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Characterization of Seizure Suppression in *Drosophila*

by

Iris Christine Howlett

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy
in

Molecular and Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, BERKELEY

Committee in charge:

Professor Mark Tanouye, Chair
Professor Gian Garriga
Professor Iswar Hariharan
Professor Patrick O'Grady

Spring 2013

Characterization of Seizure Suppression in *Drosophila*

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by

Iris Christine Howlett

Abstract

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University of California, Berkeley

Professor Mark Tanouye, Chair

Epilepsy and seizure disorders affect a large portion of the population. These disorders are complex in both cause and in phenotypic manifestation. Genetic factors have been shown to play a role in seizure sensitivity. Much research has been focused on the genetics underlying the mechanisms of seizure sensitivity and the development of treatments for seizure disorders. Despite this, many questions remain unanswered. Using *Drosophila* as a genetic model for epilepsy and seizure disorders has given us some insight into the mechanisms of seizure susceptibility, particularly in suppression of seizures. The following dissertation, details a forward genetics screen for suppressors of seizures in $para^{bss1}$ and identification of *gilgamesh (gish)* as a voltage-gated sodium channel specific seizure suppressor mutant. Additionally, the utility of dorsal vessel injection of antiepileptic drugs is discussed in detail. Following discussion of these findings, future plans for the use of *Drosophila* as a genetic model for intractable epilepsies and discovery of additional seizure suppressor mutants are laid out. While many questions regarding the mechanisms of seizure suppression remain, this work and any future work from this point will help to unravel the complexities of epilepsy and seizure disorders.

To my family: None of this would have been possible without your endless support.

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List of Abbreviations

APN - Aminopeptidase N
AED - antiepileptic drug
ADNFLE - Autosomal dominant nocturnal frontal lobe epilepsy
BS - Bang-sensitive
BFNC - Benign familial neonatal convulsions
BFNIS - Benign familial neonatal infantile seizures
DLM - Dorsal longitudinal muscle
ECT - Electroconvulsive shock treatment
EMS - Ethylmethanesulfonate
FDA - United States Food and Drug Administration
GABA - Gamma-aminobutyric acid
GEFS+ - Generalized epilepsy with febrile seizures plus
GF - Giant fiber
HFS - High frequency electrical stimulation
ICEGTC - Intractable childhood epilepsy with generalized tonic-clonic
ILAE - International League Against Epilepsy
MRT - Mean Recovery Time
nMRT - Normalized mean recovery time
SMEI - Severe myoclonic epilepsy in infancy
SUDEP - Sudden unexplained death in epilepsy
TLE - Temporal lobe epilepsy

Acknowledgements

It was six years ago that I began my studies as a graduate student at the University of California – Berkeley. During these years, I have pursued several projects relating to the fascinating and wholly important field of seizure disorders. While not all of them produced meaningful results, each and every project has helped to shape my graduate career and make me who I am today. Needless to say, I did not get here alone, and there are many people who deserve much thanks for helping me along the way.

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Chapter 1
Introduction to the *Drosophila* Model of seizure disorder

General introduction of epilepsy and seizure disorders

Seizures are events resulting from abnormal, involuntary, rhythmic neuronal firing within the brain. They are unpredictable and can occur at any time. Patients cannot normally control their onset or termination. Seizures are typically short, lasting less than 5 minutes and usually end spontaneously. Spontaneous recurrence of unprovoked seizures is known as epilepsy. Epileptic syndromes are defined by many factors, including type of seizure, age of onset, family history, etc (Shneker and Fountain 2003). Approximately 1-2% of the worldwide population are affected by epilepsy, and 10% of the population will have at least one seizure within their lifetime (McNamara 1994; Engel 2002). While the cause for every individual case of epilepsy cannot always be identified, there are multiple conditions or factors that may contribute. Symptomatic epilepsy is known to be caused by infections, high fever, brain malformations, brain tumors, head trauma; and genetic factors are known to underlie several idiopathic epilepsies.

Hyperexcitability of neurons and hypersynchrony of neural networks are hallmarks of seizures. Hypersynchrony refers to a population of neurons firing at a similar rate. Synchronization of neuronal responses allows for coordination of activity patterns and is critical for proper brain function. In most cases, this synchrony is tightly controlled. Loss of this control however may lead to disruption in brain function. Impairment of neuronal synchronization has been noted in Alzheimer patients. Additionally enhancement of synchronization is linked to Parkinson's disease. The most significant example of neuronal hyper synchronization is epilepsy. In epilepsy, hypersynchrony relies on neuronal hyperexcitability (Margineanu 2010).

Neuronal hyperexcitability is characterized by enhanced responsiveness to stimuli. Spontaneous and post discharge activity has also been noted. Studies have demonstrated that hyperexcitability correlates with upregulation and increased activation of glutamate receptor and sodium channels. Increases in activation of glia and decreases in inhibitory activity are also noted (Gwak and Hulsebosch 2011). Hyperexcitability in epilepsy suggests the concept of a seizure threshold: for a seizure to occur, there must be a certain level of excitability that is exceeded.

Classification of seizures and epilepsies

Epileptic seizures are varied and can manifest themselves in different ways depending on the site or origin and subsequent spread. This complexity is a reflection of the many routes of seizure genesis and spread, both on a cellular and network level. Seizures are classified into two broad categories: 1) focal or partial seizures, and 2) generalized seizures. Partial seizures involve small regions of the brain usually localized to a single hemisphere. Partial seizures are sometimes capable of generalizing into seizures involving the entire cortex. According to the International League Against Epilepsy (ILAE) classification system, partial seizures are designated as either complex or simple based on whether consciousness is altered after seizure initiation (Shneker and Fountain 2003). Simple partial seizures do not result in alteration of consciousness but usually manifest motor, sensory, psychiatric, or autonomic symptoms based on the affected region of the brain. Complex partial seizures are the most common form of epileptic seizure in adults and can manifest a variety of symptoms. These include

stereotyped movements, anxiety; and changes in cognition, hallucinations and delusions (Roffman and Stern 2006).

The second broad category of seizures is made up of generalized seizures. These seizures start throughout the entire cortex at the same time and result in loss of consciousness. There are seven types of generalized seizure recognized by the ILAE. These classifications are *tonic-clonic, absence, myoclonic, clonic, tonic, atonic and atypical absence* (Shneker and Fountain 2003). Tonic-clonic seizures, previously known as grand mal seizures, have two phases. They begin with a tonic phase in which the entire body stiffens. This is followed by the clonic phase, which consists of repetitive contractions. These seizures last 2-3 minutes and are generally followed by a period of confusion or unresponsiveness. The next two categories of generalized seizures, tonic seizures and clonic seizures, consist only of one of the phases of tonic-clonic seizures. Myoclonic seizures are brief, muscular jerks. They can affect any body region, but bilateral hand and arm jerks are the most common. While the first four classifications of seizures are characterized by motor manifestations, the last three are characterized by their lack of motor symptoms. Absence seizures, formerly known as petit mal seizures, manifest as brief episodes of staring and unresponsiveness. There are generally no other symptoms, but those episodes lasting more than 7-10 seconds may also be associated with eye blinking or other automatisms. Atypical absence seizures are similar to absence seizures but last longer and may include some motor involvement. Lastly, atonic seizures are characterized by a sudden loss of muscle tone. They can be referred to as "drop attacks," as patients subsequently fall or drop to the floor (Shneker and Fountain 2003).

Epileptic syndromes can be classified further as either idiopathic or symptomatic epilepsies. Idiopathic epilepsies have no clear etiology. Recent advances in molecular biology and genetics have shown that many idiopathic epilepsies are generally genetic abnormalities in neurotransmission. Symptomatic epilepsies result from known causes or structural disease. Brain tumors and trauma are examples of structural disorders. Anoxia and infection may result in brain damage leading to epilepsy. Epileptic disorders that are suspected to be of symptomatic origin that cannot be identified are classified as cryptogenic epilepsies (Shneker and Fountain 2003).

Treatment of epilepsy

Epilepsy is a diverse collection of disorders and treatment of epilepsy is a complex problem. The most common method of treatment is antiepileptic drug (AED) therapy. The primary goal of AED therapy is to keep a patient seizure free without disrupting normal brain function (Löscher and Schmidt 2002). AEDs are believed to produce their effect by one or more of four mechanisms: 1) inhibition or blockage of sodium channels, 2) inhibition or blockage of calcium channels, 3) potentiation of gamma-aminobutyric acid $(GABA)_A$-mediated inhibition, and 4) limitation of glutamatergic excitation (Bazil and Pedley 1998; Löscher and Schmidt 2002). To date there are 24 drugs approved for use in epilepsy by the United States Food and Drug Administration (FDA), and in the past three years, five new AEDs have been approved (Sirven et al. 2012). In general, therapy with one or more AEDs effectively controls seizures for the majority of patients. Despite this, there are many challenges remaining for the treatment of epilepsy. A number of patients will develop tolerance to multiple

AEDs, leading to the inability to control seizures. The mechanisms behind the development of AED tolerance are not clearly understood, but it has been suggested that genetic factors may cause variations in the way patients respond to treatment. Additionally, disease progression may lead to alteration of the drug's targets, reducing drug efficacy (Löscher and Schmidt 2002). AEDs have also been associated with a range of chronic adverse effects. Vigabatrin, for example has been linked to peripheral vision loss in 30% or more of patients and thus has been limited for usage in only severe cases of epilepsy. Rufinamide has been linked to an increased potential for cardiac conductivity disturbances. In 2008, the FDA issued a safety alert regarding an association between many AEDs and suicidal behavior and ideation (Sirven et al. 2012).

In addition to AED treatment, patients with intractable epilepsy may be able to consider neurosurgical treatment or a ketogenic diet as alternatives to drugs. Neurosurgical treatment of epilepsy usually consists of ablation of an epileptogenic region of the brain. Surgery can be classified into two categories, curative and palliative. Palliative surgery is intended to lessen seizure severity or frequency and may prevent occurrence of certain seizure types. This type of surgery may reduce the frequency of seizures but does not necessarily eliminate all seizures. Curative surgeries on the other hand, aim to completely eradicate seizures (Kunieda, Kikuchi, and Miyamoto 2012). The success of surgical treatments for epilepsy is dependent on complete resection of the epileptogenic region. It is not always possible to identify and/or remove the epileptogenic region. For these patients, surgery may not be a viable option. Another alternative method of epilepsy treatment is the ketogenic diet. The ketogenic diet has been shown to be effective in treating multiple epilepsy syndromes. It is a high fat, high protein diet that excludes carbohydrates. The mechanism of seizure control is unknown. The ketogenic diet has been shown to be most effective at treating West Syndrome, Lennox-Gastaut Syndrome and epilepsies with myoclonic-atonic seizures (Nangia et al. 2012). Even with advances in AED therapy and alternative treatments, approximately 30% of all epilepsy patients suffer from intractable disorders. For these patients, no current anti-epileptic drug (AED) therapy, diet, or surgical treatment offers relief from seizures.

Genetics of human epilepsies

Partly because of their heterogeneity, inheritance of epilepsy disorders is thought to be due to interactions between environmental factors and susceptibility genes. Many idiopathic epilepsies are considered to be genetic in origin (Gardiner 2005). The inheritance of epilepsy can be either monogenic, caused by a single gene, or polygenic, caused by a combination of alleles. Monogenic epilepsies follow Mendelian inheritance while polygenic epilepsies do not. Due to advances in molecular genetics, a number of genes linked to epilepsy have been identified. However, the search for genes involved in genetic epilepsies is complicated by several factors. Many mutations involved in monogenic epilepsies are incompletely penetrant. Single gene mutations show variable expressivity and gene-environment interactions complicate things further. Additionally, it is now clear that the majority of genetic epilepsies have a complex inheritance involving different genes each with a small contribution to the overall seizure susceptibility (Michelucci et al. 2012).

Ion channel defects, or channelopathies are the most common cause of monogenic epilepsies. Multiple ion channel mutations have been identified with links to epileptic syndromes, including mutations in voltage-gated Na^+, K^+ and Cl^- channels and ligand-gated channels such as nicotinic acetylcholine and $GABA_A$ receptors (Gardiner 2005; Rodrigues-Pinguet et al. 2003). Several genetic defects underlying different epilepsies have been identified. Mutations in the voltage-gated potassium channels KCNQ2 and KCNQ3 have been linked to benign familial neonatal convulsions (BFNC). Mutations affecting voltage-gated sodium channel subunits SCN1A, SCN2A and SCN1B have been found to cause generalized epilepsy with febrile seizures plus (GEFS+). In the voltage-gated chloride channel CLCN2, mutations have been linked to multiple epilepsies, including juvenile absence epilepsy and epilepsy with grand-mal seizures on awakening. The $GABA_A$ receptor subunit genes GABRG2 and GABRA1 have been shown to cause childhood absence epilepsy and juvenile myoclonic epilepsy (Gardiner 2005). Five mutations in subunits of the neuronal nicotinic acetylcholine receptor (three mutations in α4, and two in β2) cause autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) (Rodrigues-Pinguet et al. 2003).

Ion channels are important in regulating excitability in the nervous system. The brain specific form of the nicotinic acetylcholine receptor is thought to negatively regulate release of the excitatory neurotransmitter glutamate. Impairment of nicotinic cholinergic transmission in epilepsy increases glutamate release, resulting in hyperexcitability (Rodrigues-Pinguet et al. 2003). Potassium channels in the nervous system are critical for maintaining resting membrane potentials and enabling repolarization after action potentials. KCNQ2 and KCNQ3 assemble into potassium channels responsible for the M current which regulates excitability of neurons by reducing the tendency for repetitive firing (Wang et al. 1998). Voltage-gated sodium channels initiate and propagate action potentials in excitable cells and will be discussed in greater detail in a later section. The voltage gated Cl^- channel CLCN2 acts as a chloride-efflux pathway, maintaining the necessary chloride gradient for inhibitory GABA responses. Epilepsy can be caused by loss-of-function or gain-of-function mutations in CLCN2. Loss-of-function lowers the chloride gradient essential for GABA inhibition while gain-of-function mutations potentially cause hyperexcitability due to recurrent membrane depolarization (Haug et al. 2003) $GABA_A$ receptors mediate inhibitory neuronal activity. Loss of inhibition can cause hyperexcitability and seizures.

