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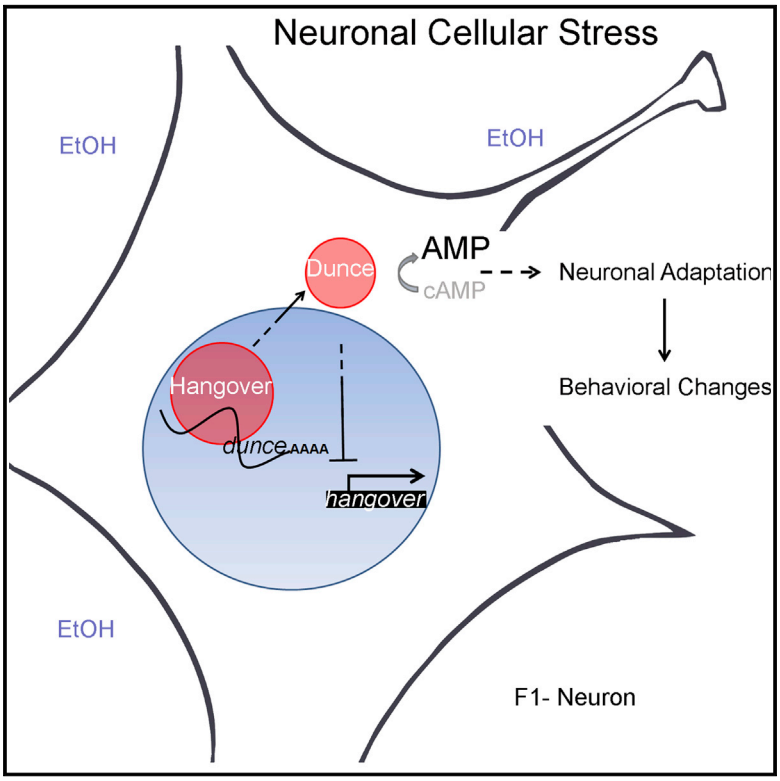
Hangover Links Nuclear RNA Signaling to cAMP Regulation via the Phosphodiesterase 4d Ortholog *dunce*

Graphical Abstract



Highlights

- The nuclear stress protein Hangover binds RNA.
- The Pde4d ortholog *dunce* is a target of Hangover signaling.
- Both mediate experience-dependent behavioral changes in the same neurons.
- Hangover links two cAMP-dependent mechanisms to regulate ethanol tolerance.

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In Brief

Cellular stressors like ethanol cause specific behavioral changes. Ruppert et al. show that the nuclear stress RNA-interacting protein Hangover is broadly expressed but only required in neurons mediating experience-dependent changes in behavior. At the cellular level, Hangover links nuclear signaling to cAMP regulation via the phosphodiesterase 4d ortholog Dunce.

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Hangover Links Nuclear RNA Signaling to cAMP Regulation via the Phosphodiesterase 4d Ortholog *dunce*

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SUMMARY

The *hangover* gene defines a cellular stress pathway that is required for rapid ethanol tolerance in *Drosophila melanogaster*. To understand how cellular stress changes neuronal function, we analyzed Hangover function on a cellular and neuronal level. We provide evidence that Hangover acts as a nuclear RNA binding protein and we identified the phosphodiesterase 4d ortholog *dunce* as a target RNA. We generated a transcript-specific *dunce* mutant that is impaired not only in ethanol tolerance but also in the cellular stress response. At the neuronal level, Dunce and Hangover are required in the same neuron pair to regulate experience-dependent motor output. Within these neurons, two cyclic AMP (cAMP)-dependent mechanisms balance the degree of tolerance. The balance is achieved by feedback regulation of Hangover and *dunce* transcript levels. This study provides insight into how nuclear Hangover/RNA signaling is linked to the cytoplasmic regulation of cAMP levels and results in neuronal adaptation and behavioral changes.

INTRODUCTION

In mammals and insects alike, repetitive ethanol exposure leads to the development of tolerance (Scholz and Mustard, 2013). Tolerance is defined as an increased resistance to the behavioral effects of ethanol upon previous exposure. Tolerance is also used as a criterion to diagnose alcohol use disorders (American Psychiatric Association, 2013). Previously, we established *Drosophila melanogaster* as a suitable model system to analyze the molecular and neural bases of rapid ethanol tolerance (Scholz et al., 2000). At least two different mechanisms contribute to the rapid development of tolerance. One requires the monoamine octopamine, which shares functional similarities to noradrenaline in vertebrates, whereas the other relies on a cellular stress response uncovered by the phenotypic characterization of *hangover* (*hang*) mutants (Scholz et al., 2000, 2005). The *hang* gene encodes a large, approximately 210-kDa nuclear zinc finger protein that is expressed in most, if not all, neurons in the adult brain (Scholz et al., 2005). A Hang-related protein, ZNF699, is associated with alcoholism in humans (Riley et al., 2006), supporting the idea that a cellular stress mechanism underlying ethanol tolerance is evolutionarily conserved between humans and *D. melanogaster*. *Hang* mutants develop reduced ethanol tolerance and show defects in their response to oxidative stress and heat shock-induced ethanol resistance. However, the cellular signaling process upon which Hang acts remains unclear, and how the broadly expressed Hang protein mediates specific behavioral changes in response to global increases of cellular stressors such as ethanol remains an open question.

In neuronal cell lines, ethanol exposure reduces cyclic AMP (cAMP) levels, which results in cellular tolerance (Diamond et al., 1991). cAMP-dependent phosphodiesterases (PDEs) are essential components of the cAMP signaling cascade and fine-tune cyclic nucleotide signaling (Conti and Beavo, 2007). In *Drosophila*, three different cAMP-dependent PDEs have been identified (Day et al., 2005); two of them play a dual role in cyclic guanosine monophosphate (cGMP) and cAMP signaling, whereas only Dunce (Dnc) is cAMP specific (Davis and Kiger, 1981; Houslay, 1998). The *dnc* gene encodes for at least eight isoforms with high homology to the human phosphodiesterase 4 class of PDEs (Bolger et al., 1993). In general, PDE4s can be divided into two classes based on the presence or absence of the two highly conserved N-terminal domains. The long forms contain the upstream conserved region 1 and 2 (UCR1 and UCR2, respectively) domains, whereas the short forms lack the UCR1 domain and have either a partial or complete UCR2 domain (Bolger et al., 1993). The Dnc isoforms share a common PDE domain but differ in their N termini, suggesting that they may exhibit different regulatory and cellular functions (Bolger et al., 1993; Qiu and Davis, 1993). This hypothesis has been further

supported by the phenotypic characterization of various *dnc* mutants. For example, dnc^{1} and dnc^{M11} share similar initial learning and memory defects (Tully and Gold, 1993) but differ at the level of the neuroanatomical structure of the larval neuromuscular junction. Nerve terminal growth is increased in dnc^{1} mutants, whereas the nerve terminals grow normally in dnc^{M11} mutants (Zhong et al., 1992). These phenotypic differences correlate with differences in PDE activity. dnc^{1} mutants show reduced PDE activity, and dnc^{M11} mutants display no detectable PDE activity; as a result, the cAMP level is elevated in both mutants (Byers et al., 1981). While the function of PDE4s has been analyzed in terms of the cellular stress response to oxidative stress and in non-neuronal tissues such as pulmonary endothelial cells (Ortiz et al., 2013), the function of PDE4s in ethanol-induced cellular stress in neurons has not been addressed.

Here, we provide evidence that Hang functions as a nuclear RNA binding protein and we identify *dnc* as a target gene. To confirm the role of the PDE4 ortholog Dnc in ethanol tolerance, we investigated the consequence of altered cAMP signaling by analyzing the dnc^{1} mutant for defects in ethanol-induced behaviors. Because multiple Dnc isoforms are altered in this *dnc* mutant, we generated a new dnc^{RA} transcript-specific mutant, $dnc^{\Delta 143}$, which enabled isoform-specific analysis of ethanol tolerance. To identify the neurons required for Dnc-dependent ethanol tolerance, we generated a dnc^{RA}-specific promoter-GAL4 driver (dnc^{RA}-GAL4). Phenotypic characterization revealed that both the $dnc^{\Delta 143}$ and *hang* mutants exhibited similar impairment in the cellular stress response to ethanol. Both genes were required in the same neurons, enabling them to participate in the same cellular signaling process. In addition, Dnc^{PA} regulates Hang function during the development of ethanol tolerance. Furthermore, cytoplasmic Dnc, rather than nuclear Dnc, is required for tolerance development. The identification of the PDE4 ortholog Dnc as an interaction partner of Hang links nuclear Hang function to the regulation of cytoplasmic cAMP levels. These results substantiate the conserved nature of the signaling process underlying the neuronal stress response to ethanol.

