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CREB regulation of BK channel gene expression underlies rapid drug tolerance

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Abstract

Pharmacodynamic tolerance is believed to involve homeostatic mechanisms initiated to restore normal neural function. *Drosophila* exposed to a sedating dose of an organic solvent, such as benzyl alcohol or ethanol, acquire tolerance to subsequent sedation by that solvent. The *slo* gene encodes BK type Ca²⁺-activated K⁺ channels and has been linked to alcohol- and organic solvent-induced behavioral tolerance in mice, *C. elegans* and *Drosophila*. The cAMP response element binding (CREB) proteins are transcription factors that have been mechanistically linked to some behavioral changes associated with drug addiction. Here we show that benzyl alcohol sedation alters expression of both *dCREB-A* and *dCREB2-b* genes to increase production of positively acting CREB isoforms and to reduce expression of negatively acting CREB variants. Using a CREB-responsive reporter gene we show that benzyl alcohol sedation increases CREB-mediated transcription. Chromatin immunoprecipitation assays show that the binding of dCREB2, with a phosphorylated kinase-inducible domain, increases immediately after benzyl alcohol sedation within the *slo* promoter region. Most importantly, we show that a loss-of-function allele of *dCREB2* eliminates drug-induced up-regulation of *slo* expression and the production of benzyl alcohol tolerance. This unambiguously links *dCREB2* transcription factors to these two benzyl alcohol induced phenotypes. These findings suggest that CREB positively regulates the expression of *slo*-encoded BK type Ca²⁺-activated K⁺ channels, and that this gives rise to behavioral tolerance to benzyl alcohol sedation.

Keywords

Drosophila; transcription factor; CREB; slowpoke; *slo*; BK channel; potassium channel; drug addiction; tolerance

Introduction

Changes in the neural expression of the *slo* Ca²⁺-activated K⁺ channel gene have been linked to the production of rapid drug tolerance in the fly. It has been shown that benzyl alcohol sedation induces neural expression of *slo*, that *slo* mutations block the acquisition of behavioral tolerance and that transgenic induction of *slo* phenocopies the tolerant phenotype

(Ghezzi et al., 2004; Cowmeadow et al., 2005). However, the molecular pathways that mediate the up-regulation of *slo* transcription are still unknown.

Previously we have shown that sedation with the anesthetic benzyl alcohol produces a specific histone H4 hyperacetylation pattern within the *slo* promoter region (Wang et al., 2007). Histone acetylation is a common early step in gene activation. Histone acetylation stimulates transcription because it loosens the interaction between DNA and histones—making the DNA more available for recognition by other transcription factors—and because the bromodomains of a variety of general transcription factors bind acetylated histones (Berger, 2007).

The *slo* promoter region contains binding motifs for the cAMP response element binding protein (CREB) transcription factor. CREB can recruit histone acetyl transferases (HATs) to the promoter region. In mammals, CREB is a key factor in producing neuronal changes associated with drug tolerance and addiction (Widnell et al., 1996; Misra et al., 2001; Brunzell et al., 2003; McClung and Nestler, 2003).

Two CREB gene family members, *dCREB-A* and *dCREB2*, were discovered in *Drosophila* more than a decade ago (Smolik et al., 1992; Usui et al., 1993). Based on sequence similarity, *dCREB2* is thought to be the homolog of the mammalian CREB and cAMP response element modulator (CREM) genes (Usui et al., 1993; Yin et al., 1995). In *Drosophila*, both the *dCREB-A* and *dCREB2* genes are expressed in the adult brain (Smolik et al., 1992; Yin et al., 1995). *dCREB-A* has not yet been linked to behavioral phenotypes, however, this may merely be because appropriate mutant alleles have not yet been isolated. The *dCREB2* gene, which is also referred to as *Crebb-17A* in the literature, has been shown to have a role in the production of circadian rhythms, in learning, in memory, and in sexual behavior (Yin et al., 1994; Belvin et al., 1999; Sakai and Kidokoro, 2002). *dCREB2* transcripts are alternatively spliced. Most splice variants have a consensus cAMP-dependent PKA phosphorylation site, (Ser-231, equivalent to Ser133 in mammals) also known as the kinase-inducible domain which has been associated with CREB activation. In mammals, CREB family members have been shown to work as positive and as negative regulators of transcription (Shaywitz and Greenberg, 1999; Lonze and Ginty, 2002). Furthermore, mammalian CREB has been shown to stimulate transcription by recruiting the histone acetylase CBP to the region and by stabilizing the binding of other transcription factors at the promoter (Ogryzko et al., 1996; Conkright et al., 2003). In *Drosophila*, the *dCREB2-b* splice variant has been shown to be a negative regulator of CREB-mediated transcription. Other *dCREB2* splice variants are postulated to be activators of transcription, however, not all investigators agree that *dCREB2* activator isoforms exist (Yin et al., 1995; Perazzona et al., 2004).