Polygenic epilepsies are more complex epilepsies in origin. They are caused by a combination of susceptibility alleles. Complex epilepsies can arise when meiotic reshuffling creates a combination of susceptibility alleles in an individual that push neuronal hyperexcitability over the seizure threshold. Each susceptibility gene alone may not cause seizures, but it requires the effects of other susceptibility alleles to give rise to epilepsy (Mulley et al. 2005). Two susceptibility genes have been identified for complex epilepsies. Variants in the T-type calcium channel CACNA1H have been associated with childhood absence epilepsy and other idiopathic generalized epilepsies. Mutants exhibited altered channel properties that were consistent with increased seizure susceptibility (Mulley et al. 2005). The gene encoding the δ-subunit of the $GABA_A$ receptor (GABRD) is a susceptibility gene for complex idiopathic generalized epilepsy and GEFS+. The δ-subunit mediates tonic inhibition suggesting that this mechanism is involved in epilepsy (Mulley et al. 2005).

Mouse models of epilepsy

For any mutant animal to be considered as a potential model of human epilepsy, it is first necessary to assess the mutant against certain criteria. There are several characteristics that are necessary for a good model of epilepsy (modified from Löscher & Schmidt 1988). 1) The development of spontaneously occurring seizures, ideally with an age-dependent onset similar to that in generalized human epileptic syndromes. 2) The genetic mutant and change of function in the encoded protein should correspond to that in humans. 3) Behavioral and electrical seizure is similar to seizures occurring in human epilepsy. 4) Where applicable, the occurrence of pathological changes resembles the human condition. 5) The effect of different AEDs should be similar to that in human epilepsy. While no model exactly matches every criteria, the mouse does fit many of those listed above. Molecular experiments such as modifying the expression of single genes, are relatively easy to perform on mice. Thus it has become a widely used and powerful tool for studying genetic aspects of epilepsy.

There are several homologous mouse models of epilepsy. One such example is a mouse model of the human epileptic disorder Dravet Syndrome. Dravet syndrome is classified as a severe myoclonic epilepsy in infancy (SMEI). It has been linked to defects in voltage-gated sodium channels. Research has shown that a heterozygous deletion of the voltage-gated sodium channel SCN1A is sufficient to produce phenotypes of SMEI in mouse and in humans. Mutant mice display a similar temperature- and age-dependence of onset and progression of seizures to that seen in human patients (Oakley, Kalume, and Catterall 2011).

In addition to homologous models, targeted mutagenesis in mouse has led to the discovery that mutations in many distinct genes can cause epilepsy. Targeted knockout of these genes has been important to the genetic dissection of epileptogenesis and have provided new possibilities for investigation of how alterations in normal gene function lead to seizures. One such example is the knockout of the $GABA_{B1}$ receptor. Loss of this receptor results in spontaneous generalized seizures with an onset of approximately 12 days. The severe epileptic phenotype led to a life expectancy of only 21 days for these knockout mice (Prosser et al. 2001). Another example is the knockout of the brain specific protein *jerky*, which leads to recurrent seizures similar to temporal lobe epilepsy (TLE). Jerky binds mRNA with high affinity and is associated with translationally inactive mRNAs (Liu et al. 2002). The *jerky* knockout mouse may be a model for the role of mRNA control in seizure-genesis.

Drosophila as a genetic model of epilepsy

The fruit fly, *Drosophila melanogaster*, has long been an excellent model for many fundamental problems in biology. First used in 1910 for studies of heredity, the legacy of *Drosophila* research began with the discovery of the first mutant, *white* (Rubin and Lewis 2000; Morgan 1910). Research using *Drosophila* as a model has continued since resulting in a vast collection of mutants. For genes associated with human disease, 77% have a *Drosophila* homolog (Reiter et al. 2001). For use in studying seizure susceptibility, *Drosophila* has several advantages. A collection of seizure-sensitive mutants will be discussed in detail in the next section. These mutants can be used in conjunction with various mutants to examine molecular defects that can enhance or suppress seizure susceptibility. There are many excellent molecular genetic methods available; including P-element-mediated cloning methods and a fully sequenced genome. Additionally, there are a variety of methods for electrophysiological stimulation and recording. These include recordings from the giant fiber (GF) system (Tanouye and Wyman 1980) that were used extensively in the work presented in this dissertation. *Drosophila* is an attractive model for studying seizure susceptibility. Several of the seizure-sensitive mutants are homologous to mutations associated with human epilepsy. The seizure threshold of these mutants can be affected genetically, just as in mice and human cases of epilepsy. Additionally, findings in the *Drosophila* model of epilepsy indicate that it is likely that similar mechanisms underlie changes in seizure susceptibility in both flies and humans. Despite the fact that *Drosophila* does not satisfy several of the criteria for a good animal model, the similarities presented make it a powerful model in which to investigate molecular defects that make the nervous system vulnerable to seizures, an undertaking that is difficult in other systems.

The bang-sensitive mutant class

A collection of seizure-sensitive mutants in *Drosophila* called bang-sensitive (BS) paralytic mutants is the basis for the *Drosophila* seizure model. These mutants display prominent seizure-like behaviors. Following a “bang,” or a mechanical shock, such as a hard tap of the culture vial on the bench top or a brief vortex mixing, these mutants undergo stereotyped seizure-like behaviors. These behaviors are characterized by an initial seizure lasting approximately 2 seconds, a period of temporary paralysis that lasts from 20 seconds to 5 minutes depending on genotype, and finally a recovery seizure lasting about 2 seconds (Ganetzky and Wu 1982). This behavioral activity can also be recapitulated electrophysiologically. Electrical stimulus in the form of a short wavetrain of high frequency impulses can evoke seizure like activity. An initial seizure is followed directly by a period of synaptic failure. Upon recovery from synaptic failure a second, recovery seizure occurs (Tan et al. 2004). Seizure susceptibility my electrical means is quantified by seizure threshold. That is the voltage at which high frequency stimulation becomes an electroconvulsive shock and elicits seizure like activity (Kuebler and Tanouye 2000). Electrical seizures can be evoked in any genotype of fly, but the seizure thresholds of the BS mutants are far lower than in wild type.

Seizures in flies and in humans share several key similarities that provide support for the utility of the *Drosophila* model of epilepsy. Previous investigations have shown

that in *Drosophila*: 1) all individuals have a seizure threshold, 2) genetic mutations can modulate seizure susceptibility, 3) electroconvulsive shock treatment (ECT) in flies will raise the threshold for subsequent seizure-like activity, 4) seizure-like activity spreads throughout the fly central nervous system along particular pathways dependent on functional synaptic connections and recent electrical activity, 5) seizure-like activity in flies can be spatially segregated into particular regions of the CNS, 6) *Drosophila* phenotypes can be ameliorated by human AEDs, and 7) mutations affecting *Drosophila* sodium channels are excellent seizure suppressors, consistent with the notion that many AEDs target sodium channels (Kuebler and Tanouye 2000; Kuebler and Tanouye 2002; Kuebler et al. 2001; Reynolds et al. 2004; Tan et al. 2004).

There are 11 known BS mutants. These represent a wide variety of gene products with altered function causing seizure-like behavior (Song and Tanouye 2008). Of these mutants, there are three canonical BS mutants that serve as experimental representatives of the BS class of mutants. These are *bang senseless* (*para^{bss}*)*,* *easily shocked* (*eas*)*,* and *slamdance* (*sda*). In these mutants the BS phenotype is completely penetrant and the electrical seizure threshold is much lower than that of wild type flies.

The BS mutant *slamdance* (*sda*) displays the weakest seizure-sensitivity of the canonical BS mutants. It is also the easiest mutant to suppress genetically and pharmacologically. The *Drosophila sda* gene is a homolog of the human aminopeptidase N (APN). APN is a member of a family of zinc-dependent metallopeptidases. While the exact mechanism leading to seizure-sensitivity in *sda* is unknown, it has been shown to be highly expressed within the *Drosophila* CNS (Zhang et al. 2002). Additionally, *sda* has been shown to affect the alternative splicing of the *para* voltage-gated sodium channel. Mutation in *sda* results in 100% of *para* transcripts contain an exon which causes the channels to not inactivate appropriately (Lin et al. 2012). Electrically, *sda* has a seizure threshold of approximately 6 V, significantly lower than wild type (approximately 30 V).

The BS mutant *easily shocked* (*eas*) is an intermediately seizure susceptible mutant. It has a low seizure threshold of approximately 3.4 V but is still relatively easy to genetically suppress. The *eas* gene is homologous to ethanolamine kinase, which is required for phosphatidylethanolamine synthesis (Pavlidis, Ramaswami, and Tanouye 1994). Bang sensitivity in *eas* is thought to be due to alteration of membrane phospholipid composition that results in increased excitability. Interestingly, mutation in *eas* results in changes to *para* alternative splicing in the same manner as *sda* (Lin et al. 2012).

The most severe of the BS mutants is *bang senseless* (*para^{bss1}*). First discovered in 1978 (Ganetzky and Wu 1982), this mutant has the lowest seizure threshold of the BS mutant class (~3.2 V) and is the most difficult to suppress either genetically or with AED treatment. For many years, *bang senseless* eluded molecular identification. Recently, it was shown to be an allele of the *Drosophila* voltage-gated sodium channel *paralytic* (*para*). The mutation leading to the *para^{bss1}* phenotype affects a segment implicated in channel inactivation. Indeed, mutant channels display altered voltage dependence of inactivation (Parker et al. 2011). Seizures associated with *para^{bss1}* resemble those of pharmacologically resistant epilepsies associated with human voltage-gated sodium channels.

Modification of seizure susceptibility

Genetic factors can increase or decrease seizure susceptibility, making flies more or less seizure-prone (Kuebler and Tanouye 2000). The ability to identify these genetic factors is much less limited in *Drosophila* than in other models. Generation of double mutants allows for the testing of a variety of mutations for effects on seizure-susceptibility in the *Drosophila* BS mutants. Identification of each single mutant affecting seizure susceptibility presents a new possible target for drug development and allows determination of how various physiological processes contribute to overall seizure-susceptibility in the fly. Discovery of these mutants brings us closer to understanding the complexities of epilepsy.

Drosophila BS mutants have been used previously in several screens for mutants altering seizure-susceptibility. Using *sda* as a sensitized background has led to the identification of several seizure-enhancer mutants and also at least one novel BS mutant. This mutant, *jitterbug* (*jbug*), displays temperature- and age-dependent seizure-susceptibility, though the specific gene has not yet been identified. Screens for seizure-suppressor mutants using *eas* have yielded several seizure-suppressor mutants including $para^{JS1}$ and $top1^{JS}$ (Song and Tanouye 2007; Song, Hu, and Tanouye 2007). A screen for seizure-enhancers and –suppressors of $para^{bss1}$ and the results thereof will be detailed in Chapter 2.

Voltage-gated sodium channels and epilepsy

As previously stated, many genetic defects associated with epilepsy are channelopathies, or defects in ion channels. Voltage-gated sodium channels play a critical role in the genetic causes of epilepsy. Voltage-gated sodium channels play an essential role in initiation and firing of action potentials. All voltage-gated sodium channels share some key characteristics. At resting potential, they are in a closed state and do not pass sodium ions. Upon membrane depolarization, they open and allow inward flow of sodium, resulting in further depolarization. Following channel opening, rapid inactivation stops the flow of sodium and the channels become closed and unavailable for opening. Action of voltage-gated potassium channels allows the voltage-gated sodium channels to recover and become available again for opening (Oliva, Berkovic, and Petrou 2012). The pore-forming α subunit of voltage-gated sodium channels consists of four domains (DI-IV). Each of these domains contains six transmembrane segments. The fourth transmembrane segment is responsible for voltage-sensing and activation of the channel. A linker between DIII and DIV is thought to contain inactivation machinery for the channel. Each α subunit associates with two accessory β subunits. The β subunits are thought to establish contact with the cytoskeleton as well as affect gating and trafficking of the voltage-gated sodium channels. Mutations in both the α and β subunits have been associated with epilepsy.

In the human brain, there are 4 types of voltage-gated sodium channel expressed. These are SCN1A, SCN2A, SCN3A and SNC8A (Oliva, Berkovic, and Petrou 2012). The expression of these channels varies with development. In neonates, SCN3A is more highly expressed, suggesting a developmental role. In adults, SCN3A is found in several specific areas of the brain. SCN1A, SCN2A and SCN8A are all present in high amounts in the adult brain. Deletion of any of these genes results in lethality. SCN2A and SCN8A are predominantly found in excitatory neurons. SCN1A is expressed mainly in GABAergic neurons, though it sometimes co-localizes with SCN8A in spinal cord neurons. There are 4 types of β subunit expressed in the adult brain. These are SCN1B, SCN2B, SCN3B and SCN4B. They show a broad distribution within the brain (Oliva, Berkovic, and Petrou 2012).

There have been nearly 700 voltage-gated sodium channel mutations identified in epilepsy. Of these mutations, over 650 of them have been identified in SCN1A in patients with Dravet syndrome. More than 20 SCN1A mutations are associated with GEFS+ epilepsies. Because of this large number of mutations, SCN1A has been called a "super-culprit" gene in epilepsy. Approximately 20 SCN2A mutations have been identified in patients with Dravet syndrome, GEFS+ and benign familial neonatal infantile seizures (BFNIS). A single mutation in SNC8A has been identified in a patient with absence epilepsy. There have been four mutations in SCN1B identified in patients with GEFS+

(Oliva, Berkovic, and Petrou 2012).

The sheer number of epilepsy linked mutations discovered in voltage-gated sodium channels and existence of multiple channel types in humans leads to tremendous complexity in understanding how these individual mutations affect normal neurological function. The *Drosophila* genetic model of epilepsy may prove to be useful in unraveling some of this complexity. Where the human brain has 4 types of voltage-gated sodium channels, *Drosophila* has only one, *paralytic* (*para*). The most severe of the *Drosophila* BS mutants corresponds to a defect in *para*. This mutant closely resembles intractable epileptic disorders, as it is very difficult to suppress either genetically or by AED treatment. The seizure-sensitive phenotype of $para^{bss1}$ is due to a leucine to phenylalanine substitution at position 1699 of the ParaPA sequence. L1699 is highly conserved across mammalian neuronal voltage-gated sodium channels (Parker et al. 2011). In addition to $para^{bss1}$, another mutation in *para* has been shown to cause seizure-sensitivity. Knock in of the amino acid substitution K1270T which is associated with GEFS+ in humans, causes a temperature induced seizure phenotype (Sun et al. 2012). This evidence suggests that the *Drosophila para* gene could prove to be an invaluable tool in understanding the genetics of voltage-gated sodium channel associated epilepsies.

