

Brief Report

Inducible repression of CREB function disrupts amygdala-dependent memory

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Abstract

Evidence from *Aplysia*, *Drosophila*, mice, and rats indicates that the CREB (cAMP/Ca²⁺ responsive element binding protein) family of transcription factors is critical for long-term memory. Recent findings, however, suggest that performance abnormalities may contribute to the memory deficits attributed to CREB manipulations in mammals. To clarify the role of CREB in memory, we used a paradigm, conditioned taste avoidance, that places few performance demands on the subject. We show that lesioning or blocking protein synthesis in the basolateral amygdala of mice disrupts conditioned taste aversion. Furthermore, either chronically or acutely disrupting CREB function in two different types of genetically modified mice blocks memory for conditioned taste aversion measured 24 h following training. Together, these findings indicate that CREB-mediated transcription and protein synthesis are required for conditioned taste aversion memory.

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1. Introduction

Long-term memory (LTM; memory lasting a day or longer) is thought to involve gene transcription, protein synthesis, and the formation of new synaptic connections (Bailey & Kandel, 1993; Davis & Squire, 1984). Extensive evidence from *Aplysia* and *Drosophila* indicates that the CREB (cAMP/Ca²⁺ responsive element binding protein) family of transcription factors is important for LTM in these invertebrate species (Dash, Hochner, & Kandel, 1990; Yin, Del Vecchio, Zhou, & Tully, 1995). The first study to examine the role of CREB in mammalian LTM, tested memory in mice with a targeted mutation in the two main isoforms (α and δ) of CREB (CREB ^{$\alpha\delta$} mice). These mutant mice showed intact short-term memory (STM) but deficits in LTM for contextual conditioned fear and spatial

learning as well as unstable hippocampal long-term potentiation, a cellular model of synaptic plasticity thought to be required for learning and memory (Bourtchuladze et al., 1994). However, the memory phenotype of CREB ^{$\alpha\delta$} mutant mice appears to be sensitive to gene dosage and genetic background (Gass et al., 1998), since the phenotype is less severe when the mutation is present in specific mice strains. In addition, a recent study using both CREB ^{$\alpha\delta$} and conditional CREB knock-out mice suggests that performance abnormalities (e.g., thymotaxis in the watermaze) could contribute to the deficits previously attributed to CREB ^{$\alpha\delta$} mice (Balschun et al., 2003).

To clarify the role of CREB in mammalian memory we assessed memory in two very different types of genetically modified mice with distinct genetic backgrounds using conditioned taste aversion (CTA). In the CTA paradigm, ingestion of a novel taste is paired with transient sickness. Memory for this association is demonstrated by the animal avoiding that taste on

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subsequent presentations (Garcia, Kimeldorf, & Koelling, 1955). We chose to study memory using the CTA paradigm for two reasons. First, previous studies in rats shows that CTA critically depends on the amygdala (Yamamoto & Fujimoto, 1991) and amygdala function may be especially comprised in CREB^{Δδ-/-} mutant mice (Graves, Dalvi, Lucki, Blendy, & Abel, 2002). Second, unlike memory tasks such as the watermaze, CTA places few performance demands on the subject. Therefore conclusions regarding the role of CREB in memory may be drawn independently from potential effects on motor behaviour.

To determine whether the amygdala is critical for CTA in mice, we first examined the effect of pre-training electrolytic lesions of the basolateral amygdala (including lateral, basal, and accessory basal nuclei) in wild-type (WT) mice. Mice (3–5 months, 23–40 g, C57Bl/6 Taconic Farms) were group-housed and maintained on a 12 h light/dark cycle with food and water available ad libitum prior to the experiment. Experiments were conducted in accordance with UCLA Animal Care and Use Committee Guidelines. Mice were anesthetized with chloral hydrate (400 mg/kg, ip) and placed in a stereotaxic frame. An electrode (Kopf Model NE-300 insulated to within 0.5 mm of the tip) was lowered into the basolateral amygdala (AP = -1.3, ML = ± 3.3, DV = -4.8 and AP = -1.5, ML = ± 3.0, DV = -4.8, Paxinos & Franklin, 2000) and anodal current (3 mA, 6 s) passed. CTA training took place in the light part of the cycle 7–10 days following surgery. During the 5-day habituation, singly housed water-restricted mice were trained to drink their daily water ration from two bottles containing tap water (presented for 40 min). On the conditioning day, a novel