Previously we have shown that a CREB transcription factor binds the *slo* promoter region using the chromatin immunoprecipitation (ChromIP) assay. Over-expression of a dominant negative CREB (*dCREB2-b*) blocked benzyl alcohol-induced histone acetylation within the *slo* promoter region, *slo* induction, and drug tolerance (Wang et al., 2007). These data were interpreted to mean that up-regulation of the *slo* gene represents a homeostatic response that counters the effects of drug sedation and that CREB was likely to mediate *slo* up-regulation after benzyl alcohol sedation (Wang et al., 2007). However, high level expression of a dominant negative transcription factor could perturb gene expression in a way not related to its normal function.

Here we establish the function of CREB in the regulation of *slo* expression and in the development of rapid tolerance as produced by benzyl alcohol sedation. Using a reporter gene assay we show that benzyl alcohol sedation induces CREB-stimulated gene expression

and that this appears to be mediated by down-regulation of a CREB repressor isoform. Furthermore, we use a ChromIP assay to show that benzyl alcohol sedation increases the occupancy of Ser-231-phosphorylated CREB in the *slo* promoter region. Finally we show that the *dCREB2*^{S162} mutant, which carries a premature stop codon in *dCREB2* gene, fails to develop the rapid benzyl alcohol tolerance phenotype that has been associated with the *slo* gene expression.

Methods

Fly stocks

Drosophila stocks were Canton S (wild type), CRE-luciferase transgenic flies (Iijima-Ando and Yin, 2005), and *dCREB2*^{S162}/FM6 (Bloomington Stock Center, Indiana University). The *dCREB2*^{S162} allele is a recessive mutation in the *dCREB2* gene (Belvin et al., 1999). It carries a C to T transition that substitutes a stop codon for a glutamine in exon 7 just upstream of the bZIP domain of the *dCREB2* (Hendricks et al., 2001). In order to obtain *dCREB2*^{S162} hemizygous flies, *dCREB2*^{S162}/FM6 virgin females were mated to CS males. As described previously. *dCREB2*^{S162} hemizygous male escapers represent less than 1% of overall progeny (Belvin et al., 1999). Flies stocks were raised on standard cornmeal/molasses/agar medium and housed in a room with constant temperature at 22 °C in a 12/12 hour light and dark cycle. For the tolerance assay, newly eclosed flies were collected over a 2-3 day window, transferred to fresh food, and studied 5-6 days after eclosed.

Tolerance assay

Age and sex matched flies were treated in triplicate with benzyl alcohol (0.4%) or vehicle as described previously (Ghezzi et al., 2004). Each replicate contains 15 flies. Twenty-four hours later, both treated and mock treated control flies were simultaneously sedated with benzyl alcohol (0.4%). Five minutes after sedation, flies were transferred to an anesthetic-free tube for recovery and snapshots of the flies were taken every 30 seconds during both the sedation and recovery stage. Flies were scored as recovered when they resumed climbing. The number of recovered flies were plotted as a percentage of the population in each tube (average of three tubes) against time at 30-sec intervals. The log-rank test was used to determine whether the recovery time of the two populations differ significantly.

Luciferase reporter assay

The CRE-luciferase reporter gene construct is described in Belvin et al. (1999). Briefly, the construct contains three copies of the CRE (TGACGTCA) site followed by luciferase gene. This entire cassette is flanked by insulator elements. Age matched (4-6 days old) CRE-luc females were separated into eight groups each of which contains 15 flies. Four groups of flies, 15 flies in each group, were sedated with benzyl alcohol (0.4%) and the other four groups were mock sedated. Four hours after sedation, flies from each group were snap-frozen in liquid nitrogen and decapitated by vortexing the frozen animals. The heads were collected by sieving and were homogenized in cell lysis buffer (The Luciferase Assay System, E1500, Promega, WI) and debris were eliminated by spinning in a microcentrifuge. The luminescence in the cell lysis was measured using luciferase assay system (E1500, Promega, WI) with a luminometer (Mithras LB 940, Berthold technologies, Germany). Serial dilutions of cell lysate were used to confirm that the measurements are in the linear range. Luciferase signals were measured in triplicate and normalized with total protein concentration in the extract. Protein concentration were measured with RC DC protein assay kit (cat# 500-0120, Bio-Rad, CA).

