Behavioral/Cognitive

A Small Group of Neurosecretory Cells Expressing the Transcriptional Regulator *apontic* and the Neuropeptide *corazonin* Mediate Ethanol Sedation in Drosophila

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In the fruit fly *Drosophila melanogaster*, as in mammals, acute exposure to a high dose of ethanol leads to stereotypical behavioral changes beginning with increased activity, followed by incoordination, loss of postural control, and eventually, sedation. The mechanism(s) by which ethanol impacts the CNS leading to ethanol-induced sedation and the genes required for normal sedation sensitivity remain largely unknown. Here we identify the gene *apontic* (*apt*), an Myb/SANT-containing transcription factor that is required in the nervous system for normal sensitivity to ethanol sedation. Using genetic and behavioral analyses, we show that *apt* mediates sensitivity to ethanol sedation by acting in a small set of neurons that express Corazonin (Crz), a neuropeptide likely involved in the physiological response to stress. The activity of Crz neurons regulates the behavioral response to ethanol, as silencing and activating these neurons affects sedation sensitivity in opposite ways. Furthermore, this effect is mediated by Crz, as flies with reduced *crz* expression show reduced sensitivity to ethanol sedation. Finally, we find that both *apt* and *crz* are rapidly upregulated by acute ethanol exposure. Thus, we have identified two genes and a small set of peptidergic neurons that regulate sensitivity to ethanol-induced sedation. We propose that Apt regulates the activity of Crz neurons and/or release of the neuropeptide during ethanol exposure.

Introduction

Alcohol is a commonly abused drug, capable of altering the function of the CNS in humans and numerous animals. Studies indicate a strong genetic component to the risk for alcohol use disorders (AUDs), although few genes contributing to this risk have been identified (Goldman et al., 2005; Mayfield et al., 2008). Individuals with reduced sensitivity to the sedative effects of alcohol show increased risk for developing AUDs (Schuckit and Smith, 1996; Schuckit, 2000). Thus, identifying genes that influence alcohol sedation is critical for understanding the molecular basis for how alcohol impacts the CNS, as well as possibly mediating AUDs. In the fruit fly *Drosophila melanogaster*, as in mammals, acute exposure to high doses of alcohol initiate a series of behavioral changes beginning with increased locomotion, followed by incoordination and eventual sedation (Moore et al., 1998; Singh and Heberlein, 2000). Using genetic and neuroanatomical approaches, we identify two genes and a small set of peptidergic neurons that regulate ethanol-induced sedation: the transcriptional regulator *apontic* (*apt*), the neuropeptide *corazonin* (*crz*), and Crz-expressing neurosecretory cells.

Apt was first identified in *Drosophila* as a transcription factor involved in embryonic tissue morphogenesis (Eulenberg and Schuh, 1997; Gellon et al., 1997; Su et al., 1999). More recently, Apt was shown to promote synaptic function at the neuromuscular junction (Takasu-Ishikawa et al., 2001), and as a feedback inhibitor of the Jak/Stat pathway (Baeg et al., 2005; Starz-Gaiano et al., 2008). The human protein with the highest sequence homology to Apt is Fibrinogen silencer binding protein (FSBP), a negative regulator of transcription of the gamma chain of *fibrinogen* (Mizuguchi et al., 1995; Starz-Gaiano et al., 2008).

Crz is a neuropeptide that in insects and crustaceans is highly conserved with respect to structure and neuronal expression (Boerjan et al., 2010). However, the physiological functions of Crz vary considerably; for example, while cardiostimulatory effects have been observed in cockroaches (Veenstra, 1989), Crz induces cuticle pigmentation during the gregarious phase in locusts (Tawfik et al., 1999). In *Drosophila*, adult flies lacking Crz show altered trehalose homeostasis and triglyceride levels (Lee et al., 2008; Zhao et al., 2010), implicating Crz in fly metabolism and energy allocation. Crz function has also been linked to the fly's stress response, as *crz* transcription is inhibited by various stres-

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sors (Zhao et al., 2010). Additionally, Crz is thought to be the mammalian homolog of gonadotropin-releasing hormone (GnRH) (Cazzamali et al., 2002), which is directly regulated by stress hormones (Nikolarakis et al., 1986; Tellam et al., 1998; Li et al., 2004).

We identify Apt as a regulator of sensitivity to ethanol sedation. We show that Apt function in a small number of neurons, those expressing the neuropeptide Crz, is both necessary and sufficient for normal sensitivity to ethanol sedation. Ethanol sensitivity is also dependent on the activity of Crz neurons and the expression of the neuropeptide. We postulate that Apt and Crz, and/or the neurons in which they function, may be among the initial targets of ethanol that regulate sensitivity to its sedative effects.

Materials and Methods

Animals. Flies were raised on standard cornmeal/molasses food at 25°C and 70% relative humidity and were grown in constant light, unless otherwise noted. Flies used for behavioral testing were backcrossed for five generations to the w^{1118} Berlin genetic background; exceptions are noted below. All behavioral experiments used 2- to 4-d-old males tested at ~25°C; exceptions are noted below. For behavioral testing, each different genotype was tested over multiple days, from at least two independent sets of crosses.

Fly stocks. apt^{13-66} flies were among the collection of ~2000 mutant strains carrying P-element (PGawB; contains a GAL4 enhancer trap) insertions generated in the Heberlein lab. apt^{167}/*CyO*, $apt^{P\Delta3}$/*CyO*, $apt^{P\Delta3}$/*CyO*, and *UAS-apt (III)* flies were obtained from Denise Montell (The Johns Hopkins University, Baltimore, MD; Eulenberg and Schuh, 1997; Starz-Gaiano et al., 2008). The apt^{167}/*CyO*, $apt^{P\Delta3}$/*CyO*, and $apt^{P\Delta4}$/*CyO* flies were in the w^{1118} background on the X and III chromosomes. The $apt^{GS11714}$ flies were obtained from the Drosophila Genetic Resource Center in Kyoto, Japan. $elav^{C155}$-GAL4 (*elav-GAL4*), *crz-GAL4*, *tub-GAL80^{ts}*, *UAS-dcr2*, *UAS-Kir2.1*, *UAS-dTrpA1*, *UAS-apt^{RNAi2}*, and *UAS-Crz^{RNAi2}* flies were obtained from the Drosophila Stock Center in Bloomington, Illinois. *UAS-apt^{RNAi}* (v4289) and *UAS-crz^{RNAi}* (v106876) were obtained from the Vienna Drosophila RNAi Center (Dietzl et al., 2007). Both of the *apt* RNAi constructs (*UAS-apt^{RNAi}* and *UAS-apt^{RNAi2}*) have several predicted off-target effects. Ethanol sedation sensitivity was observed expressing *UAS-apt^{RNAi}* with several pars intercerebralis (PI)-specific GAL4 drivers (12–10, 10–227, 10–260, and 7–56) of unknown cellular identities; these GAL4 drivers were generated in our laboratory. The PI is a region of the brain containing several peptidergic neurons (Siegmund and Korge, 2001).

Ethanol sedation assays. Ethanol sedation assays were performed as previously described (Corl et al., 2009), with minor modifications. Briefly, 20 2- to 4-d-old males of each genotype raised at 25°C were allowed to equilibrate to room temperature (21−23°C) for 30 min, then introduced into "booze" tubes (long clear tubes) and placed into the booz-o-mat chamber. After 2–5 min exposure to humidified air to equilibrate the flies to the apparatus, a continuous stream of ethanol vapor (110 U ethanol/40 U air; 73% ethanol) was applied to the tubes for 30 min, unless noted otherwise. During the 30 min exposure period, flies were mechanically stimulated (by twirling the tubes) at 5 min intervals (seven time points). At each time point, the numbers of immobile flies were counted. The time to 50% sedation (ST50) was calculated as previously described (Rothenfluh et al., 2006).

All sedation assays were performed at ~21−23°C; the only exceptions to this were experiments with TrpA1 activation and inactivation performed at 29 and 22°C, respectively. To avoid acute neuronal activation in the TrpA1 experiments, both the experimental and control flies were raised at 18°C and only removed from this temperature-controlled environment for ~5–10 min before behavioral testing.

Real-time quantitative RT-PCR. Quantitative PCR (qPCR) was performed as previously described (McClure et al., 2011). Primers used to amplify the five alternative *apt* transcripts, *apt-RA*, *apt-RB*, *apt-RC*, *apt-RD*, and *apt-RE* were as follows: *apt-RA*, 5′-TGCTTACACAACCAAGAAACCA-3′ and 5′-GGTGGCCGAATACTGTTTGT-3′; *apt-RB*, 5′-CAGTTCCTTTGGTTCTTGGAA-3′ and 5′-GGTGGCCGAATACTGTTTGT-3′; *apt-RC*, 5′-ATTCAAAAGGCTCGCGAATA-3′ and 5′-GCGGCAATCTTCATAAAAGC-3′; *apt-RD*, 5′-ACTTTTGCCGGAATTGTGTC-3′ and 5′-CCGAAAAGCGAGAGTTTTTG-3′; *apt-RE*, 5′-AGCTGCCAACCCCTTTTTAT-3′ and 5′-GGTGGCCGAATACTGTTTGT-3′. *apt* (common to all five transcripts), *crz*, and *rp49* probe and primer sets (Dm01820107_g1, Dm02135830_g1, and Dm02151827_g1, respectively) were obtained from Applied Biosystems.

