Memory Extinction, Learning Anew, and Learning the New: Dissociations in the Molecular Machinery of Learning in Cortex

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The rat insular cortex (IC) subserves the memory of conditioned taste aversion (CTA), in which a taste is associated with malaise. When the conditioned taste is unfamiliar, formation of long-term CTA memory depends on muscarinic and β-adrenergic receptors, mitogen-activated protein kinase (MAPK), and protein synthesis. We show that extinction of CTA memory is also dependent on protein synthesis and β-adrenergic receptors in the IC, but independent of muscarinic receptors and MAPK. This resembles the molecular signature of the formation of long-term memory of CTA to a familiar taste. Thus, memory extinction shares molecular mechanisms with learning, but the mechanisms of learning anew differ from those of learning the new.

Experimental extinction is the decline in the frequency or intensity of a conditioned response following the withdrawal of reinforcement (1). It does not reflect forgetting due to the obliteration of the original engram, but rather "relearning," in which the new association of the conditioned stimulus with the absence of the original reinforcer comes to control behavior (2, 3). Only a few studies so far have addressed cellular mechanisms of experimental extinction (4). We approached the issue by analyzing the extinction of CTA. In CTA, the subject learns to associate a taste with delayed malaise (5, 6). In the rat, the formation of long-term CTA memory depends on multiple mechanisms, including protein synthesis, in the IC, which contains the gustatory cortex (7–9). The requirement for protein synthesis is a universal of long-term memory consolidation (10–14). If experimental extinction of CTA is indeed a relearning process that generates a long-term trace, it should be blocked by inhibition of protein synthesis in the IC during the retrieval session of the extinction protocol.

Rats were subjected to CTA training on an unfamiliar taste, saccharin (15), followed by repeated presentation of the conditioned taste in the absence of the malaise-inducing agent.

This led to the extinction of CTA (Fig. 1B, part a). However, when the protein synthesis inhibitor anisomycin was microinfused into the IC before the first retrieval test (16), 3 days after training, under conditions that inhibit >90% of protein synthesis for ~2 hours (7), extinction was blocked, to be resumed only after the subsequent extinction session (Fig. 1B, part b). A second application of anisomycin, before the subsequent extinction session, again blocked extinction (Fig. 1B, part c). Administration of anisomycin 30 min after retrieval had no effect (Fig. 2A). The transient inhibition of protein synthesis during the retrieval of CTA to saccharin had no deleterious effect on acquisition of CTA to another taste 4 days later (17). It could be argued that anisomycin did not attenuate ex-

tinction but rather reinstated the original aversion by functioning as a negative reinforcer in association with the taste [as in unconditioned stimulus reexposure (2)]. This, however, was ruled out. First, under the conditions used in this experiment, anisomycin in the IC does not serve as a negative reinforcer in CTA (7). Second, introduction of anisomycin into the IC after extinction has further proceeded did not reinstate the original memory (Fig. 2B). Inhibition of protein synthesis during or immediately after an extinction trial therefore blocks extinction. This resembles the dependence of consolidation of a newly acquired memory on protein synthesis (7, 10–14).

The formation of long-term CTA memory requires multiple neuromodulatory and signal-transduction mechanisms in the IC (8, 9, 18, 19). These involve cholinergic and β-adrenergic receptors, the MAPK ERK1-2 cascade, and the transcription factor Elk-1. If extinction is a learning process, does it require the same cellular machinery as the initial learning? When microinfused into the IC at concentrations that block long-term CTA memory (8, 9, 18, 19), the N-methyl-D-aspartate (NMDA) receptor antagonist D,L-2-amino-5-phosphonovaleric acid (APV), the muscarinic antagonist scopolamine, and the ERK-kinase inhibitor PD98059, had no effect on extinction of CTA (Fig. 3). In contrast, the β-adrenergic receptor blocker propranolol, which blocks long-term CTA memory (9), also blocked its extinction. Thus only a fraction of the molecular systems that subserve CTA acquisition is required for the learning process that is manifested in extinction. None of these systems is essential for retrieval (Table 1).

If extinction is indeed an instance of learning, why does it not share with the original learning the activation of the muscarinic recep-

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Table 1. Comparison of the contribution of identified molecular entities to the acquisition (with a novel or a familiar taste), retrieval and extinction of long-term CTA. Symbols: +, indispensable for the process; −, dispensable. NMDAR, the NMDA receptor; mAChR, the muscarinic acetylcholine receptor; β-AR, the β-adrenergic receptor. The compilation is based on the present work, as well as on data from (8, 9, 18, 19).

