susceptibility of fly mutants in which seizure-like activity is induced by either mechanical or temperature stress (Lucey *et al.*, 2015). Taken together, these results indicate that, as observed in human epilepsy patients, sleep and seizure activity are functionally related in *Drosophila*.

The most common mutations associated with human epilepsy occur in the voltage-gated sodium channel (VGSC) gene *SCN1A*. VGSCs are essential for the generation and propagation of action potentials, making them integral players in defining the excitability states of neurons under both physiological and pathological conditions (Eijkelkamp *et al.*, 2012). So far, more than 600 different *SCN1A* mutations of varying deleterious effect have been found to result in a broad spectrum of epileptic disorders, including generalized epilepsy with febrile seizures plus (GEFS+) (Catterall *et al.*, 2010; Escayg and Goldin, 2010). GEFS+ is typically an autosomal dominant disorder hallmarked by febrile seizures (short tonic-clonic attacks during a >38°C fever) that persist beyond childhood and can eventually manifest regardless of temperature. Also, human VGSC mutations and GEFS+ *Scn1a* mouse models have been linked to various sleep defects (Kalume *et al.*, 2015; Martin *et al.*, 2010; Papale *et al.*, 2013; Papale *et al.*, 2010). These findings highlight the importance of VGSC function in the relationship between sleep and epilepsy.

In *Drosophila*, VGSCs are encoded by a single gene *paralytic (para)*, and Sun *et al.* recently created a knock-in fly model of GEFS+ by inserting the human GEFS+-causing *SCN1A* mutation (*SCN1A^{K1270T}* into the corresponding fly locus (*para^{K1353T}* (Sun *et al.*, 2012). *Drosophila* GEFS+ mutants exhibited semidominant heat-induced seizure-like behavior, likely
due to reduced GABAergic inhibitory activity in the central nervous system at high temperatures (>35°C). Electrophysiological analysis revealed that upon temperature elevation the gain-of-function GEFS+ mutation increased sodium currents, leading to sustained depolarization of GABAergic neurons and reduced inhibitory activity. GEFS+ channels also showed reduced activation thresholds regardless of temperature, which could enhance GABAergic signaling at room temperature (Sun et al., 2012). Schutte et al. also created and characterized knock-in flies with a severe loss-of-function channel mutation associated with Dravet syndrome (para^{s1231R}).

Here, we characterized the sleep/wake activity of a fly model of GEFS+, revealing the effects of altered VGSC activity on sleep and probing the possible interactions between sleep and seizure behavior. The GEFS+ gain-of-function VGSC mutation significantly increased nighttime sleep and hastened sleep onset after light off. Both pharmacologic and genetic manipulations implicate increased GABAergic signaling in the enhanced sleep of GEFS+ flies. We also observed that the GEFS+ mutation dominantly disrupted homeostatic sleep regulation, and unexpectedly found that sleep deprivation reduced the susceptibility of GEFS+ mutants to heat-induced seizures. Lastly, it was unexpectedly found that Dravet mutants showed many of the same sleep abnormalities as GEFS+ flies, which adds an unclear component to our model of GABAergic control in sleep and seizure behavior. Overall, our study describes the unique sleep profiles of seizure-prone Drosophila VGSC mutants, and demonstrates the value of fly models for investigating the sleep-seizure interaction.

Materials and Methods

Fly Husbandry

Flies were raised under the conditions of a 12 hr light/dark cycle, at 25°C and 65% humidity on standard cornmeal agar food. For behavioral analyses, newly eclosed flies were collected under CO₂ anesthesia over a two-day period, housed 20/vial (all virgin females or 10 female/10 males), and aged for 3-4 days prior to experimentation. GEFS+ (w{para^{GEFS+}, UAS-GFP}, Dravet
(w para\textsuperscript{DS}, UAS-GFP), and control flies (w; UAS-GFP) were obtained from Dr. Diane O'Dowd (University of California, Irvine) (Sun et al., 2012). GEFS+ (K1270T), Dravet (S1231R), and control (K1270K, S1231S) were homologously recombined in parallel (Schutte et al., 2014; Sun et al., 2012). Canton-S and w\textsuperscript{1118} flies were collected from our common lab stock. The pdf-GAL4 strain was shared by Dr. Bridget Lear (University of Iowa) and the UAS-Rdl-RNAi (v41103) line was acquired from the Vienna Drosophila Resource Center.

**Basal Sleep Analysis**

The *Drosophila Activity Monitor* (DAM) system (TriKinetics, Waltham, MA) was used to record locomotor activity (infrared beam breaks) of individual flies in 1 min bins, and sleep was defined as any inactive bout lasting ≥5 min (Shaw et al., 2000). Basal sleep analyses were carried out using DAM2 monitors in a Fisher Scientific incubator (56 x 61 x 71 cm) at 25°C and ~40% humidity. Flies were gently aspirated into DAM tubes containing 5% sucrose, 1% agar around Zeitgeber time (ZT) 6, and acclimated overnight. Baseline sleep/activity was determined by averaging three consecutive days of data. Sleep and wake parameters were calculated using a custom Microsoft Excel-based file.

**Nighttime Video Tracking**

Flies were gently aspirated into DAM tubes containing 5% sucrose, 1% agar around ZT 6. The tubes were then placed on an infrared light box (140 LED Night Vision Illuminator Lamp) with a light diffuser to observe overnight locomotion in an environmental chamber maintained at 25°C and 65% humidity. A night vision web camera (Agama V-132 1.3M Pixel) was mounted ~15 cm above the flies, and 640X480 resolution still images were taken every 5 sec using Yawcam (free Java software available at yawcam.com). pySolo software (Gilestro and Cirelli, 2009) was used to analyze locomotion, and nighttime sleep/wake parameters were calculated.
Drug Treatment

Carbamazepine (CBZ) was obtained from Sigma-Aldrich (St. Louis, MO) and CBZ treatment was adapted from a previously published protocol (Agosto et al., 2008). CBZ was solubilized in 45% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich) to produce a 40 mg/ml stock solution. Following a baseline day, flies were transferred to 5% sucrose, 1% agar medium containing vehicle or CBZ at ZT 8, and nighttime sleep/wake parameters were calculated.