Molecular biology. Genomic DNA flanking the apt^{13-66} and $apt^{GS11714}$ insertions was isolated using inverse PCR. Comparison with the *Drosophila* genome sequence on Flybase (www.flybase.org) revealed that the insertions were located 295 and 180 base pairs upstream, respectively, of the *apt-RA* transcription start site. The P-element in apt^{13-66} flies was precisely excised using the *Δ2–3Sb* transposase (Robertson et al., 1988). Primers used to confirm the precise excision were as follows: 5′-AGCGAGAAAAGCAAAGACGA-3′ and 5′-TTCGCACTGCTTCACGTTTA-3′.

Immunohistochemistry. Whole-mount brains were fixed at room temperature in 4% paraformaldehyde in PBS. The neuropile-specific antibody NC82 was used at 1:50 (The Jackson Laboratory), mouse-anti-Fasciclin II was used at 1:200 (Drosophila Hybridoma Bank), rabbit-anti-GFP was used at 1:200 (Clontech), mouse-anti-GFP was used at 1:200 (Roche), rabbit-anti-Apontic was used at 1:100 (Eulenberg and Schuh, 1997), and rabbit-anti-Crz was used at 1:500 (Veenstra and Davis, 1993).

Ethanol concentration assays. Internal ethanol concentrations were measured in homogenized adult extracts from *wB* and apt^{13-66} flies exposed to a sedating dose of ethanol (73% ethanol). Animals were exposed to vaporized ethanol for 0, 5, 10, 20, and 30 min then snap-frozen in liquid nitrogen and assayed for ethanol content using a colorimetric enzymatic kit (Diagnostic Chemicals) (Moore et al., 1998).

Statistics. Statistics were performed using JMP 7.0.1 2007 (SAS Institute). Statistical significance was established using one-way ANOVA, followed by Tukey–Kramer *post hoc* tests for multiple comparisons. Error bars indicate SEM. Statistical significance ($p < 0.05$) is given when the experimental group differs both from the *GAL4* and *UAS* transgene controls. In all graphs, $^{***}p < 0.0001$, $^{**}p < 0.001$ and, $^{*}p < 0.01$.

Results

13–66 flies show reduced sensitivity to ethanol sedation

We screened a collection of mutant strains, each carrying individual P-element insertions, for altered sensitivity to ethanol-induced sedation (data not shown). Flies exposed to a high concentration of ethanol (73% ethanol vapor) lose postural control and exhibit a loss of righting reflex (LORR) (Rothenfluh et al., 2006). To determine the fly's ethanol sedation sensitivity, we measured the time needed for 50% of the flies to lose their LORR (time to 50% sedation, ST50). One mutant fly strain, *13–66*, displayed a dramatic reduction in ethanol sedation sensitivity, with a significantly increased ST50 compared with control flies (Fig. 1*A*,*B*; $F_{(1,51)} = 42.59$, $p < 0.0001$). The reduced sensitivity to ethanol sedation displayed by *13–66* flies was not due to altered ethanol pharmacokinetics, as control and mutant flies contained similar internal ethanol concentration when measured after different times of ethanol exposure (data not shown).

Molecular characterization of the *apt* locus and *apt* mutants

The P-element in *13–66* flies was mapped by inverse PCR and DNA sequence analysis to the gene *apontic* (*apt*) (Fig. 1*D*). The P-element insertion in *13–66* flies is responsible for the reduced sedation sensitivity, since precise excision of the transposon caused complete reversion to the wild-type phenotype (Fig. 1*C*). Several additional alleles of *apt* also displayed reduced ethanol sedation sensitivity, including another P-element insertion, $apt^{GS11714}$ ($F_{(1,42)} = 13.05$, $p = 0.0008$), and two alleles carrying genomic deletions in the gene, $apt^{P\Delta3}$/+ ($F_{(1,43)} = 54.52$, $p < 0.0001$) and $apt^{P\Delta4}$/+ ($F_{(1,42)} = 35.90$, $p < 0.0001$); the latter were