RESULTS

Hang Function as an RNA Binding Protein

To identify the potential mechanism through which Hang regulates rapid ethanol tolerance, we first analyzed the nuclear expression of Hang in the adult brain using an anti-Hang antibody (Figure 1A). Hang is expressed in a punctate pattern in the nucleus, resembling the expression pattern of RNA-processing nuclear factors such as heterogenous nuclear ribonucleoproteins (hnRNPs) (Lamond and Spector, 2003). To assess the intra-nuclear distribution of Hang in more detail, we generated transgenic flies that expressed a GFP::Hang fusion protein under UAS control. Ectopic expression of this construct in the salivary glands using the D42-GAL4 driver (Yeh et al., 1995) revealed punctate expression in the nucleus that did not co-localize with DNA (Figure 1B). Hang expression was excluded from nucleoli (Figure 1B). In addition, we failed to detect the association of GFP::Hang with late larval polytene chromosomes. These observations suggest that Hang might be involved in RNA processing.

The Hang protein contains two conserved zinc finger motifs that are commonly found in RNA binding proteins in addition to 13 others (Figure 1C; Scholz et al., 2005). To test whether these motifs can bind RNA directly, we generated a 6xHis-tagged Hang fusion protein containing the RNA zinc finger binding motifs (F2; Figure 1C) and analyzed whether this protein bound RNA in vitro. As a negative control, we used a 6xHis-tagged Hang fusion protein of a similar size (F1; Figure 1C). As a positive control, we used a 6xHis-tagged poly(A) RNA binding protein (pAbp) (Lefrère et al., 1990). The F2-Hang and pAbp fusion proteins bound RNA (Figure 1D), whereas the F1-Hang fusion protein did not. Its subcellular localization and RNA interaction in vitro are consistent with the function of Hang as a RNA-interacting protein. To identify putative transcripts that might interact with Hang, we performed a cDNA microarray experiment in which we compared transcription levels in the heads of control and $hang^{AE10}$ mutants. We identified the PDE4 ortholog *dnc* as a putative transcript reduced in the $hang^{AE10}$ mutants (for additional transcripts, see Table S1). To validate this result, we performed qRT-PCR analysis to compare *dnc* transcriptional levels in $hang^{AE10}$ and control flies' heads using primers recognizing the shared sequence of all *dnc* transcripts (Figure 1E). As expected, *dnc* was downregulated in the $hang^{AE10}$ mutants. To investigate whether Hang binds *dnc* transcripts in vitro, we performed RT-PCR analysis on the RNA pulled down with the F2-Hang fusion protein (Figure 1F). The *dnc* transcript was detected in the RNA bound to the F2-Hang fusion protein. Taken together, the results suggest a common function of *hang* and *dnc* in the same signaling pathway.

Altered Ethanol-Induced Behavior in dnc^{1} Mutants

To determine whether *dnc* as *hang* is required for ethanol tolerance, we first tested the known mutant dnc^{1} for ethanol-induced behaviors in an inebriometer, an apparatus that measures the effects of ethanol on postural control (Figure 2A). The initial exposure to ethanol is described as the mean elution time (MET1) and measures the sensitivity/resistance of flies in response to ethanol (Figure 2B; Cohan and Hoffmann, 1986; Moore et al., 1998). To analyze their ability to develop tolerance, flies were re-exposed to ethanol after a recovery period of 4 hr (MET2). Tolerance is defined as an increase in MET after the initial exposure and is measured as the relative difference between MET2 and MET1 (Scholz et al., 2000). The dnc^{1} mutants did not differ from controls in terms of alcohol sensitivity even though the tolerance was reduced by 43% (Figure 2B), suggesting that Dnc plays a role in normal tolerance development. The dnc^{1} allele is an EMS-induced hypo-morph with respect to PDE activity, but the mutation responsible for this reduction is unknown (Byers et al., 1981; Dudai et al., 1976). Genomic annotation of the *dnc* locus predicts several transcripts. We confirmed eight transcripts by expressed sequence tag (EST) and RT-PCR analysis (Figure 2C). Because we could not detect any evidence for the annotated dnc^{RR} or dnc^{RE} transcripts using RT-PCR under normal conditions, we concluded that this transcript subgroup contains only dnc^{RA}. To identify the *dnc* transcript responsible for tolerance, we analyzed the expression of five different *dnc* transcript subgroups in dnc^{1} mutants through transcript-specific qRT-PCR

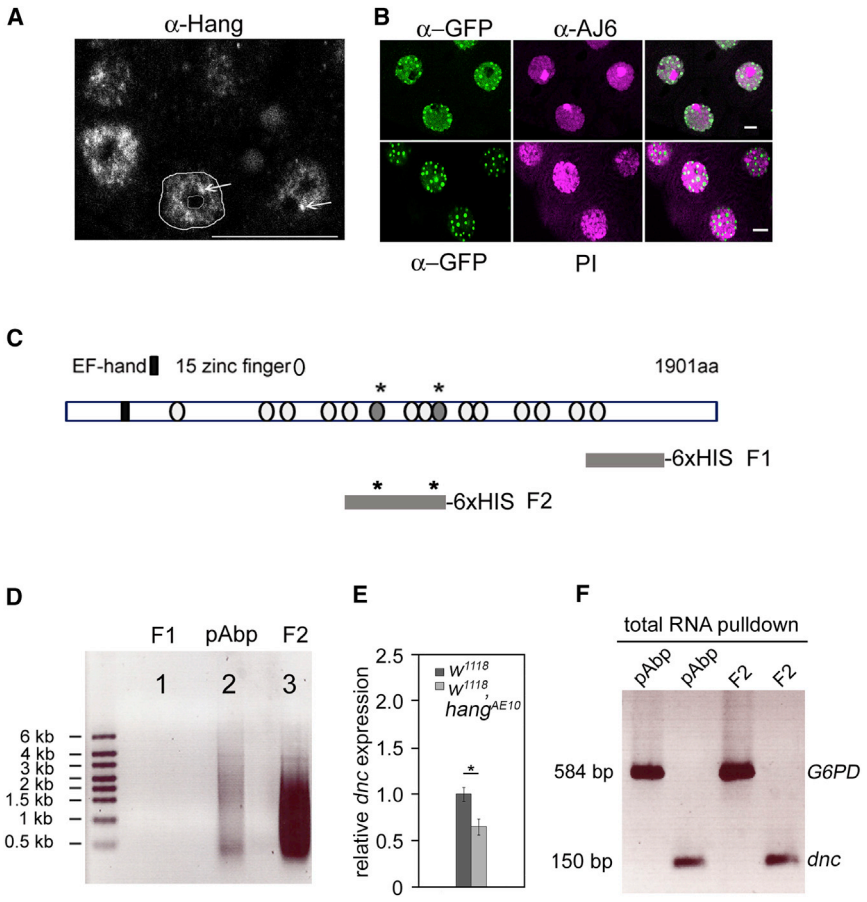

Figure 1. Hang Binds RNA

(A) Hang expression in single cells was visualized via α-Hang antibody staining of 10-μm-thick cryostat sections of the adult brain. Arrows indicate punctate Hang expression in the nucleus but not in the nucleoli. The circles indicate the boundary of the nucleus and nucleolus.

(B) In the salivary glands of D42;*UAS-GFP::hang* third instar larvae, ectopic expression of GFP::Hang was detected inside the nucleus but not in the nucleoli or on the DNA. The AJ6 antigen was used as a marker for the nucleolus and propidium iodide (PI) was used for DNA. The scale bars represent 10 μm.

(C) The Hang protein structure (not to scale) and the 6xHis-tagged fusion proteins are shown. The black bar indicates a Ca^{2+}-binding EF hand motif and the ovals indicate the C2-H2-like zinc finger domains. The asterisk indicates the C2-H2-like zinc finger motif found in RNA binding proteins (gray ovals).

(D) In an RNA pull-down experiment, total RNA was applied to a column loaded with the 6xHis-tagged Hang fusion proteins F1 or F2 or with a 6xHis-tagged pAbp. Bound RNA was eluted and analyzed by gel electrophoresis. The F2-Hang fusion protein and the control pAbp bound to RNA.

(E) *dnc* transcript levels were significantly reduced in $hang^{AE10}$ mutants. qRT-PCR analysis of *poly(A)*-selected head RNA using primers specific for the shared sequence of all *dnc* transcripts were performed in triplicate (n = 3–5 independent RNA samples). *Su(Tpl)* gene primers were used as a reference. (*$p < 0.05$, Student's t test, error bars show SD).

