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Inducible molecular switches for the study of long-term potentiation

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This article reviews technical and conceptual advances in unravelling the molecular bases of long-term potentiation (LTP), learning and memory using genetic approaches. We focus on studies aimed at testing a model suggesting that protein kinases and protein phosphatases balance each other to control synaptic strength and plasticity. We describe how gene 'knock-out' technology was initially exploited to disrupt the Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα) gene and how refined knock-in techniques later allowed an analysis of the role of distinct phosphorylation sites in CaMKII. Further to gene recombination, regulated gene expression using the tetracycline-controlled transactivator and reverse tetracycline-controlled transactivator systems, a powerful new means for modulating the activity of specific molecules, has been applied to CaMKIIα and the opposing protein phosphatase calcineurin. Together with electrophysiological and behavioural evaluation of the engineered mutant animals, these genetic methodologies have helped gain insight into the molecular mechanisms of plasticity and memory. Further technical developments are, however, awaited for an even higher level of finesse.

Keywords: gene targeting; inducible gene expression; protein kinase; protein phosphatases; long-term potentiation; learning and memory

1. INTRODUCTION

Learning about learning is a great challenge for neuroscientists because it deals with one of our most essential and intimate skills. Not only the pursuit of knowledge *per se*, but also its potential application to the therapeutic benefit of memory dysfunctions after stroke or post-traumatic disorder, and the improvement of skills in children with learning disabilities and of ageing-related memory decline, justify the urge to elucidate the fundamental bases of learning and memory. The phenomenon of LTP (Bliss & Collingridge 1993) and, by extension, of other kinds of plasticity including LTD (Lynch *et al.* 1977) and depotentiation (Staubli & Lynch 1990) are experimental models of choice to study these processes. Although a direct parallel between plasticity and memory formation has not been firmly established, multiple evidence suggests that they do share common features (Martin *et al.* 2000).

The groundbreaking character of the discovery of LTP in the early 1970s (Bliss & Gardner-Medwin 1973; Bliss & Lømo 1973) and the recognition that the discovery has brought about a new dimension to memory research are now important milestones of neuroscience history. The celebration of the 30th birthday of LTP acknowledges the extent to which it has yielded a mass of new, sometimes unexpected, knowledge about the basic physiological rules of brain plasticity and the fundamental functioning of neurons (Malenka & Nicoll 1999). It also stresses, to a large extent, the intellectual stimulation it prompted and the resulting new concepts about learning and memory.

2. A CALCIUM–CALMODULIN SWITCH

One of the concepts that addressed the potential molecular mechanisms of LTP was first formulated by John Lisman in the 1980s, and has largely been adjusted and perfected since then (see Lisman 2003). This model suggests that synaptic weight is bi-directionally controlled by protein kinases and protein phosphatases in a Ca^{2+}-dependent fashion (Lisman 1985, 1989) and that the balance between protein phosphorylation and dephosphorylation dynamically sets physiological synaptic strength (Wang & Kelly 1996). CaMKII is believed to play a central role in this model and has a number of properties that make it a strong candidate for being a memory molecule (Lisman *et al.* 2002). CaMKII is extremely abundant in the brain and is particularly enriched in postsynaptic terminals of neurons in hippocampus, neocortex, amygdala and basal ganglia. These brain structures are known to experience plastic changes upon stimulation (LTP, LTD) and to support some aspects of learning and memory. Further, after initial stimulation by calcium, CaMKII has the ability to maintain itself in an active state for long periods of time by autophosphorylation on threonine 286 (Hanson & Schulman 1992; Ouyang *et al.* 1997), a process thought to leave a molecular trace of previous Ca^{2+}-induced activity. A counterpart to CaMKII is the protein phosphatase CN, the only known Ca^{2+}/calmodulin-dependent protein phosphatase in the brain. CN would oppose CaMKII by activating PP1, a downstream phosphatase belonging to the same Ser/Thr protein phosphatase family. PP1 is able to dephosphorylate CaMKII

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One contribution of 30 to a Theme Issue 'Long-term potentiation: enhancing neuroscience for 30 years'.

