The Regulation of *para*^{bss1} by s*pen* and *brm* and its Role in Seizure Susceptibility and Nociception Sensitivity in *Drosophila*

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Abstract

Seizures are defined by abnormal and simultaneous firing of neurons within the central nervous system, and epilepsy is persistent, spontaneous seizures. Epilepsy can interfere with daily life, cause physical injury, and alter brain function. Single gene mutations have been linked to several epileptic disorders with most mutations occurring in genes which encode proteins necessary for regulating neuronal excitability including voltage-gated and ligand-gated channels. Many of these voltage-gated and ligand-gated channels have highly conserved homologs in *Drosophila melanogaster*, fruit flies. There are also extensive shared mechanisms of neural function between *Drosophila* and mammalian nervous systems making flies a highly relevant model for studying human seizure disorders. While there are currently many therapies for the symptomatic treatment of epilepsy, symptoms are not adequately controlled in one-third of all affected individuals and comorbidity still imposes a major burden on the quality of life. Identifying and understanding the mechanisms of mutations that contribute to seizure susceptibility is important to provide avenues for treatment of seizure disorders. In flies, mutations in the *paralytic* (*para*) voltage-gated sodium channel gene can cause seizure susceptibility. We are interested in understanding how this mutation might impact other neuronal functions such as nociception as well as how this mutation might be affected by other genes that modify its function. Flies with the $para^{bss1}$ mutation experienced a significant increase in seizure sensitivity compared to wild-type flies. $para^{bss1}$ mutants carrying the *brm* mutation were found to retain almost no seizure susceptibility while mutants carrying the *spen* mutation retained their seizure susceptibility, but it was significantly reduced. Flies with the $para^{bss1}$ mutation were also tested for nociceptive defects to both thermal and mechanical stimuli. Mutant flies expressed a decrease in sensitivity to

thermal stimuli at 46°C. From our results we determined that increased neuronal excitability in *para*^{bss1} mutants may have led to a decrease in synapse size to control for this increase in neuronal excitability. Upon a mechanical stimulus to produce seizures or a nociceptive mechanical or thermal stimulus, this decreased synapse size might have led to a weaker synaptic output.

Introduction

Human seizure disorders are a highly studied, but still not completely understood field. They pose a significant health concern due to the large number of individuals impacted and the current limitations in available treatments. While it is estimated that 10% of the population will experience a seizure sometime during their lifetime, 1% of people will suffer persistent, unprovoked seizures that define epilepsy (Kroll et al., 2015; Parker et al., 2011). Approximately 50 million people worldwide have epilepsy and in the United States alone, it is ranked the fourth most common neurological disorder with nearly 150,000 new cases diagnosed annually (World Health Organization, 2012; Hirtz et al., 2007).

Seizures can be caused by a variety of brain injuries including trauma, fever, illness, and electroconvulsive shock (Parker et al., 2011). However, a main cause of seizure susceptibility is due to genetic predisposition with more than 70 genes linked to epilepsies (Noebels, 2003). These genes can encode a range of products such as ion channel proteins which play a direct role in neuron functioning and tRNAs which may have a more complex role that has yet to be fully elucidated (Parker, Howlett, Rusan, & Tanouye, 2011). To further confound the understanding of the processes behind the development of epilepsy, many of these genes have no obvious functional relationship between their mutation and seizure susceptibility (Parker, Howlett, Rusan, & Tanouye, 2011). Therefore, our ability to understand the mechanisms behind epilepsy is further impeded.

Model organisms are one potential strategy scientists have adopted to help us gain a mechanistic understanding of seizure disorders. *Drosophila melanogaster* is a major model system used to study the mechanisms behind seizure disorders because it has a genome that shares many functions conserved evolutionarily with humans. $para^{bss1}$ is a gain of function

mutation in the *Drosophila* Para voltage-gated sodium channel that exhibits a seizure susceptible phenotype (Parker, Howlett, Rusan, & Tanouye, 2011). Using this as a model of seizure susceptibility can serve as a basis for studying the mechanisms of seizure activity. Furthermore, determining factors that modify seizure susceptibility in a $para^{bss1}$ background offers a potential avenue for pinpointing possible cellular and molecular pathways by which seizure susceptibility is regulated. It can also elucidate novel targets for the development of anticonvulsant drugs to treat seizure disorders.

Drosophila *paralytic* encodes a voltage-gated sodium channel

Voltage-gated ion channels are ion specific, and they open and close due to changes in electrical potential across a cell membrane. Voltage-gated channels are essential for membrane excitability and the subsequent propagation of action potentials along neuronal axons (Hodgkin and Huxley, 1952). The clearest cases of functional relationships between mutations and seizure susceptibility are epilepsies that are caused by mutations within ion channel genes (Parker, Howlett, Rusan, & Tanouye, 2011). The *SCN1A* gene, for example, encodes the voltage-gated sodium channel $Na_v1.1$, and more than 1,250 mutations in this gene have been identified to cause epilepsy (Meng et al., 2015). The sodium channel structure encoded by *para* in *Drosophila melanogaster* is almost identical to that of the vertebrate sodium channel (Schutte et al., 2016). These sodium channels also possess conserved structural features, like four homologous transmembrane domains, found in the Para polypeptide which are imperative for channel function (Schutte et al., 2016; Loughney et al., 1989).

Voltage-gated sodium channels are generally similar in structure across species. The para sodium channel is a large alpha subunit polypeptide made of four homologous domains (I-IV) (Figure 1; Loughney et al., 1989). Each domain contains six hydrophobic transmembrane alpha-helical domains (S1-S6) which are connected by an intracellular or extracellular loop of amino acid sequences (Figure 1; Loughney et al., 1989; Kroll et al., 2015). Several structure-function analyses show that a short alpha-helical loop connecting S5 and S6 of each homologous domain transverses through the membrane and this combined association forms the channel pore (Figure 1; Kroll et al., 2015). Unlike *Drosophila*, humans express functionally distinct voltage-gated sodium channels ($Na_v1.1$-$Na_v1.9$) in their neurons and muscles. This diversity arises from the differential expression of nine different alpha subunit genes (*SCN1A*-*SCN9A*). As aforementioned, *Drosophila* only have a single voltage-gated sodium channel alpha subunit, Para, and its channel diversity arises from alternative splicing (Kroll et al., 2015).

Studies of *para* mutants have been used to elucidate the sodium channel function. Depending on where the mutation in the *para* gene appears, it can lead to either a loss or gain of function. The *bss1* allele is a lesion that causes a single nucleotide change from C to T in the coding sequence (Parker, Padilla, Du, Dong & Tanouye, 2011). This leads to an amino acid substitution from leucine to phenylalanine corresponding to the L1699 residue which lies within the S3 alpha-helical domain of the para channel (Figure 1; Parker, Padilla, Du, Dong & Tanouye, 2011.). When the *bss1* allele is present, it leads to an increase in ion channel excitability thereby increasing electrical excitability of the neuron and eliciting a seizure phenotype (Parker, Padilla, Du, Dong & Tanouye, 2011). As such, *bss1* can be used as a model for understanding seizures.

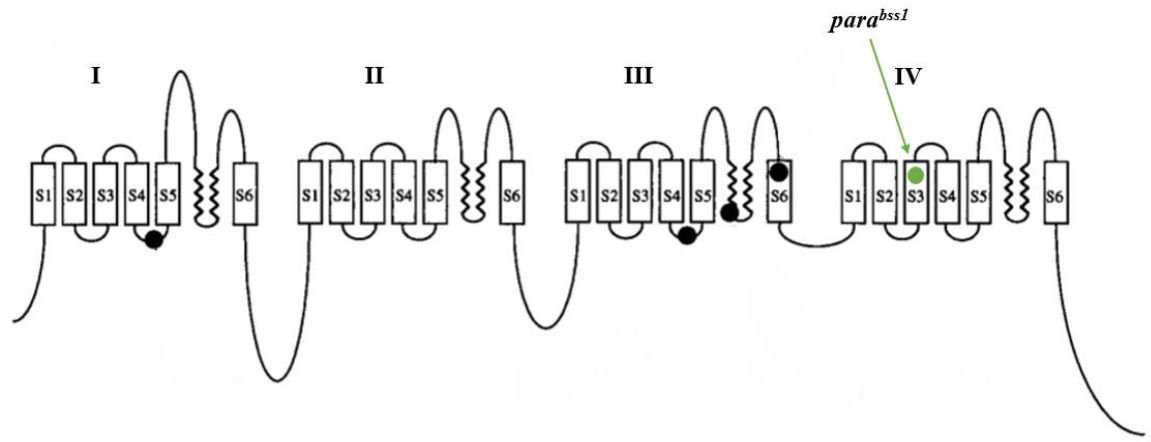

Figure 1: Diagram adapted from ffrench-Constant et al. of the para voltage gated sodium channel with the *para*^{bss1} mutation represented (ffrench-Constant et al., 1998).

Bang-Sensitive mutants and *para*^{bss1}

Bss1 is an allele of the *para* gene. When present, the *para*^{bss1} allele produces a lower seizure threshold, causing mutants to be more sensitized to seizures (Parker, Padilla, Du, Dong & Tanouye, 2011). The *para*^{bss1} mutation is a part of a bang-sensitive (BS) behavioral class of mutations in *Drosophila* genes. This set of mutations encode proteins that reduce the threshold for electrically-induced seizure onset and cause flies to become seizure-sensitive due to a mechanical shock such as a tap to a culture vial or brief vortex mixing (a 'bang') (Ganetzky and Wu, 1982; Jan and Jan, 1978; Parker, Padilla, Du, Dong & Tanouye, 2011)). Mutants of the BS behavioral class were independently isolated by different labs and found to be consistently seizure sensitive (Benzer, 1971, Kuebler et al., 2001; Pavlidis and Tanouye, 1995). Their behavior could be characterized by an initial seizure, temporary paralysis, and a recovery seizure. (Benzer, 1971; Judd, Shen, and Kaufman, 1972; Grigliatti et al., 1973; Homyk and Sheppard 1977, Homyk, Szidonya and Suzuki, 1980; Wu and Ganetzky, 1982). The BS class has 14 mutant alleles that represents 12 genes and several

gene products (Table 1). The BS behavioral phenotype is completely penetrant in most mutants compared to normal flies (Song and Tanouye, 2008).

