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Happyhour, a Ste20 Family Kinase, Implicates EGFR Signaling in Ethanol-Induced Behaviors

Ammon B. Corl,[1] Karen H. Berger,[3] Galit Ophir-Shohat,[2] Julie Gesch,[3] Jeffrey A. Simms,[3] Selena E. Bartlett,[3] and Ulrike Heberlein[1,2,3,*]
[1]Program in Neuroscience
[2]Department of Anatomy
University of California, San Francisco, San Francisco, CA 94143-2822, USA
[3]Ernest Gallo Research Center, Emeryville, CA 94608, USA
*Correspondence: ulrike.heberlein@ucsf.edu
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SUMMARY

The consequences of alcohol use disorders (AUDs) are devastating to individuals and society, yet few treatments are currently available. To identify genes regulating the behavioral effects of ethanol, we conducted a genetic screen in *Drosophila* and identified a mutant, *happyhour* (*hppy*), due to its increased resistance to the sedative effects of ethanol. Hppy protein shows strong homology to mammalian Ste20 family kinases of the GCK-1 subfamily. Genetic and biochemical experiments revealed that the epidermal growth factor (EGF)-signaling pathway regulates ethanol sensitivity in *Drosophila* and that Hppy functions as an inhibitor of the pathway. Acute pharmacological inhibition of the EGF receptor (EGFR) in adult animals altered acute ethanol sensitivity in both flies and mice and reduced ethanol consumption in a preclinical rat model of alcoholism. Inhibitors of the EGFR or components of its signaling pathway are thus potential pharmacotherapies for AUDs.

INTRODUCTION

Alcohol (ethanol) is one of the most popularly consumed and abused drugs in the world. Its pleasurable and disinhibiting effects have been enjoyed by humankind for thousands of years. For some, however, alcohol consumption leads to addiction, a devastating illness with enormous medical and societal costs. A better understanding of the genetic and environmental factors that contribute to the development of alcohol use disorders (AUD) would, therefore, provide considerable benefits to those who suffer its consequences and to society. Although the cognitive and behavioral changes associated with alcohol consumption are familiar to many, our knowledge concerning the mechanisms through which ethanol acts in the central nervous system to produce these effects is still far from complete. Rather than acting on a single molecular target, ethanol affects the function of multiple targets—most commonly, voltage- and ligand-gated ion channels (Diamond and Gordon, 1997; Lovinger, 1997). Studies of genetically engineered mice have provided further insight into molecules that regulate the behavioral response to ethanol in vivo, demonstrating roles for serotonin, dopamine, and cannabinoid systems, as well as several signal transduction pathways (Crabbe et al., 2006).

Family, adoption, and twin studies strongly support a genetic component to alcoholism, although identifying specific genes underlying alcoholism has proved difficult (Reich et al., 1999; Dick et al., 2004; Edenberg et al., 2004; Schuckit et al., 2004). Human studies also indicate that the level of response to intoxicating doses of ethanol acts as a predictor of future alcoholism, with a lower initial response correlated with increased risk (Schuckit, 1994; Schuckit and Smith, 1996). Thus, the identification of genes and pathways mediating acute responses to ethanol promises to offer insight into the genetic factors contributing to the much more complex process of addiction.

The fruit fly, *Drosophila melanogaster*, has proven to be a useful model system in which to study the genes and pathways that mediate acute and chronic behavioral responses to ethanol (Guarnieri and Heberlein, 2003). Upon acute ethanol exposure, flies exhibit behaviors similar to those observed in mammals: low ethanol doses result in hyperactivity, whereas higher doses cause decreased activity and eventual loss of postural control and sedation (Singh and Heberlein, 2000; Wolf et al., 2002). Unbiased genetic approaches and candidate gene analyses have provided insight into various molecules and biochemical pathways that regulate the ethanol response in *Drosophila* (Moore et al., 1998; Park et al., 2000; Corl et al., 2005; Wen et al., 2005; Rothenfluh et al., 2006) as well as the responsible neuroanatomical loci (Rodan et al., 2002; Urizar et al., 2007). Several of the molecules implicated in ethanol-related behaviors in *Drosophila*, such as protein kinase A (PKA), calcium-sensitive adenylate cyclase (Moore et al., 1998), and the fly ortholog of neuropeptide Y, NPF (Wen et al., 2005), have been shown to have similar roles in mammals (Thiele et al., 2000, 2002; Maas et al., 2005), corroborating *Drosophila* as a valuable model.

To identify molecules and pathways regulating the behavioral response to ethanol, we conducted a genetic screen for

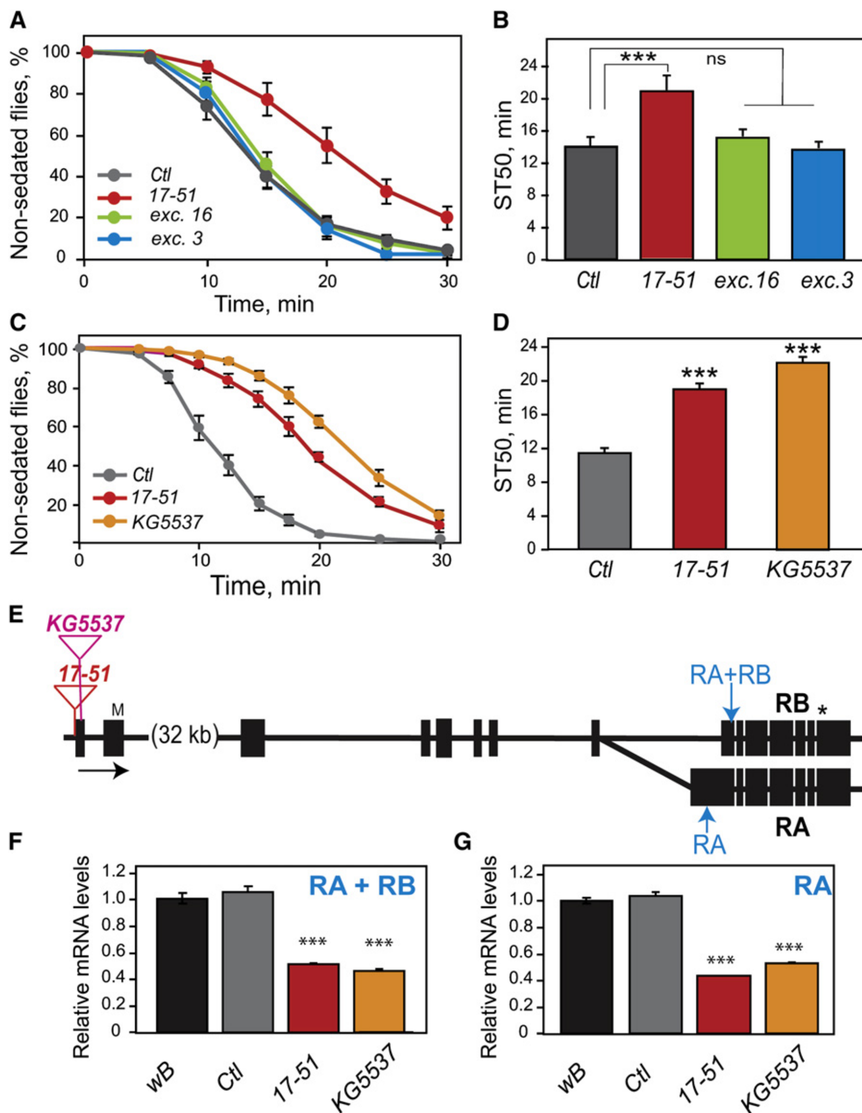

Figure 1. *hppy* Mutants Display Increased Resistance to Ethanol Sedation

(A) *hppy*$^{17\text{-}51}$ flies show increased resistance to ethanol-induced sedation in the loss of righting (LOR) assay, and precise excision of the *P* element in *hppy*$^{17\text{-}51}$ (exc. 3 and 16) reverted the sedation resistance phenotype. Ethanol exposure (110/40 E/A) commenced at 0 min and was continuous thereafter (n = 8).

(B) The median sedation time (ST50)—the time required for half of the ethanol-exposed flies to show LOR—was calculated by linear interpolation.

(C and D) *hppy*^{KG5537} flies also showed increased resistance to ethanol-induced sedation. (C) Sedation profiles and (D) ST50 values were calculated for *hppy*$^{17\text{-}51}$, control, and *hppy*^{KG5537}. Error bars represent SEM, and asterisks denote statistical significance by one-way ANOVA followed by post hoc Newman-Keuls testing. n = 8–12.

(E) Diagram of the *hppy* transcription unit, with exons represented as boxes. M indicates the translation start site, and an asterisk indicates the stop codon. Blue arrows indicate regions amplified for QPCR analysis. Structures of the two transcripts, *hppy-RA* and *hppy-RB*, and the insertion sites of *hppy*$^{17\text{-}51}$ and *hppy*^{KG5537} are diagrammed.

