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Genetic Dissection of Neural Circuits and Behavior in *Mus musculus*

Robbert Havekes and Ted Abel

Department of Biology, University of Pennsylvania, Philadelphia, PA, USA

Abstract

One of the major challenges in the field of neurobiology is to elucidate the molecular machinery that underlies the formation and storage of memories. For many decades, genetic studies in the fruit fly (*Drosophila melanogaster*) have provided insight into the role of specific genes underlying memory storage. Although these pioneering studies were groundbreaking, a transition to a mammalian system more closely resembling the human brain is critical for the translation of basic research findings into therapeutic strategies in humans. Because the mouse (*Mus musculus*) shares the complex genomic and neuroanatomical organization of mammals and there is a wealth of molecular tools that are available to manipulate gene function in mice, the mouse has become the primary model for research into the genetic basis of mammalian memory. Another major advantage of mouse research is the ability to examine *in vivo* electrophysiological processes, such as synaptic plasticity and neuronal firing patterns during behavior (e.g., the analysis of place cell activity). The focus on mouse models for memory research has led to the development of sophisticated behavioral protocols capable of exploring the role of particular genes in distinct phases of learning and memory formation, which is one of the major accomplishments of the past decade. In this chapter, we will give an overview of several state of the art genetic approaches to study gene function in the mouse brain in a spatially and temporally restricted fashion.

I. CONVENTIONAL GENE TARGETING STRATEGIES

In 2007, the Nobel Prize in physiology or medicine was awarded to Mario Capecchi, Martin Evans, and Oliver Smithies for the development of specific gene modification techniques and stem cell technology that led to the creation of the first “knockout mouse.” To date, thousands of knockout mouse models have been developed that have revolutionized our understanding of the molecular mechanisms responsible for various biological processes, including the signaling pathways underlying memory storage. Recently, the mouse knockout project (KOMP, see <http://www.knockoutmouse.org>), based on reverse genetics (from gene to phenotype), was established to produce and phenotype knockouts for all mouse genes. In addition to knockout mouse models, knock-in mouse models have been used as an alternative approach to determine the function of specific functional domains within proteins of interest. Knockout models are based on the deletion of an entire gene leading to complete inactivation, but knock-in models are based on a subtle alteration of a targeted gene sequence, for example, by introducing one or more point mutations to alter specific functional domains of proteins rather than deleting the entire protein. One example of the successful use of a knock-in strategy, was the recent discovery that a single transcription factor-binding domain of the CREB-binding protein (CBP), a transcriptional coactivator, is pivotal for the formation of long-term memories (Wood *et al.*, 2005, 2006). Knock-in mice also enable researchers to model disease-causing mutations found in human patients.

As an alternative to knockout and knock-in mutations, transgenic methods have been used for more than 25 years as a reverse genetic approach to study the role of particular gene products in biological processes. The first transgenic mouse was generated in 1982 by Palmiter and

colleagues via injection of a metallothionein-growth hormone fusion gene directly into the pronucleus of a fertilized single cell mouse embryo. This fusion gene incorporated into the genome and was subsequently expressed in essentially every cell in the embryo throughout development and adulthood (Palmiter *et al.*, 1982). Such a ubiquitous expression pattern could make the functional interpretation of specific gene functions difficult due to various confounding side effects even including premature death. For example, the role of the NR1 subunit of the *N*-methyl-D-aspartate receptor (NMDAr) in learning and memory could not be unraveled using a classical knockout approach because mice lacking the NR1 subunit of the NMDAr die as neonates (Forrest *et al.*, 1994). In another example, initial studies on mice with a mutation in the tyrosine kinase gene *fyn* revealed that loss of the *fyn* gene resulted in deficits in long-term potentiation (LTP, the persistent increase in synaptic strength following high-frequency stimulation of a chemical synapse) and spatial learning (Grant *et al.*, 1992). However, *fyn* deletion also caused developmental abnormalities in various brain structures, for example, an increased number of cells in hippocampus area CA3 and the dentate gyrus (DG). Similarly, studies with mice exhibiting a mutant form of the α -calcium-calmodulin-dependent kinase II (α -CaMKII), a synaptic protein that is strongly enriched in forebrain neurons including those in the hippocampus, indicated that loss of α -CaMKII-function resulted in impaired spatial memory and LTP (Silva *et al.*, 1992a,b). However, simultaneous studies indicated that loss of functional α -CaMKII resulted in behavioral abnormalities unrelated to memory formation including a decreased fear response and increased aggression (Chen *et al.*, 1994). Another risk with conventional gene knockout is that loss of a particular gene may result in compensation by other related genes. For example, loss of the alpha and delta isoform of the cAMP response element binding protein (CREB) results in a compensatory upregulation of the beta isoform of CREB (Blendy *et al.*, 1996). This beta isoform is expressed in low levels in wild-type mice, but is dramatically upregulated in mutant mice. The fact that beta CREB is expressed in several but not all regions where CREB function has been implicated and that the specific phenotype observed in these mutants was different from the phenotype observed in mice expressing a dominant-negative mutant form of CREB (Struthers *et al.*, 1991), suggest that the beta isoform at least partly compensates for the loss of the alpha and delta isoforms (Blendy *et al.*, 1996; Gass *et al.*, 1998). Thus, although the initial approach to study the role of particular genes were groundbreaking, the interpretation of the phenotypes of these first mutant mice remain difficult not just for the molecular reasons described here, but also for difficulties in interpreting the limited behavioral experiments that were carried out in the initial studies (Deutsch, 1993).