Chromatin immunoprecipitation

Three groups of flies, about 1500 flies in each group, were independently sedated with benzyl alcohol and three groups were independently mock-sedated. Four hours after benzyl alcohol sedation fly heads were collected from each group as described above. Heads were cross-linked with 2% formaldehyde and the chromatin immunoprecipitation assay was performed as described in Wang et al 2007 using the anti-phospho-dCREB2 antibody described in Horiuchi et al. (2004). This antibody is specific for dCREB2 that is phosphorylated at Ser231 (activated KID domain) and was used at 1:200 dilution. Both co-immunoprecipitated and input DNA was recovered by reverse cross-linking, phenol/chloroform extraction and ethanol precipitation. Realtime PCR was performed using the ABI SYBR Green PCR protocol (Applied Biosystems) to determine the relative amount of DNA fragment from the *slo* transcriptional control region that co-immunoprecipitated with phospho-dCREB2. Within the *slo* transcriptional control region (Figure 3A), primers which were designed to amplify approximately 200-bp fragments are : C0 (5'-ATCGAACGAAGCGTCCAG-3', 5'-CGACGCGCTCAAACG-3'), 6b (5'-CCAGCAGCAATTGTGAGAAA-3', 5'-CGAAGCAGACTTGAAAGCAA-3'), cre1 (5'-GATGGGAAAGCGAAAAGACAT-3', 5'-CATGTCCGTCAAAGCGAAAC-3'), S2 (5'-CATTGCTATCCCTTCCCATC-3', 5'-ATGCAATGAAGCGAAGAACC-3'), 55b (5'-TACCCAATTGAATTGCCTTGTCTT-3', 5'-CCCACTCTCCGGCCATCTCT-3'), cre2 (5'-TGGATTGCGACCGAGTGTCT-3', 5'-ATCAATACGATAACT GGCGGAAACA-3'). As a control, we used primers for *Cyp1* (*Cyclophilin 1*) promoter region (5'-TCTGCGTATGTGTGGCTCAT-3', 5'-TACAGAACTCGCGCATTTCAC-3'). Each PCR reaction generated only the expected specific amplicon that was proved by running the melting temperature profiles of the final products (dissociation curve). The relative abundance of each co-immunoprecipitate fragment (IP DNA) was expressed as a percentage of the total amount of DNA fragment used in the immunoprecipitation (input DNA) in both experiment and mock sedated control with the equation: $IP/Input=2^{-(Ct^{input}-Ct^{IP})}$. Significance was determined with the two-way ANOVA. Independent repetitions of entire procedure yielded essentially the same binding profile.

Real-time RT PCR

Total RNA was extracted from the heads of three groups of independently benzyl alcohol sedated and three mock treated groups of flies (15 flies per group). RNA was isolated, 6 hours after benzyl alcohol sedation using a single-step RNA isolation protocol as described previously (Ghezzi et al., 2004; Cowmeadow et al., 2006) and quantified in a NanoDrop spectrophotometer (NanoDrop Technologies). Two step reverse transcription and real time PCR were performed in triplicate with specific primers for *dCREB-A*, *dCREB2-b*, *slo* C1 exon and *cyclophilin 1*. *Cyclophilin 1* was used as an internal control gene for normalization. First-strand cDNA was synthesized from total RNA with gene-specific reverse primers with Superscript II reverse transcriptase (Invitrogen). The cDNA was amplified by real-time PCR in an ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the ABI SYBR Green PCR protocol (Applied Biosystems). mRNA abundance was calculated using the standard-curve method (Applied Biosystems manual) and Significance was calculated using the Student t-test.

The following primers were used to quantify the transcripts of interest: dCREB-A forward primer (5'-TTCAACTACCTCAGCACCTATACGA -3'), dCREB-A reverse primer (5'-TCTCGATGTCCGAGCAAATG- 3'), dCREB2-b forward primer (5'-ACTGCAGGTGGCAGTCCG -3'), dCREB2-b reverse primer (5'-TAGACCACCTTCTGCATCGCT-3), *slo* (C1) forward primer (5'-AAACAAAGCTAAATAAGTTGTGAAAGGA-3'), *slo* (C1) lower reverse primer (5'-GATAGTTGTTTCGTTCTTTTGAATTTGA-3'). Transcripts from *Cyp1* (internal control)

were detected using upstream primer 5'-ACCAACCACAACGGCACTG-3' and the downstream primer. 5'-TGCTTCAGCTCGAAGTTCTCATC- 3'.

Results

Expression of CREB mRNA is altered by anesthetic sedation

To address the role of CREB in the production of tolerance we asked whether sedation altered expression of either of the CREB genes. The dCREB-A transcription factor is thought to be a positive regulator of transcription. Transcripts from the dCREB2 gene are alternatively spliced. The dCREB-2b splice variant has been clearly shown to be a repressor isoform with important effects on behavior (Yin et al., 1995; Perazzona et al., 2004). We measured the relative abundance of dCREB-A and dCREB2-b transcripts in fly heads by real-time RT-PCR using message specific primers. To account for variability in RNA purification efficiency, the abundance of CREB mRNA was expressed relative to the abundance of mRNA from the *cyclophilin 1* gene (*Cyp1*). *Cyclophilin 1* mRNA was chosen as an internal control because its abundance was not affected by a single benzyl alcohol sedation (Ghezzi et al., 2004). We observed that a single benzyl alcohol sedation selectively up-regulated the *dCREB-A* transcript and down-regulated the *dCREB2-b* splice variant. Based on these changes, one would predict that benzyl alcohol sedation produces a net increase in the abundance of the stimulatory dCREB-A transcription factor and a net decrease in the activity of the dCREB2-b negatively acting transcription factor (Fig.1). Thus, benzyl alcohol sedation is predicted to increase CREB-stimulated gene transcription.

Sedation affects CRE-dependent gene expression

To test the hypothesis that sedation enhances CREB-mediated transcription, we used the CRE-luc transgene to compare the level of CREB-mediated gene expression before and after sedation. In the CRE-luc transgene, a series of cAMP response elements (CRE) modulate expression of the bioluminescent luciferase reporter gene. Expression of luciferase from this transgene increases in response to activation by CREB (Iijima-Ando and Yin, 2005). The level of luciferase bioluminescence was measured in lysates prepared from the heads of benzyl alcohol sedated and mock sedated animals. Fly heads are primarily comprised of neural tissue and are easily isolated in mass. As compared to mock sedated animals CRE-luc flies showed a significant increase in bioluminescence 4 hours after a brief (15-30 min) benzyl alcohol sedation (Fig. 2). This indicates that benzyl alcohol sedation resulted in an increase in CREB-mediated transcription. Non-transgenic control animals produced only trace levels of bioluminescence.