(F and G) Expression of *hppy* is reduced in *hppy* mutants. RNA was isolated from whole flies and subjected to QPCR. Similar results were obtained with mRNA isolated from heads and bodies separately (data not shown). Relative mRNA levels are expressed as fold difference relative to *w* Berlin (*wB*) control RNA. QPCR on whole-fly mRNA using a primer/probe set recognizing both *hppy-RA* and *hppy-RB* transcripts (F) or only the *hppy-RA* transcript (G) is shown. One-way ANOVA with post hoc Newman-Keuls tests revealed a significant difference between *hppy*$^{17\text{-}51}$ and both *wB* and Ctrl and between *hppy*^{KG5537} and both controls. n = 3.

***$p < 0.001$.

Drosophila mutants with altered sensitivity to ethanol's sedative effects. Here, we describe the identification and characterization of mutants in the *happyhour* (*hppy*) gene, which exhibit a marked resistance to ethanol-induced sedation. A series of genetic, behavioral, and biochemical experiments suggest that *hppy* modulates ethanol sedation by regulating EGFR signaling in the nervous system. These experiments also strongly suggest that *hppy* acts as an inhibitor of the EGFR pathway. Finally, acute pharmacological inhibition of the EGFR significantly perturbed ethanol sensitivity in both adult flies and mice and reduced ethanol consumption in rats.

RESULTS

hppy Mutants Display Increased Resistance to Ethanol-Induced Sedation

To identify molecules mediating the sedative effects of ethanol in *Drosophila*, we screened a collection of strains carrying random insertions of the P{GawB} transposable element by using a locomotor tracking device (Wolf et al., 2002). When exposed to a relatively high concentration of ethanol (see Supplemental Experimental Procedures available online), *Drosophila* exhibit a transient startle response to the smell of ethanol, followed by a decrease in locomotor activity associated with gradual loss of postural control and, finally, akinesis (sedation) (Wolf et al., 2002). We isolated one mutant, line 17-51, that displayed increased resistance to ethanol-induced sedation (Figure S1A). Direct observation of sedation responses using a modified loss-of-righting (LOR) assay (Rothenfluh et al., 2006) confirmed that 17-51 flies displayed marked resistance to ethanol-induced sedation (Figures 1A and 1B), a phenotype evident at all ethanol concentrations tested (Figure S2). This was not simply due to altered ethanol pharmacokinetics, as ethanol absorption was normal in 17-51 flies (Figure S1B). In addition, 17-51 flies showed normal locomotor behavior and negative geotaxis (Figure S3 and data not shown).

Inverse PCR and DNA sequencing analysis revealed that the P{GawB} element in 17-51 is inserted in the gene CG7097, affecting its expression (see below). We decided to name the gene *happyhour* (*hppy*) because its mutation results in flies being able to imbibe significantly more alcohol than wild-type controls before succumbing to its sedating effects. The transposon inserted in *hppy*$^{17\text{-}51}$ is responsible for the sedation resistance, as precise excisions of the element reverted the mutant phenotype (Figures 1A and 1B). Database searches (http://www.flybase.org) identified a second *P* element insertion in CG7097, KG5537. When tested in the LOR assay, this strain, *hppy*^{KG5537}, also showed increased resistance to ethanol-induced sedation (Figures 1C and 1D). In addition, *hppy*$^{17\text{-}51}$ and *hppy*^{KG5537} failed to complement each other's ethanol sedation phenotypes (data not shown).

Molecular Characterization of the *hppy* Locus and *hppy* Mutants

The *hppy* (CG7097) locus covers ~48.5 kb and encodes two transcripts, *hppy-RA* and *hppy-RB* (Figure 1E; http://www.flybase.org). Both transcripts, generated by alternative splicing of the eighth intron, share the same transcription start site, but the longer 5.1 kb *hppy-RA* transcript contains an additional ~800 bp in its ninth exon, not included in *hppy-RB*. Both *hppy* mutants harbor *P* element insertions in the 5′ gene region. In *hppy*$^{17\text{-}51}$, the transposon is inserted 10 bp upstream of the transcription start site; the *hppy*^{KG5537} transposon is inserted in the first noncoding exon (Figure 1E). The *hppy-RA* and *hppy-RB* transcripts are predicted to encode proteins with an N-terminal serine/threonine kinase domain and a citron-homology domain near the C terminus. The closest mammalian homologs of Hppy are members of the germinal center kinase-1 (GCK-1) family of Ste20-related kinases, including GLK (germinal center-like kinase) and GCK itself (Dan et al., 2001; Findlay et al., 2007). GCK-1 family members have been shown previously to act as mitogen-activated protein kinases (MAPKs) upstream of the Jun N-terminal kinase (JNK)-signaling pathway (Chen and Tan, 1999), although studies in *Drosophila* cell culture have failed to show such a role for CG7097 (*hppy*) (Findlay et al., 2007).

To determine how these mutations affect *hppy* expression, we measured *hppy* transcript levels in the mutant and control strains by quantitative RT-PCR (QPCR). Using a primer and probe set recognizing both *hppy-RA* and *-RB* transcripts, we examined *hppy* expression in adult flies and during development. The relative expression of *hppy* in the *hppy*$^{17\text{-}51}$ and *hppy*^{KG5537} mutants was reduced to approximately half that of controls in adult flies (Figure 1F); relative *hppy* levels were also decreased in *hppy* mutant flies during development (Figure S4). A similar reduction in *hppy* expression was seen in the mutants with a primer and probe set recognizing specifically the *hppy-RA* transcript (Figure 1G). In summary, we have identified two mutations in the CG7097/*hppy* locus that share an increased resistance to ethanol-induced sedation and show reduced levels of *hppy* transcripts.

Neuronal *hppy* Expression Is Sufficient for Normal Ethanol Sensitivity

To conclusively demonstrate that the increased sedation resistance observed in *hppy* mutants was due to decreased *hppy* expression, we tested rescue of the mutant phenotype by expressing a *UAS-hppy* transgene in *hppy* mutants. We generated a *UAS-hppy*^{RB} construct by inserting the *hppy-RB* cDNA into the pUAST vector (see Supplemental Experimental Procedures) and introduced this transgene into *hppy*$^{17\text{-}51}$ homozygous mutant flies. The *hppy*$^{17\text{-}51}$ P[GAL4] insertion drives widespread GAL4 expression in many tissues, including the central nervous system, as visualized with a *UAS-green fluorescent protein* (*UAS-GFP*) reporter (Figures 2A and 2B). *hppy*$^{17\text{-}51}$ homozygous mutant flies carrying the *UAS-hppy*^{RB1} transgene showed increased expression of specifically *hppy-RB* (QPCR) (Figures S5A and S5B). When tested in the LOR assay, these flies displayed normal sedation sensitivity (Figure 2C), indicating complete rescue of the mutant phenotype by *hppy-RB* expression. Partial rescue was achieved with a second, more weakly expressed insertion of *UAS-hppy*^{RB} (Figures S5C and S5D). Importantly, introduction of the *UAS-hppy*^{RB} transgene into the *hppy*^{KG5537} homozygous mutant, in which GAL4 is not expressed, did not rescue the *hppy*^{KG5537} sedation resistance phenotype (Figure 2D). These data confirm that the reduction in *hppy* expression is responsible for the resistance to ethanol-induced sedation observed in *hppy* mutant flies.

To determine whether expression of *hppy* specifically in the nervous system was sufficient to restore normal ethanol-induced sedation to *hppy* mutants, we expressed the *UAS-hppy*^{RB1} transgene in neurons using the *elav-GAL4*^{c155} driver in *hppy*^{KG5537} homozygous flies. Neuronal expression of *hppy-RB* completely rescued the sedation resistance of *hppy*^{KG5537} (Figure 2E). Conversely, expressing the *UAS-hppy*^{RB1} transgene under the control of *elav-GAL4*^{c155} in an otherwise wild-type background caused increased sensitivity in the LOR assay (Figure 2F). Thus, *hppy* functions in neurons to control ethanol-induced sedation, and the pathway whose function is regulated by *hppy* can operate bidirectionally to enhance or suppress the response to the sedating effects of ethanol.

JNK Signaling Does Not Regulate Ethanol-Induced Sedation

Because previous work had shown that a human homolog of *hppy*, GCK, acts as a MAP4K in the JNK pathway (Pombo et al., 1995; Dan et al., 2001), we investigated whether perturbation of the JNK pathway in *Drosophila* would alter ethanol sensitivity (Figure S6). Panneuronal expression of various transgenes that activate or inhibit the JNK pathway did not alter ethanol sensitivity. For example, flies expressing a constitutively activated form of the JNKK *hemipterous* or a dominant-negative form of the JNK homolog *basket*, showed wild-type ethanol sensitivity (Figures S6A and S6B). Similarly, neuronal manipulations of the JNK pathway transcription factor dJUN, through overexpression of wild-type or a dominant-negative form, failed to affect ethanol-induced sedation (Figures S6C and S6D). We also tested the effects of perturbing the p38 pathway, with equally negative results (Figure S6E and data not shown).

