Ethanol Intoxication in *Drosophila*: Genetic and Pharmacological Evidence for Regulation by the cAMP Signaling Pathway

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Summary

Upon exposure to ethanol, *Drosophila* display behaviors that are similar to ethanol intoxication in rodents and humans. Using an inebriometer to measure ethanol-induced loss of postural control, we identified *cheapdate*, a mutant with enhanced sensitivity to ethanol. Genetic and molecular analyses revealed that *cheapdate* is an allele of the memory mutant *amnesiac*. *amnesiac* has been postulated to encode a neuropeptide that activates the cAMP pathway. Consistent with this, we find that enhanced ethanol sensitivity of *cheapdate* can be reversed by treatment with agents that increase cAMP levels or PKA activity. Conversely, genetic or pharmacological reduction in PKA activity results in increased sensitivity to ethanol. Taken together, our results provide functional evidence for the involvement of the cAMP signal transduction pathway in the behavioral response to intoxicating levels of ethanol.

Introduction

Alcohol is one of the most widely abused drugs in the world, yet little is known about the molecular regulation of ethanol-induced responses in the brain. While ethanol does not act through a specific receptor, there is increasing evidence that neural responses to ethanol are due to alterations of specific brain proteins rather than to nonspecific changes in membrane function. For example, a specific region in the γ subunit of the GABA$_A$ receptor is required for the potentiating effects of ethanol on the channel's function in heterologous systems (Wafford et al., 1990). It has been difficult, however, to relate these specific effects of ethanol, observed in isolated cells or tissues, with ethanol-induced behaviors in animals.

Humans exhibit responses to ethanol that can range from disinhibition and euphoria at low doses to uncoordination and lethargy at higher doses. It has been shown that the degree of response to ethanol is genetically influenced: young men with a family history of alcoholism are less sensitive to the biochemical, motor, and perceptual changes induced by intoxicating levels of ethanol than those from families without alcoholism (Schuckit and Gold, 1988). Moreover, when reexamined a decade later, a significantly higher proportion of subjects with reduced ethanol sensitivity had developed alcoholism (Schuckit, 1994). Thus, the level of response to ethanol appears to be influenced genetically and may be a strong predictor of risk for alcoholism. However, a causal relationship between ethanol sensitivity and risk for alcoholism or the biological bases for this correlation remain unknown.

As in humans, acute ethanol sensitivity in rodent models is also influenced genetically. Strains of mice and rats that differ substantially in their responses to an acute ethanol dose have been obtained through selective breeding (for a review, see Crabbe et al., 1994). More recently, mice lacking the 5-HT$_{1B}$ serotonin receptor or the γ isoform of protein kinase C have been shown to display decreased ataxic and hypothermic responses to ethanol, respectively (Harris et al., 1995; Crabbe et al., 1996). Conversely, mice lacking Fyn-tyrosine kinase are hypersensitive to the hypnotic effects of ethanol (Miyakawa et al., 1997). These studies have provided important clues that specific genes can modulate ethanol-induced behaviors in animals.

Drosophila melanogaster, with its accessibility to genetic analysis, is an attractive model system to investigate the molecular mechanisms underlying ethanol-induced behaviors. We have found that the behavior of flies upon exposure to ethanol is remarkably similar to that of inebriated humans and rodents (see below). In addition, many of the neurotransmitter systems and signal transduction cascades whose functions are altered by ethanol in mammalian cells or tissues have been identified in *Drosophila*. To learn about the mechanisms that regulate ethanol sensitivity and to search for potential ethanol targets, we initiated a genetic screen for *Drosophila* mutants that respond abnormally to an acute ethanol exposure. Here we report the phenotypic and molecular characterization of *cheapdate* (*chpd*), a P element–induced mutation isolated on the basis of increased sensitivity to ethanol-induced loss of postural control. *chpd* was found to be an allele of the memory mutant *amnesiac* (*amn*) (Quinn et al., 1979). The *amn* gene encodes a putative neuropeptide believed to act through adenylate cyclase to increase cAMP levels (Feany and Quinn, 1995). Using a combination of genetic and pharmacological approaches, we demonstrate that proper regulation of the cAMP signaling pathway is central to establishing ethanol sensitivity in *Drosophila*. Together with the fact that this signaling pathway has been implicated in the cellular response to ethanol (for a review, see Diamond and Gordon, 1997), our study establishes *Drosophila* as a viable model system in which to study the molecular bases underlying ethanol-induced behaviors.

Results

chpd Is a Mutant with Enhanced Ethanol Sensitivity
Upon exposure to ethanol vapor, adult *Drosophila* display many behaviors resembling acute intoxication in

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mammals. Within minutes of exposure to ethanol vapor, flies first become hyperactive and disoriented and then uncoordinated and sedated. After approximately 20 min of exposure they become inmobile, but nevertheless recover 5–10 min after ethanol is withdrawn (these be-haviors and the assays used to measure them will be described in detail elsewhere; C. M. S. and U. H., unpub-lished data). To measure the ethanol sensitivity of a population of flies, we used an inebriometer, which is a device that allows a quantitative assessment of ethanol-induced loss of postural control (Cohan and Hoffman, 1986; Weber, 1988). Briefly, the inebriometer is an ap-proximately 4 ft long glass column containing multiple oblique mesh baffles through which ethanol vapor is circulated. To begin a "run," about 100 flies are intro-duced into the top of the inebriometer. With time, flies lose their ability to stand on the baffles and gradually tumble downward. As they fall out of the bottom of the inebriometer, a fraction collector is used to gather them at 3 min intervals, at which point they are counted. The elution profile of wild-type control flies follows a normal distribution (Figure 1A); the mean elution time (MET), approximately 20 min at our standard ethanol vapor concentration (see Experimental Procedures), is in-versely proportional to their sensitivity to ethanol.

A genetic screen was carried out to isolate P element–induced mutants with altered sensitivity to ethanol intox-ication using the inebriometer as the behavioral assay (see Experimental Procedures). One X-linked mutation isolated in this screen was named *cheapdate* (*chpd*) to reflect the increased ethanol sensitivity displayed by hemizygous mutant male flies. *chpd* males elute from the inebriometer with a MET of 15 min compared with 20 min for the wild-type controls (Figures 1A and 1B). This increased ethanol sensitivity of *chpd* males was observed at all ethanol vapor concentrations tested (Fig-ure 1C).

Neither *chpd* nor control flies eluted from the inebrio-meter in the absence of ethanol, indicating that *chpd* mutants had no major difficulty in clinging to the inebrio-meter's mesh baffles. *chpd* mutants also had apparently normal geotaxis and locomotor activity and showed no obvious anatomical defects (see Experimental Proce-dures). Thus, *chpd* flies display a specific increase in ethanol sensitivity rather than a generalized weakness.

chpd Displays Normal Ethanol Absorption and Metabolism

A trivial potential explanation for the ethanol-sensitive phenotype of *chpd* might have been that ethanol ab-sorption and/or metabolism is altered by the mutation. For example, mutants in which ethanol absorption is increased display enhanced ethanol sensitivity (see Ex-perimental Procedures). To evaluate this possibility, we measured the concentration of ethanol in extracts pre-pared from flies exposed to ethanol for defined periods of time. Ethanol levels detected in *chpd* and control males were indistinguishable after 10, 20, and 30 min exposures (Figure 2). Residual ethanol levels, measured at various recovery times after a 30 min exposure, were also comparable in *chpd* and controls (Figure 2). Hence, the ethanol-sensitive phenotype of *chpd* did not appear to be caused by an alteration in the rate of ethanol absorption or metabolism.
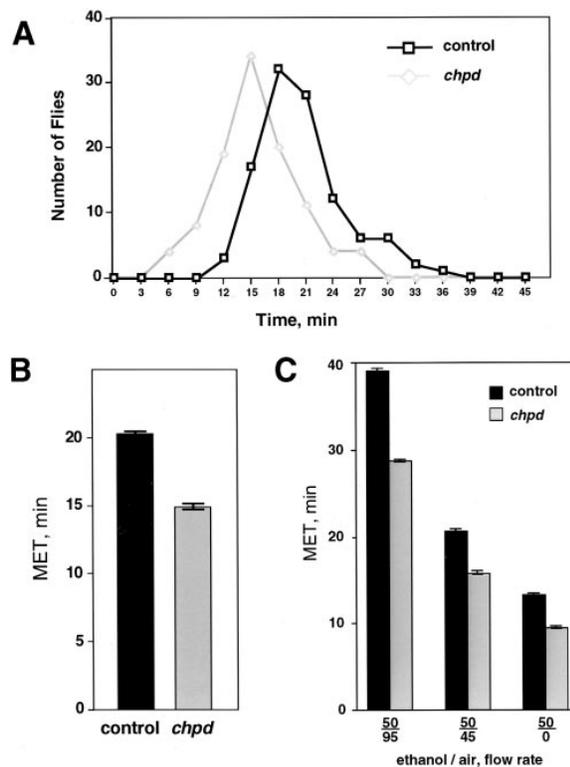