Circadian Rhythm Analysis

Circadian rhythmicity was analyzed under nearly the same conditions as basal sleep; the only exception was the experimental lighting conditions. Specifically, female flies were subjected to 5 days of 12 hr LD conditions, then 7 days of constant darkness. Free running-period length (tau, \( \tau \)) was calculated using ChronoShop, a software package developed for period analysis (Steiger et al., 2013).

Sleep Deprivation Analysis

Sleep deprivation experiments were performed using DAM5 monitors in an environmental chamber maintained at 25°C and 65% humidity. To perform sleep deprivation, we used the apparatus and protocol described in (Huber et al., 2004). Briefly, the monitors were housed inside a framed box that rotates 180° at a speed of 2-3 revolutions/min once every ~5 min. The monitors dropped approximately 6 cm each rotation, producing a mechanical shock. After one baseline day, 24 hr sleep deprivation commenced at ZT 0. The flies were then given 24 hr to recover. Cumulative sleep loss and rebound were determined relative to each fly’s baseline day. Percent change in sleep for each fly was determined by the formula

\[
\frac{[(24 \text{ hr recovery day sleep} - 24 \text{ hr baseline day sleep})]}{24 \text{ hr baseline day sleep}} \times 100.
\]
Heat-Induced Seizure Assay
Following a 24 hr of no treatment or sleep deprivation, 3-5 day old flies were individually transferred to empty glass vials (15 mm x 45 mm). After 15-30 min of acclimation, vials were submerged in a 40°C waterbath for 2 min. Occurrence or absence of seizing in individual flies was determined every 5 sec, and the proportion of flies seizing at each time point was calculated. Heat-induced seizures were defined as a failure to maintain standing, twitching of the leg, flapping of a wing(s) or curling of the abdomen (Sun et al., 2012).

Statistics
All data presented in this study were generated from two or more independent sets of experiments, with the exception of the longevity analysis, which was carried out once. Unless otherwise stated, “n” represents number of total flies examined. Statistical analyses were performed using SigmaPlot 13.0 (Systat Software, Inc., Point Richmond, CA). ANOVA on Rank (Kruskal-Wallis) and Mann-Whitney Rank Sum tests were employed for multiple and pairwise comparisons, respectively. Most data are represented as box plots with “X” denoting mean values.

Results
GEFS+ mutants show increased nighttime sleep and decreased sleep latency
To examine how an epileptogenic mutation in the Drosophila VGSC gene affects fly sleep, we assessed the sleep/wake behavior of the knock-in GEFS+ mutants and their genetic controls generated by Sun et al. (Sun et al., 2012). Using the Drosophila Activity Monitoring (DAM) system, activities of virgin females, mated females and males were monitored for three days at 25°C under standard 12 hr light/dark (LD) conditions. Fig. 10A shows daily activities of the mutants and control flies averaged over three days. Homozygous GEFS+ virgin and mated females, and hemizygous males exhibited increased daytime activity relative to controls. In contrast, nighttime activity counts were markedly reduced in GEFS+ flies (Fig. 11A, B). Notably,
the activities of GEFS+ mutants sharply dropped upon light off (Fig. 11A).

**Figure 11.** GEFS+ mutation affects sleep/wake behavior. **A)** The 24 hr activity profiles, **B)** 12 hr LD activity counts, and **C)** 24 hr sleep profiles of virgin females (♀), mated females (♀), and males (♂) for knock-in controls (n = 85, 93, 95) and GEFS+ mutants (n = 88, 87, 94). GEFS+ mutants particularly sleep more than controls at night. **D)** Nighttime 12 hr sleep/activity parameters of mated females for control (n = 93), GEFS+ heterozygotes (n = 44), and GEFS+ homozygotes (n = 87). GEFS+ mutation dominantly increases sleep reducing sleep latencies, increasing sleep bout lengths, and reducing wake bout lengths. Data are presented as averages with SEM or boxplots with means ("X"), ANOVA on Ranks, Dunn’s vs control; *p < 0.05, ***p < 0.001.
Despite the greater total daytime activity of flies with the GEFS+ mutation, the effect on daytime sleep was relatively minor (Fig. 11C). In contrast, the reduced nighttime activity of GEFS+ mutants coincided with a dramatic increase in sleep amount (Fig. 11C). For subsequent analyses of GEFS+ sleep, we focused on mated females and their nighttime sleep unless otherwise noted. To further characterize the effect of the GEFS+ mutation on nighttime sleep, various sleep-related parameters during the scotophase (dark phase) were calculated for homozygous mutants, heterozygous mutants, and control flies (Fig. 11D). Corresponding to the rapid decline in activity of GEFS+ mutants after light off, sleep latencies (time to first sleep episode after light off) were considerably reduced in the mutants. The exaggerated nighttime sleep of GEFS+ mutant flies was primarily attributed to longer duration of sleep bouts and shorter duration of wake bouts (Fig. 11D). Together, these observations indicated that both the onset and maintenance of sleep were promoted by the GEFS+ mutation. Despite spending less time awake at night than controls, GEFS+ mutant flies actually had increased waking activity (counts per waking minute), showing that increased nighttime sleep was not simply caused by a general reduction in locomotor capacity.

We also analyzed sleep in the GEFS+ and control flies at higher resolution using a video tracking system, in which the positions of each fly were assessed every 5 sec (Fig. 12). As expected, the video analysis estimated total sleep time at lower values compared to the DAM-based analysis, but recapitulated many of the characteristic features of GEFS+ nighttime sleep, i.e. increases in total sleep time and sleep bout length, and decreases in sleep latency and wake bout length (Fig. 12B).

*Drosophila* GEFS+ mutants display a semi-dominant heat-induced seizure phenotype, with significantly less severe seizure events in heterozygous compared to homozygous mutants (Sun et al., 2012). In contrast, the effect of the GEFS+ mutation on nighttime sleep is fully dominant, with sleep parameters for GEFS+ heterozygotes and homozygotes being indistinguishable (Fig. 11D). Dominantly inherited human *SCN1A* mutations associated with
GEFS+ vary with respect to both penetrance and expressivity (Abou-Khalil et al., 2001; Escayg and Goldin, 2010; Hawkins et al., 2011; Xu et al., 2012). To determine whether the sleep phenotype of the GEFS+ mutant observed here occurs in other genetic backgrounds, control and GEFS+ homozygous females were out-crossed once to two standard laboratory strains (Canton-S and w^{1118}). Heterozygous female progeny from both crosses also displayed increased nighttime sleep and reduced sleep latency compared to those of genetic controls.
albeit with reduced severity (Fig. 13). Thus, although genetic backgrounds significantly influence sleep, the GEFS+ mutant sleep phenotype was detectable in heterozygotes of different genetic backgrounds.