taste (the conditioned stimuli, CS; 0.2% saccharin sodium salt w/v or Cherry Kool-Aid) was paired with the malaise-inducing agent lithium chloride (LiCl; 0.15 M, 2% body weight ip). The CS fluid was presented for 20 min and, 40 min later, mice were treated with LiCl or a similar volume of vehicle (VEH). Testing occurred 24 h later when two bottles (one bottle containing tap water and the other containing the CS fluid) were presented for 40 min. The intake of each fluid was measured and the relative consumption of the CS fluid calculated (amount of CS fluid consumed/total fluid consumed). It is important to note that for all studies, mice in different treatment groups consumed similar total levels of fluid (CS + water) in the test session (data not shown).

An analysis of variance (ANOVA) performed on the test day CS consumption scores from the 4 groups (Sham-VEH, Sham-LiCl, Lesion-VEH, and Lesion-LiCl) revealed a significant interaction between *Lesion* and *LiCl-Treatment* [$F(1, 18) = 10.73, P < .05$]. Sham-LiCl mice drank less CS fluid (saccharin) than Sham-VEH mice, indicating significant CTA (Fig. 1A; post hoc Newman–Keuls analyses comparing Sham-VEH to Sham-LiCl; $P < .05$). However, there was no difference in CS consumption between Lesion-LiCl and Lesion-VEH groups ($P > .05$). Therefore, pairing the CS fluid with LiCl produced robust CTA in sham-operated, but not amygdala-lesioned, mice. This result indicates that the amygdala is critical for CTA in mice, in agreement with previous studies in rats (Yamamoto & Fujimoto, 1991). However, since the lesion is present both at the time of training and testing, it is unclear whether the amygdala is critically involved in the learning/memory or performance aspects of CTA.

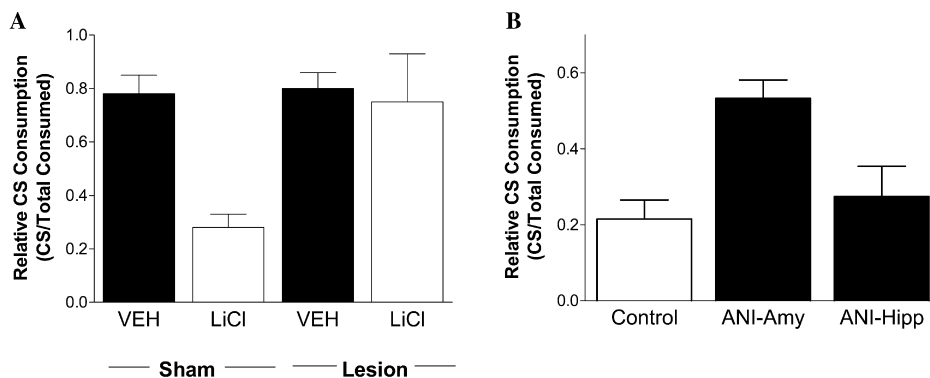


Fig. 1. Manipulations of amygdala function impair CTA. (A) Mean (\pm SEM) CS consumption scores (intake of saccharin/total fluid) 24 h following pairing with LiCl or VEH in WT mice with electrolytic lesions of the basolateral amygdala or sham surgery. Sham-operated mice in which the CS fluid was paired with VEH consume high levels of CS fluid in the test whereas similarly treated mice in which the CS fluid was paired with LiCl subsequently avoid the CS fluid, showing significant CTA. Mice with lesions of the basolateral amygdala consume equally high quantities of the CS fluid regardless of pairing with LiCl or VEH, showing impaired CTA. Sham-VEH $n = 4$, Sham-LiCl $n = 8$, Lesion-VEH $n = 4$, and Lesion-LiCl $n = 6$. (B) Mean (\pm SEM) CS consumption scores (intake of saccharin/total fluid) 24 h following pairing with LiCl in WT mice infused with the protein synthesis anisomycin (ANI) or PBS into amygdala (Amy) or dorsal hippocampus (Hipp). Mice infused with PBS into the amygdala or hippocampus are combined into one Control group. Inhibition of protein synthesis in the amygdala but not dorsal hippocampus at the time of training disrupts LTM for CTA. Control $n = 6$, ANI-Amy $n = 8$, and ANI-Hipp $n = 6$.