(F) The RNA pulled down with the F2-Hang fusion protein was transcribed into cDNA and analyzed for expression of the *dnc* transcripts. The pAbp-bound RNA and the glucose-6-phosphate dehydrogenase (*G6PD*) transcript were used as controls.

See also Table S1.

analysis (Figure 2C; Table S2 for primers; Qiu and Davis, 1993). In the dnc^{1} mutant, the transcripts dnc^{RB}, dnc^{RJ}, and dnc^{RA} were downregulated, whereas the $dnc^{RG\text{-}RN}$ transcript group was upregulated (Figure 2D). The dnc^{RG} and dnc^{RN} transcripts were grouped based on their similar protein coding sequence. Primers specific for the conserved sequence shared by all of the *dnc* transcripts showed no overall up- or downregulation. Thus, the altered tolerance in dnc^{1} mutants might be due to reduced dnc^{RB}, dnc^{RJ}, or dnc^{RA} transcripts or increased $dnc^{RG\text{-}RN}$ transcripts. Because multiple isoforms were affected, it was difficult to identify the isoform required for ethanol tolerance; therefore, we aimed to generate a transcript-specific mutant. Because the development of ethanol tolerance exhibits similarities with mechanisms underlying learning and memory (Cunningham et al., 1983; Fadda and Rossetti, 1998) and because the dnc^{RA} transcript group is associated with learning in *Drosophila* (Qiu and Davis, 1993), we focused our analysis primarily on this transcript group.

Requirement for PDE Activity for Ethanol Tolerance

To investigate whether the reduction of tolerance is indeed due to defects in dnc^{RA} function, we generated a dnc^{RA} transcript-specific mutant using P-element mutagenesis generating imprecise excisions by remobilization of the P-element insertion. Beginning with the dnc^{EP973} insertion line as the parental chromosome, we generated the $dnc^{\Delta 143}$ mutant, which has a deletion in the first exon of the dnc^{RA} transcript (Figure 3A). The deletion reduced the dnc^{RA} transcription level to almost zero, as confirmed by qRT-PCR. The deletion also resulted in a slight reduction in dnc^{RL} expression but did not affect the expression of the other transcript groups (Figure 3B). While $dnc^{\Delta 143}$ mutants showed normal ethanol sensitivity, their tolerance was reduced by approximately 36% (Figure 3C). To confirm the necessity of Dnc for ethanol tolerance, we restored Dnc expression using a UAS-dnc^{all} transgene during development containing the coding sequence for the PDE domain and UCR2 present in all *dnc* transcripts (Qiu and Davis, 1993). At the larval neuromuscular junction (NMJ), the expression of this variant can efficiently reduce cAMP levels (Cheung et al., 1999). To ensure that PDE activity was restored in the neurons in which dnc^{RA} is normally expressed, we generated a dnc^{RA}-GAL4 driver carrying a genomic fragment with the putative dnc^{RA} promoter region (Figure 3A). Expression of UAS-dnc^{all} in the $dnc^{\Delta 143}$ mutant using the dnc^{RA}-GAL4 driver during development restored ethanol tolerance to control levels

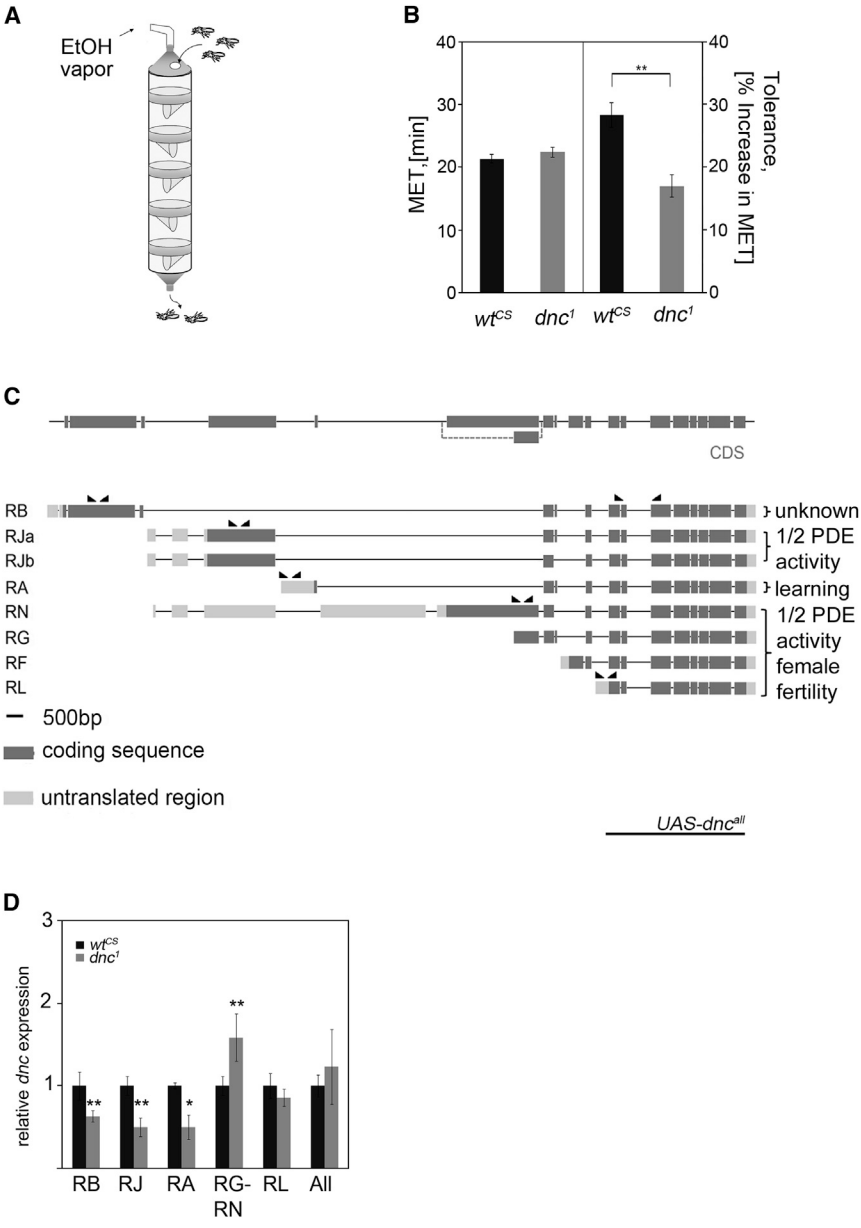

Figure 2. Altered Ethanol-Induced Behavior in the *dnc*^{1} Mutant

(A) The effect of ethanol on behavior was measured in an inebriometer, a column filled with ethanol vapor (2.5 L with 95% EtOH saturated air: 2.25 L humidified air). Sober flies show negative geotaxis, whereas intoxicated flies lose postural control and fall down.

(B) The initial sensitivity, measured as MET1, did not differ between the *dnc*^{1} mutants and the controls, but the mutants reduced their tolerance (n = 8–16; **p < 0.01, Student's t test). Error bars are the SEM.

(C) The genomic region encoding the *dnc* gene spans 160 kb. Triangles indicate primers used for qRT-PCR. The UAS-*dnc*^{all} transgene contains the coding sequence of *dnc*-^{RL} transcript (following Cheung et al., 1999). Brackets indicate functional groups following Qiu and Davis (1993).

(D) qRT-PCR analysis of *poly(A)*-selected head RNA of *dnc*^{1} mutants with *dnc* transcript group-specific primers revealed a significant difference in *dnc*^{RB}, *dnc*^{RJ}, *dnc*^{RA}, and *dnc*$^{RG-RN}$ transcript levels (n = 3–5 independent RNA samples with three replicates each; *p < 0.05, **p < 0.01, Student's t test). Error bars indicate the SD. *actin5C* gene primers were used as a reference.

See also Tables S2 and S3.

(Figure 3D). Thus, the reduced tolerance in *dnc*$^{\Delta 143}$ flies is caused by the loss of PDE activity and Dnc function.