The GEFS+ mutation has no effect on lifespan

The sleep phenotypes of GEFS+ mutants are the opposite of those of other seizure-prone fly mutants such as Shaker (Sh), quiver/sleepless (qvr/sss) and Hyperkinetic (Hk), all of which display significantly reduced sleep [18-20]. Because these other mutants all have decreased longevity, we examined our GEFS+ model for effects on lifespan. Under normal rearing conditions (12 hr LD cycle at 25°C and 65% humidity), there was no significant effect of GEFS+ mutation on the lifespan of virgin females (Fig. 14).

Figure 13. Nighttime sleep parameters of control and heterozygous GEFS+ mated females from an outcross with wild-type genetic backgrounds (Canton-S and w1118). Canton-S control and GEFS+/+ (n = 32, 64), w1118 control and GEFS+/+ (n = 30, 64). Data presented as boxplots with means (“X”), ANOVA on Ranks, Dunn’s multiple comparisons; **p < 0.01, ***p < 0.001.

Figure 14. Percent survival of control and GEFS+ virgin females. Flies were raised in ~20 flies/vial, at 25°C 65% humidity, and transferred to new vials every 3-4 days; control (n = 141), GEFS+ (n = 95). Data presented as daily averages of surviving flies in each vial with SEM, Survival Log Rank analysis.
Sleep is differentially affected by GABAergic manipulation in GEFS+ and control flies

As observed in mammals, the inhibitory GABAergic system significantly regulates sleep in *Drosophila* (Agosto et al., 2008; Chung et al., 2009; Cirelli, 2009; Crocker and Sehgal, 2010; Parisky et al., 2008). Specifically, previous studies have demonstrated that circadian-regulated GABAergic inhibition promotes the initiation and maintenance of sleep (Agosto et al., 2008; Chung et al., 2009; Parisky et al., 2008). In fact, *RdlMDRR* mutants, which have enhanced GABAergic transmission due to altered channel properties of the GABA_A receptor (Resistant to dieldrin; Rdl), show sleep phenotypes similar to those of GEFS+ mutants — shorter sleep latency and increased sleep. Electrophysiological analyses at room temperature (23°C) have demonstrated that the *Drosophila* GEFS+ sodium channels display reduced activation thresholds as compared to controls in adult brain GABAergic interneurons, indicating that GABAergic inhibition is increased in the context of the mutant channel (Sun et al., 2012). Therefore, we hypothesized that enhanced GABAergic transmission was the primary cause of the GEFS+ sleep phenotypes we observed. To investigate this possibility, we pharmacologically targeted the *Drosophila* Rdl GABA_A receptor using carbamazepine (CBZ) (Fig. 15). In mammals, CBZ is thought to act as an anticonvulsant by allosteric agonism of GABA_A and stabilizing the inactive state of voltage-gated sodium channels. In flies, CBZ actually reduces GABAergic transmission by accelerating the desensitization of Rdl, and dose-dependently decreases total sleep and increases sleep latency (Agosto et al., 2008). As expected, CBZ feeding reduced nighttime sleep and extended sleep latency in a dose-dependent manner in both control and GEFS+ flies (Fig. 15A). However, while the lowest concentration of CBZ (0.1 mg/ml) significantly influenced nighttime sleep and sleep latency in control flies, its effects did not reach statistical significance in GEFS+ mutants (Fig. 15B, D). Furthermore, when the nighttime sleep of CBZ-fed flies was normalized to that of vehicle-fed flies of the same genotype, GEFS+ mutants showed significantly less percent change in nighttime sleep relative to control flies at every CBZ concentration (Fig. 15C). Based on these results, GEFS+ mutants
appear to be unusually resistant to pharmacologic suppression of GABAergic transmission.

GABAergic transmission regulates fly sleep largely through the inhibition of wake-promoting clock neurons that express the neuropeptide pigment dispersing factor (PDF) (Chung et al., 2009; Parisky et al., 2008; Shang et al., 2008). To examine the role of GABA and PDF neurons in control and mutant flies we knocked down the expression of Rdl GABA\textsubscript{A} receptors specifically in PDF neurons using pdf-GAL4 and UAS-Rdl-RNAi. Total nighttime sleep was reduced in both control flies and GEFS+ heterozygous mutants upon PDF neuron-specific Rdl knockdown (Fig. 16A). This result was expected in light of the decreased inhibition of wake-

Figure 15. Pharmacologic suppression of GABA\textsubscript{A} receptor function differentially affects sleep in control and GEFS+ flies. A) Sleep profiles of control (n = 63, 60, 62, 60) and GEFS+ (n = 62, 61, 63, 62) flies fed vehicle or various concentrations of CBZ starting at ZT 8 (arrow). B) CBZ feeding decreased nighttime sleep; ANOVA on Ranks, Dunn’s within genotype compared to vehicle-fed flies. C) The percent change of nighttime sleep normalized within genotype to vehicle-fed flies revealed that GEFS+ mutants were more resistant to CBZ as compared to control flies at each CBZ concentration. Data are presented as averages with SEM or boxplots with means ("X"), Rank Sum tests; *p < 0.05, **p < 0.01, ***p < 0.001.
promoting PDF neurons resulting from Rdl knockdown, and consistent with the results from previous studies (Chung et al., 2009; Parisky et al., 2008). The extent of nighttime sleep reduction caused by Rdl knockdown, which was calculated by finding the difference between experimental data (pdf-GAL4/+ ; UAS-Rdl-RNAi/+ ) and the average of control data (+/+ ; UAS-Rdl-RNAi/+ ) for each genotype, was not significantly different between control flies and GEFS+ heterozygous mutants (Fig. 16A).