To address this question and to examine whether protein synthesis in the amygdala is critical for CTA memory, we injected the protein synthesis inhibitor anisomycin (ANI) into the amygdala of mice during the conditioning phase of the task. As an anatomical control, another group of mice received ANI infusions into the dorsal hippocampus. WT mice were implanted with cannula (26-gauge cannula, Plastics One, VI) aimed at the basolateral amygdala or dorsal hippocampus (AP = -2.0, ML = ± 2.0, V = -2.0). Following recovery, mice were presented with the CS fluid (saccharin) as above and, 5 min later, infused with ANI (62.5 µl/0.5 µl over 2 min; dissolved in PBS with the pH adjusted to 7.0–7.4; Sigma) or PBS. LiCl was administered to all groups (PBS-amygdala, PBS-hippocampus, ANI-amygdala, and ANI-hippocampus) 35 min later.

During the subsequent test, mice infused with PBS in the amygdala or dorsal hippocampus consumed equally low levels of the CS fluid [$F(1, 4) = 0.110, P > .05; n = 3$ for each group]. Therefore, PBS infusions into the amygdala or hippocampus did not disrupt the CTA produced by pairing saccharin with LiCl. As there was no difference between these two groups, they were combined into a single PBS-Control group in subsequent analyses. An ANOVA performed on the CS consumption scores for PBS-Control, ANI-amygdala, and ANI-hippocampus groups showed a significant effect of *Group* [$F(2, 17) = 8.84, P < .05$; Fig. 1B]. Post hoc analyses revealed that mice in the ANI-amygdala group drank more CS fluid than mice in the ANI-hippocampus or the PBS-Control groups, while the latter two groups did not differ. These results indicate that protein synthesis in the amygdala, but not hippocampus, is important for CTA memory. These data are consistent with findings that disrupting the function of the amygdala (Bahar, Samuel, Hazvi, & Dudai, 2003), but not hippocampus (Yamamoto & Fujimoto, 1991), blocks CTA memory in rats. In addition, these results are in agreement with data showing that long-term, but not short-term, memory for CTA is blocked by brain-wide inhibition of protein synthesis in rats (Haupt & Berlin, 1999).

To assess the role of CREB in CTA memory, we first used genetically modified mice with a targeted deletion in the two main isoforms of CREB (CREB^{αδ-/-} mice; Hummler et al., 1994). Levels of CREB protein are reduced by roughly 90% in these mice (Walters & Blendy, 2001). Experiments were performed on F2 mice homozygous for the CREB deletion (CREB^{αδ-/-}) and WT littermate controls derived from a cross between CREB^{αδ+/-} mice in the C57Bl/6NTac background (N10) and WT 129S6/SvEvTac mice. Genotypes were determined by PCR analysis of tail DNA samples.

The CS fluid (Cherry Kool-Aid) was paired with LiCl or VEH in separate groups of CREB and WT mice. An ANOVA performed on the CS consumption scores

during the test session for each group (WT-VEH, WT-LiCl, CREB-VEH, and CREB-LiCl) showed a significant interaction between *Genotype* and *LiCl-Treatment* [$F(1, 14) = 7.51, P < .05$; Fig. 2A]. Post hoc comparisons revealed that WT-LiCl mice drank less CS fluid than WT-VEH ($P < .05$), indicating that WT mice showed significant memory for CTA (Fig. 2A). However, CREB-LiCl mice consumed similar amounts of the CS fluid to CREB-VEH mice ($P > .05$), indicating that the CREB-mutant mice showed disrupted memory for CTA. Therefore, mice with a chronic disruption of CREB function show memory deficits in this task. However, since the disruption in CREB function is present at both the time of training and at testing, the interpretation of this deficit may be complicated. To address this potential limitation, we performed a similar experiment using mice with an inducible and reversible disruption of CREB function.