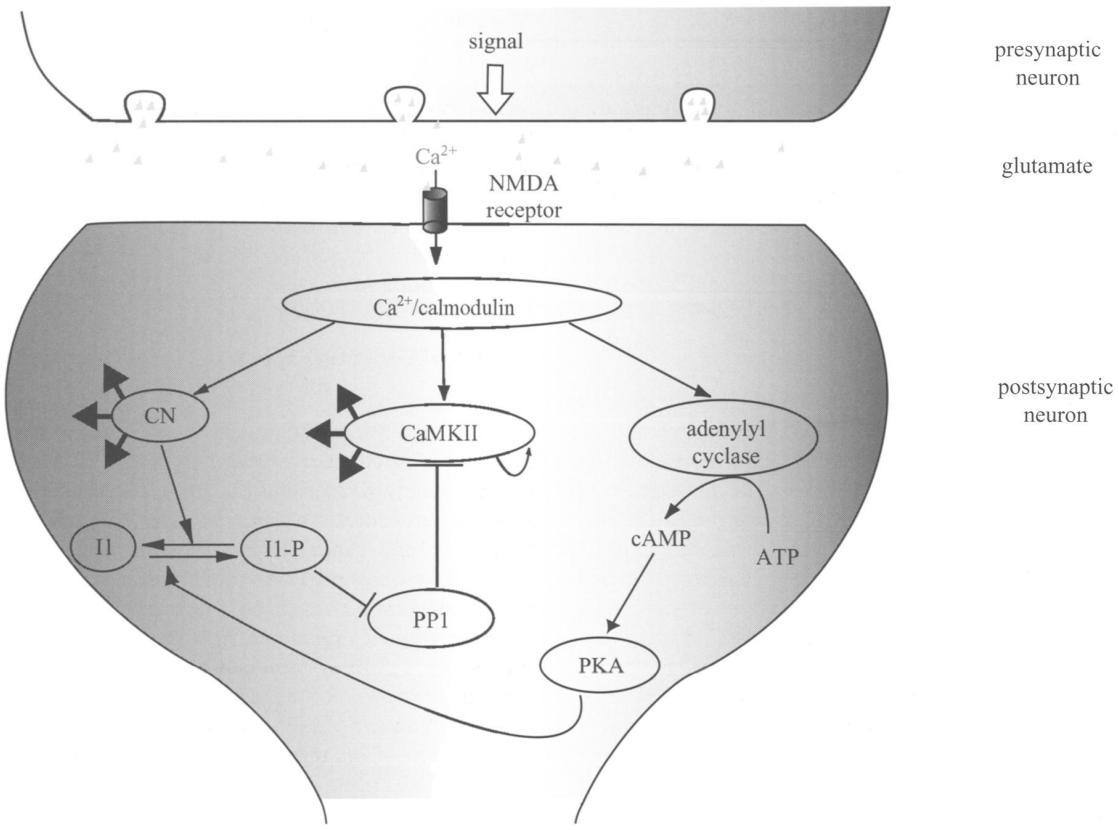

Figure 1. Scheme of the balance between protein kinases/phosphatases. CaMKII and CN are activated by Ca^{2+} and act on multiple targets (represented by small arrows) including CaMKII itself. CN dephosphorylates inhibitor-1 (I1), an inhibitor of PP1 that is phosphorylated by PKA, a cAMP-dependent protein kinase. When relieved from inhibition, PP1 dephosphorylates CaMKII. Although not represented, a similar balance may also be operating presynaptically.

presumably through CN-dependent dephosphorylation of inhibitor-1, an inhibitor of PP1 that is activated by phosphorylation by the cAMP-dependent PKA (figure 1).

Numerous pharmacological, electrophysiological, biochemical and genetic studies have been carried out to challenge this model and determine the functions of CaMKII and CN in plasticity, learning and memory. We discuss studies that have exploited gene targeting and transgenic technologies, and we try to describe how the use of inducible approaches or systems have helped gain insight into the role of these molecules in LTP.

3. INITIAL STRATEGY AND RECENT ELABORATIONS ON THE GENE TARGETING APPROACH TO STUDY THE ROLE OF CAMKII IN LTP

The development of refined genetic techniques such as knockout and transgenesis significantly advanced the understanding of the mechanisms of plasticity, learning and memory. Analyses of *in vitro* LTP and cross-comparison with behaviour in genetically modified animals has been a useful means to investigate the molecular pathways underlying these processes and their potential commonalities.

