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Rapid Erasure of Long-Term Memory Associations in the Cortex by an Inhibitor of PKMζ

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Little is known about the neuronal mechanisms that subserve long-term memory persistence in the brain. The components of the remodeled synaptic machinery, and how they sustain the new synaptic or cellwide configuration over time, are yet to be elucidated. In the rat cortex, long-term associative memories vanished rapidly after local application of an inhibitor of the protein kinase C isoform, protein kinase M zeta (PKMζ). The effect was observed for at least several weeks after encoding and may be irreversible. In the neocortex, which is assumed to be the repository of multiple types of long-term memory, persistence of memory is thus dependent on ongoing activity of a protein kinase long after that memory is considered to have consolidated into a long-term stable form.

Persistent phosphorylation by the atypical protein kinase C isoform PKMζ is required for maintenance of long-term potentiation (LTP) in hippocampus and for sustaining hippocampus-dependent spatial memory (*1*). It is neocortex, however, which is assumed ultimately to store multiple types of long-term memory in the mammalian brain (*2*, *3*). We set out to determine whether persistent phosphorylation by PKMζ is critical for storage of long-term memory in cortex. We investigated taste memory in the insular cortex (IC), which contains the gustatory cortex (*4*).

We trained rats on conditioned taste aversion (CTA) (*5*) using saccharin as the conditioned stimulus (CS), and 3 days later, microinfused the selective PKMζ pseudosubstrate inhibitor ZIP (*1*, *6*) bilaterally into the IC. Controls received vehicle only. We tested one ZIP group 1 week later and another 1 month later. ZIP in the IC blocked CTA memory in both groups [one-way analysis of variance (ANOVA), $F(2,16) = 7.61$, $P < 0.005$] (Fig. 1A). Post hoc comparisons unveiled no difference between the ZIP groups; however, each was different from the control ($P < 0.05$). The difference persisted in extinction [repeated-measures ANOVA, group effect, $F(2,16) = 6.17$, $P < 0.01$, test effect, $F(2,32) = 8.91$, $P < 0.001$]. The ZIP groups did not differ from each other, but each was different from control ($P < 0.05$).

Although consolidation of memory in the IC is considered to be over within hours, judged by loss of vulnerability to amnesic agents (*7*), we wondered whether the vulnerability to ZIP reflects a longer consolidation process (*8*). We administered ZIP at various times 3 to 25 days after training, followed by CTA testing. The PKMζ inhibitor blocked memory at all time points tested

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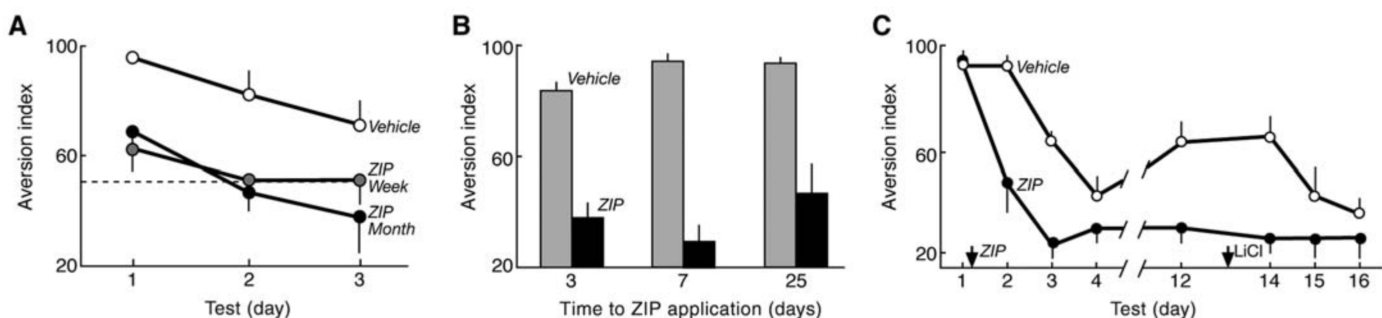

