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Conditional Transgenesis and Recombination to Study the Molecular Mechanisms of Brain Plasticity and Memory

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Abstract In the postgenomic era, a primary focus of mouse genetics is to elucidate the role of individual genes *in vivo*. However, in the nervous system, studying the contribution of specific genes to brain functions is difficult because the brain is a highly complex organ with multiple neuroanatomical structures, orchestrating virtually every function in the body. Further, higher-order brain functions such as learning and memory simultaneously recruit several signaling cascades in different subcellular compartments and have highly fine-tuned spatial and temporal components. Conditional transgenic and gene targeting methodologies, however, now offer valuable tools with improved spatial and temporal resolution for appropriate studies of these functions. This chapter provides an overview of these tools and describes how they have helped gain better understanding of the role of candidate genes such as the NMDA receptor, the protein kinase CaMKIIα, the protein phosphatases calcineurin and PP1, or the transcription factor CREB, in the processes of learning and memory. This review illustrates the broad and innovative applicability of these methodologies to the study of brain plasticity and cognitive functions.

Keywords Conditional transgenesis · Gene targeting · Learning · Memory · Synaptic plasticity

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Why Employ Conditional Transgenesis or Recombination in Memory Studies?

Cognitive functions are highly complex processes whose molecular mechanisms involve multiple genes with tightly regulated but yet highly dynamic expression profiles. Because most of these genes generally act in a cell- and time-specific fashion, their study requires controllable and flexible genetic tools. Transgenic and gene targeting approaches have been developed to provide such versatility and have been instrumental for studies of gene functions in the nervous system. In their most sophisticated versions, they allow spatial and temporal control over gene manipulations and provide a means to up- or down-regulate specific molecules in selected areas of the brain at will. These features have allowed exquisite analyses of the involvement of candidate genes in specific brain areas, and in distinct types and temporal phases of memory formation and storage.

1.1
Spatial Control over a Genetic Manipulation

The brain is an extremely complex organ with multiple distinct neuroanatomical and functional regions. To investigate the functions of specific genes in the brain using genetic approaches, it is essential that any manipulation of the gene be spatially highly precise. For this, specific promoter sequences have been cloned and used for transgenesis. Most of these promoters were picked for their brain specificity and for their broad, i.e. nestin (Cheng et al. 2004), prion protein (PrP) (Fischer et al. 1996) or neuron-specific enolase (NSE) (Forss-Petter et al. 1990) promoter, or restricted, i.e., Purkinje cell-specific L7 (Oberdick et al. 1990), forebrain neuron-specific Ca^{2+}/calmodulin-dependent protein kinase α (CaMKIIα) (Mayford et al. 1996b), oligodendrocyte-specific proteolipid protein (PLP) (Fuss et al. 2001) or astrocyte-specific GFAP promoter (Brenner et al. 1994) pattern of activity. Although generally stable and reliable, the selectivity of some promoters can vary and be influenced by the site of transgene integration. For instance, a more restricted pattern of gene expression has been observed in transgenic mouse lines carrying the CaMKIIα promoter, a promoter that is normally active in all forebrain neurons but is sometimes restricted to hippocampal CA1 (Tsien et al. 1996a) or striatal (Kellendonk et al. 2006) neurons (see Sect. 2). However, to date, the choice of promoter sequences truly selective for brain sub-regions or nuclei is still slim, and there is no promoter specific for cortical subdivisions such as the frontal cortex, or for hypothalamus or amygdala nuclei. However, promoters can be combined with expression systems to manipulate genes in these regions. For instance, a broadly expressed and drug-dependent transgene can be activated by local stereotactic injection of the drug (see Sect. 4). Such method is however

more invasive and depends on the availability of thus far rare ligand-dependent molecules with appropriate pharmacokinetic properties.

1.2 Temporal Control over a Genetic Manipulation

Higher-order brain functions such as learning and memory have multiple temporal phases that may recruit different signaling pathways and distinct cellular and molecular processes (i.e., spine growth/retraction, structural rearrangements, receptor trafficking, etc.). To distinguish the temporal phase(s) in which a given component of these pathways may act, it is essential to restrict any manipulation of its coding gene to the temporal phase of interest. For this, conditional expression or recombination systems have been developed to allow the induction or inactivation of gene expression at will, often with the possibility for reversal. These systems circumvent many drawbacks of constitutive transgenesis or knockout such as early lethality or developmental defects often induced when genes are manipulated early in life. For conditional transgenesis, systems based on the tetracycline-responsive transactivator (tTA) or its reversed versions (rtTA and rtTA2), whose transactivation activity can be controlled by doxycycline (administered in food or drinking water), have been developed and adapted to the brain. Their inducibility and reversibility were useful to dissect out some of the molecular mechanisms of specific phases of memory such as memory retrieval or consolidation (see Sect. 3). Likewise, conditional gene recombination was developed based on the Cre recombinase, an enzyme that recombines and excises a DNA fragment flanked by two loxP sites (floxed). Cre-dependent recombination was further made inducible by combination with inducible expression systems, i.e., tTA-based, or by fusion of Cre with a tamoxifen-dependent mutated human ligand-binding domain of the estrogen receptor (CreER). Spatial restriction of recombination can be achieved by placing Cre expression under the control of a tissue- or cell-specific promoter or for CreER, by local injection of tamoxifen.

This chapter aims at illustrating how conditional transgenesis and recombination have improved the understanding of the molecular mechanisms of learning and memory. Studies of major proteins suggested to be implicated in learning and memory will be presented (Fig. 1). Mutant models created to investigate the functions of the N-methyl-D-aspartate receptor (NMDAR), a glutamate receptor essential for the initiation of intracellular responses to neuronal activation, will be described. Furthermore, as part of the cascades relaying NMDAR-mediated signaling, the calcium/calmodulin-dependent kinase II (CaMKII), the protein phosphatases calcineurin and PP1, and the cAMP-responsive element binding (CREB) protein involved in synaptic and nuclear events will also be covered. The chapter will end with future perspectives on conditional transgenesis and recombination, and on how further technical improvements may aid forthcoming studies.