Figure 1. *chpd* Is an Ethanol-Sensitive Mutant

(A) Representative inebriometer elution profile of *chpd* and control flies. Approximately 100 male *chpd* or control flies were exposed to ethanol vapor (concentration 50/45, see Experimental Proce-dures) in the inebriometer. The number of *chpd* (gray line) or control (black line) flies eluting from the inebriometer as a function of time (minute) is shown.
(B) Average mean elution time (MET) of *chpd* and control males. The MET of *chpd* is significantly lower than that of control flies ($p < 0.0001$; $n = 31$).
(C) Dose response curve. *chpd* and control flies were exposed in the inebriometer to three different concentrations of ethanol vapor achieved by mixing a constant flow of ethanol vapor (50 flow units) with humidified air at different flow units (95, 45, and 0). Increasing the relative ethanol concentration lowers the MET of both *chpd* and control flies. At every ethanol concentration tested, the average MET of *chpd* was significantly lower than that of control flies ($p < 0.0001$; $n = 4$). Error bars in all figures correspond to SEM.

chpd Is an Allele of *amn*

Precise excision of the P element in *chpd* restored the wild-type phenotype, implying that the insertion is re-sponsible for the increased ethanol sensitivity (see Ex-perimental Procedures). The *chpd* insertion was mapped cytologically to band 19A1–2 on the X chromosome, a location to which *amnesiac* had been mapped pre-viously (Tully and Gergen, 1986). *amn* was first isolated in a genetic screen for mutants with associative memory defects (Quinn et al., 1979). To determine whether *chpd* was allelic to *amn*, we tested additional *amn* alleles for ethanol sensitivity in the inebriometer. Hemizygous males carrying either the original EMS-induced *amn[1]* allele or the P element–induced allele *amn[28A]* (see Experi-mental Procedures), showed an ethanol-sensitive phe-notype indistinguishable from that of *chpd* (Figure 3A). In addition, *amn[X8]* males, which carry a deletion of the
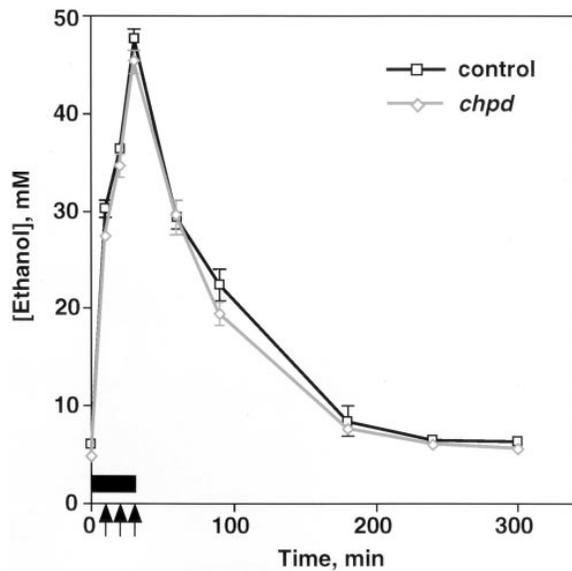
Figure 2. *chpd* Flies Absorb and Metabolize Ethanol Normally

To measure ethanol absorption, the concentration of ethanol was determined in *chpd* and control flies exposed to ethanol vapor (50/45) for 0, 10, 20, or 30 min (black arrows). The ability of *chpd* and control flies to metabolize ethanol was assessed by exposing flies to ethanol vapor (50/45) for 30 min (black box) and by measuring the ethanol remaining in the flies 30, 60, 150, 210, and 270 min after the exposure. Thus, the ascending and descending parts of the curve reflect ethanol absorption and metabolism, respectively. The concentration of ethanol is measured in extracts from whole flies using an alcohol dehydrogenase–coupled spectrophotometric assay (see Experimental Procedures). No significant differences in ethanol absorption or metabolism were detected between control and *chpd* flies (n = 4).

amn locus (see below), were even more sensitive to ethanol (Figure 3A).

To confirm that lesions in the *amn* gene confer increased ethanol sensitivity, we carried out genetic complementation tests (Figure 3B). We found that all *amn* alleles tested (*amn¹*, *amn²⁸ᴬ*, and *amnˣ⁸*) and *chpd*, when assayed in homozygous females, display a phenotype indistinguishable from that of hemizygous males. In addition, all *amn* alleles and *chpd* were completely recessive. Most importantly, *chpd* failed to complement the ethanol sensitivity of *amn¹*, *amn²⁸ᴬ*, and *amnˣ⁸* (Figure 3B). These data demonstrate that *chpd* is an allele of

amn and that the *amn* locus is involved in controlling ethanol sensitivity. The fact that *amnˣ⁸* had a more extreme phenotype than *amn¹*, *amn²⁸ᴬ*, or *chpd* suggests that the latter are not complete loss-of-function alleles.

chpd Contains a P Element Insertion in *amn*

amn was cloned previously and shown to encode a novel protein with weak homology to two mammalian neuropeptides, pituitary adenylyl cyclase activating peptide (PACAP) and growth hormone releasing hormone (GHRH) (Feany and Quinn, 1995), both of which are known to activate receptors that couple positively with adenylate cyclase (AC) to increase cAMP levels. We recovered and sequenced approximately 1.1 kb of the genomic DNA flanking the *chpd* P element insertion site. A search of the NCBI database revealed that the P element had inserted within the open reading frame (ORF) of the *amn* gene C-terminal to the PACAP and GHRH homology regions (Figures 4A and 4B). The P element in *amn²⁸ᴬ* was found to be inserted in the putative 5' untranslated region (*amn²⁸ᴬ* carries an additional DNA duplication in the ORF). The *amnˣ⁸* allele, which was derived by imprecise excision of the P element insertion in *amn²⁸ᴬ*, contains a deletion that removes most of the P element and the putative *amn* coding region (Figure 4A).

Our sequence analysis of genomic DNA isolated from several strains of wild-type flies revealed a discrepancy with the previously published *amn* cDNA sequence; the latter contains an extra T at position 2300 (Feany and Quinn, 1995). The impact of this modification is 2-fold. First, the corrected wild-type sequence alters part of the predicted ORF and implicates an upstream AUG translation start site instead of the previously proposed CUG (Figure 4B). Hydrophobicity analysis of the new putative *amn* ORF suggests that the amino terminus of the protein (amino acid 4–28) can encode a signal sequence or a transmembrane domain. This finding, therefore, does not detract from the proposed neuropeptide function of the encoded product. Second, the sequence of genomic DNA isolated from *amn¹* and wild type are now identical in the ORF. It was therefore crucial to prove that this genomic region generates a transcript that is disrupted in known *amn* alleles. Previous screening of cDNA libraries representing various *Drosophila* developmental stages suggested that the *amn* mRNA is extremely rare (Feany and Quinn, 1995). Using RT–
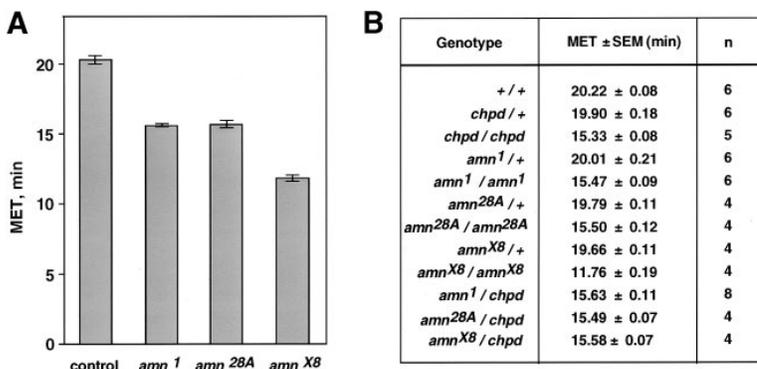

Genotype	MET ± SEM (min)	n
+/+	20.22 ± 0.08	6
chpd/+	19.90 ± 0.18	6
chpd/*chpd*	15.33 ± 0.08	5
amn¹/+	20.01 ± 0.21	6
amn¹/*amn¹*	15.47 ± 0.09	6
amn²⁸ᴬ/+	19.79 ± 0.11	4
amn²⁸ᴬ/*amn²⁸ᴬ*	15.50 ± 0.12	4
amnˣ⁸/+	19.66 ± 0.11	4
amnˣ⁸/*amnˣ⁸*	11.76 ± 0.19	4
amn¹/*chpd*	15.63 ± 0.11	8
amn²⁸ᴬ/*chpd*	15.49 ± 0.07	4
amnˣ⁸/*chpd*	15.58 ± 0.07	4

Figure 3. *chpd* Is an Allele of *amn*

(A) Two known alleles of *amn*, the EMS-induced *amn¹* and the P element–induced *amn²⁸ᴬ* alleles, and an excision derivative of *amn²⁸ᴬ*, *amnˣ⁸*, display significantly increased ethanol sensitivity compared to control (p < 0.0001; n = 4). *amnˣ⁸* was significantly more sensitive than *amn¹* and *amn²⁸ᴬ* (p < 0.0001; n = 4).