Chapter 2
Drosophila as a model for intractable epilepsy: *gilgamesh* suppresses seizures in *para*^{bss1} heterozygote flies

Introduction

A fundamentally important problem in biomedical research is the tragedy of intractable epilepsy. For more than one million Americans suffering from this disorder, seizures do not respond to any currently available medication, and surgery is not a viable option. We are not aware of any especially promising new research approaches for dealing with intractable epilepsy. For example, Dravet syndrome, known as Severe Myoclonic Epilepsy of Infancy (SMEI, MIM 182389), is a rare and catastrophic intractable epilepsy beginning in infancy (Dravet 1978). The syndrome is an autosomally-inherited dominant disorder associated with mutations in the voltage-gated Na^+ channel gene SCN1A (Claes et al. 2001; Wallace et al. 2003; Fukuma et al. 2004). Initial seizures in Dravet are tonic, clonic, and tonic-clonic, often prolonged and generalized. These are fever-associated beginning early in life, before age 6 months. Psychomotor and speech development stagnates about age 2 y with subsequent mental deterioration; there is also a higher incidence of SUDEP (sudden unexplained death in epilepsy). As Dravet progresses, seizures are afebrile with variable manifestation including myoclonic, tonic-clonic, absence, and simple and complex partial seizures. Convulsive status epilepticus is frequent. Dravet epilepsy is resistant to all pharmacotherapy. SCN1A mutations are also associated with ICEGTC (intractable childhood epilepsy with generalized tonic-clonic), an atypical SMEI that does not show myoclonic seizures (Fujiwara et al. 2003).

In this study, we examine genetic complexities that may underlie intractable epilepsy using, as a model, genetic combinations of single-gene mutations in the fruitfly Drosophila: seizure-sensitive, seizure-enhancer, and seizure-suppressor mutations. The study is based on genetic interactions that modify phenotypes in $para^{bss1}$, a model for intractable epilepsy (Parker et al. 2011). The $para^{bss1}$ mutant is due to a gain-of-function mutation in the voltage-gated Na^+ channel gene that causes extreme seizure-sensitivity. In our Drosophila collection the $para^{bss1}$ mutant: a) displays the lowest threshold to evoked seizure-like activity; b) exhibits the longest paralytic behavior recovery time with prominent episodes of seizure and paralysis that resemble tonic-clonic-like activity; and c) is the most difficult mutant to suppress by suppressor mutations or antiepileptic drugs (Pavlidis and Tanouye 1995; Kuebler and Tanouye 2000; Kuebler et al. 2001; Song and Tanouye 2006).

Materials and methods

Fly stocks

Drosophila strains were raised on standard cornmeal-molasses agar medium at room temperature (23-25°C). The *para* gene is located at map position 1-53.5 and encodes a voltage-gated Na^+ channel (Loughney, Kreber, and Ganetzky 1989; Ramaswami and Tanouye 1989). The BS allele used in this study, *para*^{bss1}, previously named *bss*^{1}, is the most seizure-sensitive of fly mutants, the most difficult to suppress by mutation and by drug, and is a model for human intractable epilepsy (Ganetzky and Wu 1982; Parker et al. 2011). The *para*^{bss1} allele is a gain-of-function mutation caused by a substitution (L1699F) of a highly conserved residue in the third membrane-spanning segment (S3b) of homology domain IV (Parker et al. 2011). In this study, we use *para*^{bss1} and *para*^{bss1}/+ as genetic backgrounds to screen for enhancers and suppressors of seizure, respectively. The *eas* gene is located at 14B on the cytological map and encodes an ethanolamine kinase (Pavlidis, Ramaswami, and Tanouye 1994). The BS allele used in this study is *eas*^{PC80}, which is caused by a 2-bp deletion that introduces a frame shift; the resulting truncated protein lacks a kinase domain and abolishes all enzymatic activity (Pavlidis and Tanouye 1994). Df(2R)Exel7135=51E2-51E11 contains approximately 22 genes. Df(2R)Exel6056=44A4-44C2 contains approximately 39 genes. Df(2R)Exel6078=58B1-58D1 contains approximately 35 genes. *UAS-gishRNAi* and other *UAS-RNAi* lines were obtained from the Vienna Drosophila RNAi Center. All other lines, including Gal4 drivers and deletion lines were obtained from the Bloomington Drosophila Stock Center.

Haplo-deficiency screen for seizure-enhancers and -suppressors

A screen was designed to detect novel seizure-suppressors and -enhancers based on haplo-induced changes in *para*^{bss1} seizure-susceptibility. The screen examined 200 stocks, each carrying a different Df(2) or Df(3) chromosomal deletion with appropriate CyO, TM3, or TM6 balancer in a *para*^{bss1} background. For Df(2) deletions: female *para*^{bss1}*;+;*+ flies were crossed to +/Y;Df(2)/CYO;+ males. Male progeny of the genotype: *para*^{bss1}/Y;Df(2)/+;+ were tested for enhancement of BS phenotype compared to their sibling controls (*para*^{bss1}/Y;CYO/+;+). Female progeny arising from the same cross: *para*^{bss1}/+*;Df(2)/+;*+ were tested for suppression of the BS phenotype compared to their control siblings (*para*^{bss1}/+;CYO/+;+). Df(3) deletions were tested similarly. Thus, *para*^{bss1}/Y;+;Df(3)/+ male flies were examined for enhancement and *para*^{bss1}/+*;+;Df(3)/*+ flies were tested for suppression of BS phenotypes relative to their respective control siblings.

Behavior and electrophysiology

Behavioral testing for BS paralysis was performed on flies 2-3 d after eclosion, as described previously (Kuebler and Tanouye 2000). Flies were anesthetized with CO_2 before collection and tested the following day. For testing, 15-20 flies were placed in a food vial and stimulated mechanically with a VWR vortex mixer at maximum speed for

10 s. For analysis, recovery time was measured for each fly from the end of the vortex stimulation until it resumed an upright standing position. Mean Recovery Time (MRT) was the average time taken for a fly exhibiting BS behavior to recover in a population. Pools of flies are combined (in total, n ≈ 100 for each genotype). For the purposes of comparisons, these are expressed here as Normalized Mean Recovery Time (nMRT), which is the MRT of the experimental flies divided by MRT of their control siblings. For genotypes that display only partial penetrance of BS paralysis, only those flies that displayed paralysis were used for recovery time analysis. A simpler measure of recovery time is RT_{50} (50% Recovery Time), the time at which half of BS flies have recovered from paralysis. RT_{50} was used in some analyses and especially to facilitate initial identification of enhancers and suppressors.

In vivo recording of seizure-like neuronal activity and seizure threshold determination in adult flies was performed as described previously (Kuebler and Tanouye 2000; Lee and Wu 2002). Flies 2-3 d post-eclosion were mounted in wax on a glass slide, leaving the dorsal head, thorax, and abdomen exposed. Stimulating, recording, and ground metal electrodes were made of uninsulated tungsten. Seizure-like activity was evoked by high-frequency electrical brain stimulation (0.5-ms pulses at 300 Hz for 400 ms) and monitored by dorsal longitudinal muscle (DLM) recording. During the course of each experiment, the giant fiber (GF) circuit was monitored continuously as a proxy for holobrain function. For each genotype tested, n ≥ 10, and unless otherwise noted, all flies were female. Comparisons of paralytic recovery time and seizure threshold were Student's *t*-test. For all figures, error bars represent standard error of the mean, and statistical significance is indicated by * $P<0.01$ and ** $P<0.0001$.

Results

Screening for *para^{bss1}* enhancers with deficiencies

The *para^{bss1}* mutant displays phenotypes that are similar to other mutants of the bang-sensitive (BS) paralytic class such as *eas^{PC80}*, *sda$^{iso7.8}$*, and *tko^{25t}* (Ganetzky and Wu 1982; Royden, Pirrotta, and Jan 1987; Pavlidis, Ramaswami, and Tanouye 1994; Zhang et al. 2002), albeit more severe. BS seizure-like behaviors and paralysis are observed in response to mechanical shock ("a bang") (Figure 1). The time of BS paralysis for *para^{bss1}* is much longer than for other mutants and exhibits unusual tonic-clonic-like behaviors. For example, total paralytic time for *para^{bss1}* is about 240 s, longer than for *sda$^{iso7.8}$* about 25 s (Zhang et al. 2002; Parker et al. 2011). The *para^{bss1}* mutant also has a low threshold for seizure-like activity evoked by high-frequency electrical stimulation (HFS) of the brain. For example, seizure threshold for *para^{bss1}* is 3.2 ± 0.6 V HFS, lower than the threshold for *sda$^{iso7.8}$* which is 6.2 ± 0.8 V HFS; wild type Canton-Special flies have a seizure threshold of 30.1 ± 3.8 V HFS, for comparison (Figure 2) (Kuebler et al. 2001).

Despite the existing severity of *para^{bss1}* phenotypes, we explored the possibility that these might be exacerbated further by enhancer mutations. We have previously found that recovery time from BS paralysis for *para^{bss1}* varies with genetic background, age, and other factors (Parker et al. 2011). The length of time required for recovery appears to be primarily dependent on the number of bouts of tonic-clonic-like activity. We exploited this in an initial screen, investigating the possibility that potential enhancers may reside in chromosomal segments made haploid by deletions, and these would become manifest by a change in the time required to recover from BS paralysis. We then examined enhancers for effects on other *para^{bss1}* phenotypes. We measured BS paralytic recovery times in *para^{bss1}/Y; Df/+* flies compared to their control siblings of genotype *para^{bss1}/Y; Balancer/+* (Table 1). Several deficiency chromosomes consistently showed increased recovery times for *para^{bss1}* males (Table 1). For example, Df(2R)Exel7135 had a mean recovery time (MRT) of 363 s for experimental males, compared to 234 s for their sibling controls yielding an nMRT of 1.55. Other notable deficiencies included: Df(2R)Exel6078 and Df(2R)Exel6056 with nMRTs of 2.27 and 2.53, respectively.

The Df(2R)Exel7135 is a representative of our findings on *para^{bss1}* enhancers. We found that BS enhancement in the segment is accounted for by reduced expression of the *charlatan (chn)* gene. The enhancement of *para^{bss1}* by *chn* was limited to BS paralysis recovery time phenotype, that is, an increase in the severity of this phenotype; there was no apparent enhancement of the other major phenotype: threshold for evoked seizure. Further detail on seizure enhancement by *chn* is part of another student's work and beyond the scope of this dissertation.

Screening for *para^{bss1}* suppressors with deficiencies

The *para^{bss1}* mutant is severely seizure-sensitive, and has been presented as a Drosophila model for intractable human epilepsy. As with intractable human epilepsies, such as autosomal dominant disorder Dravet syndrome, *para^{bss1}* mutant phenotypes are difficult to suppress by antiepileptic drug feeding. Also, Drosophila seizure-suppressor mutations thus far identified have been ineffective at alleviating *para^{bss1}* phenotypes. The

$para^{bss1}$ mutation is semi-dominant with seizure-like behaviors and BS paralysis reduced in heterozygous $para^{bss1}$/+ flies, but still present at high penetrance (>95%) (Figure 1) (Ganetzky and Wu 1982; Parker et al. 2011). We exploited this feature to screen for suppressor mutations inferring that heterozygotes would provide a genetic background that is sensitized for detecting putative suppressors. As an initial screen, we investigated the possibility that potential suppressors may reside in chromosomal segments made haploid by deletions and that these would become manifest by a change in BS paralysis. That is, we compared $para^{bss1}$/+; *Df*/+ females with their control sisters of genotype $para^{bss1}$/+; *Balancer*/+ for differences in the percentage of flies undergoing BS paralysis. Several deletion chromosomes consistently reduced the BS phenotype in $para^{bss1}$/+ females (Table 2). For example, only 13% of $para^{bss1}$/+; *Df(3R)ED10639*/+ females showed BS paralysis compared to their sibling controls, an apparent phenotypic suppression of about 87%. Other notable deletions included *Df(2R)Exel6285* and *Df(3L)ED4502* that caused 97% and 93% suppression, respectively. Here, we focus on *Df(3R)ED10639* as representative of our findings on $para^{bss1}$ suppressors.

Reduced expression of *gilgamesh (gish)* contained in the *Df(3R)ED10639* chromosomal segment suppresses $para^{bss1}$/+ BS paralysis

The *Df(3R)ED10639* deficiency is a deletion spanning from 89B7 to 89D5 and contains approximately 57 genes. In this section, we describe analyses showing that $para^{bss1}$/+ suppression in the segment is accounted for by reduced expression of the *gilgamesh (gish)* gene (Figure 3). $para^{bss1}$/+ BS suppression phenotype was mapped to a small region on chromosome 3R between 89B9 and 89B12 using overlapping deficiencies. In particular, localization of the suppression phenotype is based on its inclusion in the *Df(3R)Exel7329* deletion, which affects the number of animals paralyzed (Figure 3) (89B9-89B13), and its exclusion from the *Df(3R)Exel6269* deletion which has no effect on paralysis (Figure 3) (89B12-B18). This localization is consistent with the combined findings from other overlapping deletions in the region (Figure 3).

The 89B9-89B12 segment contains six genes (Figure 3). We found that an allele of *belphegor (bor)* did not appear to cause suppression based on flies of the genotype: $para^{bss1}$/+;bor^{c05496}/+ which showed similar BS paralysis compared to control siblings (9% reduction in BS paralysis). Also, an allele of *taranis (tara)* did not appear to cause suppression based on flies of the genotype $para^{bss1}$/+;$tara^{1}$/+, with BS paralysis similar to their sibling controls (0% reduction in BS paralysis). In contrast, an allele of *gilgamesh (gish)* caused substantial suppression based on flies of the genotype $para^{bss1}$/+;$gish^{04895}$/+, which showed a 57% reduction in BS paralysis compared to their $para^{bss1}$/+;*TM3*/+ control siblings.

The *gish* gene

The *gish* gene of Drosophila is homologous to mammalian casein kinase CK1g3, both members of the CK1 family of serine-threonine kinases (Zhai et al. 1995). The Drosophila gene is about 30 kb and alternatively-spliced to express 12 different isoforms in 4 main classes (Figure 4; Hummel et al. 2002; Tan et al. 2010). These arise from two initiation sites: two classes of long transcript (~3 kb) arise from an upstream initiation

site; two classes of short transcript (~2.5 kb) from a downstream initiation site (Figure 4; Hummel et al. 2002; Tan et al. 2010). The *gish*^{04895} mutation is a P-element insertion in exon 2, present in long, but not short *gish* transcripts. RT-PCR analysis (Tan et al. 2010) has shown that long *gish* transcripts are apparently undetectable in *gish*^{04895} mutants. Interestingly, in contrast, short transcripts appear to be more abundant in *gish*^{04895} mutant than in wild type flies (Tan et al. 2010). In the present experiments, *gish*^{04895} acts as a recessive lethal, in contrast to previous reports suggesting that it is a viable (Tan et al. 2010). We are unclear on the reasons for this apparent difference in viability. We find that precise excision of the *gish*^{04895} P-element completely reverted the BS suppressor phenotype (Figure 3), restored viability, but did not appear to revert the male sterility phenotype seen among *gish* mutant alleles (Castrillon et al. 1993).

