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CREB regulation of BK channel gene expression underlies rapid drug tolerance

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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 Ca^{2+}-activated K^+ channels and has been linked to alcohol- and organic solvent-induced behavioral tolerance in mice, *Caenorhabditis elegans (C. elegans)* and *Drosophila*. The cyclic AMP 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 upregulation 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 Ca^{2+}-activated K^+ channels and that this gives rise to behavioral tolerance to benzyl alcohol sedation.

Keywords: BK channel, CREB, *Drosophila*, drug addiction, potassium channel, *slo*, *slowpoke*, tolerance, transcription factor

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Changes in the neural expression of the *slo* Ca^{2+}-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 (Cowmeadow *et al.* 2005; Ghezzi *et al.* 2004). However, the molecular pathways that mediate the upregulation 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 cyclic AMP (cAMP) response element-binding (CREB) protein transcription factor. Cyclic AMP response element-binding protein can recruit histone acetyltransferases to the promoter region. In mammals, CREB is a key factor in producing neuronal changes associated with drug tolerance and addiction (Brunzell *et al.* 2003; McClung & Nestler 2003; Misra *et al.* 2001; Widnell *et al.* 1996).

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 genes (Usui *et al.* 1993; Yin *et al.* 1995). In *Drosophila*, both the *dCREB-A* and the *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, learning, memory and sexual behavior (Belvin *et al.* 1999; Sakai & Kidokoro 2002; Yin *et al.* 1994). *dCREB2* transcripts are alternatively spliced. Most splice variants have a consensus cAMP-dependent protein kinase A (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 (Lonze & Ginty 2002; Shaywitz & Greenberg 1999). Furthermore, mammalian CREB has been shown to stimulate transcription by recruiting the histone acetylase CREB-binding protein (CBP) to the region and by stabilizing the binding of other transcription factors at the

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promoter (Conkright *et al.* 2003; Ogryzko *et al.* 1996). 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 (Perazzona *et al.* 2004; Yin *et al.* 1995).

Previously, we have shown that a CREB transcription factor binds the *slo* promoter region using the chromatin immunoprecipitation (ChromIP) assay. Overexpression 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 upregulation of the *slo* gene represents a homeostatic response that counters the effects of drug sedation and that CREB was likely to mediate *slo* upregulation 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 downregulation 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 (CS) (wild type), cAMP response element (CRE)-luciferase transgenic flies (Iijima-Ando & Yin 2005) and *dCREB2*^{S162}/FM6 (Bloomington Stock Center, Indiana University, Bloomington, IN, USA). 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). 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-h light and dark cycle. For the tolerance assay, newly eclosed flies were collected over a 2- to 3-day window, transferred to fresh food and studied 5–6 days after eclosed.

Tolerance assay

Age-matched 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 the recovery stage. Flies were scored as recovered when they resumed climbing. The number of recovered flies was plotted as a percentage of the population in each tube (average of three tubes) against time at 30-second intervals. The log-rank test was used to determine whether the recovery time of the two populations differs significantly.

Luciferase reporter assay

The CRE-luciferase (CRE-luc) 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, Madison, WI, USA), and debris were eliminated by spinning in a microcentrifuge. The luminescence in the cell lysis was measured using Luciferase Assay System (E1500; Promega) with a luminometer (Mithras LB 940; Berthold Technologies U.S.A. LLC, Oak Ridge, TN, USA). 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 was measured with RC DC Protein Assay kit (catalog number 500-0120; Bio-Rad, Hercules, CA, USA).