2 NMDA Receptor-Dependent Processes

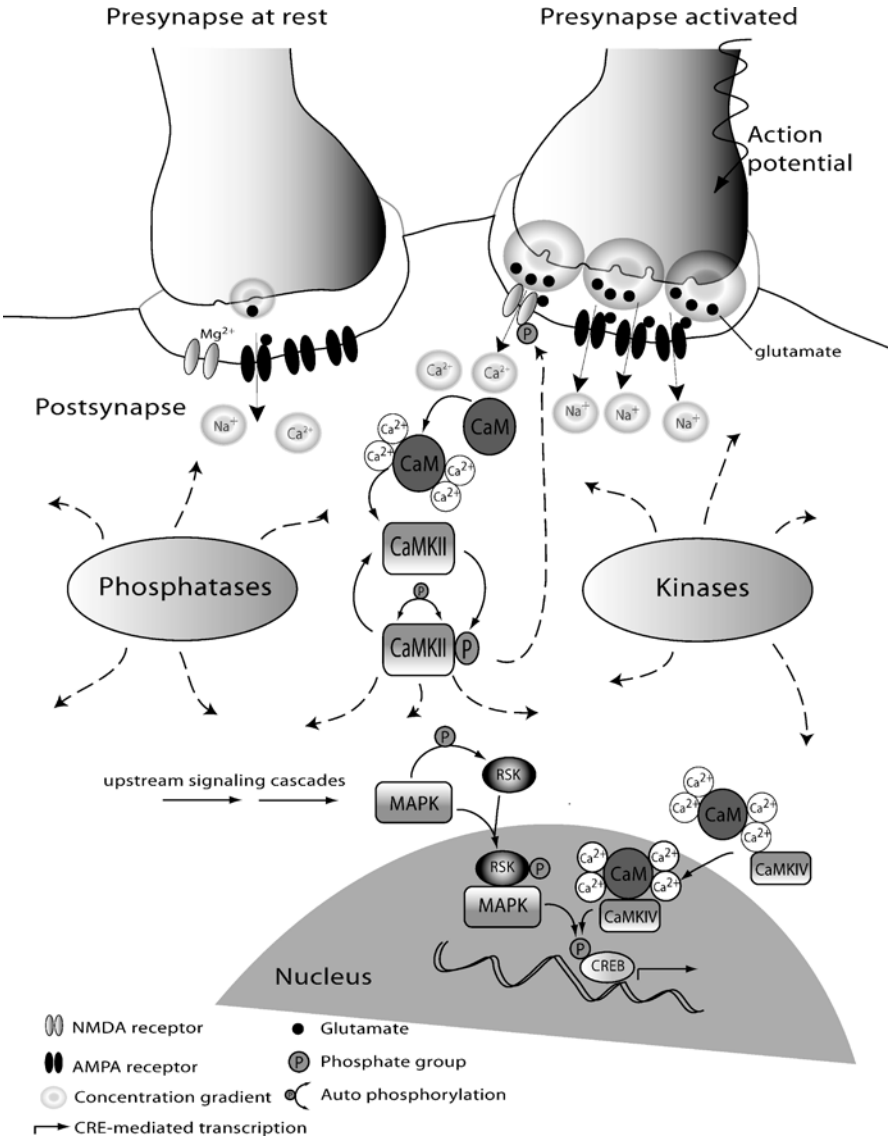
The NMDAR is a glutamate-activated membrane receptor that functions as an ion channel highly permeable to Ca^{2+} and is present essentially on postsynaptic neurons. The NMDAR assembles as a hetero-tetramer of two obligatory NR1 subunits that are ubiquitously expressed and are essential for channel function, and two NR2 subunits, NR2A, B, C or D. NR2 subunits have different profiles of expression and different properties that modulate the characteristics of the NMDAR, for instance its sensitivity to magnesium block, channel conductance or glutamate affinity. The NMDAR is critical for developmental processes in the brain such as neuronal survival (Balazs et al. 1989), differentiation (Blanton et al. 1990), migration (Marret et al. 1996), and for the formation, stabilization, and modulation of synapses and neuronal circuits (Constantine-Paton 1990).

The NMDAR was first implicated in learning and memory when receptor antagonists were found to block the induction of long-term potentiation (LTP) in hippocampal synapses (Collingridge et al. 1983). LTP is a well-characterized form of synaptic plasticity reflecting an increase in synaptic efficacy that is observed in many excitatory synapses in the mammalian brain, in particular CA3-CA1 hippocampal synapses (Bliss and Collingridge 1993). During LTP in CA1 neurons, the NMDAR acts as a coincidence detector that senses simultaneous pre- and post-synaptic activity and ensures efficient and reliable neuronal activity (Fig. 1). LTP in CA3 neurons, however, does not require the NMDAR but rather other types of glutamate receptors such as kainate or metabotropic receptors. The coincidence detector property of the NMDAR was first postulated to be a mechanism for the strengthening of synaptic connections by Donald Hebb (Hebb 1949), and is now widely accepted as one of the prerequisites for the formation and the storage of major forms of memory. However, the precise role of the NMDAR in synaptic plasticity and memory was poorly understood until transgenic and recombination

Fig. 1 Major molecular components of signaling cascades during synaptic transmission. This figure schematically outlines a synapse at rest (*left*) or after activation (*right*) in the brain. When an action potential (AP) reaches a glutamatergic presynaptic terminal, glutamate is released and diffuses across the synaptic cleft to bind to specific receptors on the postsynaptic membrane. This results in an influx of sodium (Na^+) leading to membrane depolarization, which is required for the subsequent influx of calcium (Ca^{2+}) ions. The level of intracellular Ca^{2+} determines which intracellular cascade predominates: a protein kinase cascade activated by high levels of Ca^{2+} that enhances synaptic strength and promotes signal transmission, or a protein phosphatase cascade responsive to low Ca^{2+} that weakens synaptic strength and down-regulates signal transmission. These intracytoplasmic cascades may then transmit the signal to the nucleus where gene expression may be activated for long-term processes

technologies were exploited to manipulate the different NMDAR subunits *in vivo*.

Mutant mice carrying null alleles of NR1 (Forrest et al. 1994), NR2A (Sakimura et al. 1995), NR2B (Kutsuwada et al. 1996) or NR2C (Ebralidze et al. 1996) were first created by classical knockout in the mid-1990s, followed by mice carrying inactivating point mutations or interrupting loxP sites. NR1 null mutants are not viable and die shortly after birth. Likewise, mice with



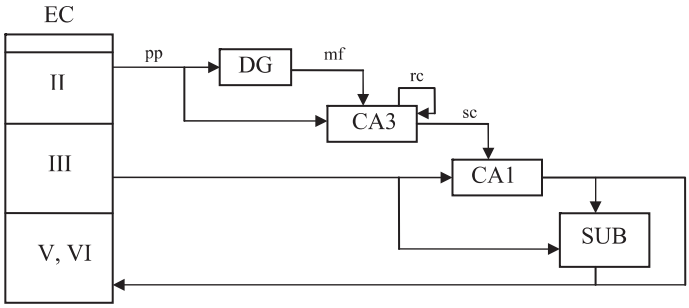


Fig. 2 Main connections of the hippocampal/cortical circuit. *EC,* entorhinal cortex; *DG,* dentate gyrus; *SUB,* subiculum; *pp,* perforant pathway; *mf,* mossy fibers; *rc,* recurrent collateral axons of CA3 pyramidal neurons; *sc,* Schaffer collateral axons

an inactive NMDAR due to a point mutation at asparagine N598, an amino acid required for correct voltage-dependent Mg^{2+} block and Ca^{2+} permeability, die prematurely (Single et al. 2000). Other mutant lines are viable (although NR2B knockout pups need manual feeding to survive) but have impaired NMDAR functions and NMDAR-dependent plasticity. To circumvent the lethality of NR1 inactivation, a conditional manipulation was designed using the late onset forebrain-specific CaMKIIα promoter and the Cre recombinase in transgenic mice. Several mouse lines expressing Cre under the control of the CaMKIIα promoter (CaMKIIα promoter-Cre) were generated and the pattern of Cre-dependent recombination was tested in reporter mice. As expected, gene recombination was induced postnatally but surprisingly in one line of mice, it was restricted to the hippocampus CA1 area (Tsien et al. 1996a). In rodents and human, the CA1 area is part of a tri-synaptic loop that together with the dentate gyrus, CA3 area, and subiculum, constitutes the hippocampal formation. The hippocampal formation receives input from the entorhinal cortex, then the signal is successively processed through dentate gyrus, CA3 and CA1 areas and sent back to the cortex (Amaral and Witter 1989) (Fig. 2). In this circuit, CA1 neurons are particularly important because they express NMDAR-dependent forms of synaptic plasticity including LTP and LTD, and are essential for memory formation (Zola-Morgan et al. 1986). The unusual CA1 restriction of Cre-dependent gene recombination obtained with that CaMKIIα promoter-Cre transgenic line was fortuitous but extremely useful. It allowed the elimination of NR1 not only late in development but also selectively in CA1 neurons, providing convenient temporal and spatial restriction.