(B) Genetic complementation of *chpd* and *amn¹*, *amn²⁸ᴬ*, and *amnˣ⁸* were performed by assaying virgin females. All *amn* alleles and *chpd* are completely recessive and *amn¹*, *amn²⁸ᴬ*, and *amnˣ⁸* failed to complement the ethanol sensitivity of *chpd* (n = 4).

A

B

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1982 TTT TGC ATG TCA CCA ACA GAA ACA GAA AAA AAA AAC GAA ACG AAA CAC ACT GAA AAA CGC
2042 GCG CGT ACA CTT GTA TAT ATG TGA ATG TGC GCT GGT ATT GGC GCG CGT GTG AGT GTG AGC

2102 GAA TTT GCA AGA GAC GGG GCG TAT GAG CAA TGC CGT GGC GAA AAC TTT GCC ACT TTT GTT
2162 GTC TGC GAC GAA AGC AAA AAC AGC GAG ACA CCG TAA AAA AAA AAA CAA GTA ATA TAA ACA
2222 CAG CTT TAC ATG CAC AGT TTT TGT TGT TGT TTT TAT CCG GCT GCT GTG GCG TTG CAC TGC
      1 met arg ser phe cys cys cys phe tyr pro ala ala val ala leu his cys
                                                    *
2282 GTA TTA CTG TTT TAC ACT TTT TTT TTA CTT TTT AGA GCG TCC GCG TTG CGG CGA CGC GTT
  18   val leu leu phe tyr thr phe phe leu leu phe arg ala ser ala leu arg arg arg val

2342 GTA AGC GGT TCG AAA GGT AGC GCA GCA CTG GCG CTC TGC CGC CAA TTC GAA CAG CTG AGC
  38   val ser gly ser lys gly ser ala ala leu ala leu cys arg gln phe glu gln leu ser

2402 GCC AGC CGA AGA GAG AGA GCC GAA GAG TGC CGA ACG ACG CAG CTC CGC TAC CAC TAC CAC
  58   ala ser arg arg glu arg glu glu cys arg thr thr gln leu arg tyr his tyr his

2462 CGC AAT GGC GCT CAG TCG CGG TCG CTG TGC GCT GCT GTG CTC TGC TGT AAG CGC TCT TAC
  78   arg asn gly ala gln ser arg ser leu cys ala ala val leu cys cys lys arg ser tyr

2522 ATT CCG CGC CCG AAT TTT TCG TGC TTT TCC CTT GTT TTC CCT GTG GGC CAG CGT TTT GCT
  98   ile pro arg pro asn phe ser cys phe ser leu val phe pro val gly gln arg phe ala

2582 GCT GCT CGG ACT CGG TTT GGG CCA ACA CTT GTC GCC TCT TGG CCA TTA TGC AAC GAC AGC
 118   ala ala arg thr arg phe gly pro thr leu val ala ser trp pro leu cys asn asp ser

2642 GAA ACG AAG GTG CTC ACG AAG TGG CCA AGC TGC TCT CTG ATT GGT CGG CGG TCA GTG CCA
 138   glu thr lys val leu thr lys trp pro ser cys ser leu ile gly arg arg ser val pro

2702 CGA GGC CAG CCG AAG TTC TCT CGC GAG GAC AAC CCA AGA GCA CTC TCT CCC TCT CTT TTG GGA
 158   arg gly gln pro lys phe ser arg glu asn pro arg ala leu ser pro ser leu leu gly

2762 GAG ATG AGA TAG CAA AGA AAA GCC AAA ACA CTA GAA CAA TCC AGA AGC CAA GTT TTC CGG
 178   glu met arg  *

2822 CTT GCA CAT ACG CCG TAT AAT CCG CAT TTT TTG CGA ACT CTA AAC TCG AGT CTA TCA GCT
2882 ATA ATA TTA CAA TTC GTT CAC TAT AAA CAA ATA AAC TAA TAT AGT TTT GCT CAG AAA TCG
2942 ATG CAG TAT GAT GAG AGA ATA GTG AAA AAC AAT TCT CAG TTA ATA GCT TTT AAA CTG CGT
3002 CTG CAA ATG GGA AAA CAA ATT AAA ATA TAC ACA TTT TCA TGC CAA ATA ATA GGA TAT CCT
3062 ATC CTT CAT TAT ATA AGC ATA AAC AAT AAA AAT ATT CTA GCC CGT CGC TTT GAT CTT TTA ATG
3122 TGT GTA TAA TGT ATT AGT ACG TGT ACT TTA GTC AAA GAA CTA TGC ACA AAT ACC GGT TGA
3182 ACT TTT TTT GGG TAT GGA AAA GCA ACC GCA TGT GAG CGA AAA TGT TTG GGG ATT GAA
3242 GGC GTG ATA TTG GGC GGA GTG ACC AAA ACA TTG GCA AGG GAG CCA TCA GCA TCC ACA TCC
3302 ACA TCC ACA TCA TCG CCA GCG TCG AGA GCT CTT TCG ATC GAA ATA AAT TCG AAT CGA GTG
3362 CGA TTC GTC AAC AAG GCG AAG ATA AAG ATT TCA GAG CAA TTA AAC AGA AAA ATA TTG TTA
3422 TTC AAT CAA AAG CCA ATC GAA AAT TGC TTT GCA CTC TGG CAC ACT GCT ATG TAC ATA TGT
3482 AAA GTG AGA GAT TCT ACT TTT ACC AAA TTC TAT TTT CCA AGT CCA AGT AAA TAT TTT AAA
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3602 TCG TGA ATT
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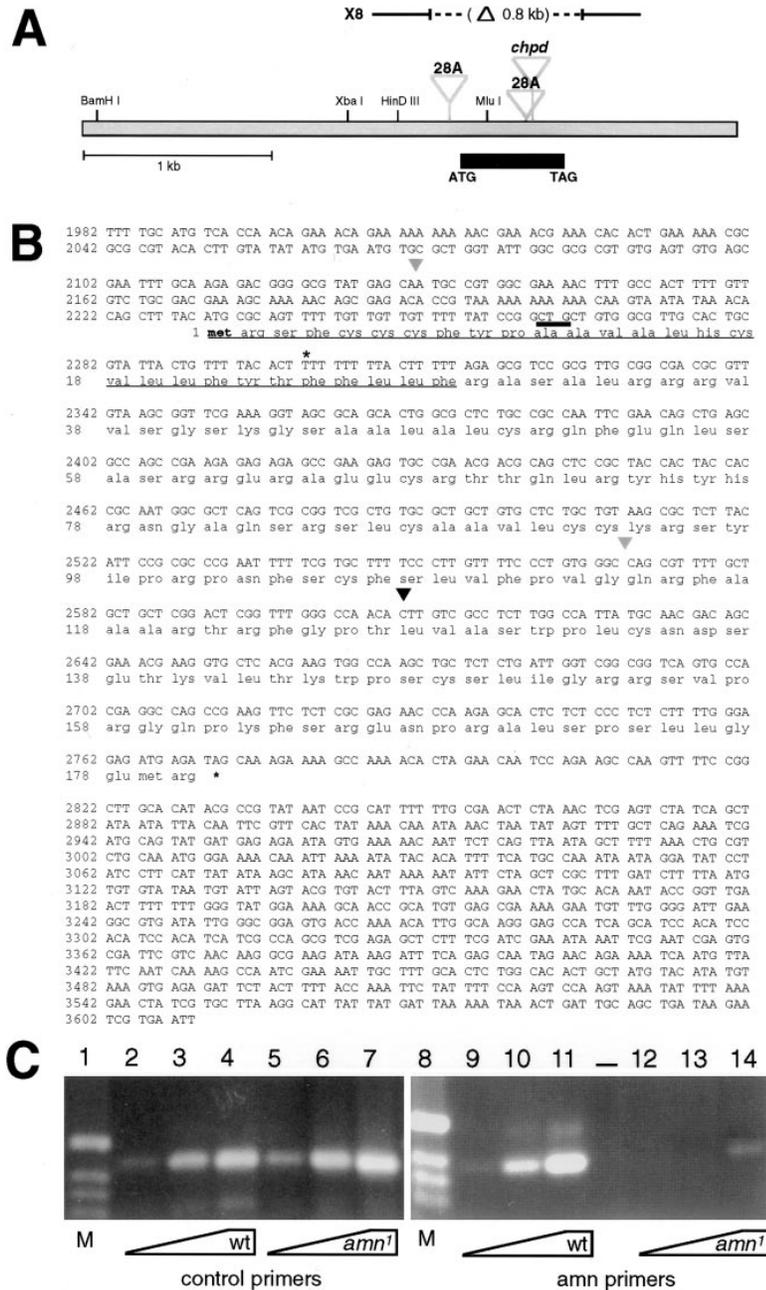
C

Figure 4. *chpd* Contains a Single P Element Insertion in the ORF of *amn*

(A) A genomic map of the *amn* locus displaying the location of the P element insertions in *chpd* and *amn^28A* (triangles). *amn^28A* contains, in addition to the P[w⁺] element in the 5' untranslated region, an additional DNA duplication of approximately 1.4 kb in the ORF, as shown. *amn^X8* is an excision of *amn^28A* and contains a deletion of approximately 800 bp that removes the *amn* ORF (dotted line).

