Glycogen synthase kinase-3/Shaggy mediates ethanol-induced excitotoxic cell death of *Drosophila* olfactory neurons

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Communicated by Richard Axel, Center for Neurobiology and Behavior, New York, NY, October 12, 2009 (received for review September 30, 2008)

It has long been known that heavy alcohol consumption leads to neuropathology and neuronal death. While the response of neurons to an ethanol insult is strongly influenced by genetic background, the underlying mechanisms are poorly understood. Here, we show that even a single intoxicating exposure to ethanol causes non-cell-autonomous apoptotic death specifically of *Drosophila* olfactory neurons, which is accompanied by a loss of a behavioral response to the smell of ethanol and a blackening of the third antennal segment. The *Drosophila* homolog of glycogen synthase kinase-3 (GSK-3)β, Shaggy, is required for ethanol-induced apoptosis. Consistent with this requirement, the GSK-3β inhibitor lithium protects against the neurotoxic effects of ethanol, indicating the possibility for pharmacological intervention in cases of alcohol-induced neurodegeneration. Ethanol-induced death of olfactory neurons requires both their neural activity and functional NMDA receptors. This system will allow the investigation of the genetic and molecular basis of ethanol-induced apoptosis in general and provide an understanding of the molecular role of GSK-3β in programmed cell death.

ethanol-induced apoptosis | lithium | olfactory system | NMDA receptors

Heavy alcohol consumption leads to neuropathological changes and neuronal cell death (1–4). Brains of alcoholics are reduced in weight and volume, and ≈10% of alcoholics develop a severe cognitive disorder, such as alcoholic dementia or Wernicke-Korsakoff Syndrome (1, 2, 4). Chronic ethanol consumption in humans and rats leads to cholinergic neuron loss in the basal forebrain, which causes impairment of memory (4). In addition, alcoholics display diminished olfactory sensitivity, with one study finding that more than half of alcohol-dependent patients are hyposmic (5). This result is mirrored in rodent models, where 2 days of acute ethanol exposure causes the death of olfactory neurons, followed by retrograde degradation in the temporal dentate gyrus and regions of the hippocampus known to be involved in olfaction and memory (6, 7).

Several mechanisms have been proposed to explain ethanol-induced brain damage (4). One of these mechanisms involves thiamine deficiency (4, 8), another involves the induction of reactive oxygen species and increased production of polyamines (4, 9). Many of ethanol's neurotoxic effects are mediated through cellular excitability and interactions of ethanol with NMDA receptors. Ethanol binds to and inhibits the function of NMDA receptors in many types of neurons (10, 11), although chronic ethanol exposure results in a compensatory increase in glutamatergic neurotransmission (4). Upon ethanol withdrawal, neurons are hyper-excitable, and the resultant excess of intracellular Ca^{2+} can lead to mitochondrial damage and activation of apoptotic pathways (4). Finally, ethanol can induce acute excitotoxic cell death, as shown by enhancement of ethanol cytotoxicity in cortical neurons treated with the NMDA receptor agonist MK-801 (12) and enhancement of NMDA excitotoxicity in aminergic neurons treated with ethanol (13).

Glycogen synthase kinase 3β (GSK-3β) is a multifunctional protein that can both inhibit and activate apoptosis (14). This protein has been implicated as a mediator of cell death in a variety of systems, including tau-mediated neurodegeneration (15), β-amyloid-associated neurotoxicity (16), excitotoxic cell death (17, 18), and, most intriguingly for the present work, ethanol-induced apoptosis of cultured neurons (17).

Here we describe a model for ethanol-induced neuronal apoptosis in *Drosophila melanogaster*. We show that a single sedating dose of ethanol causes widespread apoptosis in the antennae. This apoptosis is dependent on *shaggy* (*sgg*), the *Drosophila* homolog of GSK-3. Ethanol-induced neuronal death requires electrical activity and is mediated by NMDA receptors in olfactory receptor neurons (ORNs). Our system will allow the use of powerful genetic tools available in *Drosophila* to begin identifying the genes and mechanisms involved in predisposition to ethanol-induced neuronal death.

Results and Discussion

Ethanol Vapor Causes Death of Olfactory Receptor Neurons. When *Drosophila* are exposed to ethanol vapor, they display an immediate and transient increase in locomotor activity (19). We found that preexposure to a sedating dose of ethanol vapor diminished this startle response (Fig. 1*A*) and was accompanied by an obvious and specific phenotype: blackening of the third antennal segments, the primary olfactory organs of the fly (Fig. 1 *B* and *C*). Visible damage was restricted to third antennal segments. The maxillary palps, secondary olfactory organs that do not respond to the odor of ethanol (19), were unaffected (see Fig. 1 *B* and *C*).