Double mutant mice carrying a floxed NR1 gene (exons 3–22 flanked by loxP sites) and expressing the CA1-specific recombining Cre were obtained. Unlike plain knockout animals, these conditional knockout mice were viable, grew and developed normally. However, when adult, they exhibited severe impairments in NMDAR- and hippocampal-dependent functions. They

showed reduced NMDAR-mediated synaptic currents and deficient NMDAR-dependent LTP in area CA1 but normal plasticity in other hippocampal regions such as dentate gyrus. This selective impairment in plasticity was accompanied by a severe deficit in spatial learning, shown by an inability to acquire and remember the location of a hidden platform in a water maze (Tsien et al. 1996b). Nonspatial learning was also affected and performance was severely impaired in both trace- and contextual-fear conditioning, two hippocampal-dependent tasks based on learning of an association between a sound or a context and a foot shock (Huerta et al. 2000; Rampon et al. 2000). In other hippocampal-dependent tests such as object recognition, olfactory discrimination or olfaction-based transverse patterning tasks, performance was also impaired (Rampon et al. 2000; Rondi-Reig et al. 2001). In contrast, nonhippocampal-dependent learning such as cued fear conditioning (the association between a tone and foot shock) was not altered, highlighting overall the essential role of NR1 in hippocampal LTP and multiple forms of learning.

These initial results, however, did not determine whether NR1 is needed for processes following learning needed for the establishment, the consolidation and the storage of memory traces since these processes cannot take place when learning is blocked. To answer this question, it was necessary to inactivate NR1 only after training (allowing normal learning) and examine performance thereafter. This was achieved with a combined conditional approach, with which NR1 deficiency in the CA1-specific knockout animals was rescued by inducible expression of an NR1 transgene in CA1 neurons. The inducible NR1 transgene was assembled with a triple construct composed of a CaMKIIα promoter-Cre transgene, a Cre-dependent tTA gene interrupted by a floxed stop sequence and placed under the control of a β-actin promoter, and a tTA-dependent NR1 transgene fused to a tetO promoter (Fig. 3) (Shimizu et al. 2000). When combined with the endogenous floxed NR1 gene, this system allowed the inducible and reversible rescue of NR1 through tTA-dependent expression of transgenic NR1 in CA1 neurons during learning. In the resulting animals, dox treatment induced NR1 deficiency in CA1 neurons by suppression of transgene expression resulting in a similar defect as in the conditional knockout animals. Transgene expression induced by dox withdrawal fully restored LTP in area CA1, indicating that the NR1 transgene compensated for the absence of endogenous NR1. Suppression of NR1 rescue during learning, however, prevented the animals from acquiring information on the fear conditioning task or the water maze and induced a similar performance deficit as in the knockout animals (Tsien et al. 1996b). Strikingly, when the rescue was suppressed only after learning (for 1 or 2 weeks), performance was similarly impaired, suggesting that NR1 is required not only during but also after learning. This defect may have resulted from a failure in the consolidation of the acquired information and the formation of memory, or in the retrieval of a consolidated memory. To distinguish between these possibilities, NR1 was rescued both during and after learning to allow proper acquisition and

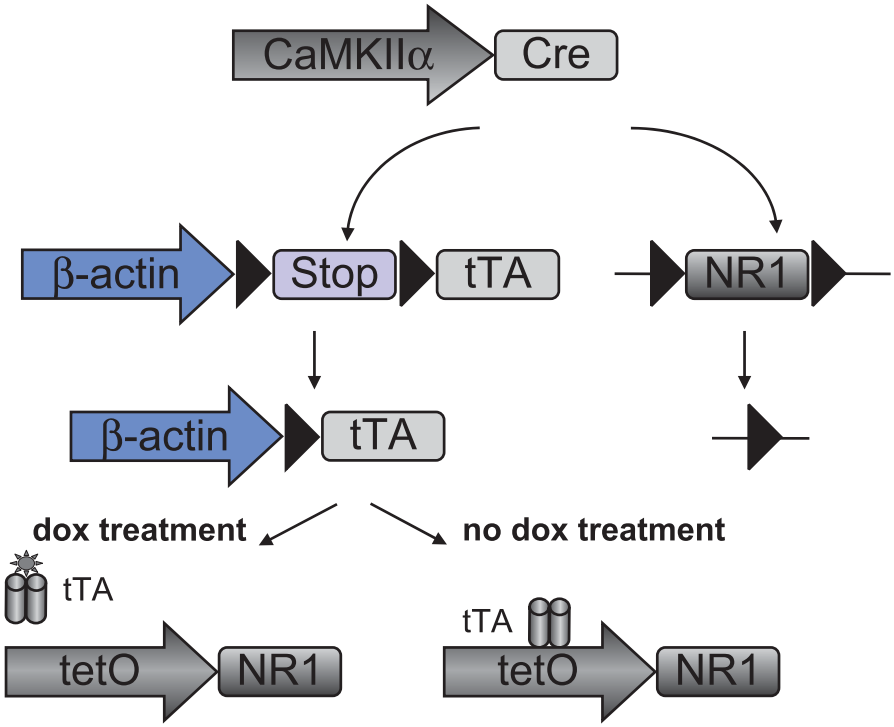


Fig. 3 Strategy to obtain an inducible, reversible, and CA1-specific NR1 knockout in the mouse. Cre expression under the control of the CaMKIIα promoter leads to simultaneous expression of tTA, after excision of a stop cassette 5′ to the tTA transgene by Cre, and inactivation of the endogenous NR1 gene through loxP-directed recombination in CA1 neurons. Regulation of the system is possible through the administration/withdrawal of dox. tTA binds to the tet operon (tetO) in the absence of dox and induces the expression of the NR1 transgene. Dox administration prevents the binding of tTA to tetO and switches NR1 transgene expression off (derived from Shimizu et al. 2000)

memory consolidation, then NR1 deficiency was re-instated by dox-induced NR1 transgene expression selectively during retrieval, i.e. shortly before the memory test that took place 4 weeks after training. This transient deficiency in NR1 at the time of retrieval did not impair retrieval and the animals correctly remembered the learned information, indicating that NR1 is not needed for retrieval *per se* but is required for the consolidation of memory, whether spatial, associative or even gustatory (Shimizu et al. 2000; Cui et al. 2004, 2005).

Since NR1 appeared to be required for the acquisition and the consolidation of memory traces, it was important to determine whether it is also needed after the initial consolidation to maintain memory traces for long periods of time. The conditional NR1 knockout model was ideal to test this possibility (Cui et al. 2004). After NR1 inactivation (for 1 month) by dox treatment 6 months after

learning (when consolidation is generally complete), the content of contextual and cued fear memory, both long-lasting forms of memory, was examined (Shimizu et al. 2000). This prolonged NR1 deficiency impaired performance in both contextual and cued-fear conditioning tests. However, it had no effect when induced for only 7 days, indicating that only prolonged but not transient NR1 absence interferes with the storage of memory. The continuous presence of NR1 is thus indispensable for the stability of stored remote fear memories. Overall, these results provided firm evidence that NR1 is required not only for the acquisition of information but also for the consolidation and the storage of memory traces.