To temporally disrupt CREB function, we used a line of transgenic mice that inducibly and reversibly express a dominant negative form of CREB (α CREB^{S133A}) in forebrain regions, including the amygdala, hippocampus, and cortex (Kida et al., 2002). In these CREB^{IR} transgenic mice, a CREB repressor is fused to a mutated ligand-binding domain (LBD) of a human estrogen receptor, the activity of which is regulated not by estrogen, but by the synthetic ligand, tamoxifen (TAM) (Feil et al., 1996). In the absence of TAM, the CREB repressor is bound to heat shock proteins and inactive (Feil et al., 1996). Administration of TAM activates the inducible CREB repressor, allowing it to compete with endogenous CREB and disrupt CREB-mediated transcription (see Fig. 2B). These mice are ideal to study the behavioral effects of acutely disrupting CREB function because the kinetics of CREB disruption in the CREB^{IR} mice following a single TAM injection is rapid (approximately 6 h) and reversible (Kida et al., 2002). Importantly, unlike CREB^{αδ-/-} (Hummler et al., 1994) and CREB knock-out mice (Balschun et al., 2003), CREB^{IR} mice do not have upregulated levels of CREM.

Separate groups of CREB^{IR} and WT mice (F1, C3H × C57Bl/6; Taconic Farms) were pre-treated with TAM (4-hydroxytamoxifen; 16 mg/kg, ip; Sigma) or peanut oil (PO, the vehicle for TAM) 6 h prior to CTA training in which the CS fluid (saccharin) was paired with LiCl or VEH (see Kida et al., 2002). Memory was assessed 24 h later in drug-free mice. All groups of mice in which the CS fluid was paired with VEH consumed equally high levels of the CS fluid in the test [CREB^{IR}/TAM ($n = 3$), CREB^{IR}/PO ($n = 4$), WT/TAM ($n = 4$), WT/PO ($n = 6$); *Genotype* by *Pre-treatment*, $F(1, 13) = 0.17, P > .05$; *Genotype*, $F(1, 108) = 0.37, P > .05$; *Pre-treatment*, $F(1, 13) = 2.21, P > .05$]. Therefore, neither the presence of the transgene, pre-treatment with TAM, nor the combination of both, affected the preference for