Benzyl alcohol sedation increases the occupancy of phosphorylated dCREB2 in the *slo* promoter region

The *slo* gene has been shown to be essential for the acquisition of rapid benzyl alcohol tolerance. It has been previously shown that that benzyl alcohol sedation enhances the binding of a CREB isoform, within the *slo* promoter region, and that this binding is correlated to increased *slo* expression (Wang et al., 2007). However, the antibody used in the prior experiment does not distinguish between *dCREBA* and *dCREB2* products nor between activated and non-activated states. The kinase-inducible domain of CREB family members becomes competent to stimulate transcription when it is phosphorylated. Here we performed the chromatin immunoprecipitation (ChromIP) assay using an antibody specific for the phosphorylated kinase-inducible domain of dCREB2 (Horiuchi et al., 2004). We measured the relative occupancy of activated phosphorylated dCREB2 at six positions across the *slo* transcriptional control in chromatin isolated from fly heads. The relative abundance of the co-immunoprecipitated genomic DNA was measured by real time PCR. We observed that benzyl alcohol sedation increased phospho-dCREB2 occupancy at three

sites known to contain the DNA motif for a CRE-site (cre1, 55b and cre2) but not at positions that do not contain recognizable CRE-sites (C0, 6b, S2, Cyp). The ChromIP assay showed that phospho-dCREB2 binds the *slo* promoter region and that the binding of this form of activated CREB is enhanced by sedation (Fig.3).

A *dCREB2* loss-of-function mutation blocks sedation-induced *slo* induction

The *slo* gene is up-regulated by benzyl alcohol sedation, and increased *slo* expression is capable of producing the tolerant phenotype. Previously, we had shown that over-expression of the *dCREB2-b* repressor could block benzyl alcohol induced *slo* gene expression (Wang et al., 2007). To determine if *dCREB2* produces a positively acting factor that is required for *slo* induction we tested the response of animals carrying the *dCREB2*^{S162} loss-of-function mutation. This mutant allele carries a stop codon just upstream of the bZIP motif that abolishes dCREB2 activity (Hendricks et al., 2001). *dCREB2*^{S162} is homozygous lethal mutation which shows only partial penetrance. We observed that less than 1% of hemizygous *dCREB2*^{S162} males survived to adulthood, as described previously (Belvin et al., 1999). While these “escaper” males are about three-fourths the size of wild-type flies but are appear healthy. Previous studies showed that *dCREB2*^{S162} escaper males have circadian arrhythmicity (Belvin et al., 1999).

If *dCREB2* participates in drug-induced *slo* expression then the *dCREB2*^{S162} mutation should interfere with drug-evoked enhancement of *slo* expression. To evaluate this idea, *slo* transcripts were quantified six hours after benzyl alcohol sedation in both mutant *dCREB2*^{S162} flies and wild-type Canton S flies. In *dCREB2*^{S162} males, *slo* mRNA levels decreased after benzyl alcohol sedation (Fig.4). As has been previously reported, wild-type Canton S males showed a significant increase in *slo* mRNA level six hours after benzyl alcohol sedation (Ghezzi et al., 2004). This shows that benzyl alcohol-induced *slo* expression is coordinated by the dCREB2 transcription factor.

A *dCREB2* loss-of-function mutation eliminates the capacity to acquire rapid tolerance

To determine if the *dCREB2* gene produces a product that is required for the development of drug tolerance, we examined flies carrying the *dCREB2*^{S162} loss-of-function mutation. We observed that a single benzyl alcohol sedation failed to induce tolerance in *dCREB2*^{S162} hemizygous males. However, the sibling FM6 male flies (data not shown) and wild-type CS male flies can develop tolerance to the sedative effect of benzyl alcohol after a single exposure (Fig.5).

It is possible that the truncated polypeptide generated from the *dCREB2*^{S162} allele might interfere with the capacity of the flies to acquire tolerance in a dominant negative manner (Hendricks et al., 2001). To test this possibility, we measured the ability of *dCREB2*^{S162/+} females to acquire tolerance. Heterozygous flies can develop tolerance (Fig.5), indicating that the loss of tolerance is not due to a dominant phenotype associated with the *dCREB2*^{S162} mutation.

We also noted another drug-related phenotype of the *dCREB2*^{S162} mutants. At low concentrations, benzyl alcohol acts as a stimulant. In wild-type animals, a hyperactive phase precedes the sedative phase of benzyl alcohol exposure. This short stimulatory period is thought to represent the time in which the drug concentration within the fly is still relatively low. We noted that the *dCREB2*^{S162} hemizygous males did not exhibit a hyperactive phase in response to benzyl alcohol. This most certainly is a consequence of low dCREB2 activity during development since the speed of the response precludes a role for changes in gene expression.