Unlike total nighttime sleep, sleep latency of control flies and GEFS+ heterozygous

![Graph showing sleep latency](image)

**Figure 16.** Rdl GABA$_A$ knockdown in PDF-positive neurons differentially influences sleep latency behavior in GEFS+ mutants. A,B) Rdl knockdown in the PDF neurons of control and heterozygous GEFS+ mutants; UAS-Rdl-RNAi (n = 55), pdf-GAL4/Rdl-RNAi (n = 49), GEFS+/+ ; Rdl-RNAi (n = 56), GEFS+/+ ; pdf-GAL4/Rdl-RNAi (n = 38). A) Rdl knockdown in PDF neurons reduced sleep to the same extent in both control and GEFS+ flies, but B) specifically increased sleep latencies in heterozygous GEFS+ mutants and not in control flies; ANOVA on Ranks, Dunn’s Multiple Comparisons. Extent of change caused by Rdl knockdown was calculated by subtracting experimental data from the averages of RNAi only controls. C) Representative 20x confocal z-stack images suggest that GEFS+ mutants had no gross morphological abnormalities in PDF neurons. All data presented as boxplots with means (“X”), Rank Sum tests; **p < 0.01, ***p < 0.001.
mutants were differentially influenced by *Rdl* knockdown in PDF neurons (Fig. 16B). Under the conditions we used, PDF neuron-specific *Rdl* knockdown did not increase sleep latency in control flies, which is inconsistent with a previous observation (Parisky et al., 2008). This is probably due to the fact that only a single copy of each transgene (*pdf-GAL4* and *UAS-Rdl-RNAi*) was used here. We found, however, that *Rdl* knockdown did significantly increase the sleep latency of GEFS+ heterozygous mutants, restoring sleep latencies in GEFS+ mutants to levels comparable to those in their genetic controls. Further, the extent of change caused by *Rdl* knockdown between the sleep latencies within control flies and GEFS+ mutants was statistically significant (Fig. 16B).

To verify that GEFS+ mutants do not simply have reduced or abnormal PDF-positive neuron morphology, we examined control and mutant brains expressing GFP under the *pdf* promoter (*pdf-GAL4 > UAS-GFP*). Confocal auto-fluorescent images indicate that GEFS+ mutants have grossly normal PDF+ neurons, suggesting that abnormal neurodevelopment of wake-promoting neurons does not appear to underlie the GEFS+ sleep phenotype (Fig. 16C).

**Effects of altered nighttime lighting conditions on GEFS+ sleep phenotypes**

GEFS+ mutants showed phase-specific abnormalities in locomotor activity — an increase during photophase and a decrease in scotophase as compared to genetic controls. Their sleep abnormalities were primarily observed during the scotophase (Fig. 11). Furthermore, the effect of the GEFS+ mutation on short sleep latency depended on the level of *Rdl* in light-activatable PDF-positive neurons (Fig. 16B). These observations indicate that light may play an important role in the expressivity of the GEFS+ sleep phenotype. To better understand how light influences GEFS+ sleep, sleep/wake activity was analyzed under abnormal nighttime lighting conditions. When exposed to constant light following a baseline light/dark day, both control flies and GEFS+ mutants dramatically reduced subjective nighttime sleep (Fig. 17A, B). Under this constant light condition, GEFS+ mutants still spent considerably more time asleep than controls.
Sleep latencies at subjective dusk (36 hr from start of baseline day) of both control and GEFS+ flies were lengthened in response to constant lighting conditions, eliminating the short sleep latency phenotype of GEFS+ mutants (Fig. 17C).

Nocturnal light interruption has been shown to promptly induce waking (Liu and Zhao, 2014), so we examined behavioral responses of control and mutant flies to light during the scotophase. Following a baseline light/dark day, flies were subjected to a 1 hr light stimulus 5 hr after light off (41-42 hr from start of baseline day). Both control and GEFS+ flies robustly suppressed sleep in response to the acute light exposure (Fig. 17D). Interestingly, GEFS+
mutants were significantly more active during the light stimulus relative to controls as judged by the total number of activity counts (Fig. 17E). Upon light removal (42 hr), sleep latencies in control and mutant flies were consistent with those seen at normal light off times (12 hr and 36 hr), and, therefore, the effect of GEFS+ mutation on rapid sleep onset was still observed (Fig. 17F). These results show that GEFS+ mutants can be aroused from their exaggerated nighttime sleep by light stimuli, become hyperactive during scotophase light, and maintain the propensity to rapidly initiate sleep in response to a light off signal during the night.

**GEFS+ mutants have normal circadian locomotor rhythms**

Fly sleep is regulated by circadian and homeostatic mechanisms. To explore whether the GEFS+ mutation alters circadian regulation, free-running activity rhythms of control flies and GEFS+ mutants were examined during seven days of constant darkness following five days of 12 hr LD. As determined by $\chi^2$ periodogram analyses, both GEFS+ and control flies displayed robust rhythmicity in dark/dark conditions and maintained comparable mean circadian periods (Fig. 18), indicating that the GEFS+ mutation does not have a significant effect on the central circadian clock.

![Figure 18. Circadian regulation is intact in GEFS+ mutants.](image) Locomotor activity profiles of control ($n = 22$) and GEFS+ ($n = 30$) flies during 5 light/dark (LD) days then seven constant dark (DD) days. GEFS+ flies show normal circadian rhythmicity under both LD and DD treatment (inset). Data presented as averages with SEM.
**GEFS+ mutants are defective in homeostatic regulation of sleep**

Next, we examined whether the GEFS+ mutation affects homeostatic sleep regulation.

Following a baseline day, flies were mechanically deprived of sleep for 24 hr using the protocol described in (Huber et al., 2004) and then given one day to recover. Control flies exhibited shorter sleep latency following sleep deprivation and recovered all of their sleep loss by the end of the recovery period (Fig. 19). In striking contrast, GEFS+ mutants had a severe impairment in homeostatic regulation, and showed almost no sleep rebound after 24 hr of deprivation. The defects in homeostatic regulation of sleep were also observed, at similar levels, in heterozygous GEFS+ mutants (data not shown).