(B) DNA sequence of the *amn* locus predicts an ORF of 541 bp initiating at the AUG at position 2231 and terminating at position 2771. Conceptual translation predicts a 180–amino acid product containing a signal sequence or transmembrane domain at the N terminus (underlined) (Kyte and Doolittle, 1982). The exact location of the P element insertions in *amn^28A* and *chpd* are shown (gray and black triangles, respectively). The previously proposed translation initiation codon (CUG) is underlined and the position of the extra T is shown with an asterisk. Repeated sequencing of the *amn* locus by S. Waddell and W. G. Quinn (personal communication) is in agreement with the amendment reported here.

(C) Expression of the *amn* transcript is reduced in *amn^1* mutants. Transcripts were detected by RT–PCR using mRNA isolated from wild-type (lanes 2–4 and 9–11) and *amn^1* (lanes 5–7 and 12–14) third instar larvae. The mRNA encoded by the *amn* locus is significantly reduced in *amn^1* larvae (compare lanes 12–14 with lanes 9–11); a quantitative assessment of this reduction, however, could not be established by this technique. RT–PCR reactions with control primers from the *Roi* locus (lanes 2–7) were used to control for mRNA concentration. Addition of RNase, but not of DNase, before the RT reaction completely abolished the *amn* and control signals (data not shown). M corresponds to φx-HaeIII molecular weight markers.

PCR with nested primers, however, we were able to detect an *amn* transcript in wild-type larvae and adult heads (Figure 4C; data not shown). This transcript is strongly reduced in *amn^1* mutant flies. Taken together, these data provide support for the correct identification of the *amn* gene and argue the *amn^1* is a regulatory mutation that causes a reduction in steady-state transcript levels from this locus. On the basis of the genetic and molecular data shown above, *chpd* will henceforth be referred to as *amn^chpd*.

Induced Expression of a *hs-amn* Transgene Rescues the Ethanol-Sensitivity Defect of *amn^chpd* Mutants

The genetic and molecular data presented above suggested that disruption of the *amn* gene was responsible for the ethanol-sensitive phenotype of *amn^chpd*. To demonstrate this hypothesis conclusively, a 744 bp genomic fragment containing the *amn* ORF was fused to the heat shock protein-70 (hsp-70) promoter to generate an inducible *hs-amn* gene. Several transgenic fly lines carrying independent chromosomal insertions of this construct were generated and crossed into the *amn^chpd* genetic background. *amn^chpd* males carrying a *hs-amn* transgene on one of the autosomes were grown in the presence or absence of a daily heat shock administered throughout development; upon eclosion, adult males were heat shocked once more and tested 24 hr later in the inebriometer (see Experimental Procedures).

In the absence of heat shock, ethanol sensitivity of *amn^chpd* males carrying any of the four autosomal *hs-amn* transgenes was similar to that displayed by *amn^chpd*

mutants (Figure 5A; data not shown). In contrast, normal ethanol sensitivity was restored after these transgenic flies were subjected to heat shock (Figure 5A). The heat shock regimen had no effect on ethanol sensitivity of *amn^chpd* mutants or wild-type controls. In addition, induction of *hs-amn* did not further reduce the ethanol sensitivity of control males, arguing that induced overexpression of *amn* does not make flies generally more resistant to ethanol (Figure 5A). The observed failure of *hs-amn* to alter the increased ethanol sensitivity of *lightweight* (*ltwt*), another mutant with increased ethanol sensitivity (see Experimental Procedures), further supports the specificity of the *amn^chpd* rescue (Figure 5A).

The above described heat shock regimen served to induce the *hs-amn* transgene throughout development, which precluded us from distinguishing whether the observed phenotypic rescue resulted from a restoration of normal development or from an acute requirement for Amn in adult flies during ethanol exposure. To address this issue, male *amn^chpd* flies carrying *hs-amn* were heat shocked as described above and then tested for ethanol sensitivity at various times after the last heat shock. As shown above, this heat shock regimen completely rescued the *amn^chpd* mutant phenotype when flies were assayed 24 hr after the last heat shock (Figure 5C). Full rescue was still evident after 48 hr. When flies were tested 72 hr after the last heat shock, however, their phenotype had reverted to the ethanol-sensitive phenotype of *amn^chpd*. Therefore, heat shock–induced *amn* expression throughout development does not rescue the adult mutant phenotype permanently.

We also found that induction of the *hs-amn* transgene only in adult flies was sufficient to rescue the *amn^chpd* mutant phenotype. A single heat shock resulted in a partial rescue, while three heat shocks, administered at 24 hr intervals, completely rescued the ethanol-sensitive phenotype of *amn^chpd* (Figure 5D). Similarly, three adult heat shocks completely rescued the stronger ethanol-sensitive phenotype displayed by *amn^X8* (Figure 5D). This finding rules out the possibility that in *amn^X8*, disruption of a neighboring gene contributes to its stronger ethanol-sensitive phenotype. Taken together with the fact that the *amn* ORF is deleted in *amn^X8* (see above), these data indicate that *amn^X8* reveals the complete loss-of-function phenotype.

To ensure that the behavioral rescue described above resulted from an increase in transcription of the *hs-amn* transgene, we used RT–PCR to assess the amount of transcript induced after heat shock. In the absence of heat shock, the *hs-amn* transcript was not detected (Figure 5B, lane 6), indicating little or no leaky expression. However, 4 hr after a single heat shock, the level of transcript increased significantly (Figure 5B, lane 7). In addition, the transgene-encoded transcript was still detectable in RNA isolated from phenotypically rescued *amn^chpd* flies (carrying *hs-amn*, heat shocked three times as adults, and tested 24 hr after the last heat shock) (Figure 5B, lane 8).

Mutations that Impair cAMP Signaling Also Alter Ethanol Sensitivity
Genetic data have implicated *amn* in activation of the cAMP pathway (Feany and Quinn, 1995). To further explore a potential role of the cAMP pathway in regulating

ethanol sensitivity, we extended our behavioral analysis to flies carrying mutations in three molecules involved in cAMP signaling: (1) *rutabaga* (*rut*), encoding the Ca²⁺-calmodulin-sensitive AC (Livingstone et al., 1984; Levin et al., 1992); (2) *dunce* (*dnc*), encoding the major cAMP-phosphodiesterase (PDE) (Chen et al., 1986; Qui et al., 1991); and (3) *DCO*, encoding the major catalytic subunit of cAMP-dependent protein kinase (PKA-C1) (Lane and Kalderon, 1993).

Males hemizygous for *rut^1*, *rut^2080*, or *rut^769* (Livingstone et al., 1984; Han et al., 1992) displayed an ethanol-sensitive phenotype similar to that of *amn* mutants (Figure 6; data not shown). Flies heterozygous for the loss-of-function *DCO^B3* and *DCO^H2* alleles, which show reduced cAMP-stimulated PKA activity (Lane and Kalderon, 1993), also displayed increased ethanol sensitivity (homozygotes cannot be tested because they die as embryos). Ethanol sensitivity of males hemizygous for the *dnc^1* or *dnc^M11* mutations (Dudai et al., 1976; Mohler, 1977), however, was nearly normal. These data show that flies unable to increase cAMP levels normally (such as *rut* and possibly *amn*) or to respond properly to increased cAMP levels (such as *DCO/+*) are more sensitive to ethanol-induced loss of postural control. The converse, however, is not observed; *dnc* flies, whose cAMP levels are several times higher than wild type (Byers et al., 1981; Davis and Kiger, 1981), display nearly normal ethanol sensitivity, a phenotype that is also observed in males doubly mutant for *dnc* and *amn*. Unexpectedly, whereas both *rut* and *amn* are ethanol sensitive, males doubly mutant for *rut* and *amn* are not significantly different from control (Figure 6; see Discussion).