Discussion

Benzyl alcohol is an organic solvent that is used in dyes, inks and cleaning solutions (Mookherjee and Wilson, 1992). Most organic solvents are potent central nervous system depressants that produce sedation if inhaled or consumed in sufficient quantities (Giovacchini, 1985). These properties have led to the use of organic solvents both as anesthetics and as drugs of abuse. When flies are exposed to benzyl alcohol, they first become hyperactive and then become sedated, all within 10–15 minutes. It has been shown that flies develop tolerance to benzyl alcohol sedation after a single exposure to the drug (Ghezzi et al., 2004). Rapid tolerance to benzyl alcohol sedation is dependent on the presence of a functional *slo* gene (Ghezzi et al., 2004). The *slo* gene encodes the BK-type calcium-activated potassium channel (Atkinson et al., 1991; Adelman et al., 1992). In this study we use benzyl alcohol as a model organic solvent to study the neuronal basis of rapid tolerance to organic solvents. Our findings indicate that benzyl alcohol sedation increases CREB signaling to up-regulate the *slo* promoter activity which gives rise to rapid benzyl alcohol tolerance in flies.

A role for CREB in benzyl alcohol-induced responses was indicated by the observation that benzyl alcohol sedation enhanced expression from a CREB-responsive luciferase transgene in fly heads. Furthermore, we observed that benzyl alcohol sedation produces a rapid change in the abundance of mRNAs produced from both CREB genes. The relative abundance of *dCREB-A* transcript increased and the abundance of *dCREB2-b* transcript dropped. The *dCREB-A* transcription factor has been shown to activate transcription, while *dCREB2-b* has been shown to be a *dCREB2* splice variant that acts as a transcriptional repressor. Both have been shown to be expressed in the adult nervous system (Smolik et al., 1992; Yin et al., 1995). An increase in the abundance of a transcript encoding a CREB activator and a reduction in the abundance of the transcripts encoding a repressor isoform are predicted to cause a net increase in expression from genes regulated by CREB. Both *dCREB-A* and *dCREB2* are expressed in the nervous system and may both contribute to the regulation of the *slo* gene. Because of the availability of well-described mutant alleles in *dCREB2* we were able to directly test the role of *dCREB2* in *slo* regulation. A similar analysis can not yet be done for *dCREB-A* and it is possible that it also contributes transcription factors that regulate *slo* gene expression.

In a previous study we showed that expression of a *dCREB2-b* dominant-negative transgene from a heat shock promoter could suppress benzyl alcohol induced responses including 1) histone acetylation changes across the *slo* promoter region, 2) the induction of *slo* gene expression and 3) the acquisition of tolerance to benzyl alcohol sedation (Wang et al., 2007). This suggested that *dCREB2* was involved in regulating *slo* gene expression after sedation. However, over-expression of a transcription factor might affect transcription of genes not normally considered to be targets of its regulation.

To independently verify that *dCREB2* plays a central role in benzyl alcohol responses we examined the phenotype of the *dCREB2*^{S162} loss-of-function mutation (Belvin et al., 1999). The loss of *dCREB2* expression prevented both *slo* induction and the acquisition of benzyl alcohol tolerance. This result unambiguously links *dCREB2* to these benzyl alcohol responses. Benzyl alcohol sedation also causes a specific pattern of histone acetylation changes across the *slo* promoter region (Wang et al., 2007). We could not, however, determine if the *dCREB2*^{S162} mutation affected this latter benzyl alcohol-associated phenotype because it was not possible to isolate a sufficient number (~1000) of hemizygous *dCREB2*^{S162} “escaper” males to perform the ChromIP assay.

The hypothesis that dCREB2 regulates *slo* expression was also supported by ChromIP assays performed using an antibody raised against a phosphorylated dCREB2 kinase-inducible domain (Horiuchi et al., 2004). Using this antibody, we observed that benzyl alcohol sedation increased CREB occupancy at the three CRE sites within the *slo* promoter region. A product of the dCREB2 gene must be the entity detected by this antibody because the dCREB-A gene does not encode the hapten targeted by the antibody. Phosphorylated kinase-inducible domains have been associated with CREB isoforms that stimulate transcription (Shaywitz and Greenberg, 1999).

CREB activators have been shown to promote gene expression through at least three mechanisms. One mechanism is the binding of the glutamine rich domain Q2 domain of CREB to TAFII130, which is a component of the transcription pre-initiation complex (Quinn, 1993; Ferreri et al., 1994). A second means of interacting with TAFII130 is provided by the TORC (Transducers of Regulated CREB activity) cofactors, which bind the bZIP domain of CREB at one end and TAFII130 on the other end (Lonze and Ginty, 2002; Konkright et al., 2003). Both mammalian and *Drosophila* genomes encode TORCs (Bittinger et al., 2004). These interactions with TAFII130 may stimulate transcription by stabilizing or modifying this transcription factor.

Phosphorylation of the kinase-inducible domain is not thought to be required for gene activation by the latter two mechanisms (Takemori and Okamoto, 2008). However, CREB, with a phosphorylated kinase-inducible domain can stimulate expression by binding the histone acetylase CBP (CREB-binding protein). The resultant acetylation of local histones makes the DNA more accessible and enhances the affinity of the chromatin for other transcription factors required for transcription (Gonzalez and Montminy, 1989; Brindle et al., 1993; Struhl, 1998).

