

# Pharmacologically Regulated Induction of Silent Mutations (PRISM): Combined Pharmacological and Genetic Approaches for Learning and Memory

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Mouse transgenic and knock-out approaches have made fundamental contributions to our understanding of the molecular and cellular bases of learning and memory. These approaches have successfully identified a large number of molecules with either a central or modulatory role in learning and memory. However, there are limitations associated with first-generation mutant mice, which include, for example, the lack of temporal control over the mutation. Recent technical developments have started to address some of these shortcomings. Here, the authors review a newly developed inducible approach that takes advantage of synergistic interactions between subthreshold genetic and pharmacological manipulations. This approach is easily set up and can be used to study the functional interactions between molecules in signaling pathways. *NEUROSCIENTIST* 9(2):104–109, 2003. DOI: 10.1177/1073858403252225

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## Molecules and Plasticity

The introduction of gene targeting approaches in mice has revolutionized the study of many biological systems, including learning and memory. In the summer of 1992, the first “learning and memory” knock-out papers were published (Silva and others 1992a, 1992b). Ten years later, published papers describing mutant mice with learning and memory phenotypes number in the thousands. These studies have played a central role in the identification of signaling molecules involved in synaptic and behavioral plasticity: At the present time, nearly 100 different molecules—playing either essential or at least modulatory roles—have been identified (or confirmed) using genetic approaches (Sanes and Lichtman 1999). In some cases, the molecules were known (e.g., the n-methyl-d-aspartate [NMDA] receptor), having previously been identified using traditional pharmacological approaches. However, in many other cases, genetic techniques uncovered novel molecular components of memory (e.g., by targeting molecules that are inaccessible to traditional pharmacological tools, such as neurofibromin and PSD-95).

These studies have not only been instrumental in generating a list of molecules involved in synaptic and behavioral plasticity, but they have also furthered the

understanding of how molecular and cellular mechanisms modulate learning and memory. However, the usefulness of this first generation of mutants (where the targeted gene is knocked out in all tissues and throughout development) has been limited for a number of reasons, two of which we shall focus on in this review. First, the absence of temporal control over the mutation means that it is difficult to determine in which precise memory process (or phase) a given molecule participates. Second, using the first generation of knock-out (KO) mice, it is not possible to probe the functional relevance of interactions between two molecules. For example, although we know a considerable amount about the role of NMDA receptors and calcium/calmodulin kinase II (CaMKII) in plasticity and learning, the importance of NMDA receptor-dependent CaMKII activation during learning is less well understood.

## Temporal Control

The fact that in traditional knock-outs the targeted gene is deleted throughout the life of the animals poses several problems for studies of learning and memory. Besides developmental concerns, with traditional knock-out strategies, it is impossible to target specific temporally distinct processes in a complex sequence of biological events. Memory, for example, involves multiple phases regulated by distinct gene products (Dudai 2002). With global knock-outs, it is difficult to determine whether a gene is required for a specific memory phase. To address this problem, a second-generation of mice has been developed with the aim to gain temporal control over

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molecular manipulations. So far, three different types of genetic systems have been used to temporally restrict gene function in studies of learning and memory.

The first system used a tetracycline-controlled transactivator (tTA) to regulate the expression of a constitutively active form of CaMKII ( $\alpha$ CaMKIIT286D). With the tTA system, a tetracycline analogue (doxycycline) represses the expression of a transgene (e.g., the  $\alpha$ CaMKIIT286D transgene) under the regulation of the tet operon promoter. With this system, it was possible to repress the  $\alpha$ CaMKIIT286D transgene during development and simply lift the repression at appropriate experimental times. These studies confirmed a role for CaMKII in plasticity and learning and demonstrated that even after training, expression of activated CaMKII disrupts memory (Mayford and others 1996).

The second approach to temporally restrict gene expression uses a modified tetracycline-inducible system (Mansuy and others 1998). In this system, the tTA has been mutated so that instead of repressing transcription, the mutant transactivator activates it. With this reverse transactivator (rtTA), doxycycline induces the transgene (e.g., calcineurin), and therefore there is no need to keep the animals on doxycycline for extended periods of time, which is problematic because this drug may itself have effects on behavior.

A third temporally controlled transgenic system used in the study of memory involves a mutant ligand-binding domain (LBD) of the estrogen receptor. To better define when and where CREB is required for memory in mice, this system was used to regulate the function of a transgenic CREB repressor (Kida and others 2002). The CREB repressor was fused to the LBD. This mutant LBD binds tamoxifen instead of estrogen, a critical property that prevents circulating estrogen from confounding the experiments with this inducible system (Logie and Stewart 1995). The utility of this approach is restricted because only proteins that can be regulated in fusions with LBD may be used (Feil and others 1996). In contrast, the tTA system can be potentially used to regulate the expression of any gene. However, this fusion protein is fully inducible in less than 6 h, and this induction is fully reversible in less than 24 h. By comparison, the tTA system requires days or weeks for full induction and repression (Feil and others 1996).