EGFR/ERK Signaling Regulates Ethanol Sensitivity

Because manipulations of the JNK and p38 pathways failed to alter ethanol sensitivity, we tested the role of the extracellular signal-regulated protein kinase (ERK) pathway. Specifically, we

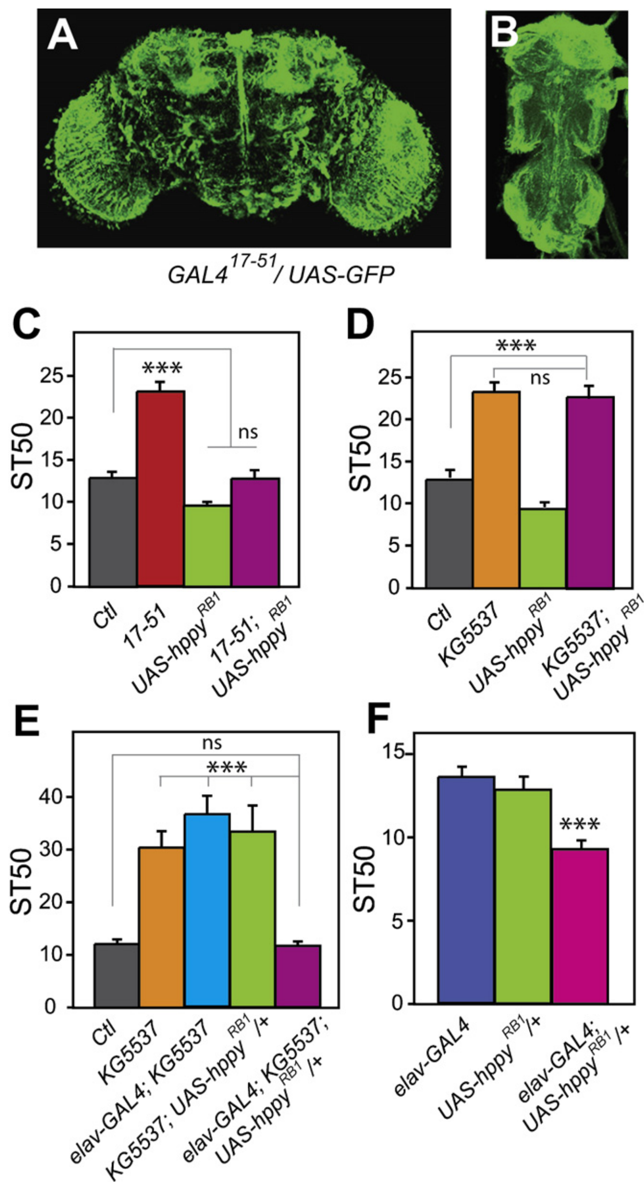

Figure 2. Phenotypic Rescue and Overexpression of *hppy*

(A and B) The $hppy^{17\text{-}51}$ GAL4 expression pattern is widespread in the fly CNS. Pictured is an image of the adult brain (**A**) and ventral nerve cord (**B**) of a fly harboring $hppy^{17\text{-}51}$ and *UAS-GFP*.

(C) The $hppy^{17\text{-}51}$ sedation resistance can be rescued by expression of the *UAS-*$hppy^{RB1}$ transgene in the $hppy^{17\text{-}51}$ homozygous mutant background. A significant difference was observed between $hppy^{17\text{-}51}/hppy^{17\text{-}51}$;*UAS-*$hppy^{RB1}$/+ and $hppy^{17\text{-}51}$/$hppy^{17\text{-}51}$. ST50 of $hppy^{17\text{-}51}/hppy^{17\text{-}51}$;*UAS-*$hppy^{RB1}$/+ was not significantly different than control ($p > 0.05$) or *UAS-*$hppy^{RB1}$/+ ($p > 0.05$) (n = 8–12).

(D) Introduction of the *UAS-*$hppy^{RB1}$ transgene into the $hppy^{KG5537}$ mutant background, which lacks GAL4 activity, did not rescue the $hppy^{KG5537}$ sedation resistance. Significant differences were observed when comparing $hppy^{KG5537}/hppy^{KG5537}$;*UAS-*$hppy^{RB1}$/+ with control or *UAS-*$hppy^{RB1}$/+ flies. ST50 of $hppy^{KG5537}/hppy^{KG5537}$;*UAS-*$hppy^{RB1}$/+ was not significantly different than $hppy^{KG5537}/hppy^{KG5537}$ ($p > 0.05$) (n = 8).

(E) Panneuronal expression of *UAS-*$hppy^{RB1}$ under the control of the *elav-*$GAL4^{c155}$ driver rescued the sedation resistance of $hppy^{KG5537}$ flies. Significant differences were observed between *elav-*$GAL4^{c155}$;$hppy^{KG5537}/hppy^{KG5537}$;*UAS-*$hppy^{RB1}$/+ and (1) $hppy^{KG5537}/hppy^{KG5537}$, (2) *elav-*$GAL4^{c155}$;$hppy^{KG5537}/hppy^{KG5537}$, and (3) $hppy^{KG5537}/hppy^{KG5537}$;*UAS-*$hppy^{RB1}$/+. ST50 of *elav-*$GAL4^{c155}$; $hppy^{KG5537}$/ $hppy^{KG5537}$; *UAS-*$hppy^{RB1}$/+ was not significantly different than control ($p > 0.05$) (n = 8).

(F) Neuronal overexpression of *UAS-*$hppy^{RB1}$ using the *elav-*$GAL4^{c155}$ driver increased sensitivity to ethanol-induced sedation. Significant differences were observed when comparing *elav-*$GAL4^{c155}$;*UAS-*$hppy^{RB1}$/+ with *elav-*$GAL4^{c155}$ or *UAS-*$hppy^{RB1}$/+ (n = 8).

***$p < 0.001$, one-way ANOVA with post hoc Newman-Keuls tests. Error bars represent SEM.

asked whether perturbation of the ERK pathway activated by the EGFR affected ethanol-induced sedation by expressing various EGFR-pathway transgenes using panneuronal drivers (*elav-*$GAL4^{c155}$, *elav-*$GAL4^{3E1}$). Using the ubiquitous driver *Tub-GAL4* resulted in lethality in all cases except when driving expression of a secreted form of the EGFR ligand encoded by the *spitz* (*spi*) gene.

Manipulations that enhanced EGFR signaling at several levels in the pathway potently increased resistance to ethanol-induced sedation (Figure 3). Increasing expression of an activated form of the EGFR ligand Spitz (*UAS-*spi^{SEC}) strongly increased resistance to ethanol-induced sedation (Figure 3A and data not shown). Marked resistance was also produced by neuronal overexpression of a wild-type EGFR transgene (*UAS-*$egfr^{WT}$, Figure 3B), a gain-of-function Raf MAP3K (data not shown), or a constitutively active form of the ERK *rolled* (*rl*) (*UAS-*rl^{ACT}, Figure 3C). Conversely, inhibiting EGFR signaling resulted in the opposite effect: enhanced sensitivity to ethanol-induced sedation. For example, a mutant in *rhomboid* (*rho*), encoding a protease that activates Spitz (Lee et al., 2001), displayed enhanced sensitivity to ethanol-induced sedation (Figure 3D). In this mutant, which harbors a *P* element insertion in the promoter region of *rho* (Figure S7A), mRNA levels were reduced to ~30% of wild-type (Figure S7C). We also tested a mutant in *Star* (*S*), which encodes a chaperone required for trafficking of Spitz (Lee et al., 2001). S^{d01624} flies, which carry a *P* element insertion in the *Star* gene (Figure S7B) that reduces *Star* function as ascertained by complementation analysis with a null allele of *Star* (see Supplemental Experimental Procedures), also showed enhanced sensitivity to ethanol-induced sedation (Figure 3E). Finally, we utilized an RNAi transgene that targets the *egfr*, *UAS-*$egfr^{RNAi}$, which strongly reduces *egfr* transcript levels when expressed with *elav-*$GAL4^{c155}$ (Figure S7D); this also resulted in increased sensitivity to ethanol-induced sedation (Figure 3F). Taken together, our data strongly support a role for the EGFR pathway in regulating ethanol-induced sedation in *Drosophila*, where inhibition of the pathway leads to enhanced sensitivity to ethanol-induced sedation, whereas its activation leads to the opposite phenotype.