Identification of *gish* as a *para*^{bss1}/+ BS suppressor by mutant analysis was supported further by RNAi analysis. Flies of the genotype *ELAV-Gal4*^{C155} *para*^{bss1}/+*;UAS-gishRNAi/+* showed a 75% reduction in BS paralysis compared to their *ELAV-Gal4*^{C155} *para*^{bss1}/+*;+/+* control siblings, showing that BS suppression occurred when *gish* expression was reduced in all neurons with the *ELAV-Gal4* pan-neuronal driver. We propose that *gish* is a suppressor of *para*^{bss1}/+ based on reversion of phenotypes by *gish*^{04895}/+, by *ELAV-Gal4*^{C155}*-driven UAS-gishRNAi*, by *Df(3R)ED10639/+,* and by *Df(3R)Exel7329/+*. Several mutant alleles of *gish* that failed to suppress *para*^{bss1}/+ BS paralytic phenotypes were also found in these analyses. Thus, suppression was not observed for 3 P-element mutations with inserts in the second intron of *gish* which is spliced out of the long transcripts (genotypes: *para*^{bss1}/+*;gish*^{KG03891}*,* *para*^{bss1}/+*;gish*^{KG16412}, and *para*^{bss1}/+;*gish*^{EY06457}) (Figures 3, 4). No suppression was seen in *para*^{bss1}/+*;gish*^{e01759}/+ flies, which has an insert upstream of the first transcript initiation site (Figure 3, 4).

The *gish*^{04895} mutation raises the threshold for evoked seizures in *para*^{bss1}/+ flies

The mutation *gish*^{04895} is a recessive lethal. As a heterozygote, in a wild type background, it displays a seizure-resistant phenotype. Thus, the seizure threshold of *gish*^{04895}/+ flies is about twice that of wild type Canton-Special flies, 63.4± 5.8 V HFS and 33.8 ± 3.2 V HFS, respectively (Figure 5). The *gish*^{04895}/+ flies have no other apparent phenotypes: electrophysiology, behavior, and morphology are all wild type.

Seizure-suppression for *gish* is seen with flies of the genotype: *para*^{bss1}/+*;* *gish*^{04895}/+ which show a seizure threshold of 15.6 ±2.42 V HFS, higher than the threshold of their *para*^{bss1}/+*;TM6*/+ siblings (9.8 ± 1.09 V HFS seizure threshold) (Figure 5). This seizure-suppression is due to a loss of *gish* function as seen most clearly in deletion flies: *para*^{bss1}/+*;Df(3R)ED10639*/+ show a seizure threshold nearly in the wild type range (22.0 ±2.62 V HFS) (Figure 5). Their *para*^{bss1}/+*;TM3*/+ siblings show a low seizure threshold (10.3 ± 1.73 V HFS). The loss of *gish* function finding was confirmed further by RNAi analysis. Flies of the genotype *ELAV-Gal4*^{C155} *para*^{bss1}/+*; UAS-gishRNAi/+* showed an increased seizure threshold of 29.28 ± 6.78 V HFS compared to their *ELAV-Gal4*^{C155} *para*^{bss1}/+*; +/Tm6* control siblings (8.19 ± 0.355 V HFS) (Figure 5).

Seizure-suppression by *gish* is specific to *para*^{bss1}/+ heterozygotes

Seizure suppressor mutations that have been identified previously have been general suppressors, each suppressing several Drosophila BS mutants. In contrast, *gish*^{04895}/+ suppression is found here to be specific: it appears to only suppress *para*^{bss1}/+ heterozygotes. We tested for *gish*^{04895}/+ suppression against BS mutant, *eas*: *gish* was ineffective as a suppressor. Thus, *eas* mutants showed 100% BS paralysis in a *gish*^{04895}/+ background; electrophysiology also showed minimal increases in seizure threshold (Figure 6). We also find that *gish/*+ does not suppress phenotypes of *para*^{bss1} homozygous females and *para*^{bss1}/*Y* hemizygous males (Figure 4). Thus, *para*^{bss1} homozygotes and hemizygotes showed 100% BS paralysis in a *gish* background: BS paralysis could not be suppressed by *gish*^{04895}/+, by *Df(3R)ED10639*/+, or by *UAS-gishRNAi.* In addition, a *Df(3R)ED10639*/+ background caused no reductions of BS paralytic recovery time in *para*^{bss1} homozygotes and hemizygotes, a phenotype of *para*^{bss1} that is ordinarily easier to suppress than BS paralysis (Figure 6). Electrophysiology showed no increases in seizure threshold (data not shown).

Seizure-suppression by *gish* does not appear to be dependent on Wingless signaling

The *prickle* gene functions in non-canonical Wg/Wnt signaling, and mutations have been found to cause myoclonic seizures in humans and BS paralytic behavior in Drosophila (Tao et al. 2011). CK1g casein kinases subserve a large number of cellular processes with diverse substrates (Knippschild et al. 2005), and one prominent role for *gish* is to phosphorylate *arrow*, a co-receptor for Wg (Zhang et al. 2006). To test if seizure-suppression by *gish* might be via Wg signaling, we examined other components of the pathway by RNAi. To test *arrow* loss-of-function, flies of the genotype *ELAV-Gal4*^{C155} *para*^{bss1}*/+;; UAS-arrRNAi/+* showed a slightly lower, but not significant percentage of BS paralysis compared to control *ELAV-Gal4*^{C155} *para*^{bss1}*/+; +/Tm6* flies (data not shown). To test *Wg* and *pangolin* loss-of-function, flies of the genotypes *ELAV-Gal4*^{C155} *para*^{bss1}*/+; UAS-WgRNAi/+* and *ELAV-Gal4*^{C155} *para*^{bss1}*/+; UAS-panRNAi/+* were comparatively equal in percentage of BS paralysis as their *ELAV-Gal4*^{C155} *para*^{bss1}*/+; tft/+* controls (data not shown). Thus, we conclude that seizure-suppression by *gish* is not directly linked to Wg/Wnt signaling.

Discussion

Epilepsies considered to be intractable are refractory to pharmacotherapy. Clinically, this is defined as a failure to respond to two or more anticonvulsants, that is drugs known to ameliorate seizure severity (Kwan et al. 2010; Kossoff 2011). Polypharmacy is the employment of several medications at once to treat the same condition. Some epileptic individuals require more than one drug to control their epilepsy, but additional drugs rarely lead to complete freedom from seizures. Patients taking polypharmacy may have so many side effects that it is difficult to increase dosage for any of their drugs to an effective level. Also, polypharmacy can include drug interactions limiting effectiveness or increasing side effects of another drug. Some non-medication treatment options exist for intractable epilepsies. In limited cases, surgery is an option; and occasionally there is improvement with ketogenic diet, vagus nerve stimulation, or herbal treatment (alternative medicine) (Kossoff 2011). Generally, however, intractable epilepsies represent severe seizure disorders with severity that is difficult to modulate by available treatment options.

A number of fundamental questions exist regarding intractable epilepsies. For example, true intractable epilepsy results from seizures that are thought to be "too strong" to be controlled by medication. However, it is unclear what "too strong" actually means in terms of underlying physiology dysfunction. There are also examples of "spontaneous" changes in seizure severity. One common observation in refractory epilepsy is the development of medication tolerance. In this, a new drug works for a few months and then becomes ineffective. This cycle can repeat over several new medications with each showing initial effectiveness followed by ineffectiveness. Another example of spontaneous change comes from the observation that intractable epilepsy does not always remain intractable. For example, in one study of 246 patients with intractable epilepsy, the mean duration of epilepsy was 25 years and the mean duration of intractability was 20 years (Callaghan et al. 2007). Over a three-year period, 5% of patients each year became seizure-free for at least six months.

In the present paper, we examine severe seizure phenotypes and explore the possibility that severity may be modulated by genetics. We use as substrate the Drosophila $para^{bss1}$ mutation which models human Dravet syndrome. Like Dravet, $para^{bss1}$ is a mutation affecting the voltage-gated Na^{+} channel. Severe seizure-sensitivity is observed in Dravet and $para^{bss1}$; severity that is unresponsive to available drug treatment. In addition, $para^{bss1}$ has not responded to seizure-suppressor mutations identified in screens based on the Drosophila mutants *eas* and *sda*. The present study is based on an unbiased, forward genetics screen for mutations that interact with $para^{bss1}$ by either exacerbating seizure phenotypes (seizure-enhancer mutations) or reducing the severity of phenotypes (seizure-suppressor mutations).

The search for $para^{bss1}$-enhancers and -suppressors identified several candidates. Analysis of *chn* was representative of an enhancer. We found that the time of paralysis of $para^{bss1}$ individuals was increased (the phenotype screened for), but there was otherwise no obvious enhancement of seizure-sensitivity or severity. Analysis of *gish* was representative of a $para^{bss1}$ suppressor. We found that seizure-sensitivity of heterozygous $para^{bss1}$/+ individuals was greatly reduced by *gish* loss-of-function mutation and by RNAi. The suppression by *gish* is the first and only example (drug or mutation) we have identified that can modulate the percentage of flies exhibiting behavioral seizure and

paralysis. Also, electrophysiological threshold is increased, a further indication that seizure-susceptibility has been reduced in *para*^{bss1}/+ individual flies. A further unusual finding of *gish* suppression is that it is specific for *para*^{bss1}/+ flies: neither *para*^{bss1} homozygote, nor *eas* mutant phenotypes were suppressed by gish loss-of-function.

It is clear from this study that *gish* is capable of suppressing *para*^{bss1}/+ phenotypes; and from other deletions identified in our screen that additional suppressor mutations may be found. These findings suggest a compelling novel approach for developing options for intractable epilepsy therapeutics depending on exactly how well *para*^{bss1} models Dravet syndrome or other intractable epilepsies and how well these findings transfer to mammalian models. At present, available data show that the *para*^{bss1} model is a good one. Further experiments of this type as well as the isolation of new suppressors may bring us closer to unraveling the complexity of seizure disorders, especially intractable disorders.

Figures and tables for Chapter 2

DEFICIENCY	Experimental (Df) MRT (s)	Control (Balancer) MRT (s)	nMRT
Df(2R)Exel7135	363	234	1.55
Df(2R)Exel6078	306	135	2.27
Df(2R)Exel7094	232	102	2.27
Df(2R)Exel6071	217	118	1.84
Df(2R)Exel6056	215	85	2.53

Table 1. Chromosomal deletions that enhance the behavioral bang-sensitive (BS) paralytic phenotype of *para*^{bss1}/+ flies. Values of the length of time that hemizygous *para*^{bss1}*/Y* males remained paralyzed are depicted as mean recovery time (MRT). To minimize the effects of genetic background, experimental males of the general genotype: *para*^{bss1}*/Y;Df/+* were compared directly to sibling control brothers arising from the same cross (genotype: *para*^{bss1}*/Y;Balancer/+*). The ratio of MRT for experimental males with that of their control siblings is listed as normalized mean recovery time (nMRT).

DEFICIENCY	BS
WILD TYPE	0.00
Df(2R)Exel6285	0.03
Df(3L)ED4502	0.07
Df(3R)ED10639	0.13
Df(3L)ED224	0.19
Df(3L)ED201	0.29
Df(3L)ED4502	0.42
Df(2R)BSC427	0.49
Df(3R)ED5518	0.50
Df(3L)ED4486	0.50
$para^{bss1}$/+	0.95

Table 2. Chromosomal deletions that revert the behavioral bang-sensitive (BS) paralytic phenotype of $para^{bss1}$/+ flies. Ordinarily, about 95% of $para^{bss1}$/+ flies show a BS paralytic phenotype: paralysis following mechanical stimulation. Wild type never show BS paralysis. The number of flies showing BS paralysis is greatly reduced by the deficiency chromosomes listed in the table. Flies tested carried the heterozygous deficiency and were of the general genotype: $para^{bss1}$/+; *Df*/+. In all cases, to control for genetic background, experimental flies were compared directly to sibling control flies arising from the same cross (genotype: $para^{bss1}$/+; *Balancer*/+).

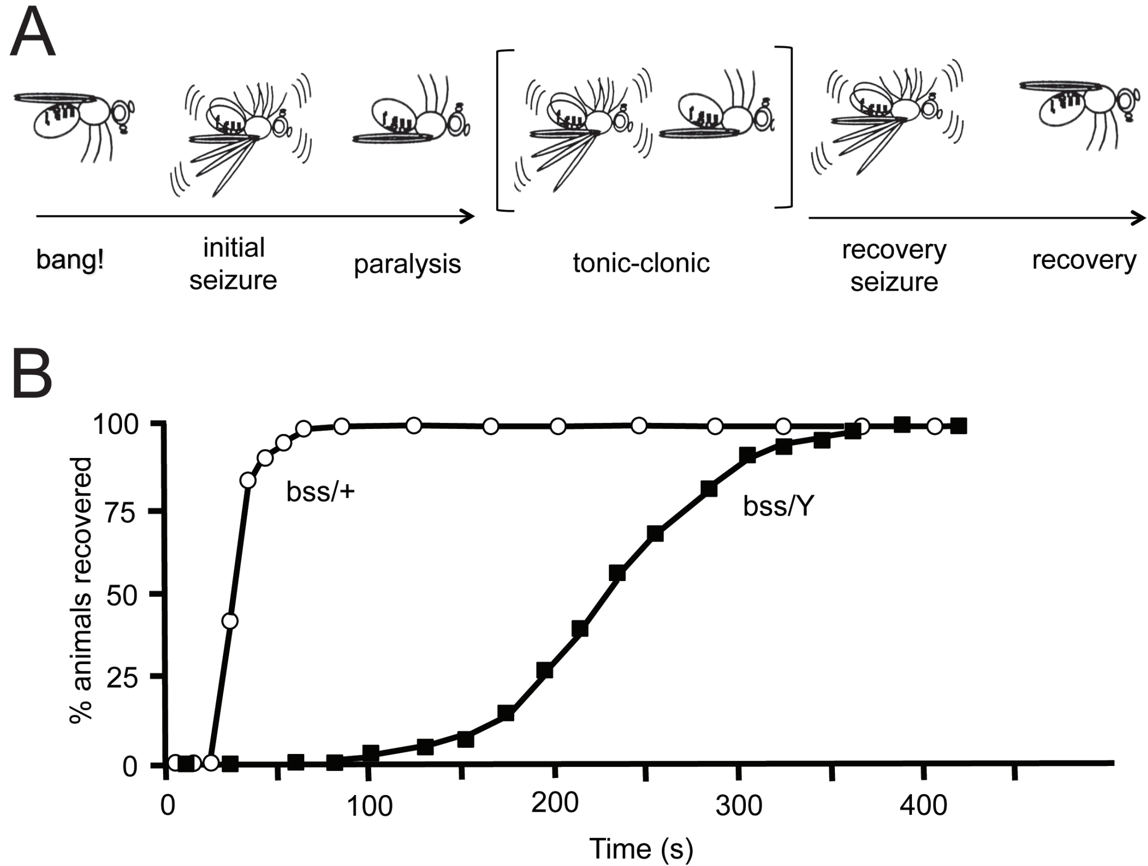

Figure 1. Behavior phenotypes for para^{bss1} mutants. A. Cartoon depicting stereotype behavioral phenotype of para^{bss1} flies subjected to a mechanical shock (10 s vortex: "bang!"): initial seizure-like behavior, followed by complete paralysis and then a tonic-clonic period that is unique to para^{bss1} and not evident in other BS mutant genotypes. One clonus-like event is depicted, but the number can vary, as can the duration of the period. The tonic-clonic-like period is followed by a recovery seizure, and the fly then recovers. Not depicted is a quiescent period of variable duration often observed between the recovery seizure and recovery, as well as the refractory period during which flies are resistant to further seizures that occurs immediately following recovery. B. Recovery times from behavioral paralysis for para^{bss1}/Y hemizygous males (labeled "bss/Y") is substantially longer than for para^{bss1}/+ heterozygous females (labeled "bss/+"). For the enhancer screen described in the text, heterozygous deletions were selected that prolonged the para^{bss1}/Y recovery time compared to sibling controls. For the suppressor screen described in the text, heterozygous deletions were selected that reduced the percentage of para^{bss1}/+ females paralyzed by the mechanical shock compared to sibling controls. (Figure adapted from Parker et al. 2011).

