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Alcohol resistance in Drosophila is modulated by the Toll innate immune pathway

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Abstract

A growing body of evidence has shown that alcohol alters the activity of the innate immune system and that changes in innate immune system activity can influence alcohol-related behaviors (Cui et al., 2014; Vetreno & Crews, 2014). Here we show that the Toll innate immune signaling pathway modulates the level of alcohol resistance in Drosophila. In humans, a low level of response to alcohol is correlated with increased risk of developing an alcohol use disorder (Schuckit, 1994). The Toll signaling pathway was originally discovered in, and has been extensively studied in Drosophila. The Toll pathway is a major regulator of innate immunity in Drosophila, and mammalian Toll-like receptor signaling has been implicated in alcohol responses. Here, we use Drosophila-specific genetic tools to test eight genes in the Toll signaling pathway for effects on the level of response to ethanol. We show that increasing the activity of the pathway increases ethanol resistance while decreasing pathway activity reduces ethanol resistance. Furthermore, we show that gene products known to be outputs of innate immune signaling are rapidly induced following ethanol exposure. The interaction between the Toll signaling pathway and ethanol is rooted in the natural history of *Drosophila melanogaster*.

Keywords

Alcohol Resistance; Alcohol Sensitivity; Innate Immunity; Neuroimmune; Addiction; Alcoholism; NF-κB; Tolerance; Mutations; RNAi; Ethanol

Introduction

Alcohol use is pervasive in our society, and alcohol abuse has been estimated to cost the United States economy $223.5 billion per year (Bouchery et al., 2011). A survey of more than 36,000 American adults found that 29.1% of respondents met the criteria for DSM-5 diagnosis of an alcohol use disorder at some time in their life, and 13.9% had met the criteria in the past 12 months (Grant et al., 2015). A 25 year longitudinal study in humans found that baseline resistance to alcohol was the strongest predictor of future alcoholism (Schuckit,

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1994; Schuckit & Smith, 2011). Alcoholism has a strong genetic component, and while no single alcoholism gene has been identified, large networks of genes with small individual effects sum to generate predisposition for addiction (Enoch, 2013).

A growing body of evidence shows that chronic alcohol consumption changes the expression of conserved gene networks in the human brain, members of which have been demonstrated to regulate alcohol behaviors in model systems (Ponomarev et al., 2012; Zhou et al., 2011; Liu et al., 2007; Liu et al., 2006; Iwamoto et al., 2004; Flatscher-Bader et al., 2005; Liu et al., 2004; Sokolov et al., 2003; Mayfield et al., 2002; Lewohl et al., 2000; Farris et al., 2014). One such network contains a set of genes that control the innate immune system. Studies in fruit flies, rodents, and humans have all shown that innate immune system genes increase expression after alcohol exposure (Crews et al., 2013; Liu et al., 2006; Kong et al., 2010; Zou & Crews, 2014). Recent rodent work has shown that numerous innate immune system pathways affect alcohol consumption, including chemokines, interleukins, peroxisome proliferator-activated receptors (PPARs), and Toll-like receptor pathways (Robinson et al., 2014). Furthermore, it has been reported that Toll-like receptor signaling can modulate neural activity. In brain slice preparations from the central amygdala of mice, treatment with lipopolysaccharide, the activator of Toll-like receptor 4 (TLR4), can directly modulate GABAergic signaling, and ethanol and lipopolysaccharide treatment can have additive effects on GABAergic signaling (Bajo et al., 2014).

The innate immune system is a branch of the immune system that invokes a rapid, preprogrammed, and generalized response to pathogens. Whereas the adaptive immune system recognizes, responds to, and remembers essentially any foreign antigen, the innate immune system is hardwired to respond to the antigens stereotypical of pathogens. Innate immune system responses include inflammation to seal off a site of infection, recruitment of immune cells, and production of antimicrobial peptides (Turvey & Broide, 2010). In Drosophila, there are two major branches of the innate immune system: the Toll pathway and the immune deficiency (IMD) pathway (Buchon et al., 2014).

The Toll pathway was initially described by the Nüsslein-Volhard lab for its role in the establishment of the dorsal-ventral axis during embryonic development of *Drosophila melanogaster*. In larvae and adult flies however, the same pathway is reused to regulate the innate immune system (Lemaitre et al., 1996). The Toll pathway is conserved across metazoans, from sponges to humans (Song et al., 2012), and the Drosophila Toll pathway is related to the mammalian Toll-like receptor Myd88-dependent pathway. As depicted in Fig. 1, in Drosophila the Toll ligand is a protein called Spätzle, which circulates in the hemolymph as an inactive precursor. Upon fungal or Gram-positive bacterial infection Spätzle is cleaved, binds to Toll, and activates the pathway. Myd88, Tube, and Pelle are adaptor proteins that associate with Toll. After activation, Pelle phosphorylates the NF-κB inhibitor Cactus, which causes Cactus degradation and allows NF-κB homologs Dorsal and Dif to enter the nucleus and activate transcription of target genes (reviewed in Imler, 2014).

The other main branch of the Drosophila innate immune system is the IMD pathway, which responds to Gram-negative bacterial infection. There is evidence for cross-talk between the Toll and IMD signaling pathways: simultaneous stimulation of the Toll and IMD pathways

has an additive effect on expression of some antimicrobial peptide genes (Tanji et al., 2007) and infection with some pathogens leads to activation of both pathways (Mansfield et al., 2003; Hashimoto et al., 2009; Luce-Fedrow et al., 2008; Lau et al., 2003). Relish is the Drosophila NF-κB that is primarily associated with and activated by the IMD pathway (Buchon et al., 2014). All three NF-κB proteins (Dif, Dorsal, and Relish) can form heterodimers with one another, providing a means for integration of information from the Toll and IMD pathways (Tanji et al., 2010).

In recent years, an intriguing connection between ethanol consumption and the innate immune pathway has become apparent in Drosophila. In experiments detailed in Milan et al. (2012) and Kacsoh et al. (2013) it was found that fruit flies use alcohol to help fight infection by parasitic wasps. There are several species of endoparasitoid wasps of the genus *Leptopilina* that inject their eggs into fruit fly larvae. The wasp offspring then develop within and feed on the fruit fly larva, eventually killing them during pupariation. The Toll innate immune pathway is a regulator of the anti-parasite immune response in Drosophila (Paddibhatla et al., 2010; Schlenke et al., 2007; Sorrentino et al., 2004; Small et al., 2012). Under standard culture conditions fly survival is quite low after infection by these wasps. However, when standard fly food is replaced with food containing 6% ethanol, wasp survival decreases and fly survival increases. When given a choice between standard food and ethanol-containing food, a greater portion of infected larvae chose ethanol food than did uninfected larvae (Milan et al., 2012). A subsequent study showed that, after seeing a female wasp, female Drosophila were more likely to lay eggs on ethanol-containing food. This reaction was sex-specific and did not occur in response to a male wasp (Kacsoh et al., 2013). Indeed, flies respond to a parasitic infection in a way that suggests that the fly innate immune system modulates ethanol-related behaviors. Here we ask whether genetically manipulating the Toll pathway affects resistance to ethanol.

Materials and Methods

Stocks and Fly Husbandry

All flies were maintained on a standard cornmeal/molasses/agar medium under 12:12 light:dark conditions. In order to collect age-matched flies, a bottle with eclosing flies is cleared of adult flies and three days later the adult females are harvested and allowed to age for three more days, yielding a group of flies that are 3–6 days old. The full genotype of stocks used in this study and additional information about alleles or transgenes can be found in Table S2. Canton S was used as the wild-type control where appropriate. Heterozygous animals were produced by crossing stocks to our wild-type Canton S stock before testing. All transgenes were tested as heterozygotes.

Stocks were from the Bloomington Drosophila Stock Center (NIH P40OD018537) or the Tübingen Drosophila Stock Collection (provided by Dr. David Stein at the University of Texas at Austin). The *UAS-V5-Dif*, *UAS-V5-RelN*, and *UAS-Toll^{10B}-FLAG* stocks were provided by Dr. Y. Tony Ip (University of Massachusetts Medical School). Animals carrying the *Actin-GeneSwitch* transgene were derived from the stock BSC# 9431. This stock carries other mutations that did not interest us. A stock bearing only the w^{1118} allele and the *Actin-GeneSwitch* transgene was generated by genetic crossing. Each of the UAS overexpression

transgenes and the *Actin-GeneSwitch* line were backcrossed to our wild type Canton S stock seven times to remove any second-site mutations and to ensure they are in the same genetic background. RNAi lines acquired from the TRiP consortium (Transgenic RNAi Project) were used to knock down expression of eight Toll pathway genes. The TRiP stocks have the same genetic background and all carry their respective RNAi construct at the same attP insertion site (except for the *cactus* RNAi line). A stock with no RNAi construct inserted at the attP insertion site was used as a control for RNAi experiments (BSC# 36303). Dif^{1} was acquired in a background that contained *Dipt* and *Drs* reporter constructs, but was separated from these transgenes by crossing.