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 ChromIP 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 Ser-231 (activated KID domain) and was used at 1:200 dilution. Both co-immunoprecipitated and input DNA were recovered by reverse cross-linking, phenol/chloroform extraction and ethanol precipitation. Real-time polymerase chain reaction (PCR) was performed using the ABI SYBR Green PCR protocol (Applied Biosystems, Foster City, CA, USA) 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 (Fig. 3a), primers that 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′-TACCCAATTGAATTCGCCTTGTCTT-3′, 5′-CCCACTCTCCGGCCATCTCT-3′) and cre2 (5′-TGGATTGCGACCGAGTGTCT-3′, 5′-ATCAATACGATAACT GGCGGAAACA-3′). As a control, we used primers for *Cyclophilin 1* (*Cyp1*) promoter region (5′-TCTGCGTATGTGTGGCTCAT-3′, 5′-TACAGAACTCGCGCATTCAC-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: $\text{IP/input} = 2^{(\text{Ctinput} - \text{CtIP})}$. Significance was determined with the two-way analysis of variance. Independent repetitions of entire procedure yielded essentially the same binding profile.

Real-time reverse transcriptase–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 h after benzyl alcohol sedation using a single-step RNA isolation protocol as described previously (Cowmeadow *et al.* 2006; Ghezzi *et al.* 2004) and quantified in a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Two-step reverse transcription and real-time PCR were performed in triplicate with specific primers for *dCREB-A*, *dCREB2-b*, *slo* C1 exon and *Cyp1*. *Cyclophilin 1* was used as an internal control gene for normalization. First-strand complementary DNA (cDNA) was synthesized from total RNA with gene-specific reverse primers with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). 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). Messenger RNA (mRNA) abundance was calculated using the standard-curve method (Applied Biosystems manual), and significance was calculated using the Student's *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′-TCTCGATGTCGGAGCAAATG-3′), *dCREB2-b* forward primer (5′-ACTGCAGGTGGCAGTCCG-3′), *dCREB2-b* reverse primer (5′-TAGACCACCTTCTGCATCGCT-3′), *slo* (C1) forward primer (5′-AAACAAAGCTAAATAAGTTGTGAAAGGA-3′) and *slo* (C1) lower reverse primer (5′-GATAGTTGTTCGTTCTTTT-GAATTTGA-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 *dCREB2-b* splice variant has been clearly shown to be a repressor isoform with important effects on behavior (Perazzona *et al.* 2004; Yin *et al.* 1995). We measured the relative abundance of *dCREB-A* and *dCREB2-b* transcripts in fly heads by real-time reverse transcriptase–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 *Cyp1* gene. *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 upregulated the *dCREB-A* transcript and downregulated 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.

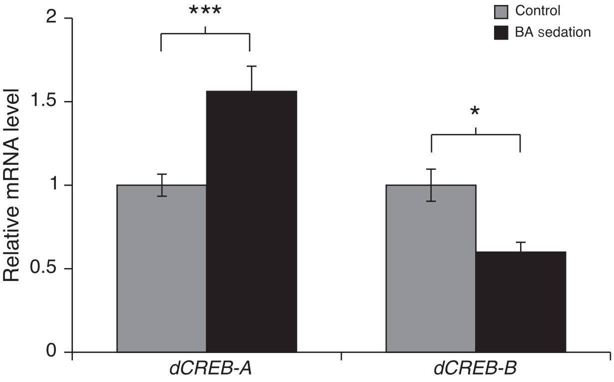

Figure 1: Benzyl alcohol (BA) sedation enhances *dCREB-A* and reduces *dCREB2* repressor splice variant (*dCREB2-b*) mRNA abundance. The mRNA levels of CREB transcripts in fly heads were measured using real-time reverse transcriptase–PCR with *Cyp1* as the internal control. RNA levels are normalized with respect to untreated animals. Four hours after sedation with BA, the *dCREB-A* messenger level was induced about 50% ($n = 11$, $^{***}P < 0.001$, Student's *t*-test). A single BA sedation causes a reduction in the abundance of the *dCREB2-b* repressor isoform ($n = 4$, $^{*}P < 0.05$, Student's *t*-test).

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 CREs modulate expression of the bioluminescent luciferase reporter gene. Expression of luciferase from this transgene increases in response to activation by CREB (Iijima-Ando & 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 composed of neural tissue and are easily isolated in mass. Compared With mock-sedated animals, CRE-luc flies showed a significant increase in bioluminescence 4 h 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.