The formation of hippocampus-dependent memory involves the association of complex configurations of stimuli into a memory trace that can be later recalled or recognized. The studies mentioned above examined the role of the NMDAR in different memory phases on the basis of full-cue conditions, meaning that retrieval occurred in the presence of all cues available during learning. In real life, however, memory recall often relies on incomplete or degraded sets of cues and requires that entire memory patterns be reconstructed from these sets. Recall based on associations must then be engaged to optimize retrieval. The CA3 region of the hippocampus has been proposed to be an anatomical basis for building such associations because it has an extensive recurrent connection network. This network has associative features due to its massive recurrent CA3 collaterals (auto-connections of pyramidal neurons) that provide major feedback to CA3, and the associated excitatory input coming from the dentate gyrus through mossy fibers, and from the entorhinal cortex through the perforant pathway (Fig. 2). The involvement of NR1 in pattern completion in CA3 neurons was tested by conditional recombination using the floxed NR1 animals and CA3-specific Cre transgenic mice. In these latter mice, Cre is expressed under the control of a kainate receptor 1 (KA1) promoter active essentially in CA3 neurons (Nakazawa et al. 2002). In mutant mice carrying this Cre transgene and the floxed NR1 gene, NR1 could be eliminated selectively in CA3 neurons about 5 weeks after birth. The loss of NR1 severely impaired LTP at CA3 synapses but not in other hippocampal synapses. In the water maze, it did not perturb spatial learning or the recall of spatial information when memory was tested under conditions of full extramaze cues (all cues used during training were available during memory test). In contrast, when most of the cues were removed, recall was severely impaired and the animals were no longer able to locate the hidden platform. The animals were also impaired in one-trial learning on a delayed matching-to-place version of the water maze, another form of memory thought to implicate the recurrent CA3 network (Nakazawa et al. 2003). The selective deficiency of NR1 in CA3 neurons thus demonstrated the NMDAR in these neurons is required for proper associative memory recall, as well as for rapid hippocampal encoding of novel information and fast learning of one-time experience.

3 Intracellular Signaling Cascades

The major functions of the NMDAR in neuronal transmission, synaptic plasticity, and memory are largely mediated by downstream intracellular cascades activated by Ca^{2+} ions flowing through the receptor. In postsynaptic neurons, Ca^{2+} stimulates numerous Ca^{2+}-sensitive enzymes that relay the signal conveyed by the NMDAR. Among them, several Ca^{2+}-dependent protein kinases and phosphatases are activated depending on their affinity for Ca^{2+} and the level of ambient Ca^{2+}. These enzymes dynamically regulate common or distinct targets in the postsynaptic neuron, and thereby modulate the efficacy of signal transmission (Fig. 1). CaMKII is a moderately Ca^{2+}-sensitive Ser/Thr protein kinase recruited by synaptic stimulation to the postsynaptic density (PSD), an electron-dense structure directly apposed to presynaptic terminals in excitatory glutamatergic synapses. After initial activation by Ca^{2+}, CaMKII has the ability to autophosphorylate at Thr286 to become Ca^{2+}-independent and remain active for long periods of time. Its persisting activity is required for the maintenance of high synaptic efficacy and for synaptic plasticity, in particular for LTP (Lisman and Goldring 1988; Lisman 1994; Pettit et al. 1994; Lisman and McIntyre 2001; Lisman and Zhabotinsky 2001). CaMKII is in part deactivated by dephosphorylation by PP1, whose activity and local distribution are themselves controlled by several regulators such as specific inhibitors and scaffolding proteins (Cohen 2002; Gibbons et al. 2005). One potent PP1 inhibitor is inhibitor-1 (I-1), a peptide activated by phosphorylation by the cAMP-dependent protein kinase A (PKA) and blocked by dephosphorylation by the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (PP2B), a highly Ca^{2+}-sensitive phosphatase. Altogether, CaMKII, PP1, I-1, PKA, and calcineurin form a balance of kinases and phosphatases differentially activated by Ca^{2+}. In this balance, kinases and phosphatases compete and antagonize each other to control intra-cytoplasmic and nuclear signal transduction pathways during neuronal activity. Predominant kinase activity is thought to favor signaling and enhance synaptic activity while predominant phosphatase activity weakens signaling and synaptic efficacy.

Several components of the kinase/phosphatase balance have been investigated *in vitro* and *in vivo* using conditional approaches and were shown to be required for synaptic plasticity, learning, and memory. Initial evidence for a contribution of CaMKII to plasticity and memory was provided when the gene coding for CaMKIIα, a predominant isoform in hippocampus and cortex, was permanently inactivated in the mouse by conventional knockout. The resulting CaMKIIα deficiency diminished NMDAR-dependent LTP in hippocampus area CA1 (Silva et al. 1992b, 1992c). However, this defect was not observed in heterozygous mice expressing half the amount of CaMKIIα, indicating that partial CaMKII activity is sufficient for proper LTP. Moreover, the effect of CaMKIIα deficiency was found to depend on the genetic make-up

of the animal as LTP was only slightly altered in this line of knockout mice with a different genetic background (Hinds et al. 1998). This may be due to compensatory mechanisms such as the recruitment of CaMKIIβ (Elgersma et al. 2002) activated to counteract the lack of CaMKII activity. Such mechanisms, however, may not operate or be less effective in visual or temporal cortex, two brain areas important for long-term memory (LTM), in which LTP was abolished whether CaMKIIα was fully (in homozygous mice) or partially (heterozygous mice) eliminated (Kirkwood et al. 1997; Frankland et al. 2001). Thus overall, CaMKIIα activity is required for cortical plasticity but is dispensable for plasticity in the hippocampus.

Importantly, a lack of Ca^{2+}-dependent kinase activity rather than a CaMKIIα protein deficiency in itself appeared to be the primary cause for the impairment in plasticity. When Ca^{2+}-dependent CaMKII activity was inhibited by blockade of Thr286 autophosphorylation through a Thr to Ala point mutation (CaMKII-T286A introduced by gene recombination), NMDAR-dependent LTP was prevented whether stimulated at high or intermediate frequency (100 or 10 Hz), or by theta bursts (two 100 Hz bursts of four stimuli, 200 ms intervals) (Giese et al. 1998). Likewise in the barrel cortex, *in vitro* LTP was absent whether induced by theta-burst stimulation, spike pairing, or postsynaptic depolarization paired with low-frequency presynaptic stimulation, and sensory-evoked potentials were impaired in vivo (Hardingham et al. 2003). The effect on LTP was dose-dependent and was only observed in the total absence of wild-type CaMKII-Thr286; mice heterozygous for the CaMKII-T286A allele had normal hippocampal LTP (Frankland et al. 2001). Interestingly however, in these mice the LTP defect could be induced in the adult hippocampus when the heterozygous mutation was combined with a drug-dependent approach. When activation of the remaining endogenous CaMKII was prevented by partial blockade of NMDAR-dependent Ca^{2+} influx (see Fig. 1) with a sub-threshold dose of the NMDAR antagonist CPP, LTP was impaired similarly to that in homozygous mice (Ohno et al. 2001, 2002). This effect was observed only when CPP was applied prior to LTP induction but not after. Further, the administered dose of CPP did not alter LTP in control slices whether applied before or after its induction. These results clearly indicated that the LTP impairment is a direct effect of Ca^{2+}-dependent CaMKIIα deficiency and not of a developmental anomaly, confirming the requirement of Ca^{2+}-dependent CaMKII activity for the induction of LTP in the hippocampus.