Fig. 1. Erasure of long-term CTA memory by a single application of the PKMζ inhibitor ZIP into the IC. (**A**) ZIP was administered 3 days after training, and memory was tested 1 week or 1 month later. Controls were tested at 1 month. Data are shown for three successive tests, 1 day apart. The dashed line indicates equal preference for the CS and water, i.e., AI = 50 (*5*). A preference for the CS may develop over time in naïve or CTA-extinguished rats, but AI usually does not decline below 20 to 30 even in naïve rats. For statistics, see text. (**B**) ZIP was microinfused into the IC at the indicated times after training, which was a single conditioning session (3 and 7 days groups) or two successive conditioning sessions, a day apart (25 days group). Memory was tested 2 hours (3 and 7 days groups) or 1 day (25 days group) later. (**C**) Rats were trained on CTA and tested once 3 days later, followed 1 to 4 min later by microinfusion of ZIP into the IC. Although spontaneous recovery was seen in the no-test interval between days 4 and 12 in the control group, the ZIP-treated rats showed neither spontaneous recovery nor any indication for UCS-reinstatement (LiCl, day 13).

[(Fig. 1B); $P < 0.001$ for the difference between ZIP groups at 3 days and 7 days and the controls, $P < 0.005$ for the difference between the 25 days group and control]. There is no evidence, therefore, for closure of a consolidation window even after several weeks. The effect of ZIP on long-term memory is rapid [within 2 hours at most; (Fig. 1B), at 3 and 7 days] and is not eliminated by intensifying training [(Fig. 1B), at 25 days after two successive CTA trainings to the same taste].

To exclude the possibility that the effect of the inhibitor is unique to the CS used, we replaced saccharin with glycine. ZIP was administered 3 days after training. Scrambled inactive ZIP was used as control (*1*). A test 1 day later showed an aversion index (AI) = 74.7 ± 6.5 in the ZIP group, and 98.2 ± 1.05 in the control ($n = 8$ each, $P < 0.005$). The effect is thus not unique to the CS used and requires inhibition of PKMζ activity.

In the experiments above, ZIP was administered before the first test. Because reactivation-induced reconsolidation may reinforce the memory trace (*9–11*), we wondered whether retrieval under conditions that promote reconsolidation (*8*) might render the trace immune to PKMζ inhibition. We subjected rats to two CTA training sessions, a day apart, followed by a test a week later [before ZIP, AI = 94.5 ± 2.76, $n = 10$; before vehicle, AI = 95.5 ± 2.24, $n = 8$; $F(1,16) < 1$]. We then delivered ZIP a day after the test, and retested a day later. The effect of ZIP was not eliminated [after ZIP, 58.68 ± 7.9; after vehicle, 92.1 ± 3.8; $F(1,16) = 12.27$, $P < 0.005$].

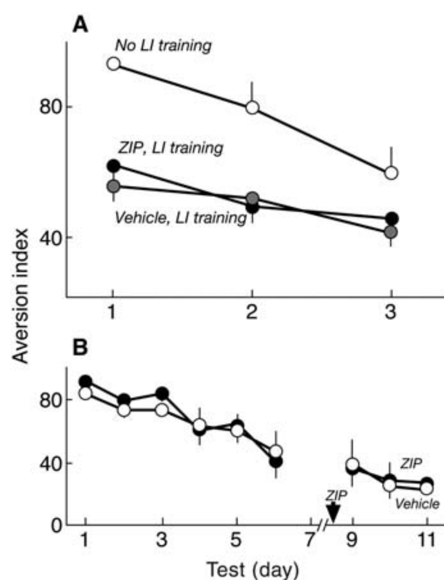

Fig. 2. (**A**) In LI, familiarity with the tastant attenuates the potency of that tastant to serve as CS in subsequent CTA training (compare vehicle, LI training to no LI training). Microinfusion of ZIP into the IC after the exposure to the tastant in the LI protocol (ZIP, LI training) has no effect on familiarity. (**B**) Neophobia declines over repeated nonreinforced exposures to the tastant (days 1 to 6). Application of ZIP into the IC has no effect on familiarity in this protocol either.