The first important experiments in the field were carried out by the laboratory of Susuma Tonegawa who developed a line of mutant mice in which gene coding for the alpha subunit of CaMKII (CaMKIIα), a predominant CaMKII isoform in the brain, was inactivated by homologous recombination. Disruption of the CaMKIIα gene resulted in a defect in the induction of LTP in area CA1 of the hippocampus (Silva *et al.* 1992*a*; Hinds *et al.* 1998) and in impaired experience-dependent plasticity in sensory cortex *in vivo* (Glazewski *et al.* 1996; Gordon *et al.* 1996). These deficits in plasticity were accompanied by an impairment of spatial and associative learning and memory (Silva *et al.* 1992*b*). Interestingly, later on, the extent of these deficits was found to depend on the degree of elimination of CaMKIIα expression. When only one of the mutated alleles was introduced in heterozygous mice, sufficient CaMKIIα was still present in hippocampal neurons to sustain the induction of LTP (although LTP magnitude was attenuated). By contrast, in the neocortex, this partial reduction in CaMKIIα was substantial enough to prevent the induction of LTP. This selective defect in neocortical plasticity was associated with impaired long-term memory but short-term hippocampal-dependent memory was intact in heterozygous animals (Frankland *et al.* 2001). These results highlighted the critical role of CaMKIIα in both hippocampal and neocortical LTP and corroborated the hypothesis that normal plasticity is required in the hippocampus for the initial encoding of memory while plasticity in the neocortex is needed for the establishment of permanent memory traces. These data indicated, in turn, that LTP, depending on the brain structure that sustains it, appears to accompany distinct forms and phases of cognitive functions, possibly by using

different mechanisms that share the recruitment of CaMKIIα.

The gene targeting approach as used in these studies, however, suffers from a number of pitfalls that limit the interpretation of the results. These include the lack of spatial restriction, the all-or-none nature of the mutation, and the irreversibility and the early occurrence of the genetic mutation that is associated with the likelihood that compensatory pathways are activated (Tonegawa *et al.* 1995; Gerlai 1996, 2000; Gingrich & Hen 2000). To provide spatial restriction to gene recombination, the Cre-loxP system was adapted to the brain and has allowed the disruption of genes in specific brain areas, for instance, in hippocampal sub-fields (Tsien *et al.* 1996*a*,*b*; Nakazawa *et al.* 2002; see Tonegawa *et al.* 2003). These approaches are a considerable improvement, but not without new difficulties, for example, the regional restriction may be age dependent, being present up to a certain age but not thereafter. Additional refinements were introduced by inserting point mutation(s) in the coding sequence of target genes by knock-in specifically altering the function of the encoded protein. With this technique, the role of distinct phosphorylation sites on CaMKIIα was examined by placing inhibitory or activating point mutations in selected residues. Replacement of threonine 286 with a non-phosphorylatable alanine (T286A) revealed that autophosphorylation of CaMKIIα at this site is required for hippocampal LTP and learning (Giese *et al.* 1998). In addition to Thr286, Thr305/306, sites that undergo inhibitory autophosphorylation after Thr286 is activated, were also shown to be essential for CaMKIIα function, specifically for its translocation to PSDs. Blockade of phosphorylation at Thr305/306 by valine and alanine substitutions increased the association of CaMKIIα with PSDs resulting in a reduction of the threshold for hippocampal LTP induction and a diminished flexibility of learning and memory. Conversely, simulating phosphorylation by replacing Thr305 with Asp decreased the level of CaMKIIα in terminals and impaired LTP and learning (Elgersma *et al.* 2002). Although not inducible, nor yet regionally specific, these genetic manipulations provided a high level of sophistication in the dissection of the mechanisms of CaMKIIα function and demonstrated the distinct role of independent residues in LTP and memory.

Another technical advance was recently achieved by combining this approach with pharmacology to spatially circumscribe the genetic manipulation and, additionally, confer inducibility. This method is based on the use of a drug that, when administered at a sub-threshold dose to animals or derived tissue slices carrying a recessive null mutation, leads to full or partial inactivation (depending on the dose) of a gene (Ohno *et al.* 2001). With this method, the effect of the loss of a gene product or function similar to that provoked by a homozygous mutation can be induced in heterozygous animals. Thus, in CaMKIIα T286A hippocampal slices from heterozygous mice, blockade of CaMKII activation by pretetanic application of a low dose of the NMDA-receptor antagonist (CPP) induced a deficit in LTP similar to that observed in slices from homozygous mice. The dose of CPP necessary to reveal this defect had no effect in wild-type slices. Further, in heterozygous slices, the drug did not affect LTP once induced, confirming that CaMKIIα is involved in induction rather than expression or maintenance mechanisms. Finally, because LTP was normal before CPP treatment, it can be concluded that its impairment resulted from the drug-induced blockade of CaMKII activation and not from a developmental anomaly (Ohno *et al.* 2001, 2002). This combined approach therefore constitutes a valuable and flexible tool to gain temporal and spatial control over recessive genetic mutations. Its rapidity, dose dependence and reversibility may allow analyses of distinct phases of LTP *in vitro* and *in vivo*.