Requirement of Cytoplasmic Dnc^{PA} for Tolerance

The different Dnc isoforms share a common PDE domain but differ in their isoform-specific motifs present in the N-terminal region (Figure 4A; Bolger et al., 1993). For example, the Dnc^{PG} and Dnc^{PA} isoforms belong to the same class of long isoforms, but Dnc^{PG}, in contrast to Dnc^{PA}, contains a nuclear localization signal (NLS) (Figure 4A). To determine whether different Dnc isoforms display different subcellular localization patterns, we generated the *UAS-dnc*^{RG}*::GFP*, *UAS-dnc*^{RA}*::GFP*, and *UAS-dnc*^{RL}*::GFP* transgenes with different N termini. A GFP tag was added to the C terminus to visualize expression of the transgenes. The functionality of the transgenes was verified by a lethality test (Figure S1). To have a better resolution of subcellular compartments, GFP-tagged isoform expression was analyzed in the well-structured large epithelial follicle cells of the ovaries using *traffic-jam*-GAL4 (*tj*-GAL4; Pancratov et al., 2013) (Figure 4B). Dnc^{PA} expression was found mainly in the cytoplasm in a punctate pattern that extended from the nucleus outward, resembling the endoplasmic reticulum (compare Figure 1; Doumanis et al., 2007). In contrast, Dnc^{PG} expression was found in the nucleus (Figure 4B). The expression of Dnc^{PL} was similar to that of Dnc^{PA}. The different Dnc isoforms were used to restore the reduced tolerance phenotype of the *dnc*$^{\Delta 143}$ mutants to normal levels using *dnc*^{RA}-GAL4 (Figures 4C–4E). The expression during development of the *UAS-dnc*^{RA}::GFP transgene significantly improved the reduced tolerance phenotype of *dnc*$^{\Delta 143}$ mutants (Figure 4C), whereas the Dnc^{PG} or Dnc^{PL} isoforms did not (Figures 4D and 4E). Together with the anatomical data, these behavioral data support the hypothesis that the N terminus of Dnc isoforms is important for their subcellular localization and function. In addition, cytoplasmic Dnc^{PA} function is required for tolerance.

Mediation of Dnc-Dependent Tolerance by Central Complex Neurons

To identify the brain structure involved in mediating ethanol tolerance in a Dnc-dependent manner, we characterized the

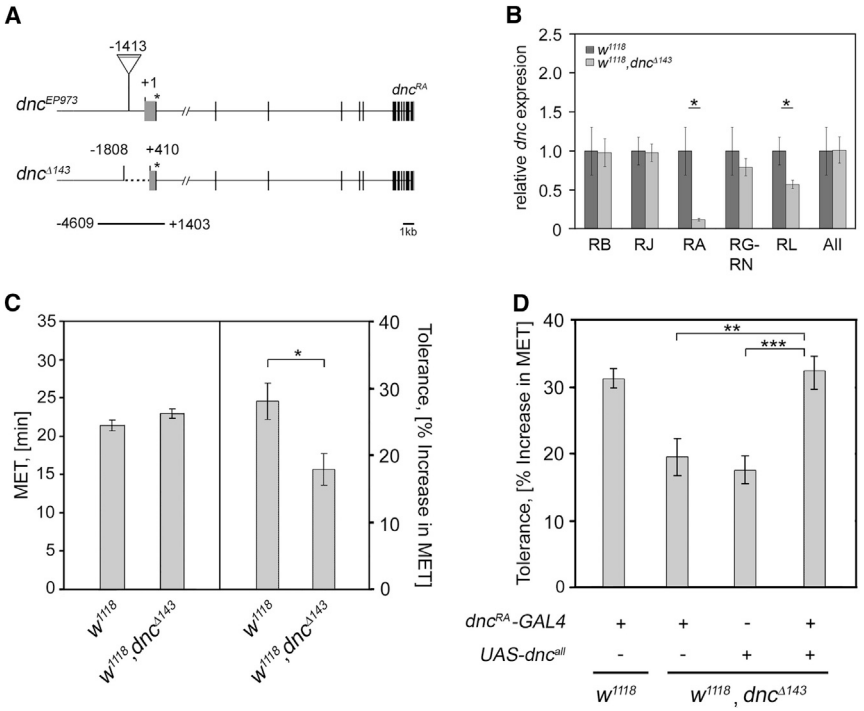

Figure 3. Requirement for Dnc in Ethanol Tolerance

(A) The triangle indicates the transposable element of the dnc^{EP973} insertion site 1.4 kb upstream of the transcriptional start site. The dotted line represents the 2,219-kb deletion of the $dnc^{\Delta 143}$ mutant, which deletes the 5′ UTR but not the translational start site (indicated by a star). The black boxes represent exons. An approximately 6-kb fragment of the dnc^{RA} promoter region (indicated by a black line) was used to generate the dnc^{RA}-GAL4 driver.

(B) qRT-PCR analysis of *poly(A)*-selected head RNA of $dnc^{\Delta 143}$ mutants using specific primers for different *dnc* transcript groups showed specific downregulation of dnc^{RA} and dnc^{RL} transcripts (n = 3–5 independent RNA samples with three replicates each; Student's t test, *p < 0.05). Error bars indicate the SD. *actin5C* gene primers were used as a reference.

(C) The $dnc^{\Delta 143}$ mutant develops normal ethanol sensitivity, as shown in the first panel, and reduced tolerance, as shown in the second panel (n = 7; Student's t test, *p < 0.05).

(D) The dnc^{RA}-GAL4-mediated expression of dnc^{all} in $dnc^{\Delta 143}$ mutants restored the reduced mutant phenotype to control levels (n = 8; **p < 0.01, ***p < 0.001, ANOVA, Tukey honest significance difference [HSD] test). For behavioral data, error bars are the SEM.

See also Tables S2 and S3.

expression pattern of dnc^{RA}-GAL4 in the adult brain using a *UAS-mCD8::GFP* reporter transgene (Figure 5; Movies S1 and S2) (Lee and Luo, 1999). GFP expression was primarily found in the mushroom body, the antenna lobes, in parts of the central complex, and in the subesophageal ganglion. Furthermore, the dnc^{RA}-GAL4 drives transgene expression in Hang-positive neurons (Figure S3). Because the central complex has been implicated in the regulation of ethanol tolerance in *Drosophila* (Scholz and Mustard, 2013), we performed a detailed expression analysis of this brain region (Figures 5Ai–5Aviii). In the central complex, GFP expression was found in neurons that project to the glomeruli of the protocerebral bridge, tangentially within the fan-shaped body (FB) and specifically into the posterior nodules (N), avoiding the anterior nodules. Only faint GFP expression was detected in the inner ring of the ellipsoid body; this expression did not co-localize with FasII expression (Figure 5Aviii). GFP expression in the FB resembled the projection pattern of F1 neurons that can be targeted by NP6510-GAL4 (Figure 5B; Movies S3 and S4; Li et al., 2009). To test whether these neurons were indeed mediating Dnc-dependent ethanol tolerance, we used NP6510-GAL4 to express Dnc in the $dnc^{\Delta 143}$ mutants (Figure 5C). The developmental expression of Dnc^{all} restored the reduced ethanol tolerance to control levels. Since the NP6510-GAL4 and the dnc^{RA}-GAL4 drivers do not drive transgene expression in the fat bodies, the major organ for ethanol metabolism (Figure S3), neuronal Dnc expression is required for normal tolerance development. The NP6510-GAL4 driver also drives the expression of GAL4 in dopaminergic PAM cluster neurons (Figure 5D) (Aso et al., 2012). To exclude a potential contribution of these neurons for tolerance, we restricted the expression of dnc^{RA}-GAL4 during development by co-expressing *Tyrosine hydroxylase*-GAL80 (*TH*-GAL80) (Figures 5D and 5E) (Sitaraman et al., 2008). Under this restriction, Dnc^{all} in the $dnc^{\Delta 143}$ mutants still restored ethanol tolerance to control levels, but not Dnc^{all} expression in the PAM neurons only using R58E02-GAL4 (Figure S4) (Liu et al., 2012). Therefore, a role of the TH-positive PAM neurons in Hang- and Dnc-dependent tolerance can be ruled out. The comparison of the expression patterns in different drivers indicates that Dnc expression in the F1 neurons of the central complex is required for ethanol tolerance.