The genetic complexity of these approaches has limited their applicability, however. Although tet-transgenic techniques have been available for nearly 8 years, there have been relatively few learning and memory papers published using this technique. Tet systems involve multiple transgenic lines, and each of these needs to be analyzed and characterized independently. Neurophysiological and behavioral experiments with these inducible mice are difficult because of the large number of experimental groups required to control for the effects of each of the transgenes used and because of the cost and difficulty of generating the mice required for these studies. Therefore, it would be desirable to develop a tool with similar or better temporal resolution than the current

genetic systems but that is more affordable and more widely accessible.

## Molecular Interactions during Learning

A second limitation of traditional knock-out approaches, including those described above, is that they have focused on the function of single molecules. However, molecular function is meaningful only in the context of the pathways in which they participate. Nonlinear interactions between mutations have been used to delineate functional pathways in fields as diverse as development, immunology, and cell cycle. Unfortunately, it would be prohibitively expensive and difficult to explore the function of molecular interactions in pathways with inducible genetic approaches because of the complexity of the genetic manipulations required.

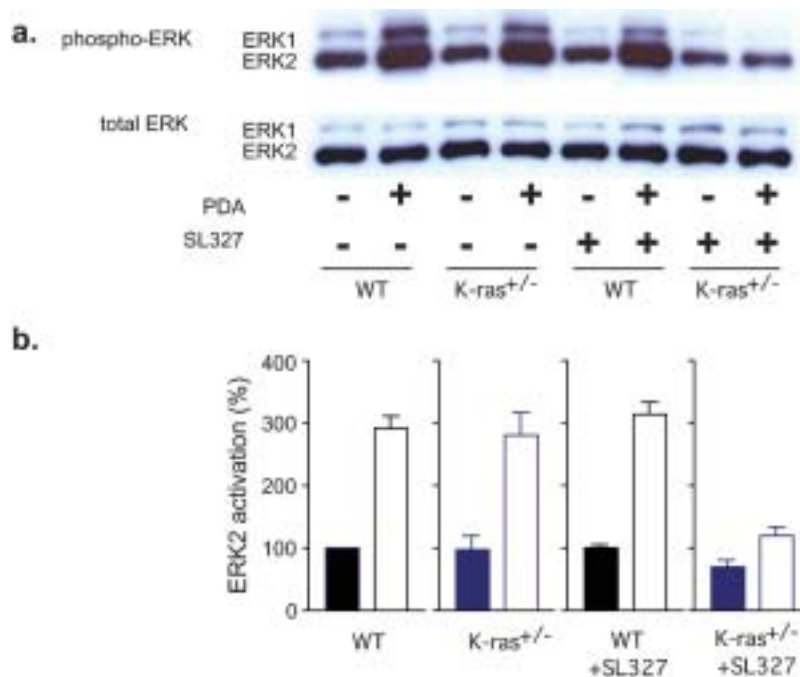
## PRISM: A New Inducible Approach to Learning and Memory

Here we review a novel inducible approach that examines synergistic interactions between pharmacological and genetic manipulations. Using this approach, it is possible to study the function of molecular interactions in a temporally restricted manner (Ohno and others 2001, 2002). We have termed this approach *PRISM*: the pharmacologically regulated induction of silent mutations. Previously, we have used the term *pharmacogenetics* to describe this approach (Ohno and others 2001, 2002). However, we believe this term is somewhat misleading because it strictly refers to the study of the genetic basis for variability in response to drug treatments across individuals (Roses 2000).

PRISM-based approaches combine the molecular specificity of genetics with the temporal control offered by pharmacological manipulations. The key concept is that neither manipulation on its own is sufficient to disrupt learning. However, if these two subthreshold manipulations target molecules within the same signaling cascade, and that cascade is important for learning, then the combination of these two treatments will lead to a disruption of learning. This way, the effects of mutations may be induced or revealed in a temporally controlled manner. Furthermore, by using this method to study the interactions between different molecular manipulations, it is possible to start to define how various signaling molecules interact during learning.