Table 1: Seizure-sensitive mutants and their gene products

Seizure-Sensitive Mutant	Gene Product
bang senseless (*para^{bss1}*, *para^{bss2}*)	Na channel (Parker et al., 2011)
para^{GEFS+}	Na channel (Sun et al., 2012)
para^{DS}	Na channel (Schutte et al., 2014)
easily shocked (eas)	ethanolamine kinase (Pavlidi et al., 1994)
slamndance (sda)	aminopeptidase N (Zhang et al., 2002)
bang sensitive (bas^{1}, bas ^{2})	unknown product
technical knockout (tko)	ribosomal protein S12 (Royden et al., 1987)
jitterbug (jbug)	unknown product
couch potato (cpo)	RNA-binding protein (Glasscock and Tanouye, 2005)
kazachoc (kcc)	K^+ Cl^- cotransporter (Hekmate-Scafe et al., 2006)
knockdown (kdh)	citrate synthase (Fergestad et al., 2006)
stress-sensitive (sesB)	adenine translocase (Zhang et al., 1999)
rock-n-roll (rnr)	unknown product
prickle (pk)	planar cell polarity (Tao et al., 2011)
wild type *(CS)*	N/A (Kuebler et al., 2001)

The *para*^{bss1} mutant is behaviorally and electrophysiologically the most sensitive to seizures and shows the most extreme phenotype of the BS mutants (Parker, Padilla, Du, Dong, & Tanouye, 2011). These mutants are only ameliorated and not suppressed when treated with anti-epileptic drugs (AEDs) resembling AED resistant epilepsies caused by mutations in the human *SCN1A* gene (Parker, Padilla, Du, Dong, & Tanouye, 2011). As such *para*^{bss1} mutants can serve as model for intractable epilepsy. When exposed to a mechanical shock (a "bang"), this mutant was recorded to undergo an abnormal behavioral phenotype with six distinguishable phases: seizure; initial paralysis; tonic-clonic like activity; recovery seizure; refractory recovery; and complete recovery (Parker, Padilla, Du, Dong, & Tanouye, 2011). Researchers found that the initial seizure was similar to other BS mutants. It could be characterized by leg shaking, abdominal muscle contractions, wing flapping, scissoring, and proboscis extensions followed by initial paralysis characterized by flies that were immobile and unresponsive to mechanical stimulus (Ganetzky and Wu 1982, 1982; Parker, Padilla, Du, Dong, & Tanouye, 2011). A novel phenomenon, however, was following initial paralysis, *para*^{bss1} homozygotes experienced an extended period of tonic-clonic like activity (Parker, Padilla, Du, Dong, & Tanouye, 2011). Flies were mainly quiescent resembling a tonic phase and this quiescence was disturbed by clonus-like activity. Resembling other BS mutants, flies would then show a recovery seizure, refractory period, and then complete recovery. The recovery time for *para*^{bss1} mutants was longer than other BS mutants with a mean recovery time of ~240 sec, compared to BS mutants *sda* or *eas* at 38s and 81s respectively (Parker, Padilla, Du, Dong, & Tanouye, 2011).

Upon electorophysiological analysis of the larval neuromuscular junction in *para*^{bss1} mutants, researchers also found that motor neurons were hyperexcitable and displayed an

abnormally long-term facilitation of excitatory synaptic responses after repeated stimulation (Parker, Padilla, Du, Dong, & Tanouye, 2011). This resulted in multiple action potentials and a large, prolonged excitatory junction potential (EJP) in $para^{bss1}$ flies, whereas wild-type flies showed only a single action potential and a single small EJP (Jan and Jan 1978; Ganetzky and Wu, 1982). Epilepsy, as previously mentioned, is a disorder of electrical activity defined by multiple neurons firing uncontrollably and synchronously. Therefore, it would follow that multiple action potentials would cause a large EJP which can summate with repeated stimuli to depolarize muscles resulting in the convulsive phenotype typical of epilepsy.

Pumilio as a *para* regulator

Pumilio is a well-studied RNA-binding protein that has been shown to play an active role in neural plasticity (Baines, 2005; Mee et al., 2004). The Pumilio protein is a member of the Pum and FBF (PuF) RNA-binding family that is also evolutionarily conserved across many species including flies and mammals, meaning that results found from studying Pumilio in a fly model can have very real implications for humans (Wickens, Bernstein, Kimble, & Parker, 2002; Zamore, Williamson, & Lehmann, 1997). In *Drosophila*, Pum plays a role in regulating dendritic structure, synaptic growth, neuronal excitability, and the formation of long-term memory (Baines, 2005). Due to Pum's role in regulating neuronal excitability, it would follow that it also plays a crucial role in neuronal homeostasis. In fact, research has shown that Pum maintains action potential firing within physiologically-appropriate limits (Baines, Mee, Pym, & Moffat, 2004; Muraro et al., 2008).

The regulation of translation plays a crucial role in gene expression. When Pumilio binds to a Pum Response Element in mRNA, it represses translation and reduces protein

synthesis (Arvola et al., 2017; Wharton et al., 1998; Wreden et al., 1997). Pumilio itself is regulated by neuronal depolarization. Increased synaptic excitation elevates Pum expression and increases translation repression of voltage-gated sodium channel transcripts (Lin, He, Fan, & Baines, 2018). This regulation produces a feedback loop that is sufficient to reduce the neuron sodium current which leads to a reduction in action potential firing in order to maintain neuronal homeostasis (Mee et al., 2004; Muraro et al., 2008). Past research also found an identical mechanism that is mediated by the homologue Pum2 gene which acts to repress translation of mammalian sodium channels, specifically in *SCN1A* and *SCN8A* (Driscoll, Muraro, He, & Baines, 2013; Vessey et al., 2006). In a more recent study, researchers found that a pan-neuronal up-regulation of the *pum* gene was sufficient to dramatically reduce seizure duration in bang-sensitive mutations (*para*^{bss}, *easily shocked, and slamdance*) (Lin, Giachello, & Baines, 2017). Genes that regulate Pum expression are therefore an interesting avenue for the study of *para* regulation.

Mutations in *spen* and b*rm* genes regulate Pum expression

A screen done by researchers using an *actin* promoter driven firefly-luciferace (luc) reporter construct (FF-PRE) to provide a fluorescent readout of Pum activity identified 467 genes that reduced Pum activity upon knockdown (Lin, He, Fan, & Baines, 2018). Among the genes identified were *split ends* (*spen*) and *brahma* (*brm*) (Lin, He, Fan, & Baines, 2018). As mentioned above, Pumilio acts as a translational repressor of voltage-gated sodium channels. Therefore, genes such as *spen* and *brm* which reduce Pum activity offer an avenue for research of their potential effects on *para* expression. It is expected that loss of *spen* and *brm* function will upregulate *para* expression.

The gene *spen* is a predominantly nuclear protein with three RNA recognition motifs (RRM) and a c-terminal SPOC (spen paralog and ortholog c-terminal) domain (Lin et al., 2003). Previous studies have implicated *spen* in neuronal cell fate, survival and axonal guidance, and cell cycle regulation (Chen and Rebay, 2000; Kuang, Wu, Shin, & Kolodziej, 2000, Wiellette et al., 1999). s*pen*, at the genetic level, has also been suggested to act in the Epidermal Growth Factor/RAS signaling pathway which is a key cell growth pathway (Chen and Rebay, 2000, Rebay et al, 2000).

The *spen* gene was originally identified in a screen for mutations impacting axonal outgrowth in the nervous system in *Drosophila* (Jan, Jan, and Kolodziej 1995). Recent studies have found that *spen* may also participate in the transduction of the Wingless (Wg) signal (Mace and Tugores, 2004; Lin et al., 2003). In *Drosophila* the Wg signaling pathway regulates crucial parts of cell fate determination, cell migration, neural patterning, and organogenesis during embryonic development (Habas and Komiya, 2008). De-regulation in the Wg pathway can have devastating ramifications for the developing embryo such as cancer and birth defects such as spina bifida (Habas and Komiya, 2008). As previously mentioned, *spen* acts to reduce Pum activity. This coupled with its role as a positive regulator of Wg signaling makes it an interesting gene for the study of the gain of function $para^{bss1}$ mutation. The *spen* gene also has a human orthologue, *SHARP*, making results found in *Drosophila* possibly applicable to a human model.

The Brahma (Brm) complex is a yeast Switch/Sucrose Non-Fermentable (SWI/SNF)-related chromatin remodeling complex required to correctly maintain proper states of gene expression (Marenda, Zraly, & Dingwall, 2004). ATP-dependent chromatin remodeling is required to help establish and maintain patterns of gene expression through the disruption of

DNA-histone contacts and higher order chromatin remodeling (Merenda, Zraly, & Dingwall, 2004). Research showed that these Brm complexes were found in chromosomes where gene expression was high and the loss of Brm function disrupted transcription via RNA polymerase II significantly (Armstrong et al., 2002). The *brm* gene was shown to encode the catalytic ATPase subunit of the Brm complex by researchers suggesting that it plays a role in chromatin remodeling (Marenda, Zraly, & Dingwall, 2004).