To circumvent these problems, two major improvements in experimental approach were needed. The first was to avoid the occurrence of developmental and nonspecific abnormalities in mice with permanent and global gene manipulations. Thus, more sophisticated molecular approaches were needed to allow researchers to affect gene function in a temporally and spatially restricted fashion, as opposed to constitutively and globally. The second major issue was the lack of sensitive behavioral paradigms that could distinguish between learning-specific and learning-unrelated phenotypes. In human patients with brain lesions, this is done by using “double dissociation approaches.” For example, Bechara and colleagues (1995) showed that a patient with bilateral damage to the amygdala failed to acquire conditioned autonomic responses to visual or auditory stimuli, although the patient could learn which visual or auditory stimuli were paired with the unconditioned stimulus (indicative for a normal declarative memory). In contrast, a patient with bilateral hippocampal lesions was impaired in the formation of declarative memories but acquired the autonomic conditioning in the same task. In mice, contextual and tone-cued fear conditioning are used in parallel to suggest whether the manipulation of a specific gene affects either hippocampal function or amygdala function. Contextual fear conditioning relies on both the hippocampus and amygdala. The hippocampus is required to make an association between the context (e.g., the shock box, the conditioned stimulus) and a negative stimulus (e.g., a mild electrical shock, the unconditioned stimulus).

If the mice make this association, then they will show freezing behavior when reexposed to the same context. Freezing behavior, defined as the lack of movement except for respiration, is initiated by the context-shock association and mediated by the amygdala. Tone-cued fear conditioning is a paradigm in which mice make an association between a tone, that is presented during training and a mild foot shock (LeDoux, 2000). In contrast to contextual fear conditioning, tone-cued fear conditioning does not require the hippocampus. Using both behavioral paradigms, Abel *et al.* (1997) showed that mice with reduced activity of the cAMP dependent protein kinase (PKA) in forebrain neurons were impaired in contextual fear conditioning but not in tone-cued fear conditioning, indicating that memory defects in this mouse line are likely due to defects in hippocampal PKA function. Although tone-cued fear conditioning in combination with contextual fear conditioning provides strong support for a hippocampal site of biochemical malfunction, this is not a true double dissociation as described by Bechara and colleagues (1995). This is particularly due to the lack of sophisticated behavioral paradigms and the inability to finely regulate spatial resolution of gene manipulation (e.g., hippocampus or amygdala specific manipulations).

In addition to the generation of mutant mice using reverse genetics, the use of forward genetics (from phenotype to gene) has been a successful approach to uncover genes that drive the molecular mechanism of specific behaviors. For instance, forward genetic screens using the point mutagen *N*-ethyl-*N*-nitrosourea have yielded great success in the discovery of genes that control certain behaviors. This approach has been especially successful in elucidating genes that control circadian rhythms, so-called “clock genes” (Takahashi *et al.*, 2008), because highly defined behavioral protocols exist to determine the activity profiles of mice with great precision and high throughput. Applying this approach to learning in mice has been more difficult, in part because of the behavioral complexity of learning tasks and because of the fact that animals cannot be repeatedly tested in learning paradigms (Reijmers *et al.*, 2006).