To investigate this ethanol-induced olfactory damage, we visualized ORNs and glia by expression of GFP using the GAL4-UAS system (20). Analysis of flies that express GFP under the control of *Or83b-GAL4*, which is expressed in ≈80% of ORNs (21), revealed that in antennae from unexposed flies, the nuclei of the ORNs were clearly visible, as was the olfactory nerve (ON) (Fig. 1*D*). In contrast, antennae from flies preexposed to ethanol displayed strongly reduced or undetectable GFP expression and the ON was not visible (Fig. 1*E*). Analysis of flies that express GFP in glia under the control of the *repo-GAL4* driver (22) revealed similar cellular loss upon ethanol exposure (Fig. 1 *F* and *G*). By contrast, maxillary palp ORNs and glia were unaffected (Fig. 1 *H–K*).

The *Drosophila* olfactory system includes three morphologically distinct types of sensory hairs, basiconic, coeloconic, and trichoid sensilla (23), which are distinguished by the expression of subsets of odorant receptor genes (24). The antenna contains all three types, whereas the maxillary palp contains only basi-

Author contributions: R.L.F. and U.H. designed research; R.L.F. performed research; R.L.F. analyzed data; and R.L.F. and U.H. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0910813106/DCSupplemental.

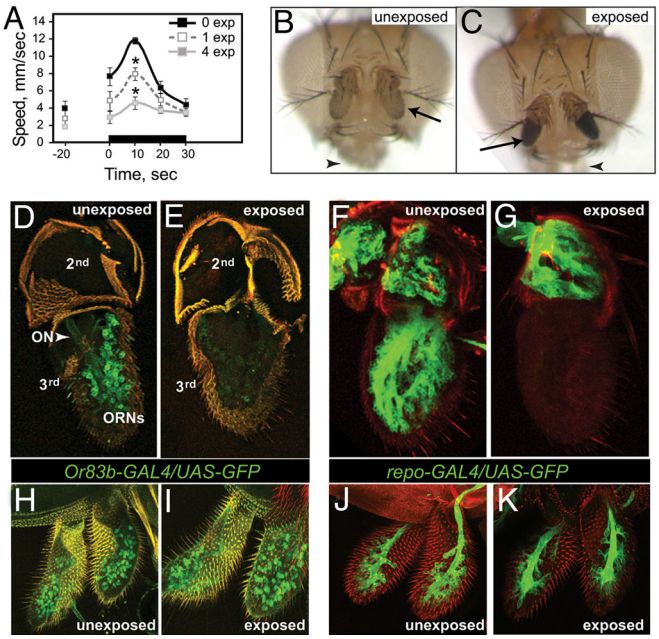

Fig. 1. Ethanol causes death of olfactory cells. (*A*) Locomotor activity profile showing olfactory startle of unexposed and ethanol preexposed flies. Ethanol exposure started at time 0. Male flies were preexposed to a 100:50 ratio of ethanol vapor to air flow rate (E/A) for 25 min once or four times (once per day). Olfactory behavior was measured 2 days after ethanol preexposure. Preexposed flies showed a significantly reduced startle ($n = 6$, $*P < 0.01$ at 10 s). (*B* and *C*) Heads from unexposed (*B*) and ethanol-exposed (*C*, single exposure) flies. The third antennal segments (*arrows*) are blackened in the ethanol-exposed fly, while the maxillary palps (*arrowheads*) are unaffected. (*D* and *E*) Confocal reconstructions of antennae from flies expressing GFP (*green*) in ORNs under the control of *Or83b-GAL4*. In unexposed flies (*D*), ORN nuclei and the olfactory nerve (ON) are clearly visible. Antennae from ethanol-exposed flies (*E*) show a strong reduction in ORN GFP expression, and the ON is no longer detectable. (*F* and *G*) Confocal reconstructions of antennae from flies expressing GFP in glia under the control of *repo-GAL4*. Glial expression of GFP is seen in both the second and third antennal segments in unexposed flies (*F*), while expression in the third antennal segment is specifically lost in ethanol-exposed flies (*G*). (*H–K*) Confocal reconstructions of maxillary palps from flies expressing GFP (*green*) in ORNs (*H*, *I*) or glia (*J*, *K*). The maxillary palps of ethanol-exposed flies (*I*, *K*) are unaffected by ethanol exposure, as compared with unexposed maxillary palps (*H*, *I*).

conic sensilla. To ask if the sensitivity of antennae, compared to maxillary palps, can be explained by the differential sensitivity of different hair types to ethanol, we generated flies expressing GFP in basiconic or trichoid sensilla (using *Or22a-GAL4* or *Or67d-GAL4*, respectively (Fig. S1) (25). Analysis of GFP expression after ethanol preexposure revealed that basiconic and trichoid sensilla survived in 53 and 56% of exposed antennae, respectively. For comparison, the ORN population at large, as defined by *Or83b-GAL4*-driven expression, survived in 55% of exposed antennae. Because the sensory hair subtypes are equally sensitive to ethanol, the differential response of antennae and maxillary palps cannot be attributed to the presence of different sensillar subtypes.