Activation of AC by Forskolin Reverses the Increased Ethanol Sensitivity of *amn* and *rut* Mutants
To further investigate the relationship between cAMP signaling and ethanol sensitivity, we used the AC activator forskolin to manipulate cAMP levels in adult flies. Control and *amn^chpd* males were fed a 10 μM forskolin solution (see Experimental Procedures) for 2 or 4 hr prior to assaying their ethanol sensitivity in the inebriometer. Whereas forskolin treatment had no effect on the behavior of control flies, the ethanol sensitivity defect of *amn^chpd* flies was reversed by a 2 hr forskolin treatment (Figure 7A). Likewise, treatment of *rut^1* males with forskolin for 2 hr led to normal ethanol sensitivity (Figure 7A), a result likely due to the activation of another AC (Iourgenko et al., 1997). Interestingly, a 4 hr forskolin treatment of *amn^chpd* males further reduced ethanol sensitivity (Figure 7A), suggesting that one or more components of the cAMP pathway may have undergone compensatory up-regulation in *amn^chpd* mutants, thereby increasing the system's ability to respond to pharmacologically induced increases in cAMP levels. Taken together, these data indicate that the effects of *amn* and *rut* on ethanol sensitivity are directly related to their ability to modulate cAMP levels.

Inhibition or Activation of PKA Leads to Alterations in Ethanol Sensitivity of Wild Type and *chpd*
A reduction of PKA-C1 function, as observed in males heterozygous for *DCO* alleles (Lane and Kalderon, 1993),
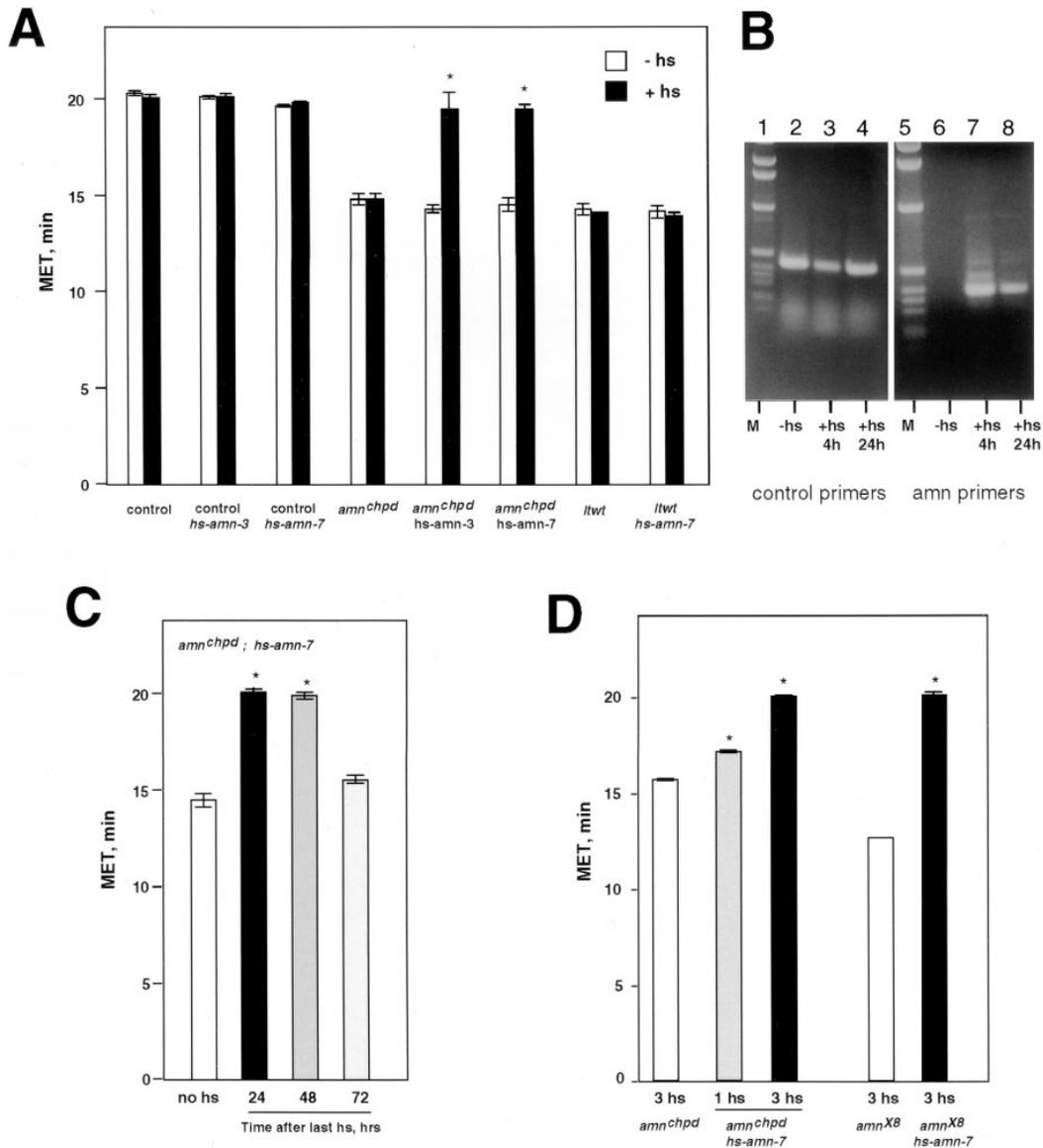
Figure 5. Induced Expression of a *hs-amn* Transgene Specifically Rescues the Ethanol Sensitivity of *amn*[chpd]

(A) Male flies of the genotypes indicated were raised in the absence (open bars) or presence (black bars) of daily heat shocks (see Experimental Procedures for heat shock protocol) and assayed in the inebriometer. In the absence of a *hs-amn* transgene, the heat shock regimen had no effect on wild-type or *amn*[chpd] males (p = 0.67 and 0.66, respectively). In the absence of heat shock, *amn*[chpd] males carrying any of the autosomal *hs-amn* transgenes displayed average METs similar to those of *amn*[chpd] (p = 0.04 and 0.10 for *amn*[chpd] carrying *hs-amn-3* and *-7*, respectively, when compared to *amn*[chpd]). Repeated heat shock induction of the *hs-amn* transgenes rescued the ethanol sensitivity of *amn*[chpd] (p = 0.60 and 0.19 for heat shock–treated *amn*[chpd] carrying *hs-amn-3* and *-7*, respectively, when compared to control untreated males). Induction of the *hs-amn* transgenes had no effect in control flies (p = 0.97 and 0.17 for control flies carrying *hs-amn-3* and *-7*, respectively, when compared to untreated males of the same genotype). Heat shock–induced expression of *hs-amn-7* had no effect on the ethanol sensitivity of *ltwt* (p = 0.58). The experiments were performed blind with respect to genotype (n = 4). Stars indicate a p < 0.0001 when comparing the corresponding genotypes in the absence and presence of heat shock treatment.

(B) The *amn* transcript is induced following heat shock treatment. Total RNA was isolated from untreated adult *amn*[chpd]; *hs-amn-7* males (lanes 2 and 6), from adult flies of the same genotype 4 hr after a single 1 hr heat shock (lanes 3 and 7), or from flies that had been behaviorally rescued by three adult heat shocks (lanes 4 and 8). Only the transcript encoded by the *hs-amn* transgene can be detected with the *amn* PCR primers used as the P element is inserted between the priming sites. The *amn* and the control transcripts were detected as described in Figure 4C.

(C) The phenotypic rescue of *amn*[chpd] by *hs-amn* is reversible. Male *amn*[chpd] flies carrying the *hs-amn-7* transgene were subjected to the heat shock regimen described above. The flies were tested in the inebriometer 24, 48, or 72 hr after the last heat shock. The MET of the flies assayed after 24 or 48 hr was significantly different from untreated flies of the same genotype (p < 0.0001; n = 2). However, after 72 hr the MET was similar to that of untreated flies (p = 0.38; n = 4).