**Figure 19. GEFS+ mutants lack homeostatic sleep regulation.**

A) The 24 hr sleep profiles of baseline day and recovery day following 24 hr sleep deprivation in control (n = 81) and GEFS+ mutants (n = 86). B) The 24 hr percent time asleep and C) subjective sleep latencies for baseline and recovery days. D) Cumulative sleep loss during 24 hr sleep deprivation and recovery. Sleep debt is presented relative to each genotype’s baseline sleep. E) Percent change in 24 hr rebound sleep relative to baseline. Data presented as averages with SEM or boxplot with means (“X”), ANOVA on Ranks, Dunn’s vs baseline data within genotype, Rank Sum test; ***p < 0.001.
Sleep deprivation affects seizure susceptibility

Since sleep deprivation is a common trigger of seizure in epileptic patients (Ellingson et al., 1984; Malow, 2004), we investigated whether 24 hr sleep deprivation affects the heat-induced seizure susceptibility of control and GEFS+ flies. Seizure susceptibility between ZT 0 and ZT 1 was assessed using the protocol described in Sun et al. (2012). Without sleep deprivation, control flies did not show any seizure-like behavior during a 2 min exposure to 40°C (0%, n = 57). However, a significant proportion of control flies had at least one heat-induced seizing episode after sleep deprivation (14%, n = 63; p < 0.01 Fisher’s exact test). In contrast, the severity of the GEFS+ seizure phenotype was reduced after being sleep deprived. Sleep-deprived GEFS+ mutants were less likely than untreated GEFS+ mutant counterparts to have a seizure during a 2 min 40°C exposure (Fig. 20). Also, while all untreated GEFS+ mutants displayed at least one seizure, a portion of the sleep-deprived mutants never had a seizure during the observation period (100%, n = 69 vs 94%, n = 68).

Dravet mutants show sleep abnormalities similar to that of GEFS+ flies

There exists another epilepsy knock-in fly that models severe myoclonic epilepsy of infancy, also known as Dravet syndrome (DS). DS flies were homologously recombined into the same genetic background as GEFS+ flies, so are therefore directly comparable. Unlike GEFS+ channels, DS VGSCs show significantly reduced function regardless of temperature, resulting in reduced GABAergic neuron firing (Schutte et al., 2014). At first, based on our GEFS+ findings,
we predicted that DS mutants would sleep much less than control flies. However, DS fly sleep was remarkably similar to GEFS+ mutants (Fig. 21).

DS mutants were somnolent in the day and night, and heterozygotes showed incomplete penetrance, as compared to controls (Fig. 21A). Like the nighttime sleep phenotype of GEFS+ flies, DS mutants also had reduced sleep latencies, increased sleep bout lengths, and reduced wake bout lengths (Fig. 21B). Unlike GEFS+ flies, though, DS mutants also had a significant reduction in the number of nighttime sleep bouts. Considering that the Dravet mutation renders a severe loss-of-function VGSC, these findings suggest that even in the context of reduced GABAergic inhibition, wake-promoting PDF neurons still show reduced activity, which is likely because these neurons also express mutant Dravet channels. Interestingly, constant light conditions reduced DS mutant sleep, though not to the same extent as controls (Fig. 21C). This is consistent with the idea that light-activatable PDF neurons in DS flies are intact but less responsive than normal.

To further dissect the similarities and differences between GEFS+ and DS sleep, we turned again to pharmacological manipulation of GABAergic transmission. Recall that GEFS+ mutants were more resistant than controls to the wake-promoting effects of carbamazepine (CBZ), supporting the model that enhanced GABAergic transmission underlies the GEFS+ sleep phenotype (Fig. 15). In Dravet mutants, CBZ feeding had little effect on reducing nighttime sleep, even at high concentrations (Fig. 21D). In fact, whereas 0.4 mg/ml CBZ nearly abolished all nighttime in control flies, DS mutants only decreased their sleep by about 5% (Fig. 20E) – this concentration reduced GEFS+ sleep by about 70%. Thus, despite having similar basal sleep phenotypes, GEFS+ and DS mutants have differing pharmacologic responses to GABAergic suppression.

We then assessed the response of DS mutants to 24 hr mechanical sleep deprivation. Like GEFS+ flies, DS mutants failed to show homeostatic rebound (Fig. 21F). Additionally,
Figure 21. Dravet mutants have sleep problems similar to GEFS+ mutants, but seizure susceptibility does not change after sleep deprivation. A) Sleep and B) nighttime sleep/activity parameters of mated female controls (n = 51), DS heterozygotes (n = 38), and homozygotes (n = 59). C) Sleep and nighttime percent change in sleep of control (n = 57) and DS (n = 64) flies under constant light. D) Sleep of control (n = 52, 28, 56) and DS (n = 48, 49, 50) flies fed CBZ at ZT 8 (arrow). E) Vehicle-normalized nighttime percent change in sleep due to CBZ feeding. F) Cumulative sleep during 24 hr sleep deprivation and 24 hr recovery, and percent sleep change during recovery. G) Percentage of seizing flies for untreated (n = 12, 30) and 24 hr sleep-deprived (n = 13, 20, 30) GEFS+ mutants when exposed to 36°C. Data presented as averages with SEM or boxplot with means (“X”), Rank Sum test, ANOVA on Ranks, Dunn’s vs control; *p < 0.05, **p < 0.01, ***p < 0.001.
after sleep deprivation treatment, DS flies showed no change in heat-induced seizure susceptibility (Fig. 21G), albeit when examined at a lower 36°C temperature. This phenotype is strikingly different than the seizure resistance that was observed in GEFS+ flies after deprivation. The mechanism for this difference is still unclear; therefore, future experiments are needed to further elucidate this interesting phenomenon.

**Discussion**

**Basal Sleep Abnormalities**

To investigate the effects of epilepsy-causing VGSCs on sleep, we characterized the sleep/wake behavior of flies harboring human *SCN1A* mutations for GEFS+ (*para*<sup>K1270T</sup>) and DS (*para*<sup>S1231R</sup>). We found that both GEFS+ and DS flies displayed abnormalities in sleep regulation; compared to their genetic controls, mutants fell asleep significantly faster at lights off and slept significantly longer at night (Fig. 11, 21). The sleep phenotype of GEFS+ flies was robust enough to be observed regardless of sex, mating state or genetic background, and was completely dominant in heterozygotes (Fig. 11-13). Homozygous DS flies slept even more than GEFS+ flies, and showed similar sleep abnormalities to GEFS+ flies (Fig. 21). Our working model (Fig. 22) is that both GEFS+ and DS voltage sodium channel mutants fail to properly promote wakefulness by different means.