4. TRANSGENESIS FROM THE TEST-TUBE TO THE BEHAVING ANIMAL

In parallel to the knockout and knock-in approach, a number of molecular switches have been developed to provide alternative strategies to modulate gene expression and activity in a spatially and temporally controlled fashion in the brain. The emergence of tissue-specific, inducible promoters for restricting genetic manipulations to specific brain structures during selected temporal windows in higher organisms have allowed these developments. Inducible systems for gene expression benefited from the expertise of the group of Hermann Bujard, who developed an inducible promoter—based on the regulatory elements of the tetracycline resistance operon of *E. coli* (Furth *et al.* 1994; Gossen *et al.* 1994, 1995). In this operon, the tetracycline-controlled repressor (*tetR*) binds to its operator to repress the expression of resistance genes conferring survival in the presence of the antibiotic. This system was made functional in eukaryotic cells by fusion to the virion protein 16 (VP16) of the herpes simplex virus. The resulting hybrid tTA after binding to the tetracycline operator (tetO), the expression of a gene fused to tetO in a tetracycline- or doxycycline (dox)-dependent manner. A rtTA factor was later engineered by chemical mutation of tTA. The rtTA factor has the exclusive property of requiring dox for binding to tetO and therefore constitutes a truly inducible system for gene expression.

The tTA system was first applied to the mouse brain by Mark Mayford to temporally restrict the expression of a Ca^{2+}-independent active mutant of CaMKIIα mutated on Asp286 (Mayford *et al.* 1996*a*). For spatial restriction, Mayford combined the tTA factor with a fragment of the CaMKIIα promoter known to be active in forebrain neurons postnatally (Mayford *et al.* 1996*b*). The increase in CaMKII activity in hippocampus provided by tTA-controlled expression of CaMKII-Asp286 was found to alter the induction of LTP in response to a 10 Hz stimulation and to impair spatial and associative memory. The power of the tTA system in this study was to demonstrate that the transgene itself mediated these defects and not a developmental anomaly resulting from CaMKII-Asp286 transgene expression early in life. Thus, by suppressing CaMKII-Asp286 transgene expression with dox, normal LTP and memory could be fully restored in adult mutant animals, confirming the specificity of the effect (Mayford *et al.* 1996*a*). Although consistent with previous findings that the constitutive expression of CaMKII-Asp286 shifts the threshold for LTP in favour of LTD (Mayford *et al.* 1995) and impairs memory (Bach *et al.* 1995), these results did not corroborate the model suggesting that autophosphorylation of CaMKII is an essential trigger for

LTP and contradicted the previous knockout data (Silva *et al.* 1992*a*,*b*).

It is only recently that an explanation for this discrepancy was proposed after re-analysis of the CaMKII-Asp286 animals based on a feature of the combined CaMKIIα promoter/tTA system (Bejar *et al.* 2002).

Bejar *et al.* (2002) used the original line of transgenic mice that expressed high levels of CaMKII-Asp286 to produce a group of low-expressor animals. For this, transgene expression was suppressed during gestation and postnatal development and then restored in adulthood. This manipulation was taking advantage of a previous observation that long-term transgene suppression often reduces the level of expression when reactivated in adult animals. The difference in CaMKII-Asp286 levels achieved that way in the same line of mice helped to reveal a dose-dependent effect of CaMKII-Asp286 on LTP. While high levels were again found to impair low-frequency LTP, low levels enhanced LTP such as expected by the model and in accordance with the results obtained in the CaMKII T286A mice (Giese *et al.* 1998). Since compensatory mechanisms were suspected to be responsible for the LTP impairment in the high expressors, gene chip analyses were carried out to identify the affected genes. These analyses revealed that several genes were upregulated in response to high CaMKII activity, some of which were already known to be activated by LTP-inducing stimuli. Thus, CaMKIIα over-activation during development appeared to prompt compensatory changes that altered LTP. These changes may also possibly have led to an enhanced potentiation that occluded further tetanic LTP. Regardless of the mechanisms involved, this evidence stresses the potentially deleterious effect of excessive overexpression or overactivation of a protein which, in the case of CaMKII, is consistent with the fact that physiologically, only a small increase (15%) in CaMKII levels is triggered by LTP (Ouyang *et al.* 1997). By extension, this confirms that complete or even partial downregulation of a gene may impose non-physiological conditions and engage non-specific responses obscuring the expected effect. Finally, it also underscores the confounding effect of the early occurrence of a genetic mutation, implying that systematic genetic analyses should be considered in plain knockout animals for verification, and that tight and temporally controlled systems should be more widely used to regulate genetic manipulations.