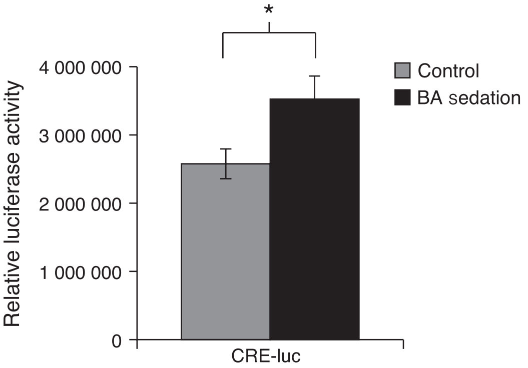

Figure 2: Positive regulation by CREB increases after benzyl alcohol (BA) sedation. CRE-luc transgenic flies were used to measure CRE-dependent gene expression. The relative amount of bioluminescence in fly head extracts was measured and normalized to total protein (specific activity). Four hours after BA sedation, luminescence activity was increased in CRE-luc transgenic flies ($n = 4$, $^{*}P < 0.05$, Student's *t*-test). Luciferase activity was not detected in flies that did not contain the transgene (data not shown).

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 benzyl alcohol sedation enhances the binding of a CREB isoform, within the *slo* promoter region, and that this binding is correlated with increased *slo* expression (Wang *et al.* 2007). However, the antibody used in the prior experiment does not distinguish between dCREB-A and dCREB2 products or 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 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 and *Cyp1*). 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 upregulated by benzyl alcohol sedation, and increased *slo* expression is capable of producing the tolerant phenotype. Previously, we had shown that overexpression 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 that 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). These 'escaper' males are about three-fourths the size of wild-type flies but 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 6 h after benzyl alcohol sedation in both mutant *dCREB2*^{S162} flies and wild-type CS flies. In *dCREB2*^{S162} males, *slo* mRNA levels decreased after benzyl alcohol sedation (Fig. 4). As has been previously reported, wild-type CS males showed a significant increase in *slo* mRNA level 6 h after benzyl alcohol sedation (Ghezzi *et al.* 2004). This shows that benzyl alcohol-induced *slo* expression is co-ordinated by the dCREB2 transcription factor.

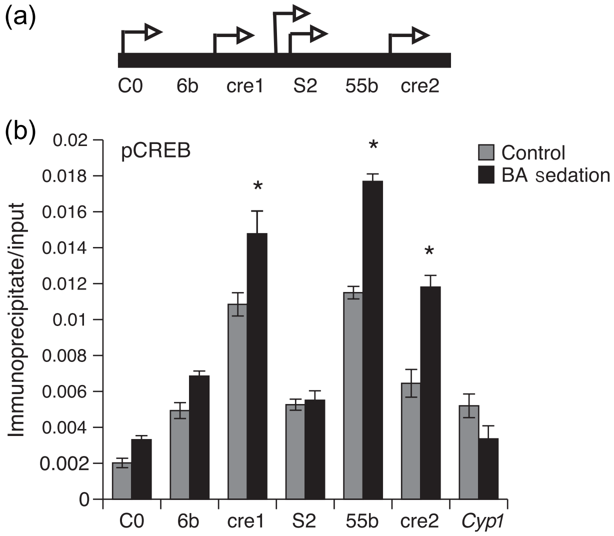

Figure 3: Benzyl alcohol (BA) sedation enhances the occupancy of activated CREB in the *slo* promoter region. (a) Map of the transcriptional control region of the *slo* gene. The arrowheads indicate the transcription start sites of five tissue-specific promoters. From left to right: the first two promoters are neural specific, the next two are known to be active in epithelial cells of the larval digestive tract and the rightmost 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 (pCREB) within the *slo* promoter region was measured by ChromIP in fly heads 4 hours after a BA 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 *Cyp1* gene. This fragment is an internal control that does not contain a CRE site and whose expression is not upregulated by BA treatment (Cowmeadow *et al.* 2005; Ghezzi *et al.* 2004). Signals obtained from PCR amplification of immunoprecipitated chromatin were normalized to the amount of input chromatin. Increased pCREB occupancy was observed around three putative CRE sites (cre1, cre2 and 55b). Significance was determined by two-way analysis of variance ($n = 3$, $^*P < 0.05$). Error bars are SEM.