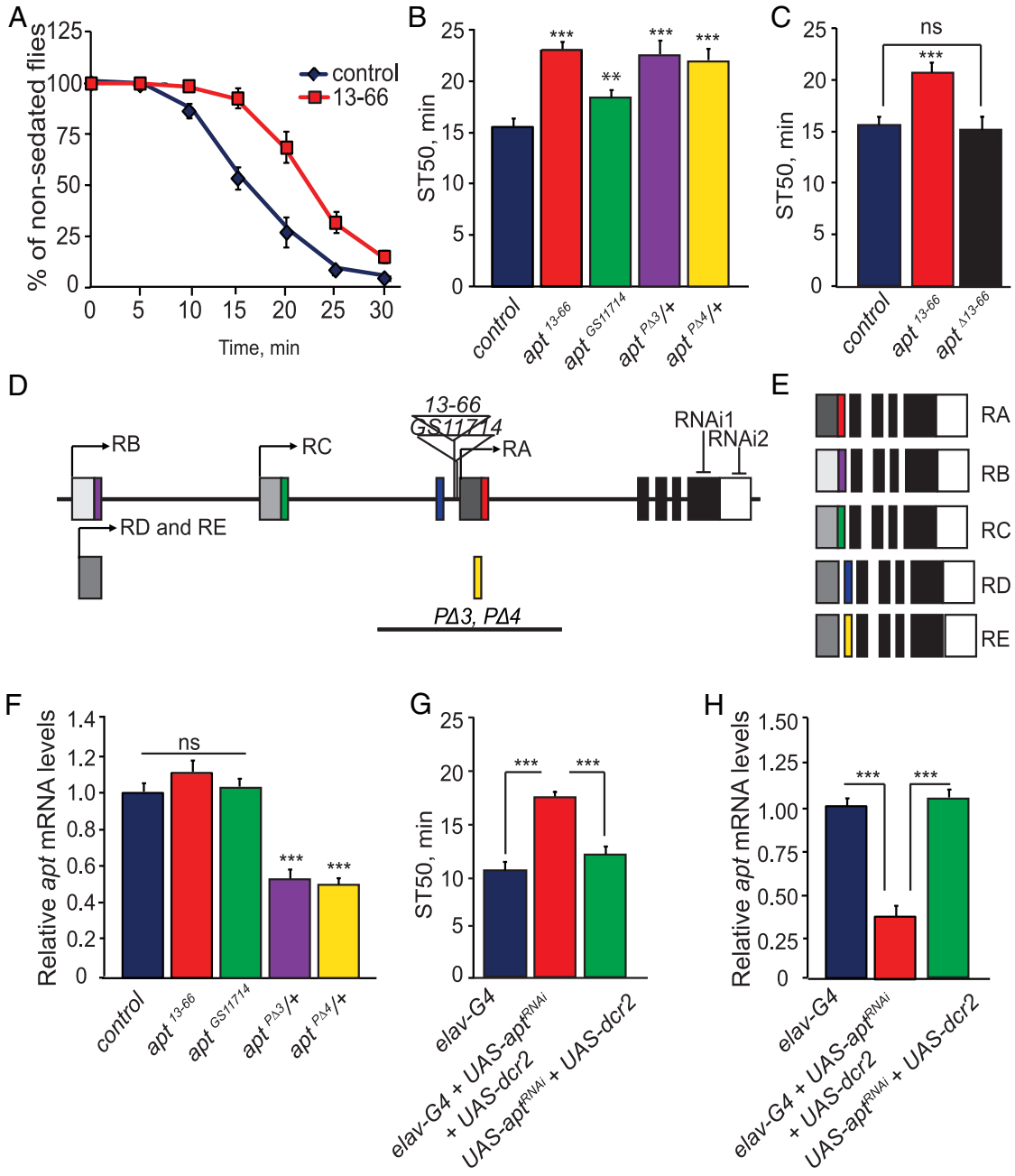

Figure 1. *apt* mutants display reduced ethanol sedation sensitivity. ***A***, Control (*wB*) and apt^{13-66} flies were exposed to a sedating dose of ethanol (73% ethanol vapor) for 30 min. The number of sedated flies was measured over the course of the exposure. ***B***, Quantification of ethanol sedation sensitivity in different *apt* mutant strains. The time at which 50% of the flies were sedated, the mean sedation time (ST50), was calculated by linear interpolation. ST50 values of *apt* mutant strains differed significantly from control (*wB*) flies (one-way ANOVA, Tukey–Kramer *post hoc*, $n = 16$). ***C***, Precise excision of the P-element in apt^{13-66} flies ($apt^{\Delta 13-66}$) completely reverted the ethanol sedation sensitivity phenotype. (Student's *t* test, $n = 12$) ***D***, ***E***, Schematic of the *apt* gene. The *apt* gene is predicted to encode for five alternative transcripts, producing five distinct protein isoforms. Transcription start sites for each of the transcripts are indicated by arrows; the same transcription start site is used for both the *apt-RD* and *apt-RE* transcripts. Translated regions are indicated in colors and black, while untranslated regions are in shades of gray. Locations of the P-element insertions in apt^{13-66} and $apt^{GS11714}$ mutant flies are indicated; both insertions are upstream of the *apt-RA* transcript. Locations of the inverted-repeat sequences used to generate RNAi knockdown of *apt* and the location of sequences deleted in the $apt^{P\Delta 3}$ and $apt^{P\Delta 4}$ mutant flies are also shown. ***F***, Expression of *apt* in control flies (*wB*) and apt^{13-66}, $apt^{GS11714}$, $apt^{P\Delta 3}/+$, and $apt^{P\Delta 4}/+$ mutant flies as quantified by qPCR. No difference in *apt* expression was observed between control (*wB*) and apt^{13-66} and $apt^{GS11714}$ mutant flies, while decreased *apt* expression to that of 50 and 55% was observed in $apt^{P\Delta 3}/+$ and $apt^{P\Delta 4}/+$ mutant flies, respectively. (Student's *t* test, $n = 3$). ***G***, RNAi knockdown of *apt* specifically in neurons reduces ethanol sedation sensitivity. ST50 values of flies coexpressing *dcr2* and apt^{RNAi} using pan-neuronal driver $elav^{C155}$-*GAL4* (*elav*^{C155}*-GAL4;UAS-dcr2/+;UAS-apt*^{RNAi}*/+*) differs significantly from the *GAL4* (*elav*^{C155}*-GAL4*) and *UAS* (*UAS-dcr2/+;UAS-apt*^{RNAi}*/+*) control flies (one-way ANOVA, Tukey–Kramer *post hoc*, $n = 16$). ***H***, *apt* expression upon RNAi knockdown. Pan-neuronal expression of apt^{RNAi} (*elav*^{C155}*-GAL4;UAS-dcr2/+;UAS-apt*^{RNAi}*/+*) reduces *apt* expression by 65% compared with the GAL4 (*elav*^{C155}*-GAL4*) and *UAS* (*UAS-dcr2/+;UAS-apt*^{RNAi}*/+*) controls. One-way ANOVA, Tukey–Kramer *post hoc*, $n = 3$). For this figure and all other figures, error bars indicate SEM and *p* values are ***$p < 0.0001$, **$p < 0.001$, and *$p < 0.01$.

tested as heterozygotes since homozygous mutant flies are not viable as adults (Fig. 1*B*) (Eulenberg and Schuh, 1997). To determine whether *apt* function is required in neurons for normal ethanol sedation sensitivity, we downregulated neuronal-specific expression of *apt* by RNA interference (RNAi). Pan-neuronal expression (using *elav-GAL4*) of a transgene (*UAS-apt*^{RNAi}) that targets *apt* by RNAi, along with *UAS-dicer2* (*UAS-dcr2*), a component of the RNAi machinery shown to increase RNAi efficacy

(Dietzl et al., 2007), caused a significant reduction in ethanol sedation sensitivity relative to control flies (Fig. 1*G*; $F_{(2,27)} = 46.96$, $p < 0.0001$). Importantly, *apt* transcript levels in flies with pan-neuronal expression of apt^{RNAi} (*elav-GAL4*;*UAS-dcr2*/+; *UAS-*apt^{RNAi}/+) were reduced by ~65% compared with control flies expressing the individual transgenes (Fig. 1*H*; $F_{(2,27)} = 49.06$, $p < 0.0001$). In addition, pan-neuronal expression of a second, nonoverlapping apt^{RNAi2} transgene without *dcr2* also caused the same phenotype (data not shown; Fig. 1*D*). These data demonstrate that neuronal expression of *apt* is required for normal ethanol sedation sensitivity.

The *apt* locus encompasses 35 kb and is predicted to encode five transcripts and five distinct protein isoforms (Fig. 1*D*,*E*). The five Apt proteins differ in their N termini, but share the same Myb/SANT motif, a DNA binding domain; previous work has shown that Apt binds DNA (Liu et al., 2003). Three of the *apt* transcripts use unique transcription start sites, while the *apt-RD* and *apt-RE* transcripts use a common start site (Fig. 1*D*). Locations of the P-element insertions in apt^{13-66} and $apt^{GS11714}$ flies are 295 and 180 base pairs upstream, respectively, of the *apt-RA* transcript (Fig. 1*D*). The two *apt* genomic DNA deletion mutants, $apt^{P\Delta3}$/+ and $apt^{P\Delta4}$/+, remove an exon from the *apt-RA*, *apt-RD*, and *apt-RE* transcripts (Eulenberg and Schuh, 1997; Starz-Gaiano et al., 2008). To determine how these mutations affect *apt* expression, we measured *apt* transcript levels in all mutant and control strains by qPCR. Primer sets recognizing each of the five *apt* transcripts revealed that *apt-RA* is the sole *apt* transcript expressed in the adult fly head (data not shown). Using primers specific to the *apt-RA* transcript and a primer/probe set that amplifies all five *apt* transcripts, we found similar expression of *apt* in the adult heads of apt^{13-66} (*t* test, $p = 0.1433$), $apt^{GS11714}$ (*t* test, $p = 0.167$), and wild-type control flies (Fig. 1*F*). However, the *apt* deletion mutants, $apt^{P\Delta3}$/+ and $apt^{P\Delta4}$/+, showed a near 50% reduction of *apt* expression compared with control flies (Fig. 1*F*; $apt^{P\Delta3}$/+, $p = 0.00093$ and $apt^{P\Delta4}$/+, $p = 0.00089$ by *t* test). The apparent lack of reduction of *apt* transcript levels in two of the *apt* mutant strains (apt^{13-66} and $apt^{GS11714}$), both showing reduced ethanol sedation sensitivity phenotypes, may be explained by *apt* expression being affected in only a small subset of neurons in the brain, a difference that would be undetectable by qPCR (see below). Importantly, both apt^{13-66} and $apt^{GS11714}$ fail to complement the embryonic lethality of a known null allele of *apt*, apt^{167} (Eulenberg and Schuh, 1997), indicating that apt^{13-66} and $apt^{GS11714}$ indeed affect *apt* and are likely hypomorphic mutations.

In summary, we have shown that reduced *apt* expression correlates with decreased ethanol sedation sensitivity. To establish a causal relationship we performed phenotypic rescue experiments that are described below.

apt is required in neurons during both metamorphosis and adulthood for normal ethanol sedation sensitivity

We have shown that neuronal expression of *apt* is required for normal ethanol sedation sensitivity (Fig. 1*G*). To determine when *apt* function is required in neurons to mediate this effect, we inhibited neuronal *apt* expression in a temporally specific manner using $GAL80^{ts}$, which represses GAL4 function at 18°C but not at 29°C (McGuire et al., 2003). We used RNAi to inhibit *apt* expression specifically during development (by raising flies at 29°C and shifting them to 18°C after adult eclosion) or specifically during adulthood (by raising flies at 18°C and shifting them to 29°C after eclosion). Interestingly, both manipulations decreased sensitivity to ethanol sedation, suggesting that *apt* function during both development and adulthood is required for normal sedation sensitivity (Fig. 2*A*,*B*; development-specific inhibition of *apt*, $F_{(2,28)} = 14.08$, $p < 0.0001$; adult-specific inhibition of *apt*, $F_{(2,49)} = 8.02$, $p = 0.001$). Thus, *apt* may have a role in regulating the development of the adult nervous system as well as its function. To more precisely define the developmental period during which *apt* function is required, we inhibited *apt* expression during specific stages of development (Fig. 2*C*,*D*). Neuronal inhibition of *apt* expression solely during metamorphosis, a period of intense remodeling of the developing adult fly CNS (Truman, 1990), caused a striking decrease in ethanol sedation sensitivity (Fig. 2*D*; $F_{(2,32)} = 25.55$, $p < 0.0001$). Remodeling of the adult fly CNS occurs mainly during metamorphosis; however, changes to the nervous system continue after adult eclosion (Truman, 1990). To determine whether *apt* function is specifically required during remodeling of the adult CNS (both during development and after eclosion) and/or in the mature adult CNS, we inhibited its expression during development and the initial 3 d after adult eclosion (by raising flies at 29°C and shifting them to 18°C 3 d after eclosion); we also inhibited *apt* expression during days 3–7 of adulthood (by raising flies at 18°C and shifting them to 29°C on the third day after eclosion). Both manipulations caused decreased sensitivity to ethanol sedation, suggesting that *apt* function in sedation sensitivity is required during remodeling of the adult CNS and in the mature adult CNS (Fig. 2*E*,*F*; inhibition of *apt* during CNS remodeling, $F_{(2,24)} = 6.08$, $p = 0.0034$; inhibition of *apt* during adulthood, $F_{(2,24)} = 5.03$, $p = 0.01$). Importantly, flies in which *apt* expression was presumably normal (maintained at 18°C during both development and adulthood) showed normal sedation sensitivity (Fig. 2*G*; $F_{(2,25)} = 1.58$, $p = 0.23$), while flies with continuous neuronal inhibition of *apt* expression (maintained at 29°C throughout development and adulthood) had significantly reduced ethanol sedation sensitivity (Fig. 2*H*; $F_{(2,28)} = 22.95$, $p < 0.0001$). From these results we conclude that *apt* function is required in neurons during two distinct life stages, metamorphosis and adulthood, to promote normal sensitivity to ethanol sedation.

apt^{13-66}-*GAL4* and Apt expression in the adult fly brain

To begin identifying the *apt*-expressing cells that regulate ethanol-induced sedation, we examined the expression of Apt in the adult brain. The transposon inserted into the *apt* locus in apt^{13-66} flies contains the transcriptional activator GAL4; thus, the cellular expression of GAL4 in apt^{13-66} flies likely reflects endogenous Apt expression. Flies carrying apt^{13-66}-*GAL4* as well as GFP reporters that allow localization of nuclei and neuronal projections [*UAS-GFP* (nls), *UAS-mCD8GFP*], showed GFP expression in the adult CNS that was limited to antennal lobe interneurons (AL-in), ventral neurons in the subesophageal ganglion (SOG), neurons in the PI, pars lateralis (PL), and some lateral neurons (Fig. 3*A*,*B*). The apt^{13-66}-*GAL4* expression pattern in the brain partially overlapped with Apt immunoreactivity in the AL-in, SOG, PI, PL, and lateral neurons (Fig. 3*C–F*). However, the Apt antibody detected additional cells in all of these brain regions, indicating that the apt^{13-66}-*GAL4* expression pattern, although more restricted, reproduces in part the endogenous Apt expression pattern.