Hppy Is a Negative Regulator of the EGFR/ERK Pathway

Based on our observations that enhanced EGFR signaling and reduced *hppy* function both led to increased ethanol resistance, whereas reduced EGFR signaling and *hppy* overexpression produced the opposite effect, we reasoned that *hppy* may function as an inhibitor of the EGFR pathway. To test this hypothesis, we first resorted to the fly eye, where the developmental role of EGFR signaling has been thoroughly studied (Dominguez et al.,

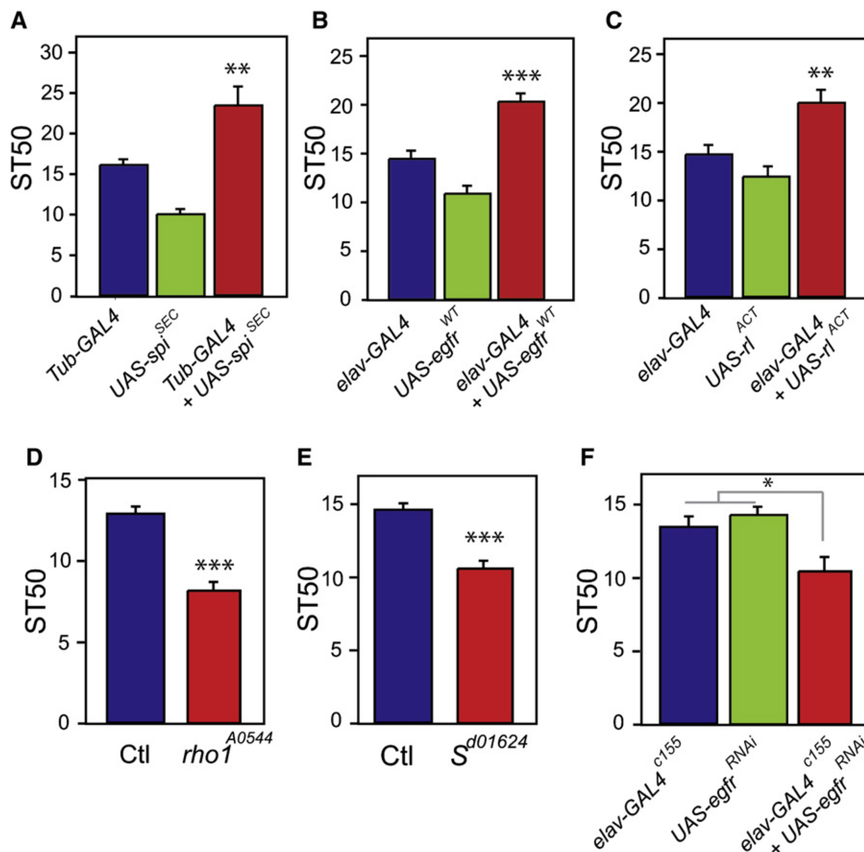

Figure 3. Activation or Inhibition of EGFR/ERK Signaling in Neurons Alters Ethanol Sensitivity

(A) Flies expressing a secreted form of the EGFR ligand Spitz, *UAS-spi*^{SEC}, under the control of the panorganismal driver *Tub-GAL4* were resistant to ethanol-induced sedation. Significant differences were observed between *Tub-GAL4/+;UAS-spi*^{SEC}/+ and *Tub-GAL4*/+ (**$p < 0.01$) as well as between *Tub-GAL4/+;UAS-spi*^{SEC}/+ and *UAS-spi*$^{SEC/}$+ (***$p < 0.001$) ($n = 8$).

(B) Flies overexpressing a wild-type form of the EGFR, *UAS-egfr*^{WT}, under the control of the panneuronal driver *elav-GAL4*^{c155} were resistant to ethanol-induced sedation. Significant differences were observed between *elav-GAL4*^{c155}*;UAS-egfr*^{WT}/+ and *elav-GAL4*^{c155}, as well as between *elav-GAL4*^{c155}*;UAS-egfr*^{WT}/+ and *UAS-egfr*^{WT}/+ (***$p < 0.001$) ($n = 8$).

(C) Flies expressing a constitutively active form of the ERK *rolled*, *UAS-rl*^{ACT}, under the control of the panneuronal driver *elav-GAL4*^{c155} displayed increased resistance to ethanol-induced sedation. Significant differences were observed between *elav-GAL4*^{c155}*;UAS-rl*^{ACT}/+ and *elav-GAL4*^{c155} (**$p < 0.01$) as well as between *elav-GAL4*^{c155}*;UAS-rl*^{ACT}/+ and *UAS-rl*^{ACT}/+ (***$p < 0.001$) ($n = 7–8$).

(D) The *P* element-induced loss-of-function *rho* mutant, *rho*^{A0544}, displayed enhanced sensitivity to ethanol-induced sedation (***$p < 0.0001$) ($n = 8$).

(E) The *P* element-induced *Star* mutant, *S*^{d01624}, showed enhanced sensitivity to ethanol-induced sedation (***$p < 0.0001$) ($n = 8$).

(F) Flies expressing RNAi against the EGFR, *UAS-egfr*^{RNAi}, under the control of the panneuronal driver *elav-GAL4*^{c155} were sensitive to ethanol-induced sedation. Significant differences were observed between *elav-GAL4*^{c155}*;UAS-egfr*^{RNAi}/+ and *elav-GAL4*^{c155} (*$p < 0.05$) as well as between *elav-GAL4*^{c155}*;UAS-egfr*^{RNAi}/+ and *UAS-egfr*^{RNAi}/+ ($p < 0.01$) ($n = 12$).

(A–C and F) One-way ANOVA with post hoc Newman-Keuls tests.

(D and E) Student's unpaired t test assuming equal variance.

Error bars represent SEM.

1998). Specifically, we tested whether overexpression of *hppy-RB* would modify the rough-eye phenotypes induced by expression/overexpression of EGFR pathway components using the retinal *GMR-GAL4* driver (Moses and Rubin, 1991). Expression of *UAS-hppy*^{RB1} under the control of *GMR-GAL4* had little, if any, effect on eye morphology (compare Figures 4A and 4B). As expected, overexpressing the EGFR using *UAS-egfr*^{WT} resulted in a very strong rough-eye phenotype with prominent blistering in the dorsal anterior section of the eye (Figure 4C). A rough-eye phenotype was also observed when expressing *rl*^{ACT} under the control of *GMR-GAL4* (Figure 4E). Notably, *hppy* overexpression suppressed the rough-eye and blistering phenotypes induced by EGFR overexpression (compare Figures 4C and 4D) but had no effect on the eye phenotype caused by expression of *rl*^{ACT} (compare Figures 4E and 4F). We next asked whether *hppy* overexpression would enhance the rough-eye phenotype caused by inhibition of the EGFR pathway. We found that, whereas expression of wild-type *yan*, which encodes a transcription factor that acts downstream of *rolled* to inhibit the transcription of EGFR pathway-regulated genes (Rebay and Rubin, 1995), produced an overall normal-looking eye (Figure 4G), the combined expression of *hppy* and *yan* under *GMR-GAL4* control produced a severe rough and "glossy" eye phenotype (Figure 4H). In addition, *hppy* expression potently enhanced the mild rough-eye phenotype induced by *GMR-GAL4*-driven expression of *UAS-egfr*^{RNAi} (Figure S7E). Thus, retinal overexpression of *hppy* ameliorated the effects of EGFR pathway overactivation and enhanced the effects of pathway inhibition. These data are consistent with *hppy* acting as an inhibitor of the EGFR pathway. The fact that *hppy* overexpression did not alter the phenotype produced by expression of *rl*^{ACT} suggests that Hppy functions upstream of this MAPK.

When expressing various EGFR pathway components with the *GMR-GAL4* driver, we observed that expression of either a dominant-negative form of the EGFR (*UAS-egfr*^{DN}) or an activated form of yan (*UAS-yan*^{ACT}) resulted in reduced viability and that coexpression of *hppy* (*UAS-hppy*^{RB1}) potently enhanced this lethality (Figure 4I). These data provide evidence that *hppy* can also modulate the EGFR pathway during earlier developmental processes needed for viability and further strengthen our hypothesis that *hppy* functions as an inhibitor of the pathway. These experiments do not, however, allow us to exclude the possibility that Hppy may function in a parallel pathway (Figure S8).