Hang Is Required in the Same Neurons as Dnc

To test whether Hang functions in the same neurons as Dnc (a prerequisite for their function in a similar signaling process), we restored Hang function in the $hang^{AE10}$ mutants using dnc^{RA}-GAL4 and NP6510-GAL4. Under these conditions, Hang expression was lethal. Thus, we limited its expression using the inducible $GAL80^{ts}$ system (Figure 6) (McGuire et al., 2003). Flies were raised at 18°C, the temperature that allows functional expression of the GAL4 repressor $GAL80^{ts}$. In adult flies, the repressor was removed with a 33°C heat shock and flies were tested for their ethanol-induced behavior after a recovery period of 12 hr. Expressing Hang in the adult brain under the control of dnc^{RA}-GAL4; *Tub*-$GAL80^{ts}$ restored ethanol tolerance to wild-type levels, similar to Hang expression under the control of NP6510-GAL4; *Tub*-$GAL80^{ts}$ (Figures 6A and 6B). In addition, Hang is expressed in neurons targeted by the dnc^{RA}-GAL4 (Figure S2). The lack of expression of the used drivers in tissue associated with ethanol metabolism and observed normal ethanol metabolism in *hang* mutants further supports that neuronal expression of Hang and Dnc is crucial for proper tolerance

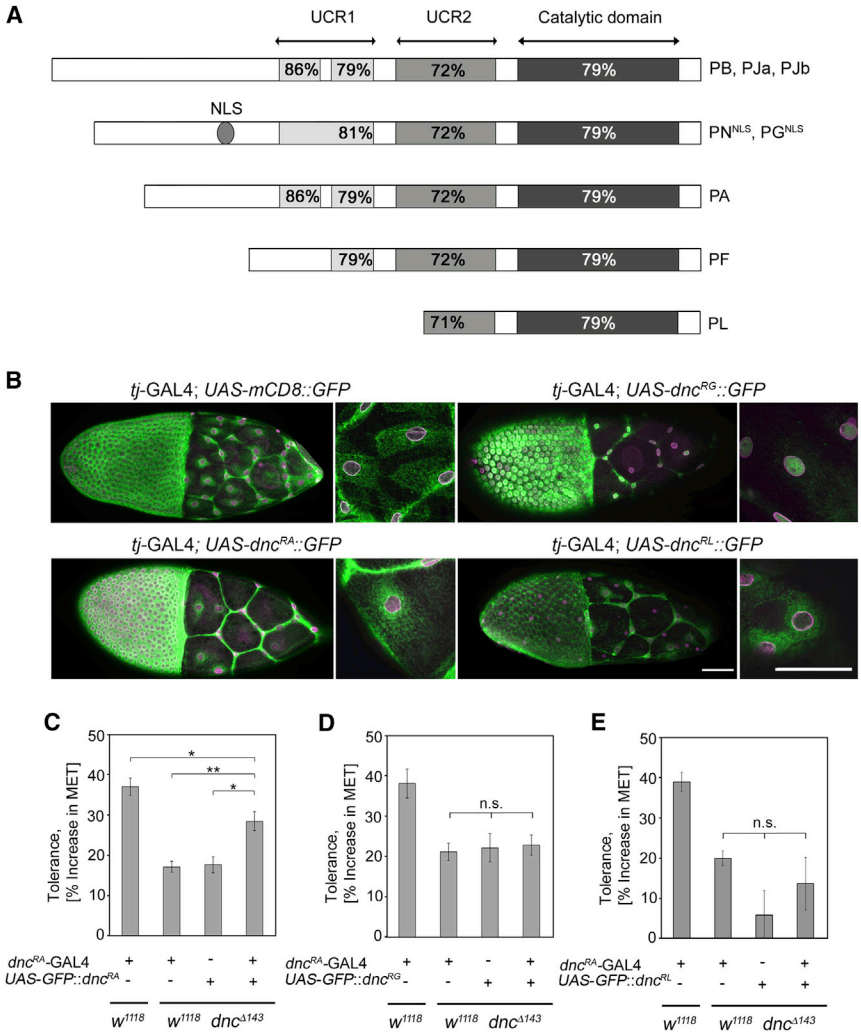

Figure 4. Subcellular Localization of Dnc Isoforms

(A) Dnc protein structures and percentage similarities of the different domains compared to the human PDE4d protein are shown. The homology of the domains is in part higher than the overall similarity of the full-length proteins. Across the isoforms, the highest degree of identity with the human PDE4d protein ranges from 59% to 61%. All isoforms contain the catalytic domain (dark gray box) and different parts of the UCR1 (light gray box) and UCR2 (medium gray box) domains. The variable domain of each protein is shown in white. The letter P indicates that the isoform is a protein.

(B) Expression of GFP-tagged Dnc transgenes (green) under the control of *tj*-GAL4 in comparison to the nuclear envelope marker Lamin (magenta) in stage 10 ovaries are shown in an overview and at higher magnification. The arrows mark the Dnc^{PG}::GFP expression in the nucleus. Scale bars indicate 50 μm. To visualize the cellular membranes for comparison, the expression of the *UAS-mCD8::GFP* transgene is shown in the first panel.

(C) Dnc^{PA} expression in $dnc^{\Delta 143}$ flies improved ethanol tolerance significantly (n = 11–39).

(D and E) The expression of neither (D) Dnc^{PG} nor (E) Dnc^{PL} improved the tolerance defect of the $dnc^{\Delta 143}$ mutants. [n = 6–14 for (D) and n = 10–22 for (E); *p < 0.05, **p < 0.01, ANOVA, Tukey HSD test]. Error bars indicate the SEM. n.s., not significant. See also Figure S1 and Table S3.

development (Figure S3). Therefore, Hang is required in the adult brain but not in the developing central nervous system (CNS). Moreover, Hang and Dnc are required in the same neurons for tolerance.

Requirement of a Balance between Hang and Dnc for Tolerance

To test whether Dnc acts in a similar manner as Hang, we analyzed whether $dnc^{\Delta 143}$ flies share additional phenotypic similarities with *hang* mutants, such as cellular stress defects (Figure 7A) (Scholz et al., 2005). In control flies, a 37°C heat shock for 30 min increased ethanol resistance 4 hr later. Heat shocking control flies led to an average 35% increase in heat-induced ethanol resistance compared to untreated flies (Figure 7A). In contrast, the heat-induced ethanol resistance that develops in $hang^{AE10}$ mutants was strongly reduced (Figure 6C; Scholz et al., 2005). A similar, albeit milder, reduced-response phenotype was observed in $dnc^{\Delta 143}$ mutants, whereas $hang^{AE10}$ and $dnc^{\Delta 143}$ double mutants also exhibited the strongly reduced heat ethanol resistance. Thus, the *hang* and *dnc* mutants share similar a cellular stress phenotype in addition to a shared function in similar neurons. To uncover further similarities, we investigated the kinetics of tolerance development in $dnc^{\Delta 143}$ and $hang^{AE10}$ mutants (Figure S5). The $dnc^{\Delta 143}$ and $hang^{AE10}$ mutants developed reduced tolerance 2 and 4 hr after the initial exposure to ethanol. In contrast, 16 hr later, the tolerance in the control flies was similar to that in the $dnc^{\Delta 143}$ mutants, whereas tolerance failed to develop in the $hang^{AE10}$ mutants. To investigate whether Hang and Dnc act together during the initial phase of tolerance development, we determined the tolerance of $dnc^{\Delta 143}$ and $hang^{AE10}$ double mutants (Figure 7B). Surprisingly, the double mutants developed a normal tolerance, suggesting that two counteracting mechanisms regulate tolerance. To test whether both mechanisms require cAMP, we assayed cAMP levels in the mutants (Figure 7C). The single and the double mutants had similarly significantly increased cAMP levels. Therefore, the absolute cAMP level does not determine the behavioral outcome, such as the degree of tolerance formed. These results further suggest that both mutations affect cAMP levels directly or indirectly and might influence each other in an opposing manner.