## Ras Signaling and Learning-Related Plasticity

To illustrate the general strategy, we can consider the role of Ras signaling in plasticity and learning. Ras is a small GTP-binding protein that has a well-established role in cell proliferation and differentiation. The activity of Ras is regulated by both receptor tyrosine kinases and intracellular calcium levels (Cullen and Lockyer 2002). In its active state, where Ras is bound to GTP, it activates a number of signaling cascades, including the MAPK ERK, PI3 kinase, and Ral pathways (Cullen and Lockyer



**Fig. 1.** A subthreshold dose of the MEK inhibitor SL327 disrupts Ras-MEK-ERK signaling in K-ras<sup>+/-</sup> but not wild-type (WT) mutants (from Ohno and others 2001). (a) Representative Western blots indicating protein bands visualized with antibodies to dually phosphorylated ERK1/2 and total ERK1/2. To activate Ras-MEK-ERK signaling, hippocampal slices from WT and in K-ras<sup>+/-</sup> mutants were exposed to 10 mM phorbol diacetate (PDA), a PKC agonist, in the presence or absence of 1 mM SL327, the MEK antagonist. The total ERK1/2 levels indicate equal protein loading. (b) Averaged levels of phosho-ERK2 (normalized with respect to the WT control group). Slices were treated with either normal ACSF (control; closed bars) or PDA (open bars). SL327 significantly reduced phosho-ERK2 levels in the K-ras<sup>+/-</sup> group and not in the WT group.

2002; Lowes and others 2002). Because previous studies indicated a role for ERK in learning and memory (Adams and Sweatt 2002), we focused our studies on the role of Ras-ERK signaling in hippocampal plasticity. Ras activates Raf kinase, which phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. The activation and subsequent translocation of ERK to the nucleus leads to the activation of a number of different transcription factors including CREB and Elk-1.

We found that a heterozygous mutation of one of the three isoforms of Ras, K-ras, does not disrupt Ras-MEK-ERK signaling in the hippocampus. Similarly, pharmacological agents targeting the downstream molecule MEK do not disrupt Ras-MEK-ERK signaling at low concentrations in wild-type (WT) tissue. However, the combination of these two treatments does lead to a disruption of Ras-ERK signaling (Ohno and others 2001) (Fig. 1). That is, only the combination of the two subthreshold treatments (K-ras<sup>+/-</sup> mutation plus MEK inhibitor) leads to a disruption of Ras-MEK-ERK signaling; neither treatment alone was sufficient to cause a disruption.

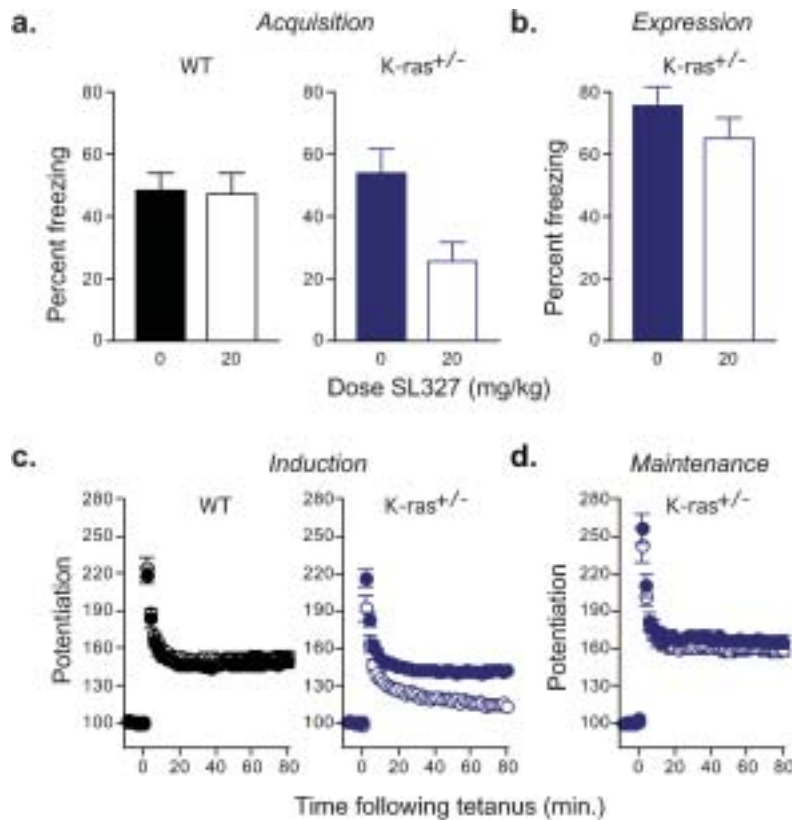
We next wanted to ask whether we could apply this approach to study the role of Ras-ERK signaling in learning and memory in behaving animals. To do this, we examined acquisition of contextual fear conditioning in K-ras<sup>+/-</sup> mice and their WT littermate controls. K-ras<sup>+/-</sup> mutants learn contextual fear conditioning normally. However, pretreatment of these mice with an MEK inhibitor, at a dose that is ineffective in WT mice, produces a learning deficit in K-ras<sup>+/-</sup> mutants. Similar treatment given to K-ras<sup>+/-</sup> mutants 2 h posttraining, rather than 30 min before training, is ineffective (Ohno and others 2001) (Fig. 2). This finding suggests that Ras-ERK signaling at around the time of training (and not

thereafter) is important for the formation of contextual fear memories.