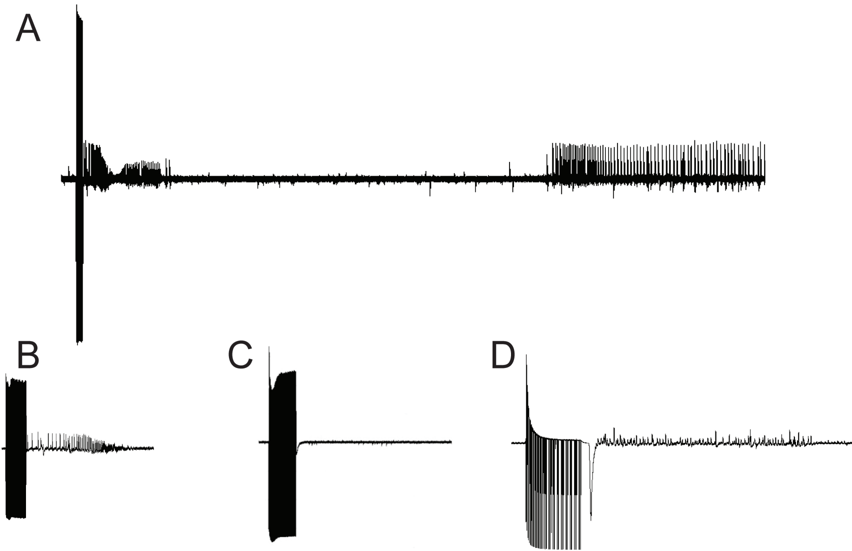

Figure 2. Electrophysiology phenotype of *para*^{bss1} mutants. Seizure-like electrical activity in *para*^{bss1} and wild type flies. The mutant fly is more susceptible to seizures and has a lower threshold. A. Seizure-like activity displayed at a slow sweep speed showing initial seizure, period of synaptic failure, and recovery seizure. B. Seizure-like activity is evoked by 4 V HFS stimulus and displayed at high sweep speed. The mutant is susceptible to low-voltage evoked seizures indicating extreme seizure-sensitivity. C. A low voltage 4 V HFS stimulus delivered to a wild type fly is ineffective at eliciting seizure-like activity because it is below the seizure threshold. D. A higher voltage 30 V HFS stimulus delivered to a wild type fly elicits seizure-like activity because it is above threshold for seizure initiation.

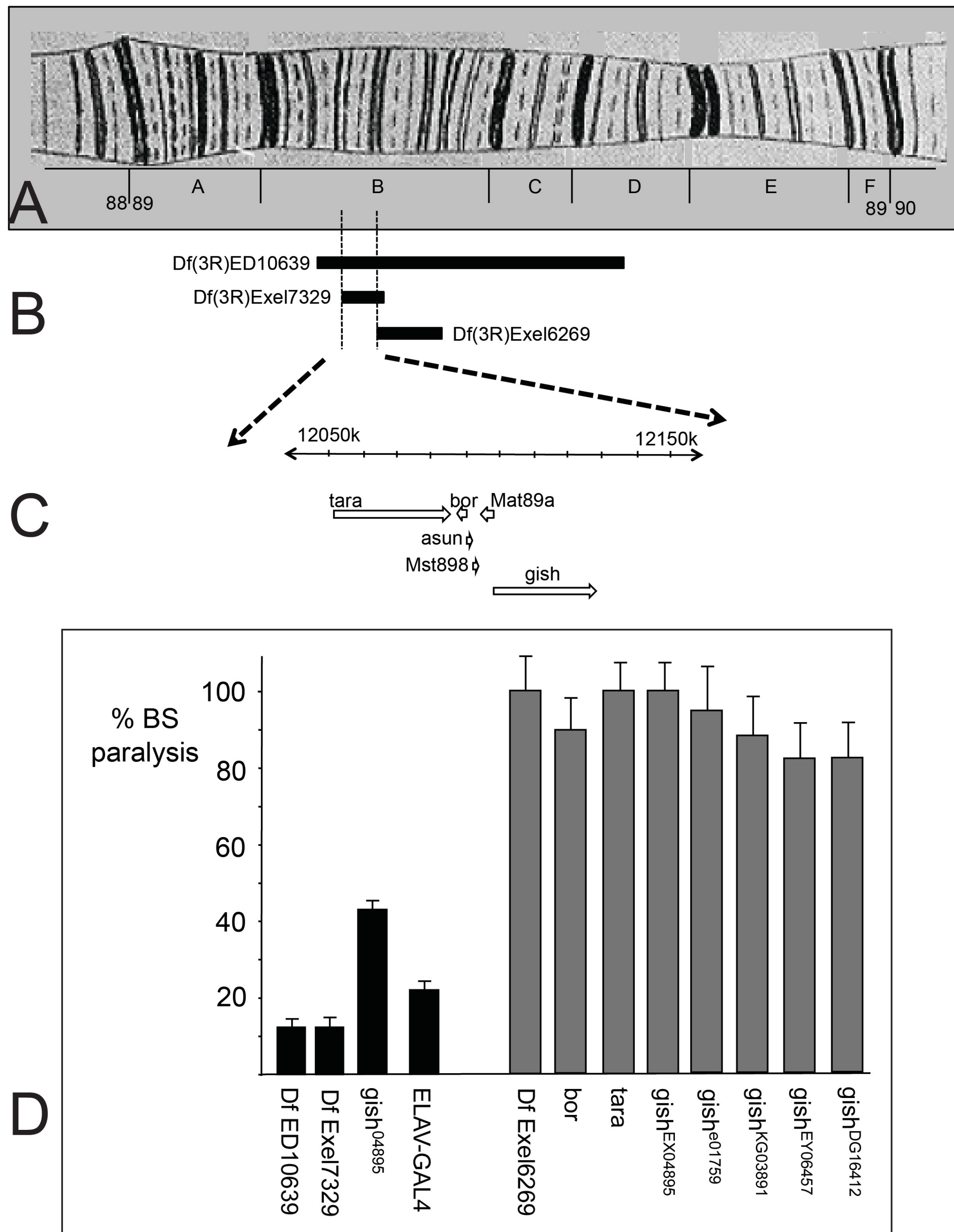

Figure 3. Suppression of $\boldsymbol{para^{bss1}}$**/+ BS paralytic phenotype by a heterozygous chromosomal segment deleted in 89B.** A. Depicted is polytene chromosome map of region 89 on 3R. B. The segment deleted in *Df(3R)ED10639* causes suppression of $para^{bss1}$/+ BS paralysis, as described in the text. Additionally, *Df(3R)Exel7329* causes suppression, and *Df(3R)Exel6269* does not cause suppression. The breakpoints of these rearrangements delimit a small region (89B9 to 89B12) responsible for seizure-suppression. C. Six genes are contained in the 89B9 to 89B12 chromosomal segment including *tara*, *bor*, and *gish*. D. BS

paralytic phenotypes (% BS paralysis) of several genotypes in a *para*^{bss1}/+ background, as described in the text. Genotypes showing BS suppression are depicted as black bars; gray bars are used in genotypes showing no suppression. In each case, the experimental genotype shown is normalized relative to sibling controls. Df ED10639 is the genotype *para*^{bss1}/+; *Df(3R) ED10639/+* showing 13% BS paralysis (87% suppression of BS phenotype). This indicates the apparent presence of a gene that acts as a haplo-seizure-suppressor. Df Exel7329 is *para*^{bss1}/+;*Df(3R)Exel7329/+* showing 13% BS paralysis and providing one boundary for suppressor location at 89B9 based on inclusion within the deleted segment. Df Exel6269 is *para*^{bss1}/+;*Df(3R)Exel6269/+* showing 100% BS paralysis and providing a second boundary for suppressor location at 89B12 based its exclusion from the deletion. Flies that are *para*^{bss1}/+;*bor*^{c05496}/+ and *para*^{bss1}/+;*tara*^{1}/+ (labeled bor and tara) show no suppression with 91% and 100% BS paralysis, respectively. Flies that are *para*^{bss1}/+;*gish*^{04895}/+ (labeled gish^{04895}) show 43% BS paralysis indicating suppression of the BS paralytic phenotype. Flies that are *para*^{bss1}/+;*gish*^{EX04895}/+ (labeled gish^{EX04895}) are a line with a remobilized, precise excision of the *gish*^{EX04895} P-element; they show no suppression with 98% BS paralysis. Flies that are *ELAV-Gal4*^{C155} *para*^{bss1}/+; *UAS-gishRNAi/+* (labeled ELAV-GAL4) show 25% BS paralysis indicating suppression of the BS paralytic phenotype. Several *gish* alleles as heterozygotes show no suppression of *para*^{bss1}/+ BS paralytic phenotypes. Thus, gish^{e01759}/+, gish^{DG16412}/+, gish^{KG03891}/+, gish^{EY06457}/+ heterozygous combinations in a *para*^{bss1}/+ background show 95%, 88%, 84%, and 83% BS paralysis, respectively.

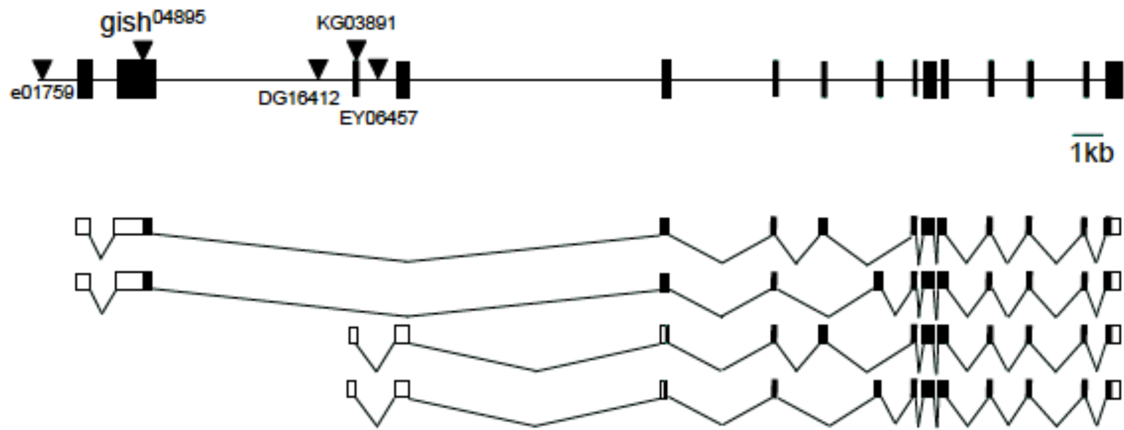

Figure 4. Organization of the *gish* gene and location of P-element mutations. A. P element insertion sites (inverted black triangles) in the *gish* gene for various mutant alleles. Black rectangles indicate exons. B. Four major types of transcripts arising from the *gish* gene. Transcripts initiate from exon for long transcripts and initiate from exon 3 for the short transcripts. Black exons correspond to coding sequence. (Figure adapted from Tan et al. 2010).

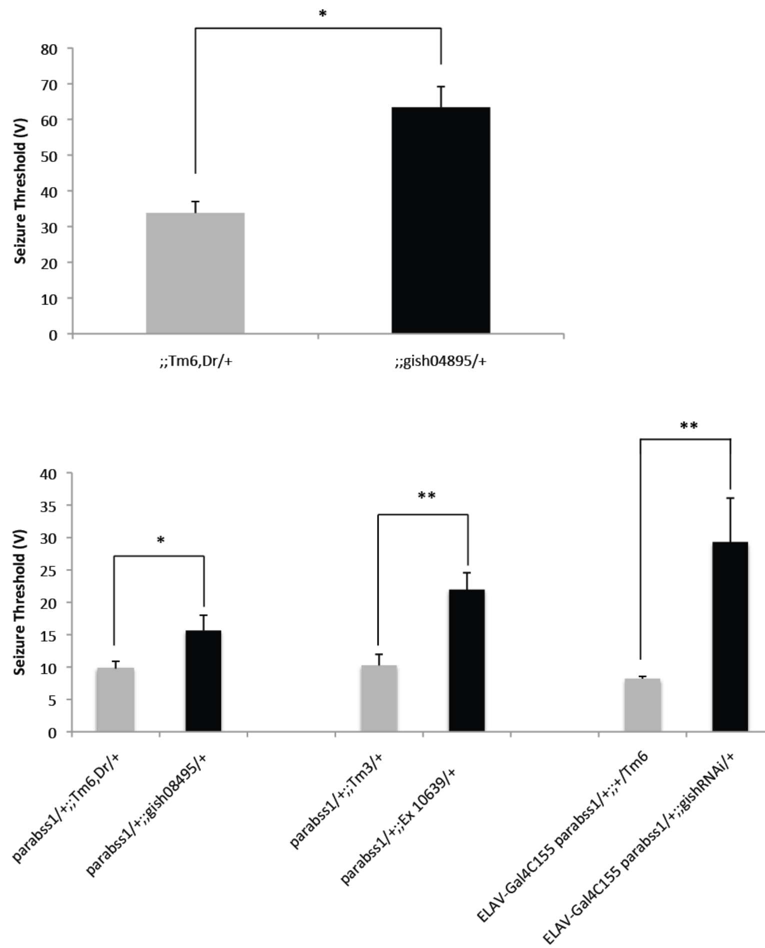

Figure 5. Suppression of seizure threshold by *gish*^{04895} and Df Ed10639. A. Seizure threshold of *gish*^{04895} as compared to Canton-Special. B. Seizure thresholds of *para*^{bss1} heterozygotes in *gish*^{04895}, Df Ed10639, and *ELAV-Gal4* driven *gish*RNAi.