To further investigate the dnc^{RA}-*hang* relationship, we analyzed *hang* and dnc^{RA} transcript levels in the double mutants using qRT-PCR (Figure 7D). As expected, *hang* was downregulated in the double mutants, while the cDNA sequence recognized by the dnc^{RA}-specific primers was upregulated. Because

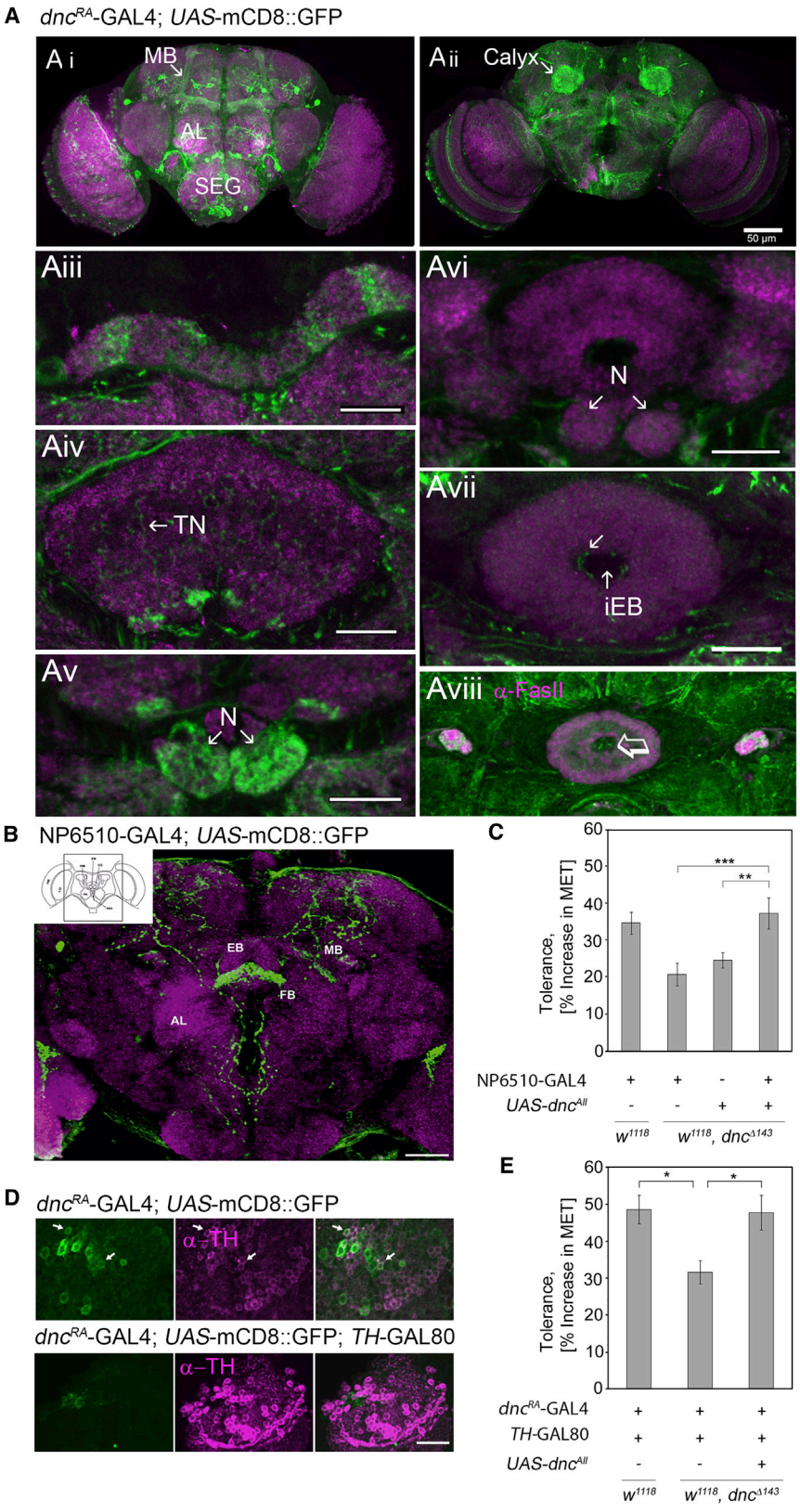

Figure 5. Dnc Is Required in F1 Neurons for Tolerance Development

(A) The GAL4 expression pattern of the *dnc*^{RA}-GAL4 driver is visualized via the expression of a *UAS-mCD8::GFP* transgene (green) and combined with the neuropil marker nc82 (magenta). A Z-projection of the anterior (Ai) and posterior (Aii) part of a confocal stack is shown. Expression is detected in the mushroom body lobes (MBs), the calyx, the antennal lobes (ALs), and in the ventral neurons of the subesophageal ganglion (SEG). (Aiii) In the central complex, GAL4-expressing neurons innervate the protocerebral bridge, the fan-shaped body tangentially (TN) (Aiv, marked with an arrow), and the posterior noduli (N) (Av) but avoid the anterior N (Avii). In the ellipsoid body, only faint GFP expression is found in the inner ring (iEB) (Avii). (Aviii) This expression does not co-localize with FasII (magenta, marked with an empty arrow). The scale bars represent 10 μm.

(B) NP6510-GAL4 drives GFP expression in the F1 neurons of the central complex. The scale bar represents 50 μm.

(C) Expression of Dnc^{all} using the NP6510-GAL4 driver restored ethanol tolerance to control levels (n = 13–18).

(D) Targeted GFP expression (green) obtained using the *dnc*^{RA}-GAL4 driver and TH expression (magenta), a marker for dopaminergic neurons, co-localizes (white) in a subset of PAM cluster neurons. Co-expression is highlighted with arrows. *TH*-GAL80 suppresses co-expression in PAM neurons. The scale bar represents 20 μm.

(E) Restriction of Dnc expression to the non-TH-positive neurons using the *dnc*^{RA}-GAL4 driver in *dnc*$^{\Delta 143}$ mutants restored ethanol tolerance (n = 10). Significance for behavioral experiments was determined with ANOVA and the Tukey HSD test (*p < 0.05, **p < 0.0,1 ***p < 0.001). The error bars represent the SEM.

See also Figures S2–S4 and Table S3 and Movies S1, S2, S3, and S4.

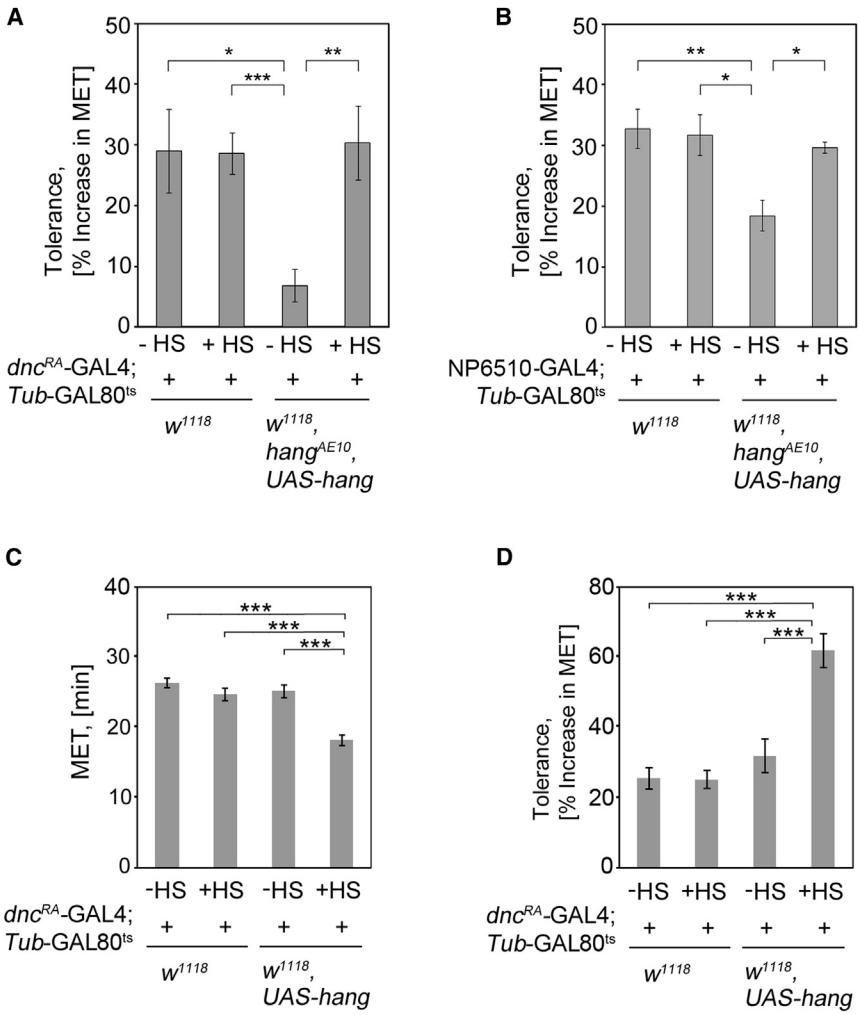

Figure 6. Hang Is Required for Tolerance

(A) Expression of *hang* in adult flies under the temporal control of *Tub*-GAL80^{ts} and spatial control of *dnc*^{RA}-GAL4 completely restored the *hang*^{AE10} mutant phenotype to control levels. The heat shock treatment did not alter ethanol tolerance, as controls developed normal tolerance (n = 6).

(B) The expression of Hang in adult flies under control of NP6510-GAL4, Tub-GAL80^{ts} restored ethanol tolerance in *hang*^{AE10} flies (n = 15–21).