II. SPATIALLY RESTRICTED GENE MANIPULATION

To avoid side effects induced by overall gene manipulation and to achieve a better understanding of a particular gene function in a specific brain region, more sophisticated methods have been developed that result in spatially restricted manipulation of a gene. One powerful approach to locally manipulate gene function uses gene promoters to restrict transgene expression. For example, the use of promoters like Nestin (Cheng *et al.*, 2004), PrP (Fischer *et al.*, 1996), and neuron-specific enolase (Forss-Petter *et al.*, 1990) result in transgene expression throughout the brain but not in other parts of the body. For further restriction to specific brain regions or cell types, researchers used more specific promoters, including the α -CaMKII promoter for postnatal forebrain specific expression in excitatory neurons (Abel *et al.*, 1997; Mayford *et al.*, 1996), glial fibrillary acidic protein (GFAP) for astrocyte-specific expression (Brenner *et al.*, 1994; Casper *et al.*, 2007; Halassa *et al.*, 2009; Pascual *et al.*, 2005), proteolipid protein (PLP) oligodendrocyte-specific expression (Fuss *et al.*, 2001), and L7 for expression in cerebellar Purkinje cells and retinal bipolar neurons (Oberdick *et al.*, 1990). Large-scale screening projects have been initiated to determine the brain expression patterns of a tremendous number of specific promoters, allowing researchers to choose a promoter that targets a particular class of neurons, such as those that produce a specific neurotransmitter, or express a specific receptor. Examples of these screening projects are GENSAT (<http://www.gensat.org>) and the Allen Brain Atlas (<http://www.brain-map.org>). Although the transgenic approach allows for region-specific overexpression of a gene, it is possible that information from transgenic mouse lines may not translate into the requirement for a mutated gene or pathway in memory. Interfering with gene function through transgenesis is necessarily through overexpression of a protein, which can potentially interfere with genes other than the intended targets. Therefore, other methods have been developed that delete single genes in a tissue or cell-type specific manner as described below.

A. The Cre/loxP system

In contrast to transgenic methods that spatially restrict overexpression of an interfering transgene, the Cre/loxP system allows for the knockout of a single gene in a cell-type or site-specific manner. The Cre/loxP system is based on a phage P1-derived site-specific recombination system (Sauer and Henderson, 1988). This system is based on the ability of Cre recombinase to induce the recombination of 34 bp loxP recognition sequences. The loxP sites can be inserted into the genome of embryonic stem cells using homologous recombination, flanking one or more exons of the target gene (referred to as a “floxed” gene). Mice homozygous for the floxed gene are then crossed to a transgenic mouse line that expresses Cre recombinase under control of a spatially restricted promoter. In offspring that are homozygous for the floxed gene and carry the Cre transgene, the floxed gene will be excised. Depending on the promoter used to drive Cre expression, the loss of the floxed gene can be more or less restricted. Gu *et al.* (1994) generated the first inducible KO mice using this method by generating a T-cell specific knockout mouse for the DNA polymerase beta gene, thus showing proof of principle.

B. Investigating NMDA receptor function in specific subregions of the brain using the Cre/loxP system

The NMDAr is an ionotropic glutamate receptor that allows flow of calcium ions (Ca^{2+}) into the cell. Gating of the channel is voltage dependent and can be controlled by binding its ligand glutamate. The influx of Ca^{2+} into neurons plays a pivotal role in synaptic plasticity thought to underlie learning and memory formation. The first indication that NMDAr could play a pivotal role in the processes underlying memory storage came from a study by Collingridge and colleagues, demonstrating that NMDAr are required for the persistent change in strength of synaptic connections, a process referred to as LTP (Collingridge *et al.*, 1983). Activity-dependent changes in synaptic strength is a form of synaptic plasticity that is a model for certain types of long-term memory (Bliss and Collingridge, 1993; Hebb, 1949; Martin *et al.*, 2000). LTP in the CA1 region of the hippocampus has distinct temporal phases (Nguyen and Woo, 2003): an early transient phase (E-LTP) and a late persistent phase (L-LTP) that lasts for up to 8 h in hippocampal slices (Frey *et al.*, 1993), and for days in the intact animal (Abraham *et al.*, 1993). Both E-LTP and L-LTP in the CA1 region of the hippocampus depend on NMDAr and on the activation of kinases such as CaMKII, whereas L-LTP (but not E-LTP) shares with long-term memory, a requirement for transcription and translation as well as cAMP and PKA activity (Abel *et al.*, 1997; Duffy and Nguyen, 2003; Frey *et al.*, 1996; Scharf *et al.*, 2002; Woo *et al.*, 2000).