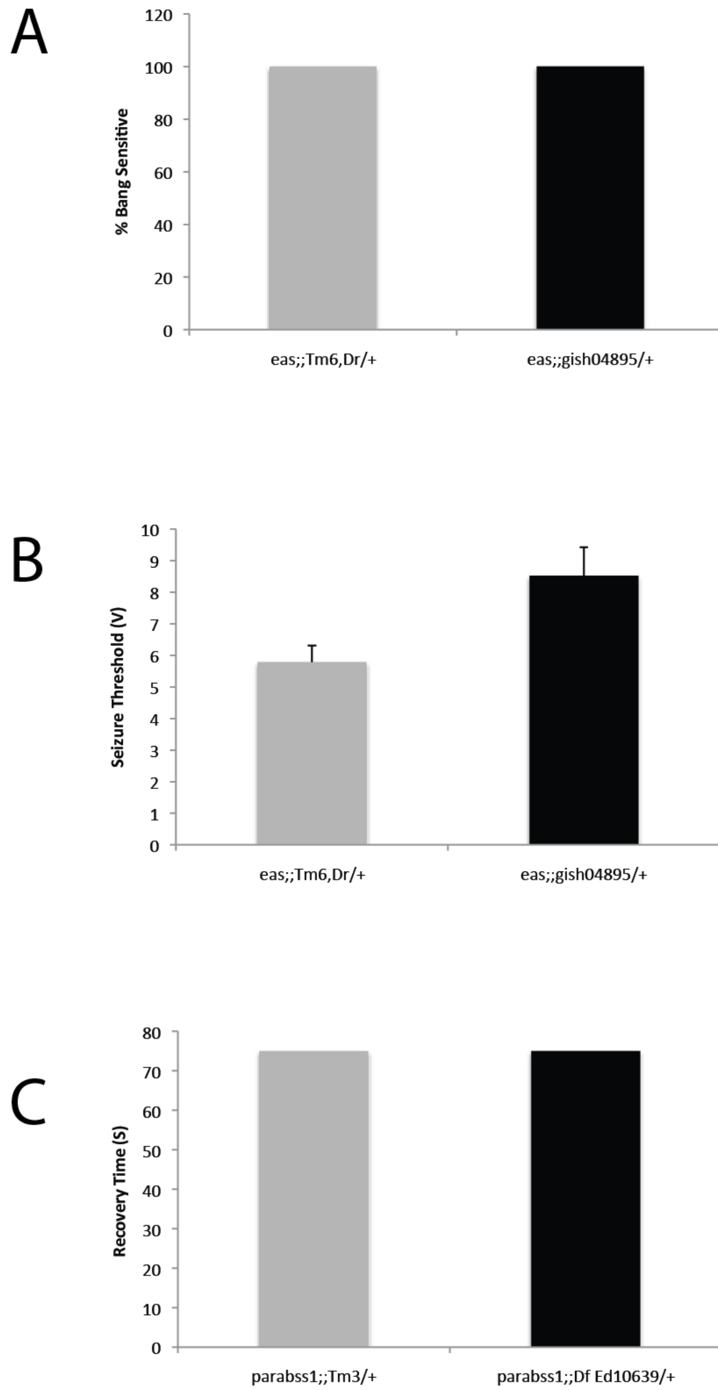

Figure 6. Suppression of seizure sensitivity by *gish* is specific to *para*^{bss1} heterozygotes. A. The percentage of *eas* flies showing a bang-sensitive paralytic phenotype is not reduced by *gish*^{04895}/+. B. Seizure threshold of *eas* is only slightly increased by *gish*^{04895}/+. C. Recovery time of *para*^{bss1} homozygotes and hemizygotes is not altered by *gish*^{04891}/+.

Chapter 3
Seizure-sensitivity in *Drosophila* is ameliorated by dorsal vessel injection of the antiepileptic drug valproate

Introduction

There is considerable interest in exploiting the power of Drosophila genetics for the study of human pathologies, especially genetic-based diseases (Lu and Vogel 2009; Nedelsky et al. 2010, for example). Study of *Drosophila* mutants has provided models for many problems in biology, particularly developmental biology and neurobiology (Rubin and Lewis 2000, for example). These have shown that fundamental processes and essential gene products are conserved across species, allowing findings to be generally applicable to other biological systems including mouse and human (Veraksa, Del Campo, and McGinnis 2000). Drosophila-based models are especially attractive in their potential for developing new therapeutics through identification of disease-causing genes followed by targeted drug development, or by development of high-throughput drug screening platforms (Song and Tanouye 2009).

Efficient drug delivery methods are required to identify a potential drug candidate from a library of chemical compounds. Most commonly utilized are feeding methodologies: flies are starved for a short time and then fed drug in sucrose solution (Tan, Lin, and Tanouye 2004; Song et al. 2008, for example). Drug feeding is simple and straightforward: large numbers of flies can be tested, and similar methods for delivering chemical mutagens such as EMS (ethylmethanesulfonate) have been utilized historically in most fly laboratories (Watanabe and Yamazaki 1976). A difficulty is that flies have an efficient detoxification system evolved for protection against plant phytochemicals, adaptable to insecticides and other compounds, which can greatly reduce drug efficacy and interfere with determination of effective and lethal dosages (Chung et al. 2009; Willoughby et al. 2006). For neurological drugs, there are often solubility difficulties with non-polar compounds and there is a formidable blood-brain barrier (Carlson et al. 2000; Stork et al. 2008; Mayer et al. 2009). Some drugs may be tested as volatilized compounds similar to methods used to screen for alcohol and cocaine -resistant or -sensitive mutants (Weber and Diggins 1990; McClung and Hirsh 1998), and for other uses (Scharf, Nguyen, and Song 2006). Neurological drug testing may also be performed by direct brain injection of drug (Kuebler and Tanouye 2002). This method has the advantage that it tests drug effects directly on the brain, bypassing detoxification mechanisms and blood-brain barrier problems. However, it is considerably more difficult than other methods and there are limits on analysis following injection. At this time, no single drug testing method is ideal for testing every drug. As an example, comparisons have been made of the effectiveness of valproate, an antiepileptic drug (AED), in a *Drosophila* model of human epilepsy (Kuebler and Tanouye 2002; Song et al. 2008). Valproate was effective at reducing seizures following brain injection; it was considerably less effective in drug feeding tests.

Here, we examine another method of neurological drug testing, direct injection of drug into the circulatory system of *Drosophila*. The *Drosophila* heart is a simple pump consisting of striated muscle just a few cell layers thick. It is responsible for transport of hemolymph. The dorsal vessel in adult flies is located medially and dorsally and extends from the sixth abdominal segment to the dorsal anterior region of the brain (Curtis, Ringo, and Dowse 1999). Here we examine the utility of injection of AED valproate into the heart to deliver the drug to the brain with subsequent testing for amelioration of seizure-sensitivity with comparisons to previously reported methods of feeding and brain

injection. This method is found to be especially effective in some instances when used in combination with a genetic background that includes the mutation Mdr65 (*Multidrug resistance*), which affects the functional integrity of the fly blood-brain barrier (Mayer et al. 2009).

Materials and methods

Fly Stocks

Drosophila melanogaster strains were raised on standard cornmeal medium at room temperature (23°C). Bang-sensitive (BS) paralytic mutants were *paralyzed*^{bss1} *(para*^{bss1}*), easily shocked (eas),* and *slamdance (sda)*. The BS mutants are more susceptible to seizures than normal flies. The behavioral and corresponding electrophysiological phenotypes have been described (Pavlidis and Tanouye 1995; Kuebler and Tanouye 2000). The *eas* gene encodes a protein homologous to human ethanolamine kinase (Pavlidis, Ramaswami, and Tanouye 1994). The *sda* gene encodes an aminopeptidase N homolog (Zhang et al. 2002). The *para*^{bss1} mutation is a gain-of-function mutation in the voltage-gated Na^+ channel gene, previously named *bss*^{1} (Parker et al. 2011). The mutant *Multidrug resistance (Mdr65)* is the fly homolog of the human ATP binding cassette transporter; in both fly and human, the transporter is localized in the blood-brain barrier and functions in efflux transport (Mayer et al. 2009). The Mdr65 stock used was obtained from the Bloomington Stock Center. Control flies used throughout were the *w*^{1118} strain.

Electrophysiology

Electrophysiology experiments on unanaesthetized adult male flies were performed as described previously (Kuebler et al. 2001). In brief, flies were immobilized by mounting them in a layer of dental wax affixed to a glass slide (Allen et al. 1999). Tungsten stimulating electrodes were inserted into the head, with single-pulse stimuli (0.2 ms in duration, 0.8 Hz) utilized to drive the giant fiber (GF) pathway as a proxy to assess general nervous system function, and high frequency electrical stimulus (HFS) wavetrains (0.5 ms pulses at 200 Hz for 400 ms) utilized to determine seizure-susceptibility (Kuebler and Tanouye 2000; Lee and Wu 2002). Muscle potentials were monitored in the dorsal longitudinal muscles (DLMs) using tungsten recording electrodes. Threshold for the GF was the lowest stimulation voltage at which single-pulse stimulation reliably evoked a GF pathway response. Following frequency of the GF circuit was determined by 20 suprathreshold stimulus pulses (1.2–1.4 times the GF threshold) delivered to the GF. The value for following frequency was the highest frequency at which the DLM responded to at least 19 of the 20 pulses. During the following frequency analysis, flies were rested >1 minute between stimulus trials. Seizure-like activity was seen as uncontrolled, high-frequency (>100 Hz) motoneuron activity evoked by HFS stimulation and recorded in the DLM (Kuebler and Tanouye 2000; Lee and Wu 2002). Seizure threshold was the lowest HFS voltage that evoked seizure-like activity. For seizure threshold determination, flies were rested 20 minutes between each delivery of HFS stimulation. The number of animals examined for each experimental group was $n \geq 10$.

Drug Delivery

Saline solution composition was (in mM): 100 NaCl, 25 KCl, 6 $CaSO_4$, 10 $MgSO_4$, 4 $NaHCO_3$, 1 NaH_2PO_4, 5 trehalose, 75 sucrose, 5 HEPES at pH 7.2 (Trimarchi and Murphey 1997). Saline solution containing valproate (Na-valproate, Sigma), was made fresh each day to the appropriate concentration utilizing a valproate stock solution (25 mM). A drop of green food coloring was added to 1 ml of the injected solution to allow visualization of the drug post injection. Concentrations of valproate are indicated in the text and refer to the value of Na-valproate in saline solutions used in the injections. For injecting solutions, a sharpened tungsten wire was used to puncture the cuticle in the space between the second and third abdominal tergites. A glass micropipette (1 μm tip diameter) filled with test or control solution was then inserted into the puncture site. Solution was pressure injected until food coloring was visible in the abdomen. If food coloring did not reach the head capsule within several seconds, the preparation was discarded. Following drug injection, the fly was rested for 10 minutes before electrophysiological analysis commenced.

An Eppendorf microloader pipette tip was used to estimate the volume of drug solution injected into each fly which was approximately 0.75 μl/injection. Fly hemolymph volume was estimated by comparing the wet and dry weights of 100 adult males with a value of 0.60 μl/fly. These considerations, taken together with dilution due to dye (approximately 45 μl/ml) would indicate that effective drug concentrations in the fly are somewhat lower than the value of the injected solution by about a factor of 0.53. Thus, 25 mM valproate saline injected into the fly is closer to about 13.3 mM valproate, effective dose.

Data Analysis

Comparisons of paralytic recovery time and seizure threshold were Student's *t*-test and ANOVA, as appropriate. For ANOVA, where the null hypothesis was rejected by overall F-ratio, multiple comparisons of data sets was performed by Fisher's Least Significant Difference (LSD) with *t*-test significance set at $P<0.05$. For all figures, error bars represent standard error of the mean, and statistical significance is indicated by * $P<0.01$ and ** $P<0.0001$.

Results

Solutions Delivered to the Fly Head by the Circulatory System

The circulatory system of insects is an open system with much of the blood flow occurring in the body cavity, a hemocoel, which is divided into three main compartments, the pericardial sinus, the perivisceral sinus, and the perineural sinus (Klowden 2002). The closed portion of the circulatory system is the dorsal vessel usually divided into heart and aorta. Pumping of hemolymph is mainly by the heart aided by various pulsatile organs and aliform muscles. Pressure injection of a colored solution into the dorsal part of the abdomen results in a marked change of color in the abdomen. In some cases, the color change was restricted to the abdomen. For these preparations, we inferred that the injected solution did not enter the dorsal vessel, and the fly was therefore discarded. In most instances, the abdominal color change was accompanied by a corresponding change in color of the head capsule, which occurred within a few seconds of injection. We inferred that in these animals, injected solution entered the dorsal vessel and was actively pumped to the head capsule by the heart (Figure 1). We examined these flies for evidence of drug effectiveness.

Drug Injection and Neuronal Excitability: No Effect on GF Threshold; Modest Influence on Following Frequency

Valproate is a wide-spectrum AED used to treat generalized absence and tonic-clonic seizures, and a variety of other non-epileptic neurological disorders. Valproate leads to a decrease in neuronal excitability probably through a combination of effects: block of voltage-gated Na+ channels and enhanced inhibition (Rogawski and Löscher 2004; Landmark 2007; White, Smith, and Wilcox 2007; Greenhill and Jones 2010). We tested for changes in neuronal excitability due to valproate injection using the Drosophila adult giant fiber (GF) neural circuit (Tanouye and Wyman 1980; Allen et al. 2006). The GF response is evoked by single-pulse electrical stimulation and the value of its response threshold has been taken as a measure of single neuron excitability. For example, the *maleless-no-action potential* (mle^{napts}) mutant has a higher GF threshold indicating a reduction in neuronal excitability (Kuebler et al. 2001). This is consistent with the reduced Na+ channel expression that underlies mle^{napts} mutant paralytic phenotypes (Wu et al. 1978; Kauvar 1982; Jackson et al.; Kernan et al. 1991; Lee and Hurwitz 1993). By this measure, injection of valproate into the dorsal vessel was found to cause no major changes in single neuron excitability. For experimental w^{1118} flies injected with 25 mM valproate saline, the mean GF threshold was 1.78±0.051 V (n= 20). This is similar to the mean GF threshold values seen in control flies: uninjected w^{1118} flies had a GF threshold of 1.875±0.071 V (n= 20), and the threshold of w^{1118} flies injected with saline without drug was 2.04±0.087 V (n= 20) ($F_{x,y}= 4.136$; P= 0.018; ANOVA). In all cases, the GF threshold remained stable throughout the course of the experiment, about 30 minutes. Our impression was that in experiments of longer duration, flies injected with 25 mM valproate saline were more susceptible to deterioration, however, this was not examined in detail. These results suggest that there are not major changes in single neuron

excitability caused by saline injection or 25 mM valproate saline, within the resolution of these measurements.