(C and D) *hang* overexpression in the adult flies in a *dnc*^{RA}-GAL4, *Tub*-GAL80^{ts}-dependent manner leads to (C) increased ethanol sensitivity and (D) increased ethanol tolerance. Expression was activated 12 hr before the flies were tested in the inebriometer by a 1-h 33°C heat shock (n = 16; ***p < 0.001, ANOVA, Tukey HSD test). Error bars indicate the SEM.

See also Figure S6 and Table S3.

genomic deletion of *dnc*$^{\Delta 143}$ does not affect this region, the upregulation of this sequence could be due to the loss of Hang-mediated repression. To test whether *dnc*^{RA} regulates *hang* expression, we analyzed *hang* expression in *dnc*$^{\Delta 143}$ mutants (Figure 7E). The expression of the *hang* transcript was upregulated in the *dnc*$^{\Delta 143}$ mutants, suggesting that *dnc* acts as a negative regulator of *hang*. In addition, the observed increase in *hang* expression in the *dnc*$^{\Delta 143}$ mutants suggests that Hang overexpression can also influence the level of tolerance formed. We therefore investigated whether *dnc*^{RA}-GAL4-mediated *hang* overexpression during development affects ethanol tolerance. However, no viable offspring emerged. To circumvent the lethality, we combined the overexpression with the inducible *Tub80*^{ts} system (Figures 6C and 6D). *dnc*^{RA}-GAL4-dependent Hang overexpression in the adult increased ethanol sensitivity and increased ethanol tolerance. The overexpression also increased *hang* and *dnc*^{RA} transcript levels, showing that Hang regulates the level of *dnc*^{RA} transcripts (Figure 7F). The Hang overexpression-mediated increase in tolerance is consistent with the reduced tolerance phenotype of the *hang*^{AE10} mutants that was caused by the loss of *hang*. In summary, two cAMP-dependent mechanisms balance the degree of tolerance formed. This balance might be maintained through feedback regulation of *hang* and *dnc*^{RA}.

DISCUSSION

Here, we show how the nuclear Hang protein, which is broadly expressed in nearly all neurons of the CNS, mediates specific behavioral changes in response to a global increase in the stressor ethanol by identifying the neurons required for Hang-dependent ethanol tolerance. In these neurons, Hang can bind RNA and modify the transcript levels of the phosphodiesterase 4d ortholog *dnc*, linking nuclear signaling to the regulation of cAMP levels. Consistently, *hang* mutants show increased cAMP levels. In addition, we show that tolerance development requires the presence of the specific Dnc isoform Dnc^{RA} in the cytoplasm of the same neurons. The functional relationship between *dnc* and *hang* depends on feedback regulation that balances the degree of tolerance formed.

Central Complex Neurons Regulate Experience-Dependent Motor Output

Although emerging evidence in mice suggests that pharmacological inhibition of PDE4s reduces alcoholism-associated behaviors such as ethanol intake and preference (Blednov et al., 2014), what type of cellular process this inhibition of PDE4 activity is embedded in and whether this intervention acts on a neuronal level remains completely unclear. Every cell in organisms uses cAMP signaling as a second messenger system, and during ethanol exposure, the concentration of ethanol increases more or less everywhere in the brain. Nevertheless, the specificity of cAMP signaling for Hang- and Dnc^{PA}-dependent tolerance development can be ascribed to a pair of F1

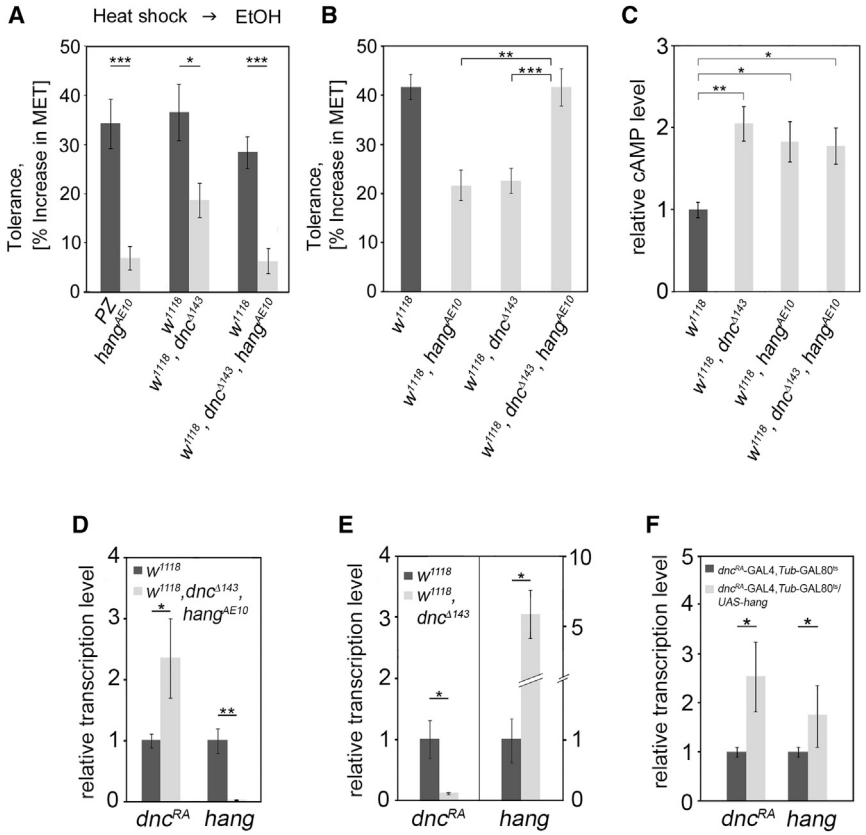

Figure 7. A Balance between *hang* and *dnc* Is Required for Tolerance

(A) Control flies show normal heat ethanol cross-tolerance, whereas the $dnc^{\Delta 143}$ mutant, the $hang^{AE10}$ mutant, and the $dnc^{\Delta 143}$, $hang^{AE10}$ double mutant developed a reduced heat ethanol response (n = 7–13).

(B) The $dnc^{\Delta 143}$, $hang^{AE10}$ mutants developed normal tolerance (n = 11).

(C) cAMP levels were analyzed in the control w^{1118}; $dnc^{\Delta 143}$ and $hang^{AE10}$ mutants and double mutants. All mutants showed increased cAMP levels (n = 10, independent protein samples with three replicates each; ANOVA, Tukey HSD test). Error bars indicate the SEM. Levels of significance are *p < 0.05, **p < 0.01, and ***p < 0.001 for all presented data.

(D and E) Differences in dnc^{RA} and *hang* transcript levels were determined by qPCR in the (D) $dnc^{\Delta 143}$, $hang^{AE10}$ double mutant and the (E) $dnc^{\Delta 143}$ mutant. dnc^{RA} is upregulated and *hang* is reduced in the double mutant, whereas dnc^{RA} is reduced and *hang* is upregulated in the *dnc* mutant. For better comparison, the same data from Figure 3B for dnc^{RA} in *dnc* mutants are shown. The reference gene was *actin5C* for *dnc* transcripts and *Su(Tpl)* for *hang*.

(F) qRT-PCR analysis of *poly(A)*-selected head RNA from flies with *hang* overexpression in adult flies showed a significant increase in *hang* and dnc^{RA} expression. The *Su(Tpl)* gene primers were used as the reference primers. For qRT-PCR analysis, three to five independent RNA samples with three replicates each were used. The Student's t test was performed for significance in qRT-PCR and ANOVA and the Tukey HSD test was used for behavioral experiments. Error bars indicate the SD for qRT-PCR and SEM for behavioral data.

See also Tables S2 and S3.

neurons. In the neurons, other participants in the cAMP signaling cascade, such as the adenylyl cyclase Rutabaga, are present and functional, as shown by the fact that Rutabaga expression in the F1 neurons ameliorates visual learning defects in those mutants (Li et al., 2009; Liu et al., 2006). The specific requirement of cAMP signaling in these neurons for tolerance might be linked to the more general function of the F1 neurons in motor control. At first glance, visual memory learning, particularly contour learning, and ethanol tolerance may not seem to have much in common because their sensory input differs. However, learning is measured as a change in the direction of flight of a tethered fly, linking visual information processing to motor output (Liu et al., 2006). In addition, there is evidence that the activity of the F1 neurons is required for the maintenance of high walking motivation (Martin et al., 1999), again linking the function of these neurons to motor output regulation. Behavioral ethanol tolerance is also measured by analyzing changes in motor output, such as the ability to maintain or regain posture after a previous ethanol experience (Scholz et al., 2000). The $dnc^{\Delta 143}$ mutants showed no difference in ethanol sensitivity, and therefore, the basic requirements of the behavior are normal. Considering the other observed functions of F1 neurons in locomotion, we propose a more general function for F1 neurons that involves linking previous motor experience to new information and changes in behavioral output. All cells within the brain are exposed to an increasing ethanol concentration and might respond with a cellular stress response, but only defects in neurons in the specific neuronal networks uncovered here impact performance on the assayed task.