The *brm* gene also plays a role in maintaining appropriate levels of gene expression. Specification and maintenance of cell fates is necessary for the development of multicellular organisms (Dingwall et al., 1995). Patterns of homeotic gene transcription are established by DNA-binding regulatory proteins encoded by segmentation genes early in embryogenesis (Harding and Levine, 1988; Ingham, 1988). These patterns are maintained later in development by two opposing trans-acting regulatory genes including the Polycomb group of repressor and the trithorax group of activators. The *brm* gene is a member of the trithorax group thus it is needed to maintain the expression of homeotic genes (Kennison, 1993). The possible role of *brm* in transcription coupled with its regulation of Pum makes it another interesting gene for the study of the possible regulation of the $para^{bss1}$ mutation. The *brm* gene also has a human orthologue, *SMARCA2*, making findings in this study possibly applicable to a human model. **Due to the roles of *spen* and *brm* mutation in decreasing *pumilio* function, it is expected that mutations in these genes will both enhance *para* expression.**

Paralytic's role in nociception

Nociception is defined by the ability of sensory neurons to detect potentially harmful stimuli such as elevated temperature, harsh mechanical force, or noxious chemicals and generate a behavioral response. When exposed to noxious stimuli, *Drosophila* exhibits a distinct behavior defined by nocifensive escape locomotion (NEL; Caldwell and Tracey, 2010). NEL is when a distinct cork-screw or barrel-roll is exhibited along the longitudinal axis of the *Drosophila* larvae (Bautista et al., 2006, Caldwell and Tracey, 2010; Robertson, Tsubouchi, and Tracey, 2013). The NEL response is triggered in response to potentially harmful mechanical, thermal, and chemical stimuli (Brierley et al., 2009; Hwang, Stearns, and Tracey, 2012).

As previously mentioned, *para* encodes for voltage-gated sodium channels in *Drosophila*. These voltage-gated sodium channels are needed for the propagation of action potentials along the axon of neurons. Therefore, it would follow that the *para* gene also plays a role in other processes that require neuronal firing like nociception. This function was seen when Dyson et al. knocked down *para* expression in nociceptor neurons which led to an insensitivity to thermal and mechanical nociception (Dyson, 2017). ***para^{bss1}*** **is gain of function mutation that causes an increase in electrical excitability to produce a seizure phenotype. We expect that neuron firing will also increase leading to a mutant that has a hypersensitive nociception phenotype.**

Methods

Fly Husbandry

Flies used in this study (Table 2) were raised in standard cornmeal molasses fly food at room temperature for 7-14 days. Following the 7-14 days, flies were transferred to a new vial of food. All flies were ordered from Bloomington *Drosophila* Stock Center at Indiana University and $para^{bss1}$ flies were a gift from Dr. Daniel Kuebler at Franciscan University.

Table of stocks used

Table 2**:** List of Drosophila stocks used

Bloomington Stock ID	**Flyabase ID**	**Gene**	**Genotype**
#5808	FBst0005808	*spen*	*spen[1401]/CyO ; P{w[+mW.hs]=sE-raf[torY9]}475*
#3619	FBst0003619	*brm*	*brm[2] e[s] ca[1]/TM6B ; Sb[1] Tb[1] ca[1]*
N/A	FBal0018186	*wild-type* (w1118)	w^{1118}
N/A	FBal0001325	$para^{bss1}$	$para^{bss1}$

Fly Crosses

The crosses delineated here were used for both the seizure assays and for nociception assays. Homozygotes of the $para^{bss1}$ mutation were made ($para^{bss1}/para^{bss1}$ or $para^{bss1}$/Y). Vials of five to six $para^{bss1}$ females were mated in cross food with three $para^{bss1}$males. Homozygotes of the control w^{1118} were also made as a negative control (w^{1118}/w^{1118} or w^{1118}/Y). Three w^{1118} male flies were crossed with five to six w^{1118} female flies. These crosses were set up in duplicates for each experiment. The flies were subsequently placed in an incubator for forty-eight hours at 25°C and ~40-70% humidity. Following forty-eight hours, flies were transferred (flipped) to a fresh vial on days three and four after the cross

was established. Approximately the same number of flies and larvae were tested for each genotype on each test day to account for any possible environmental differences that may have occurred across genotypes such as daily variations in room temperature or incubator humidity and temperature.

The goal of crosses with the *spen* mutation was to make animals that were heterozygous for the gain of function $para^{bss1}$ mutation and heterozygous for the loss of function $spen^{1401}$ allele ($para^{bss1}$/+; $spen^{1401}$/+ or $para^{bss1}$/Y; $spen^{1401}$/+). To test the effects of a mutation in the *spen* gene on the $para^{bss1}$ phenotype, five to six virgin $para^{bss1}$ females were mated with three #5808 males to produce a progeny with the following genotype: $para^{bss1}$/+; $spen^{1401}$/+ or $para^{bss1}$/Y; $spen^{1401}$/+ and $para^{bss1}$/+; *CyO*/+ or $para^{bss1}$/Y; *CyO*/+.

Before testing, flies were sorted based on the presence of the *spen* allele. This was determined based on their wing phenotype. Flies with a curly wing phenotype were discarded because they did not possess the *spen* allele.

The goal of the crosses with the *brm* mutation was to make animals that were heterozygous for the gain of function $para^{bss1}$ mutation and heterozygous for the loss of function *brm* allele ($para^{bss1}$/+;; *brm[2] e[s] ca[1]*/+ or $para^{bss1}$/Y;; *brm[2] e[s] ca[1]*/+). To test the effects of a mutation in the *brm* gene on the $para^{bss1}$ phenotype, five to six virgin $para^{bss1}$ females were mated with three #3619 males to produce the following progeny: $para^{bss1}$/+;; *brm[2] e[s] ca[1]*/+ or $para^{bss1}$/Y;; *brm[2] e[s] ca[1]*/+ and $para^{bss1}$/+;; *TM6B Sb[1] Tb[1] ca[1]*/+ or $para^{bss1}$/Y;; *TM6B Sb[1] Tb[1] ca[1]*/+.

Before testing flies were also sorted based on the presence of the *brm* allele. This was phenotypically determined by the absence of the stubble phenotype ($para^{bss1}$/+;; *brm[2] e[s] ca[1]*/+ or $para^{bss1}$/Y;; *brm[2] e[s] ca[1]*/+), which contained the *brm* gene, or the stubble

phenotype (*para*^{bss1}/+;; *TM6B; Sb[1] Tb[1] ca[1]*/+ or *para*^{bss1}/Y;; *TM6B; Sb[1] Tb[1] ca[1]*/+), which did not have the *brm* gene. Both phenotypes were kept, and flies exhibiting stubble were used as an additional control.

The same positive control was used for both *spen* and *brm.* Two vials were set up, each with five to six virgin *para*^{bss1} females mated with three male w^{1118} flies to act as the positive control (*para*^{bss1}/w^{1118} or *para*^{bss1}/Y). The same negative control was used for both *spen* and *brm*. For the negative control, two vials were set up, each with three w^{1118} males crossed with five to six w^{1118} females (w^{1118}/w^{1118} or w^{1118}/Y). The procedure for cross maintenance was the same as outlined above. The flies were subsequently placed in an incubator for forty-eight hours at 25°C and ~40-70% humidity. Following forty-eight hours, flies were transferred (flipped) to a fresh vial on days three and four after the cross was established. Approximately the same number of flies were tested for each genotype on each test day to account for any possible environmental differences that may have occurred across genotypes.

Thermal Nociception Assay

This study followed the protocol for thermal nociception outlined by Caldwell et al., 2010. Larvae from the crosses outlined above for nociception were used. Larvae were not ready for testing until they developed into wandering third instar larvae. These larvae were identified as those that had left the food and crawled up the walls of the vial. Deionized water was first added to a petri dish with a sprinkle of yeast to break the surface tension. The DI water was then poured down the sides of a cross vial and larvae were washed into the petri

dish. Water was removed from the petri dish until just enough remained allowing larvae to crawl but not swim.

A soldering iron was heated to either 42°C (between 41.5°C and 42.5°C) or 46°C (between 45.5°C and 46.5°C) to test *para*^{bss1}and the negative control. This iron was used to gently apply a thermal stimulus to the lateral wall of the larval body until either a nociceptive response occurred or 11s elapsed. To record the behavioral response, a video camera mounted to a dissecting microscope was used and this video was analyzed using Adobe Premiere Pro for a precise start and stop time for nocifensive escape locomotion (NEL). NEL is the barrel roll larvae display when trying to escape a noxious stimulus. Latency is defined as the amount of time in seconds that it takes the larvae to make one complete barrel roll and it was determined by subtracting start time from stop time. Enough larvae were tested to get a sample size greater than forty-five per genotype. Descriptive statistics were determined using Minitab Express. Statistical significance of differences between genotypes was determined by a Mann-Whitney test using Minitab Express.

Mechanical Nociception Assay

Mechanical assays were set up using the same protocol as the thermal assay. Larvae were visualized under a light microscope and the stimulus applied using a 50 mN length Von Frey filament. The stimulus was applied as a quick poke along the dorsal midline of the larvae. Three trials were performed per larvae and they were scored as either 0 = no NEL or 1 = NEL (Hwang et al., 2007). Enough larvae were tested to get a sample size larger than forty-five per genotype. Descriptive statistics were determined using Minitab Express.

Statistical significance of differences between genotypes was determined by a two-sample proportions test using Minitab Express.

Seizure Assay

A baseline seizure susceptibility of *para*^{bss1} flies was established to create a comparison point for experiments predicted to modify this susceptibility. The protocol for testing flies followed the procedure outlined by Saras et al. with some modifications (Saras and Tanouye, 2016). Flies were anesthetized with CO_2 after eclosion and transferred to a fresh cross food vial where they matured for the needed amount of days. Flies that needed to mature for 10 or 14-15 days were initially transferred to a fresh food vial using CO_2. They were then transferred to another cross food vial five days later to ensure younger flies that might have eclosed during that time were not tested. On the day of testing, flies were anesthetized with CO_2 and transferred from food vials into a clean empty vial. They were left undisturbed for at least one hour prior to testing. For testing, 6-10 flies were placed in a clean, empty, vial and stimulated mechanically with a VMWR analog vortex mixer at maximum speed for 10s. Recovery from BS paralysis was determined as the time that fifty percent of flies were up and walking (recovered). Enough flies were tested to yield ∼100 flies per genotype. Descriptive statistics were determined using Minitab Express. Statistical significance for seizure susceptibility was determined by a descriptive statistics test and a two-way (assay for age) or one-way (all other seizure susceptibility assays and assays with *spen* and *brm*) ANOVA test. Differences between individual groups were also determined using a post hoc Tukey test.