The extent of the behavioral and anatomical damage was dependent on the dose of ethanol, as well as the number of exposures (Fig. 2). Neither a moderate concentration of ethanol (70:80 ethanol:air ratio, E/A), nor a relatively low concentration (50:100 E/A) led to changes in the olfactory startle response after a 20-min preexposure, while a high ethanol concentration (100:50 E/A) caused a consistent but not statistically significant decrease in startle (see Fig. 2*A*). Longer exposure times led to a stronger phenotype: at the high concentration, 40- and 60-min exposures reduced startle magnitude to 54 and 46% of control levels, respectively (see Fig. 2*A*). Furthermore, with longer exposure times, the moderate concentration of ethanol caused olfactory damage.

The cumulative effect of multiple ethanol exposures was greater than a single dose (see Fig. 1*A* and Fig. 2*B*). On average, 45% of flies subjected to a single high-concentration preexposure showed blackening of at least one-third antennal segment (see Fig. 2*B*), whereas 92% of flies that received four doses over the course of 2 days showed blackened antennae (see Fig. 2*B*). Similarly, a single ethanol exposure reduced startle by 30%, while four exposures reduced it by 53% (see Fig. 1*A*). Finally, the damage caused by high-concentration ethanol exposure was irreversible (see Fig. 2*C*). The proportion of flies with black antennae did not change in up to 9 days of recovery time, and flies did not recover their olfactory startle within this time period (Fig. S2).

Ethanol-Induced Death of ORNs Occurs by Apoptosis. Acute ethanol exposure can cause neuronal death by either apoptotic or necrotic mechanisms, depending on the experimental conditions (3, 7, 26). To determine if ethanol-induced ORN death was caused by apoptosis, we asked if protection could be achieved by expressing baculovirus p35, a caspase inhibitor (27). We used *Or83b-GAL4* to drive expression of *UAS-p35* in ORNs, exposed the flies to ethanol, and examined them after 2 days. Flies expressing p35 in ORNs (*Or83b-GAL4*/*UAS-p35*) retained 81% of their unexposed startle response, whereas genetic controls retained only 48 to 54% (Fig. 3*A*). Analysis of GFP expression confirmed that the ORNs survive and appear normal in 80% of ethanol-exposed antennae expressing p35, while surviving ORNs were seen in only 40% of control antennae (Fig. 3 *C–E*). To confirm that cell death occurs by apoptosis, we undertook TUNEL, which labels apoptotic cells. Control antennae showed

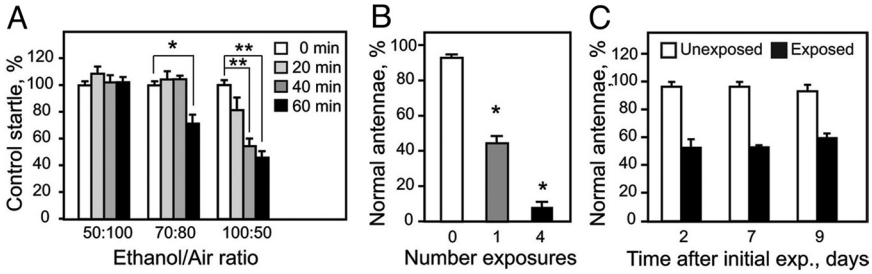

Fig. 2. Parametric analyses. (*A*) Ethanol preexposure-induced loss of olfactory startle increases with both ethanol concentration and length of a single exposure. Flies were exposed to increasing E/A of 50:100, 70:80, or 100:50 for 0, 20, 40, or 60 min, respectively, and the degree of startle in response to ethanol was measured 2 days later. (*, $P < 0.01$, **, $P < 0.001$). (*B*) The degree of morphological damage of antennae also increased with exposure number. Flies were given a single 100:50 E/A exposure once, or once per day, for 4 consecutive days and analyzed 2 days later (*, $P < 0.01$ for all comparisons). (*C*) Olfactory damage is permanent. Flies were assayed 2, 7, and 9 days after a single exposure to ethanol. Recovery was never observed.