Although the GEFS+ and DS mutants have seizures at high temperatures, their sleep phenotypes are opposite of other seizure-prone, hyperexcitable fly mutants such as *Sh*, *Hk* and *qvr/sss*, all of which have reduced sleep (Bushey et al., 2007; Cirelli et al., 2005; Koh et al.,
The GEFS+ and DS sleep phenotype likely reflects their gain- and loss-of-function channel properties in regulating sleep circuitry (Fig. 22). Previous electrophysiological analyses of GABAergic interneurons in the mutant brain indicated that at room temperature (23°C) the GEFS+ mutation leads to a decrease in the threshold voltage for activating sodium currents, resulting in increased GABAergic inhibitory activity as compared to controls (Sun et al., 2012). Whereas at high temperature (35°C), GEFS+ mutation has a deleterious gain-of-function effect in GABAergic interneurons, significantly increasing sodium currents due to defective channel inactivation and prolonging post-stimulus depolarization, which reduces GABAergic activity and induces seizures (Sun et al., 2012). DS mutation at either room or high temperature resulted in severe loss-of-function VGSC currents, resulting in reduced GABAergic transmission and seizure susceptibility (Schutte et al., 2014). Our findings here support the involvement of the GABAergic inhibitory system particularly in the manifestation of GEFS+ sleep phenotypes, but not in DS sleep. As shown in Fig. 15, GEFS+ mutants were more resistant than control flies to the sleep-suppressing effect of CBZ, a drug that accelerates desensitization of the Drosophila GABA_A receptor Rdl. We can neither rule out the possibility that CBZ differentially affects voltage-gated sodium channel activity, nor rule out whether GEFS+ mutants ate less CBZ-containing food than control flies, but our results together with the previous electrophysiological findings suggest that GABAergic transmission is indeed enhanced in GEFS+ mutants. Resistance to CBZ has also been observed in gain-of-function Rdl^{A302S} mutants, which have increased total sleep and short sleep latencies (Agosto et al., 2008) — sleep phenotypes similar to those of GEFS+ mutants. In Fig. 21, DS mutants show extreme resistance to CBZ, suggesting that failed activity of the wake-promoting PDF neurons, and not reduced GABAergic transmission, underlies their basal sleep phenotype.

PDF-positive neurons are essential targets of GABAergic inhibition in Drosophila sleep regulation (Chung et al., 2009; Li et al., 2013; Liu et al., 2014; Parisky et al., 2008; Sheeba et al., 2008). We found that total sleep time decreased and sleep latencies increased in GEFS+
mutants when \( Rdl \) expression was knocked down using RNAi specifically in PDF-positive neurons (Fig. 16). Interestingly though, under the conditions we used, the sleep latencies of control flies were not significantly affected by this genetic manipulation, indicating the increased sensitivity of sleep latencies to knockdown of \( Rdl \) expression in GEFS+ mutants as compared to control flies. These findings seem contradictory to our results with CBZ feeding, where GEFS+ mutants were more resistant than controls to functional suppression of Rdl. However, we think that these data are consistent with the idea that enhanced inhibitory GABAergic transmission to PDF-positive neurons underlies GEFS+ sleep phenotypes. Our interpretation is that PDF neurons express extra Rdl channels, which, although not necessary for normal GABAergic transmission, are required to respond to increased GABA release from GABAergic neurons. Thus, CBZ must block extra Rdl channels in GEFS+ mutants to compete with their increased GABAergic transmission. Further, mild genetic knockdown of \( Rdl \) (one copy of \textit{pdf-GAL4} and \textit{UAS-Rdl-RNAi} transgenes) removes the extra Rdl channels, preferentially affecting enhanced GABAergic transmission in GEFS+ mutants, but having less effect on normal GABAergic transmission in control flies. This model would explain why sleep is more resistant to CBZ feeding; yet, sleep latencies are more sensitive to \( Rdl \) knockdown in PDF neurons, in GEFS+ compared to control flies.

Based on the response of DS mutants to CBZ, we expect that knocking down \( Rdl \) in PDF neurons would not reduce the exaggerated sleep of these mutants. Future experiments will instead test whether expressing wildtype \textit{para} cDNA particularly in PDF neurons can restore or significantly reduce sleep in DS mutants. Similar genetic rescue experiments in GEFS+ flies using \textit{para-RNAi} in GABAergic neurons can also be pursued.

**Effects of Light Cue**

A characteristic feature of GEFS+ and DS mutants is their photophase- and scotophase-specific abnormalities in locomotor activity and sleep (Fig. 11, 21). Namely, locomotor activity of GEFS+
mutants is increased during the photophase, but decreased during the scotophase, when compared to control flies (Fig. 11). GEFS+ mutation also preferentially affects nighttime sleep (Fig. 11C, D). DS mutants showed somnolence in both day and night (Fig. 21A, B). While mutant VGSCs could affect the neuronal outputs of endogenous rhythm behaviors, our experiments under different nighttime light conditions suggest that the light stimulus is a major factor in mutants’ phase-specific phenotypes (Fig. 17, 21C). We found that a light off signal was required to observe the rapid sleep onset of GEFS+ and DS mutants at dusk. In addition, turning on the light in the middle of subjective nighttime resulted in hyperactive GEFS+ mutants (Fig. 17E), and removal of the light caused rapid sleep initiation similar to sleep onset at normal dusk. Together these findings indicate that the VGSC mutation may increase the sensitivity of relevant neuronal circuits and narrow the buffering of behavioral responses to light on and off stimuli.