Results

The first goal of this experiment was to determine a baseline seizure susceptibility of *para*^{bss1} mutants for future comparisons of conditions predicted to modify this susceptibility. To accomplish this, crosses homozygous for the gain of function *para*^{bss1} mutation and homozygous for w^{1118} were set up. Flies with the *para*^{bss1} mutation had significantly longer immobilization times indicating they were immobile with seizures longer than wild-type flies. The mean time for homozygous *para*^{bss1} mutants was 211.7s while the mean time for homozygous w^{1118} flies was 2.1s. Figure 2 shows with significance that the *para*^{bss1} mutation does produce a seizure phenotype that we are able to characterize and quantify compared to the negative w^{1118} control (One-way ANOVA test, $p<0.001$).

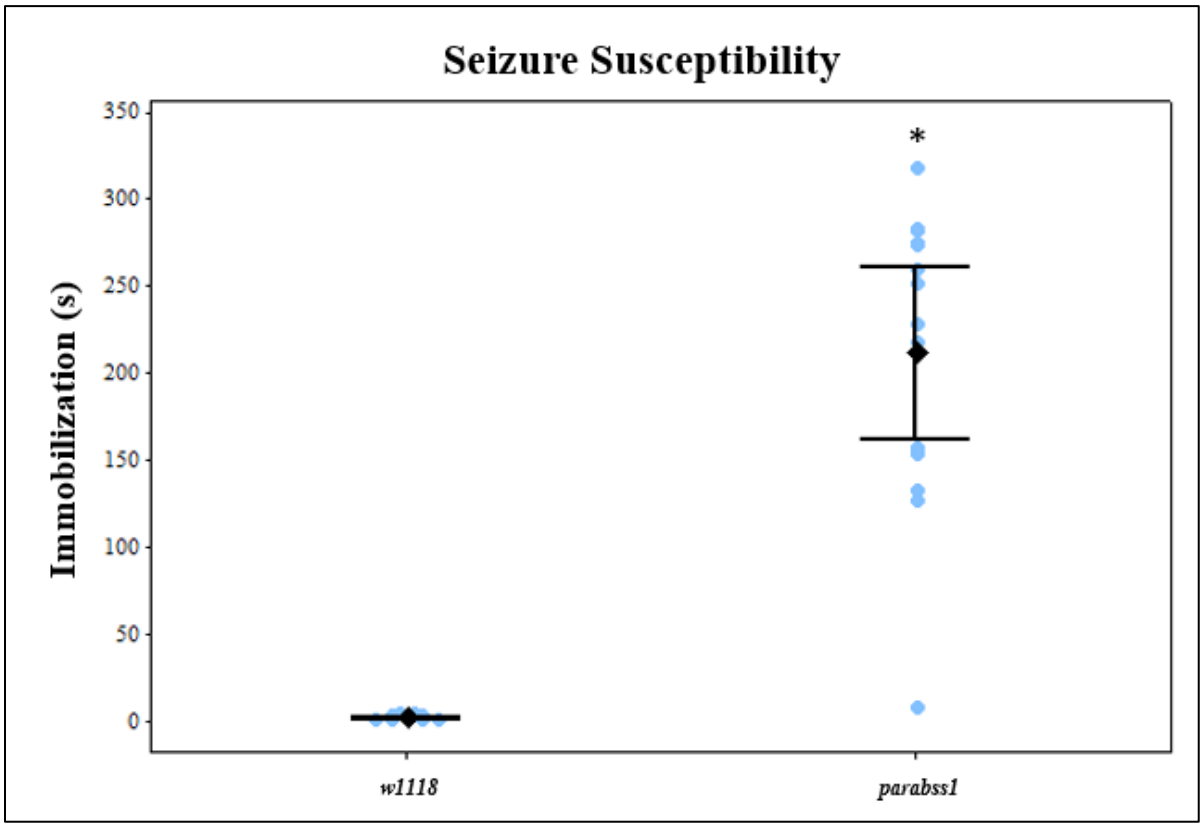

Figure 2: *para*^{bss1} mutants produced a significant seizure phenotype as denoted by longer immobilization times compared to negative control w^{1118}.

(n=13–14; One-way ANOVA, $p<0.001$; error bars=95% confidence interval)

Furthermore, we wanted to ensure the original seizure assays were sensitive enough to identify changes in the $para^{bss1}$ seizure phenotype. This led us to use age as a parameter to determine if it would have a significant effect on seizure susceptibility. Crosses homozygous for the gain of function $para^{bss1}$ mutation and homozygous for w^{1118} were set up. Flies were tested 3 days, 10 days, and 14-15 days following eclosion. Flies tested at ten days old exhibited a mean immobilization time of 391.7s while flies at 14-15 days old exhibited a mean immobilization time of 439.2s (Figure 3). Flies at 14-15 days, however, did not produce a significant increase in seizure susceptibility compared to 10 days old flies, but it did trend in that direction suggesting that as age increase its impact on seizure susceptibility is reduced. Figure 3 shows that age plays a significant role in seizure susceptibility with older flies exhibiting longer immobilization times in $para^{bss1}$ mutants (Two-way ANOVA test, $p<0.001$; Figure 3). Both age and genotype were determined to significantly interact with each other to impact recovery time (Two-way ANOVA test, $p<0.0001$; Figure 3).

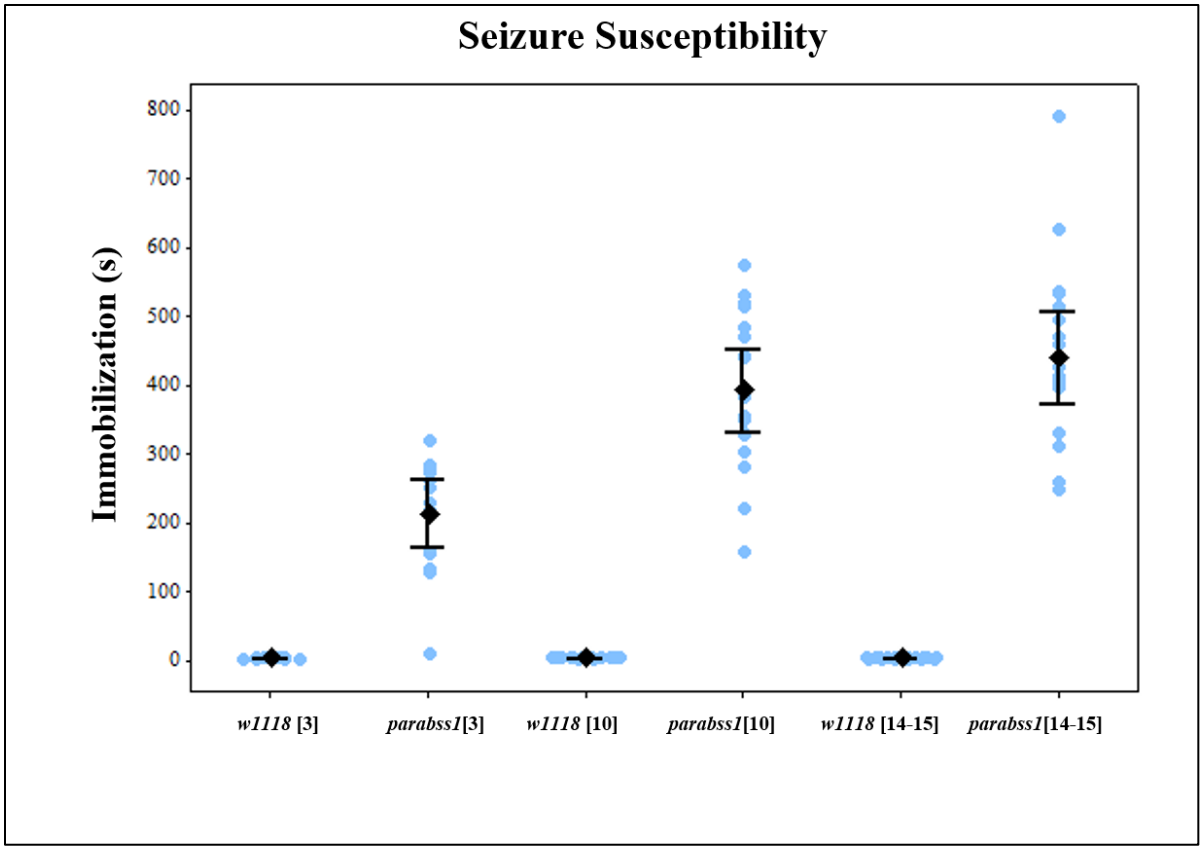

Figure 3: Age produced a significant increase in seizure phenotype on *para*^{bss1} mutants as denoted by longer immobilization times.

(n=13–18; Two-way ANOVA test, p<0.001; error bars=95% confidence interval)

Following our initial experiments to get a baseline for seizure susceptibility, we wanted to see how a mutation in the *brm* gene might impact *para* expression and modify the seizure phenotype. To do this we set up experimental crosses with progeny heterozygous for the *para*^{bss1} gain of function mutation and heterozygous for the *brm* loss of function mutation and compared them to crosses heterozygous for *para*^{bss1} and heterozygous for *w*^{1118} which acted as our positive control. We also compared our experimental group to crosses homozygous for *w*^{1118} which served as our negative control. We had to sort our experimental cross based on the absence of the stubble phenotype (*para*^{bss1}/+;; *brm[2] e[s] ca[1]*/+ or *para*^{bss1}/Y;; *brm[2] e[s] ca[1]*/+), which contained the loss of function mutation in the *brm*

gene, or the presence of the stubble phenotype (*para*^{bss1}/+;; *TM6B; Sb[1] Tb[1] ca[1]*/+ or *para*^{bss1}/Y;; *TM6B; Sb[1] Tb[1] ca[1]*/+), which did not have the loss of function mutation in the *brm* gene. Mean immobilization times for the group in which the stubble phenotype was absent was 6.2s. The mean immobilization time for homozygous *w*^{1118} flies was 1.1s. The mean immobilization time for the group in which the stubble phenotype was present was 160.2s. Lastly, the mean immobilization time for the positive control group was 80.5s. Figure 4 shows with significance that genotype effected immobilization times in flies with the *brm* mutation ($p<0.0001$) and flies without the *brm* mutation ($p<0.0144$; One-way ANOVA test). Following a post hoc Tukey test, which allowed us to compare individual groups, we found that flies heterozygous for the *para*^{bss1} mutation and heterozygous for the *brm* mutation was not significantly different from the negative control flies and in fact showed a high similarity to these homozygous *w*^{1118} flies suggesting these heterozygous flies lost their seizure sensitivity (Tukey test, $p=0.9967$; Figure 4). However, we also found using the same post hoc Tukey test, that the group that did have the stubble phenotype, while it maintained its seizure sensitivity, was significantly different from the positive control (Tukey test, $p=0.0066$; Figure 4).