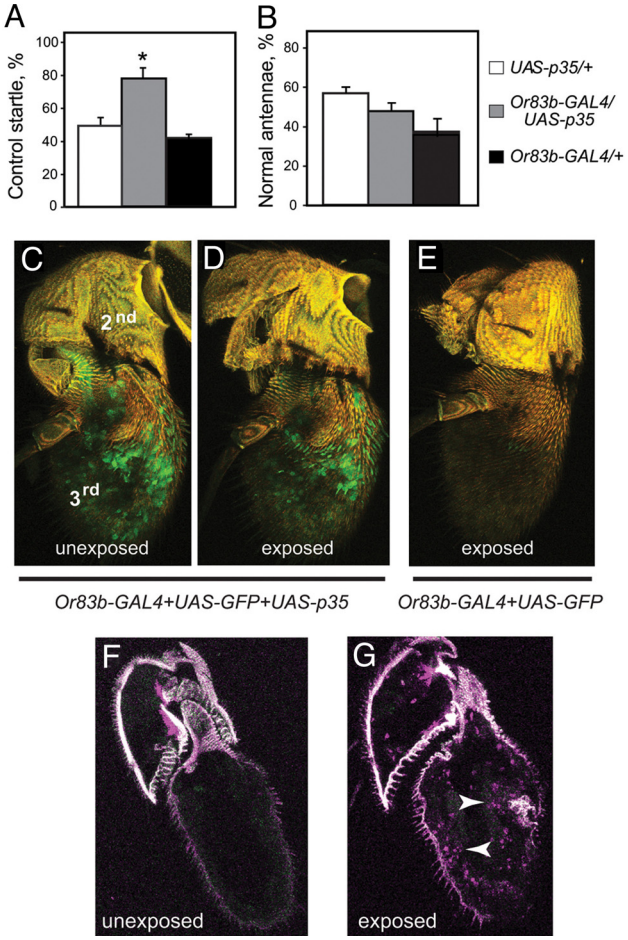

Fig. 3. Ethanol-induced olfactory damage occurs by apoptosis. (*A*) Expression of p35 in ≈80% of ORNs using the *Or83b-GAL4* driver protects flies from loss of olfactory startle caused by a single ethanol preexposure delivered 2 days before testing (n = 8, *, $P < 0.01$). Data are presented as the % of maximum startle response in preexposed flies relative to that of unexposed controls of the same genotype. (*B*) Expression of p35 in ORNs does not rescue antennal morphology in flies exposed to ethanol as in (*A*) (n = 8). (*C* and *D*) Confocal reconstruction of antennae from flies carrying *Or83b-GAL4*, *UAS-GFP*, and *UAS-p35*. Ethanol exposure did not cause loss of ORNs (*D*) in the third antennal segment as is seen in control flies not expressing p35 (*E*). Green, GFP; yellow, autofluorescence of the cuticle. (*F* and *G*) Sections of antennae from a fly preexposed to ethanol shows an increase in TUNEL-positive nuclei (*arrowheads* in *G*) when compared to the unexposed control (*F*).

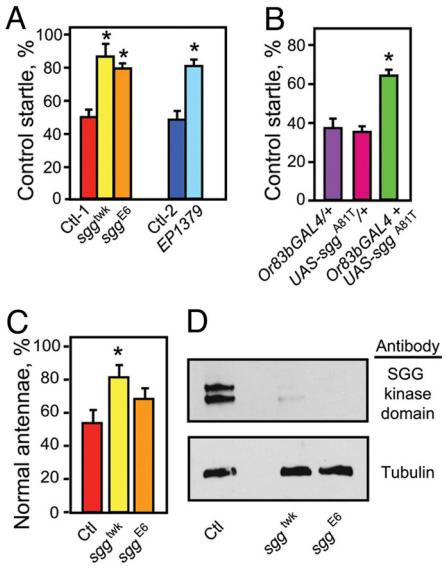

Fig. 4. Ethanol-induced ORN death requires Sgg/GSK3β. (*A*) Reduction of *sgg* function results in protection of the olfactory startle. Three loss-of-function alleles of *sgg* (*twk*, *E6*, and *EP1379*), all of which have a ≈90% reduction in the adult-specific forms of SGG (28), are resistant to ethanol-induced startle loss when compared with controls (Ctl-1 is a precise excision of the *twk* transposon and Ctl-2 is EP1576) (n = 7, *, $P < 0.01$ for *twk* and E6; *, P = 0.0013 for *EP1379*, Student's t test). (*B*) Expression of a dominant-negative allele of *sgg* (*UAS-sgg^{A81T}*) in the ORNs (using the *Or83b-GAL4* driver) results in significant protection against ethanol-induced loss of olfactory startle (n = 4, *, $P < 0.05$). (*C*) *sgg^{twk}* also demonstrates resistance to the ethanol-induced antennal-blackening phenotype (n = 7, *, $P < 0.01$), while *sgg^{E6}* shows a trend toward resistance, although the data did not achieve statistical significance. (*D*) Western analysis demonstrates that adult-specific Sgg proteins are expressed in antennae, and that both *sgg^{twk}* and *sgg^{E6}* result in a strong reduction of these proteins. Each lane was loaded with protein from 30 dissected antennae.