Considering that dysregulation of PDF neurons likely causes the abnormal sleep in GEFS+ mutants, it was interesting to find that mutants did not display rhythmicity problems during free-running dark/dark conditions (Fig. 18). Among PDF-expressing cells, the small ventrolateral neurons (sLNvs) are considered the main pacemakers for circadian activity (Grima et al., 2004; Stoleru et al., 2004), while large ventral lateral neurons (ILNvs) distinctly mediate light-dependent arousal (Shang et al., 2008). Our results indicate that VGSC modification by the GEFS+ mutation does not affect the circadian sLNvs function, but significantly influences ILNv activity. Dravet mutants have not yet been examined for rhythmicity under dark/dark conditions, however, a recent study shows that some clock neurons are inherently regulated in a biphasic manner (Flourakis et al., 2015). Thus, we suspect that DS mutants, like GEFS+ flies, also display normal circadian rhythmicity.
Homeostatic Rebound and Seizure Susceptibility

In addition to basal sleep abnormalities, the GEFS+ and DS mutants showed a severe defect in homeostatic sleep rebound after 24 hr sleep deprivation (Fig. 19, 21F). Although the neural mechanisms underlying sleep homeostasis remain unclear, it is thought that the central nervous system monitors and evaluates the quality and quantity of sleep, and then facilitates compensatory sleep by altering the sleep rheostat. A recent study demonstrated that chronic sleep deprivation enhances the excitability of PDF-positive ILNvs (Tabuchi et al., 2015). This finding is counterintuitive since ILNvs are wake-, but not sleep-, promoting neurons. Perhaps the ILNv excitability induced by sleep deprivation serves as a signal of sleep loss, which subsequently results in the activation of sleep-promoting circuits. It is possible that GEFS+ and DS mutants are defective in such a signal. Alternatively, the VGSC mutation may affect the output arm of the sleep homeostat where gain- or loss-of-function control disrupts proper homeostasis. Another recent study has shown that sleep deprivation increases the neuronal excitability of sleep-promoting neurons that project to the dorsal fan-shaped body (FB) (Donlea et al., 2014). Therefore, VGSC mutations may directly or indirectly impact the compensatory actions of sleep-promoting dorsal FB neurons, thus preventing sleep restoration. Future electrophysiological, genetic, and live imaging studies are needed to determine the relevant circuitry in GEFS+ and DS mutants, which underlies their homeostatic sleep failure.

Sleep deprivation often exacerbates the onset, frequency and severity of seizures in epileptic patients and rodent models of epilepsy. We found that control flies showed a greater susceptibility to heat-induced seizures after experiencing 24 hr sleep deprivation. Unexpectedly, in GEFS+ mutants, sleep deprivation actually reduced the probability of heat-induced seizures (Fig. 20) and in DS mutants, sleep deprivation had no effect on their seizure susceptibility (Fig. 21). These results are contrary to a new study showing that sesB and sel^{st1} mutants increase their bang-sensitivity (vortex and recover assay) following 12 hr of sleep deprivation (using a SNAP device (Shaw et al., 2002)) (Lucey et al., 2015). Their study used both a different
deprivation method as well as non-VGSC mutants, but found a seemingly more relevant result. However, we feel that our unexpected findings raise interesting possibilities for understanding sleep deprivation-induced changes in seizure susceptibility. For example, sleep deprivation may alter the properties of particularly neural circuitry involved in seizure susceptibility. We are currently investigating the role of wake-promoting PDF-positive ILNvs in heat-induced seizing since they have broad neuronal arbors and exhibit increased excitability following deprivation (Tabuchi et al., 2015). Our findings also suggest that proper VGSC function is necessary for the mechanism by which sleep deprivation affects seizure propensity. The differing responses of GEFS+ and DS mutants further support a prominent role of VGSCs in this behavior. And thus, using our methodology, we propose that studying more electrophysiologically characterized VGSC and non-VGSC mutants will help resolve the sleep/seizure relationship.

**Translational Implications**

Mouse models of GEFS+ or Dravet mutations in *Scn1a* generally display reduced activity in inhibitory GABAergic neurons, but not in glutamatergic excitatory neurons. This phenomenon is thought to be due to isoform specific or preferential expression of *Scn1a* in inhibitory neurons (Escayg and Goldin, 2010). Unlike mammals, which have multiple VGSC genes, *Drosophila* has only one (*paralytic*). The GEFS+ and Dravet mutation described here are in constitutively included exons, yet multiple splice forms of *paralytic* are expressed throughout the fly. In spite of this, Sun *et al.* and Schutte *et al.* inferred stronger effects of GEFS+ and Dravet mutation on inhibitory GABAergic neurons rather than on excitatory cholinergic or motor neurons (Schutte et al., 2014; Sun *et al.*, 2012). Our study shows the critical role of GABAergic inhibition in manifestation of GEFS+ and Dravet sleep-like behavior. These findings suggest that neurons controlling sleep and seizures exhibit diverse sensitivity to altered neuronal excitability due to VGSC mutation. We propose that further studies using VGSC mutants will help elucidate how sleep is regulated through interactions of multiple neural circuits with distinct
electrophysiological properties. It is also important to remember that the classical mammalian definition of sleep and arousal thresholds was not measured in our study. Therefore, our results must be interpreted with care since altered levels of arousal and sleep quality differences could better explain how VGSCs influence the stabilization of neuronal function and behavioral activity of flies.

Many human studies, as well as research on murine models of epilepsy, have reported various sleep deficits including abnormal NREM/REM, disrupted circadian rhythm and poor homeostatic rebound (Kalume et al., 2015; Papale et al., 2013). For example, Papale et al. reported that heterozygous mouse Scn1a mutants carrying a hypomorphic loss-of-function GEFS+ mutation (R1848H) exhibit increased wakefulness and reduced amounts of NREM and REM sleep during the scotophase, but show normal sleep recovery after sleep deprivation (Papale et al., 2013). More recently, Kalume et al. documented the profound disruption of sleep during the photophase and impaired homeostatic sleep rebound in mice heterozygous for a Scn1a null allele, a model of Dravet syndrome (DS) — a severe, childhood-onset and often refractory epilepsy syndrome (Kalume et al., 2015). These studies show that epilepsy-related VGSC mutants could display significantly different sleep phenotypes depending on the severity of reduction in sodium channel function. Although GEFS+ VGSC mutations in both fly and mouse are shown to induce febrile seizures and modulate sleep preferentially through GABAergic inhibitory neurons, there are a number of neuroanatomical and physiological (e.g., body temperature) differences that could account for the differences in their respective sleep phenotypes. Nonetheless, our study using fly GEFS+ and Dravet models provides a unique opportunity to further investigate the basic biological mechanisms underlying the effect of an epileptogenic mutation on sleep regulation, as well as on the bidirectional relationships between sleep and seizures.
CHAPTER IV: SUMMARY AND DISCUSSION

During this thesis work I was able to study the rich history of using *Drosophila* to explore how genes ultimately impact organismal behavior (Chapter I). Using both traditional and innovative techniques, I was able to pursue two projects with similar goals (Chapter II and III). One goal was to identify gaps in our knowledge about particular genes and their function in specific behaviors. Another goal was to address the mechanisms of these processes using various *Drosophila* genetic tools and behavioral assays. And the last goal was to relate my findings to previously published literature and describe their translational importance. Here, I will briefly summarize and discuss the overarching picture and future direction of each research investigation.