To examine the possibility that the inhibitor blocks memory performance only transiently, we continued testing ZIP-treated rats over time, to unveil spontaneous recovery, and in addition, after about 2 weeks, reapplied the unconditioned stimulus (UCS) to elicit potential reinstatement. None of these manipulations yielded evidence for recovered memory in the ZIP group (Fig. 1C). Repeated-measures ANOVA on days 4 and 12 (the no-test interval, in which spontaneous recovery might occur) shows significant group effect, $F(1,12) = 5.79$, $P < 0.05$, and a trend toward group × test interaction, $F(1,12) = 4.29$, $P = 0.06$. Post hoc comparisons reveal nonsignificant difference between groups on test day 4 ($P = 0.28$), but significant difference on day 12 ($P < 0.01$). All in all this indicates spontaneous recovery in the control but not in the ZIP group. The lack of expected extinction in the control between days 12 and 14 (paired t test, $P = 0.84$) suggests a reinstatement effect. No evidence for an effect from UCS reapplication was observed in the ZIP group.

Can ZIP disrupt more than one association at a time? Rats were trained on CTA to saccharin (CS1), and 2 days later to glycine (CS2). These tastants are perceived differently (*12*). One week later, ZIP was microinfused into the IC, and a day later, a test schedule was initiated in which the rats ($n = 8$) were tested on CS1 and CS2, consecutively, 1 day apart over 6 days. Both CS1-UCS and CS2-UCS associations were disrupted: AI on the first test for CS1 association was 94.0 ± 3.16 in the control ($n = 7$), 70.3 ± 7.09 in the ZIP group [$F(1,13) = 8.41$, $P < 0.05$]. AI on the first test for CS2 association was 97.6 ± 0.97 in the control, 78.9 ± 5.8 in the ZIP group [$F(1,13) = 8.61$, $P < 0.05$]. No significant difference was detected among groups in extinction rate, indicating lack of recovery from the ZIP effect on repetitive testing [repeated-measures ANOVA, group × test interactions, $F(2,26) < 1$, not significant].

We tested the effect of the PKMζ inhibitor on the ability to encode, as opposed to retain, CTA memory in the IC. First, we microinfused ZIP into the IC 2 hours before exposure to a glycine CS in CTA training and tested 3 days later. We found no effect of ZIP on acquisition of CTA [ZIP group, 85.4 ± 5.0, $n = 8$; vehicle, 87.4 ± 5.9, $n = 7$; one-way ANOVA, $F(1,13) < 1$, $P = 0.81$]. Similar results were obtained using saccharin as the CS [ZIP group, 79.6 ± 3.9, $n = 6$; vehicle, 80.0 ± 3.8, $n = 10$; one-way ANOVA, $F(1,14) < 1$, $P = 0.95$]. Second, rats that were trained on CTA to saccharin and then treated with ZIP, were subjected a week later to a new CTA training to glycine. There was no difference between the ZIP and the control rats in their ability to reacquire CTA [ZIP group 93.2 ± 2.3, $n = 9$; vehicle 95.0 ± 2.9, $n = 5$, tested 3 days after retraining; one-way ANOVA, $F(1,12) < 1$, $P = 0.62$].

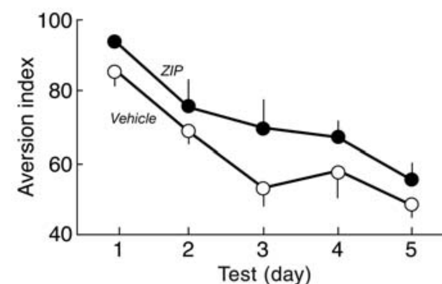

Fig. 3. PKMζ inhibitor in the hippocampus does not impair CTA memory. ZIP was microinfused bilaterally into the hippocampus 3 days after CTA training, and memory was tested starting a day later. These data also demonstrate that the effect of ZIP on CTA memory in the IC is region-specific.