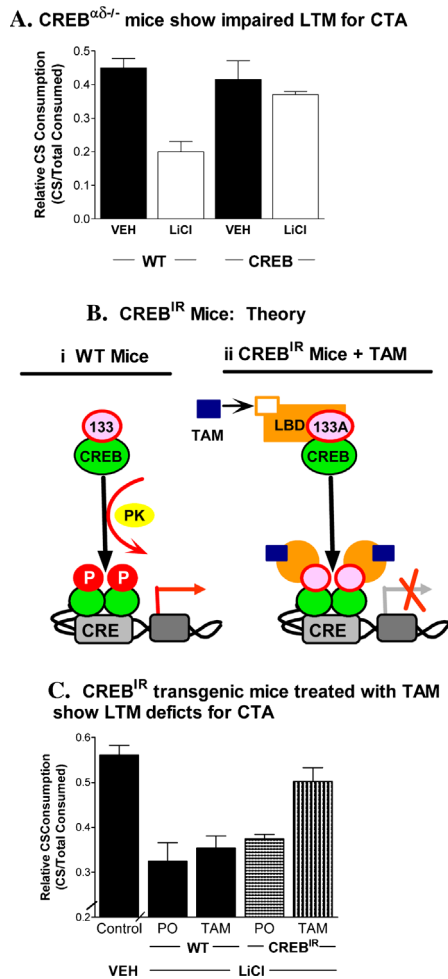


Fig. 2. Disrupting CREB function impairs LTM for CTA. (A) Chronically disrupting CREB function disrupts LTM for CTA. Mean (\pm SEM) CS consumption scores (intake of Kool-Aid/total fluid) 24 h following pairing with LiCl or VEH in CREB^{ΔΔ} mice or WT littermate controls. WT mice show LTM for CTA (relative CS consumption in CS-LiCl paired mice is significantly less than in CS-VEH paired mice) whereas CREB mice show impaired LTM for CTA (relative CS consumption in CS-LiCl paired mice is no different from that of CS-VEH paired mice). WT-VEH $n = 5$, WT-LiCl $n = 4$, CREB-VEH $n = 4$, and CREB-LiCl $n = 5$. (B) Schematic diagram depicting strategy to inducibly and reversibly inhibit CREB-mediated transcription by administration of tamoxifen (TAM) in CREB^{IR} mice. In WT mice CREB binds to CRE-sites, and when phosphorylated at Ser133 (S133) by protein kinases (PK), initiates the transcription of target genes. In CREB^{IR} mice, a CREB repressor (CREB^{S133A}) is fused to the ligand-binding domain (LBD) of a mutated estrogen receptor. Upon administration of TAM, the CREB repressor is activated and competes with endogenous CREB to disrupt CREB function. (C) Acutely disrupting CREB function during training disrupts subsequent LTM for CTA. Mean (\pm SEM) CS consumption scores (intake of saccharin/total fluid) 24 h following pairing with LiCl or VEH in CREB^{IR} transgenic mice or WT littermate controls pre-treated with TAM or PO (peanut oil). Mice in which the CS was paired with VEH are combined into a single VEH-Control group. All groups of mice in which the CS was paired with LiCl show significant LTM for CTA except the CREB^{IR} mice that were pre-treated with TAM. Activating the CREB repressor by administering TAM to CREB^{IR} transgenic mice, therefore, disrupts LTM for CTA. VEH-Control $n = 17$, WT/PO-LiCl $n = 15$, WT/TAM-LiCl $n = 22$, CREB^{IR}/PO-LiCl $n = 9$, and CREB^{IR}/TAM-LiCl $n = 12$.

saccharin or produced a taste aversion. In subsequent analyses, these Control groups (CS paired with VEH) were combined into a single VEH-Control comparison group. An ANOVA performed on the CS consumption scores in the VEH-Control, CREB^{IR}/PO-LiCl, WT/PO-LiCl, WT/TAM-LiCl, and CREB^{IR}/TAM-LiCl groups revealed a significant effect of *Group* [$F(4, 70) = 8.96$, $P < .05$]. Post hoc analyses showed that WT/PO-LiCl, WT/TAM-LiCl, and CREB^{IR}/PO-LiCl groups drank less CS fluid than the VEH-Control group, indicating significant memory for CTA (Fig. 2C, $P_s < .05$). In contrast, the CREB^{IR}/TAM-LiCl group consumed a similar amount of CS fluid to the VEH-Control group ($P > .05$), indicating a deficit in LTM for CTA. Therefore, treating CREB^{IR} mice with TAM prior to training, thereby activating the CREB^{IR} transgene and acutely interfering with CREB function, produced a memory deficit for CTA.

The present results show that memory for CTA in mice critically depends on protein synthesis in the amygdala, but not the dorsal hippocampus. Previous findings suggest that CREB may mediate the synthesis of new proteins necessary for LTM (Bourtchuladze et al., 1994; Dash et al., 1990; Yin et al., 1995). Consistent with this, the present results show that CREB is important for CTA memory in mice. We found that mice with two types of genetic disruptions of CREB function in two different background strains showed memory deficits for CTA. Both chronic (CREB^{ΔΔ} mice, in which the two main isoforms are deleted throughout development) and acute (by administering TAM to CREB^{IR} mice) disruptions of CREB function impaired memory for CTA. Previous findings show that disrupting CREB function using antisense (Lamprecht, Hazvi, & Dudai, 1997) or in a CREB knock-out mouse in which all isoforms of CREB are deleted throughout the brain during development (Balschun et al., 2003) also attenuates LTM for CTA. Furthermore, CTA training (pairing the CS with LiCl) induced robust CREB activation (phosphorylation) in the lateral nucleus of the amygdala (Swank, 2000). Together, these findings suggest that CREB function is critically involved in LTM for CTA.

Recent findings suggest that performance abnormalities and background genetics may contribute to the memory phenotype observed in mutant mice with disrupted CREB function (Balschun et al., 2003). Here, we directly assessed the role of CREB in memory using a task with few performance requirements in two types of genetically modified mice. Together with data from previous research, these findings provide compelling and convergent evidence that CREB plays an important role in mammalian memory (Josselyn et al., 2001; Kogan et al., 1997). Overall these findings demonstrate that the role of CREB in memory is conserved throughout phylogeny from *Aplysia* and *Drosophila* to mammals.