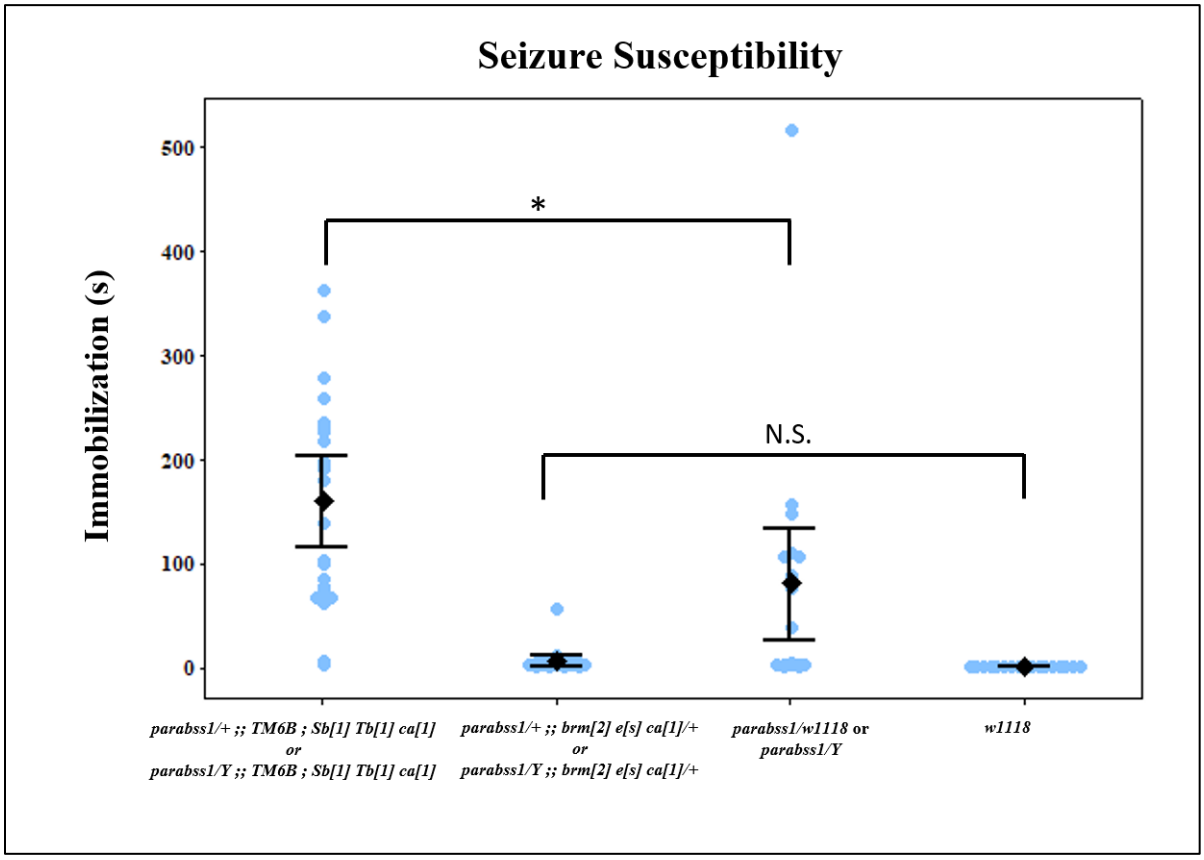

Figure 4: *brm* suppresses the *para*^{bss1} mutation for the seizure sensitivity phenotype.

(n=18–23; One-way ANOVA test, p<0.05; post hoc Tukey test, $^{N.S.}$p<0.9967, *p<0.0066; error bars=95% confidence interval)

We also wanted to see how a mutation in the *spen* gene might impact *para* expression and modify the seizure phenotype. To do this we set up experimental crosses with progeny heterozygous for the *para*^{bss1} gain of function mutation and heterozygous for the *spen* loss of function mutation and compared them to crosses heterozygous for *para*^{bss1} and heterozygous for *w*^{1118} which acted as our positive control. We also compared our experimental group to crosses homozygous for *w*^{1118} which served as our negative control. We sorted flies based on the presence of the *spen* allele. This was determined based on their wing phenotype. Flies with a curly wing phenotype were discarded because they did not possess the *spen* allele. The mean immobilization times for our experimental group was 43.3s while the mean

immobilization time for our positive control group was 104.9s. Finally, the mean immobilization time for our negative control group was 1.1s. Figure 5 shows with significance a difference in recovery time driven by genotype (One-way ANOVA test, p=0.0016; Figure 5). However, following a post hoc Tukey test we found that flies with the *spen* gene were significantly similar to the positive control flies (Tukey test, p=0.0521) and the negative control flies (Tukey test, p=0.2692). While statistically, flies with the *spen* gene were similar to both controls they trend in similarity more towards the negative control (homozygous w^{1118} flies) suggesting a decrease in seizure sensitivity in flies with the *spen* gene.

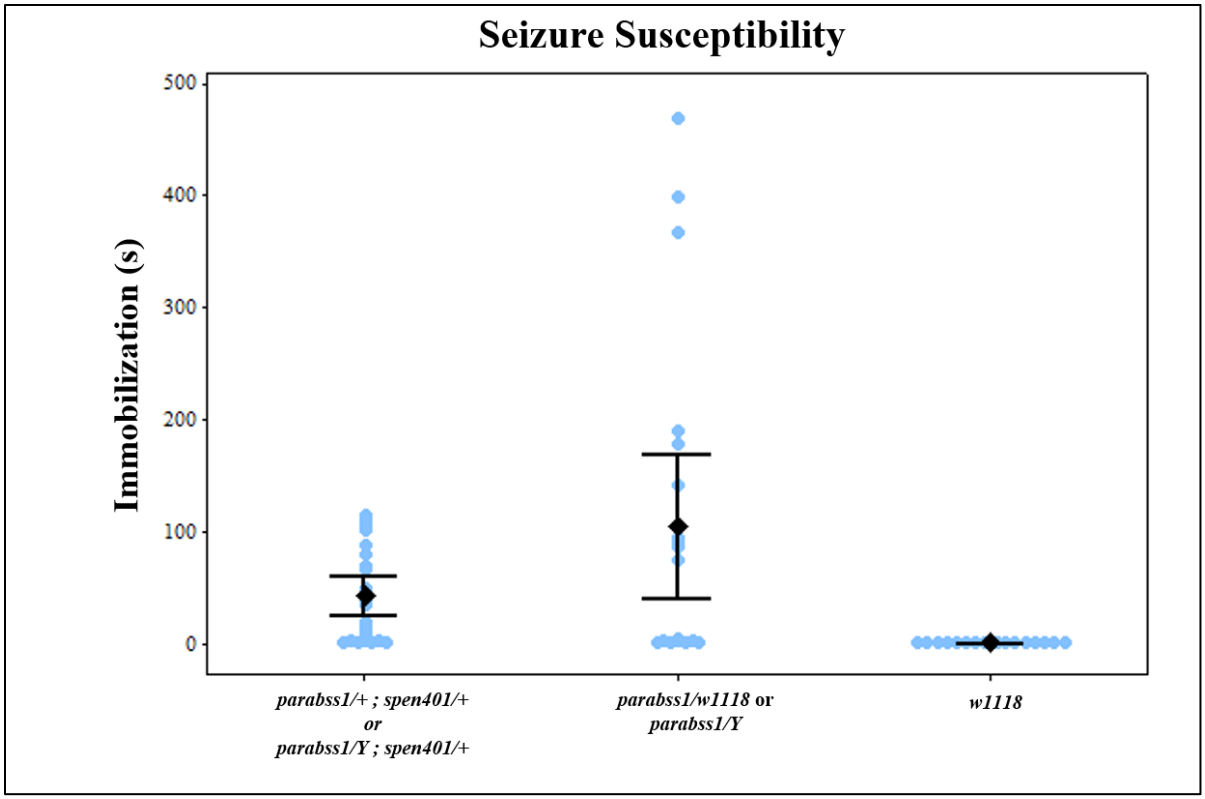

Figure 5: a *spen* suppresses the $para^{bss1}$ mutation for the seizure sensitivity phenotype (n=18–24; One-way ANOVA test, p<0.0016; post hoc Tukey test, p=0.0521 (comparison between flies with mutant *spen* gene and positive controls), p=0.2692 (comparison between flies with mutant *spen* gene and negative controls), error bars=95% confidence interval)

We next shifted our focus to nociception. To accomplish this, crosses that produced larvae homozygous for the gain of function *para*^{bss1} mutation and homozygous for w^{1118} were set up. Figure 6 displays thermal nociceptive behavior for homozygous *para*^{bss1} mutants compared to homozygous w^{1118} flies at 46°C. The mean NEL latency time for homozygous *para*^{bss1} mutants was recorded at 4.2s while homozygous w^{1118} flies had a mean NEL latency of 3.4s A significant increase in latency of homozygous *para*^{bss1} larvae was observed compared to the negative control homozygous w^{1118} flies suggesting that there was a decrease in sensitivity to thermal stimulation (Man-Whitney test, p=0.0067; Figure 6). This result was further quantified by testing at a lower temperature of 42°C shown in Figure 7.