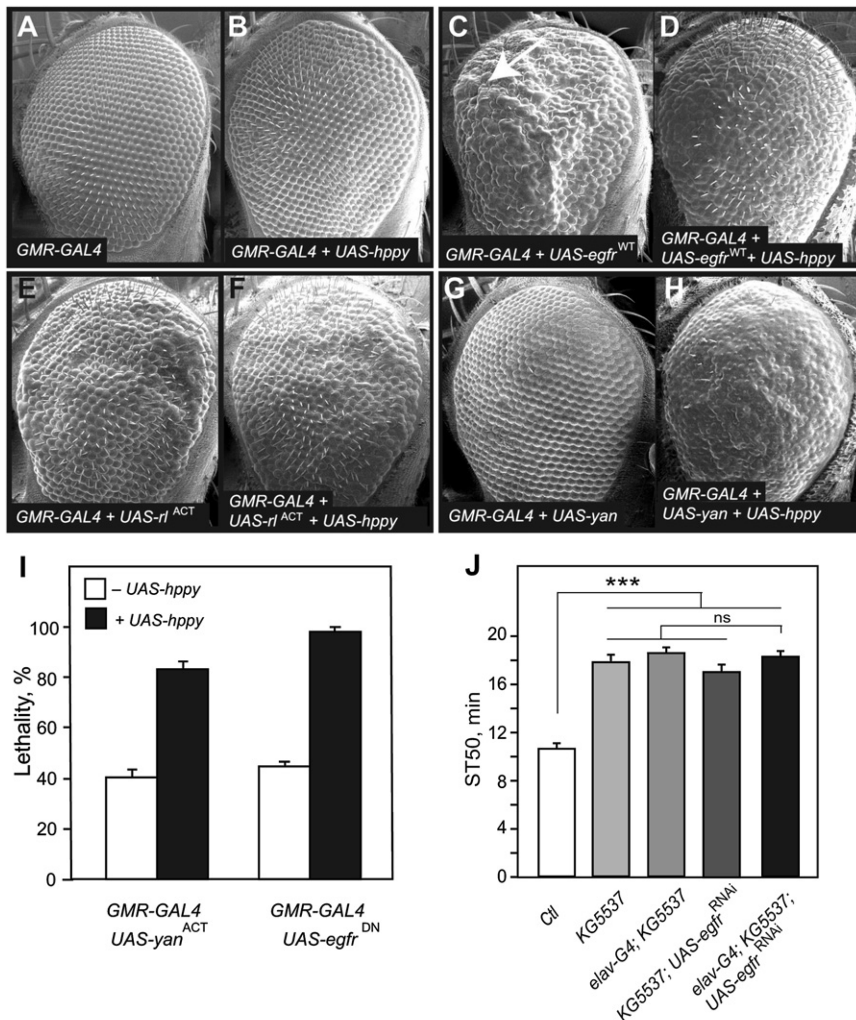

Figure 4. Genetic Interactions between the EGFR Pathway and *hppy*

(A–H) Genetic interactions in the fly eye. *GMR-GAL4*-driven *hppy-RB* expression suppressed and enhanced the rough-eye phenotype caused by overexpression of EGFR and Yan, respectively; *hppy* overexpression did not affect the rough eye of flies expressing an activated *rolled* transgene. Scanning electron micrographs of adult eyes of the following genotypes: (A) *GMR-GAL4*; (B) *GMR-GAL4;UAS-hppy*^{RB1}; (C) *GMR-GAL4;UAS-egfr*^{WT}, arrow points to blister; (D) *GMR-GAL4; UAS-egfr*^{WT}*;UAS-hppy*^{RB1}; (E) *GMR-GAL4;UAS-rl*^{ACT}; (F) *GMR-GAL4;UAS-rl*^{ACT}*;UAS-hppy*^{RB1}; (G) *GMR-GAL4;UAS-yan*; (H) *GMR-GAL4;UAS-yan;UAS-hppy*^{RB1}. Flies were heterozygous for all transgenes. Anterior is to the right, and dorsal is up.

(I) Genetic interactions with regard to viability. Expression of *hppy-RB* enhanced the semilethality induced by *GMR-GAL4*-driven expression of a dominant-negative form of the EGFR, *UAS-egfr*^{DN}, as well as expression of an activated form of the EGFR/ERK pathway inhibitor Yan, *UAS-yan*^{ACT}. Student's paired t test assuming equal variance revealed a significant difference between *GMR-GAL4/+;UAS-egfr*^{DN}/+ and *GMR-GAL4/+; UAS-egfr*^{DN}*/+;UAS-hppy*^{RB1}/+ ($p = 0.0027$) as well as between *GMR-GAL4/+;UAS-yan*^{ACT}/+ and *GMR-GAL4/+;UAS-yan*^{ACT}*/+;UAS-hppy*^{RB1}/+ ($p = 0.0064$) ($n = 3$).

(J) Genetic interactions in ethanol-induced sedation. Flies expressing the RNAi transgene targeting the EGFR, *UAS-egfr*^{RNAi}, under the control of the panneuronal driver *elav-GAL4*^{c155} in the *hppy*^{KG5537} homozygous mutant background did not display sensitivity to ethanol-induced sedation. One-way ANOVA of ST50 values with post hoc Newman-Keuls tests revealed a significant difference between *elav-GAL4*^{c155}*;hppy*^{KG5537}*/hppy*^{KG5537}*;UAS-egfr*^{RNAi}/+ and control (***$p < 0.001$) but failed to reveal a significant difference between *elav-GAL4*^{c155}*;hppy*^{KG5537}*/hppy*^{KG5537}*;UAS-egfr*^{RNAi}/+ and (1) *elav-GAL4*^{c155}*; hppy*^{KG5537}*/ hppy*^{KG5537} ($p > 0.05$), (2) *hppy*^{KG5537}*/hppy*^{KG5537}*;UAS-egfr*^{RNAi}/+ ($p > 0.05$), or (3) *hppy*^{KG5537}*/hppy*^{KG5537} ($p > 0.05$) ($n = 8$).

Error bars represent SEM.

To determine whether *hppy* interacts with the EGFR pathway in the context of ethanol-induced behaviors, we tested the ethanol sedation sensitivity of flies expressing the *UAS-egfr*^{RNAi} transgene panneuronally in the homozygous *hppy*^{KG5537} and *hppy*$^{17-51}$ mutant backgrounds. In contrast to the enhanced ethanol sensitivity seen in wild-type flies expressing the *UAS-egfr*^{RNAi} transgene panneuronally (Figure 3F), the *hppy*^{KG5537} and the *hppy*$^{17-51}$ mutants completely suppressed this sedation sensitivity (Figure 4J and data not shown). This finding is consistent with our hypothesis that *hppy* functions as an inhibitor to the EGFR pathway to regulate ethanol-induced sedation.

Acute Ethanol Exposure Leads to ERK/Rolled Phosphorylation in *hppy*, but Not Wild-Type, Flies

To ask whether ethanol has an acute effect on EGFR/ERK signaling, we examined levels of ERK (Rolled) phosphorylation in head extracts of flies exposed to ethanol vapor as in our behavioral assays (Figure 5A). Although ethanol did not detectably affect the levels of P-ERK in wild-type control flies, two distinct changes were observed in *hppy* flies. First, basal levels of P-ERK were substantially lower in *hppy* flies compared to controls (Figure 5B). We speculate that chronic upregulation of the EGFR/ERK pathway in *hppy* flies leads to compensatory downregulation of basal P-ERK levels; similar results were observed in brain extracts of flies in which the EGFR was overexpressed panneuronally (data not shown). Second, and more importantly, P-ERK levels were rapidly (within 5–10 min), highly, and transiently induced by ethanol exposure in *hppy*, but not in control flies (Figures 5A and 5C). This finding is consistent with our hypothesis that *hppy* functions as an inhibitor of the pathway, likely acting upstream of the ERK Rolled.

EGFR Signaling in Insulin-Producing Cells and Dopaminergic Neurons Regulates Ethanol Sensitivity

Because panneuronal activation of the EGFR pathway resulted in increased resistance to ethanol-induced sedation, we wished

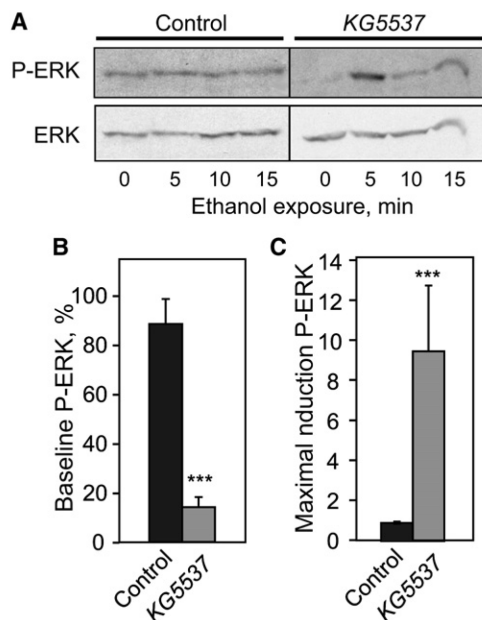

Figure 5. Acute Ethanol Exposure Leads to ERK Phosphorylation in *hppy*, but Not Wild-Type, Flies

(A) ERK phosphorylation levels in head extracts of flies exposed to ethanol vapor. Wild-type control and *hppy* flies were exposed to sedating levels of ethanol vapor for various times as indicated. Equal levels of total brain extract were subjected to western blot analysis using an anti diphosphoERK antibody (top) and reprobed with an anti-ERK antibody (bottom). A representative experiment is shown.

(B) Quantification of basal (time 0) P-ERK levels normalized to total ERK protein levels. Basal P-ERK levels were significantly lower in $hppy^{KG5537}$ compared to control flies. p = 0.004, Student's t test.