The IC subserves detection and consolidation of taste familiarity (*13–17*). We used two paradigms to determine whether the PKMζ inhibitor disrupts taste familiarity once formed. The first is latent inhibition (LI) (*17*). Preexposure to the CS in a LI protocol attenuates later CTA training to the same CS; hence, CTA performance serves as a familiarity detector (*17*). Introduction of ZIP into the IC after the preexposure to the taste in the LI protocol had no effect on LI [(Fig. 2A); one-way ANOVA for the first test, $F(2,37) = 7.77$, $P < 0.005$]. Post hoc comparisons showed no significant difference between the ZIP-LI and the vehicle-LI groups; however, both groups were significantly different from the no-LI group ($P < 0.01$). Repeated-measures ANOVA showed significant group effect, $F(2,37) = 6.83$, $P < 0.005$, and significant test effect, $F(2,74) = 15.94$, $P < 0.001$. Again, post hoc comparisons showed no significant difference between the two LI groups, and each of these groups was significantly different from the no-LI group ($P < 0.01$).

We also examined attenuation of neophobia (*18*). Here, rats are presented with an unfamiliar tastant that invokes fear-of-the-new and then repeatedly presented with the same tastant. Over time, the neophobia decreases, serving as a measure of familiarity. PKMζ inhibition had no effect in this paradigm [(Fig. 2B), repeated-measures ANOVA, significant attenuation of neophobia in the repeating tests, $F(8,112) = 21.01$, $P < 0.001$]; however, no significant difference was seen between the groups, $F(1,14) < 1$, $P = 0.85$].

PKMζ has been previously shown to maintain LTP and spatial memory in the hippocampus (*1*). The role of the hippocampus in CTA is still unsettled (*19*). Hippocampal lesions do not impair CTA and were even reported to enhance it (*20*). Microinfusion of ZIP into the dorsal hippocampus 3 days after CTA did not impair CTA memory when tested a day after ZIP administration (Fig. 3). If at all, there was a trend toward memory enhancement [repeated-measures ANOVA, $F(1,16) = 2.98$, $P = 0.1$]. Besides demonstrating that PKMζ in the hippocampus is not essential for long-term CTA memory, these data also indicate that the effect of ZIP on memory in the IC is region-specific.

The effect of the PKMζ inhibitor on long-term CTA memory in IC is consistent with

reports that the IC is critical for consolidation, storage, and extinction of CTA (*7*, *13*, *21*, *22*). Although the map of CS-UCS association sites in CTA encoding is still incomplete and probably includes subcortical structures (*23*), once the association is formed, the IC is likely to store elements of the associative hedonic or incentive value of the CS (*24*). In contrast, whereas the IC is documented to detect taste novelty that facilitates encoding of CTA (*4*, *7*, *13*, *14*, *21*), the present data are in line with the possibility that taste familiarity per se is not stored in the IC.

So far, we have found no evidence that the effect of ZIP on associative taste memory in cortex is reversible; hence, we heuristically propose that the PKMζ inhibitor might practically erase some long-term memory associations. We are aware of the difficulties in concluding that a memory trace is erased from the lack of ability to detect a change in performance that is attributed to that trace. This inherent difficulty haunts the long-standing debate on whether amnesia is a storage or a retrieval deficit, yet does not preclude the assumption that amnesia is a storage deficit (*8*, *9*, *25*).