Isoform-Specific Requirement for Dnc in Ethanol Tolerance

At the behavioral level, the cAMP second messenger system has been implicated in the regulation of ethanol sensitivity in both mammals and invertebrates. The *Drosophila* cAMP mutant *rutabaga* ethanol sensitivity increase (Moore et al., 1998) correlates with reduced cAMP levels (Cheung et al., 1999). Therefore, the increase in cAMP levels caused by the loss of PDE function should result in increased resistance to ethanol; however, neither the dnc^{1} nor the $dnc^{\Delta 143}$ mutants, which have increased cAMP levels, showed a defect in ethanol sensitivity. In addition, other *dnc* mutants, such as dnc^{M11}, show normal ethanol sensitivity (Moore et al., 1998). To date, a role for Dnc in sensitivity seems likely, given the increased sensitivity of *rutabaga* mutants; however, this role might not depend on the analyzed isoforms, as the tested *dnc* mutants showed normal ethanol sensitivity. In addition to its potential role in the regulation of ethanol sensitivity, Dnc-dependent cAMP function is required for the regulation of

rapid ethanol tolerance. The $dnc^{\Delta 143}$-like dnc^{1} mutants showed reduced ethanol tolerance but normal ethanol sensitivity, supporting the dissociation between cAMP function in ethanol sensitivity and tolerance. On a cellular level, differences in cAMP regulation might result from subcellular differences in PDE activity due to differences in PDE localization (Conti and Beavo, 2007). The mouse *PDE4D* gene, which exhibits the highest homology with *dnc*, encodes at least four isoforms that are expressed in different subcellular domains (Chandrasekaran et al., 2008). In addition, in the mollusk *Aplysia*, three different PDE4 isoforms localize to different subcellular membranes (Jang et al., 2010). Dnc^{PA} function in a specific subcellular compartment during tolerance development is consistent with the finding that nuclear Dnc^{PG} did not improve the reduced tolerance of the $dnc^{\Delta 143}$ mutants. Dnc^{PA} appears functionally similar to Dnc^{all} because both isoforms restored or improved the ethanol tolerance of the $dnc^{\Delta 143}$ mutants.

Feedback Regulation of *hang* and *dnc*

At first glance, the prolongation of cAMP signaling due to the elevation of cAMP levels seems to reduce ethanol tolerance. Consistently, the $dnc^{\Delta 143}$ and $hang^{AE10}$ mutants, with reduced tolerance, had elevated cAMP levels. These observations indicate that under normal circumstances, the termination of cAMP signaling plays a critical role in determining the level of tolerance formed. However, the true situation is more complex because the level of tolerance in the $hang^{AE10}$, $dnc^{\Delta 143}$ double mutants with increased cAMP levels was normal. Therefore, the dynamics of the cAMP changes are more crucial than the absolute cAMP level. The importance of dynamic changes of cAMP levels was already implicated by the observed similar impairments of learning and memory in *rutabaga* mutants with reduced cAMP levels and *dnc* mutants with increased cAMP levels (Davis et al., 1995). The dynamical change in ethanol tolerance might be achieved through the regulation of each gene's transcript level. In the $hang^{AE10}$ mutants, *dnc* was reduced, whereas temporally restricted expression of *hang* in the adult brain in a dnc^{RA}-GAL4-dependent manner increased tolerance and *dnc* levels, consistent with the idea that Hang is a positive regulator of *dnc* transcript levels. In contrast, Dnc^{RA} is a negative regulator of *hang* expression because in the $dnc^{\Delta 143}$ mutants, *hang* was upregulated. The existence of a feedback regulation between *dunce* and *hang* is further supported by the observation that overexpression of *hang* in a dnc^{RA}-GAL4 dependent manner produces similar changes in dnc^{RA} transcript levels and tolerance as found in the $dnc^{\Delta 143}$; $hang^{AE10}$ double mutants. However, the interaction is even more complicated. Overexpressing Hang throughout development was lethal, and temporally restricted Hang expression in the adult increased tolerance. That Hang function is extremely dose sensitive was also demonstrated by previous results showing that the loss or gain of Hang function at the NMJ results in similar morphological defects (Schwenkert et al., 2008). Taken together, these results indicate that there is complex feedback regulation between *hang* and *dnc*.

In summary, at the cellular/systems level, a reduction in cAMP level is required for behavioral tolerance. Crucial regulators at the cellular level include two conserved proteins, the PDE4d ortholog Dnc and the cellular stress regulator Hang. The subcellular function of Dnc and spatially and temporally controlled cAMP levels is important. cAMP regulation is linked via a feedback mechanism to the nuclear RNA binding protein Hang under tight temporal control. Furthermore, the identification of the requirement of Hang and the PDE4d ortholog Dnc in the same neurons for regulation of experience-dependent motor output in *Drosophila* provides insight into how specific behavioral changes are achieved in response to global increases in cellular stressors such as ethanol.

EXPERIMENTAL PROCEDURES

Fly Stocks and Genetics

Flies were raised on ethanol-free standard cornmeal/molasses/yeast/agar medium on a 12-hr/12-hr light/dark cycle at 25°C with 60% humidity. The $dnc^{\Delta 143}$ mutant flies carry a deletion from −2,148 to +71 relative to the starting point of the dnc^{RA} transcript. The $dnc^{\Delta 143}$ flies and the dnc^{RA}-GAL4 flies were backcrossed for at least six generations to w^{1118}. dnc^{1} and $hang^{AE10}$ fly stocks (Dudai et al., 1976; Scholz et al., 2005) were used in behavioral experiments, along with the corresponding control flies w^{1118}, *Canton S* (wt^{CS}), and *PZ*[+]. The *PZ*[+] control line carries the same *PZ*[ry+] transposable P-element as the P-element insertion line $hang^{AE10}$ and was isolated in the same genetic screen. The *PZ*[+] control did not show any changes of ethanol sensitivity or tolerance (Scholz et al., 2005).

Microarray Experiment and Data Analysis

For each of the five replicate experiments, separate hybridization probes were produced from approximately 1,000 fly heads. Experimental samples were generated from male $hang^{AE10}$ flies, while control samples were generated from PZ[+] flies. Following Trizol (Invitrogen)-based RNA isolation, samples were labeled with Cy3 and Cy5 fluorophores (Amersham) in a reverse transcription reaction, as described previously (Klebes et al., 2002). For more details and data analysis, see the Supplemental Experimental Procedures.

Quantitative Real-Time PCR

Total RNA was isolated from fly heads through guanidinium thiocyanate-phenol-chloroform extraction using Trizol. For further details, see the Supplemental Experimental Procedures. The optimal control primers for qRT-PCR for different genes were selected using NormFinder software (Andersen et al., 2004). In the *dnc* mutants, expression was normalized to the reference gene *actin5C*. In $hang^{AE10}$ and $dnc^{\Delta 143}$, $hang^{AE10}$ mutants, the reference gene was *Su(Tpl)*. Data are shown as fold changes in *dnc* or *hang* expression relative to that in control flies. Sequences of the primers used are listed in Table S1.

Behavioral Analysis

Ethanol tolerance and heat ethanol cross-tolerance were tested in an inebriometer as previously described (Scholz et al., 2000, 2005). Table S3 includes the behavioral data.

Statistical Analysis

For behavioral analysis, significance was evaluated using the Student's t test or one-way analysis of variance (ANOVA) with post hoc Tukey adjustment.

Immunohistochemistry

Dissection, fixation, and staining of the nervous system and ovaries were performed as described previously (Schneider et al., 2012; Cáceres and Nilson, 2005). Cryosectioning was performed following Buchner et al. (1988). The antibodies used were as described in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession numbers for the data reported in this paper are GEO: GPL22680 and GSE91049.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, three tables, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.12.048.

AUTHOR CONTRIBUTIONS

Conceptualization, M.R., M.F., and H.S.; Methodology, M.R., M.F., and H.S.; Investigation and Formal Analysis, M.R., M.F., A.S., L.V.E., O.H., L.M.G., A.K., and H.S.; Resources, I.S.; Data Curation, A.K. and H.S.; Writing—Original Draft, H.S.; Writing—Review & Editing, M.R., M.F., O.H., A.K., and H.S.; Supervision, Project Administration, and Funding Acquisition, H.S.

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