Activation of GeneSwitch Transgenes

The GeneSwitch system has been described in Osterwalder et al. (2001) and Roman et al. (2001), and makes use of a fusion of Gal4 and progesterone receptor domains to generate a transcription factor that activates UAS transgenes in the presence of RU-486 (a.k.a. mifepristone, Cayman Chemical, Ann Arbor, MI, USA). To generate drug-laced food, a stock solution of 25 mM RU-486 in 80% ethanol was added to molten fly food to produce food with a final concentration 200 μM RU-486, alongside control food that was melted and mixed with carrier (modified from McGuire et al., 2004). The RU-486 food was distributed to fly vials and allowed to cool and dry for at least one hour. Flies were kept on drug- or carrier-containing food for three days. Because RU-486 has poor solubility in water, the stock solution used 80% ethanol as the solvent, and therefore both the RU-486 and carrier-fed flies were housed on food that initially contained 0.64% ethanol v/v.

Ethanol Resistance Assay

Experiments were performed in the inebriator as described in Krishnan et al. 2012 and Cowmeadow et al. 2005. All experiments are performed with age-matched female flies being sedated with ethanol for the first time. Groups of 10 flies are placed in plastic vials and exposed to a stream of concentrated ethanol vapor until all flies are sedated (typically 15-18 minutes). Then the ethanol-saturated air stream is replaced with a humidified air stream and recovery from sedation is recorded. Flies are considered recovered when they regain postural control. n=4–6 vials for each group. All experiments were performed between 11:00 and 16:00 (zeitgeber time 3–8).

Determination of Ethanol Concentration in flies

The Enzymatic Ethanol Assay Kit (Diagnostic Chemicals Ltd. Oxford, CT) was used to measure internal ethanol concentration of flies. Flies were sedated in the inebriator, and then collected at the designated time. 10 flies were ground with a plastic pestle in 500μl of 50mM TRIS pH 7.5, vortexed, centrifuged for 2 minutes, and the supernatant was transferred to a fresh tube. 6μl of this solution was incubated in 370μl of reagent solution for 10 minutes at 37°C. 340nm absorbance was then recorded with a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE) and plotted against a standard curve. The ethanol concentration in fly hemolymph was then calculated assuming a 0.85μl volume per fly (Cowmeadow et al., 2005).

Statistical Analysis

Behavioral recovery data was entered into GraphPad Prism 6 for graphing and statistical analysis. Statistical significance was determined using the log rank test. Error bars represent standard error of the mean in all figures. Ethanol absorption and metabolism data were analyzed in Prism 6 using linear regression to compare the slope and intercepts of the data sets, and using multiple Student *t*-tests and the Holm-Sidak method to correct for multiple comparisons.

RNA Isolation and Sequencing

RNA was extracted from the heads of 3–5 day old adult female flies that were either treated with ethanol or left untreated as controls. Approximately 180 fly heads were used per group. The ethanol treated group was exposed to an ethanol-saturated air stream until all flies were sedated (15 minutes), followed by a 30 minute ethanol-free air stream. For the untreated control group, flies were exposed to an ethanol-free air stream for the entire 45 minutes. At the end of the treatment (30 minutes post-sedation), both groups of flies were transferred to a 50 ml conical tube and snap frozen in liquid nitrogen. Heads were snapped off from the body by briefly vortexing the tubes. The frozen heads were sorted from the bodies using a series of cooled metal mesh sieves. Total RNA was isolated from the heads using the guanidinium thiocyanate single-step method (Ausubel, 1994). After isolation, RNA was treated with DNAse I (Life Technologies, Grand Island, NY) and purified by acid-phenol/chloroform extraction and ethanol precipitation. RNA concentration and quality was assessed using a 2100 Bioanalyzer RNA 6000 Pico Chip (Agilent Technologies, Inc., Santa Clara, CA). RIN values for the control and ethanol-treated samples were 6.80 and 6.40, respectively.

Poly(A)+ RNA was prepared from an aliquot of each total RNA sample with magnetic oligo-(dT) beads (Dynabeads® Oligo (dT), Life Technologies, Grand Island, NY). cDNA synthesis and Illumina library construction were performed with the TruSeq RNA Library Preparation Kit using standard Illumina protocols and sequenced to at least 20M reads in an Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA) using paired-end chemistry and 100-bp cycles. Raw sequences have been deposited in the public functional genomics data repository from NCBI: Gene Expression Omnibus (GEO). Data can be found on the GEO website (http://www.ncbi.nlm.nih.gov/geo/) using accession number GSE77792. All essential sample annotation and experimental design information including sample data relationships have been included in the repository according to the Minimum Information About a Microarray Experiment (MIAME) guidelines (Brazma et al., 2001).

RNA-Seq Data Processing and Statistical Analysis

RNA-seq reads were aligned and mapped to the Drosophila reference genome (BDGP Release 5) using SOAPaligner/SOAP2, allowing no more than 5 bp mismatches. Low-quality reads (containing adapters or high content of unknown bases) were filtered out. Expression levels for genes were calculated using RPKM (reads per kilobase transcriptome per million mapped reads) method (Mortazavi et al., 2008) using the CLC Genomics Workbench (CLC bio, Boston, MA). Differential expression analysis was conducted using Cluster 3.0, and Java TreeView software (Eisen et al., 1998; Saldanha, 2004) and expressed

as $\log_2$ Ratios (EtOH/Ctrl). We used FDR ≤ 0.0001 and an absolute value of $\log_2$ Ratio ≥ 1 as the threshold to judge the significance of expression difference. Gene ontology analysis was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID) web-accessible tool, version 6.7 (Huang et al., 2009b; Huang et al., 2009a). For gene ontology annotation search and clustering, significant gene categories for each cluster were identified using default High Classification Stringency parameters (Kappa Similarity Term Overlap: 3; Similarity Threshold: 0.85; Initial Group Membership: 3; Final Group Membership: 3; Multiple Linkage Threshold: 0.5) and official gene symbols as input. The statistical significance of over-representation of antimicrobial peptide genes was determined using a binomial test in GraphPad Prism. The observed frequency of eight antimicrobial peptide genes in the 137 differentially expressed genes was compared to the 124 Humoral Immune Response genes (GO:0006959) in the *Drosophila melanogaster* genome (17,717 genes in FlyBase release 6.06).

Results

To test whether the Toll pathway modulates ethanol-induced behaviors in *Drosophila melanogaster*, we tested the effect of mutations, RNAi knockdown, and overexpression of Toll pathway genes on the rate of recovery from ethanol sedation. In this study, experimental and control animals were simultaneously exposed to vaporized ethanol until sedated, then switched to a humidified air stream to recover and the rate of recovery was measured (Krishnan et al., 2012; Cowmeadow et al., 2005). If the experimental group recovered from sedation faster than the control group, the experimental group is said to be more resistant to ethanol. Conversely, flies that recovered significantly slower than the control are said to be less resistant (or more sensitive). There is day-to-day variability in resistance to ethanol, for this reason all of the direct comparisons that we make are sedated in tandem on the same day. As per convention, in the text, gene names are italicized while the encoded protein is capitalized and typeset in roman.

Spätzle is the *Drosophila* ligand of Toll, and Spätzle binding to Toll activates the pathway (Lemaitre et al., 1996; Weber et al., 2003). Transheterozygotes are often used when studying maternal effect lethal mutations. This is necessary because the stocks are maintained over a balancer chromosome, and it is thought that the chromosome of interest accumulates secondary lethal mutations, making homozygotes unobtainable without first backcrossing the mutation. Thus, animals lacking functional *spätzle* were obtained as a heteroallelic combination of two null alleles, spz^2 and spz^4 (Lemaitre et al. 1996).These *spätzle* null animals were less resistant to ethanol than wild-type controls, as can be seen by their slower recovery rate ($p<0.0001$, Fig. 2A). Additionally, animals heterozygous for a null allele of *spätzle* (spz^2) and a wild-type chromosome were less resistant to ethanol than wild-type controls ($p<0.0001$, Fig. 2B). If the *spätzle* mutant is less ethanol resistant because of reduced signaling down the Toll pathway, then one would expect that reducing signaling at subsequent steps in the pathway would also reduce ethanol resistance. We tested this idea with a variety of genetic tools.