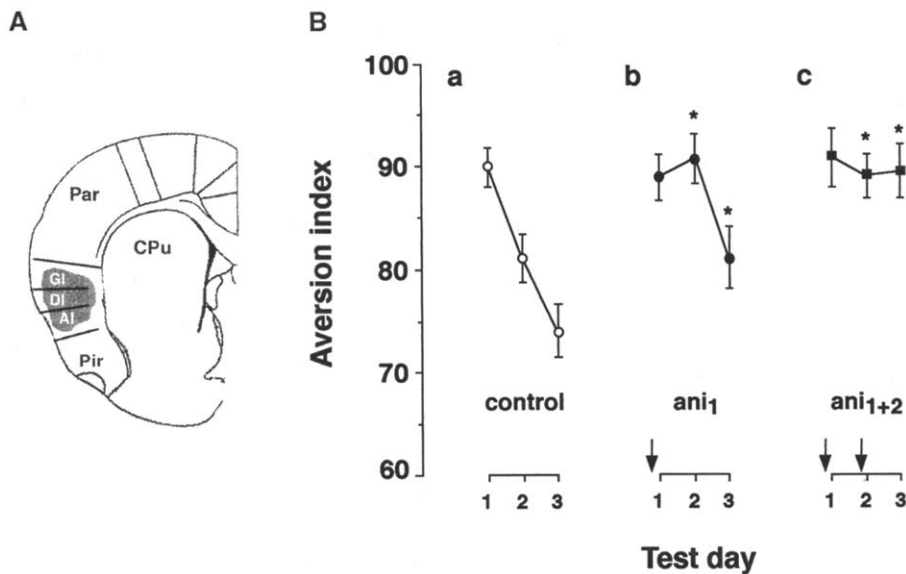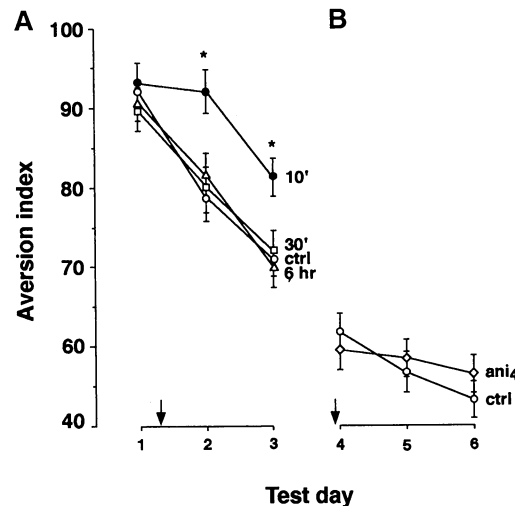| Agent	Long-term CTA			
	Acquisition		Retrieval	Extinction
	Novel	Familiar		
NMDAR	+	+	−	−
mAChR	+	−	−	−
β-AR	+	+	−	+
MAPK	+	−	−	−
Protein synthesis	+	+	−	+

A

B

Fig. 1. (A) A simplified scheme of a coronal section of a rat brain hemisphere (+1.2 AP to bregma), depicting the sphere of diffusion of the solution infused into the IC. India ink was the visible simulant. The gray area represents the superimposed sphere of diffusion in a sample of eight rats. All the drugs were infused bilaterally, and the doses indicated below are per hemisphere. AI, agranular IC; CPu, caudate-putamen; DI, dysgranular IC; GI, granular IC; Par, parietal cortex; Pir, piriform cortex. **(B, part a)**. The extinction curve of CTA in control rats, microinfused into the IC with artificial cerebrospinal fluid (the vehicle for drug infusion) 20 min before the first retrieval trial in the experimental extinction protocol (control). The first trial (1) was held 72 hours after the completion of CTA training, and the subsequent trials (2 and 3) were each a day apart. **(B, part b)** The extinction curve of rats microinfused with 100 μg anisomycin 20 min before the first extinction session (arrow, ani1). **(B, part c)**. The extinction curve of rats microinfused with anisomycin 20 min before the first extinction session and then again 20 min before the second extinction session (arrows, ani1+2). Asterisks here and in the following figures indicates P < 0.05, n = 12 each.

Fig. 2. The extinction of long-term CTA memory as a function of the time of microinfusion of anisomycin into the IC. **(A)** Whereas infusion 10 min (filled circles) after the completion of the retrieval session still had an inhibitory effect on extinction (infusion time indicated by the light gray arrow), post-retrieval infusions at 30 min (squares) and 6 hours (triangles) had no effect (infusion time indicated by dark gray and black arrows, respectively). **(B)** Microinfusion of anisomycin into the IC 20 min before the retrieval session on day 4 (diamonds) of the extinction protocol (black arrow), did not reinstate CTA memory (ani4). Ctrl (open circles), control, vehicle only; n = 11 each.