Because the formation of contextual memories depends on the hippocampus (Fanselow 2000), we conducted a series of parallel experiments to examine whether plasticity in the CA1 region of the hippocampus was similarly affected. Long-term potentiation (LTP), induced by 100 Hz stimulation, is unaffected in slices from K-ras<sup>+/-</sup> mutants. However, pretreatment of slices with a dose of the MEK inhibitor that is ineffective in WT slices produced a deficit in slices from K-ras<sup>+/-</sup> mutants. Largely consistent with the behavioral findings, similar treatment given posttetanically does not disrupt already established LTP, suggesting that Ras-ERK signaling is important for the induction rather than maintenance of LTP (Ohno and others 2001) (Fig. 2). Although a direct link between LTP and learning is difficult to establish, the fact that similar manipulations have a qualitatively similar impact on LTP and contextual learning suggests that these two processes (related or otherwise) are mediated by similar mechanisms.

### NMDAR/CaMKII Interactions and Learning-Related Plasticity

In principle, it should be possible to use this approach to study the participation of a wide variety of different signaling mechanisms in synaptic and behavioral plasticity. For example, it is well known that CaMKII plays a critical role in synaptic and behavioral plasticity (Elgersma and Silva 1999). Furthermore, structural and biochemical studies indicate that the activity of NMDA receptors and CaMKII are tightly coupled (Fink and Meyer 2002; Lisman and others 2002): CaMKII is activated following Ca<sup>2+</sup> influx via NMDA receptors. When Ca<sup>2+</sup> enters the



**Fig. 2.** Subthreshold levels of the MEK inhibitor SL327 selectively block the acquisition of contextual fear conditioning and the induction of 100 Hz long-term potentiation (LTP) in K-ras<sup>+/-</sup> mutants. (a) Contextual fear in wild-type (WT) and K-ras<sup>+/-</sup> mice trained with a single foot-shock and tested 24 h later. Prior to training, WT and K-ras<sup>+/-</sup> mice received 0 or 20 mg/kg SL327. Although this dose was ineffective in WT mice, it disrupted acquisition of contextual fear in K-ras<sup>+/-</sup> mutants. (b) A similar treatment given 2 h posttraining was ineffective, indicating that Ras-MEK-ERK signaling at around the time of training (and not thereafter) is important for the acquisition of contextual fear conditioning. (c) A subthreshold dose of SL327 (1 mM) disrupts LTP (induced by 100 Hz stimulation) in K-ras<sup>+/-</sup>, but not WT, mice. (d) Similar treatment given following tetanization was ineffective, suggesting that Ras-MEK-ERK signaling is important for the induction, rather than maintenance, of LTP.

cell, it forms a complex with calmodulin (CaM). This Ca-CaM complex binds and activates CaMKII, allowing the kinase to become autophosphorylated at a Thr286 residue. This phosphorylation event is critical because it allows the kinase to remain active, even in the absence of Ca<sup>2+</sup> influx. Once activated, CaMKII can bind NR2B subunits of the NMDA receptor (Lisman and others 2002).

Either genetic disruption of CaMKII function or pharmacological blockade of NMDA receptors impairs contextual fear conditioning and the induction of hippocampal LTP in a dose-dependent manner. For example, it has been shown that the introduction of a point mutation at the Thr286 site in  $\alpha$ CaMKII blocks the Ca<sup>2+</sup>-independent activity of the kinase and disrupts synaptic plasticity, place cell stability, and spatial learning (Cho and others 1998; Giese and others 1998). Whereas mice homozygous for this point mutation ( $\alpha$ CaMKII<sup>T286/-</sup>) exhibit severe impairments in contextual fear conditioning and hippocampal LTP, mice heterozygous for this mutation ( $\alpha$ CaMKII<sup>T286+/-</sup>) show normal contextual fear conditioning and only mild deficits in hippocampal LTP (Ohno and others 2002). Similarly, CPP (an NMDA antagonist) blocks contextual fear conditioning and LTP induction in WT mice in a dose-dependent manner (Ohno and others 2001, 2002).