We manipulated *Toll* receptor activity with mutant alleles, a gene-specific RNAi knockdown transgene, and a *Toll* overexpression transgene (Fig. 3). *Toll* mutant animals were generated

by combining two loss-of-function alleles of *Toll (Toll*^{R3} and *Toll*^{rv19}) to yield a transheterozygous animal. When compared to wild-type, these *Toll* mutant animals were less resistant than controls in a recovery from sedation assay ($p<0.0001$, Fig. 3A). Other researchers have reported similar findings for *Toll* mutants: animals with a transposon inserted near the *Toll* locus were less resistant than wild-type controls in an ethanol sedation assay (Morozova et al., 2007; Morozova et al., 2011). To confirm the involvement of *Toll*, we suppressed *Toll* expression with an RNAi transgene. For this experiment we used the *Actin-GeneSwitch* driver. An important advantage of the GeneSwitch system is that it permits adult-specific expression, which avoids disruption of normal development. In this system, a fusion protein between the Gal4 transcription factor and progesterone receptor activates UAS transgenes only in the presence of the RU-486 inducer (Osterwalder et al., 2001; Roman et al., 2001). The GeneSwitch system allowed us to perform experiments in which all flies have the same genotype, and the transgene of interest is expressed only in adults fed an inducer for three days prior to the experiment. Feeding RU-486 to animals carrying the *Actin-GeneSwitch* transgene but not a UAS responder transgene has no effect on ethanol resistance at the dose used here (Fig. S1 C & D). In concert with the *Toll* mutant analysis, animals in which *Toll* expression was suppressed with an *Actin-GeneSwitch* driven RNAi transgene also showed reduced ethanol resistance ($p<0.0001$, Fig. 3B).

Increased *Toll* activity produced the opposite response. To increase *Toll* signaling activity we used a transgene that expresses a constitutively active *Toll* variant *(Toll*^{10B}*).* Expression of the *UAS-Toll*^{10B} transgene (Hu et al., 2004) enhanced ethanol resistance in our sedation assay. Driving the *UAS-Toll*^{10B} construct ubiquitously with *tubulin-Gal4* increased behavioral ethanol resistance ($p<0.0001$ vs. either parental control, Fig. 3C). Additionally, overexpressing *Toll*^{10B} in neurons with the *Appl-Gal4* driver increased ethanol resistance ($p<0.0001$ vs. either parental control, Fig. 3D). *Appl-Gal4* is a neuron-specific, pan-neural driver (Torroja et al., 1999; Scholz et al., 2005; Fang et al., 2013). In addition, the *Actin-GeneSwitch* driver was used to overexpress *UAS-Toll*^{10B}. When these animals were fed RU-486 inducer for three days, resistance increased compared to carrier-fed controls ($p=0.03$, Fig. 3E). Increasing or reducing Toll activity transgenically does not affect ethanol absorption or metabolism (Fig. S1 A and B). In all Gal4 overexpression experiments, we also individually examine the parental Gal4 driver line (black triangles in plots) and the parental UAS-responder line (black square in plots) to verify that changes in behavior are caused by expression of the responder transgene and are not the consequence of mutational insertion of a single transgene into the genome nor are they caused by off-target effects of the Gal4 transcription factor. The parental lines were crossed to wild-type Canton S so that the parental controls carry only a single copy of the transgene, as is the case in the experimental group.

Myd88 is an adapter protein that interacts with Toll and is required for the immune response to infection (Tauszig-Delamasure et al., 2001; Marek & Kagan, 2012). Only transgenic RNAi knockdown of *Myd88* was tested in this study. When *Myd88* was knocked down for three days with the *Actin-GeneSwitch* driver, resistance to ethanol was reduced ($p<0.0001$, Fig. 4A). Tube is part of the adapter complex that assembles along with Toll, Myd88, and Pelle during Toll activation and is required for Toll signaling (Letsou et al., 1991; Moncrieffe et al., 2008). We generated *tube* null animals by a transheterozygous cross;

animals carrying the *tube*^{2} null allele were mated with animals carrying a chromosomal deficiency (deletion) that removes the *tube* locus, producing *tube*^{2}/*tube*^{DF} null animals (Hecht & Anderson, 1993). These mutant animals are less resistant to ethanol than the wild-type control (p=0.0013, Fig. 4B). Furthermore, a reduction in resistance was also produced when *tube* was knocked down in an adult-specific manner using an *Actin-GeneSwitch* driven RNAi transgene (p<0.0001, Fig. 4C).

Pelle is a kinase that assembles with Toll, Myd88, and Tube after Toll pathway activation, and there is indirect evidence that Pelle is the kinase that phosphorylates Cactus (Huang et al., 2010; Towb et al., 2001). The phosphorylation of Cactus causes its degradation, which frees the Dif and Dorsal transcription factors that were sequestered outside of the nucleus by Cactus. Blocking the destabilization of Cactus reduces downstream nuclear signaling by Dif and Dorsal (Fig. 1). When two *pelle* loss-of-function alleles *(pelle*^{2} and *pelle*^{7}*)* were combined to generate a *pelle* mutant transheterozygote (Hecht & Anderson, 1993; Anderson & Nüsslein-Volhard, 1984), ethanol resistance was reduced (p=0.0058, Fig. 5A). We also examined the effect of *pelle* knockdown using the inducible GeneSwitch system to drive ubiquitous expression of a *pelle* RNAi transgene. Similar to the *pelle* mutant, a three day *pelle* RNAi knockdown reduced resistance (p=0.0008, Fig. 5B).

When the Toll pathway is inactive, the NF-κB inhibitor Cactus sequesters Dif and Dorsal transcription factors in the cytoplasm. After stimulation of the Toll pathway, Cactus is degraded and the NF-κB proteins Dif and Dorsal are able to enter the nucleus and activate target genes (Fig. 1, Geisler et al., 1992; Roth et al., 1991). Thus, reduction of *cactus* expression via mutation or knockdown should mimic a stimulated pathway where NF-κB proteins are allowed to enter the nucleus. We show that *cactus* mutant animals (transheterozygous for two separate loss-of function alleles, *cactus*^{7} and *cactus*^{E8}) were more resistant to ethanol (p<0.0001, Fig. 6A) than the paired control, and that adult-specific RNAi knockdown of *cactus* expression increases resistance (p=0.0091, Fig. 6B). These findings align with the phenotypes seen when Toll pathway activity was stimulated with the *UAS-Toll*^{10B} transgene (Fig. 3C–E).

Dif is one of the three NF-κB family members in Drosophila, and upon activation of the Toll pathway functions in the induction of target genes including antimicrobial peptides (Petersen et al., 1995). *Dif* mutant animals *(Dif*^{1}/*Dif*^{1}*)* are less ethanol resistant than wild-type animals (p<0.0001, Fig. 7A), and RNAi knockdown of *Dif* expression in the adult decreases resistance (p<0.0001, Fig. 7B). Next we used overexpression of a *Dif* transgene to mimic active Toll pathway signaling. We observed that overexpression of *Dif (UAS-Dif*, Yagi & Ip, 2005) leads to increased resistance when driven by the ubiquitous *tubulin-Gal4* driver (p<0.0001 vs. either parental control, Fig. 7C). Resistance is also increased when the neural *Appl-Gal4* driver is used to drive expression of the *UAS-Dif* transgene (p<0.0001 vs. *UAS-Dif* alone, p=0.0005 v *Appl-Gal4* alone, Fig. 7D). However, three day overexpression of *Dif* using the *Actin-GeneSwitch* driver had no effect on ethanol resistance (p=0.4895, Fig. 7E).

dorsal is another NF-κB transcription factor, and while Dif and Dorsal are both stimulated by Toll pathway activation and are redundant in some contexts, they have some distinct roles. *dorsal*, but not *Dif* is required for embryonic development. On the other hand *Dif*

plays the greater role in the adult innate immune system (see Discussion). Nevertheless we also examined the role of *dorsal* on ethanol resistance. When we tested *dorsal* null animals – transheterozygotes carrying a null allele of *dorsal (dorsal*^{1}*)* and a deficiency removing *dorsal (dorsal*^{DF}*)* – the mutants were more resistant than the wild-type control (p=0.0079, Fig. 8A). Knockdown of *dorsal* with an RNAi transgene also led to increased resistance (p<0.0001, Fig. 8B). Conversely, when a *dorsal* transgene (Yagi & Ip, 2005) was driven in the nervous system using the *Appl-Gal4* driver we observed that the animals had reduced resistance to ethanol (p=0.003 vs. *Appl-Gal4* alone, p<0.0001 vs. *UAS-dorsal* alone, Fig. 8C). However, *dorsal* overexpression might have reduced ethanol resistance because the animals are less fit and have physical defects. We observed that these flies had crumpled, unexpanded wings. Furthermore, overexpressing *dorsal* using a *tubulin-Gal4* was lethal. We also observed lethality when the *UAS-dorsal* was combined with the *Actin-GeneSwitch* driver. Lethality occurred even when the animals were raised without inducer at 18°C to minimize expression from the transgenes (Duffy, 2002).