GF pathway following frequency, the maximum stimulation frequency that the GF pathway can reliably follow, may also be used to evaluate neuronal excitability. For example, Na+ channel mutants, mle^{napts} and *paralytic (para)* have been found to have greatly reduced GF pathway following frequencies compared to wild type flies reflecting a reduction in neuronal excitability (Kuebler et al. 2001). In the present experiments, uninjected w^{1118} flies displayed a following frequency of 133.69±3.04 Hz (n= 20), similar to wild type Canton-Special and Berlin flies reported previously (Kuebler et al. 2001). Flies injected with 25 mM valproate saline showed a following frequency of 109±4.64 Hz (n= 20), indicating that by this measure, valproate injection had a modest, but significant effect on reducing excitability (P<0.001 Student's *t*-test). In comparison with previous findings, the decrease in excitability caused by valproate injection in w^{1118} remains within the wild-type range; these animals remain slightly more excitable than Oregon-R flies (98 Hz) and much more excitable than for ion channel mutants that showed following frequency reductions ranging from 88 Hz (*para*) to 59 Hz (mle^{napts}) (Kuebler et al. 2001).

Injected valproate Increases the Seizure Threshold for Wild Type flies and Multiple Bang-Sensitive Mutants

Seizure-like activity in the *Drosophila* nervous system can be elicited by high frequency (HF) electrical stimuli delivered to the brain, with different wild-type and mutant strains exhibiting characteristic seizure susceptibilities (Pavlidis and Tanouye 1995; Kuebler and Tanouye 2000; Lee and Wu 2002). Here, HFS stimuli elicited seizures in uninjected w^{1118} control flies at a threshold of 30.1 ± 2.88 HFS V (n= 20), similar to the values reported previously for wild type CS flies (~30 HFS V; Kuebler et al. 2001). Injection of control saline showed a slight increase in seizure threshold for w^{1118} flies (45.5 ± 4.68 HFS V). In contrast, injection of 25 mM valproate saline solution caused a large increase in seizure threshold to 88.20 ± 5.1 HFS V (n= 20)(P< 0.0001, Student's *t*-test).

The seizure threshold for the seizure-sensitive mutant *sda* is much lower than wild type: 9.1±0.64 HFS V (n= 20) for saline-injected *sda* control flies, similar to uninjected mutants previously reported (Kuebler and Tanouye 2001). Injection of 25 mM valproate saline solution greatly increased seizure threshold of *sda* mutants, by about a factor of seven to 62.6±3.46 HFS V (n= 20)(P< 0.0001, Student's *t*-test). Valproate similarly increased seizure thresholds for other seizure-sensitive mutants, albeit to a lesser extent (Figure 2D). The seizure threshold for valproate-treated *eas* flies was to the wild type range 32.7±3.54 (n= 23), compared to 4.7±0.5 HFS V (n= 20)(P< 0.0001, Student's *t*-test) for saline-injected *eas* flies (Figure 2A-C). For $para^{bss1}$, valproate solution caused a more modest change in seizure threshold, by about a factor of two. Seizure threshold for drug-treated $para^{bss1}$ flies was 12.7±2.51 HFS V (n= 20) compared to 4.57±0.63 HFS V (n= 20)(P< 0.005, Student's *t*-test) for hemolymph-injected controls. Overall, these results are generally comparable to other findings on the suppression of seizure phenotypes by mutation and drugs: *sda* phenotypes have been found to be the easiest to suppress,

para^{bss1} the most difficult to suppress with *eas* in between (Kuebler and Tanouye 2001; Song et al. 2008).

We used *eas* as a convenient genotype to examine dosage-response relationships for valproate effects on seizure-susceptibility. Decreases of valproate concentration in the dorsal vessel injections were accompanied by concomitant decreases in seizure threshold. However, even in the lowest valproate concentration examined here, 2.5 mM, seizure threshold for *eas* was 12.26±81.93 HFS V (n= 20)(P< 0.0001), about twice value of seizure threshold for saline-injected *eas* controls (Figure 3).

Injected 25 mM valproate has a greater effect in a genetic background containing the blood-brain barrier mutant *Mdr65*, but no effect at lower dosages

Drosophila possesses an effective blood-brain barrier composed of subperineural glia and pleated septate junctions that provide a physical barrier; and transporters, including the ATP binding cassette transporter Mdr65, that expel lipophilic molecules (Carlson et al. 2000; Stork et al. 2008; Mayer et al. 2009). Here, we examined, with mixed results, the effect of an Mdr65 genetic background on valproate injections. The Mdr65 mutant has a seizure susceptibility similar to wildtype flies with a threshold of 48.9±5.3 HFS V (n>10). With injection of 25 mM valproate, Mdr65 mutants become seizure resistant, with a threshold above 80 HFS V. Measurements above this voltage are often more variable than at lower voltages and difficult to interpret. Interestingly, when Mdr65 is combined in an *eas* homozygous genetic background (genotype: *eas; Mdr65*), the seizure threshold of *eas* is increased to approximately 12.2 HFS V, suggesting that the *Mdr65* mutation may be a partial suppressor of *eas*. Because of this, we did not further examine valproate injections in this genetic background. We also investigated *para^{bss1}*, the most difficult of the BS mutants to suppress. The seizure threshold for the homozygous double mutant *para^{bss1};Mdr65* is 3.4±0.28 HFS V (n=10), similar to the single mutant *para^{bss1}*, and indicating that unlike for *eas, Mdr65* does not apparently act as a seizure-suppressor for *para^{bss1}* (Figure 3B). Varying the dosage of valproate in the injection shows that at lower concentrations, no great effect is seen in *para^{bss1};Mdr65* double mutants when compared to injected *para^{bss1}* flies (Figure 3C). For example, an injection of 15 mM valproate saline shows suppression with a seizure threshold of 10.3±0.44 HFS V (n=10) in *para^{bss1};Mdr65*. This is similar to the suppression seen in *para^{bss1}* flies injected with 15 mM valproate. These flies show a seizure threshold of 10.0±0.67 HFS V (n=10). Interestingly, however, the seizure threshold of the double mutant injected with 25 mM valproate saline is 47±6.4 HFS V (n=10), considerably above the wild type range (Figure 3B). The seizure-sensitivity of *para^{bss1}* has heretofore been the most difficult to suppress by suppressor mutation or by drug, and the methodology employed in the present experiments appears to provide most effective suppression that we have thus far observed; we suspect comparable to direct brain injection of valproate (Kuebler and Tanouye 2002), although the latter is difficult to quantify.

Discussion

AEDs are a heterogeneous group of compounds with various targets of action generally either: i) blocking voltage-gated ion channels (Na+ channels, Ca++ channels), ii) enhancing synaptic inhibition (GABA transmission), or iii) reducing synaptic excitation (glutamate transmission)(Rogawski and Löscher 2004; Landmark 2007; White, Smith, and Wilcox 2007). The broad-spectrum AED valproate is one of the most widely used drugs worldwide to treat both generalized and partial seizures in adults and children. It has been used increasingly in the treatment also of non-epileptic neurological sydromes, including anxiety, schizophrenia, and bipolar disorder (Löscher 2002; White, Smith, and Wilcox 2007; Johannessen 2000; Johannessen Landmark 2008; Greenhill and Jones 2010). The broad antiepileptic efficacy of valproate is thought to reflect a combination of neurochemical and neurophysiological mechanisms directed against multiple molecular targets (Löscher 2002). Valproate has been found to inhibit voltage-gated Na+ channels and block repetitive firing of action potentials, to block T-type Ca++ channels, to elevate GABA levels and potentiate GABA responses (Landmark 2007; White, Smith, and Wilcox 2007).

In the present study, we find acute application of valproate suppresses seizures in several *Drosophila* BS mutant genotypes consistent with its broad antiepileptic efficacy. The mechanism of this suppression in *Drosophila* is not completely clear. Seizure suppression by valproate is similar in effectiveness to the best genetic suppressors in our collection, the Na+ channel mutants *para* and mle^{napts}. However, whereas *para* and mle^{napts} mutants show considerable reduction in neuronal excitability, as indicated by monitoring the GF neural circuit, valproate has only a modest effect, suggesting that suppression is not due to Na+ channel block. The acute nature of the valproate effect, might suggest it unlikely that its suppression of *Drosophila* seizures could be due to metabolic changes, such as an effect on succinate semialdehyde dehydrogenase known to raise GABA neurotransmitter levels (Johannessen 2000; Löscher 2002). However, this remains a possible mechanism since in mammals, valproate can rapidly induce significant GABA increases within 5 minutes. (Löscher and Vetter 1984; Johannessen 2000). Another possibility is through a decrease in glutamatergic excitation via intracellular signaling pathways (Landmark 2007).

Dorsal vessel injection described here is a novel and valuable method for drug delivery, especially for neurological drugs. Drug feeding has the advantage that it allows a considerably greater throughput; large numbers of flies may be simultaneously fed drug allowing the possibility of designing drug-based screening. However, valproate seizure suppressive effects in dorsal vessel injection experiments are substantially more efficacious compared to drug feeding (Song et al. 2008). The analyses of dorsal vessel injection lead to quantifiable comparisons of seizure threshold, unlike the case for experiments utilizing brain injection of drug (Kuebler and Tanouye 2002).

The effectiveness of drug feeding methods in *Drosophila* neurological mutants may be limited by several factors, most saliently detoxification mechanisms, such as multifunction oxidases, and the blood-brain barrier separating the brain from the hemolymph. The present analysis suggests that both of these mechanisms are in play in seizure-suppression by valproate. We presume the greatly improved effectiveness of

dorsal vessel injection over valproate feeding analysis to be mainly due to by-passing the fly digestive system thereby avoiding detoxification enzymes.

Figures for Chapter 3

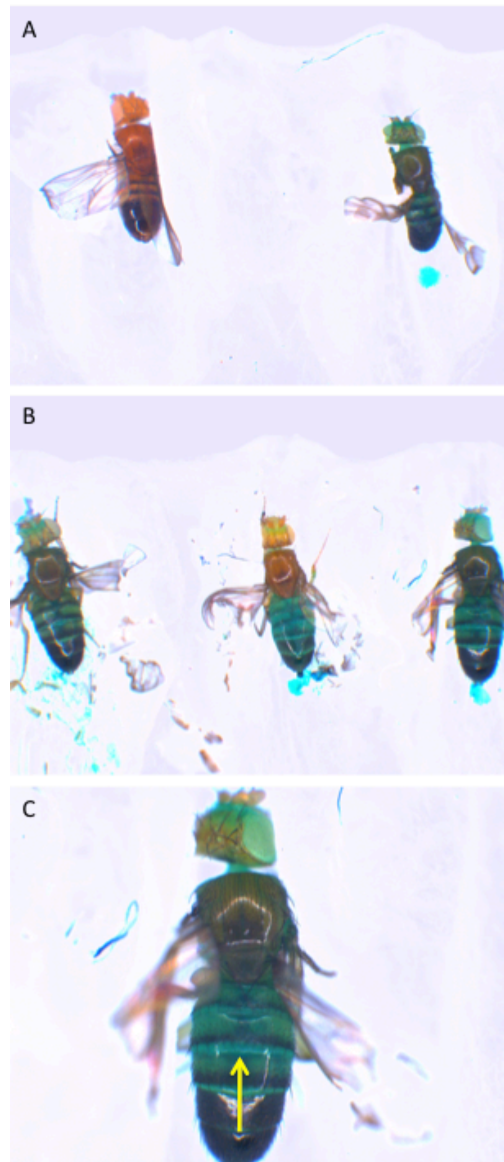

Figure 1. Dorsal vessel injection. Injection into *Drosophila* circulatory system delivers solution to the head. (A) Two flies are mounted in wax for electrophysiological recording. The fly on the right was injected in the abdomen with saline containing green food coloring and a few seconds after injection the entire fly turned green indicating spread of the solution. Our interpretation is that the solution was injected into the dorsal vessel and spread by the circulatory system throughout the fly. For comparison, the fly on the left is an uninjected control. (B) In this panel, three mounted flies are injected. For flies on the left and right, dye is spread throughout and we presume that the pipette successfully delivered solution to the dorsal vessel. For the fly in the center, dye is limited to the abdomen. We presume that solution was not delivered to the dorsal vessel in this instance and such flies are discarded. (C) Larger image of one of the flies in (B). Arrow shows the site of pipette placement.

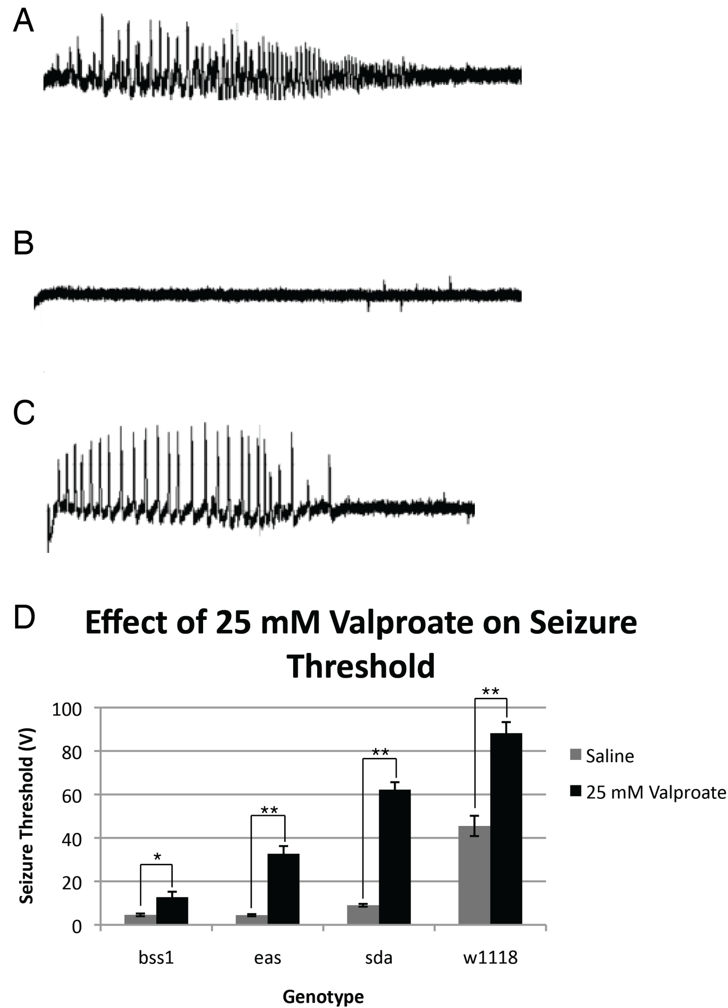

Figure 2. Effect of 25 mM valproate on BS mutants. Seizure threshold in the *eas* mutant is increased by valproate. (A) A representative seizure as recorded from the DLM following a high-frequency stimulus (HFS) delivered to the brain of an *eas* fly injected with control saline solution without valproate at 6 HFS V. During seizure, the innervating DLM motoneuron shows aberrant high-frequency firing. The initial seizure depicted is followed by synaptic failure and then a recovery seizure (not shown). (B) Failure to elicit seizure in an *eas* fly injected with saline containing valproate (25 mM) following a stimulus of 10 HFS V suggesting that the seizure threshold was increased by the valproate injection. (C) Seizure is evoked by the delivery of a 34.0 HFS V stimulus in the same fly as (B) suggesting that the higher intensity stimulus is above the seizure threshold and is effective in evoking seizure. (D) Valproate injection causes an increase in seizure threshold for three bang sensitive (BS) mutants, $para^{bss1}$ (labeled *bss1), eas,* and *sda.* Calibration: 10 mV (vertical calibration bar) and 200 ms (horizontal calibration bar) for (A), (B) and (C).