In mammals, phosphorylation of the kinase-inducible domain is thought to be a major avenue through which cells regulate CREB activity. In flies, however, most CREB activation domains exist in the phosphorylated active state (Horiuchi et al., 2004). It is believed that flies make extensive use of phosphorylation of CREB casein kinase sites in the DNA binding domain. Phosphorylation of casein kinase sites inhibits the capacity of CREB to bind CRE sites (Horiuchi et al., 2004). In flies, it has been proposed that modulation of the capacity of CREB to bind its DNA element is the more important aspect of the regulation of this transcription factor.

The protein expressed from the dCREB2-b splice variant is a known transcriptional repressor. It is thought to repress transcription by dimerizing with CREB activators and sequestering them in the cytoplasm or in the nucleus or by occupying the CRE site to prevent their recognition by CREB activators (Karpinski et al., 1992; Shaywitz and Greenberg, 1999; Lonze and Ginty, 2002). While it is clear that *dCREB2* can produce a transcriptional repressor, it is controversial whether or not *dCREB2* also produces positively-acting isoforms (Yin et al., 1995; Perazzona et al., 2004). Nevertheless, our ChromIP data shows a correlation between phospho-dCREB2 binding (phosphorylated kinase-inducible domain) within the *slo* promoter region and an induction in *slo* gene expression. This suggests that either 1) *dCREB2* expresses activator isoforms that stimulate *slo* expression or that 2) phosphorylation of the kinase-inducible domain prevents dCREB2b repressor activity or causes it to act as a transcriptional activator.

The *slo* gene has a well-described role in the response to sedation with organic solvents (Davies et al., 2003; Ghezzi et al., 2004; Cowmeadow et al., 2005; Cowmeadow et al., 2006). We have shown that the *slo* gene is a downstream target of the CREB transcription factor, and we propose the following regulatory cascade. A sedative dose of the anesthetic

benzyl alcohol activates the CREB pathway by down-regulation of the CREB repressor isoform. This frees up other CREB activator isoforms (probably dCREB2 isoforms) and the phosphorylated versions of these proteins bind to CRE sites within the *slo* promoter region and induce acetylation of the neighboring histones. This increases availability of the underlying DNA and increases the affinity of the promoter region for other required transcription factors. These changes stimulate the neural-specific promoters to increase the expression of BK type channel from the neural promoters. Increased BK channel activity has been shown to act as a neural stimulant, enhancing neural activity (Brenner et al., 2005). This regulatory cascade is summarized in figure 6. We suspect that this change directly counters some of the effects of the anesthetic on the nervous system enabling the flies to recover more rapidly from sedation - a behavioral phenotype that we classify as benzyl alcohol tolerance.

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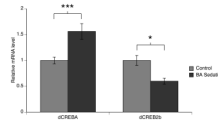


Figure 1. Benzyl alcohol sedation enhances dCREB-A and reduces dCREB2 repressor splice variant (dCREB2-b) mRNA abundance

The mRNA levels of CREB transcripts in fly heads were measured using RT-realtime-PCR with *Cyclophilin 1* as the internal control. RNA levels are normalized with respect to untreated animals. Four hours after sedation with benzyl alcohol (BA), the *dCREB-A* messenger level was induced about 50% (n=11, p<0.001 Student *t*-test). A single benzyl alcohol sedation causes a reduction in the abundance of the *dCREB2-b* repressor isoform (n=4, p<0.05 Student *t*-test).