(D) Induction of the *hs-amn* transgene in adult males is sufficient to rescue the ethanol sensitivity of *amn*[chpd]. Male flies carrying *amn*[chpd] or *amn*[X8] and the *hs-amn-7* transgene were assayed in the inebriometer after the transgene had been induced once (1 heat shock) or three times at 24 hr intervals (3 heat shocks). Induction of the *hs-amn-7* transgene during adulthood significantly decreased the ethanol-sensitivity of *amn*[chpd] and *amn*[X8] (asterisks represent p < 0.0001; n = 4).
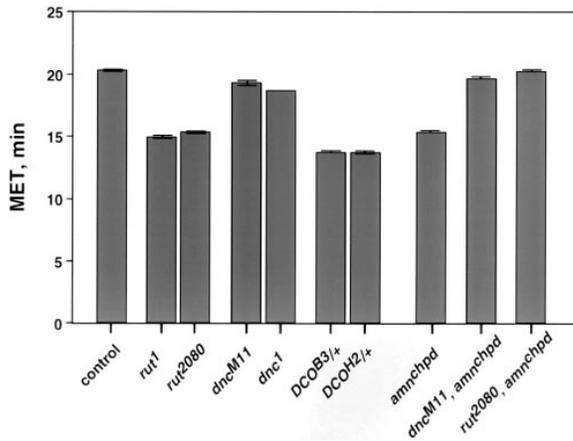
Figure 6. Mutations that Impair cAMP Signaling Alter Ethanol Sensitivity

Previously characterized alleles of *rut* (the loss-of-function EMS-induced *rut¹* allele and P element–induced allele *rut²⁰⁸⁰*) are significantly more sensitive to ethanol vapor compared to control flies ($p < 0.0001$; n = 4). Similarly, flies heterozygous for mutations in PKA-C1 (alleles *DCO^B3* and *DCO^H2*) are also ethanol sensitive ($p < 0.0001$; n = 4). The MET of *dnc¹*, but not that of *dnc^M11*, is significantly different from control ($p < 0.0001$ and $p = 0.028$, respectively; n = 4). Recombinants carrying *dnc^M11* and *amn^chpd* (*dnc^M11, amn^chpd*) or *rut²⁰⁸⁰* and *amn^chpd* (*rut²⁰⁸⁰, amn^chpd*) display a MET that is not significantly different from control ($p = 0.51$ and 0.75, respectively; n = 4).

led to increased ethanol sensitivity (Figure 6). To corroborate a role for PKA in ethanol sensitivity, we fed adult control and *amn^chpd* males solutions containing 200 μM R_p-cAMPS or S_p-cAMPS for 2 hr prior to their assay in the inebriometer. R_p-cAMPS is a competitive antagonist of cAMP that binds the regulatory subunit of PKA without releasing the catalytic subunit (Rothermel and Botelho, 1988); S_p-cAMPS is an analog of cAMP that activates PKA. S_p-cAMPS treatment of control males did not alter ethanol sensitivity. This treatment, however,

completely reversed the enhanced ethanol sensitivity of *amn^chpd* (Figure 7B). In contrast, feeding R_p-cAMPS to control males resulted in increased ethanol sensitivity. R_p-cAMPS treatment had the opposite effect on *amn^chpd* males, partially reversing their increased ethanol sensitivity (Figure 7B). While unexpected, this last observation is consistent with our finding that flies doubly mutant for *rut* and *amn* do not (unlike single mutants) display increased ethanol sensitivity (Figure 6; see Discussion).

Treatment of control flies with the PKA inhibitor R_p-cAMPS for only 2 hr led to an ethanol-sensitive phenotype similar to that of *amn*, *rut*, and *DCO/+* flies. This argues that even a relatively short-term inhibition of the cAMP pathway is sufficient to increase ethanol sensitivity.

In summary, as previously shown for activation of AC by forskolin (Figure 7A), direct activation of PKA activity reverses the ethanol sensitivity of *amn^chpd* without altering the response of control flies. Most importantly, inhibition of PKA activity in control flies causes an increase in ethanol sensitivity, thus mimicking the results obtained by genetic reduction of PKA activity.

Discussion

In a screen for *Drosophila* mutants with abnormal responses to an acute ethanol exposure, we isolated *chpd*, a mutant with increased sensitivity to ethanol-induced loss of postural control. We demonstrate that *chpd* is a mutation in the *amn* gene, which is believed to encode a neuropeptide that stimulates cAMP production (Feany and Quinn, 1995). Using a variety of genetic and pharmacological tools, we show that proper activation of the cAMP pathway plays an important role in regulating ethanol sensitivity in *Drosophila*. As the cAMP pathway has been implicated in the acute and chronic responses to ethanol in mammalian cells (for a review, see Diamond and Gordon, 1997), our findings suggest
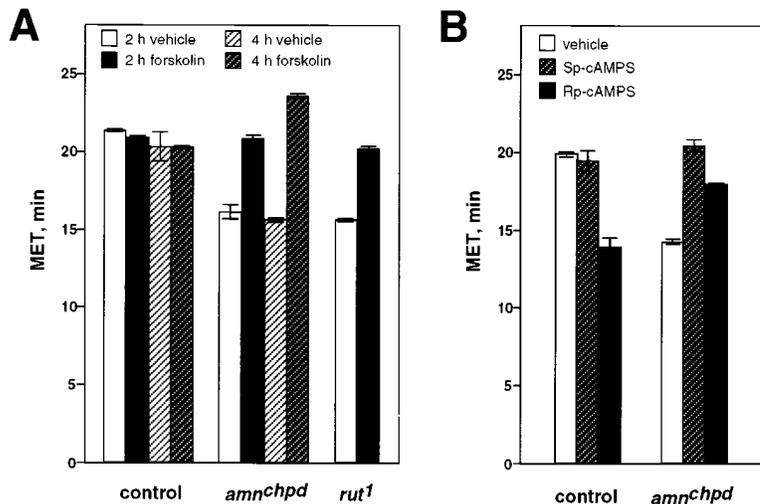

Figure 7. Pharmacological Agents that Affect AC and PKA Activity Alter Ethanol Sensitivity

(A) Forskolin treatment rescues the ethanol sensitivity of *amn^chpd* and *rut¹*. Adult control, *amn^chpd*, and *rut¹* male flies were fed a sucrose solution (vehicle) or the same solution containing 10 μM forskolin for 2 or 4 hr prior to being assayed in the inebriometer. Treatment with forskolin for 2 hr significantly increased the MET of *amn^chpd* and *rut¹* flies ($p < 0.0001$; n = 4) but had little effect on control flies ($p = 0.07$; n = 4). The MET of *amn^chpd* and that of control flies treated with forskolin for 4 hr was, however, significantly different ($p < 0.0001$; n = 5).

(B) Control and *amn^chpd* flies were fed sucrose solutions containing either no addition (vehicle), 200 μM S_p-cAMPS, or 200 μM R_p-cAMPS for 2 hr prior to being assayed in the inebriometer. Treatment with S_p-cAMPS did not alter the MET of control flies ($p = 0.47$; n = 4)

but reversed the ethanol sensitivity of *amn^chpd* ($p = 0.22$ when compared to untreated controls; n = 4). Conversely, treatment of control flies with R_p-cAMPS resulted in an ethanol-sensitive phenotype indistinguishable from that of *amn^chpd* ($p = 0.62$; n = 4). Treatment of *amn^chpd* flies with R_p-cAMPS caused a partial, but highly significant, reversal of ethanol sensitivity ($p < 0.0001$ when compared to untreated *amn^chpd* flies; n = 4).

that *Drosophila* is a powerful model system in which to study the regulation of ethanol-induced behaviors.

amn Regulates Ethanol Sensitivity in *Drosophila*

We provide three lines of evidence that *chpd*, a mutant isolated due to its increased ethanol sensitivity, is an allele of the memory mutant *amn*. First, several independently isolated alleles of *amn* also display increased ethanol sensitivity, a phenotype that is not complemented by *amn^chpd*. Second, the P element that causes the mutant phenotype is inserted in the putative ORF of *amn*. Third, induced expression of the *amn* ORF specifically rescues the ethanol sensitivity of two different *amn* mutants (*amn^chpd* and *amn^X8*).

Using a heat shock–inducible *amn* transgene, we show that *amn* is required in the adult stage to determine ethanol sensitivity. Moreover, the observation that the phenotypic rescue was reversible suggests that *amn* function is required in the adult fly, perhaps continuously, to determine normal ethanol sensitivity. Consistent with this finding is the observation that the *amn* transcript can be detected in the head of adult flies.

Several genes involved in cAMP signaling, including *rut*, *dnc*, and *PKA-C1*, are expressed preferentially in the mushroom bodies (Nighorn et al., 1991; Han et al., 1992; Skoulakis et al., 1993), two prominent brain structures that play a central role in olfactory learning and memory (de Belle and Heisenberg, 1994). Complete ablation of the mushroom bodies does not affect ethanol sensitivity (A. Y. L, M. S. M., and U. H., unpublished data), suggesting that *amn* acts elsewhere to regulate this behavior. Further experiments to determine where *amn* functions are in progress.

amn and the Regulation of cAMP Signaling

The signal transduction system generating cAMP is membrane bound and consists of three main components: (1) seven-transmembrane receptors that recognize extracellular signals, (2) heterotrimeric guanine nucleotide binding proteins (G proteins), and (3) G protein–stimulated ACs that convert ATP to cAMP. Amn has been postulated to increase cAMP levels based on the ability of *amn* mutants to suppress the sterility of *dnc* females (Feany and Quinn, 1995; data not shown) and due to its weak homology to mammalian neuropeptides (PACAP and GHRH) that couple positively with AC.