To examine the brain anatomy of apt^{13-66} mutant flies we used the NC82 antibody, which labels synapses and thus neuropil (Laissue et al., 1999), and an antibody that recognizes Fasciclin II, a cell adhesion molecule that labels neurons of the ellipsoid body and mushroom body α/β and γ lobes (Cunningham et al., 1987; King et al., 2011). The gross morphology of the CNS in apt^{13-66} flies was normal compared with control flies (data not shown).

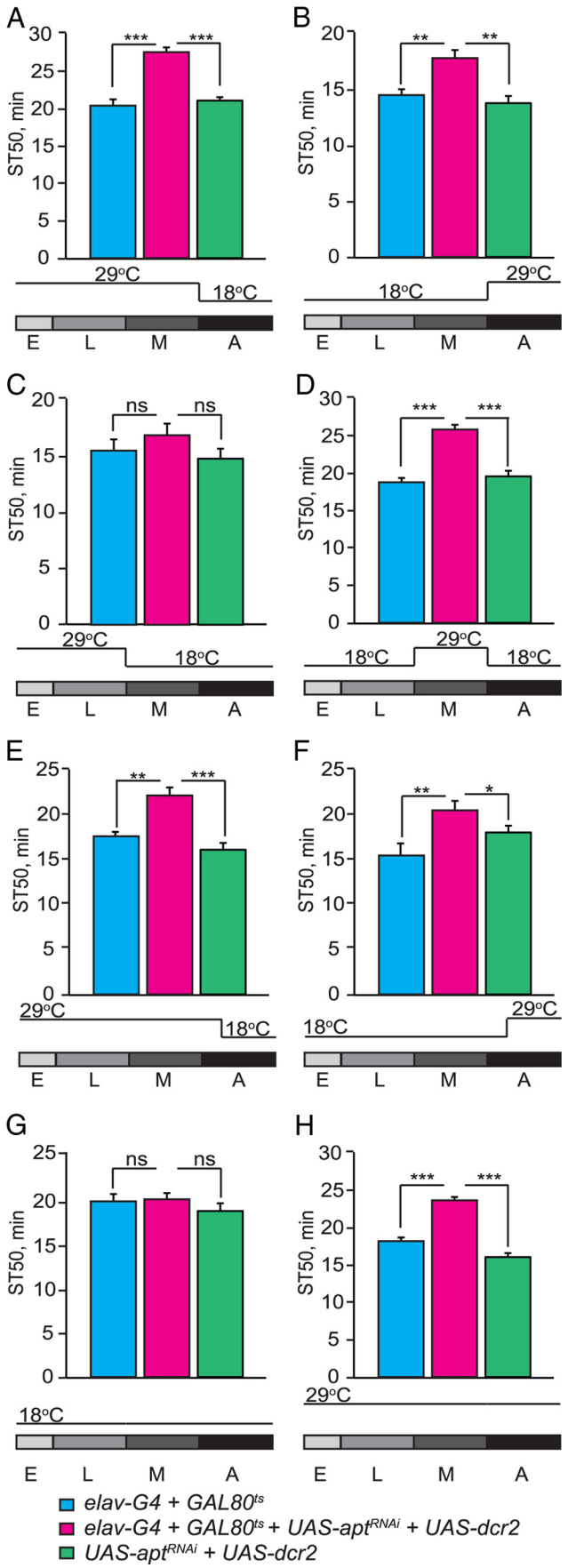

Figure 2. *apt* is required in neurons during both metamorphosis and adulthood for normal ethanol sedation sensitivity. ***A–D***, Temporal knockdown of *apt* expression was achieved by coexpressing *UAS-dcr2* and *UAS-apt*^{RNAi} with *elav*^{c155}*-GAL4* and *tub*-GAL80^{ts}. Flies were raised at 29 (GAL4 on, GAL80^{ts} off) and 18°C (GAL4 off; GAL80^{ts} on). ST50 values are shown for the experimental group with temporal inhibition of *apt* expression (*elav*^{c155}*-GAL4;UAS-dcr2/tub-GAL80*^{ts}*;UAS-apt*^{RNAi}/+), the *GAL4-GAL80*^{ts} (*elav*^{c155}*-GAL4; tub-GAL80*^{ts} /+), and *UAS* (*UAS-dcr2*/+;*UAS-apt*^{RNAi} /+) controls. ***A***, Neuronal inhibition of *apt* expression during development significantly reduces ethanol sedation sensitivity. Flies were raised at 29°C then shifted to 18°C upon adult eclosion until behavioral testing. ***B***, Neuronal inhibition of *apt* expression during adulthood reduces ethanol sedation sensitivity. Flies were raised at 18°C then shifted to 29°C upon adult eclosion until behavioral testing. ***C–F***, Temporal mapping of *apt* function during development. ***C***, Neuronal inhibition of *apt* expression during embryogenesis and larval development does not alter ethanol sedation sensitivity ($p > 0.05$, $n = 12$). Flies were raised at 29°C then shifted to 18°C from the start of metamorphosis until behavioral testing. ***D***, Neuronal inhibition of *apt* expression during metamorphosis reduces ethanol sedation sensitivity. Flies were raised at 18°C, then shifted to 29°C at the beginning of metamorphosis, and shifted back to 18°C upon adult eclosion until behavioral testing. ***E***, Neuronal inhibition of *apt* expression during development and three initial days after eclosion reduces ethanol sedation sensitivity. Flies were raised at 29°C until the third day after eclosion then shifted to 18°C for 4 d. ***F***, Neuronal inhibition of *apt* expression during adulthood, specifically days 3–7 of adulthood, decreases ethanol sedation sensitivity. Flies were raised at 18°C until the third day after eclosion, then shifted to 29°C for 4 d. ***G***, ***H***, Show temperature shift controls for mapping neuronal *apt* function. ***G***, Raising flies at 18°C during both development and adulthood (GAL4 off) leads to normal ethanol sedation sensitivity ($p > 0.05$, $n = 12$). ***H***, Raising flies at 29°C during both development and adulthood (GAL4 on) leads to decreased ethanol sedation sensitivity. (For all parts shown, one-way ANOVA, Tukey–Kramer *post hoc*, $n = 16$.)

apt is required in *corazonin*-expressing neurons for normal ethanol sedation sensitivity

To define specific neurons in which *apt* function is required to regulate ethanol-induced sedation, we screened a collection of 22 GAL4 lines using *UAS-apt*^{RNAi}. We selected GAL4 lines with expression limited to one or more of the following brain regions observed in the *apt*$^{13-66}$*-GAL4* and Apt expression patterns: AL-in; ventral neurons in the SOG; and neurons in the PI, PL, and lateral neurons. Since both the *apt*$^{13-66}$*-GAL4* and the Apt antibody reveal prominent expression in the PI and PL (Fig. 3), regions containing several peptidergic neurons (Siegmund and Korge, 2001), we screened additional GAL4 lines that drive expression in various classes of neuropeptidergic cells. A significant reduction in ethanol sedation sensitivity was observed when expressing *apt*^{RNAi} using several PI- and PL-specific drivers (see Materials and Methods) of unknown cellular specificity. More intriguingly, however, ethanol sedation sensitivity was dramatically reduced when expressing *UAS-apt*^{RNAi} with the neuropeptidergic driver *corazonin-GAL4* (*crz-GAL4*) (Fig. 4*A*; $F_{(2,24)} = 22.87$, $p < 0.0001$), indicating that Apt function is required in *crz*-expressing neurons.

To verify that Apt functions in *crz*-expressing cells, we first examined whether Apt was expressed in these neurons in the adult brain. In the Drosophila adult CNS, the neuropeptide Crz is expressed in 6–8 bilaterally symmetric neurons in the PL (Fig. 4*B*), and in a cluster of four abdominal ganglion neurons present only in male flies (Lee et al., 2008). Together, the PI and PL comprise the central neuroendocrine system in the Drosophila brain (de Velasco et al., 2007). We used the *apt*$^{13-66}$*-GAL4* driver to express a GFP reporter that drives GFP expression in nuclei [*UAS-GFP* (nls)] and immunostained the adult brains with antibodies recognizing both GFP and Crz. We observed three *apt*$^{13-66}$*-GAL4*-positive neurons, in the PL, which also express the neuropeptide Crz (Fig. 4*B*,*C*).