Fig. 3. The effect of inhibitors on the extinction of long-term CTA memory. Compounds were microinfused into the IC as described above for anisomycin, 20 min before the first retrieval session in the extinction protocol (black arrow). Prop (filled circles), propranolol (20 μg); APV (diamonds), (10 μg); scop (triangles), scopolamine (50 μg); PD98059 (squares), (8 ng); ctrl (open circles), control, vehicle only; n = 10 each.

synthesis, as well as on β-adrenergic and NMDA receptors. However, the CTA to the familiar taste was independent of cholinergic transmission and MAPK activation (Fig. 4). This indicates that the muscarinic receptors and the MAPK cascade in the IC are not essential for the taste-malaise association, further corroborating the notion that they play a role in encoding the novelty dimension of the taste stimulus in CTA. The molecular fingerprint of the extinction of taste memory in cortex hence overlaps with that of learning a new association of a familiar taste. The two processes differ in their dependency on the NMDA receptor. This could be accounted for by noting that whereas CTA involves association of the taste with a negative reinforcer, extinction is an association with the absence of the reinforcer; the NMDA receptor was indeed implicated in encoding the effects in the IC of LiCl given intraperitoneally (ip) (19).

Several treatments have been proposed to be capable of affecting the trace after its retrieval (20). Systemic administration of protein synthesis inhibitors with varying degrees of specificity disrupts retention of extinguished behavior in some associative tasks in the goldfish and the rat (21, 22), but no information is available on the identity of the brain sites and the molecular mechanisms involved. Extinction of shock-avoidance conditioning is blocked after systemic injection of scopolamine in the rat (23), and after infusion of APV into the amygdala of a fear-potentiated rat (4). In our hands, both drugs had no effect on extinction of CTA in the IC. The involvement of β-receptors in extinction in the IC is in line with the observation that locus coeruleus lesions, resulting in widespread

tor and of the MAPK cascade? This is not because of the absence of the malaise-inducing agent, because muscarinic receptors and the MAPK cascade are essential in the encoding of taste memory per se, in the absence of the reinforcer (8, 9). The reason could be that in CTA training, the taste is unfamiliar, whereas in extinction it is already familiar. This is in accordance with the suggestion that the cortex contains molecular "novelty" switches, which are turned on only on the

first, highly salient encounter with the stimulus (8, 18, 19). We familiarized rats with saccharin, and 3 days later subjected them to CTA training with the familiar taste. As expected (7), the CTA training with the familiar taste was less effective than with the novel taste (Fig. 4; compare the aversion index of the control on the first test day to that in Fig. 1B, part a). Similarly to CTA of a novel taste, the formation of long-term CTA memory of the familiar taste was dependent on protein
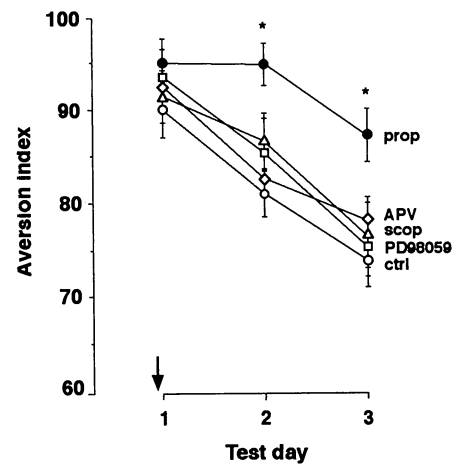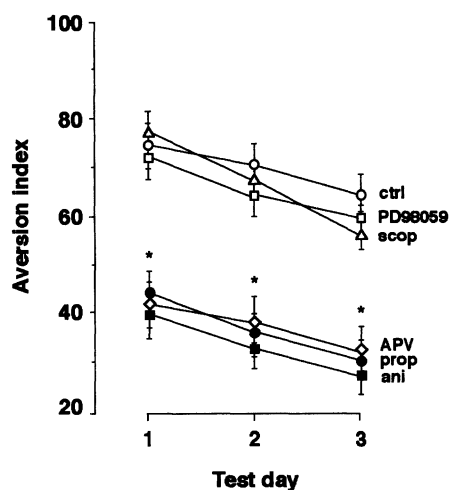
Fig. 4. Effect of the specific inhibitors on CTA to a familiar taste. Rats drank saccharin for 2 days, 10 min per day, instead of water. Three days later they were subjected to CTA training with saccharin as the conditioned stimulus (*14*). The drugs were microinjected into the IC 20 min before the exposure of the animals to the familiar taste in the CTA training session; ani (filled squares), anisomycin (100 μg); prop (filled circles), propranolol (20 μg); APV (diamonds), (10 μg); scop (triangles), scopolamine (50 μg); PD98059 (open squares), (8 ng); ctrl (open circles), control, vehicle only; *n* = 10 each.

depletion of catecholamines in brain, render classical conditioning of the nictitating membrane reflex in the rabbit resistant to extinction (*24*). The observation that β-receptors are obligatory for all the types of learning situations in our protocols is in accordance with the prominent role of these receptors in consolidation in the mammalian brain (*25*).