Relish is a third NF-κB family transcription factor in Drosophila. It is primarily activated by the IMD innate immune pathway, which responds to infection by Gram-negative bacteria. We included *Relish* in this study because there is evidence of cross-talk between the Toll and IMD pathways, and because Relish has been shown to heterodimerize with both Dif and Dorsal (Tanji et al., 2010; Dushay et al., 1996; Hedengren et al., 1999). Animals homozygous for the loss-of-function *Relish*^{E38} allele (Hedengren et al., 1999) showed reduced resistance to ethanol (p<0.0001, Fig. 9A). Knockdown of *Relish* in adults using RNAi and the *Actin-GeneSwitch* driver also reduced resistance (p=0.0023, Fig. 9B). In contrast to Dif and Dorsal, where functional regulation is achieved via sequestration by Cactus protein, Relish has an autoinhibitory domain that is cleaved after IMD pathway activation (Stöven et al., 2000). Overexpression of the active form of *Relish (UAS-RelN*, Yagi & Ip, 2005) with *tubulin-Gal4* or *Appl-Gal4* increases resistance (*tubulin-Gal4:* p<0.001, Fig. 9C; *Appl-Gal4:* p<0.0001, Fig. 9D). However, three day overexpression with the GeneSwitch system significantly reduces resistance (p<0.0001, Fig. 9E).

To establish that overexpression of *Toll* is acting through the canonical signaling pathway to increase resistance, we overexpressed a constitutively active *Toll* in a *Dif* mutant background. To do so we generated *Dif*^{1}*/UAS-Toll*^{10B}*; Actin-GeneSwitch/+* animals and fed them RU-486 inducer for three days. Feeding inducer had no effect on resistance, indicating that for the increased resistance response, *Toll* acts through *Dif* (p=0.38, Fig. 10A). This experiment utilized animals heterozygous for the *Dif*^{1} loss-of-function allele, and here we show that animals heterozygous for *Dif*^{1} over a wild-type chromosome are more sensitive to ethanol (p<0.0001, Fig. 10B), mimicking the phenotype of the homozygote reported in Fig. 7A.

The innate immune system is stimulated by ethanol sedation

To determine whether the Drosophila Toll innate immune signaling pathway is activated after ethanol sedation we performed RNA-seq 30 minutes after ethanol sedation. Activation of the Toll pathway results in increased transcription of antimicrobial genes. We observed a change in abundance of 137 mRNAs purified from adult heads (Table S1). This includes

eight antimicrobial peptide genes that represent 38.1% of the microbial peptide genes encoded in Drosophila ($p<0.0001$; Table S1; Hetru et al., 2003). Furthermore, the top two DAVID gene ontology clusters were stress-response genes and innate immune genes (Table S1; Huang et al., 2009b; Huang et al., 2009a). In addition to the enriched subset of innate immune genes, we saw induction of genes linked to heat shock response, stress response, programmed cell death, and calcium sensing. However, there is a dearth of genes whose ontology is neural specific or behavioral specific.

The Toll signaling pathway is not required for animals to acquire 24h ethanol tolerance

The observation that genetic manipulation of the Toll-signaling pathway affects resistance and the observation that ethanol sedation activates the Toll-signaling pathway led us to hypothesize that Toll signaling might be a trigger for the production of ethanol tolerance. Ethanol tolerance is a reduced response to an effect of ethanol caused by prior ethanol exposure (ethanol-induced ethanol resistance). We tested for the capacity to acquire ethanol tolerance by comparing the rate of recovery from ethanol sedation in animals that are recovering from their first ethanol sedation to the rate of recovery of animals recovering from their second ethanol sedation (24h between first and second sedation; Cowmeadow 2005). The activity of the Toll pathway was manipulated in the same way in both first and second sedation animals. We activated the Toll pathway using the constitutively active *Toll* receptor (*Actin-GeneSwitch* driven expression of *UAS-Toll*^{10B}) or blocked signaling from the pathway with loss-of-function mutations in the *Dif* transcription factor. Whereas both manipulations altered innate ethanol resistance, neither affected the capacity to acquire 24h tolerance (Supplementary Fig. S2).

Discussion

In this study we examined resistance to ethanol sedation because baseline resistance to the effects of ethanol can be used as a real world predictor of drinking problems in humans. In a 25 year study, the baseline resistance of college-aged participants was a strong predictor of alcohol use disorders later in life (Schuckit & Smith, 2011). An individual's level of response to alcohol has been shown to have a strong genetic component (Heath et al., 1999; Schuckit et al., 2004). Individuals who have a lower response to alcohol have to drink more to experience the pleasurable effects of alcohol and can also drink longer. As a result, they expose themselves to higher levels of alcohol, which in turn promotes addiction and increases their risk for alcohol toxicity. Here we show that the Toll innate-immune signaling pathway can profoundly influence resistance to ethanol sedation in adult Drosophila.

Consistent with the hypothesis that ethanol sedation rapidly promotes signaling down the Toll innate immune signaling pathway, 30 minutes after ethanol sedation, we observed increased expression of a number of antimicrobial peptide genes—these genes are outputs of innate immune signaling pathways. Eight of the 21 canonical antimicrobial peptide genes were upregulated. Previously, Kong et al. (2010) reported that the genes encoding core members of the innate immune signaling pathways, *Toll, Myd88, cactus, Imd,* and *Relish*, are induced less than two fold about 90 minutes after the start of ethanol exposure and return to baseline within three hours. We did not observe induction of these pathway genes in our

analysis and Kong et al. only reported *dro5* antimicrobial peptide gene induction. This difference may be because we did not accept changes that were less than two fold, or because of differences in the treatment protocol. Stimulation of the signaling pathway and induction of expression of the signaling pathway genes are fundamentally distinct events. However, upregulation of the pathway genes themselves is also interesting because it could sensitize this pathway to future ethanol or inflammatory stimuli and might contribute to acute tolerance (see below).

Every member of the Toll signaling pathway that we tested had an effect on resistance to ethanol in at least one paradigm. Suppressing Toll pathway signaling by mutation or by knockdown of pathway members decreases resistance to ethanol sedation, while increasing Toll pathway activity increases resistance to ethanol sedation (Table 1) with one notable exception — *dorsal*. Whereas, the effects of manipulating *Dif* expression fit the model that increased and decreased Toll pathway activity increases and decreases ethanol resistance, respectively, the manipulation of *dorsal* produced the opposite result. Overexpression of *dorsal*, however, also caused developmental defects affecting adult fitness while overexpression of *Toll*, *Dif*, or *Relish* did not obviously affect fitness. Furthermore, there is strong evidence that *Dif* and *dorsal* play distinct roles in Drosophila embryos and adults (Rutschmann et al., 2000; Lemaitre et al., 1996; Lemaitre et al., 1995; Gross et al., 1996; Meng et al., 1999). An absence of Dorsal protein in the embryo lethally disrupts dorsal-ventral patterning while the absence of Dif protein does not perturb embryogenesis. Whereas, in the innate immune system *Dif* is a strong regulator of antifungal genes, *dorsal* is not required for normal immune function. For instance, *dorsal* mutations do not affect the induction of *Drosomycin* after infection, while *Dif* mutants show a substantial reduction in the ability to induce *Drosomycin* after infection (Rutschmann et al., 2000; Lemaitre et al., 1996). In Drosophila, the fat body is a major hub of immune signaling activity, and fat body explants have been used to assay Toll and IMD pathway activity after exposure to various pathogens and pathogen components. In dissected fat bodies, the nuclear translocation of Dif protein can be stimulated by bacterial coat components, but the movement of Dorsal into the nucleus requires components of the hemolymph (Bettencourt et al., 2004). In at least one context Dorsal was shown to have the opposite effect of Dif. In the case of *Cecropin* transcriptional control, Dorsal was shown to suppress gene activation by Dif, reducing expression of a *Cecropin* reporter construct when co-expressed with Dif (Petersen et al., 1995). Finally, mutations that constitutively activate Toll signaling, such as $Toll^{10B}$ or *cactus* loss-of-function alleles, cause the formation of melanotic tumors in larvae. *dorsal* is not involved in this process, as null mutations in *dorsal* do not block the appearance of tumors (Lemaitre et al., 1995). Together these studies show that *dorsal* and *Dif* have distinct and sometimes opposing roles, and our results extend these observations to include effects on ethanol resistance.

Our experiments using *Actin-GeneSwitch* to drive overexpression of either *Dif* or *Relish* yielded results that did not match the results obtained when the UAS transgene was driven by *tubulin-Gal4* or *Appl-Gal4. Dif* overexpression led to resistance when driven by the two Gal4 drivers, but had no effect when driven by the *Actin-GeneSwitch* driver, and *Relish* overexpression driven by *Actin-GeneSwitch* decreased resistance, as opposed to increased resistance when driven by the Gal4 constructs. This conflict may result from poor

GeneSwitch induction of the *Dif* and *Rel* transgenes or may indicate that the overexpression phenotype for these transcription factors has a developmental component.