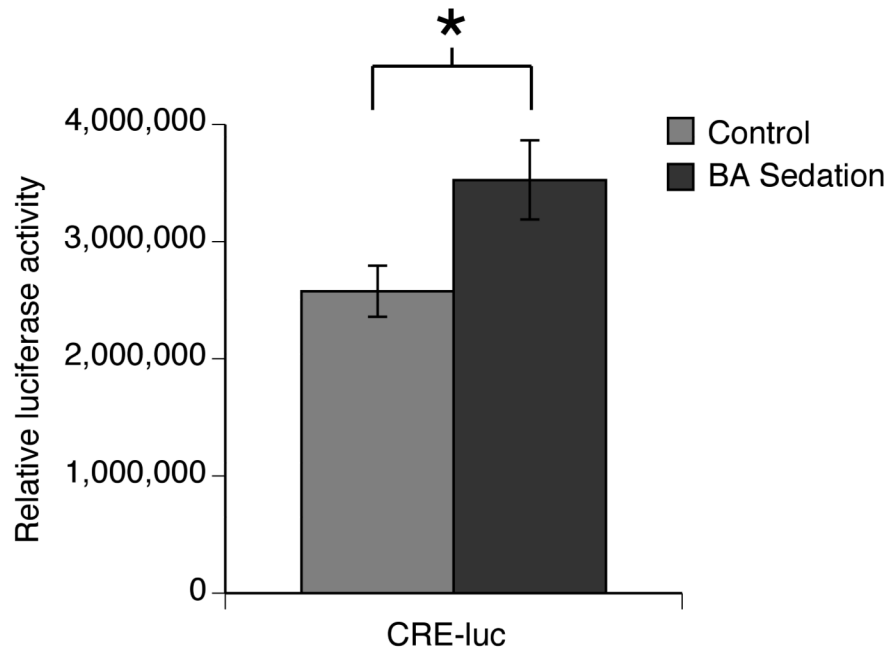


Figure 2. Positive regulation by CREB increases after benzyl alcohol sedation

CRE-luc transgenic flies were used to measure CRE-dependent gene expression. The relative amount of bioluminescence in fly head extracts was measured and normalized to total protein (specific activity). Four hours after benzyl alcohol sedation luminescence activity was increased in CRE-luc transgenic flies ($n=4$, $p<0.05$, Student t -test). Luciferase activity was not detected in flies that did not contain the transgene (data not shown).

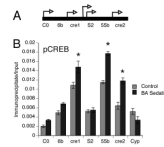


Figure 3. Benzyl alcohol sedation enhances the occupancy of activated CREB in the *slo* promoter region

A) Map of the transcriptional control region of the *slo* gene. The arrowheads indicate the transcription start sites of 5 tissue-specific promoters. From left to right: the first two promoters are neural specific, the next two are known to be active in epithelial cells of the larval digestive tract and the right-most promoter is responsible for tracheal and muscle cell expression. The labeled regions below the line represent the position of blocks of sequence that are evolutionarily conserved in the genus *Drosophila*. Conserved control elements might regulate proximal and/or distal promoters and cannot yet be determined solely by sequence analysis. This map is not to scale but is drawn to align with the histogram. **B)** The relative abundance of phospho-CREB within the *slo* promoter region was measured by ChromIP in fly heads 4 hours after a benzyl alcohol sedation (black bars) and in control animals that have never been sedated (gray bars). The antibody recognizes dCREB2 phosphorylated within the activation domain (position Ser-231). The relative abundance of the co-immunoprecipitated genomic DNA was measured at six positions (C0, 6b, cre1, S2, 55b and cre2) across the *slo* transcriptional control region by real time PCR. We also measured the relative abundance of a fragment from the *Cyclophilin 1* (*Cyp*) gene. This fragment is an internal control which does not contain a CRE site and whose expression is not up-regulated by benzyl alcohol treatment (Ghezzi et al., 2004; Cowmeadow et al., 2005). Signals obtained from PCR amplification of immunoprecipitated chromatin were normalized to the amount of input chromatin. Increased phospho-CREB occupancy was observed around three putative CRE sites (cre1, cre2 and 55b). Significance was determined by two-way ANOVA ($n=3$, $p<0.05$). Error bars are SEM.

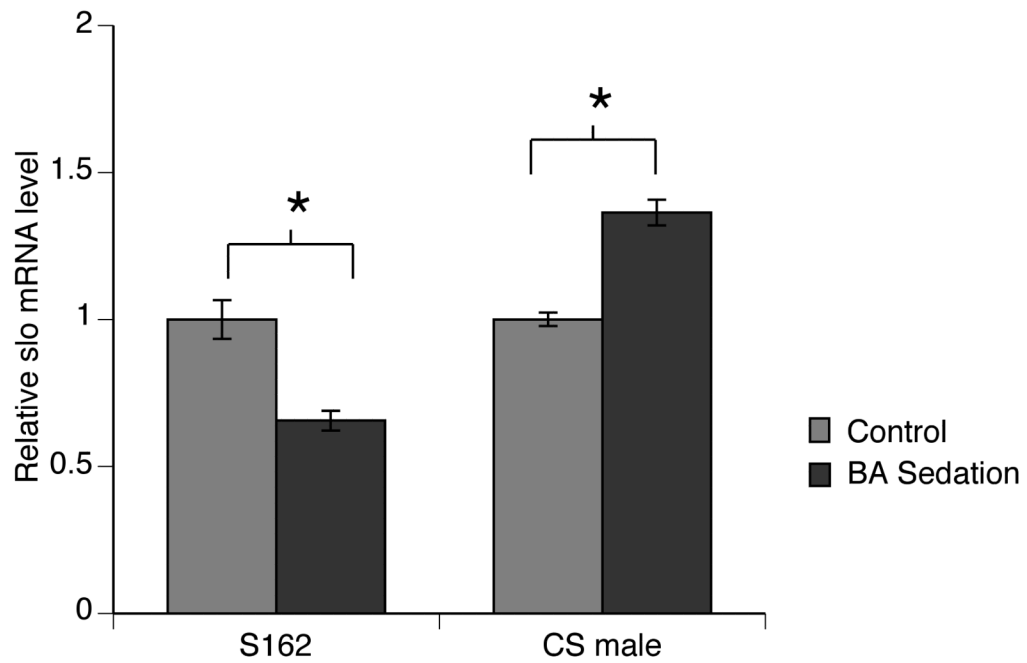


Figure 4. The dCREB2^{S162} mutation blocks induction of *slo* by benzyl alcohol sedation
slo mRNA levels in dCREB2^{S162} (S162) and Canton S (CS) males were measured by RT-real time PCR with primers specific for the neural *slo* isoforms. Six hours after benzyl alcohol sedation, *slo* mRNA decreased about 40% in the dCREB2^{S162} stock as compared to non-treated group whereas benzyl alcohol sedation produced an increase in *slo* mRNA abundance in the CS control group (n=4, p<0.05 Student *t*-test).