(C) Quantification of maximal ERK phosphorylation. P-ERK induction values were calculated by dividing the maximal phosphorylation levels reached in each experiment by the basal (time 0) ERK phosphorylation levels (p = 0.002, Student's t test). The results in (B) and (C) are the mean ± SEM of four independent experiments.

to identify the specific cells/brain regions responsible for this phenotype. We drove *egfr* overexpression using 15 GAL4 drivers whose expression patterns had been characterized previously (Table S1). We found that overexpression of wild-type EGFR ($UAS\text{-}egfr^{WT}$) in such brain regions as the mushroom body, ellipsoid body, or the ventral lateral neurons had no effect on ethanol-induced sedation (Table S1). *Egfr* expression using a muscle driver also produced no effect, whereas driving *egfr* expression using a glial driver resulted in lethality (Table S1 and data not shown). In contrast, strongly increased resistance to ethanol-induced sedation was observed when driving *egfr* expression in insulin-producing cells (IPCs) using the *dilp2-GAL4* driver (Rulifson et al., 2002) (Figure 6A) or in dopaminergic cells using the *TH-GAL4* driver (Friggi-Grelin et al., 2003) (Figure 6D). More modest resistance was observed when overexpressing *egfr* with *Ddc-GAL4*, which drives expression in most dopaminergic and serotonergic neurons (Li et al., 2000) (Table S1). Importantly, expression of GAL4 and gross morphology of cells was unaffected by *egfr* overexpression driven by *dilp2-GAL4*, *TH-GAL4*, or *Ddc-GAL4* (compare Figures 6B and 6C, Figures 6E and F, and data not shown). We also expressed $UAS\text{-}egfr^{DN}$ with *dilp2-GAL4* and observed enhanced ethanol sensitivity (data not shown; *TH-GAL4; UAS-egfr*^{DN} flies did not survive), implying that the EGFR pathway normally functions in these cells to regulate ethanol sensitivity. Thus, perturbation of the EGFR pathway in discrete subsets of CNS neurons, but not in many others, was sufficient to alter ethanol-induced sedation.

Pharmacological Inhibition of the EGFR in Adult Flies Affects Ethanol-Induced Sedation

To determine whether inhibition of EGFR signaling during adulthood would alter ethanol sensitivity, we used the well-characterized EGFR inhibitor erlotinib (Tarceva). This small molecule drug is orally bioavailable and a selective inhibitor of the tyrosine kinase activity of the mammalian EGFR/ErbB1 (Ciardiello et al., 2004). Adult flies were fed food containing erlotinib for ~40 hr and then tested in the ethanol LOR assay. Erlotinib-fed flies were more sensitive to the sedating effects of ethanol compared to vehicle-fed flies (Figures 7A and 7B). This enhanced sensitivity did not appear to be due to abnormal ethanol pharmacokinetics, fly feeding behavior, or ethanol-induced locomotion (Figures S9A–S9C). A second orally active, specific, and potent inhibitor of EGFR tyrosine kinase activity is the drug gefitinib (Iressa) (Ranson and Wardell, 2004; Ono and Kuwano, 2006; Dutta and Maity, 2007). Similar to erlotinib, adult flies fed gefitinib exhibited increased sensitivity to ethanol-induced sedation (Figures 7C and 7D). These data indicate that inhibition of the EGFR pathway during adulthood is sufficient to elicit enhanced ethanol sensitivity in *Drosophila*. A developmental role for the pathway in behavior is, however, also possible.

Acute Pharmacological Inhibition of EGFR Alters Ethanol Sensitivity and Consumption in Rodents

We next asked whether acute administration of erlotinib might alter ethanol-induced behaviors in mammals. We first determined the effects of erlotinib administration on acute ethanol sensitivity in mice using the LOR reflex assay and found that erlotinib-treated mice recovered more slowly from a sedating dose of ethanol (Figure S9D); this enhanced ethanol sensitivity is similar to that observed in erlotinib-fed flies. We next evaluated the effects of erlotinib on 10% ethanol and 5% sucrose consumption in rats using the continuous-access two-bottle-choice drinking paradigm (see Supplemental Experimental Procedures). Once rats had achieved a stable baseline consumption, erlotinib (5, 20, and 40 mg/kg i.p.) was administered 30 min prior to access to either ethanol or sucrose. Erlotinib significantly decreased ethanol consumption for up to 24 hr in a dose-dependent manner (Figure 7E). Interestingly, the effect of the drug was selective for ethanol, as it had no effect on 5% sucrose consumption (Figure 7F). There was no overall effect on water consumption (Figure 7G), indicating a reduction in ethanol preference rather than simply an alteration in overall fluid consumption. Finally, the amount of ethanol consumed between 24 and 48 hr following erlotinib administration did not differ from ethanol consumption after vehicle treatment (data not shown); thus, erlotinib did not cause a rebound increase in ethanol consumption. In summary, our data demonstrate that acute inhibition of EGFR pathway signaling is sufficient to significantly

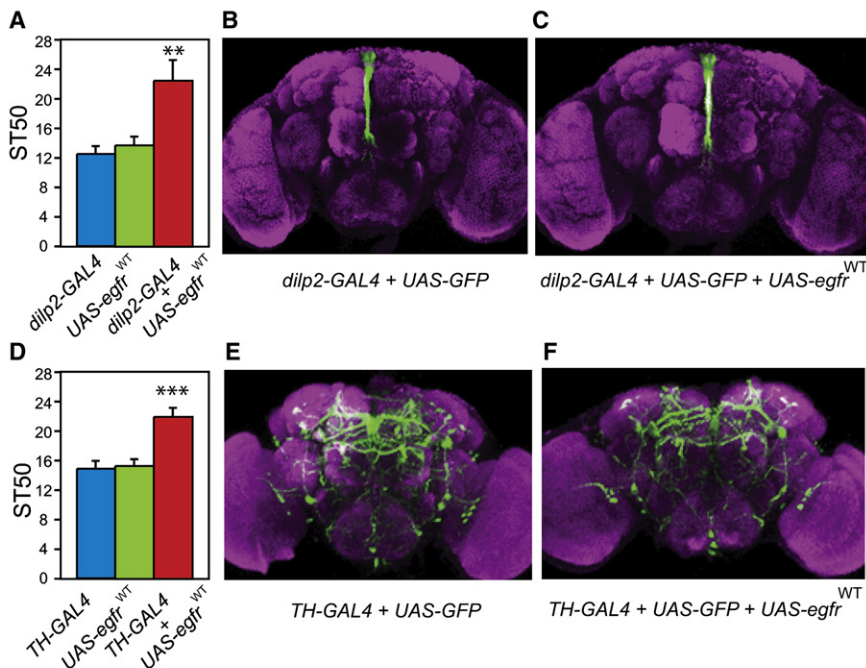

Figure 6. Activation of EGFR/ERK Signaling in Insulin-Producing Cells and Dopaminergic Cells Decreases Ethanol Sensitivity as Measured in the LOR Assay

(A) Flies overexpressing a wild-type form of the EGFR, *UAS-egfr*^{WT}, in IPCs under the control of the *dilp2-GAL4* driver were resistant to ethanol-induced sedation. A significant difference was observed between *dilp2-GAL4/+;UAS-egfr*^{WT}/+ and *dilp2-GAL4*/+ (**$p < 0.01$) as well as between *dilp2-GAL4/+;UAS-egfr*^{WT}/+ and *UAS-egfr*^{WT}/+ (**$p < 0.01$) ($n = 8$).

(B and C) The projection pattern of brain IPCs appeared unaffected by overexpression of EGFR. Compare confocal images of representative adult brains of *dilp2-GAL4/+;UAS-GFP/+* flies (B) versus *dilp2-GAL4/+;UAS-GFP/+;UAS-egfr*^{WT}/+ flies (C).

(D) Flies expressing a wild-type form of the EGFR, *UAS-egfr*^{WT}, under the control of *TH-GAL4* were resistant to ethanol-induced sedation. Significant differences were observed between *TH-GAL4/+; UAS-egfr*^{WT}/+ and (1) *TH-GAL4*/+ and (2) *UAS-egfr*^{WT}/+ (***$p < 0.001$) ($n = 8$).

(E and F) The projection pattern of TH-positive dopaminergic cells in the brain appeared to be unaffected by overexpression of EGFR. Compare the confocal images of adult brains of *TH-GAL4/+; UAS-GFP* flies (E) versus *TH-GAL4/+;UAS-GFP/+;UAS-egfr*^{WT}/+ flies (F).

(A and D) One-way ANOVA with post hoc Newman-Keuls tests.

(B, C, E, and F) Expression of GFP is green, and expression of the general neuropil marker Nc82 is purple.

Error bars represent SEM.

decrease ethanol sensitivity in mice and consumption and preference in rats.

DISCUSSION

hppy Regulates Ethanol-Induced Sedation and EGFR/ERK Signaling in *Drosophila*

We identified and characterized two *P* element mutants in the CG7097/*happyhour* (*hppy*) gene region and found that reduced *hppy* expression resulted in decreased sensitivity to ethanol-induced sedation, whereas neuronal overexpression of *hppy* caused the opposite effect. By in situ hybridization and QPCR (data not shown), we found evidence for *hppy* expression in adult brain, and behavioral rescue experiments demonstrated that neuronal expression of *hppy* was sufficient to rescue the *hppy* sedation resistance phenotype.