The observations that Toll signaling modulates resistance, and that the innate immune system is rapidly activated by ethanol exposure led us to speculate that the Toll pathway might be involved in the generation of ethanol rapid tolerance (rapid tolerance has been defined as the tolerance manifest after ethanol clearance). However, in our experiments perturbation of Toll pathway activity did not affect the ability to acquire 24h rapid tolerance, indicating that the Toll pathway is not necessary for producing 24h rapid alcohol tolerance despite the fact that modulating its activity affects ethanol resistance. This is not unusual in that mechanisms that produce resistance and rapid tolerance have been shown to be distinct before. Genes that contribute to resistance are not necessarily required for the acquisition of rapid tolerance, and genes necessary for the acquisition of rapid tolerance do not always affect baseline resistance. For instance, measurement of the magnitude of resistance and rapid tolerance in 205 inbred, sequenced Drosophila lines did not show a correlation between the magnitudes of resistance and rapid tolerance (see Fig.1 in Morozova et al., 2015). Furthermore, Drosophila experiments describing circadian fluctuation in ethanol-induced behaviors also exposed a disconnect between resistance and rapid tolerance. In a loss of righting reflex assay, baseline resistance oscillated in a circadian manner, peaking in the early evening, but rapid tolerance did not oscillate—the magnitude of rapid tolerance was the same regardless of time of day (van der Linde & Lyons, 2011). Although the innate immune signaling pathway does not appear to have a role in producing 24h rapid tolerance, our data predicts that it may contribute to the production of a transient form of tolerance called acute tolerance (defined as tolerance that appears during a drug experience). Expression data suggests that ethanol causes a sudden activation of innate immune signaling. Because increased Toll activity increases resistance, we would expect animals to become ethanol resistant during immune activation. This increase in resistance (acute tolerance) is perhaps later subsumed by a distinct mechanism responsible for 24h rapid tolerance.

Our findings mesh well with how Drosophila interact with ethanol in their natural environment. The findings in Milan *et al.* (2012) demonstrate that Drosophila seek ethanol-containing food after becoming infected by parasitic wasps, and that ethanol consumption helps kill the invading wasp. In order to promote maximum fitness, an animal that is driven to self medicate with ethanol might be expected to increase its resistance to the sedative effects of the drug, lest it become intoxicated and become easy prey. It has been shown that infection by endoparasitic wasps activates the Toll Pathway, so perhaps increasing resistance to ethanol arose as an adaptive response in anticipation of ethanol consumption. While it has not been shown that the Drosophila Toll pathway modulates drinking in the adult fly, in mammals innate immune signaling through pathways related to Drosophila Toll such as IL-1 and TLR4 have been shown to increase drinking (Robinson et al., 2014). Here we have shown that in Drosophila the innate immune system regulates how the animal responds to ethanol sedation, and that innate immune signaling is rapidly induced by ethanol exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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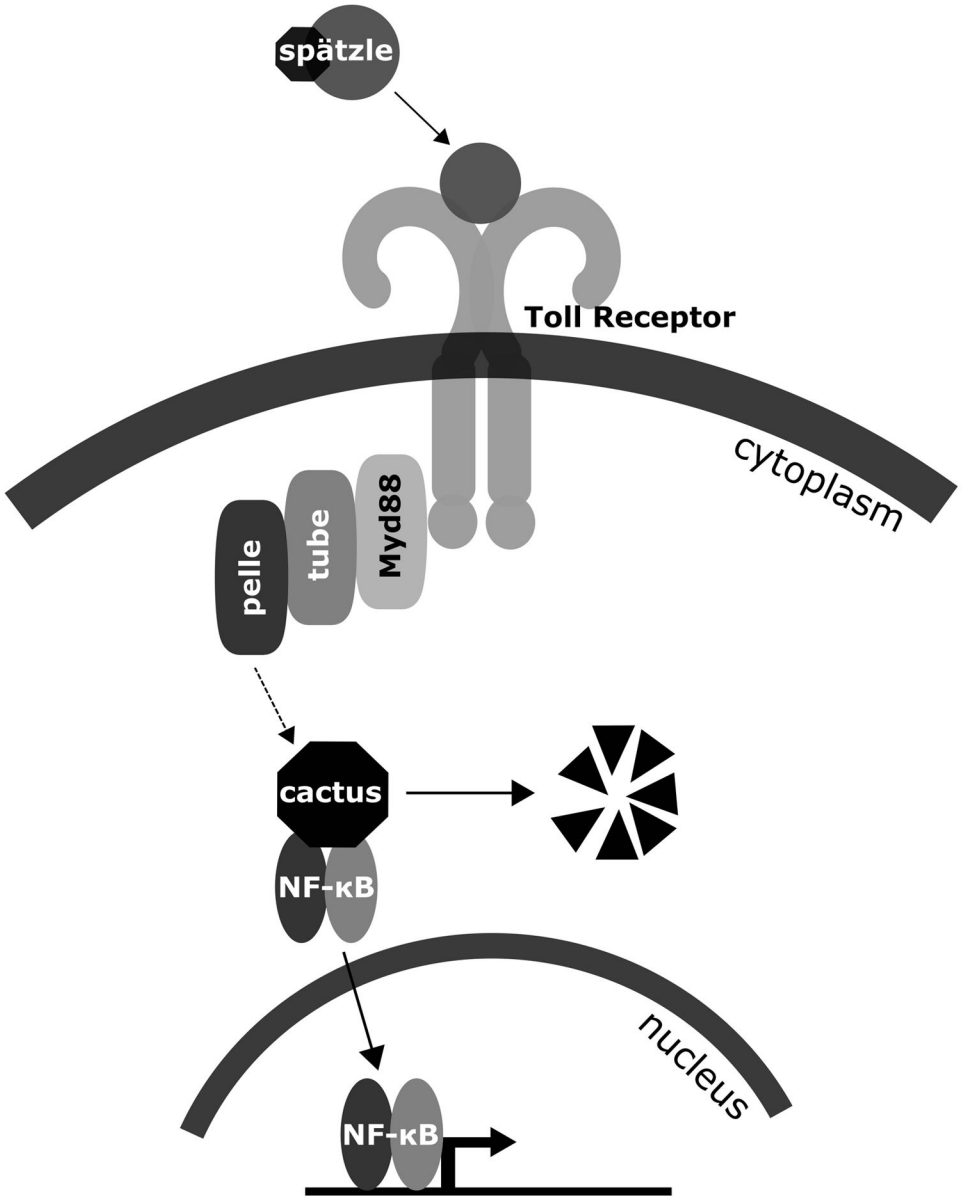

Figure 1.

Schematic diagram of the interacting proteins in the Toll signaling pathway. An inactive Spätzle precursor is cleaved after infection and binds to the Toll receptor. Upon Toll pathway activation, Myd88, Tube, and Pelle associate with Toll, and Cactus is phosphorylated. Phosphorylation of Cactus leads to degradation of Cactus at the proteasome, which releases the inhibition of NF-κB proteins. Once released, NF-κB transcription factor family members (Dif, Dorsal) enter the nucleus and can regulate target genes.

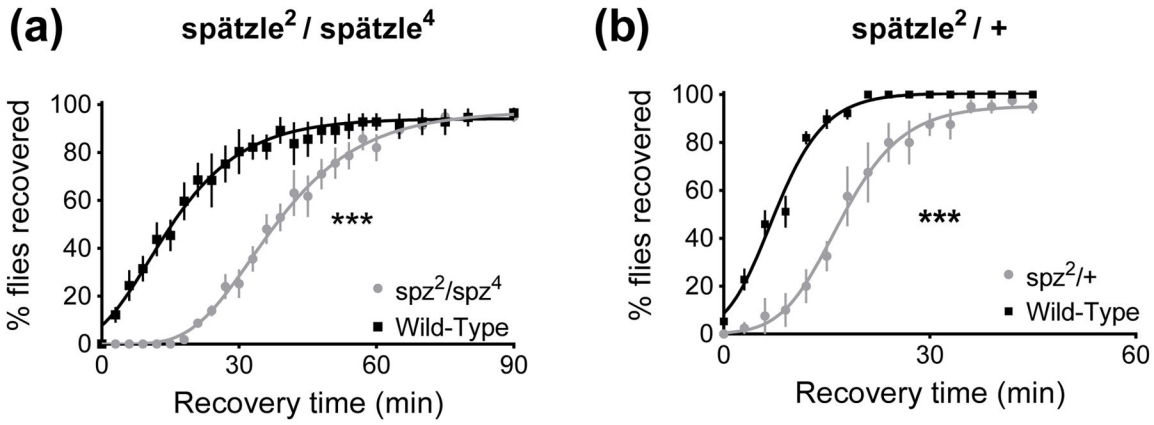

Figure 2.

spätzle mutants show reduced resistance to ethanol in a recovery from ethanol sedation assay. Age-matched females are placed in vials and exposed to ethanol vapor until sedated, and then animals are allowed to recover in a humidified air stream. 0 minutes denotes the beginning of the recovery. A) *spätzle*-null transheterozygotes recover from sedation more slowly than Canton S wild-type control animals (***, $p<0.0001$). B) Heterozygotes carrying a null allele of *spätzle* and a wild type chromosome recover from sedation more slowly than Canton S wild-type control animals (***, $p<0.0001$).