Several of our experiments provide evidence that proper regulation of the cAMP pathway is required to determine normal ethanol sensitivity (see below) and suggest that a defect in the activation of this pathway is responsible for the *amn* phenotype. First, the ethanol-sensitive phenotype of *amn^chpd* could be suppressed by mutations in the PDE-encoding *dnc* gene. Second, pharmacological stimulation of the cAMP pathway by activation of AC by forskolin or of PKA by S$_p$-cAMPS reversed the ethanol sensitivity of *amn^chpd*.

On the other hand, two additional pieces of evidence suggest that ethanol sensitivity is not correlated directly with the ability of flies to properly increase cAMP levels. First, whereas flies mutant for either *amn* or *rut* were ethanol-sensitive, double mutants displayed a nearly wild-type phenotype. Second, treatment of *amn^chpd* flies

with the PKA inhibitor R$_p$-cAMPS caused a reduction, rather than an increase, in ethanol sensitivity. While we do not understand the basis for this apparent paradox, it is possible that *amn* and *rut* function in different groups of cells or in separable biochemical pathways, both of which regulate ethanol sensitivity. Alternatively, the extent of activation of the cAMP pathway may not be related linearly to the degree of ethanol sensitivity of the fly. Finally, we cannot exclude the possibility that the cAMP analogs used in our pharmacological experiments act on multiple targets.

In summary, the combination of genetic and pharmacological evidence discussed above suggests that *amn* acts normally to increase cAMP levels and that the ethanol-sensitive phenotype displayed by *amn* mutants is caused by an impaired ability to activate this pathway. It remains possible, however, that *amn* also activates other pathways that modulate ethanol sensitivity.

Ethanol Sensitivity and cAMP Signaling

Our analysis of additional mutations that disrupt cAMP signaling strengthens our proposed role for this pathway in determining ethanol sensitivity in *Drosophila*. Mutations that reduce the ability of flies to properly increase cAMP levels (such as *rut*) or respond to increased cAMP levels (such as *DCO/+*) led to an augmentation in ethanol sensitivity similar to that displayed by *amn* mutants. Similarly, treatment with the PKA inhibitor R$_p$-cAMPS induced an ethanol-sensitive phenotype. The converse, however, was not observed. Genetic or pharmacological manipulations known to increase cAMP levels or PKA activity (as in *dnc* mutants or control flies treated with forskolin or S$_p$-cAMPS) did not reduce ethanol sensitivity from the wild-type level. Two observations indicate that this is not due to a ceiling effect of our behavioral assay. First, *amn^chpd* flies treated with forskolin for 4 hr are more resistant to ethanol than wild-type controls, and second, mutants with decreased sensitivity to ethanol can be isolated in our genetic screens (C. M. Singh and U. H., unpublished data). Thus, a component of the cAMP pathway functioning downstream of the activation of AC and PKA is rate limiting with regard to ethanol sensitivity.

In mammalian cells and tissues, ethanol potentiates receptor-mediated cAMP signal transduction (for a review, see Gordon et al., 1992); the mechanisms underlying this effect, however, remain poorly understood. While a direct link between cAMP signaling and ethanol-induced behaviors has not been established in mammals, the responses to acute ethanol are thought to be mediated by alterations in the function of various ligand-gated ion channels. Certain subtypes of GABA$_A$ and NMDA receptors are potentiated and inhibited by ethanol, respectively (for reviews, see Grant and Lovinger, 1995; Buck, 1996; Crews et al., 1996), and both these channels can be phosphorylated by PKA in cells, tissues, or heterologous expression systems (for a review, see Tabakoff and Hoffman, 1996). It is tempting to speculate that PKA phosphorylation of neurotransmitter receptors is altered by ethanol and that this contributes to the behavior of the inebriated animal.

Conclusions

We show that proper activation of the cAMP pathway plays a central role in regulating ethanol sensitivity in

flies. This signaling pathway has been implicated previously in regulating ethanol responses in mammalian cells (Diamond and Gordon, 1997). Moreover, the levels of AC are frequently reduced in lymphocytes and platelets obtained from alcoholic subjects, even after long periods of abstinence (Diamond et al., 1987; Tabakoff et al., 1988). *Drosophila* may thus provide a powerful genetic model in which to identify relevant novel genes that control ethanol sensitivity and neural responses to alcohol. As resistance to ethanol has been correlated with alcoholism in humans (Schuckit, 1994), genes and pathways identified in *Drosophila* may aid the study of the mechanisms responsible for this phenotype.

Experimental Procedures

Drosophila Strains and Genetics

amn^{chpd} was isolated in a genetic screen for P element–induced X-linked mutations displaying altered ethanol sensitivity when assayed in the inebriometer. Briefly, flies carrying the enhancer detector PZ[ry^+] (Mlodzik and Hiromi, 1992) on a CyO second chromosome were mated to flies carrying the transposase source P[ry^+, $\Delta2$–3]99B on a third chromosome marked with ry^{506} and *Ki*. Male progeny carrying *CyO* and *Ki* were mated to ry^{506} females. ry^+, Cy^+, Ki^+ male progeny were mated individually to XX/Y; ry^{506} females to establish stocks. Stocks carrying X-linked PZ[ry^+] insertions were tested for ethanol sensitivity in the inebriometer either individually or in pools. We screened ~5000 P element insertions and isolated ~12 mutants with increased or reduced ethanol sensitivity. The METs obtained for these mutants range from 11 to 28 min. This screen is ongoing and the results will be described in detail elsewhere.

Two independent alleles of *ltwt* (AF30 and V11), which disrupt a novel locus, were isolated in the above-described screen. $ltwt^{V11}$ males elute from the inebriometer with a MET of 12.38 ± 0.75 (n = 8) and display normal ethanol absorption and metabolism, locomotion, and geotaxis (M. S. M., A. Y. L., and U. H., unpublished data).

In all experiments, flies referred to as control contain the same PZ[ry^+] element as amn^{chpd} and *ltwt* in an unknown X-linked location; these flies behave in a manner that is indistinguishable from several wild-type *Drosophila* strains in all behavioral tests in which they have been assayed (ethanol sensitivity, ethanol tolerance, geotaxis, locomotion, and ethanol-induced changes in locomotor behavior) (C. M. Singh and U. H., unpublished data).

amn^{28A} (Ferveur et al., 1995) carries a mini-P[w^+] element at cytological location 19A and fails to complement the learning defect of amn^1 (J. D. and T. T., unpublished data). The EMS-generated mutants rut^1 (Livingstone et al., 1984), dnc^{M11} (Mohler, 1977), dnc^1 (Dudai et al., 1976), and the P element–induced mutants rut^{769} and rut^{2080} (Han et al., 1992) were backcrossed for at least six generations to XX/Y or to XX/Y; ry^{506} females, respectively, to eliminate potential autosomal mutations. Male flies for each behavioral experiment were generated by crossing mutant males to XX/Y females. dnc^{M11}, cv, amn^{chpd} and rut^{2080}, amn^{chpd} recombinants were generated using standard crosses; the presence of the *rut* and *amn* alleles was assayed by the expression of β-galactosidase driven by the respective enhancer-traps, and the presence of *dnc* was ensured by linked w^+ and *cv* markers (the *cv* mutation does not affect ethanol sensitivity).

To eliminate potential unlinked mutations, amn^{chpd}; $ry506$ females were crossed to ry^{506} males for five generations. Fly lines carrying excisions of the amn^{chpd} PZ[ry^+] element were generated in dysgenic females. Briefly, amn^{chpd}; ry^{506} males were crossed to $FM7c/+$; $\Delta2$–3, *Ki*, p^P females. Approximately 50 crosses were set up, each containing three dysgenic females carrying $amn^{chpd}/FM7c$; $\Delta2$–3, *Ki*, p^P/ry^{506} and several ry^{506} males. Single ry^{506} males from each dysgenic cross were mated to XX/Y; ry^{506} females for five generations prior to behavioral testing. Thirty-seven independent excision lines were generated. Among these, 9 lines reverted to a wild-type phenotype, 3 displayed more severe ethanol sensitivity, 7 behaved similarly to amn^{chpd}, and 18 had an intermediate phenotype. Sequence analysis

revealed that 4 of the 9 phenotypic revertants carry a precise excision of the P element. PCR analysis of the remaining five phenotypic revertants demonstrated that a 1.4 kb fragment of the P element still remained. Sequence analysis of three of these imprecise excisions revealed that they contained exactly the same lesion. Specifically, they carry a 5 bp deletion flanking the P element (AACAC, position 2610–2615) and retain exactly 585 bp from the 5′ and 815 bp from the 3′ of the P element. We do not understand how these imprecise excisions can lead to a wild-type phenotype. We speculate that these particular revertants still allow the synthesis of a stable *amn* transcript that encodes a functional Amn peptide, since Amn is believed to be processed into smaller peptides (Feany and Quinn, 1995) and the P element is inserted near the putative C terminus of the ORF. Molecular analysis of the seven imprecise excisions that did not revert to a wild-type phenotype revealed that they carry P elements of various lengths.