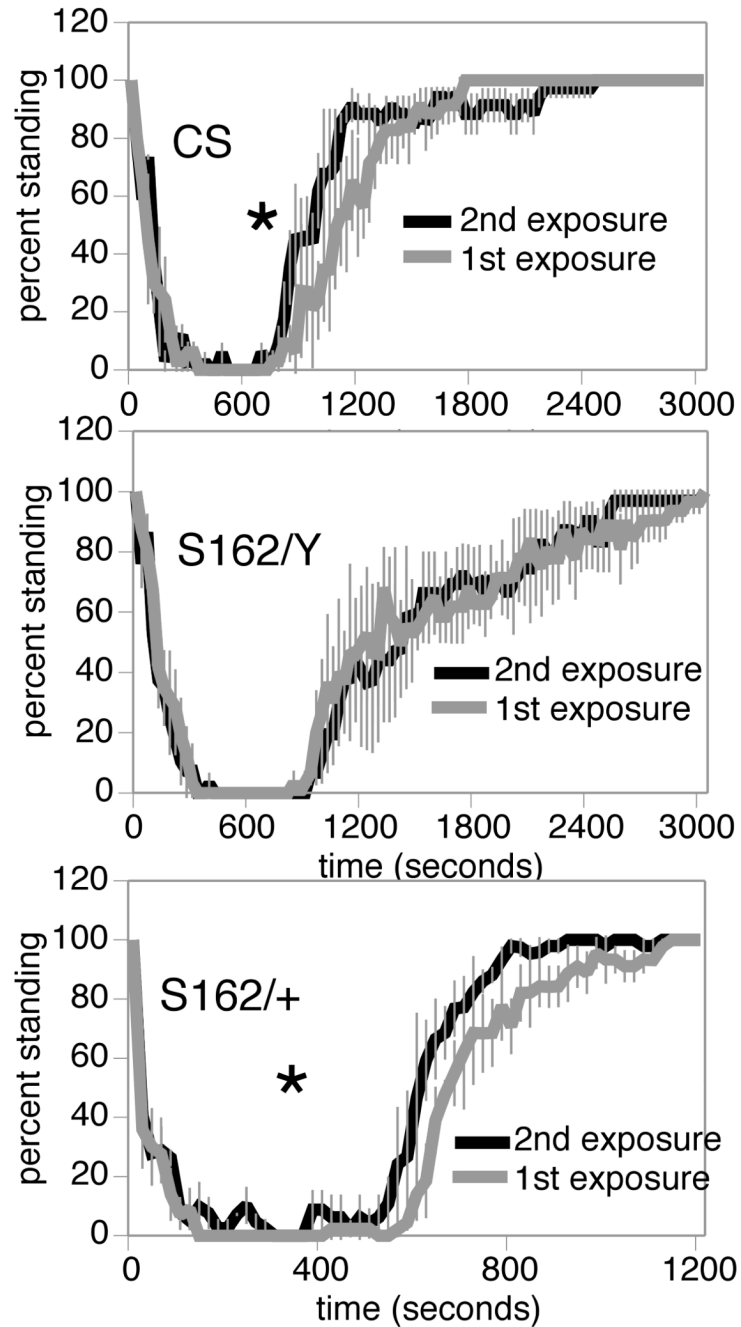


Figure 5. The $dCREB2^{S162}$ mutation blocks the acquisition of benzyl alcohol tolerance
Top) Wild-type CS males acquire rapid tolerance after a single benzyl alcohol sedation.
Middle) hemizygous $dCREB2^{S162}$ males do not acquire tolerance after one benzyl alcohol sedation.
Bottom) $dCREB2^{S162}$ heterozygous female flies acquire tolerance in response to prior benzyl alcohol sedation. Significance between recovery curves was determined by the log-rank test ($n=45$, $*p<0.05$).

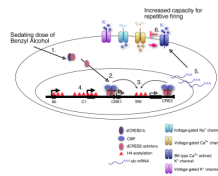


Figure 6. Proposed regulatory cascade that produces BK channel dependent tolerance to anesthetic drug sedation

Benzyl alcohol sedation down regulates the repressor form of *dCREB2* (the *dCREB2-b* isoform) and releases sequestered *CREB* activators (1). Once free, *dCREB2* activators bind to CRE sites in the *slo* transcriptional control region (2) and stimulate histone acetylation probably by recruiting CBP which has histone acetyltransferase (HAT) activity (3). CBP binding at 55b is not shown because it would obscure the symbol for acetylation.

Acetylation may expose binding sites for other transcription factors required to activate the *slo* promoter (4) to increase *slo* mRNA abundance in the nervous system (5). Up-regulation of BK channels will enhance the capacity of the neuron for repetitive firing by limiting the inactivation of voltage-gated Ca^{2+} and Na^{+} channels and/or by preventing the activation of other K^{+} channels (6).