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

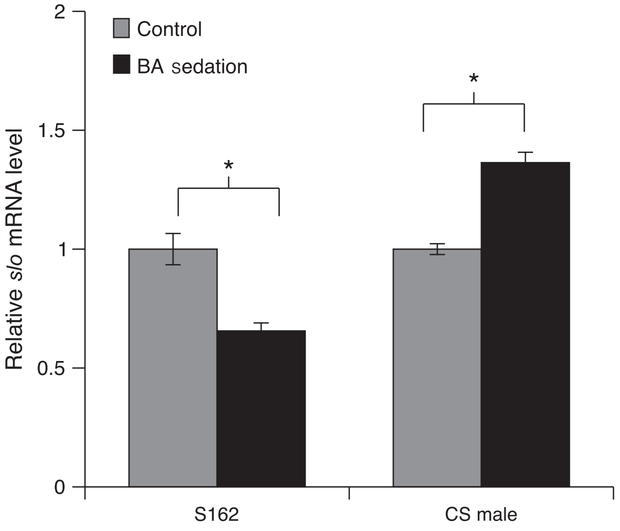

Figure 4: The *dCREB2*^{S162} mutation blocks induction of *slo* by benzyl alcohol (BA) sedation. *slo* mRNA levels in *dCREB2*^{S162} (S162) and CS males were measured by real-time reverse transcriptase–PCR with primers specific for the neural *slo* isoforms. Six hours after BA sedation, *slo* mRNA decreased about 40% in the *dCREB2*^{S162} stock compared with non-treated group, whereas BA sedation produced an increase in *slo* mRNA abundance in the CS control group ($n = 4$, $^*P < 0.05$, Student's *t*-test).

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 because of 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 because 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 & 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 (Adelman *et al.* 1992; Atkinson *et al.* 1991). 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 upregulate the *slo* promoter activity, which gives rise to rapid benzyl alcohol tolerance in flies.

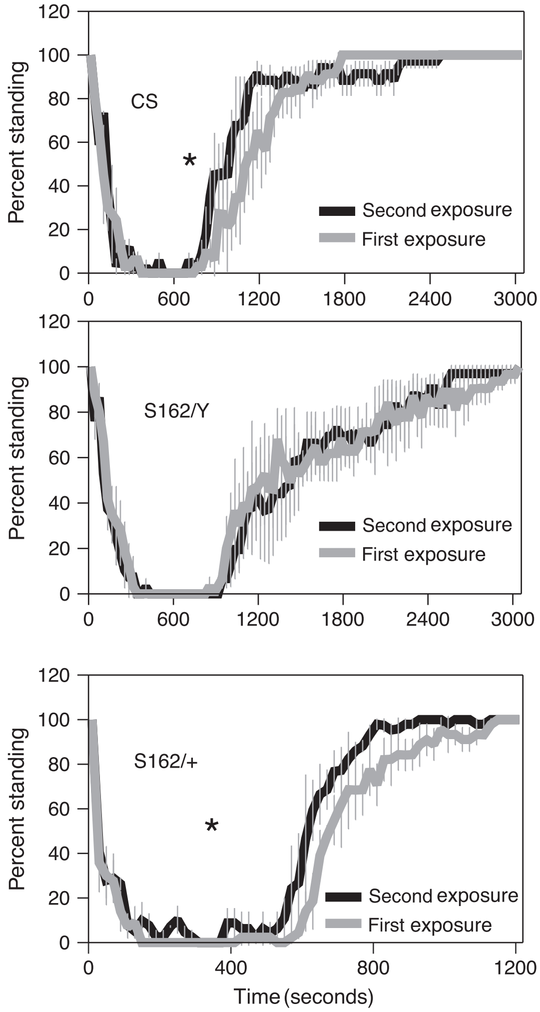

Figure 5: The *dCREB2*^{S162} mutation blocks the acquisition of benzyl alcohol tolerance. (Top) Wild-type CS males acquire rapid tolerance after a single benzyl alcohol sedation. (Middle) Hemizygous *dCREB2*^{S162} males do not acquire tolerance after one benzyl alcohol sedation. (Bottom) *dCREB2*^{S162}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

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 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 cannot yet be performed 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, overexpression 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 (approximately 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 & Greenberg 1999).