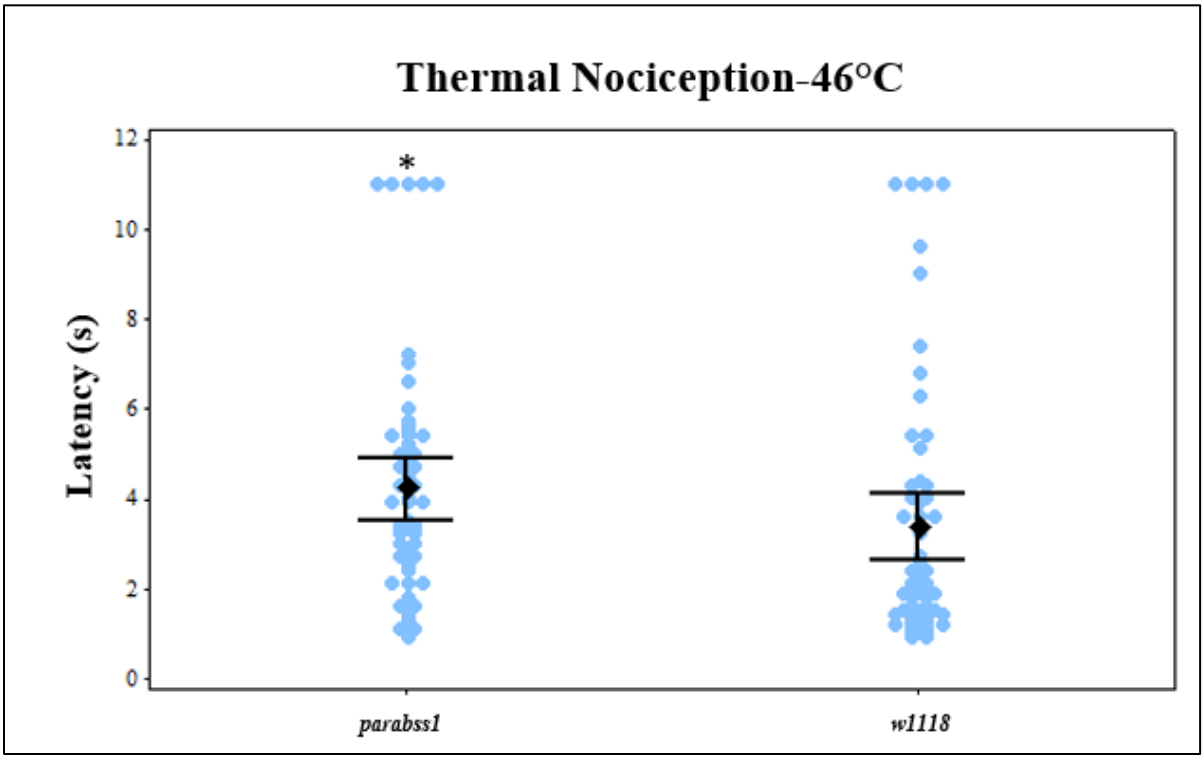

Figure 6: *para*^{bss1} mutants produced a significant decrease in sensitivity to a 46°C thermal stimulus.

(n=59–60; Mann-Whitney test, *p=0.0067; error bars=95% confidence interval)

We next tested thermal nociception at 42°C. To accomplish this, crosses homozygous for the gain of function *para*^{bss1} mutation and homozygous for w^{1118} were set up. The mean latency time for homozygous *para*^{bss1} mutants was 9.8s and the mean latency for homozygous w^{1118} flies was 9.6s. Figure 7 shows no significant difference in latency between homozygous *para*^{bsss1} mutants and homozygous w^{1118} flies (Mann Whitney test, p=0.6263; Figure 7). This suggests that because results were seen at 46°C, a thermal stimulus of 42°C might not be noxious enough to observe a measurable difference between *para*^{bss1} mutants and control w^{1118}.

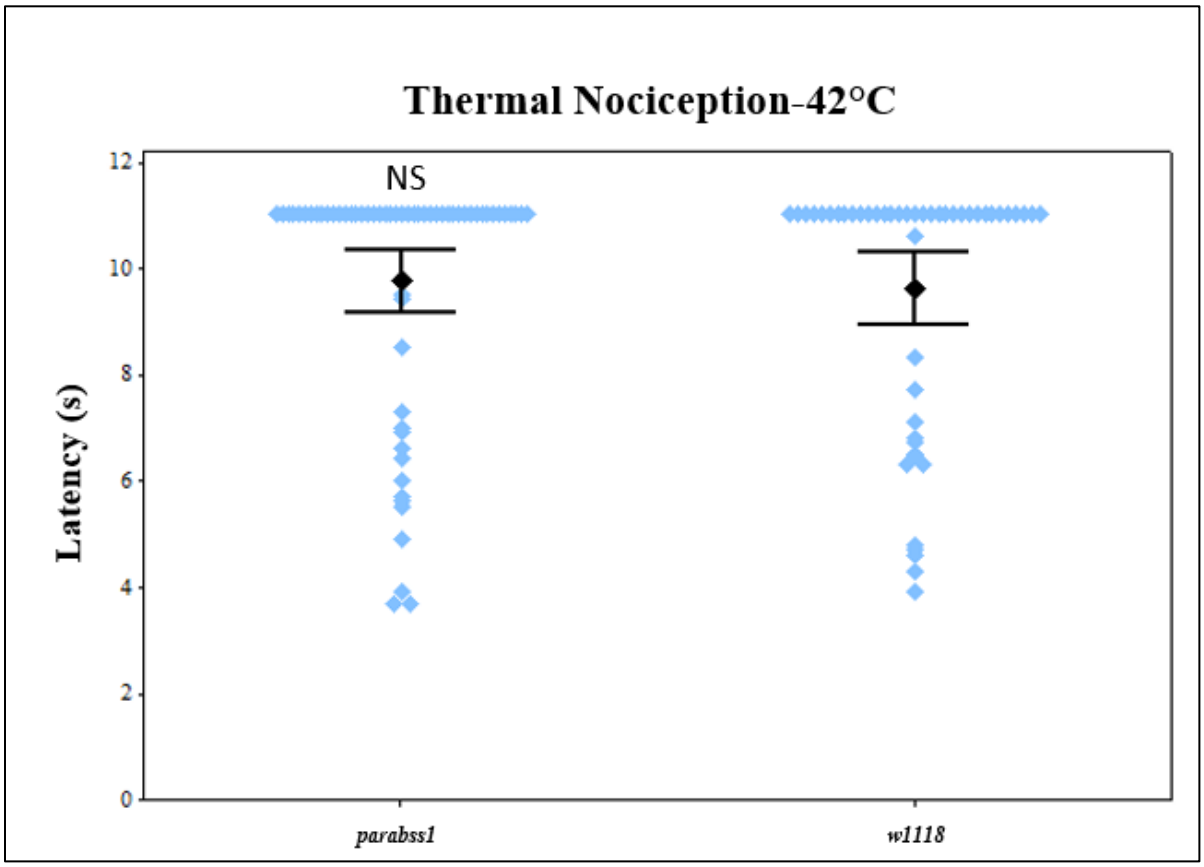

Figure 7: *para*^{bss1} mutants did not produce a significant difference in sensitivity to a 42°C thermal stimulus.

(n=48–61; Mann-Whitney test, ^{NS}p=0.6263; error bars=95% confidence interval)

Finally, we looked at mechanical nociception to determine if it would also be impacted by the *para*^{bss1} mutation. To do this, crosses homozygous for the gain of function *para*^{bss1} mutation and homozygous for w^{1118} were set up. Figure 8 shows no significant difference in the percentage of larvae responding to noxious mechanical stimuli. This suggests the *para*^{bss1} mutation in the *para* gene does not affect mechanical nociception while it does impact thermal nociception at 46°C (Two-sample proportions test, p=0.0582; Figure 8).

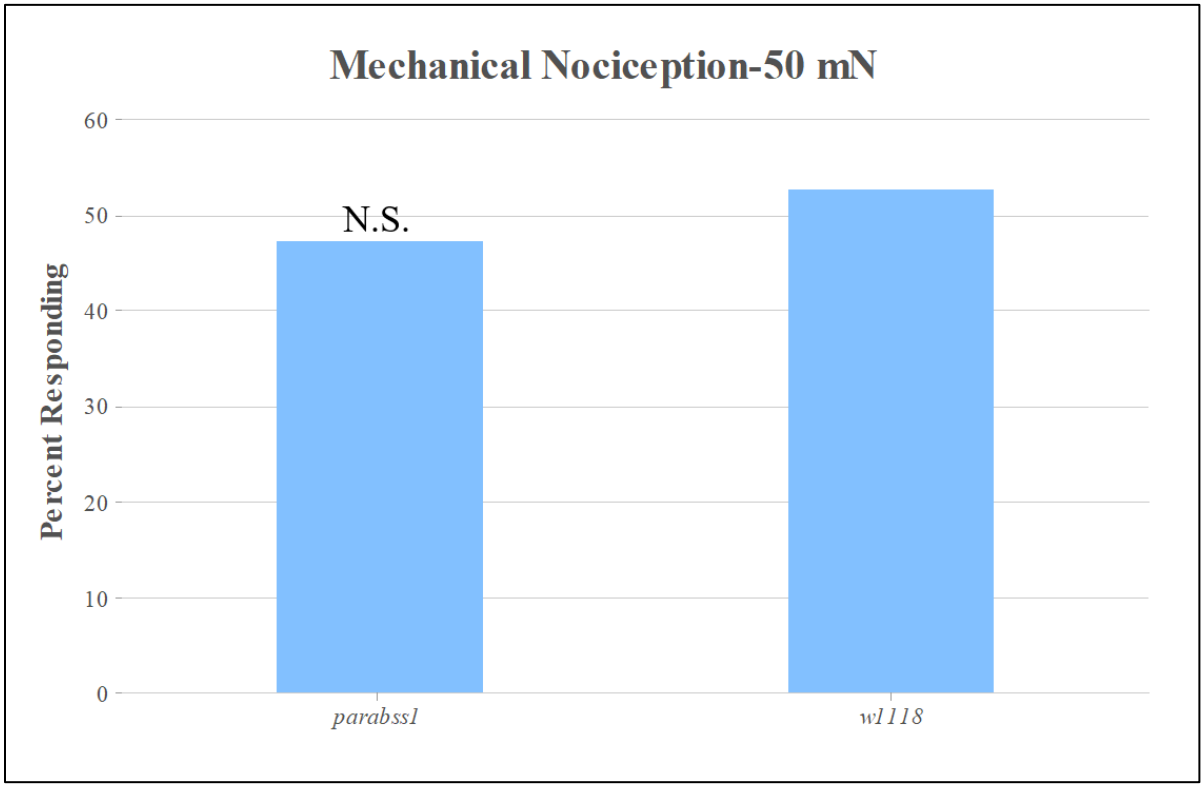

Figure 8: *para*^{bss1} mutants did not produce a significant difference in sensitivity to noxious mechanical stimuli.