The importance of Thr286-autophosphorylation for CaMKII function in the brain was further confirmed by expression of a constitutively active and Ca^{2+}-independent CaMKII mutant in the mouse brain. CaMKII-Asp286 (carrying an Asp residue instead of Thr286) was inducibly expressed in the brain of transgenic mice using the dox-dependent tTA system and the CaMKIIα promoter (Mayford et al. 1996a). In these mice, the inducibility and reversibility of the tTA system was exploited to modulate the level of CaMKII-Asp286 expression and obtain a high or low increase in Ca^{2+}-independent activity. When induced

through development via tTA-dependent transactivation, CaMKII-Asp286 expression was strong and yielded high Ca^{2+}-independent CaMKII activity in forebrain neurons. However, when suppressed by dox during development and re-activated only in adulthood by dox removal, transgene expression was lower (only about 60% of wild-type CaMKIIα levels in hippocampus and 20%–30% in striatum) yielding a low level of Ca^{2+}-independent CaMKII activity (Bejar et al. 2002). This partial silencing may have resulted from changes in chromatin structure or DNA methylation following early and prolonged transcriptional suppression that could not be fully reversed after dox removal (Bejar et al. 2002) (our own observation). Strikingly, the resulting high or low level of Ca^{2+}-independent CaMKII activity produced an opposite effect on LTP in hippocampus area CA1. LTP was enhanced when Ca^{2+}-independent CaMKII activity was moderate (Bejar et al. 2002), consistent with the hypothesis that Ca^{2+}-independent CaMKII favors neuronal signaling and enhances plasticity. However, LTP was impaired when Ca^{2+}-independent CaMKII activity was high (Mayford et al. 1996a). High activity actually provoked a general shift of synaptic plasticity toward synaptic depression and increased the threshold for long-term depression (LTD), a form of synaptic plasticity induced by low frequency stimulation reflecting a weakening of synaptic efficacy (Mayford et al. 1996a). This effect was not due to a developmental anomaly resulting from transgene expression because it could be reversed by blockade of CaMKII-Asp286 expression. This finding was unexpected and is inconsistent with the postulated strengthening function of CaMKII on plasticity. It was later explained as resulting from altered expression of dozens of secondary genes including protease inhibitors, Ca^{2+}-binding proteins, growth factors, and transcription factors due to CaMKII-Asp286 expression (Bejar et al. 2002). Such genetic compensation was observed whether transgene expression was low or high but it was much more pronounced (more genes and larger changes) with high expression. The purpose of this transcriptional compensation may be to counterbalance the increased kinase activity but when excessive, it was detrimental to plasticity.

These results were corroborated by another study in which both Ca^{2+}-dependent and Ca^{2+}-independent activity were increased inducibly but over a shorter time window, minimizing genetic compensation. This was achieved by using a mutant form of CaMKIIα designed to be selectively and reversibly inhibited by a specific inhibitor peptide (Wang et al. 2003). This mutant carries a point mutation at Phe89 (F89G) in the ATP-binding pocket that does not affect ATP binding but renders this kinase mutant sensitive to low doses of a designed inhibitor 1-naphtylmethyl-PP1 (NM-PP1, IC_{50} = 32 nM). When expressed in forebrain neurons under the control of the CaMKIIα promoter, CaMKIIα-F89G increased Ca^{2+}-dependent CaMKII activity 2.6-fold and doubled Ca^{2+}-independent activity in the hippocampus. The increase was quickly suppressed by oral administration of NM-PP1 to the animals (5 μM in drinking water) and was fully reversible. The analysis of synaptic plasticity in CaMKIIα-

F89G-expressing mice revealed the expected potentiation in LTP across a broad range of stimulation frequency (from 1 to 100 Hz), that was accompanied by a reduction in LTD (induced by 5-min 3-Hz stimulation) (Wang et al. 2003). Importantly, this effect was specific to postsynaptic CaMKII while presynaptic CaMKII, in contrast, appeared to negatively regulate neuronal efficacy. When presynaptic CA3 terminals were selectively deprived of CaMKII by Cre-dependent recombination with the KA1 promoter, basal neurotransmitter release was increased in response to neuronal activity (Hinds et al. 2003; Nakazawa et al. 2003). This indicated that CaMKIIα serves as a negative modulator of activity in CA3 hippocampal area, contrary to CA1 where it promotes synaptic activity.

Altering CaMKII activity also has a strong impact on cognitive functions. Full elimination of CaMKII by plain knockout, mild or high overexpression by transgenesis interferes with spatial and associative learning and memory. Thus, null, CaMKIIα-T286A or CaMKIIα-Asp286 mutant animals are not able to learn spatial information in the water maze (Silva et al. 1992a; Mayford et al. 1996a; Giese et al. 1998) or on the Barnes maze (Mayford et al. 1996a). Intensive training on the water maze, however, rescues spatial learning in the null mutants (Elgersma et al. 2002), possibly through molecular compensation by recruitment of CaMKIIβ. Partial reduction in CaMKII activity in heterozygous CaMKII-T286A mice impairs short-term associative memory. On the contextual fear conditioning paradigm, heterozygous animals acquire and retain information for 1 day but do not consolidate this information and lose it after 36 days (Frankland et al. 2001). This selective defect in long-term memory correlates with the strong LTP impairment in cortex, a site for remote memory, and with normal LTP in hippocampus, a site for temporary memory storage (Frankland et al. 2001, 2004). One-day fear memory was nonetheless impaired in CaMKII-T286A heterozygous mutants when CaMKII activity was fully eliminated by administration of a subthreshold dose of the NMDAR antagonist CPP (5 mg/kg) (Ohno et al. 2001). When injected before training, CPP prevented the animals from learning the association between a context and a foot shock, but had no effect when injected after learning, highlighting the selective impact of CaMKII deficiency on information acquisition. CPP also did not affect memory in control animals at a sub-threshold concentration, clearly demonstrating that its effect was conditional to partial CaMKII inhibition. Unexpectedly, CaMKII overexpression also alters associative memory. tTA-dependent expression of CaMKII-Asp286 or constitutive expression of CaMKIIα-F89G in forebrain neurons during learning impairs cued and contextual fear conditioning 1 day or even 1 month after training (Mayford et al. 1996a; Wang et al. 2003). This effect was strong and could be produced even when CaMKIIα-F89G expression was induced only after training and for only 1 week (by removal of NM-PP1). However, it had no effect when induced only 2–3 weeks after training (Wang et al. 2003). Transgene expression directly accounted for the memory defects, as restoration of normal CaMKII activity by suppression of transgene expres-

sion with dox in the CaMKII-Asp286 mutants or by NM-PP1 administration in the CaMKIIα-F89G mice fully reversed the memory impairment (Mayford et al. 1996a; Wang et al. 2003).

Altogether, these results indicated that CaMKII activity must be tightly regulated during learning for proper acquisition but also shortly after learning for memory consolidation. They also highlighted the role of autophosphorylated CaMKII as a positive regulator of synaptic plasticity, and the importance of its tight fine-tuning for cognitive functions. In neuronal cells, this tuning is largely provided by protein phosphatases, specifically calcineurin and PP1, that can counteract CaMKII and/or antagonize its activity by dephosphorylation of common targets. The function of calcineurin and PP1 has been examined by conditional transgenesis with dox-dependent expression systems and the CaMKIIα promoter. When the activity of calcineurin or PP1 was reduced (by about 50%–80%) in the mouse forebrain by tTA- or rtTA-dependent expression of selective inhibitor peptides, hippocampal LTP was enhanced both *in vitro* and *in vivo* (Malleret et al. 2001; Genoux et al. 2002). LTP was also less prone to reversal by depotentiation (Jouvenceau et al. 2003; 2006), indicating a general increase in synaptic efficacy by reduced phosphatase activity. Consistently, when calcineurin activity was increased (by 80%–100%) by regulated expression of a partially Ca^{2+}-independent active mutant in forebrain neurons, LTP was impaired in the hippocampal area CA1 (Mansuy et al. 1998b; Winder et al. 1998). These changes in LTP could be reversed in adult animals by suppression of transgene expression, confirming that they were a direct effect of the transgene. Further, reduced calcineurin or PP1 activity in adult animals facilitated spatial learning and memory in the water maze, and improved memory for objects on an object recognition test (Malleret et al. 2001; Genoux et al. 2002). Several temporal components including acquisition, short- and long-term memory were enhanced when phosphatase activity was maintained low during and after training. When normal PP1 activity was restored by transgene suppression right after acquisition, spatial memory remained normal (Genoux et al. 2002). However, when PP1 was inhibited only after acquisition, consolidation was enhanced and memory was more robust and persistent.