We previously showed that *apt* function is necessary in *crz*-expressing neurons to mediate normal ethanol sedation (Fig. 4*A*). To determine whether *apt* function in these cells is sufficient to regulate this behavior, we examined whether expression of

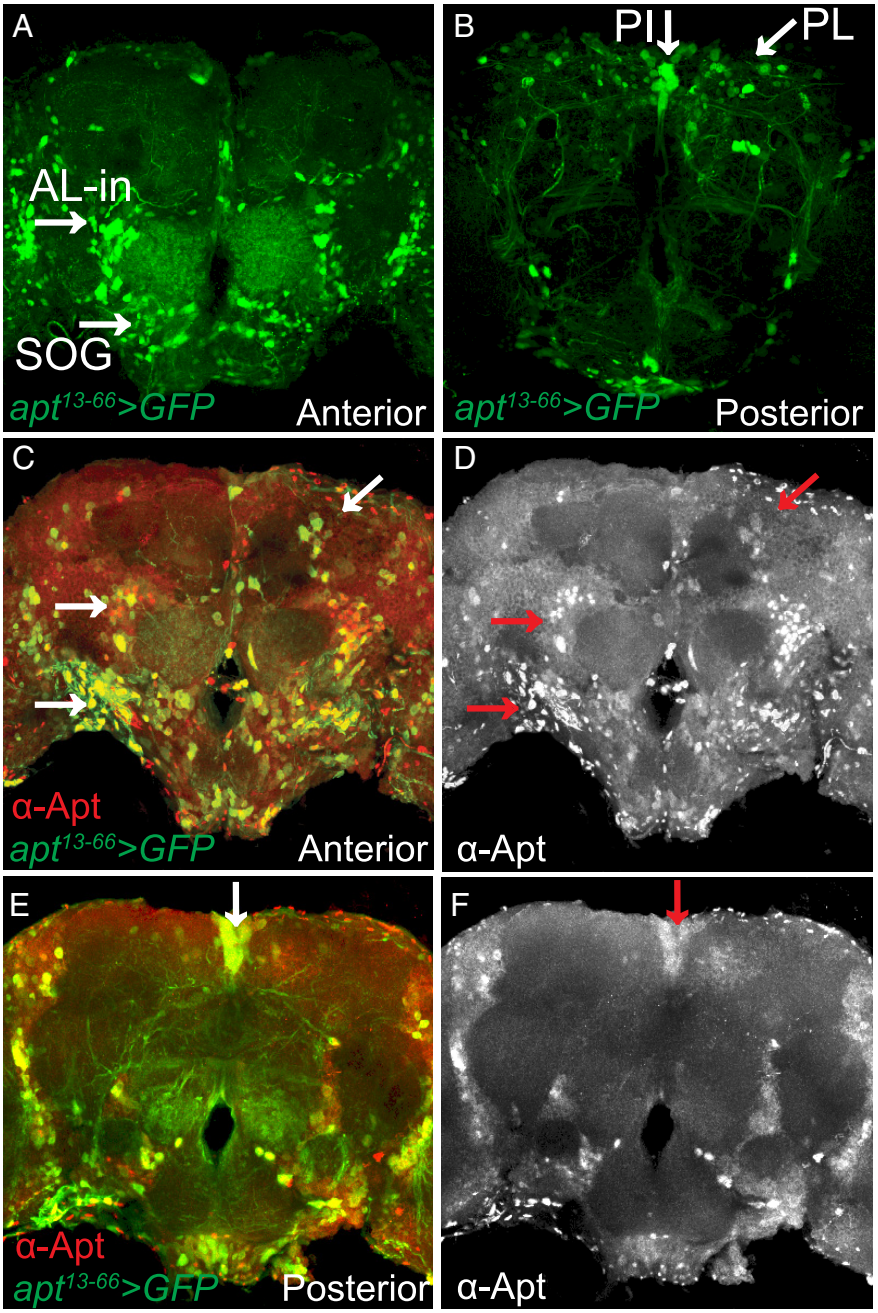

Figure 3. Apt expression in the adult CNS. ***A***, ***B***, Whole-mount brain from an adult male fly *apt*$^{13-66}$*-GAL4/UAS-GFP* (nls);*UAS-mCD8GFP/+* stained with anti-GFP. Confocal projections are from the anterior (***A***) and posterior (***B***) regions of the brain. ***A***, In the anterior brain, *apt*$^{13-66}$*-GAL4* expression (green) is found in AL-in and neurons in the SOG. ***B***, In the posterior brain, *apt*$^{13-66}$*-GAL4* expression is observed in neurons of the PL and PI. ***C–F***, Whole-mount brain from an adult male fly *apt*$^{13-66}$*-GAL4/UAS-GFP* (nls);*UAS-mCD8GFP/+* stained with anti-GFP (green) and anti-Apt (red). Projections are from the anterior (***C***, ***D***) and posterior (***E***, ***F***) regions of the brain. ***C***, ***E***, There is colocalization of *apt*$^{13-66}$*-GAL4* (green) and Apt immunoreactivity (red) in the AL-in, SOG, PL, and PI (yellow; white arrows). ***D***, ***F***, Anterior and posterior projections showing the expression pattern of Apt in the AI-in, SOG, PL, and PI (red arrows).

UAS-apt specifically in *crz*-expressing neurons would rescue the sedation resistance of *apt*$^{P\Delta3}$/+ mutant flies (*apt*$^{P\Delta3}$/+;*crz-GAL4/UAS-apt*) (Fig. 4*D*). Indeed, these flies showed normal ethanol sedation sensitivity (Fig. 4*D*; $F_{(3,61)} = 10.84$, $p = 0.839$), showing that increasing expression of *apt* in Crz neurons is sufficient for normal ethanol sensitivity. When introducing the *UAS-apt* transgene into *apt*$^{P\Delta3}$/+ mutant flies (*apt*$^{P\Delta3}$/+;*UAS-apt*/+), which lacks expression of GAL4, no behavioral rescue was achieved (Fig. 4*D*; $F_{(3,61)} = 10.84$, $p < 0.0001$). These results imply that reduced expression of *apt* specifically in *crz*-expressing neurons is responsible for the reduced ethanol sensitivity observed in *apt* mutant flies.

Activity of *crz*-expressing neurons mediates normal ethanol sedation sensitivity

We next asked whether *crz*-expressing neurons are required for normal ethanol-induced sedation. First, we tested whether ablation of *crz* neurons, using transgenes encoding the pro-apoptotic genes *reaper* (*rpr*) and *Head involution defective* (*Hid*) (*UAS-rpr and UAS-Hid*), affected ethanol sedation. Flies in which these transgenes were driven by *crz-GAL4* showed a complete loss of Crz immunolabeling in the adult brain (data not shown), and also displayed a significant reduction in sensitivity to ethanol sedation (Fig. 5*A*; $F_{(2,28)} = 75.45$, $p < 0.0001$) indicating that *crz*-expressing neurons and possibly Crz signaling regulate sensitivity to ethanol sedation.

To determine whether the activity of *crz*-expressing neurons regulates ethanol sedation sensitivity, we introduced transgenes driving the expression of either Kir2.1 or dTrpA1, which decrease or increase neuronal activity, respectively, to Crz neurons. Expression of *UAS-Kir2.1*, an inward rectifying K^+-channel that causes neuronal hyperpolarization (Baines et al., 2001), driven by *crz-GAL4*, in adult flies (using GAL80^{ts}) caused a marked reduction in ethanol sedation sensitivity (Fig. 5*B*; $F_{(2,45)} = 40.76$, $p < 0.0001$). Conversely, acute activation of *crz*-expressing neurons using the temperature-gated cation channel dTrpA1 (Hamada et al., 2008) (*crz-GAL4;UAS-dTrpA1*) led to increased ethanol sedation sensitivity compared with controls; specifically, increased sensitivity was observed when TrpA1 was active at 29°C ($F_{(2,39)} = 6.69$, $p = 0.0033$), but not when inactive at 22°C ($F_{(2,24)} = 0.52$, $p = 0.604$) (Fig. 5*C*). Together, these experiments show that silencing and activating *crz*-expressing neurons causes opposite ethanol sedation sensitivity phenotypes, suggesting that the activity of *crz*-expressing neurons directly promotes ethanol sedation sensitivity.