Excisions of the P[w^+] insertion in amn^{28A} were generated by dysgenesis in males. Briefly, amn^{28A} females were crossed to w^{1118}; *Dr*, $\Delta2$–3/TM6B males. Dysgenic progeny w, amn^{28A}/Y; *Dr*, $\Delta2$–3/+ were crossed to XX/Y females, and excision derivatives that had lost the P[w^+] element were balanced over FM7a. Of the 23 independent lines generated, 19 were analyzed and found to contain imprecise excisions. Subsequent mapping of these excisions by Southern blotting and PCR analysis led to the isolation of a single deficiency, amn^{X8}, which contained a breakpoint between nucleotides 2683 and 2819, deleting the *amn* ORF.

Inebriometer, Locomotion, and Geotaxis Assays

For behavioral testing, flies were raised on standard cornmeal food at 25°C and 70% relative humidity. For each inebriometer test, approximately 100 2–5-day-old male flies were collected and placed in a vial with fresh food for 24 hr at 25°C prior to behavioral testing. Flies were tested in an inebriometer preequilibrated with ethanol vapor. Different ethanol vapor concentrations were achieved by mixing in various proportions ethanol vapor, produced by diffusion of air through a 95% ethanol solution, with humidified air. All experiments, with the exception of the dose-response curve shown in Figure 1C, were carried out at an ethanol vapor concentration of 50/45; this corresponds to a relative flow of 50 U of ethanol vapor and 45 U of humidified air and is equivalent to approximately 15 mM ethanol. The MET corresponds to the sum of the number of flies eluting at a given time (minute) multiplied by the time of elution divided by the total number of flies. All experiments were carried out at least in duplicate and at least twice (see figure legends for specific information). In all of the experiments shown in Figures 3 and 5, the inebriometer operator was blind to the genotype of the flies; only the wild-type control was known to assess proper functioning of the inebriometers.

Spontaneous locomotor activity was measured in plastic tubes covered with lines at 1 cm intervals and was quantified as the number of lines crossed per minute. Individual flies are tested for 15 min, which is approximately the inebriometer MET for *chpd*. In this assay, control males scored 11.9 ± 1.8 and *chpd* males scored 11.7 ± 2.9 (n = 10); there was no fatigue during the time period of the assay.

Negative geotaxis was measured in a 30 cm long and 2 cm wide glass cylinder. Ten flies are banged to the bottom of the cylinder and observed as they climb up to the top; this procedure is repeated at 1 min intervals for 20 min. This assay not only measures geotaxis, but also locomotion and responsiveness to banging. The negative geotaxis score corresponds to the percentage of flies that have arrived at the top in 1 min. Control males scored $98\% \pm 2\%$ and *chpd* males scored $94\% \pm 6\%$. There was no obvious difference in the velocity with which control and *chpd* males climbed up the column and there was no fatigue during the period of the assay.

DNA and RNA Analysis

Genomic sequences flanking the amn^{chpd} and amn^{28A} insertion were isolated by plasmid rescue (Wilson et al., 1989). mRNA from third instar larvae, adult heads, adult bodies, or whole flies was isolated using the RNA-STAT60 (Tel-Test, Inc.) reagent followed by an oligo(dT)-biotin selection (Promega). The mRNA was treated with DNAse (RNAse free) or with RNAse (DNAse free) for 1 hr at 37°C

prior to being used for reverse transcription (RT). For analysis of the *amn* transcript in wild-type and *amn1* flies, the RT reaction was performed according to manufacturer's protocol (Pharmacia) using three different amounts of mRNA (0.01, 0.05, and 0.1 μg) and an *amn*-specific primer (5'-CGGATTATACGGCGTATGTGCAAGCC-3'). The RT reactions were amplified using an *amn* antisense primer (5'-CTGCTGTGGCGTTGCACTGCGTATT-3') in a final volume of 50 μl. This RT–PCR reaction (1 μl) was used for a subsequent PCR amplification with nested *amn* primers (3'-GTAAGCGGTTCGAAAGG TACGCGC and 5'-CTCTTGGGTTCTCGCGAGAGAAC-3') and the PCR product was electrophoresed in 1.5% agarose gel. For analysis of the control transcript, the RT reaction was set up using three different amounts of mRNA (0.01, 0.05, and 0.1 μg) with a control primer from the *Roi* locus (5'-CTGAGGTACCTACATCAATAACAACC ATG-3') and PCR amplified using an antisense primer (5'-CGATGAA CGGTCCCGATGGAATGC-3'). For analysis of the *amn* transcript induced upon heat shock, 5 μg of total RNA was used with the *amn* primers described above.

Generation and Analysis of *hs-amn* Flies

The *hs-amn* transgene was generated by subcloning a 744 bp PCR fragment generated with *amn* primers 5'-TGTGCGCTGCTGGTATTG GCGCG-3' and 3'-GTGATCTTGTTAGGTCTTCGG-5' into the SmaI site of Bluescript KS+. The amplified fragment extends from nucleotides 2067 to 2811 (Figure 4). The *amn* fragment was excised by digestion with EcoRI and XbaI and cloned into the P element transformation vector pCaSpeR, generating the *P[w+, amn]* construct. *amnX8* embryos were injected with a solution containing 10 μg of the *P[w+, amn]* construct and 2 μg pTurbo in 19 μl of injection buffer (5mM KCl, 0.1 M KH2PO4 [pH 6.8]) and 1 μl of food dye. Five independent transformed lines were generated and characterized: *hs-amn-1, -3, -4,* and *-7* mapped to one of the autosomes; *hs-amn-2* is X-linked. Males carrying *hs-amn-1, -3, -4,* or *-7* were crossed to *amnchpd; ry506* females and the male progeny, carrying both the *amnchpd* mutation and the *hs-amn* transgene, were used in behavioral testing. The male progeny from the cross between *amnchpd; ry506* females and *hs-amn-2* males carry only the *amnchpd* mutation and were used as a negative control; this protocol ensures that all flies have a similar genetic background. As additional controls, we also crossed males carrying *hs-amn* to control and *ltwt* flies (*ltwt* is another X-linked mutant with increased ethanol sensitivity).

The progeny of these crosses were grown in the presence or absence of a daily heat shock (1 hr at 37°C–38°C) starting in the early third larval instar and continuing throughout pupal life. Upon eclosion, adult male progeny were collected and heat shocked one more time 24–48 hr after eclosion. Alternatively, the progeny of these crosses received a single or three daily heat shock treatments starting 24–48 hr after eclosion. Flies were analyzed for ethanol sensitivity 24 hr after the last heat shock.

Ethanol Absorption and Metabolism

Ethanol absorption was measured by exposing flies to ethanol vapor for defined periods of time (0, 10, 20, or 30 min) in 50 ml Falcon tubes containing multiple small perforations to allow the flow of ethanol vapor. A mixture of ethanol vapor and humidified air was diffused through these tubes. Immediately following exposure, 30 flies were frozen in liquid nitrogen and homogenized in 300 μl of 50 mM Tris-HCl (pH 7.5). The homogenate was centrifuged at 15,000 g for 20 min at 4°C. Supernatant (5 μl) was added to 500 μl of Alcohol Reagent (Sigma) containing alcohol dehydrogenase and NAD+. The production of NADH was monitored by the increase in absorbance at 340 nm, which is directly proportional to the alcohol concentration in the sample (calculations were carried out following manufacturer's instructions). The protein concentration was determined with Comassie Plus reagent (Pierce) and used to normalize the alcohol concentration in each sample. To calculate the millimolar ethanol concentration in flies, the volume of one fly was estimated to be 2 μl. Ethanol metabolism was assessed by exposing flies to ethanol vapor for 30 min and quantitating the amount of ethanol remaining in the flies 30, 60, 150, 210, and 270 min after the end of the exposure. This assay easily discriminates mutations that alter ethanol absorption (such as flies carrying the *yellow* mutation, which are sensitive

to ethanol) or metabolism (such as flies carrying null alleles of *Adh*) (C. M. S. and U. H., unpublished data).

Pharmacological Treatment

Approximately 120 adult male flies (2–3 days old) were starved in glass vials for 5–6 hr at room temperature before being fed a sucrose solution (1.5 % sucrose, 10 mM Tris [pH 7.4]) containing 10 μM forskolin (RBI), 200 μM Rp-cAMPS (Biolog), or 200 μM Sp-cAMPS (Biolog) for 2 or 4 hr. As a control, flies were fed the sucrose solution. Flies were assayed in the inebriometer immediately after the feeding protocol.

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