Like its mammalian homologs, the GCK-1 subfamily of Ste20 family kinases, the predicted *hppy* products contain N-terminal serine/threonine kinase domains and C-terminal regulatory domains known as citron homology domains. In vitro studies of these homologs of Hppy, including GCK (Pombo et al., 1995), GCK-like kinase (Diener et al., 1997), kinase homologous to SPS1/STE20 (Tung and Blenis, 1997), and hematopoietic progenitor kinase (HPK) (Kiefer et al., 1996), have revealed that they activate JNK signaling, but not ERK or p38 signaling. HPK1 (Hu et al., 1996) and GLK (Diener et al., 1997) have both been shown to phosphorylate MAP3Ks in the JNK pathway, implying that GCK-1 kinases are MAP4Ks acting upstream of JNK signaling.

In this study, we provide evidence that Hppy, a presumed MAP4K in the GCK-1 subfamily of Ste20 kinases, can modulate EGFR/ERK signaling in a manner that is consistent with it acting as an inhibitor of the pathway. First, retinal *hppy* overexpression respectively enhanced and suppressed the rough-eye phenotypes brought about by EGFR/ERK pathway inhibition and activation. Second, increased *hppy* expression enhanced the semilethality caused by ectopic expression of transgene EGFR pathway inhibitors. Third, decreasing levels of *hppy* completely suppressed the enhanced ethanol sensitivity brought about by neuronal EGFR downregulation. Finally, ethanol induced robust phosphorylation of ERK/Rolled in *a hppy* mutant, but not in control flies. What, then, is the biochemical mechanism through which Hppy inhibits EGFR/ERK signaling? The answer to this question is still unknown. However, an in vitro study of another GCK-1 subfamily kinase, HPK1, offers an intriguing possibility (Anafi et al., 1997). This study showed that HPK1 physically associates with the EGFR adaptor protein Grb2 and that EGF stimulation recruits the Grb2/HPK1 complex to the autophosphorylated EGFR. This recruitment leads to the tyrosine phosphorylation of HPK1. It will be interesting to determine whether such a physical association exists between Hppy and components of the EGFR/ERK signaling cascade and, if so, what the consequences may be on signaling.

Our experiments cannot completely rule out a role for *hppy* in regulating JNK signaling, although JNK signaling perturbation did not affect ethanol-induced sedation. In addition, *hppy* mutant flies responded normally to a variety of stress stimuli known to activate the JNK and p38 pathways, including

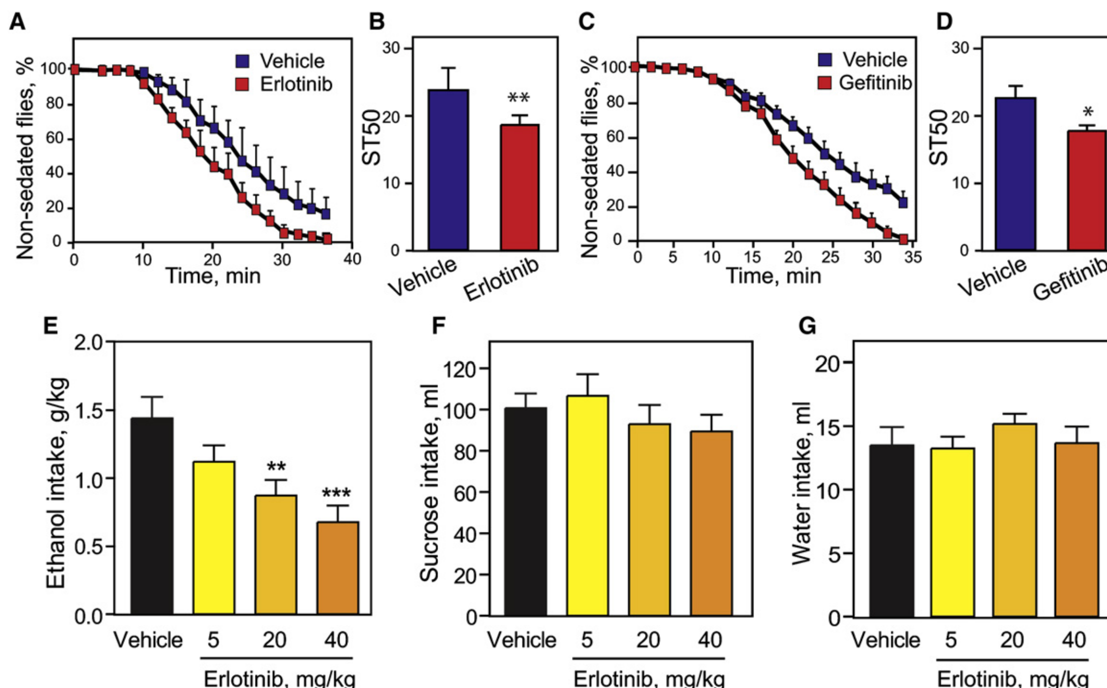

Figure 7. EGFR Inhibitors Alter Ethanol Sensitivity in Flies and Ethanol Consumption in Rats

(A–D) Samples of 25 flies each were fed a sucrose/yeast mixture containing either erlotinib (0.8 mg/ml; [A] and [B]) or gefitinib (0.5 mg/ml; [C and D]) dissolved in vehicle or vehicle alone for 40 hr and were tested in the LOR assay (100 U/50 U E/A). (A and C) Sedation profiles and (B and D) ST50 values are shown. One-way ANOVA revealed a significant difference between erlotinib- and vehicle-fed flies ($p = 0.006$; $F_{1,15} = 10.341$; $n = 8$) and between gefitinib- and vehicle-fed flies ($p = 0.022$; $F_{1,23} = 6.058$; $n = 12$).

(E–G) Erlotinib (5–40 mg/kg i.p.) was administered to rats 30 min prior to the start of the drinking session using the continuous access to 10% ethanol or 5% sucrose two-bottle-choice drinking paradigm (see Supplemental Experimental Procedures). Repeated-measures ANOVA followed by post hoc Newman-Keuls analysis revealed that erlotinib significantly decreased 10% ethanol consumption (E) ($p < 0.0009$; $F_{3,11} = 7.0$), but not 5% sucrose consumption (F) ($p > 0.05$; $F_{3,10} = 2.0$) 24 hr after onset of drinking. (G) Erlotinib treatment had no overall effect on water consumption ($F_{3,11} = 0.6$) ($n = 10$). Values are expressed as mean ethanol consumed (g/kg) or mean sucrose consumed (ml).

The values are expressed as mean ethanol consumed (g/kg) ± SEM or mean sucrose consumed (ml) ± SEM. **$p < 0.01$ and ***$p < 0.001$ compared to vehicle.

oxidative stress, heat stress, and starvation (data not shown). Indeed, studies in HeLa cells show a lack of involvement of *hppy* in JNK activation in response to osmotic stress and the protein synthesis inhibitor anisomycin (Findlay et al., 2007).

The EGFR/ERK Pathway Regulates Ethanol-Induced Sedation in *Drosophila*

In recent years, studies in vitro and in vivo have revealed an intriguing link between ethanol and the mammalian EGFR/ERK pathway, demonstrating that EGFR autophosphorylation and ERK phosphorylation are both inhibited by pharmacologically relevant concentrations of ethanol (Chandler and Sutton, 2005; Ma et al., 2005). In addition, elevated expression of several MAPKs and their regulators has been reported in the brains of mice and rats selected for high ethanol preference (Arlinde et al., 2004; Mulligan et al., 2006). In this paper, we uncover a role for the EGFR/ERK pathway in mediating the behavioral responses to ethanol in *Drosophila*. Neuronal manipulations that activate the EGFR/ERK pathway resulted in enhanced resistance to the sedative effects of ethanol, whereas neuronal inhibition of the pathway caused increased sensitivity. These effects were seen upon manipulations of several different components of the EGFR/ERK pathway. In contrast, we found no evidence for the other two major MAPK pathways, JNK and p38, in mediating the sedative response to ethanol. Our finding that EGFR activation specifically in either insulin-producing cells (IPCs) or dopaminergic cells affects ethanol sensitivity is consistent with previous studies implicating both the IPCs (Corl et al., 2005) and dopaminergic systems (Bainton et al., 2000) in the behavioral response to ethanol in *Drosophila* and suggests that the EGFR/ERK pathway may interact with the insulin- and dopamine-signaling pathways to control drug responses. Equally interesting is our observation that EGFR activation in many other brain regions, including those previously shown to play a role in ethanol-related behaviors, such as the ellipsoid body (Urizar et al., 2007) and the cells defined by the 201Y GAL4 line (Rodan et al., 2002), had no effect on ethanol-induced sedation. Thus, the EGFR pathway appears to play a role in only a subset of brain regions that regulate flies' response to ethanol.