(n=105–121; Two-sample proportions test, ^{NS}p=0.0582)

Discussion

Para as a model for seizure susceptibility

The first goal of this experiment was to determine a baseline seizure susceptibility of $para^{bss1}$ mutants for future comparisons of experiments predicted to modify this susceptibility. This was accomplished in Figure 2 where we saw that homozygous $para^{bss1}$ mutants had an increase in immobilization times compared to homozygous w^{1118} mutants pointing to an increase in seizure sensitivity. It has been suggested that $para^{bss1}$ could be used as a model for intractable epilepsy because of its high sensitivity to seizures as exhibited by its immobilization time of ~240s compared to other bang sensitive mutants *sda* and *eas* which had immobilization times of 38s and 82s respectively (Parker, Padilla, Du, Dong, & Tanouye, 2011). Our findings of a mean immobilization time of ~212s was in line with the ~240s found in Parker et al. We also wanted to ensure the original seizure assays were sensitive enough to identify changes in the $para^{bss1}$ seizure phenotype. This led us to use age as a parameter to determine if it would have a significant effect on seizure susceptibility. Flies were tested 3 days, 10 days, and 14-15 days after eclosion. Flies that were 10 days and 14-15 days old exhibited significantly increased seizure sensitivity compared to the baseline 3 days old flies (Figure 3). Thus, the $para^{bss1}$ model is appropriate to use as a model to identify changes in the $para^{bss1}$ seizure phenotype. In our subsequent experiments, we expected that *spen* and *brm* would enhance *para* expression and that increased neuronal firing would lead to a hypersensitive nociceptive phenotype in homozygous $para^{bss1}$ mutants.

The role of *para* in nociception

Parker et al. found that $para^{bss1}$ increased neuronal excitability in seizure sensitive flies leading to a highly seizure sensitive mutant making it a gain of function mutation (Parker, Padilla, Du, Dong & Tanouye, 2011). The role of *para* in both seizure sensitivity and nociceptive functioning led us to believe that the increased neuronal excitability exhibited by $para^{bss1}$ would lead to a mutant that was hypersensitive to noxious nociceptive stimuli. However, when $para^{bss1}$ flies were tested using a thermal stimulus of 46°C, we observed a decrease in sensitivity denoted by the increase in latency times of $para^{bss1}$ compared to w^{1118}. One explanation for this unexpected result is a range of excitability that neurons experience. For example, increasing action potential firing in the *para* mutants might lead to ineffective encoding of noxious stimuli by the sensory neurons. When the range of excitability is exceeded, it may lead to a decrease in function as observed in Figure 6 with the decrease in sensitivity to a thermal noxious stimulus. Following this discovery, we next tested the response of $para^{bss1}$ mutant flies to a thermal stimulus at 42°C.

At 46°C wild-type w^{1118} flies are already responding so quickly that it is hard to see mutants responding much quicker. When we test at 42°C it is easier to see mutants that might respond more quickly than control w^{1118} because w^{1118} is responding much more slowly. When we tested flies at 42°C we found that homozygous $para^{bss1}$ flies experienced no significant difference in sensitivity to a thermal stimulus compared to homozygous w^{1118} flies. One potential reason why we saw an affect at 46°C but not at 42°C might be due to homeostatic mechanisms. Synapses must possess plasticity in order to adjust to environmental challenges while regulatory mechanisms must constrain this activity within appropriate physiological ranges. The *Drosophila* neuromuscular junction (NMJ) has been

shown in past studies to exhibit a strong homeostatic response to changes in excitability with the main cause due to synapse impairment of the postsynaptic glutamate receptor function (Frank, 2014). Studies show that deletion of a *Drosophila* glutamate receptor subunit gene (DiAntonio et al., 1999; Petersen et al., 1997) and muscle-specific expression of active Protein Kinase A (PKA) (Davis et al., 1998) worked to greatly reduce muscle response to single vesicles of glutamate (Frank et al., 2014). Past studies have also shown that loss of function mutations in *Drosophila p21 activated kinase* (*Pak*) (Albin and Davis, 2004), mutations in *dorsal* and *cactus* (Heckscher et al., 2007), and loss of the translational repressor gene *nanos* (Menon et al., 2009) all diminished glutamate receptor clusters at the NMJ. In each of these cases, the NMJs of the mutants all showed reduced synaptic response in conjunction to increased neurotransmitter release (Albin and Davis, 2004; Heckscher et al., 2007; Menon et al., 2009). Parker et al. pinpointed the *bss1* allele as a gain of function mutation that leads to hyperexcitability in neurons (Parker, Padilla, Du, Dong & Tanouye, 2011).

It is possible that the size of these synapses of the nociceptor sensory neurons in $para^{bss1}$ mutants decreased during development to control for this larger response in neuronal excitability. This potential reduction in synapses and subsequent regulation of neurotransmitter release might make it more difficult to quantify a response to a weaker noxious stimulus. This might explain why we did not see a significant response to noxious thermal stimuli at 42°C, but we did see a decrease in sensitivity at 46°C (Figure 9). Homeostatic plasticity in the nervous system is used to counteract challenges that occur to neuronal function that could potentially disturb essential neuronal and circuit activities (Yeates, Zwiefelhofer, & Frank, 2017). In fact, research has shown that these homeostatic

responses can be carried out via compensatory adjustments to presynaptic neurotransmitter release (Cull-Candy et al., 1980; Peterson et al. 1997; Murthy et al. 2001), postsynaptic neurotransmitter receptor composition (O'Brien et al. 1998; Turrigiano et al., 1998; Rongo and Kaplan, 199; Turrigiano, 2008), or developmentally via changes in synaptic contact formation and maintenance (Davis and Goodman, 1998; Burrone et al., 2002; Wefelmeyer et al., 2016). This research further corroborates our assertation that perhaps a homeostatic mechanism might be leading to the reduction in synapse size and subsequent decrease in neurotransmitter release.

We next performed a mechanical assay to see if the *bss1* mutation had a similar role in mechanical nociception as thermal nociception. We found that there was no significant difference between $para^{bss1}$ and w^{1118}. This is suggestive of multiple possibilities. First, it is probable that the *bss1* mutation does not impact mechanical nociception. Past studies have shown evidence for nociceptive-specific pathways. For example, Zhong et al. demonstrated that the *pickpocket (ppk)* gene was required for mechanical nociception but not thermal nociception as larvae expressed greatly reduced nociceptive behaviors in response to harsh mechanical stimuli but no change to thermal stimuli (Zhong, Hwang, & Tracey, 2010). They further quantified this result with RNAi knockdown of the *ppk* gene and found that mechanical nociception was impaired but thermal nociceptive behavior remained unchanged (Zhong, Hwang, & Tracey, 2010). Therefore, an assertion can be made that the pathways for mechanical and thermal nociception are different. We can conclude that perhaps the $para^{bss1}$ mutation only affects thermal pathways and not mechanical pathways. Another possible explanation is that we could not observe a measurable difference in mechanical nociception phenotypes due to the earlier mentioned neuronal plasticity. We used a noxious mechanical

stimulus (50mN) but perhaps due to the increased neuronal excitability present in *para*^{bss1} mutants these synapses decreased in size to reduce the amount of neurotransmitter released making it difficult to see a change in response to noxious stimuli like mechanical.