Recent data on reactivation-dependent vulnerability of memory to amnesic agents (*8*, *9*) reemphasize the frailty of the engram—an attribute long recognized by cognitive psychologists (*26*), but somehow mostly ignored till recently by neuroscientists. Our data reinforce the notion that memory traces are prone to swift interferences long after their encoding. In contrast to these earlier studies, however, no reactivation is needed to render the trace susceptible to ZIP. The possibility that the trace reactivates implicitly is low given that classical amnesic agents, e.g., macromolecular synthesis inhibitors, have no effect on the long-term CTA trace that has not been reactivated (*7*, *13*).

The possibility cannot yet be excluded that vulnerability of memory to PKMζ inhibition in cortex might wane. If so, then the temporal window of "cellular consolidation," i.e., the stabilization process that is postulated to occur in synapses and cell bodies after memory encoding (*8*), lingers far longer than originally thought. This conclusion is even more striking given that elemental CTA seems hippocampus-independent, excluding a "systems consolidation" process in which the hippocampal trace invades neocortex over days to weeks (*8*). An alternative possibility is that PKMζ permanently maintains long-term memory and, thus, is a target for amnesic agents as long as the memory persists. In this case, defining consolidation on the basis of vulnerability to amnesic agents (*8*) requires reconsideration.

How does PKMζ inhibition disrupt memory in neocortex? If work on LTP in the hippocampus is a guide, the effect of PKMζ might be on the microstructure of preexisting synapses, resulting in a doubling of the number of functional postsynaptic AMPA-type glutamate receptors (*27*). Our results indicate, however, that these changes, even weeks after learning, are not indelible modifications of synaptic structure, but remain dependent on ongoing enzymatic activity and, thus, are capable of rapid and dynamic alterations by experimental manipulation or, perhaps, in the course of incorporation of new experience into associative knowledge schemas in cortex (*28*). The idea that persistent enzymatic activity keeps memory going has been raised on the basis of theoretical considerations (*29*–*31*). The finding that this takes place, via PKMζ, not only in LTP and hippocampus (*1*, *6*, *27*), but also in long-term memory in neocortex, has, in addition to theoretical implications, potential clinical significance, e.g., in the field of cognitive enhancement.

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Detection of Near-Atmospheric Concentrations of CO_2 by an Olfactory Subsystem in the Mouse

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Carbon dioxide (CO_2) is an important environmental cue for many organisms but is odorless to humans. It remains unclear whether the mammalian olfactory system can detect CO_2 at concentrations around the average atmospheric level (0.038%). We demonstrated the expression of carbonic anhydrase type II (CAII), an enzyme that catabolizes CO_2, in a subset of mouse olfactory neurons that express guanylyl cyclase D (GC-D$^+$ neurons) and project axons to necklace glomeruli in the olfactory bulb. Exposure to CO_2 activated these GC-D$^+$ neurons, and exposure of a mouse to CO_2 activated bulbar neurons associated with necklace glomeruli. Behavioral tests revealed CO_2 detection thresholds of ~0.066%, and this sensitive CO_2 detection required CAII activity. We conclude that mice detect CO_2 at near-atmospheric concentrations through the olfactory subsystem of GC-D$^+$ neurons.

CO_2 is an olfactory stimulus for many invertebrates (*1*, *2*). CO_2 levels fluctuate locally with biological activities, such as animal respiration, plant photosynthesis, and the decomposition of organic matter. CO_2 signals regulate many insect innate behaviors, such as seeking food and hosts, avoiding stressful environments, and ovipositioning (*3*–*6*). CO_2 has no discernable odor to humans, but at high concentrations (>30%), it produces a pungent trigeminal sensation in the nasopharynx (*7*). Carbonic anhydrase (CA), an enzyme that is implicated in CO_2 sensing by peripheral systems such as carotid chemoreceptors (*2*, *8*), is expressed in a subset of olfactory sensory neurons (OSNs) in several vertebrate species (*8*, *9*). Studies indicate that rats can detect CO_2 at levels above 0.5% (*10*, *11*). It remains unknown whether mammals can detect