5. INDUCIBLE TRANSGENESIS FOR THE STUDY OF PROTEIN PHOSPHATASES IN LTP

We have adopted an inducible approach based on the rtTA system to investigate the protein phosphatase side of the kinase/phosphatase balance thought to regulate LTP. The idea was to shift the balance either in favour of protein phosphatases by increasing CN activity, or in favour of protein kinases by decreasing CN activity. For this, the rtTA system was combined with the CaMKIIα promoter (figure 2*a*) to express either a truncated active form of the Aα catalytic subunit of CN, or the autoinhibitory domain of CNAα in forebrain neurons (Mansuy *et al.* 1998*a*; Malleret *et al.* 2001). In both cases, the shift was induced only in adulthood just a few days before experimentation to avoid any possible detrimental effect of transgene expression during development, and it could be fully reversed by suppressing transgene expression. Further, in both cases the achieved increase or reduction in CN activity was moderate, 77% and 35–45%, respectively, which more closely mimicked physiological conditions. CN overactivity was found to reversibly impair a PKA-dependent intermediate phase of LTP (I-LTP) (figure 3*a*) without affecting early-phase LTP, a phase distinguished from I-LTP by its PKA independency (Mansuy *et al.* 1998*a*; Winder *et al.* 1998). Conversely, inhibiting CN facilitated early LTP both *in vitro* in area CA1 of the hippocampus (figure 3*b*), and in anaesthetized mice in area CA1, and the dentate gyrus and made it PKA dependent (figure 3*c*; Malleret *et al.* 2001). In addition to increasing the overall magnitude of LTP, CN inhibition also significantly prolonged LTP in freely moving animals. Thus while in control mice, *in vivo* LTP started to decay soon after induction and was gone 3 days later, it remained high and persisted over 3–4 days in the mutant mice (figure 3*d*). Mechanistically, the facilitation of early LTP appeared to result from the intervention of PKA since it was blocked by a PKA inhibitor KT5720 (figure 3*b*), suggesting a failure of CN to oppose PKA. Overall, the apparent PKA dependency of both the impairment and enhancement of LTP confirmed that CN acts by interfering with PKA-controlled pathway(s) as predicted by the kinase/phosphatase balance model.

Further to its modulatory effect on LTP, CN was found to influence learning and memory. While an excess of CN perturbed spatial learning and a temporal phase of memory between short- and long-term memory (Mansuy *et al.* 1998*b*), CN inhibition facilitated learning and prolonged memory (Malleret *et al.* 2001). Recently, the downstream protein phosphatase PP1 was similarly demonstrated to improve learning efficacy and the persistence of memory (Genoux *et al.* 2002). Strikingly, with CN, comparable temporal phases of LTP and memory were affected by the genetic modulation of its activity, suggesting a temporally correlated effect of CN on plasticity and behaviour. Interestingly, the transient overexpression of CN after learning was found to reversibly impair retrieval, a specific phase of memory that allows the recovery of previously learned information (Mansuy *et al.* 1998*a*). Although the mechanisms of retrieval remain unclear, initial evidence has recently suggested that LTP in area CA3 of the hippocampus might sustain memory recall (Nakazawa *et al.* 2002).