**The Dopamine Ecdysone Receptor**

In Chapter II we provided evidence that *Drosophila* DopEcR, a unique G protein-coupled receptor (GPCR) for dopamine and ecdysone, promotes sedation and suppresses hyperactivity during ethanol exposure. We then implicated upstream ecdysone regulation and downstream EGFR/ERK and cAMP signaling in DopEcR-mediated control of sedation, and suggested a dopaminergic role for DopEcR’s role in hyperactivity. These findings help us better understand the role of non-classical steroid GPCRs in neuroendocrinology and alcohol research.

GPCRs are one of the largest evolutionarily conserved protein families. They are also necessary in virtually all physiological processes and, therefore, make extremely attractive pharmaceutical targets – cornering >30% of the drug market (Garland and Gloriam, 2011). Despite being the focus of much research, nearly 100 non-olfactory GPCRs remain “orphan receptors” with unidentified ligands, providing a tantalizingly untapped source of possible neuromodulators (Civelli, 2012). GPCRs and other membrane-associated proteins like ion channels have long been known to be allosterically or directly affected by steroid hormones. However, due to steroid- and cell-type specific actions, the response of GPCRs – orphan or
otherwise – to steroids particularly in the nervous system, has been challenging to decipher. Our investigation into *Drosophila* DopEcR sheds necessary light onto this problem and provides a model to further scrutinize. For instance, how might steroid hormones bind to non-classical steroid GPCRs? This question could be addressed by observing the consequence of mutating the three CRAC sequences (putative consensus motifs for cholesterol binding (Wang et al., 2013; Wang et al., 2014)) in *DopEcR*. How might different neuronal subtypes expressing the same non-classical GPCR respond to steroid stimulation? Employing trans-sectional genetics and live Ca$^{2+}$ imaging techniques can refine the precise spatiotemporal necessity of DopEcR. Where might non-classical steroid GPCRs reside within the cell – post/pre-synaptic, on the endoplasmic reticulum? Creating and characterizing a fluorescently tagged DopEcR transgene would help reveal the subcellular expression, trafficking, and localization of this receptor. Lastly, how does non-classical steroid GPCR signaling coincide with classical steroid processes? Since DopEcR is expressed very early in development, it would be interesting to observe whether transcriptional activity by classical ecdysteroid signaling is influenced by DopEcR activity.

When considering the translational impact of DopEcR, it is important to remember that unlike other highly conserved GPCRs or alcohol-associated genes, *Drosophila* DopEcR has no direct mammalian homolog. However, DopEcR does bear striking resemblance to the estrogen GPCR, GPER1 (formally orphan GPR30) (Evans et al., 2014). Our findings with DopEcR mediating alcohol-induced behaviors provide sufficient rationale for investigating whether GPER1 has a role in mammalian alcohol response especially since recent evidence has shown that GPER1 functions in the mouse nervous system (Kastenberger and Schwarzer, 2014; Kosaka et al., 2012). Future works should closely compare the orphan GPCRs that are predicted to be putative orthologs of DopEcR by Ensembl gene tree – GPR101, GPR161, GPR52, GPR21 (Vilella et al., 2009). Perhaps these receptors are unidentified non-classical steroid GPCRs and/or influence behavioral response to alcohol.
Epileptogenic Voltage-Gated Sodium Channels

In Chapter III we explored the relationship between sleep and seizures in *Drosophila* models of human epilepsy. Voltage-gated sodium channel (VGSC) mutants displayed various activity and sleep abnormalities as compared to control flies. Specifically, they slept more at night, lacked homeostatic rebound, and showed reduced seizure susceptibility after sleep deprivation. These findings highlight the conserved nature of a sleep/seizure relationship, and help establish an experimental approach for resolving the intricate mechanisms underlying this connection.

VGSCs are essential for the initiation and propagation of action potentials in neurons. And, thus, any changes in the properties of VGSCs – voltage gating, pore selectivity, inactivation – can, and do, have serious consequences. In fact, more than 600 hundred epilepsy-causing mutations have been reported in one of the nine human VGSC genes, *SCN1A*, which accounts for roughly 70% of children afflicted with a spectrum of seizure disorders (Catterall et al., 2010). In hopes of predicting genotype-phenotype correlations and discovering environmental factors that contribute to expressivity and penetrance, known deleterious mutations associated with the milder generalized epilepsy with febrile seizures plus (GEFS+) and the more severe Dravet syndrome (DS) are now being used to model these conditions in animals. Our investigation into how GEFS+ and DS flies sleep provides unique insight into sleep circuitry and regulation, as well as the impact of sleep deprivation on seizure susceptibility. Future experiments can help address outstanding scientific questions in the field of sleep and intractable epilepsy. Which neurons in the sleep/wake circuitry are more or less plastic to changes within an epileptic context? Performing a neuronal subset GAL4 screen with temporally controllable effector transgenes, like UAS-TrpA1 or UAS-shi ts, to identify changes in the sleep/wake activity of various seizure-prone mutants would begin to answer this question. These experiments should also focus on GABAergic circuitry since it remains unclear why these neurons are particularly affected by VGSC mutations. Contrariwise, how might sleep disorders predispose healthy or epileptic patients to seizures? Further investigation into the seizure
susceptibility of fly sleep mutants is required to address the commonality of sleep and seizure disorders observed in mammals.

The translational potential for studying genetic epilepsy and sleep behavior in *Drosophila* is tremendous. Flies provide a cheap and scalable platform from which to study disease-causing mutations. With efficient genome editing tools like CRISPR/Cas9, high-throughput behavioral assays, and rigorous electrophysiological analysis, unique VGSC mutants will individually and collectively provide better resolution of the relationship between sleep and seizures. It is important to keep in mind that *Drosophila* relies on numerous isoforms from a single voltage-gated sodium channel gene, *paralytic*. The precise nature and compensatory regulation of *paralytic* splicing will likely require RNA-seq information, preferably from specific neuronal subsets like GABAergic neurons.
REFERENCES