The neuropeptide crz is required for normal ethanol sedation sensitivity

We next asked whether the neuropeptide Crz is involved in the regulation of ethanol sedation sensitivity. To do so, we inhibited the expression of *crz* by RNAi. We found that RNAi-mediated down-regulation of *crz* in either *crz*-expressing neurons or pan-neuronally (using the *crz-GAL4* and *elav-GAL4* drivers, respectively), in the absence of *dcr2*, caused a significant reduction in ethanol sedation sen-

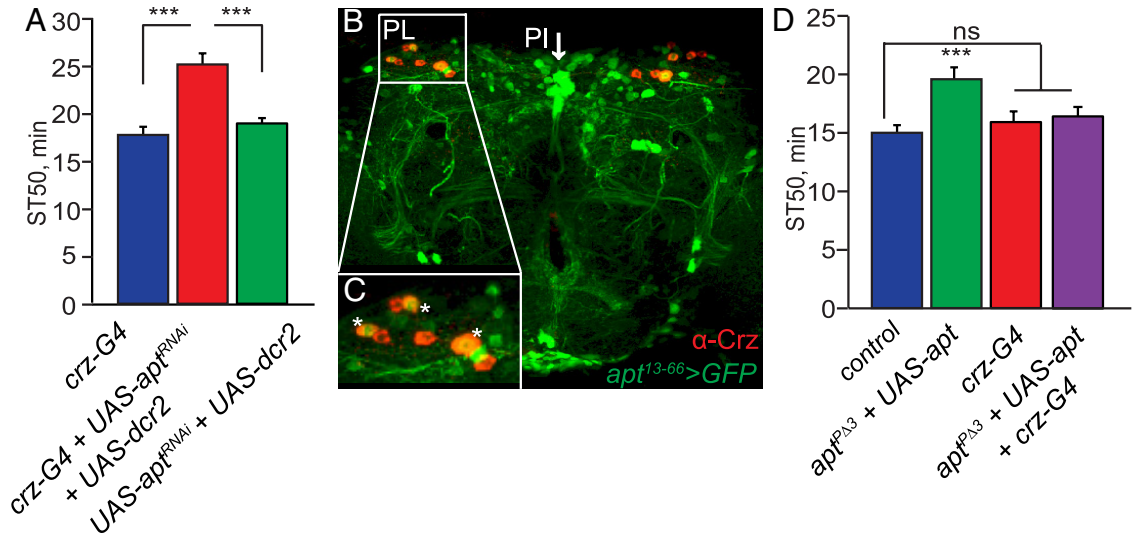

Figure 4. Requirement of *apt* function in *crz*-expressing neurons for normal ethanol sedation sensitivity. ***A***, Inhibition of *apt* expression by RNAi in *crz*-expressing neurons reduces ethanol sedation sensitivity. ST50 values of flies with knockdown of *apt* expression in *crz*-expressing neurons (*UAS-dcr2/+;crz-GAL4/UAS-apt^{RNAi}*) differ significantly from *GAL4* (*crz-GAL4/+*) and *UAS* (*UAS-dcr2/+;UAS-apt^{RNAi}/+*) control flies (one-way ANOVA, Tukey–Kramer *post hoc*, $n = 16$). ***B***, ***C***, Whole-mount brain from adult male fly *apt$^{13-66}$-GAL4/UAS-GFP* (nls) stained with anti-GFP and anti-Crz antibodies. ***B***, In the adult fly brain, Crz (red) is expressed in 6–8 neurons per lobe in the PL (Lee et al., 2008). In each lobe, there is colocalization of *apt$^{13-66}$-GAL4* (green) and Crz (red) in approximately three of the Crz-expressing neurons (yellow). ***C***, Enlargement of region marked in ***B*** (white box) showing colocalization between *apt$^{13-66}$-GAL4* and cells labeled with the Crz antibody. ***D***, Phenotypic rescue of the reduced ethanol sedation sensitivity in *apt^{PΔ3}/+* mutant flies by driving *UAS-apt* in *crz*-expressing neurons. ST50 values differ significantly between *apt^{PΔ3}/+* mutant flies with *UAS-apt* (*apt^{PΔ3}/+;UAS-apt/+*) and *apt^{PΔ3}/+* mutant flies driving *UAS-apt* in *crz*-expressing neurons (*apt^{PΔ3}/+;UAS-apt/crz-GAL4*) (one-way ANOVA, Tukey–Kramer *post hoc*, $n = 12$). ST50 values of *apt^{PΔ3}/+* mutant flies driving *UAS-apt* in *crz*-expressing neurons (*apt^{PΔ3}/+;UAS-apt/crz-GAL4*) does not differ significantly from control (*wB*) or GAL4 (*crz-GAL4/+*) alone control flies ($p > 0.05$, $n = 12$).

sitivity (Fig. 6*A*; $F_{(2,57)} = 14.86$, $p < 0.0001$; data not shown). Importantly, *crz* transcript levels in the heads of *crz-GAL4/UAS-crz^{RNAi}* and *elav-GAL4;UAS-crz^{RNAi}/+* flies were reduced by ~62 and 90% compared with control flies, respectively (Fig. 6*B*; $F_{(2,12)} = 10.97$, $p = 0.0039$; data not shown). In addition, expression of a second, non-overlapping *Crz^{RNAi2}* transgene in *crz*-expressing neurons caused the same decreased sedation sensitivity phenotype (data not shown; Fig. 6*A*). To determine the temporal requirements of *crz* function, we inhibited neuronal expression of *crz* by RNAi specifically during development or adulthood (using *elav-GAL4* and *GAL80^{ts}*) and found that only adult-specific inhibition of *crz* led to decreased sensitivity to ethanol sedation (Fig. 6*C*,*D*; $F_{(2,43)} = 9.91$, $p = 0.003$), suggesting that Crz function during adulthood is required for normal sedation sensitivity. Flies with no manipulation of *crz* expression (maintained at 18°C during both development and adulthood) showed normal sedation sensitivity (Fig. 6*E*; $F_{(2,30)} = 1.54$, $p = 0.2313$), while continuous neuronal inhibition of *crz* expression (flies maintained at 29°C throughout development and adulthood) caused significantly reduced ethanol sedation sensitivity (Fig. 6*F*; $F_{(2,32)} = 8.77$, $p = 0.005$). These data suggest that Crz may play a role in regulating adult neuronal function during acute ethanol exposure. Moreover, ethanol-induced sedation is mediated in the adult by both the

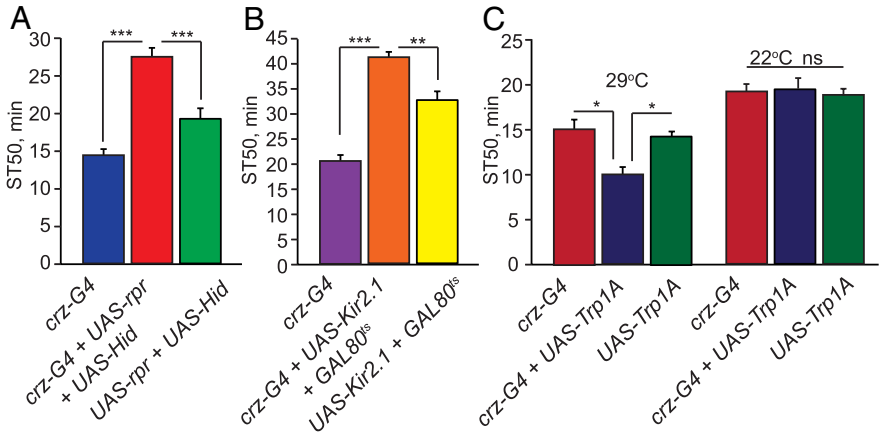

Figure 5. Activity of *crz*-expressing neurons mediates normal ethanol sedation sensitivity. ***A***, Ablation of *crz*-expressing neurons reduces ethanol sedation sensitivity. ST50 values of flies in which expression of the pro-apoptotic genes *rpr* and *Hid* is driven with *crz-GAL4* (*UAS-rpr;;UAS-Hid/crz-GAL4*) differ significantly from *GAL4* (*crz-GAL4/+*) and *UAS* (*UAS-rpr;;UAS-Hid/+*) control flies (one-way ANOVA, Tukey–Kramer *post hoc*, $n = 12$). ***B***, Adult-specific silencing of *crz*-expressing neurons reduces ethanol sedation sensitivity. *crz*-expressing neurons were silenced specifically during adulthood by driving *Kir2.1* with *crz-GAL4;tub-GAL80^{ts}*. The experimental (*tub-GAL80^{ts}/+;UAS-Kir2.1/crz-GAL4*), *GAL80^{ts}-UAS* (*tub-GAL80^{ts}/+;UAS-Kir2.1/+*), and *GAL4* (*crz-GAL4/+*) alone control groups were raised at 18°C then shifted to 29°C at eclosion until behavioral testing. ST50 values of flies with adult-specific silencing of *crz*-expressing neurons using *Kir2.1* and *crz-GAL4;GAL80^{ts}* (*tub-GAL80^{ts}/+;UAS-Kir2.1/crz-GAL4*) differ significantly from the *GAL80^{ts}-UAS* (*tub-GAL80^{ts}/+;UAS-Kir2.1/+*) and *GAL4* (*crz-GAL4/+*) control flies (one-way ANOVA, Tukey–Kramer *post hoc*, $n = 12$). ***C***, Adult-specific activation of *crz*-expressing neurons increases ethanol sedation sensitivity. The experimental flies (*UAS-TrpA/Crz-GAL4*) as well as the control *GAL4* (*Crz-GAL4/+*) and *UAS* (*UAS-TrpA*) flies were raised at 18°C until behavioral testing, then shifted to 29°C, a temperature for which *TrpA1* is activated, during the sedation assay. ST50 values of flies with adult-specific expression of *TrpA1* in *crz*-expressing neurons (*UAS-TrpA/Crz-GAL4*), tested at 29°C, significantly increased ethanol sedation sensitivity compared with the *GAL4* (*crz-GAL4/+*) and *UAS* (*UAS-TrpA/+*) alone control flies (one-way ANOVA, Tukey–Kramer *post hoc*, $n = 12$). Temperature controls for *TrpA* activation in *crz*-expressing neurons (22°C) were used. The experimental and control flies were raised at 18°C until behavioral testing then shifted 22°C, a temperature for which TrpA is not activated, during the sedation assay. At this temperature the ST50 values of the experimental group (*UAS-TrpA/Crz-GAL4*) did not differ from the *GAL4* (*Crz-GAL4/+*) and *UAS* (*UAS-TrpA/+*) alone control flies. ($p > 0.05$, $n = 8$).