no TUNEL staining, whereas antennae dissected from flies 0 to 3 h after ethanol exposure exhibited TUNEL-positive nuclei (Fig. 3 *F* and *G*). Together, these results indicate that ethanol-induced ORN death occurs by apoptosis.

Curiously, the flies that were protected by expression of p35 in ORNs still displayed ethanol-induced antennal blackening (Fig. 3*B*), indicating that olfaction can be rescued independently of antennal morphology. It may be that antennal blackening in flies that were protected by expression of p35 in ORNs is a result of the death of nonneuronal cells, such as glia.

Ethanol-Induced ORN Death Requires the GSK3β Homolog Shaggy. To investigate the mechanism by which ethanol kills olfactory cells, we carried out a genetic screen for mutations that cause flies to be resistant or hypersensitive to the toxic effects of a single ethanol exposure. We identified *shaggy* (*sgg*), encoding the *Drosophila* homolog of GSK-3β. The allele of *sgg* isolated in our screen, *sgg^{twk}* (28), was resistant to the toxic effects of ethanol on both olfactory startle and antennal morphology (Fig. 4 *A* and *C*). Two additional loss-of-function alleles of *sgg* showed similar effects on the startle response, and one of these, *sgg^{E6}*, was modestly protective against antennal blacking (see Fig. 4 *A* and *C*). Immunoblotting revealed that both *sgg^{twk}* and *sgg^{E6}* reduced antennal expression of adult-specific Sgg proteins to <10% of control values (Fig. 4*D*). Expression of a dominant-negative form of Sgg, Sgg^{A81T} (29), in ORNs produced resistance to the effects of ethanol (Fig. 4*B*). Together, these data suggest that Sgg functions in ORNs to promote ethanol-induced damage.

To test whether the effects of *sgg* were caused by developmental requirements or, alternatively, because of a change in acute response to ethanol, we tested if adult flies could be protected from the damaging effects of ethanol with the GSK-3β inhibitor lithium. We fed adult wild-type flies food containing a range of lithium chloride (LiCl) concentrations for 7 days, exposed them to ethanol on day 5, and examined them for olfactory startle and antennal morphology on day 7. LiCl had a dose-dependent protective effect on ethanol-induced antennal blackening (Fig. 5*A*). Ten millimolar LiCl also had a protective effect on the olfactory startle (Fig. 5*B*), although this effect was not increased further at higher LiCl concentrations, perhaps because of pleiotropic effects on behavior. Feeding flies equivalent concentrations of other salts, such as KCl or NaCl, had no effect, indicating that protection is specific to LiCl (Fig. S3). Consistent with the behavioral data, there were surviving ORNs in 62% of antennae from ethanol-exposed flies fed 10 mM LiCl, compared to only 22% in control antennae (Fig. 5 *C* and *D*). Thus, both the behavioral and anatomical effects of ethanol