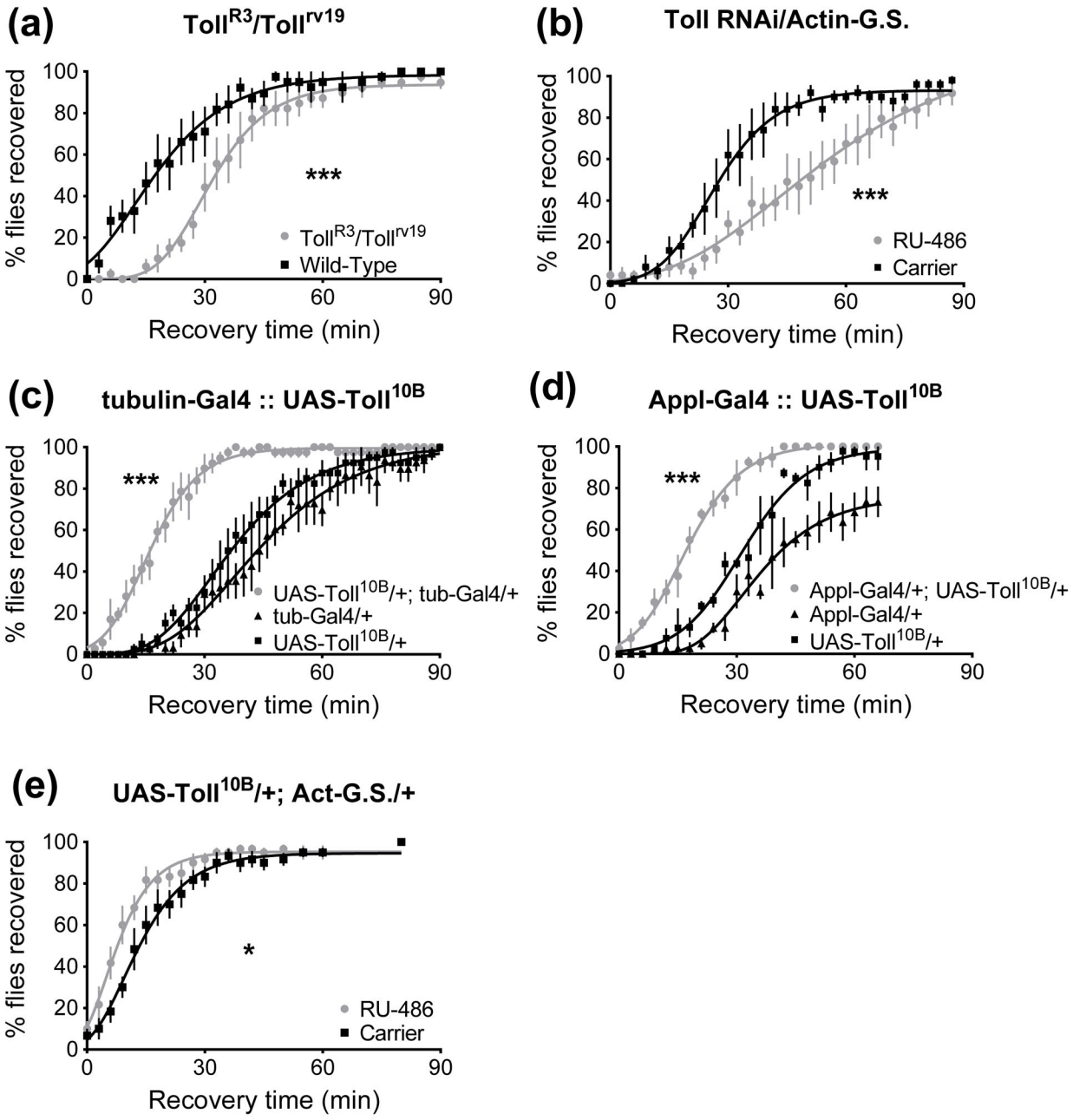

Figure 3.

A *Toll* loss-of-function mutation or a *Toll* knockdown decreases resistance, while expression of a constitutively active *Toll* increases resistance in a recovery from ethanol sedation assay. 0 minutes denotes the beginning of the recovery period. A) Transheterozygous *Toll^{R3}/Toll^{rv19}* loss-of-function animals recover more slowly than wild-type Canton S (***, p<0.0001). B) RNAi knockdown of *Toll* via a UAS transgene driven by *Actin-GeneSwitch*, in which the GeneSwitch inducer was provided for three days prior to testing. Inducer-fed animals (RU-486) recovered more slowly than carrier-fed controls (***, p<0.0001). C) Overexpression of the constitutively active *Toll^{10B}* allele using the ubiquitous *tubulin-Gal4*

driver increased resistance (***, p<0.0001 *vs.* either parental control). D) Overexpression of *Toll*^{10B} in neurons using the *Appl-Gal4* driver increased resistance (***, p<0.0001 *vs.* either parental control). E) Inducible overexpression of *Toll*^{10B} only in adults increased resistance (*, p=0.03).

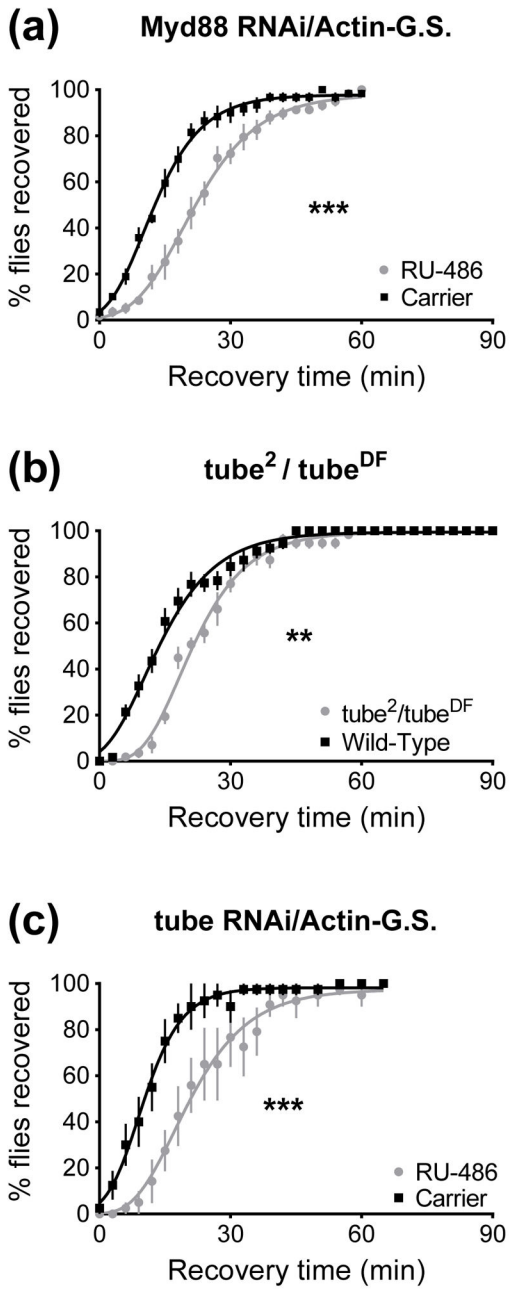

Figure 4.

Loss of *Myd88* or *tube* reduces resistance in a recovery from ethanol sedation assay. 0 minutes denotes the beginning of the recovery period. A) When an RNAi transgene is expressed in the adult to knock down *Myd88* expression, resistance to ethanol is reduced compared to carrier-fed controls (***, $p<0.0001$). B) *tube* null transheterozygotes recover from sedation more slowly than wild type flies (**, $p=0.0013$). C) Knockdown of *tube* in the adult reduces resistance (***, $p<0.0001$).