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References

- Bahar, A., Samuel, A., Hazvi, S., & Dudai, Y. (2003). The amygdalar circuit that acquires taste aversion memory differs from the circuit that extinguishes it. *European Journal of Neuroscience*, *17*, 1527–1530.
- Bailey, C. H., & Kandel, E. R. (1993). Structural changes accompanying memory storage. *Annual Review of Physiology*, *55*, 397–426.
- Balschun, D., Wolfer, D. P., Gass, P., Mantamadiotis, T., Welzl, H., Schutz, G., Frey, J. U., & Lipp, H. P. (2003). Does cAMP response element-binding protein have a pivotal role in hippocampal synaptic plasticity and hippocampus-dependent memory? *Journal of Neuroscience*, *23*, 6304–6314.
- Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., & Silva, A. J. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. *Cell*, *79*, 59–68.
- Dash, P. K., Hochner, B., & Kandel, E. R. (1990). Injection of the cAMP-responsive element into the nucleus of *Aplysia* sensory neurons blocks long-term facilitation. *Nature*, *345*, 718–721.
- Davis, H. P., & Squire, L. R. (1984). Protein synthesis and memory: A review. *Psychological Bulletin*, *96*, 518–559.
- Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D., & Chambon, P. (1996). Ligand-activated site-specific recombination in mice. *Proceedings of the National Academy of Science United States of America*, *93*, 10887–10890.
- Garcia, J., Kimeldorf, D. J., & Koelling, R. A. (1955). Conditioned aversion to saccharin resulting from exposure to gamma radiation. *Science*, *122*, 157–158.
- Gass, P., Wolfer, D. P., Balschun, D., Rudolph, D., Frey, U., Lipp, H. P., & Schutz, G. (1998). Deficits in memory tasks of mice with CREB mutations depend on gene dosage. *Learning and Memory*, *5*, 274–288.
- Graves, L., Dalvi, A., Lucki, I., Blendy, J. A., & Abel, T. (2002). Behavioral analysis of CREB alphas mutation on a B6/129 F1 hybrid background. *Hippocampus*, *12*, 18–26.
- Haupt, T. A., & Berlin, R. (1999). Rapid, labile, and protein synthesis-independent short-term memory in conditioned taste aversion. *Learning and Memory*, *6*, 37–46.
- Hummler, E., Cole, T. J., Blendy, J. A., Ganss, R., Aguzzi, A., Schmid, W., Beermann, F., & Schutz, G. (1994). Targeted mutation of the CREB gene: compensation within the CREB/ATF family of transcription factors. *Proceedings of the National Academy of Science United States of America*, *91*, 5647–5651.
- Josselyn, S. A., Shi, C., Carlezon, W. A., Jr., Neve, R. L., Nestler, E. J., & Davis, M. (2001). Long-term memory is facilitated by cAMP response element-binding protein overexpression in the amygdala. *Journal of Neuroscience*, *21*, 2404–2412.
- Kida, S., Josselyn, S. A., de Ortiz, S. P., Kogan, J. H., Chevere, I., Masushige, S., & Silva, A. J. (2002). CREB required for the stability of new and reactivated fear memories. *Nature Neuroscience*, *5*, 348–355.
- Kogan, J. H., Frankland, P. W., Blendy, J. A., Coblenz, J., Marowitz, Z., Schutz, G., & Silva, A. J. (1997). Spaced training induces normal long-term memory in CREB mutant mice. *Current Biology*, *7*, 1–11.
- Lamprecht, R., Hazvi, S., & Dudai, Y. (1997). cAMP response element-binding protein in the amygdala is required for long- but not short-term conditioned taste aversion memory. *Journal of Neuroscience*, *17*, 8443–8450.
- Paxinos, G., & Franklin, K. B. J. (2000). *The mouse brain in stereotaxic coordinates* (2nd ed.). San Diego: Academic Press.
- Swank, M. W. (2000). Phosphorylation of MAP kinase and CREB in mouse cortex and amygdala during taste aversion learning. *Neuroreport*, *11*, 1625–1630.
- Walters, C. L., & Blendy, J. A. (2001). Different requirements for cAMP response element binding protein in positive and negative reinforcing properties of drugs of abuse. *Journal of Neuroscience*, *21*, 9438–9444.
- Yamamoto, T., & Fujimoto, Y. (1991). Brain mechanisms of taste aversion learning in the rat. *Brain Research Bulletin*, *27*, 403–406.
- Yin, J. C., Del Vecchio, M., Zhou, H., & Tully, T. (1995). CREB as a memory modulator: induced expression of a dCREB2 activator isoform enhances long-term memory in *Drosophila*. *Cell*, *81*, 107–115.