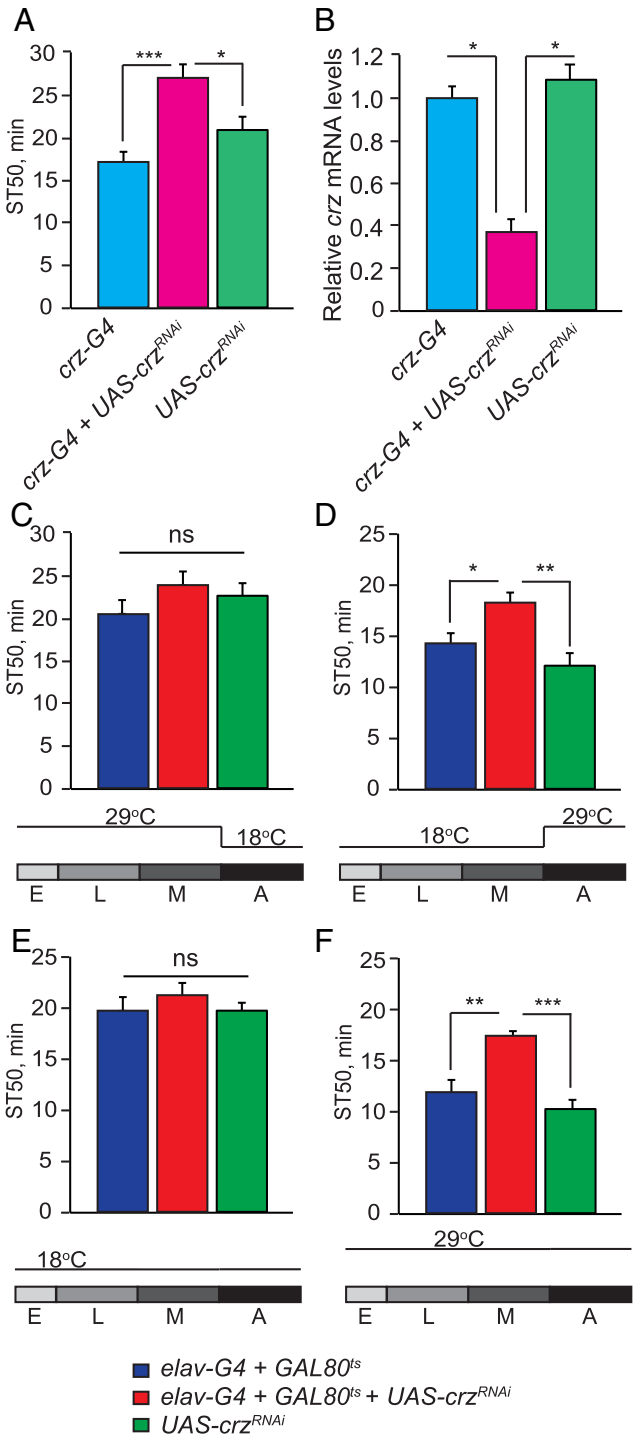

Figure 6. The neuropeptide Crz mediates ethanol sedation sensitivity. ***A***, RNAi knockdown of *crz* specifically in *crz*-expressing neurons reduces ethanol sedation sensitivity. ST50 values of flies in which *UAS-crz*^{RNAi} is driven with *crz-GAL4* (*crz-GAL4/UAS-crz*^{RNAi}/+) differ significantly from the *GAL4* (*crz-GAL4/*+) and *UAS* (*UAS-crz*^{RNAi}/+) control flies (one-way ANOVA, Tukey–Kramer *post hoc*, $n = 16$). ***B***, Expression of *crz* upon RNAi knockdown. Flies with neuronal knockdown of *crz* by RNAi, specifically in *crz*-expressing neurons (*crz-GAL4/UAS-crz*^{RNAi}/+), showed a 62% reduction of *crz* transcript levels compared with the *GAL4* (*crz-GAL4/*+) and *UAS* (*UAS-crz*^{RNAi}/+) control flies (one-way ANOVA, Tukey–Kramer *post hoc*, $n = 3$). ***C***, ***D***, Temporal knockdown of *crz* expression was achieved by coexpressing *UAS-crz*^{RNAi} with *elav*^{c155}*-GAL4;tub-GAL80*^{ts}. Flies were raised at 29 (GAL4 on, GAL80^{ts} off) and 18°C (GAL4 off; GAL80^{ts} on). ST50 values are shown for the experimental group with temporal inhibition of *crz* expression (*elav*^{c155}*-GAL4;tub-GAL80*^{ts}*/UAS-crz*^{RNAi}), and the *GAL4-GAL80*^{ts} (*elav*^{c155}*-GAL4;tub-GAL80*^{ts} /+), and *UAS* (*UAS-crz*^{RNAi} /+) controls. ***C***, Neuronal inhibition of *crz* expression during development does not alter ethanol sedation sensitivity. ($p > 0.05$, $n = 12$). Flies were raised at 29°C then shifted to 18°C at eclosion until behavioral testing. ***D***, Neuronal inhibition of *crz* expression during adulthood reduces ethanol sedation sensitivity (one-way ANOVA, Tukey–Kramer *post hoc*, $n = 12$). Flies were raised at 18°C then shifted to 29°C from the time of adult eclosion until behavioral testing. ***E***, ***F***, Temperature shift controls. ***E***, Raising flies at 18°C during both development and adulthood (GAL4 off) leads to normal ethanol sedation sensitivity. ($p > 0.05$, $n = 12$). ***F***, Raising flies at 29°C during both development and adulthood (GAL4 on) leads to decreased ethanol sedation sensitivity (one-way ANOVA, Tukey–Kramer *post hoc*, $n = 16$).

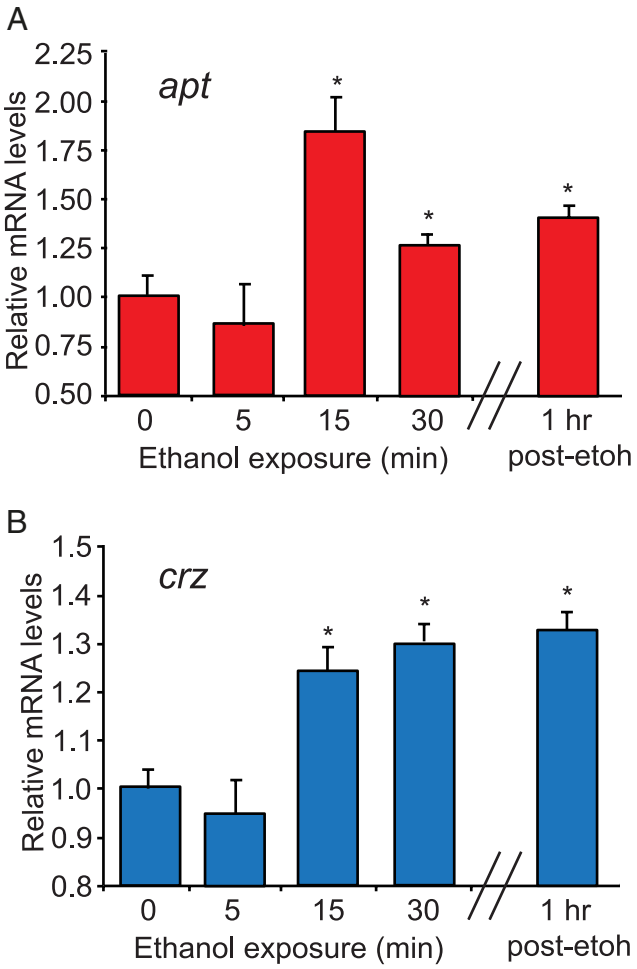

Figure 7. Acute ethanol exposure increases both *apt* and *crz* transcript levels. ***A***, ***B***, *apt* and *crz* transcript levels measured by qPCR in the heads of wild-type (*wB*) flies exposed to ethanol (73% ethanol vapor) for the times indicated. qPCR revealed that both *apt* and *crz* transcript levels increased after 15 min of ethanol exposure and remained elevated at the end of the exposure period as well as 1 h later. (Student's *t* test, $n = 3$).

neuropeptide Crz and the activity of *crz*-expressing neurons.

Acute ethanol exposure increases transcription of both the *apt* and *crz* genes

To determine whether *apt* and/or *crz* expression is acutely regulated by ethanol, we examined *apt* and *crz* transcript levels in the heads of wild-type flies exposed to ethanol as in our sedation assay. We observed an 85% increase in *apt* transcript levels after 15 min of ethanol exposure (*t* test, $p = 0.0028$), and modestly elevated transcript levels by the end of the exposure period (Fig. 7*A*; *t* test, $p = 0.0343$). A similar pattern was observed with *crz* transcript levels: after 15 min of ethanol exposure *crz* expression increased by 25% (*t* test, $p = 0.0432$) and was slightly more elevated by the end of the 30 min exposure (*t* test, $p = 0.0378$) (Fig.