Taken together, these data demonstrated that the level of CN activity is critical for determining synaptic strength and, in turn, the degree of LTP. Notably, and even more convincing than impairment, a correlated improvement of similar temporal phases of LTP and memory provides strong evidence in support of LTP being a cellular substrate of memory. In these studies, the rtTA system was instrumental in that it allowed the subtle perturbation of fine-tuning mechanisms required by the balance between kinases and phosphatases to regulate synaptic processes over only short time-windows and to only a limited extent. In contrast to such moderate change, the complete elimination of CN activity by CA1-restricted knockout of the major regulatory subunit of CN, CNB, appeared to have no effect on LTP but diminished LTD. It also impaired working memory, an immediate phase of memory, without affecting long-term memory (Zeng *et al.* 2001). These

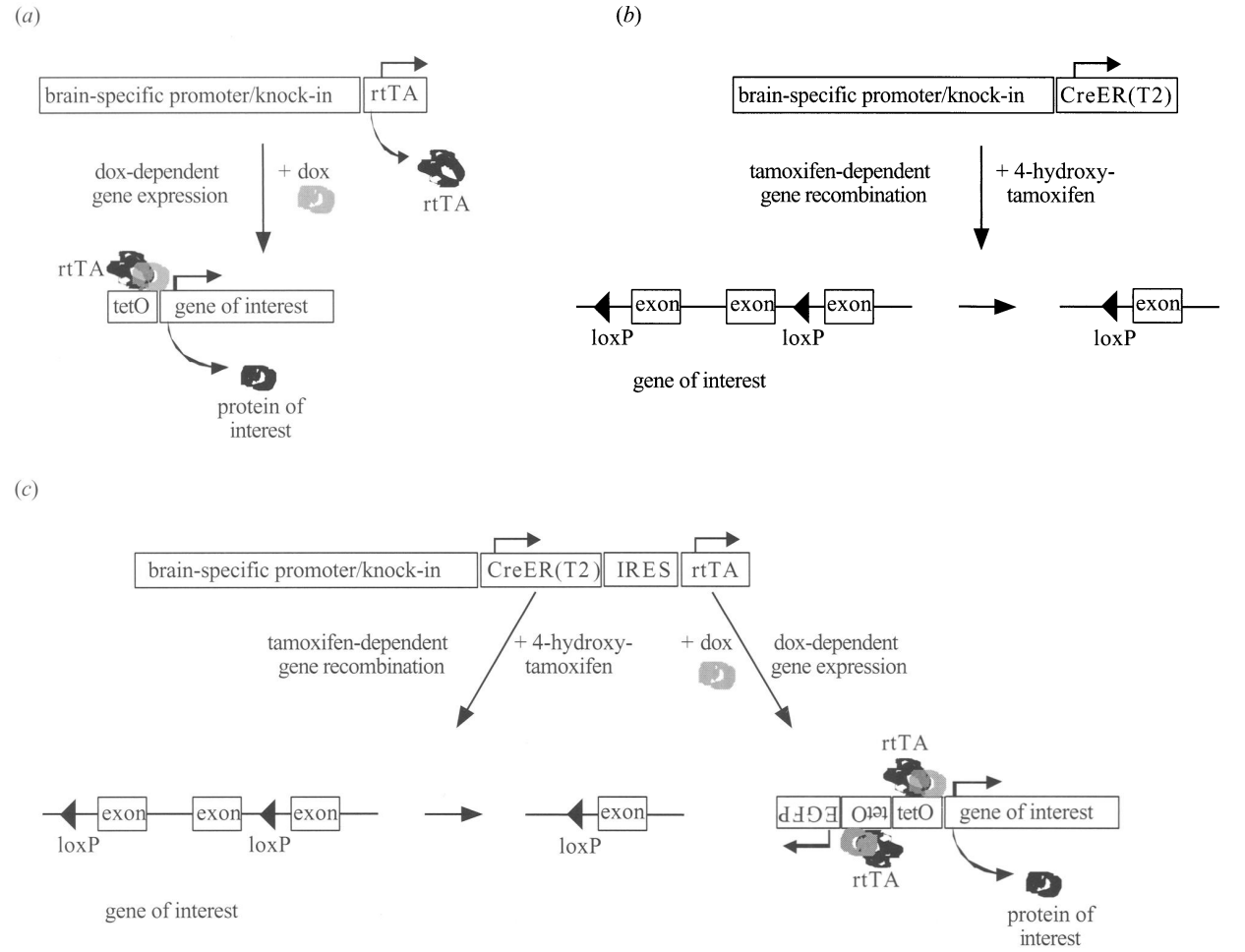

Figure 2. Strategy to achieve inducible gene expression, knockout or rescue in the mouse brain. (*a*) Inducible gene expression with the rtTA system based on the combination of a transgene expressing rtTA under the control of a brain-specific promoter or of an endogenous promoter by knock-in, with a transgene carrying a rtTA-specific tetO promoter fused to the gene of interest. Expression of the gene of interest is induced by dox and is suppressed by dox removal. (*b*) Inducible gene knockout. The inducible CreER(T2) (ER: oestrogen receptor) recombinase is expressed under the control of a brain-specific promoter or of an endogenous promoter by knock-in. It excises a DNA fragment between loxP sites in the gene of interest only in the presence of 4-hydroxy-tamoxifen. (*c*) Inducible gene knockout and rescue. CreER(T2) and rtTA expressed simultaneously under the control of a brain-specific promoter or of an endogenous promoter by knock-in through an internal ribosomal entry site (IRES) induce gene recombination and/or gene expression (gene of interest + marker such as EGFP) in the presence of 4-hydroxy-tamoxifen and/or dox.