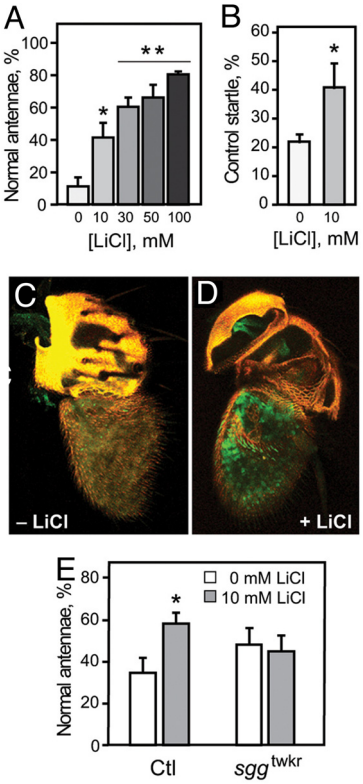

Fig. 5. Lithium prevents ethanol-induced programmed cell death in adult flies. (*A*) Flies fed LiCl-containing food for 5 days before ethanol exposure show a dose-dependent resistance to the damaging effects of ethanol on antennal morphology ($n = 6$, *, $P < 0.05$ for 0 vs. 10 mM; **, $P < 0.01$ for 0 vs. 30, 50, or 100 mM). (*B*) LiCl feeding also protects against startle loss caused by a single ethanol preexposure ($n = 6$, *, $P = 0.038$, Student's *t* test). (*C* and *D*) Confocal reconstructions of antennae from flies expressing GFP in ORNs under the control of the *Or83b-GAL4* driver. Ethanol exposure did not cause loss of ORNs in the third antennal segment in flies that were fed 10-mM LiCl for 5 days before exposure (*D*), as is seen in control flies that were not fed LiCl (*C*). GFP, green; yellow, autofluorescence of the cuticle. (*E*) The protective effect of LiCl requires *sgg*. sgg^{twk} and control (Ctl) flies were fed food containing 10-mM LiCl for 5 days and exposed to ethanol vapor for 45 min (all other experiments involved 25-min exposures). The low dose of LiCl and the longer exposure to ethanol were chosen to minimize a potential ''ceiling effect'' that might be expected in the already-resistant sgg^{twk} allele. Controls showed the expected protective effect of LiCl ($n = 7$, *, $P = 0.005$, Student's *t* test), while sgg^{twk} was not protected by LiCl (, $P = 0.39$, Student's *t* test).

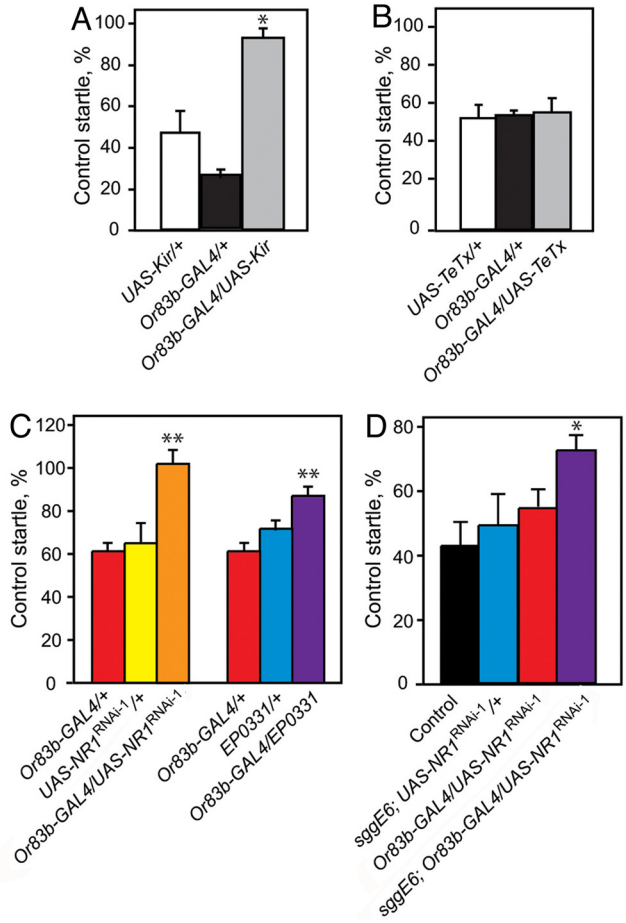

Fig. 6. Electrical silencing protects ORNs from ethanol-induced damage. (*A*) Expression of Kir2.1 (*UAS-Kir*) in ORNs using the *Or83b-GAL4* driver protects flies from ethanol-induced loss of olfactory startle ($n = 6$, *, $P < 0.01$). Data are presented as % startle retained by preexposed flies compared to unexposed flies of the same genotype. (*B*) Synaptic silencing by expression of tetanus-toxin light chain (TeTx) had no protective effect ($n = 4$). (*C*) Double-stranded RNA interference ($UAS\text{-}NR1^{RNAi}$) and antisense *dNR1* (EP0331) expression protects flies against ethanol-induced startle loss ($n = 5$, **, $P < 0.01$, *, $P < 0.05$). (*D*) Combining mutation of *sgg* with $UAS\text{-}NR1^{RNAi}$ driven by *Or83b-GAL4* results in a supra-additive protective effect. While sgg^{E6} and *Or83b-GAL4*/$UAS\text{-}NR1^{RNAi}$ flies both demonstrate weak resistance, the combination results in a synergistic effect ($n = 3$, *, $P < 0.05$).

exposure can be ameliorated by acute pharmacological intervention with a GSK-3β inhibitor.