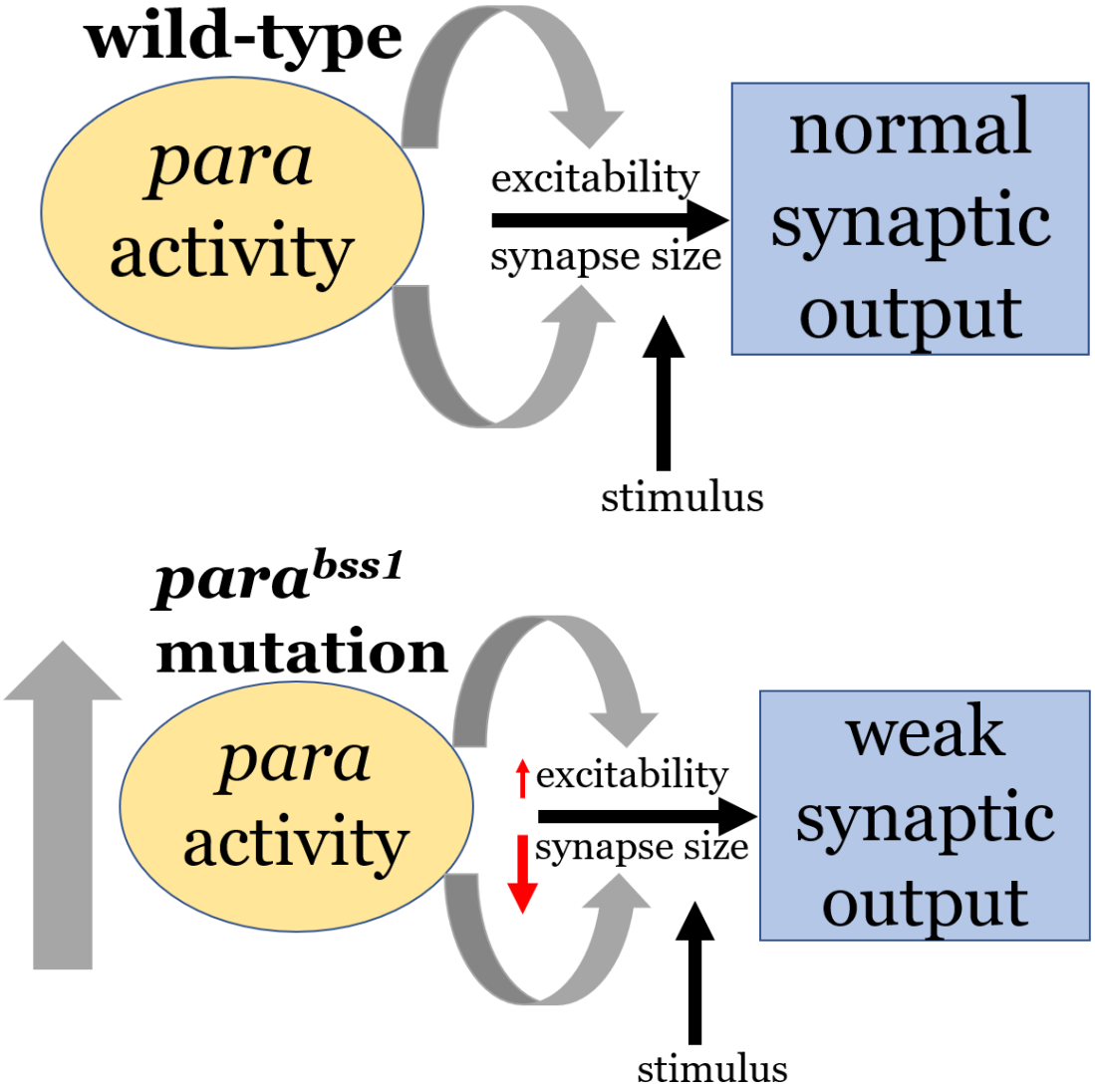

Figure 9: A proposed schematic for homeostatic mechanisms leading to reduced synapse size and in turn reduced neurotransmitter release.

Genetic modifiers of seizure susceptibility

We looked at mutations in *spen* and *brm* as possible enhancers of the gain of function *para*^{bss1} mutation. Lin et al. identified mutations in *spen* and *brm* genes to act to reduce the

expression of Pum, a translational repressor of *para* (Lin, He, Fan, & Baines, 2018). We expected mutants for these genes to therefore cause an increase in seizure sensitivity of $para^{bss1}$ mutants due to their ability to decrease Pum expression which is a translational repressor of *para*. We would thus expect an in increase in *para* translation and protein expression (Figure 10). Instead we found flies heterozygous for the $para^{bss1}$ mutation and heterozygous for the *brm* loss of function allele lost their seizure sensitivity altogether which we identified by the reduction in immobilization times compared to positive and negative controls (Figure 4). The line of *brm* that we used had a balancer chromosome (*TM6B*) carrying a visible phenotypic marker which allowed us to identify which flies had the mutation in the *brm* gene by the lack of the stubble phenotype along the dorsal side of the fly body. Along with this balancer was another gene (*ca[1]*) which was present on both the *brm* mutant chromosome and on the chromosome containing the *sb[1]* marker. Because the group with stubble maintained its seizure sensitivity and the group without stubble lost its seizure sensitivity we can rule out the role of *ca[1]* in impacting seizure susceptibility (Figure 4).

Past research showed that the *brm* gene is needed to regulate homeotic gene expression (Kennison, 1993). More recent research conducted by Merenda et al. also showed that the *brm* gene encoded a catalytic ATPase subunit of a Brm chromatin remodeling complex further emphasizing the role of *brm* gene in gene expression (Merenda, Zraly, & Dingwall, 2004). We expected that the *brm* mutation would act to enhance seizure sensitivity due to defective regulation of Pumilio, but instead it reduced seizure sensitivity. This further corroborates our assertion that perhaps there is a homeostatic mechanism taking place during development. Because *brm* is present during development and it plays a role in gene expression, it could be working during development to regulate the amount of

neurotransmitter being released that causes this larger neuronal excitability. This coupled with the possible decrease in synapse size could be why we see this loss of seizure sensitivity in flies with the mutation in the *brm* gene.

We also tested the mutation in the *spen* gene and found that there was a significant reduction in seizure sensitivity (Figure 5). Unlike the mutation in the *brm* gene, however, flies containing the *spen* mutation were found to be statistically similar to both the positive and the negative control after performing a post hoc Tukey test. However, flies with *spen* mutation trended more towards the negative control suggesting it lost more of its seizure sensitivity than it retained. The fact that *spen* mutants did not significantly increase seizure sensitivity and instead trended more towards a loss of seizure sensitivity further corroborates the assertion that it is likely that homeostatic mechanisms reduce the synapse sizes of these gain of function $para^{bss1}$ mutants to control for this larger neuronal excitability.

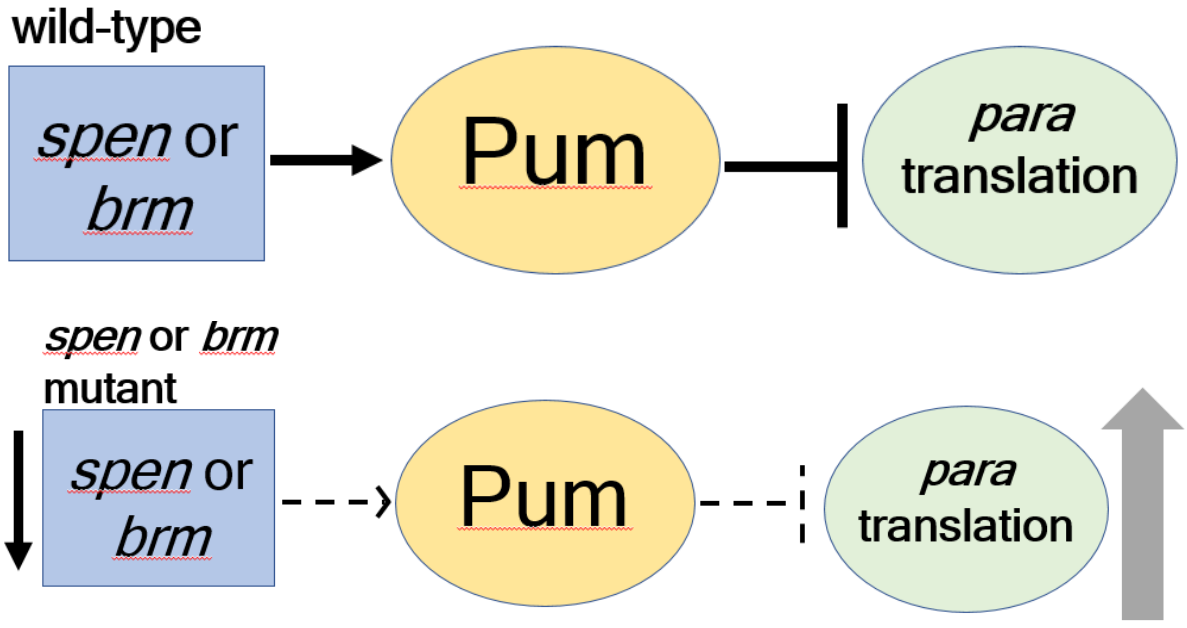

Figure 10: A proposed schematic for *para* regulation due to the mutation in the *spen* or *brm* gene.

Future directions

One prediction we have is that the homozygous *para*^{bss1} synapses are either smaller or reduced in number. A future direction for this study would be to stain synaptic proteins and motor neurons then image them using confocal microscopy to visualize the morphology of the synapses. We might also stain synaptic proteins and motor neurons in mutants heterozygous for *para*^{bss1} and heterozygous for *spen* and *brm* mutants. The data obtained from this morphological analysis of the synapse could be used to prove the role of homeostatic mechanisms in affecting synapse sizes to control for larger neuronal excitation.

Another future direction would be to investigate other lines of *spen* and *brm* because the results we observed are the opposite of what we expected. Investigating other mutants would give us the opportunity to see if these mutants lead to the same or different result. This would allow us more confidence in asserting that these genes do play a role in regulating gene expression related to the expression of *para*. Another prediction we have is that the amount of neurotransmitter that is released might be reduced. While it is difficult to directly measure neurotransmitter release, research done by Streit et al. showed that when GCaMP, a genetically encoded calcium indicator, was expressed in motor neurons, its peaks corresponded with bouts of action potentials (Streit, Fan, Masullo, & Baines, 2016). We could measure neuronal activity using GCaMP expression. Also, if there is a change *para* expression, we could also directly see if there is a change in the proteins encoded by *para* using antibody staining of *para* in *spen* and *brm* mutants.

Conclusion

In conclusion this study produced a sensitized background that can be used in future analyses to determine the effect of different parameters on seizure susceptibility. It also identified a role for the mutation in the *brm* gene in reducing seizure sensitivity while the role of the mutation in the *spen* gene is still not as clear but trends more towards a reduction in seizure sensitivity. We were also able to confirm a role for $para^{bss1}$ in thermal nociception at 46°C but not at 42°C. We were also unable to confirm a role for $para^{bss1}$ in mechanical nociception. Mutations in the *spen* and *brm* genes suppress the $para^{bss1}$ mutation and lead to a mutant that is less seizure sensitive. More research is needed to elucidate the exact roles of *spen* and *brm* in this suppression.

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