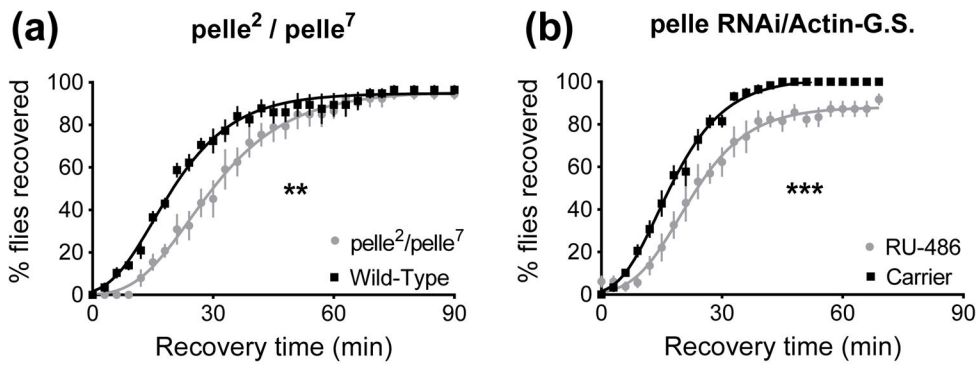

Figure 5.

Loss of *pelle* via mutation or knockdown reduces resistance in a recovery from ethanol sedation assay. 0 minutes denotes the beginning of the recovery period. A) *pelle* loss-of-function transheterozygotes recover from sedation more slowly than wild type flies (**, p=0.0058). B) Knockdown of *pelle* in the adult reduces resistance (***, p=0.0008).

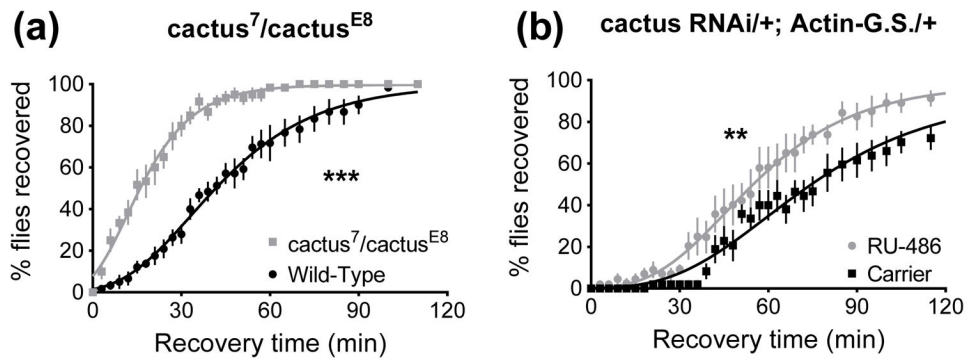

Figure 6.

Loss of *cactus* increases resistance in a recovery from ethanol sedation assay. 0 minutes denotes the beginning of the recovery period. A) Transheterozygous *cactus* loss-of-function animals (*cactus*^{7}/*cactus*^{E8}) are more resistant to ethanol than wild type (***, p<0.0001). B) RNAi knockdown of *cactus* increases resistance to ethanol (**, p=0.0091). In A and B all animals recovered.

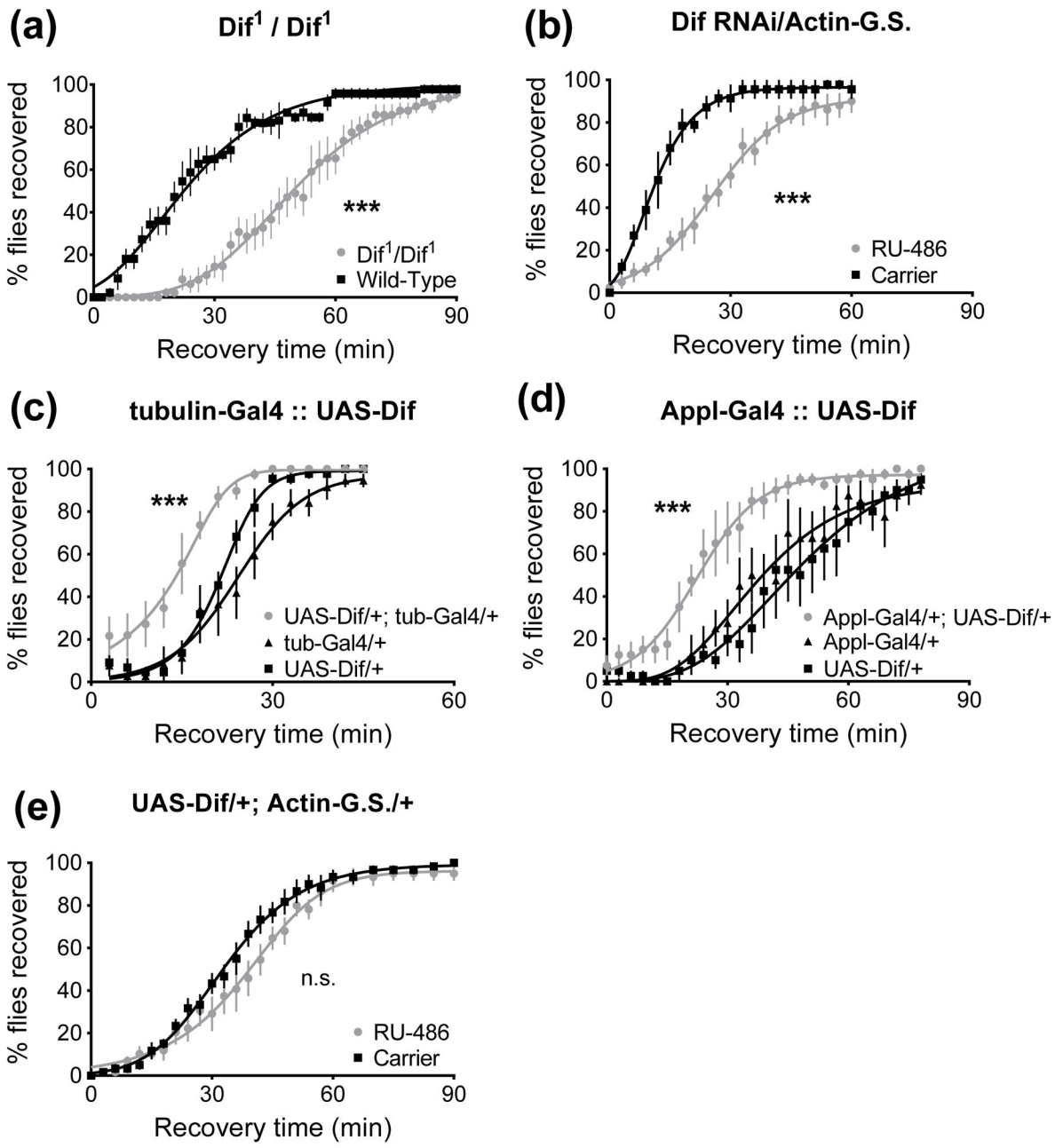

Figure 7.

Reduction of *Dif* reduces resistance, and overexpression of *Dif* can increase resistance as measured in a recovery from ethanol sedation assay. 0 minutes denotes the beginning of the recovery period. A) *Dif* mutant animals show reduced resistance (***, p<0.0001), as seen by their slower recovery from sedation. B) Three day knockdown of *Dif* using the inducible GeneSwitch system leads to decreased resistance (***, p<0.0001). C) Overexpression of a *Dif* transgene with the ubiquitous *tubulin-Gal4* driver increases resistance (***, p<0.0001 vs. either parental). D) Overexpression of *Dif* in neurons increases ethanol resistance (***,

p<0.0001 vs. *UAS-Dif* alone, p=0.0005 v *Appl-Gal4* alone). E) Three day overexpression of *Dif* using the GeneSwitch system had no effect on alcohol resistance (p=0.4895).