The mechanisms through which the EGFR/ERK cascade detects ethanol and how it might transduce those signals into a behavioral response remain unknown. We found that acute ethanol exposure, at concentrations that are behaviorally relevant, led to a rapid and transient increase in ERK/Rolled

phosphorylation in the heads of *hppy* mutants, an effect that was not observed in wild-type flies. Although this finding supports a role for Hppy as an inhibitor of the pathway, it is unclear whether it explains the increased resistance to ethanol-induced sedation observed in *hppy* flies or in flies in which the EGFR/ERK pathway was chronically upregulated. It is curious that our data do not reveal an inhibitory effect of ethanol on the EGFR/ERK pathway, as has been reported in rodents. While this may reflect a fundamental dissimilarity in the way that the pathway operates in flies and mammals, this discrepancy is more likely due to the fact that mammalian experiments used chronic ethanol exposure paradigms, whereas our experiments relied on acute exposure.

The EGFR Regulates Ethanol Sensitivity and Preference in Adult Flies and Rodents

We show that the EGFR has a role in regulating ethanol behaviors in adult flies and mammals. Administration of two well-studied EGFR inhibitors, erlotinib (Tarceva) and gefitinib (Iressa), to adult flies resulted in enhanced sensitivity in the LOR assay. Though our results do not rule out a developmental role for the EGFR/ERK pathway, they do show that this pathway can function in the adult fly to regulate the sedative effects of ethanol. Similarly, acute administration of erlotinib enhanced sensitivity of mice to the sedating effects of ethanol, implying that the role of the EGFR in this behavior is conserved among flies and rodents. Most importantly, we found that treatment of adult rats with erlotinib significantly decreased ethanol preference in a two-bottle-choice drinking paradigm. This effect appears to be ethanol specific, as preference for a second rewarding substrate, sucrose, was not altered. Together, these data reveal a potentially conserved role for the EGFR pathway in regulating ethanol behaviors in both flies and rodents. Because both erlotinib (Tarceva) and gefitinib (Iressa) (as well as many other small molecule EGFR inhibitors) are FDA-approved drugs, are known to cross the blood-brain barrier, and are well-tolerated, they offer a possible therapeutic avenue for the treatment of AUDs in humans.

EXPERIMENTAL PROCEDURES

Fly Genetics

Genetic Screen

Approximately 850 P[GAL4] homozygous viable strains (carrying the GawB element) were screened in an eight-chambered locomotor tracking apparatus at a 100 U ethanol vapor/50 U air concentration. Lines were judged to have a mutant phenotype if they differed by at least two standard deviations from the mean at two or more consecutive time points. After retesting and extensive backcrossing, five lines, including 17-51 (*hppy*), retained their mutant phenotypes (see Supplemental Experimental Procedures for details). Flies were raised on standard cornmeal/molasses food at 25°C and 70% relative humidity. All experiments used 2- to 5-day-old males at 20°C, ~25 males per behavioral run. All genotypes were tested across multiple days. For information about fly stocks, genetic background, and selection of control strains see the Supplemental Experimental Procedures.

Lethality Enhancement Test

GMR-GAL4 or *GMR-GAL4; UAS-hppy*^{RB1} virgins were crossed to *UAS-yan*^{ACT}*/CyO* or *UAS-egfr*^{DN}*/CyO* males. Percent lethality for each genotype was calculated as: % lethality = (1 – (# of non-Cy winged progeny / # of Cy winged progeny)) × 100%.

Behavioral Assays

Ethanol Sedation Assay

Assays were carried out as previously described (Rothenfluh et al., 2006), with minor modifications (see Supplemental Experimental Procedures).

Immunohistochemistry

dilp2-GAL4, *TH-GAL4*, *Ddc-GAL4*, and *GAL4*$^{17\text{-}51}$ GAL4 virgins were crossed to *UAS-GFP T2*, *UAS-Tau GFP* males (double-transgenic stock created by F. Wolf) or *UAS-GFP T2, UAS-Tau GFP; UAS-egfr*^{WT} males. Brains and ventral nerve cords were dissected from adult male progeny in 1 × PBS, fixed in 4% paraformaldehyde for 20 min, and then washed in 1 × PBS. GFP labeling was achieved by incubating specimens in a 1:200 dilution of a rabbit anti-GFP antibody (Clontech, Mountain View, CA) and with an FITC-coupled goat-anti-rabbit antibody, diluted 1:500 (Molecular Probes, Eugene, OR). Neuropil labeling was achieved by incubating specimens in a 1:10 dilution of Nc82 antibody (Laissue et al., 1999) and with a Cy3-coupled goat anti-mouse antibody (1:500; Molecular Probes, Eugene, OR). Specimens were analyzed with a Leica confocal microscope.

Molecular Biology

Hppy Characterization and Transgene Construction

The genomic DNA flanking the 17-51 (*hppy*) insertion was isolated using inverse PCR. Comparison with the *Drosophila* genome sequence on Flybase (www.flybase.org) revealed that the insertion was located 10 bp upstream of the first exon of *CG7097*. This finding was confirmed by PCR analysis (data not shown). The *UAS-hppy* transgene, *UAS-hppy*^{RB}, was generated by cloning the EST RH10407, encoding full-length *CG7097-RB*, into the pUAST vector (Brand and Perrimon, 1993). This transgene was injected into *w* Berlin flies, and two independent insertions were obtained, *UAS-hppy*^{RB1} and *UAS-hppy*^{RB2}, yielding different levels of *hppy-RB* expression.

Real-Time Quantitative RT-PCR

Adult flies 2–4 days old were collected, frozen in liquid nitrogen, and then stored at −80°C. RNA was extracted from whole flies or isolated heads, as described for each experiment, by homogenization in Trizol (Invitrogen). Quantitative RT-PCR was performed as described in Tsai et al. (2004). Primers and probes recognizing *CG7097*-RA and *CG7097*-RB transcripts are described in Supplemental Experimental Procedures.

Analysis of ERK/Rolled Phosphorylation

Twenty-five 4-day-old males of each genotype were introduced to ethanol exposure chambers. Following 12 min of humidified air, flies were given a continuous stream of ethanol vapor (110 U ethanol/ 40 U air) for restricted time periods ranging from 0 to 20 min and frozen immediately in liquid nitrogen. Frozen samples were vortexed to dissociate heads from bodies. An equal number of heads were lysed in phosphate lysis buffer (10 mM NaPO4 [pH 7.5], 5 mM EDTA, 100 mM NaCl, 1% Triton, 0.5% Na deoxycholate, and 0.1% SDS) supplemented with a mixture of protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, 100 μg/ml leupeptin, 1.5 μg/ml pepstatin A, 2 μg/ml antipain, 2 μg/ml, 10 mM NaPPi, and phosphatase inhibitor cocktail; Sigma P2850). Equal amounts of total head extracts were fractionated by SDS-PAGE and subjected to western blot analysis using anti-diphosphoMAPK antibodies (Sigma M8159) and anti-MAPK antibodies (Sigma M5670). Proteins were detected using ECL detection reagents and autoradiography. Relative phosphorylation levels were quantified by densitometric analysis and normalized according to protein level.

Feeding EGFR Inhibitors to *Drosophila*

Flies of the genotype *w Berlin* were collected at 0–2 days posteclosion, 25 males per large food vial. The following day, flies were transferred to large vials without food, each lined with a strip of Whatman 3 MM paper and containing 1 ml of 5% sucrose, 2% yeast, and 0.02 ml of 40 mg/ml Tarceva (obtained from OSI Pharmaceuticals, Melville, NY) freshly dissolved in 25% cyclodextrin/0.9% saline (vehicle); final drug concentration in food was 0.8 mg/ml. Gefitinib feeding was carried out as for erlotinib, with 1 ml of 5% sucrose, 2% yeast, and 0.01 ml of 50 mg/ml gefitinib (Biaffin GmbH & Co KG, Kassel, Germany) freshly dissolved in DMSO (vehicle); final concentration of gefitinib was 0.5 mg/ml.

Control vials contained an equivalent volume of vehicle. Flies were returned to a 25°C incubator and kept on drug for 40–41 hr until they were assayed for sedation.

Erlotinib Experiments in Rats

Continuous Access Two-Bottle-Choice 10% Ethanol or 5% Sucrose Drinking Paradigm

Rats were divided into two separate groups, of which one was trained to voluntarily consume 10% ethanol and the other to voluntarily consume 5% sucrose using a two-bottle-choice drinking paradigm, as described (Steensland et al., 2007 and Supplemental Experimental Procedures). Drug administrations began after rats maintained stable baseline drinking levels for 2 weeks.

Drugs and Treatment Schedules

Erlotinib was generously provided by OSI Pharmaceuticals. Ethanol, sucrose, and erlotinib solutions were prepared as described (Supplemental Experimental Procedures). All rats in the erlotinib experimental groups received each of the four treatments (vehicle, 5, 20, and 40 mg/kg), and each injection was given 7 days apart using a Latin square design; thus, each rat served as its own control.

Statistics

Statistical significance was established using either Student's t tests assuming equal variance or one-way analysis of variance (ANOVA) tests, followed by post-hoc Newman-Keuls testing using GraphPad Prism software, Version 4 (Graphpad, San Diego, CA). Error bars in all experiments represent SEM.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, eight figures, and one table and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00319-5.

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