7*B*). Interestingly, both *crz* and *apt* transcript levels were still elevated after 1 h of recovery from the ethanol exposure (Fig. 7*A*,*B*; *apt t* test, $p = 0.0279$ and *crz t* test, $p = 0.0321$). In summary, acute ethanol exposure rapidly upregulates the transcription of both *apt* and *crz*, and these results are consistent with our findings that both genes function to promote ethanol sedation sensitivity.

Discussion

The work presented here identifies two genes, *apt* and *crz*, which mediate the fly's sensitivity to ethanol-induced sedation. Flies with reduced expression of either gene display dramatically decreased ethanol sedation sensitivity; thus, both *apt* and *crz* normally promote ethanol sedation. We show that normal sensitivity to ethanol sedation requires *apt* expression in neurons during two distinct life stages, metamorphosis and adulthood. Apt function in a subset of *crz*-expressing neurons (approximately 6 of the 12–16 *crz*-expressing cells) is necessary and sufficient for normal sensitivity to ethanol sedation. Acute manipulations of the activity of *crz* neurons led to altered ethanol sedation sensitivity, demonstrating that these neurons play an active role in regulating the behavioral response to ethanol-induced sedation. The neuropeptide Crz is also involved in ethanol sedation, as flies with reduced *crz* expression, specifically during adulthood, show dramatically decreased ethanol sedation sensitivity. Finally, in response to acute ethanol exposure the expression of both *apt* and *crz* are rapidly upregulated during ethanol exposure. We hypothesize that the Apt-Crz system, functioning in a very small group of neurosecretory cells, may be an early target of ethanol in the fly brain whose function is crucial for normal sensitivity to ethanol.

Mechanisms of Apt function

How does Apt regulate ethanol-induced sedation? Although Apt's role could be to regulate the expression of *crz*, we were unable to observe such a function. We thus postulate that Apt functions to regulate the activity of *crz* neurons and/or neuropeptide release. For example, Apt, acting as a transcription factor, could regulate the transcription of proteins required for synthesis, packaging, and/or release of Crz and possibly other neuropeptides. Alternatively, Apt could regulate the expression of proteins required for synapse formation. In support of these two possibilities, *apt* mutant embryos were observed to have defective synaptic transmission at the neuromuscular junction, as well as fewer numbers of active zones within motoneurons, indicating a presynaptic defect (Takasu-Ishikawa et al., 2001).

Another possible function for Apt in regulating ethanol sedation behavior may be found in its neuronal requirement during metamorphosis, a time of intense remodeling to construct the adult CNS (Truman, 1990). During embryogenesis, Apt functions in multiple morphogenetic processes, including tracheal, head, CNS, and heart morphogenesis, as well as border cell migration (Eulenberg and Schuh, 1997; Gellon et al., 1997; Su et al., 1999; Starz-Gaiano et al., 2008). It is therefore possible that during metamorphosis Apt establishes proper development and neuronal connectivity of the adult CNS, and in particular the Crz neurons. However, this possibility seems somewhat unlikely given that the adult CNS in apt^{13-66} flies appeared normal, as was the number and morphology of Crz-expressing neurons. Additionally, we found that adult-specific expression of *apt* in neurons was necessary for normal sedation sensitivity. However, there may be subtle defects in the adult CNS of *apt* mutant flies, which we were unable to detect, that could contribute to their altered sedation sensitivity.

Apt shows highest sequence conservation with the human FSBP, a negative regulator of transcription of the gamma chain of *fibrinogen* (Mizuguchi et al., 1995; Starz-Gaiano et al., 2008). Sequence conservation between *apt* and *FSBP* is observed within the DNA-binding domain (Starz-Gaiano et al., 2008). Interestingly, moderate alcohol consumption in humans has been known to exert a cardioprotective effect, in part by lowering levels of circulating Fibrinogen (Wang et al., 1999). The mechanism for how alcohol consumption regulates Fibrinogen is currently unknown, but in light of our findings we speculate that it may occur at the level of transcription. We found that ethanol exposure in flies acutely upregulates *apt* expression (Fig. 7*A*). We hypothesize a similar situation may occur in humans, whereby alcohol consumption could upregulate the transcription of *FSBP*, ultimately leading to negative regulation of the gamma chain of *fibrinogen* and lowered levels of circulating Fibrinogen, which in turn would provide cardioprotection.

Regulation of ethanol sedation by *crz neurons*

Our observations implicate *crz*-expressing neurons in the regulation of ethanol sedation behavior, a function not previously attributed to these neurons. We demonstrate that adult-specific silencing of *crz* neurons significantly reduced ethanol sedation sensitivity, while increasing their activity resulted in the opposite phenotype, an increase in ethanol sedation sensitivity (Fig. 5). Based on the observation that inhibiting *crz* expression also reduced ethanol sedation sensitivity (Fig. 6), we believe that the phenotypes associated with *crz* neuronal manipulations reflect changes in the release of the neuropeptide Crz and activation of its signaling pathway. However, a few Crz neurons also express the short Neuropeptide F (sNPF) (Nässel et al., 2008). sNPF is considered to be a multifunctional neuropeptide due to its broad expression in diverse neuronal types and we have not excluded its possible role in *crz*-expressing neurons and in ethanol sedation sensitivity. However, the observation that Apt function is required in a subset of *crz* neurons to promote ethanol sedation behavior, firmly establishes the importance of these neurons in mediating the behavioral response to ethanol. Our data also suggest that the function of both genes, *crz* and *apt*, overlaps in a small set of neurons likely located in the PL to mediate the behavioral response to ethanol-induced sedation.

Mechanisms of Crz function

It has recently been hypothesized that Crz is released in response to various types of stress in insects (Veenstra, 2009; Boerjan et al., 2010), and that this could explain its pleiotropic effects. This hypothesis was bolstered by a recent study showing that flies deficient in Crz are resistant to metabolic, osmotic, and oxidative stress, as measured by survival (Zhao et al., 2010). In addition, Crz plays a role in stress physiology through its association with well characterized stress hormones. For instance, *crz*-expressing neurons in the PL also express receptors for two diuretic hormones, DH^{44} and DH^{31} (Johnson et al., 2005). By virtue of receptor similarity, DH^{44} and DH^{31} are related to corticotrophin-releasing factor (CRF) and calcitonin-gene related peptide (CGRP), respectively, both of which mediate the mammalian physiological and behavioral responses to stress (Dunn and Berridge, 1990; Bale and Vale, 2004). Interestingly, both CRF and CGRP act to inhibit secretion of GnRH in the mammalian hypothalamus (Nikolarakis et al., 1986; Tellam et al., 1998; Li et al., 2004). This is significant because Crz is thought to be the homolog of mammalian GnRH, and suggests that analogous regulation occurs in *Drosophila* (Cazzamali et al., 2002). It is thus

possible that in flies a stress signal or the animal's stress status may be relayed to Crz neurons and alters their function. Thus, based on its functional and molecular associations with stress physiology, it is tempting to speculate that the role of Crz signaling in ethanol sedation sensitivity is related to a stress response. A previous study has shown that stress, in the form of heat shock, induces tolerance to a subsequent ethanol exposure, and that ethanol tolerance relies on the gene *hangover*, a large nuclear zinc-finger protein, that mediates various other stress responses (Scholz et al., 2005). In addition, several genes related to stress responses have been shown to be upregulated by ethanol exposure in transcriptional profiling studies, including nearly half of all *Drosophila* heat shock protein genes, as well as genes involved in the regulation of oxidative stress and aging (Morozova et al., 2006; Kong et al., 2010; Awofala, 2011). Importantly, a maladaptive response to stress has been shown in humans to be a major and common element contributing to drug addiction (Nemeroff et al., 2005). Finally, an increase in ethanol self-administration has been observed in animal models with physical, social, and emotional stress (Higley et al., 1991; Mollenauer et al., 1993). In light of these findings, it will be interesting to further explore the role of Crz and its function in stress physiology and the regulation of ethanol-related behaviors.

Neuropeptides as regulators of ethanol-related behaviors

Neuropeptides are diverse signaling molecules that mediate a broad spectrum of physiological and behavioral processes (Altstein and Nässel, 2010). Several studies have linked neuropeptides to behavioral responses to ethanol. For instance, one of the first ethanol sensitivity mutants described in Drosophila, *amnesiac*, encodes a neuropeptide homologous to the vertebrate pituitary adenylate cyclase-activating peptide (Feany and Quinn, 1995; Moore et al., 1998). In addition, mice lacking either neuropeptide Y (NPY), a neuromodulator abundantly expressed in many regions of the CNS, or its Y1 receptor subtype, display increased ethanol consumption and resistance to ethanol sedation, whereas animals overexpressing NPY show the opposite behavioral phenotypes (Thiele et al., 1998, 2002; Thorsell et al., 2007). Neuropeptide F (NPF), the sole member of the NPY family in *Drosophila*, and its receptor NPFR1, has similarly been shown to mediate the fly's sensitivity to ethanol-induced sedation (Wen et al., 2005). Finally, flies with neuronal perturbations in the insulin signaling pathway displayed increased ethanol sedation sensitivity (Corl et al., 2005). These studies and our own, implicating the neuropeptide Crz in sensitivity to ethanol sedation, suggest that neuropeptides are important regulators of the behavioral response to ethanol, and it would therefore be interesting to survey all known *Drosophila* neuropeptides and their downstream signaling components for possible role(s) in ethanol-related behaviors.

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