In contrast, an increase in calcineurin activity by dox-dependent expression of an active calcineurin mutant during and after training impaired the acquisition of spatial memory in the water maze (Mansuy et al. 1998b). Calcineurin excess was also found to impair memory retrieval. Thus, when calcineurin activity was increased only before retrieval, e.g., after information was properly learned while transgene expression was turned off, the animals were not able to remember the platform position. However, when normal calcineurin activity was subsequently restored (after the failed retrieval attempt), they could find the platform position, indicating that the information had been correctly consolidated and maintained in memory but could not be recollected in the presence of an excess of calcineurin. Altogether, these results highlight the function of calcineurin as a molecular constraint on the acquisition, the con-

solidation, and the retrieval of memory. They are consistent with the model that a tightly regulated kinase-phosphatase balance controls synaptic efficacy, and is essential for learning and memory. When this balance is slightly tilted in favor of kinases or phosphatases, synaptic efficacy and performance are strengthened or weakened, respectively. But if shifted excessively or unduly in either direction, it impairs these processes. This may explain why memory is impaired in mice expressing high levels of CaMKII-Asp286, or in knockout mice deficient for the predominant calcineurin isoform (CNAα) in CA1 hippocampal neurons (Zeng et al. 2001).

4 Nuclear Events and Transcriptional Regulation by CREB

Conditional transgenesis and recombination methods have been instrumental to investigating signaling processes in the nucleus downstream of the NMDAR and intracytoplasmic kinase/phosphatase cascades. Nuclear events such as gene transcription and subsequent cytoplasmic protein translation are essential for the establishment of long-lasting forms of synaptic plasticity and memory. Both memory consolidation and re-consolidation, processes necessary to stabilize (re-stabilize) memory after initial formation or re-activation, respectively, depend on gene expression and protein synthesis. Transcriptional regulation is mediated by transcription factors such as the cAMP-responsive element (CRE) binding protein (CREB). CREB is a ubiquitous factor in mammals activated by two major Ca^{2+}-dependent signaling cascades that control CREB phosphorylation (primarily at serine 133). Upon phosphorylation, CREB binds to CREs in specific genes and recruits a complex of transcriptional activators that promote mRNA synthesis. The initial phosphorylation of CREB is triggered by Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV), but this phosphorylation is transient and not sufficient to initiate gene transcription. Additional phosphorylation by the mitogen-activated protein kinase (MAPK) is required for persistent CREB activity and transcription. MAPK is activated in the cytoplasm where it subsequently activates pp90 ribosomal protein S6 kinase (Rsk) and as a complex, these proteins translocate to the nucleus where Rsk phosphorylates CREB (Fig. 1) (for review refer to West et al. 2002).

Conditional approaches were first applied to CREB in the fruit fly *Drosophila melanogaster* to investigate the CREB-dependence of long-term forms of plasticity and memory. Olfactory memory in *Drosophila* is a major form of memory that has two distinct phases: anesthesia-resistant (ARM), that is short-term (declines after about 4 days) and independent of protein synthesis, and LTM, a long-lasting and protein synthesis-dependent phase. The importance of CREB in these phases of memory was tested in transgenic fly models taking advantage of the naturally occurring CREB transcriptional repressor dCREB2-b or the activator CREB2-a, and a heat shock promoter for temporal control

(Yin et al. 1994; Yin et al. 1995). Heat shock gene regulation is an endogenous process in *Drosophila* that allows the rapid switching of specific sets of genes upon temperature-induced stress. When combined with the repressor dCREB2-b, the heat shock promoter allowed temperature-controlled repression of CREB-dependent gene expression in adult flies. The effect of repression on memory was examined in a Pavlovian odor avoidance task after dCREB2-b expression was induced by heat shock (42°C). This resulted in a selective blockade of LTM but no alteration of ARM, an effect that was directly due to dCREB2-b expression but not to a developmental anomaly. In contrast, expression of dCREB2-a strongly activated CREB-driven gene expression and enhanced LTM (Yin et al. 1995). Transgenic flies expressing dCREB2-a needed only one training session for optimal performance when wild-type flies needed ten sessions, clearly demonstrating that CREB acts as a positive regulator of memory.

These results were confirmed in the mouse by the generation and analyses of several mutant models in which the CREB gene was inactivated by plain or conditional knockout. Mice deficient for the predominant α- and δ-isoforms (CREB $^{\alpha\delta-/-}$) developed normally and had normal short-term plasticity and memory. However, they exhibited a severe impairment in late-phase LTP in hippocampus area CA1, a form of protein synthesis-dependent plasticity, and in associative and spatial long-term memory (Bourtchuladze et al. 1994). On the fear conditioning test, they were not able to remember the association between a tone or a context and a foot-shock, while on the water maze, they could not recollect the position of an escape platform previously learned. However, the memory deficit appeared to be dependent on gene dosage and genetic background (Gass et al. 1998), and in some cases, it was compensated for by related CREB factors such as CREM or ATF-1 (Hummler et al. 1994). Further, unspecific behavioral abnormalities such as thigmotaxis in the water maze (swimming along the walls of the maze) were also observed and may have confounded the interpretation of the results (Balschun et al. 2003).

Conditional approaches were therefore employed to spatially and temporally restrict manipulations of the CREB gene. One approach based on regulated transgenesis allowed the inducible and reversible inhibition of CREB activity in selected areas of the brain. A CREB dominant-negative mutant, KCREB, carrying an amino acid substitution, was used to block the ability of CREB and related CREM and ATF1 factors to bind to CRE (Walton et al. 1992). When placed under the control of tTA and the CaMKIIα promoter, KCREB expression could be targeted to CA1 neurons in dorsal hippocampus in adult mice (again fortuitously), and interfered with CREB-dependent gene expression selectively in these neurons (Pittenger et al. 2002). The dorsal hippocampus is recognized to be critical for spatial learning and memory and for object recognition, while ventral hippocampus is rather involved in contextual fear conditioning (Moser and Moser 1998; Broadbent et al. 2004). When tested for spatial learning and memory in the water maze or for associative memory on

the contextual fear-conditioning task, mice expressing KCREB in dorsal CA1 showed a severe impairment in spatial LTM but had intact STM and contextual memory (Pittenger et al. 2002). Learning or STM on the object recognition test were also normal but not LTM after 1 day. The memory impairments were fully reversed when CREB activity was restored by suppression of KCREB expression with dox, indicating that they directly resulted from a failure in CREB activity and its related family members CREM and ATF-1. Finally, performance on hippocampal-dependent memory tests was normal in another line of mice not expressing KCREB in hippocampus, but only in striatum and piriform cortex. Consistent with the involvement of CREB in late transcriptional events, CREB inactivation did not impair an early phase of LTP (E-LTP) in hippocampal area CA1 but impaired late phase LTP (L-LTP) induced by forskolin, a drug that stimulates adenylyl cyclase and triggers a PKA-dependent form of LTP, or by pairing a single tetanus with the dopamine agonist chlo-APB. The LTP impairment was directly associated with KCREB expression since it could be reversed when KCREB was turned off by dox administration. Interestingly, KCREB did not interfere with L-LTP induced by high-frequency tetanic stimulation or theta burst potentiation, indicating that CREB-mediated transcription differentially contributes to different forms of L-LTP.