results conflict with the kinase/phosphatase balance model and with our data, and may be explained by compensatory mechanisms that would gain by being identified by genetic analyses.

6. TECHNICAL IMPROVEMENTS AND FUTURE DIRECTIONS

As illustrated by the studies reviewed above, the mechanisms that regulate LTP, learning and memory are extremely complex and are subjected to discrete regulatory mechanisms. To fully understand these mechanisms, it is thus essential that refined methodological approaches are employed. Recent developments in the use of tTA and rtTA inducible expression systems and their combination with gene recombination techniques such as the Cre-loxP system now make these approaches suitable. In a recent study for instance, an inducible genetic rescue of the NR1 gene deletion was achieved in knockout animals by expressing a NR1 transgene in a dox-dependent and brain-specific fashion with the tTA system and the CaMKIIα promoter (Shimizu *et al.* 2000). This study showed that defects in LTP and memory resulting from NR1 gene disruption (Tsien *et al.* 1996*b*) could be reversed by dox-induced expression of the NR1 transgene while reproduced by its suppression. This approach provided proof of the principle that inducible gene expression and recombination can be achieved together. However, this strategy requires a considerable labour-intensive investment for generating and breeding the multiple transgenic lines needed (floxed NR1 × Cre transgenic × tTA transgenic × NR1 transgenic). Further, it does not provide true inducibility since only the transgenic rescue and not the knockout itself can be turned on or off. Finally, the degree of rescue is contingent upon the spatial restriction and the level of transgene expression driven by the tTA system and does not optimally mimic endogenous gene expression.