It has recently been reported that in fear conditioning in the amygdala, inhibition of protein synthesis immediately after retrieval extinguishes the fear behavior (*26*). We show here that in CTA, a similar treatment in the IC strengthens the trace rather than extinguishing it. The effect of protein synthesis inhibition in retrieval on the fate of the trace might hence be task- or region-dependent.

In conclusion, we show that extinction of long-term CTA memory is subserved by the same brain region that subserves the acquisition and consolidation of that same memory. Further, we identify in the IC essential core elements of the molecular machinery of learning, with other obligatory elements added according to the stimulus dimension and context. This means, among others, that the cortex honors at the molecular level the distinction between learning anew and learning the new.

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15. Male Wistar rats (~60 days old, 200 to 250 g) were caged individually at 22° ± 2°C. The behavioral protocol detailed in (*8*) was used. Sodium saccharin (0.1% w/v) was the conditioned stimulus, and LiCl ip (0.15 M, 2% body weight) was the toxicosis-inducing agent. Rats were pretrained to get their water ration once a day for 10 min from a pipette containing 10 ml of tap water. On the conditioning day, they were allowed to drink the saccharin solution instead of water for 10 min, and 40 min after the offset of the drinking period, they were injected with LiCl ip. Three days after training, the conditioned rats preferred water to saccharin at a ratio of 9:1 in a multiple-choice situation (three pipettes with 5 ml of saccharin each, three with water), whereas nonconditioned rats preferred saccharin to water. For extinction, the conditioned rats were presented once a day with the choice situation, for three to six consecutive days. The aversion index is {[ml of water/(ml of water + ml of saccharin)] × 100} consumed in the test.
16. Microinfusion into the IC was performed via chronically implanted cannulae. Rats were implanted bilaterally with a guide stainless steel cannula (23 gauge) aimed 1.0 mm above the gustatory cortex [anteroposterior +1.2 mm, lateral ±5.5 mm, ventral 5.5 mm relative to bregma, according to G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates* (Academic Press, New York, ed. 2, 1986)]. The cannulae were positioned in place with acrylic dental cement and secured with skull screws. A stylus was placed in the guide cannulae to prevent clogging. Animals were allowed 1 week to recuperate. The stylus was removed and a 28-gauge injection cannula, extending 1.0 mm from the tip of the guide cannula, was inserted. The injection cannula was connected via PE20 tubing to a microsyringe driven by a microinfusion pump. Solution (1 μl) was delivered per hemisphere over 1 min (Fig. 1A). The injection cannula was left in position before withdrawal for an additional 1 min to minimize dragging of the injectate along the injection track.
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Activity-Dependent Transfer of Brain-Derived Neurotrophic Factor to Postsynaptic Neurons

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Neurotrophins such as brain-derived neurotrophic factor (BDNF) are thought to be transferred from post- to presynaptic neurons and to be involved in the formation and plasticity of neural circuits. However, direct evidence for a transneuronal transfer of BDNF and its relation to neuronal activity remains elusive. We simultaneously injected complementary DNAs of green fluorescent protein (GFP)-tagged BDNF and red fluorescence protein into the nucleus of single neurons and visualized expression, localization, and transport of BDNF in living neurons. Fluorescent puncta representing BDNF moved in axons in the anterograde direction, though some moved retrogradely, and transferred to postsynaptic neurons in an activity-dependent manner.

Neuronal activity modifies the formation of neural circuits in developing cerebral cortex (*1–3*). Neurotrophins such as BDNF are attractive candidates for molecular signals that translate neuronal activity into such structural and functional changes in the cortex (*4–7*). Since the initial discovery of nerve growth factor, researchers have believed that neurotrophins are released or secreted from postsynaptic neu-

rons or target cells (*4, 8–10*). However, recent studies suggested that BDNF may be supplied from presynaptic axons (*11–18*). Because these studies detected BDNF mainly with immunocytochemistry after fixation, dynamics of BDNF trafficking was not analyzed in living neurons, and the crucial question of whether anterograde, transneuronal transfer of BDNF— if it exists—is related to neuronal activity re-