To confirm that the protective effect of LiCl was a result of inhibition of Sgg/GSK-3β, rather than another target, we tested the effect of LiCl on sgg^{twk} mutant flies. While sgg^{twk} was itself resistant to the damaging effects of ethanol, significant antennal blackening was produced in the mutant by increasing the dose of ethanol, thus allowing us to assay protection by LiCl. LiCl had no effect on ethanol-induced antennal damage in sgg^{twk} mutant flies, while control flies were significantly protected (Fig. 5*E*). These data show that at least some of the protective effects of LiCl are mediated by inhibition of Sgg.

Electrical Silencing Protects the ORNs From Ethanol-Induced Apoptosis. GSK3β has been implicated in cell death in response to a variety of insults, including excitotoxicity (18, 30). To test if excessive neural activity mediates ethanol-induced apoptosis of ORNs, we silenced the majority of ORNs by expression of the mammalian inward rectifying K^+ channel Kir2.1 (31) using *Or83b-GAL4*. This manipulation fails to silence all neurons required for ethanol startle (see below), most likely because of the fact that there are two families of ORNs that do not express Or83b. Nevertheless, it almost completely abolished the loss of olfactory startle induced by ethanol preexposure. Flies expressing Kir2.1 in Or83b-positive ORNs retained 96% of their startle after exposure to ethanol, compared with 41 to 61% in control flies (Fig. 6*A*), an effect that was not observed upon synaptic silencing of ORNs by expression of tetanus-toxin light chain (32) (Fig. 6*B*). Thus, ethanol-induced apoptosis of ORNs requires their activity.

Ethanol-Induced Neuronal Death is Not Cell-Autonomous. To ask if ethanol-induced apoptosis is cell-autonomous, we used *Or67d-GAL4* and *Or22a-GAL4* to drive expression of *UAS-p35* in trichoid or basiconic sensilla, respectively. The flies also carried *UAS-GFP* to mark p35-expressing cells. We exposed the flies to ethanol and examined the survival of GFP-labeled ORNs. Expression of p35 under the control *Or67d-GAL4* was not protective (47% antennal survival, comparable to controls).

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Expression under the control of *Or22a-GAL4* was modestly protective, although lower than when p35 was expressed under the control of *Or83b-GAL4* (Table S1). Thus, expression of p35 in *Or67d-GAL4*-expressing cells is not sufficient to protect those cells, strongly suggesting that ethanol-induced cell death is not cell-autonomous. The greater protective effect seen with *Or22a-GAL4*, compared to *Or67d-GAL4*, is interesting, given that the former is expressed in a far greater number of ORNs (see Fig. S1). Thus, the protective effect is proportional to the fraction of cells expressing p35, which strengthens the hypothesis that ethanol-induced ORN death is nonautonomous.

Curiously, flies are still able to startle in response to ethanol even when ≈80% of their ORNs are inactivated by Kir2.1 or tetanus toxin expression. Similarly, *Or83b* null mutants, in which dendritic localization of OR proteins is disrupted, leading to severe defects in odor-responsive behaviors (21), display a normal startle in response to the smell of ethanol (Fig. S4). These results indicate that the Or83b-expressing ORNs are not responsible for the olfactory response to ethanol. The fact that we are nevertheless able to protect the startle behavior through expression of p35 or Kir2.1 in the Or83b-expressing ORNs further implies that the protective effect (and, by extension, the widespread ORN death caused by ethanol) is not cell-autonomous. Finally, we never observe ethanol-exposed antennae in which only a subset of the ORNs have died; thus, ethanol-induced ORN death is an all-or-none phenomenon.

Excitotoxic Death of ORNs Is Mediated By NMDA Receptors. Because many of ethanol's toxic effects on neurons are mediated by interactions with NMDA receptors, and because acute ethanol exposure can lead to NMDA receptor-mediated excitotoxic cell death in cortical (12) and aminergic (13) neurons, we hypothesized that the acute neurotoxic effects of ethanol on *Drosophila* ORNs might be caused by over-stimulation via NMDA receptors. To test this hypothesis, we examined the effects of genetic manipulation of the NR1 subunit of the NMDA receptor in ORNs.

We down-regulated *dNR1* expression with two, independently generated double-stranded RNA interference constructs, *UAS-NR1*$^{\text{RNAi-1}}$ and *UAS-NR1*$^{\text{RNAi-2}}$ (33). These constructs reduce dNR1 transcript (by 47% when driven in neurons) and protein levels (33), respectively. Flies expressing either *UAS-NR1*$^{\text{RNAi}}$ transgene under the control of the *Or83b-GAL4* driver showed significant protection of olfactory startle after ethanol exposure (Fig. 6*C* and Fig. S5). We confirmed this result by driving expression from EP0331, a UAS-containing P element inserted in the 3′ end of *dNR1*. EP0331 is inserted in an orientation to drive antisense expression (34), and it has been shown that driving EP0331 with *hsp70-GAL4* results in a significant reduction in dNR1 (35). *Or83b-GAL4/*$^{+}$; *EP (3)0331/*$^{+}$ flies, like those expressing *UAS-NR1*$^{\text{RNAi}}$, were resistant to startle loss caused by ethanol exposure (see Fig. 6*C*).