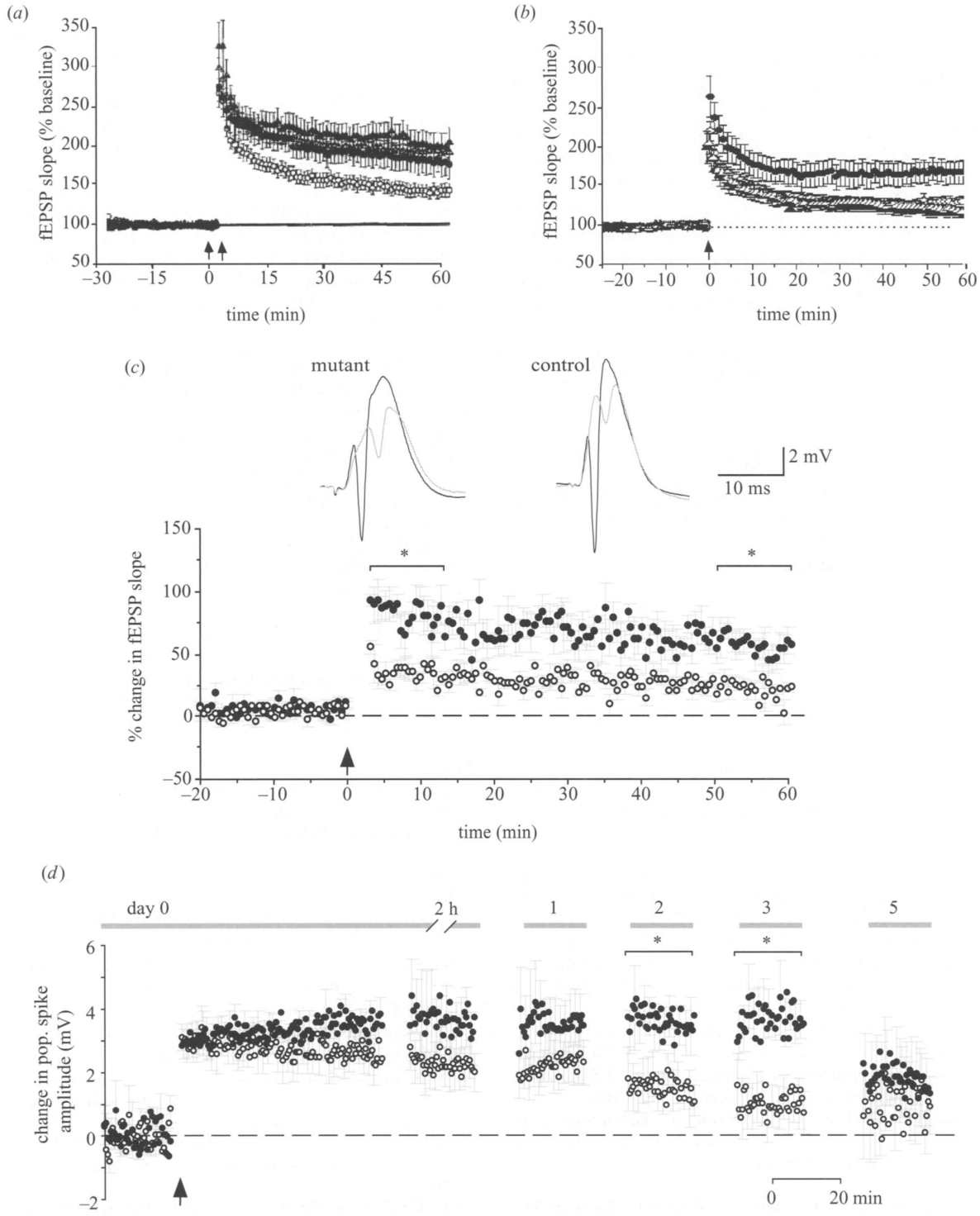

Figure 3. Impaired or facilitated LTP by overexpression or inhibition of CN. (*a*) A PKA-dependent intermediate phase of CA1 LTP induced by 2-train of high-frequency stimulation is impaired in slices from mutant mice expressing an active CN upon dox treatment. Filled triangles, control; open triangles, mutant; filled circles, control dox; open circles, mutant dox. (*b*) A PKA-independent early phase of CA1 LTP induced by 1-train of high-frequency stimulation is facilitated in slices from mutant mice expressing a CN inhibitor upon dox treatment. The PKA inhibitor KT5720 reverses this facilitation. Open circles, control dox; filled circles, mutant dox; open triangles, control dox + KT5720; filled triangles, mutant dox + KT5720. (*c*,*d*) Enhanced 1-train dentate gyrus LTP in (*c*) anaesthetized and (*d*) freely moving mutant mice expressing a CN inhibitor. Open circles, control dox; filled circles, mutant dox.

An alternative strategy to achieve inducible gene inactivation would be to express the Cre protein itself under the control of an inducible expression system. In that respect, the second generation of rtTA factors, rtTA2(s)-M2 and -S2, with increased sensitivity to dox and higher transactivation activity, may help improve the efficiency and rapidity

of such genetic manipulations (Urlinger *et al.* 2000; Lamartina *et al.* 2002; Salucci *et al.* 2002). Another powerful approach would be to use inducible versions of Cre like, for instance, CreER(T2) (Indra *et al.* 1999; figure 2*b*) or CrePR (Wunderlich *et al.* 2001). These recombinases contain the ligand-binding domain of the oestrogen or progesterone receptors, which renders their activity dependent on the synthetic ligand 4-hydroxy-tamoxifen. In combination with the rtTA system, they should allow inducible gene knockout and rescue via tamoxifen-induced gene recombination and dox-dependent reversible transgene expression (figure 2*c*). The association of various inducible recombination–expression systems is a promising means for targeting–expressing several genes and fluorescent markers simultaneously (Baron *et al.* 1999; Moser *et al.* 2001) to further advance research on LTP.

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GLOSSARY

CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II
CN: calcineurin
CPP: 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid
fEPSP: field excitatory postsynaptic potential
LTD: long-term depression
LTP: long-term potentiation
NMDA: *N*-methyl-D-aspartate
NR1: *N*-methyl-D-aspartate receptor 1
PKA: protein kinase A
PP1: protein phosphatase 1
PSD: postsynaptic density
rtTA: reverse tetracycline-controlled transactivator
tTA: tetracycline-controlled transactivator