Therefore, by combining subthreshold genetic and pharmacological manipulations of CaMKII and NMDA function, respectively, it should be possible to test whether NMDA-CaMKII interactions are important for

plasticity and learning. We found that pretreatment of  $\alpha$ CaMKII<sup>T286+/-</sup> mice with CPP, at a dose that is ineffective in WT mice, disrupts contextual fear conditioning (Ohno and others 2001). Similarly, low concentrations of CPP further exacerbate the mild LTP deficits in slices from  $\alpha$ CaMKII<sup>T286+/-</sup> mice (Ohno and others 2002). Similar treatments posttraining, or posttetanically, are ineffective. Therefore, these results provide strong evidence for NMDA receptor-dependent activation of CaMKII signaling in the initial encoding (but not subsequent consolidation) of hippocampal memories and in the induction (but not maintenance) of LTP (Otmakhov and others 1997).

## Conclusions and New Directions

With the first generation of genetically modified mice—where the gene is deleted from conception—it has proven difficult to probe molecular mechanisms other than those underlying the initial encoding of memories. Using combined pharmacological and genetic approaches, it will be possible to dissect the roles of various signaling mechanisms in different memory phases (e.g., short- vs. long-term memory) as well as those related to memory usage: For example, it will be possible to study the molecular mechanisms underlying such phenomena as reconsolidation, extinction, and forgetting (Nader and others 2000; Berman and Dudai 2001; Villarreal and others 2002). The temporal resolution of the approach is defined in terms of the pharmacokinetics of the drug

treatment. This gives a temporal resolution at least an order of magnitude greater than that achieved in currently available genetic systems. Spatial resolution may be achieved with targeted infusions of drug into relevant brain structures.

The finding that Ras, ERK, NMDA receptors, and CaMKII play central roles in the encoding of memories is not especially new. Many other groups have come to more or less similar conclusions using a wide range of different approaches and techniques (Mayford and Kandel 1999; Tsien 2000; Sweatt 2001; Fink and Meyer 2002; Lisman and others 2002). However, PRISM-based approaches offer an important additional advantage. That is, the synergistic interaction between two treatments, genetic and pharmacological, suggest a direct functional interaction between the two target molecules. It is important to recognize that this is not the only possibility: It is equally plausible that the functional interaction between these two treatments occurs at the circuit, systems, or even behavioral output levels. However, we were able to narrow down these possibilities by combining the behavioral and electrophysiological studies with biochemical studies showing that Ras-ERK signaling was compromised in K-ras<sup>+/-</sup> (but not WT) tissue following MEK inhibition.

Therefore, these combined biochemical, behavioral, and electrophysiological approaches allow us to start to identify subsets of signaling pathways within complex signaling networks that are important for synaptic and behavioral plasticity. For example, the activity of Ras is regulated both by increases in intracellular Ca<sup>2+</sup> and by growth factor stimulation (Lowes and others 2002). Therefore, one possibility is that the regulation of Ras activity by Ca<sup>2+</sup> influx via NMDA receptors is important for hippocampal learning and LTP. We tested this idea by examining whether CPP treatment preferentially disrupts contextual fear conditioning in K-ras<sup>+/-</sup> mice (Ohno and others 2001). However, the same dose of CPP that revealed the contextual deficit in the  $\alpha$ CaMKII<sup>T286+/-</sup> mice was ineffective in the K-ras<sup>+/-</sup> mice. The failure to detect a synergistic interaction between the K-ras<sup>+/-</sup> mutation and CPP treatment suggests that the activation of Ras via activated NMDA receptors is less important for contextual learning. It is possible that Ca<sup>2+</sup> released from other sources (e.g., intracellular stores, voltage-sensitive Ca<sup>2+</sup> channels) may play a more central role in the regulation of Ras signaling for synaptic and behavioral plasticity. Alternatively, the regulation of Ras signaling by growth factors (e.g., BDNF, NGF) may be more important for learning and memory.

PRISM-based approaches, therefore, allow us to start to define how single molecules behave within the context of a wider signaling network. Previous studies have shown that genetic lesions of molecules that directly regulate the activity of Ras (such as NF1, Ras-GRF) disrupt synaptic and behavioral plasticity (Brambilla and others 1997; Silva and others 1997; Costa and others 2001; Giese and others 2001). However, from these studies, it is not possible to determine which downstream effector pathways (e.g., ERK, PI3 kinase, Ral) mediate these

effects. Our data show that at least one of these pathways—Ras-ERK—plays a central role in learning and memory. Therefore, this approach allows us to move beyond a list of signaling molecules, to identifying which subset of signaling pathways contribute to learning and LTP.

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