To establish that the protective effects on olfactory startle were caused by neuroprotective effects of inhibiting dNR1 expression, we examined the ORNs of flies coexpressing *UAS-NR1*$^{\text{RNAi}}$ (or antisense) and GFP in ORNs. Down-regulation of dNR1 in ORNs led to enhanced survival after ethanol exposure: on average, 56% of antennae from flies with reduced dNR1 retained GFP expression, compared with only 35% of antennae from control flies.

Finally, it is known that lithium can protect against NMDA-receptor-mediated excitotoxic neuronal death in both cell culture and rodent models (36, 37). Thus, mutation of *sgg* and down-regulation of dNR1 may exert their protective effects by impairing the same cellular pathway. If this were the case, the combination of *sgg* mutation and dNR1 down-regulation should result in a synergistic effect. To test this hypothesis, we subjected flies of genotype *sgg*^{E6}; *Or83b-GAL4/UAS-NR1*$^{\text{RNAi-1}}$, as well as control flies bearing only one or neither of the two genetic manipulations, to a high dose of ethanol (to overcome the baseline resistance phenotypes). The experimental flies retained 74% of control startle, compared to the 65% predicted if the effects of the two manipulations were independent. This greater-than-additive resistance suggests that Sgg and dNR1 are conveying resistance by way of a common pathway.

Summary. We show that exposure of adult flies to a single sedating dose of ethanol vapor results in widespread death of cells in the third antennal segment and a consequent loss of olfaction. The effect is dose-dependent, requires Sgg/GSK-3β, and can be prevented by treatment with the GSK-3β inhibitor LiCl. Ethanol-induced death of the antennal ORNs is non-cell-autonomous, apoptotic, and dependent on electrical activity and function of the NMDA receptor. This system will allow the study of ethanol-induced neuronal apoptosis in an organism that is amenable to rapid and complex genetic manipulations, likely leading to insights into the genes involved in sensitivity to ethanol neurotoxicity and a greater understanding of the molecular processes of neuronal death in alcoholic dementia. The system will also allow screening for drugs that can prevent ethanol-induced neuronal apoptosis. Finally, neurons were protected from ethanol-induced apoptosis by inhibiting Sgg/GSK3β with LiCl, indicating the possible utility of GSK3β as a target for preventative therapy in alcoholic neurodegeneration.

Experimental Procedures

***Drosophila* Strains and Culture.** Flies were raised at 25 °C and 70% humidity on standard cornmeal/molasses medium. All experiments were carried out in a *white*^{1118} Berlin genetic background. Behavioral assays used 20 to 25 male flies aged 2 to 4 days after eclosion at the start of the experiment. Flies analyzed for behavior were subjected to brief (<5 min) CO_2 anesthesia no <24 h before behavioral assays. For source of fly strains, see the *SI Text*.

Olfactory Startle. To assay olfactory startle, we used the locomotor tracking system (19) (see the *SI Text* for details).

LiCl Feeding. Standard cornmeal molasses medium was supplemented with LiCl to the desired final concentration (0 to 100 mM). Twenty to twenty-five 2-day-old male flies were placed on the medium and allowed to feed for 5 days. In initial experiments, 0.5% FD&C blue #1 was added to the food to verify consumption. After 5 days, flies were exposed for 30 min to 100:50 E/A, then placed back on the lithium-containing food for 2 more days. Flies were then subjected to behavioral and visual assays, as described above.

Western Blots and Immunohistochemistry. Western blots and immunohistochemistry were carried out using standard procedures (see *SI Text* for details).

Statistical Analyses. All analyses are one-way ANOVA with Tukey HSD posthoc analysis, unless otherwise indicated.

ACKNOWLEDGMENTS. We thank L. Vosshall for technical advice and fly stocks, G. Ophir-Shohat for technical advice, Fred Wolf for sharing unpublished information about *sgg* alleles, and A. Rothenfluh, I. King, and D. Tran for helpful discussions. This work was supported by National Institutes of Health/National Institute of Alcohol Abuse and Alcoholism Grants F32 AA015000 (to R.L.F.), and AA10035 and AA13105 (to U.H.).

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