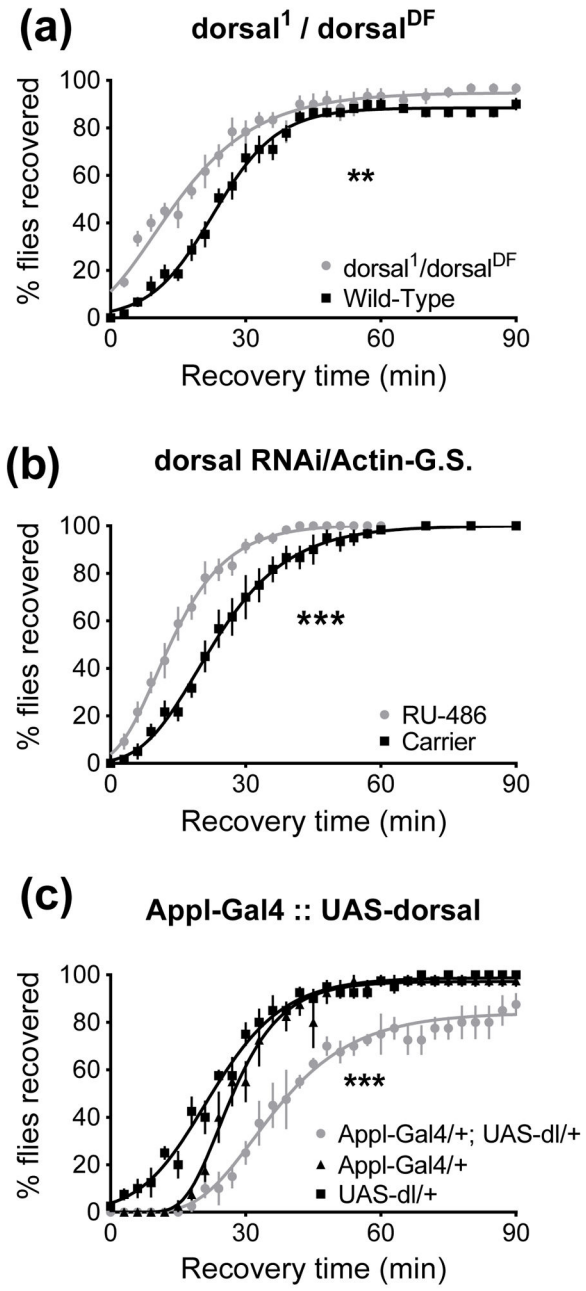

Figure 8.

Loss of *dorsal* increases resistance, while overexpression of *dorsal* decreases resistance in a recovery from ethanol sedation assay. 0 minutes denotes the beginning of the recovery period. A) *dorsal*-null animals carrying a *dorsal* null allele and a deficiency uncovering *dorsal* are more resistant than wild type animals (**, p=0.0079). B) Knockdown of *dorsal* expression in the adult increases resistance (***, p<0.0001). C) Overexpression of a *UAS-dorsal* transgene in neurons caused decreased resistance (***, p=0.003 vs. *Appl-Gal4* alone, p<0.0001 vs. *UAS-dorsal* alone).

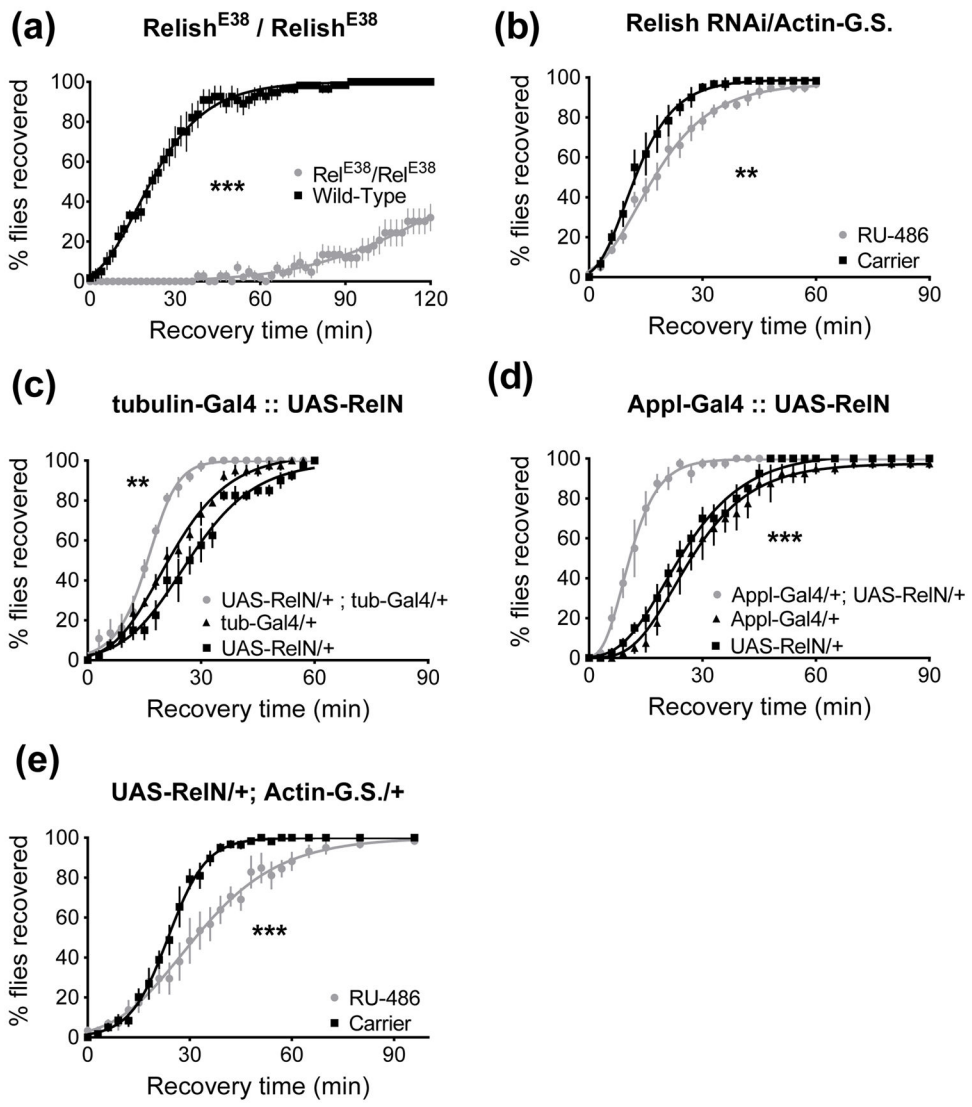

Figure 9.

Relish affects resistance to ethanol in a recovery from ethanol sedation assay. 0 minutes denotes the beginning of the recovery period. A) Homozygous loss-of-function Rel^{E38} animals recover more slowly than wild type animals (***, p<0.0001). All animals eventually recover, this graph is clipped. B) Three day RNAi knockdown of *Relish* using *Actin-GeneSwitch* reduces resistance (**, p=0.0023). C) Expression of the N-terminal active domain of *Relish (RelN)* using the *tubulin-Gal4* driver increases resistance (**, p<0.0001 vs. *UAS-RelN* alone, p=0.001 vs. *tubulin-Gal4* alone). D) Overexpression of *RelN* in the nervous system increases resistance (***, p<0.0001 vs. either parental). E) Three day overexpression of *RelN* using *Actin-GeneSwitch* reduces resistance (***, p<0.0001).

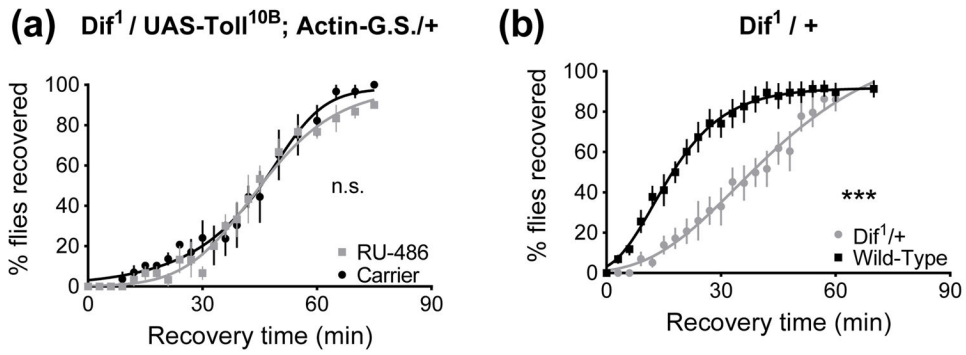

Figure 10.

Increased *Toll* activity produces ethanol resistance in a *Dif*-dependent manner. 0 minutes denotes the beginning of the recovery period. A) A Dif^{1} mutation blocks the effect of overexpressing constitutively active $UAS\text{-}Toll^{10B}$ using *Actin-GeneSwitch* (p=0.38). Overexpressing $Toll^{10B}$ with functional *Dif* increases resistance (see Fig. 3E). B) *Dif* is haploinsufficient with respect to ethanol resistance: Dif^{1} heterozygotes are less resistant to ethanol sedation (p<0.0001). The animals in Fig. 10A are heterozygous for Dif^{1}, whereas the Dif^{1} mutants reported in Fig. 7A were homozygous.

Table 1

Summary of Behavior Experiments

			Overexpression		
Gene	**Mutant Allele**	**Inducible RNAi**	**Tub-Gal4**	**Appl-Gal4**	**Actin-G.S.**
spätzle	Sensitive				
Toll	Sensitive	Sensitive	Resistant	Resistant	Resistant
Myd88		Sensitive			
tube	Sensitive	Sensitive			
pelle	Sensitive	Sensitive			
cactus	Resistant	Resistant			
Dif	Sensitive	Sensitive	Resistant	Resistant	n.s.
dorsal	Resistant	Resistant	Lethal	Sensitive	Lethal
Relish	Sensitive	Sensitive	Resistant	Resistant	Sensitive