Cyclic AMP response element-binding protein activators have been shown to promote gene expression through at least three mechanisms. One mechanism is the binding of the glutamine-rich Q2 domain of CREB to TAFII130, which is a component of the transcription pre-initiation complex (Ferreri *et al.* 1994; Quinn 1993). A second means of interacting with TAFII130 is provided by the transducers of regulated CREB activity (TORC) cofactors, which bind the bZIP domain of CREB at one end and TAFII130 on the other end (Conkright *et al.* 2003; Lonze & Ginty 2002). 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 & Okamoto 2008). However, CREB, with a phosphorylated kinase-inducible domain, can stimulate expression by binding the histone acetylase CBP. 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 (Brindle *et al.* 1993; Gonzalez & Montminy 1989; 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; Lonze & Ginty 2002; Shaywitz & Greenberg 1999). While it is clear that *dCREB2* can produce a transcriptional repressor, it is controversial whether *dCREB2* also produces positively acting isoforms (Perazzona *et al.* 2004; Yin *et al.* 1995). Nevertheless, our ChromIP data show 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 *dCREB2* expresses activator isoforms that stimulate *slo* expression or phosphorylation of the kinase-inducible domain prevents dCREB2-b 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 (Cowmeadow *et al.* 2005, 2006; Davies *et al.* 2003; Ghezzi *et al.* 2004). 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 downregulation 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*

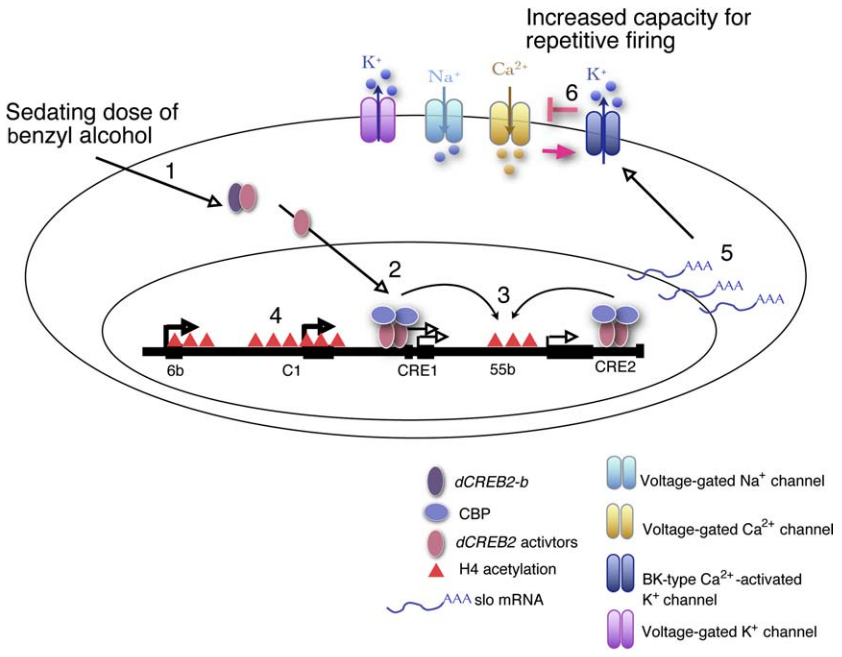

Figure 6: Proposed regulatory cascade that produces BK-channel-dependent tolerance to anesthetic drug sedation. Benzyl alcohol sedation downregulates the repressor form of dCREB2 (the *dCREB2-b* isoform) and releases sequestered CREB activators (1). Once free, dCREB2 activators bind to CRE sites in the *slo* transcriptional control region (2) and stimulate histone acetylation probably by recruiting CBP, which has histone acetyltransferase 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). Upregulation 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).

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 Fig. 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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