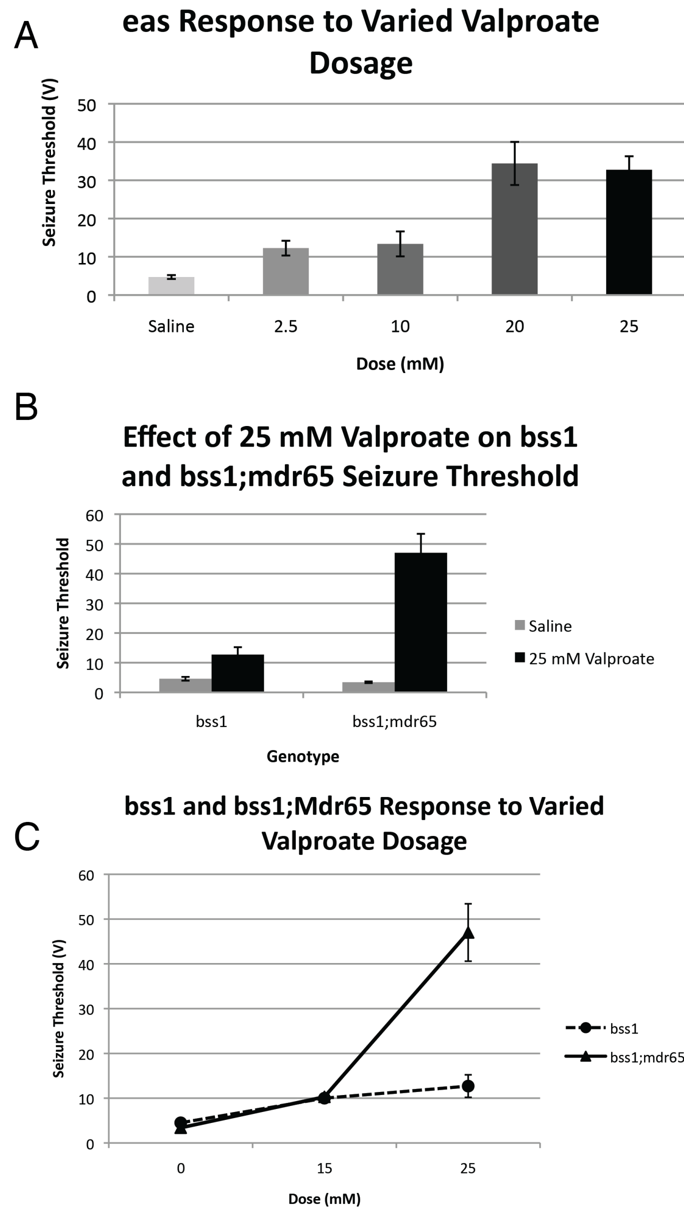

Figure 3. Effects of varied valproate concentration and blood brain barrier mutant Mdr65. (A) The seizure threshold of *eas* varies with the concentration of injected valproate. Valproate causes a significant increase in seizure threshold at the lowest concentrations examined in this study (2 mM valproate). From about 10 mM valproate until the highest concentrations examined (25 mM valproate), the value of seizure threshold appears to increase steeply with drug concentration with the effect saturating at ~20 mM. (B) Injection of 25 mM valproate saline has a modest, but significant effect on $para^{bss1}$ seizure threshold (left panel). The effect of 25mM valproate on seizure threshold is substantially greater in the $para^{bss1}$*; Mdr65* double mutant suggesting a large blood-brain barrier contribution. (C) The seizure threshold of the $para^{bss1}$*; Mdr65* double mutant is similar to that of $para^{bss1}$ at lower dosages of valproate.

Chapter 4
Summary and Conclusions

Epilepsy and seizure disorders affect a large portion of the population. Recent efforts have lead to a greater understanding of the nature of these disorders. Yet, despite recent advances, much remains unclear about the genetic and physiological mechanisms behind epilepsy. Genetic factors play a large role in regulating seizure susceptibility, either by enhancing susceptibility, or by suppressing seizures and epileptogenesis. Due to the large number of genes involved and the many pathways that these genes are associated with, epilepsy and seizure disorders are complex and difficult to fully understand. Investigation of well-characterized mutations and second-site enhancers or suppressors of seizure susceptibility has some potential advantages. First, identification of novel mechanisms underlying seizure susceptibility may elucidate some of the many questions regarding not only seizure susceptibility, but also the spread and termination of seizures. Second, such investigation may provide clues for developing novel therapeutic methods, including targets for novel antiepileptic drugs.

Dorsal vessel injection of antiepileptic drugs

One of many important areas of epilepsy research is the search for novel antiepileptic drugs. A key validation of the *Drosophila* epilepsy model is that currently available AEDs are effective on *Drosophila* seizure mutants. The antiepileptic drugs valproic acid, potassium bromide, phenytoin and gabapentin used in humans, have all been shown to reduce the severity of seizures in several of the Drosophila bang-sensitive mutants either by feeding or by direct injection to the brain. Other AEDs, including carbamazepine, ethosuximide, and vigabactrin have been ineffective in feeding experiments (Song and Tanouye, 2008). There are several issues that appear to limit the effectiveness of drug feeding experiments in *Drosophila*. First, the drugs may be metabolized within the gut, rendering them useless before they can act upon the CNS. Second, the blood brain barrier may hinder the progression of the drugs from the gut into the brain regardless of whether they have been metabolized or not. This would severely reduce the efficacy of the drug in reducing seizure sensitivity. Lastly, the drugs may be toxic to the flies, killing them at concentrations lower than would be necessary for the drug to have an effect. One way to get around these potential problems is injecting the drugs into the fly. A method to deliver drugs directly to the fly brain was developed previously in the lab. This method was used to demonstrate the usefulness of valproic acid in reducing seizure sensitivity of $para^{bss1}$ and *sda* flies (Kuebler and Tanouye, 2002). This method though, has its own problems. It is highly invasive and limits the usefulness of the flies to only a short period. Experiments cannot be complicated and must be performed quickly as the flies do not survive for long. This severely limits the ability to determine exact seizure thresholds, as there is only time to deliver a single high frequency stimulus and note whether the fly did or did not seize at this voltage.

Dorsal vessel injection of drug overcomes some of limitations imposed by brain injection. By utilizing the open circulatory system of *Drosophila*, drug can be injected in a manner that bypasses the gut and allows rapid delivery to the head of the fly. This delivery method is not as invasive as injection directly into the head, and the flies survive for a much longer time. Exact seizure thresholds can be determined for each fly. While the blood brain barrier may still be an issue, there are available mutants that may reduce its function enough to allow these drugs to pass into the brain. There are also chemical

means of disrupting the blood brain barrier. These mutants or chemicals may be adequate to allow the delivered drugs to enter the brain and have an effect on seizure susceptibility. Future work, using the dorsal vessel injection method can be aimed at screening compounds for efficacy as antiepileptic drugs, either as a primary method of screening, or as a confirmatory method following feeding.

Further study of seizure suppression by *gish*, a casein kinase mutation

Efforts in the Tanouye Lab have revealed a class of mutations that we call "seizure suppressor" mutants. Mutants such as these reduce seizure susceptibility in various members of the bang-sensitive class. The mutant *mle^{napts}* is a gain-of-function mutation in an RNA-helicase like protein that causes a reduction in voltage gated sodium channels (Jackson et al 1984). The *para^{bss1}; mle^{napts}* and *mle^{napts};sda* double mutants have an increased seizure threshold (Kuebler et al 2001). The gap junction mutant *shakB^{2}* is a seizure resistant mutant that completely suppresses seizure activity in *sda* and partially suppresses seizures in *eas* (Song and Tanouye, 2006). Additionally, the DNA topoisomerase I mutant *top1^{JS1}* partially suppresses seizure susceptibility in *sda*, *eas*, and to a much lesser extent, *para^{bss1}* (Song and Tanouye, 2007).

The seizure suppressor mutant *gish* is one of the most interesting seizure suppressors uncovered by the Tanouye Lab. It reduces the percentage of *para^{bss1}*/+ flies that undergo seizure activity following mechanical stimulus. The seizure threshold of *para^{bss1}*/+ flies in combination with either the mutant *gish^{04895}* or *gish* RNAi is significantly higher than that of sibling controls with normal expression of *gish*. What makes seizure suppression by *gish* interesting is that it is the first seizure suppressor identified that not only strongly affects *para^{bss1}*, but also is specific it. Other known suppressors are primarily general suppressors that exert their effect on a variety of bang-sensitive mutants. As stated previously, many human epilepsies have been linked to mutations in voltage gated sodium channels. That seizure suppression by *gish* appears to be specific for *para^{bss1}*, may lead us to a greater understanding of mechanisms behind seizure susceptibility due to defects in voltage-gated sodium channels.

My research, has identified *gish* as a seizure suppressor, but has not yet defined a mechanism by which this suppression occurs. In order to gain useful insight into the genetics underlying epilepsy, we must understand how *gish* functions in seizure susceptibility. While I have ruled out direct Wg/Wnt signaling involvement in seizure suppression, casein kinase (CK1) is involved in a wide range of cellular processes and has a diverse group of substrates (Knippschild 2005). There are many ways that *gish* could be functioning in seizure susceptibility. For instance, *gish* has been shown to have involvement in planar cell polarity-mediated morphogenesis via regulation of vesicle trafficking (Gault et al. 2012). It is possible that loss of *gish* function leads to a developmental defect within neurons that impedes neuronal hyperexcitability. Recently, *gish* has been implicated in *rutabaga* independent olfactory memory pathway (Tan et al. 2010). While it is unclear how learning and memory could be related to seizure susceptibility, recent evidence in our lab has identified a link between learning and memory mutants and seizure susceptibility (unpublished data). In addition to the pathways of CK1 action, it may be possible that regulation of splicing plays a role in seizure suppression by *gish*. There are 12 different isoforms of *gish* that can be classified

as either long transcripts or short transcripts. In the *gish*^{04895} mutant, long transcripts are not present, while the short transcripts appear in greater abundance (Tan et al 2010). Future work on *gish* will need to identify the specific pathway(s) involved in seizure suppression. Identification of the mechanism of action, may provide invaluable insight into the genetics of voltage-gated sodium channel associated epilepsies and possibly suggest new therapeutic methods.

Further study of *para* and its utility as a model for intractable epilepsies

Voltage-gated sodium channels play a large role in the genetic aspects of many epilepsies. With upwards of 700 mutations linked to several forms of epilepsy, including the intractable Dravet syndrome, understanding the role of voltage-gated sodium channels in epilepsy is critical. Using *para*^{bss1}, we screened for enhancers and suppressors of seizure activity. In order for *para*^{bss1} to be a good model for intractable epilepsies, it must first be proven to be useful in identifying genetic factors behind seizure susceptibility. Identification of *gish* as a suppressor of *para*^{bss1}/+ seizure phenotypes, indicates that despite its difficulty to suppress, it is a useful model for uncovering novel and effective seizure suppressor mutants. Additional novel suppressors may come from other deletions identified in our screen, or from other on-going screening efforts. There were several other deletions identified by our screen with the capability to suppress *para*^{bss1}/+ as strongly as the deletion containing *gish*. In addition to deletion screening, *Drosophila* offers a variety of other screening techniques, including P-element insertions and chemical mutagenesis. These techniques and the ability to screen in both *para*^{bss1} and *para*^{bss1}/+ offer powerful method for identifying suppressors of voltage-gated sodium channel epilepsies.

In addition to identifying second-site suppressor mutations, further investigations of *para* may provide insight into how voltage-gated sodium channels affect seizure susceptibility. The identification of *para*^{bss1} as a bang-sensitive mutant indicates that voltage-gated sodium channels are important for seizure susceptibility in *Drosophila* as well as in humans. The *para*^{bss1}mutant phenotype shares several similarities with intractable epilepsies in humans. Not only is it the most difficult BS mutant to suppress genetically, it is also the least responsive to AED treatment. Introduction of the amino acid substitution K1270T that causes GEFS+, a fever-associated epilepsy in humans, also causes a temperature induced seizure phenotype in *Drosophila* (Sun *et al*, 2012). There are other human epilepsy mutations in voltage-gated sodium channels that lie in conserved regions of these channels that may be introduced into *Drosophila para* via targeted mutagenesis; these may allow us to gain additional understanding of how mutations in voltage-gated sodium channels in *Drosophila* affect seizure susceptibility and the role of these mutations in human epilepsies.

Closing Comments

The research presented in this dissertation has allowed us to identify a novel gene associated with suppression of seizure susceptibility. While no exact mechanism has been identified, a door has been opened that may lead to an extended understanding of the mechanisms behind seizure suppression. A forward genetics screen has identified several

chromosomal segments that may contain seizure suppressors. The characterization of *gish* validates both the deletion screening strategy and the use of *para*^{bss1} as a model for intractable epilepsy. The complex nature of casein kinase 1 activity, highlights the fact that epilepsy is a complex problem that is not easy to study. Our understanding of the mechanisms of seizure susceptibility is still incomplete and many questions remain. However, despite the difficulty posed by these questions, our work, and the work of others will provide insight into the mechanisms of seizure susceptibility and the wide variety of other complexities of the nervous system.

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