Another conditional study exploited a CREB mutant carrying an amino acid substitution at Ser133 phosphorylation site ($\alpha CREB^{S133A}$) (Gonzalez and Montminy 1989) that competes with endogenous CREB (Kida et al. 2002). $\alpha CREB^{S133A}$ was made inducible by fusion with the ligand-binding domain (LBD) of the human estrogen receptor, itself mutated on G512R (LBD^{G521R}) (Danielian et al. 1993; Logie and Stewart 1995; Feil et al. 1996) to respond only to the synthetic ligand 4-hydroxy-tamoxifen and not to endogenous estrogen. Mice expressing $\alpha CREB^{S133A}$ in excitatory neurons in hippocampus and cortex were generated using the CaMKIIα promoter. In these mice, tamoxifen injection resulted in the rapid translocation of $\alpha CREB^{S133A}$ to the nucleus and disruption of CREB-mediated transcription (within 6 h). This fast inducibility was exploited to study the contribution of CREB to the encoding and the consolidation of information into LTM, and memory retrieval using contextual and cued conditioning tasks. Activation of $\alpha CREB^{S133A}$ by tamoxifen injection before training did not impair short-term contextual or cued fear memory when tested 2 h after training. However, it impaired both types of memory after 24 h, confirming that CREB-mediated transcription is required for LTM but not STM. The defect was not caused by a failure in retrieval since $\alpha CREB^{S133A}$ activation only 6 h before the memory test did not affect performance. Since memory re-consolidation after retrieval also requires protein synthesis and is thought to activate similar mechanisms as initial memory consolidation (Nader et al. 2000), it was examined after $CREB^{S133A}$ activation. For this, transgenic mice expressing $CREB^{S133A}$ were trained for contextual or cued fear conditioning, $\alpha CREB^{S133A}$ was activated 18 h later and then the animals were re-exposed to the context or the tone (alone) 24 h later. Re-exposure

is meant to reactivate memory for context or tone, a process known to make memory traces transiently unstable and susceptible to disruption, and that requires re-consolidation. CREB inactivation at the time of re-consolidation impaired performance, thus providing novel evidence that CREB is needed not only for the consolidation but also the re-consolidation of memory traces after retrieval.

5 Discussion and Future Perspectives

The advent of spatially and temporally restricted genetic manipulations in the mouse brain has been a critical step forward in the understanding of gene functions in synaptic plasticity, learning, and memory. To date, multiple transgenic lines are available for conditional transgenesis in the brain (Tables 1 and 2). The growing popularity of the approach and the need for even further spatial and/or temporal restriction will have to be accommodated in the near future, for instance by the establishment of new promoters.

The limitations and shortcomings of classical transgenesis will also have to be improved. For example, transgene expression is often different and variable in independent mouse lines. This variability generally derives from the randomness of transgene integration, i.e. integration in a region of heterochromatin often correlates with high expression variability (Pravtcheva et al. 1994). Copy number also influences expression parameters and a high number increases the risk for gene silencing (Martin and Whitelaw 1996; Garrick et al. 1998; Henikoff 1998). Although mostly undesired, transgene variability may turn extremely advantageous if the actual pattern of expression is restricted to an area of interest. As discussed in this chapter, mouse lines carrying the same promoter fragment (Table 3) have not always exhibited the same expression pattern, level, or onset depending on whether it was used alone or in combination with tTA(rtTA)- or Cre-dependent systems. That was the case for an 8.5-kb CaMKIIα promoter fragment originally described with a late onset (about 3 days after birth) and neuronal specificity in cortical structures, hippocampus, striatum, and amygdala (Mayford et al. 1996b). However, when combined with tTA or Cre, it was found to be sometimes active during embryogenesis, leading to perinatal lethality as observed with a mutant huntingtin gene (Yamamoto et al. 2000), to drive expression in most forebrain neurons except hippocampal CA1 neurons (with tTA; (Mayford et al. 1996b) or in constrast only in CA1 neurons (with Cre; Tsien et al. 1996a, 1996b).

Different approaches may be used to circumvent the variability of transgene expression. One possibility is to use large fragments of DNA carrying *cis* -regulatory elements including introns, locus control regions, and insulators that generally ensure position-independent and copy number-dependent gene expression (for review, see Giraldo and Montoliu 2001). They require chromosome-type vectors such as bacterial, plasmid, or yeast artificial chro-

Table 1 Different nervous system-specific promoters used to drive the expression of Cre-recombinase

Promoter	Specificity	Characterization	Studies in memory and synaptic plasticity
D6	Neocortex, hippocampus	van den Bout et al. 2002	-
Emx1	Cerebral cortex, hippocampus	Guo et al. 2000	-
C-kit	CA1, CA2 and CA3 regions of hippocampus, anterior region of the dentate gyrus, ganglion cell layer of retina	Eriksson et al. 2000	-
Nestin	Neuronal and glial cell precursor	Tronche et al. 1999	Fleischmann et al. 2003 Tomita et al. 2003 Golub et al. 2004
CaMKIIα	1: CA1 region of hippocampus/ Hippocampus, cortex and striatum. 2: High level in all forebrain structures; low levels in cerebellum. 3: High levels in hippocampus, cortex and amygdala; low levels in striatum, thalamus and hypothalamus.	1: Tsien et al. 1996a 2: Dragatsis and Zeitlin 2000 3: Casanova et al. 2001	Tsien et al. 1996b Huerta et al. 2000 Rampon et al. 2000 Rondi-Reig et al. 2001b Shimizu et al. 2000 Yu, Saura et al. 2001 Zeng et al. 2001 Schweizer et al. 2003 Bukalo et al. 2004 Kelleher et al. 2004 Saura et al. 2004 Knuesel et al. 2005 Saura et al. 2005
KA1	1: In embryo: most neuronal cells of CNS. In adult: CA3 region and dentate gyrus granule cells of hippocampus. 2: High level in CA3, low in dentate gyrus.	1: Kask et al. 2000 2: Nakazawa et al. 2002	Nakazawa et al. 2002 Nakazawa et al. 2003
mNF-H	Neurons of the brain and spinal cord during late stage of development	Hirasawa et al. 2001	-
NEX	Granule cells of dentate gyrus	Schwab et al. 2000	Kleppisch et al. 2003

Table 1 (continued)

Promoter	Specificity	Characterization	Studies in memory and synaptic plasticity
PrP (inducible-ERT)	Brain, retina, hippocampus cerebellum	Weber, Metzger et al. 2001	-
Thy-1	CNS and PNS (cortex, cerebellum, spinal cord, retina, dorsal root ganglion)	Campsall, Mazerolle et al. 2002	-
NSE	Embryo: forebrain, midbrain, hindbrain flexure. Adult: cortex, cerebellum, hippocampus, septum	Cinato, Mirotsou et al. 2001	-
Syn-1	Neuron specific (brain and spinal cord)	Zhu, Romero et al. 2001	-

mosomes (BACs, PACs, and YACs) with large cloning capacity (up to 1 Mb). Artificial chromosomes are either microinjected in a linearized form into the pronucleus of fertilized mouse eggs or inserted into embryonic stem cells where they randomly integrate into the host genome. The cloning, handling, and injection of these vectors requires more skill and time than conventional transgenesis because large DNA fragments are more fragile and prone to breakage, and extensive analysis is required after insertion into a host to ensure for the presence of the whole transgene. Furthermore, frequency of integration is lower than with plasmid-based transgenes. However, since the reliability of expression is higher, a smaller number of lines needs to be generated and screened to obtain one with the desired characteristics. A BAC carrying 170 kb of noncoding genomic DNA of the CaMKIIα gene, consisting of approximately 50 kb of genomic sequence upstream of the ATG and 110 kb of downstream sequences was cloned. This BAC allowed faithful expression of genes according to the pattern and onset of endogenous CaMKIIα and expression levels were copy number-dependent (Casanova et al. 2001). Incidentally, its specificity did not differ much from that normally observed with the 8.5-kb promoter, indicating that a shorter fragment in this case was sufficient (Mayford et al. 1996b). Another efficient method to faithfully express a transgene following the pattern, time course, and level of a specific endogenous gene is to knock in the gene of interest into the ORF of a selected locus by homologous recombination (Misawa et al. 2003; Korets-Smith et al. 2004). Knock-ins help prevent expression variability linked to random

Table 2 Different nervous system-specific promoters used to drive the expression of either tTA or rtTA

Promoter	Specificity	Characterization	Studies in memory and synaptic plasticity
Prnp-tTA	Cerebral cortex, hippocampus, thalamus, hypothalamus, striatum, cerebellum	Tremblay, et al. 1998	Peters et al. 2005
CaMKIIα-tTA	Forebrain, neocortex, hippocampus, amygdala, striatum	Mayford et al. 1996	Mayford et al. 1996 Mansuy et al. 1998a Pittenger et al. 2002 Chen et al. 2003 Huang et al. 2004 Santacruz et al. 2005
CaMKIIα-rtTA	Hippocampus, cortex, septum, striatum	Mansuy et al. 1998b	Mansuy et al. 1998b Malleret et al. 2001 Genoux et al. 2002
NSE-tTA	Striatum, cerebellum, CA1, neocortex	Chen et al. 1998	King et al. 2003
GFAP-rtTA	Astrocytes	Kim et al. 2003	-
GABAα6-rtTA	Cerebellar granule cells	Yamamoto et al. 2003	-

integration and selects for active euchromatin, ensuring efficient expression. However, knock-ins generally yield few positive animals and heterozygosity at the target locus may perturb the full function of the targeted gene. However, this may be alleviated by simultaneous expression of a transgene and the endogenous gene using internal ribosomal entry sequences (IRES) (Michael et al. 1999; Gorski et al. 2002; Funfschilling et al. 2004; Lindeberg et al. 2004).

Several approaches have been used to improve the inducibility and tightness of transgenic manipulations. Improved versions of the rtTA factor with optimized codon use for mammals, enhanced affinity for dox and reduced leakage have been engineered (Urlinger et al. 2000). Transgenic mice expressing one of these new factors, rtTA2S-M2, under the control of the CaMKIIα promoter, have been generated and characterized (Michalon et al. 2005) and will be useful for future studies in the brain. Furthermore, tTA or rtTA expression was made Cre-dependent by insertion of a floxed stop cassette upstream the coding region (Belteki et al. 2005; Yu et al. 2005). Another study combined rtTA with

Table 3 Studies using the CaMKIIα promoter elements in transgenic mice to examine memory and synaptic plasticity

Type of transgene expressed	1995/1997	1998/2001	2002/2005
direct	Bach et al. 1995 Mayford et al. 1995 Kojima et al. 1997 Abel et al. 1997	Tang et al. 1999 Rammes et al. 2000 Kang et al. 2001 Tang et al. 2001 Philpot et al. 2001	Wong et al. 2002 Wang et al. 2003 Wang et al. 2004 Wei et al. 2004 Wood et al. 2005
tTA	Mayford et al. 1996	Mansuy et al. 1998a Jerecic et al. 2001	Bejar et al. 2002 Hernandez et al. 2002 Pittenger et al. 2002 Chen et al. 2003 Fridmacher et al. 2003 Huang et al. 2004 Santacruz et al. 2005
rtTA	-	Mansuy et al. 1998 Malleret et al. 2001	Genoux et al. 2002
Cre	Tsien et al. 1996a	Huerta et al. 2000 Rampon et al. 2000 Rondi-Reig et al. 2001 Shimizu et al. 2000 Yu et al. 2001 Zeng et al. 2001	Vyssotski et al. 2002 Schweizer et al. 2003 Bukalo et al. 2004 Kelleher III et al. 2004 Saura et al. 2004 Knuesel et al. 2005 Saura et al. 2005

a tet repressor tTR that eliminated leakage. tTR binds to tetO promoter and actively represses expression in the absence of dox, but is displaced by rtTA in the presence of dox (Konopka et al. 2005).

Similar to transgenesis, gene targeting has been made conditional by combination with specific promoters and regulated systems. The first example of an inducible knockout made use of the interferon responsive-promoter of the *Mx1* gene fused to Cre and controlled by IFNα or β (Kuhn et al. 1995). More recent examples employed dox-dependent expression systems (Saam and Gordon 1999; Radomska et al. 2002; Schonig et al. 2002; Guo et al. 2005; Yu et al. 2005) that when fused to the CaMKIIα promoter, allowed inducible expression of Cre and recombination in forebrain, similar to the one described above with NR1 (Lindeberg et al. 2002). Interestingly in this latter example, different patterns of gene recombination could be obtained by varying the timing of

dox-mediated shutdown of Cre expression, due to different onset and expression level of tTA in different brain regions. More recently, an inducible version of Cre was designed by fusion of Cre or improved derivatives with the LBD of the estrogen receptor that can be induced with tamoxifen (Metzger et al. 1995; Kellendonk et al. 1996; Brocard et al. 1997; Feil et al. 1997; Danielian et al. 1998; Schwenk et al. 1998; Casanova et al. 2002; Guo et al. 2002; Hayashi and McMahon 2002; Shimshek et al. 2002). While protocols for tamoxifen administration still need optimization, initial reports with high recombination efficiency indicate that the method is promising (Hayashi and McMahon 2002; Zirlinger et al. 2002). The system has been successfully adapted to the brain (Weber et al. 2001; Leone et al. 2003), but its tremendous potential has not yet been exploited for studies of cognitive functions.

Alternative approaches to gene overexpression or recombination may also be based on the design of inducible proteins more amenable to rapid and flexible biochemical modulation. One example in this chapter described tamoxifen-dependent CREB based on CREB fusion with the LBD of the estrogen receptor. Such fusion has been employed with other targets such as the transcription factors c-jun, c-fos, and c-myc (Rossler et al. 2002; Jager et al. 2004) or the cytoplasmic enzyme ornithine decarboxylase (Lan et al. 2005). It was further recently combined with Cre-mediated recombination to control the expression onset of the fusion protein induced by tamoxifen, which resulted in an tightly controlled system (Jager et al. 2004).

In only a little over a decade, conditional transgenesis has evolved to provide an exquisite degree of specificity of genetic manipulations. Further improvements and alternative approaches offering higher spatial resolution, i.e. specific sub-cellular compartments such as recently achieved in the nucleus (Limback-Stokin et al. 2004), enhanced temporal control, and taking into account post-translational modifications are still needed to gain even deeper understanding of protein functions. In light of the rapid technological progress in this field and the rise in popularity of these systems, there are good reasons to believe that these requirements will be met in the near future.

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