Three years after the study by Collingridge in 1983, Morris *et al.* (1986) showed that blocking NMDAr activity using the drug (2R)-amino-5-phosphonovaleric acid (AP5) prevents the formation of memories for the location of a submerged platform in the Morris water maze, based on visual cues in the room. In addition, the authors showed that application of AP5 impairs hippocampal LTP *in vivo* demonstrating the causal role of the NMDA receptor in hippocampus-dependent memory formation. Although these pharmacological studies indicate a pivotal role of the hippocampus in the formation of long-term memories, it could not be excluded that AP5 in the study by Morris *et al.* also affected NMDAr function in regions other than the hippocampus that are also implicated in memory formation, such as the neocortex (Morris *et al.*, 1986).

To analyze the role of the NMDAr specifically in the hippocampus, a genetic approach was needed that could tightly control the loss of NMDA receptor function to a specific cell type or group of cells. The discovery of the gene that encoded the NR1 subunit, a crucial subunit of the NMDA receptor (Moriyoshi *et al.*, 1991; Nakanishi, 1992) enabled researchers to

manipulate NMDAr function genetically in a spatially restricted manner, because loss of this single receptor subunit prevents the expression of functional NMDAr.

The Tonegawa laboratory successfully applied the Cre/loxP system to study the NMDAr in distinct hippocampal subregions in learning and memory formation. They first generated several mouse lines that expressed Cre recombinase under control of the α -CamKII promoter to drive the expression of Cre recombinase specifically in neurons of the forebrain postnatally (Fig. 1.1A, Tsien *et al.*, 1996a). Several of the different founder lines showed variation in the expression pattern of Cre recombinase 3 weeks postnatally, despite the fact that the same promoter (α -CaMKII) was used to drive Cre expression (Tsien *et al.*, 1996a). This variation in Cre expression is likely due to distinct sites of transgene insertion in the genome. Some founders expressed Cre recombinase in all forebrain regions, including the three major hippocampal regions (e.g., areas CA1, CA3, and the DG), striatum and cortex, but other lines had a more restricted expression pattern. One of the founder lines was of particular interest because Cre recombinase expression was restricted to area CA1 of the hippocampus at the age of 3 weeks. This very narrow Cre expression allowed the Tonegawa laboratory to examine the role of the NMDAr specifically within the hippocampal CA1 area for learning and memory formation as well as synaptic plasticity.

By crossing this CA1-specific Cre expressing line with a floxed NR1 line, Tsien and colleagues (1996b) generated a mutant mouse in which the NR1 subunit was lost specifically in the CA1 region of the hippocampus at 3 weeks of age (Fig. 1.1B). These mice lacked NMDAr-mediated synaptic currents and LTP at CA1 synapses (Fig. 1.1C). Behavioral studies showed that loss of NMDAr function caused difficulties with learning and remembering the location of a submerged platform in the Morris water maze using distal cues (Fig. 1.1D and E). The mutants were not impaired in a nonspatial version of the same task that does not require the hippocampus, indicating that specifically hippocampus-dependent memory processing was affected by loss of NMDAr in area CA1 of the hippocampus (Tsien *et al.*, 1996b). In a study using the same mutant mice, McHugh *et al.* (1996) determined whether loss of CA1 NMDAr affected the properties of particular hippocampal cells called place cells: cells that exhibit a high rate of firing whenever an animal is in a specific location in an environment corresponding to the cell's "place field." Place cells were first described by O'Keefe and Dostrovsky (1971) and are thought to be important for spatial mapping of novel environments and thus pivotal for the formation of long-term spatial memories. They found that mice lacking NMDAr in area CA1 of the hippocampus had reduced spatial specificity of individual place fields (e.g., place cells activity was more widely distributed throughout the environment). Thus, their study indicated that CA1 NMDAr-mediated synaptic plasticity is necessary for the generation of a proper representation of space.

Using a similar approach, the Tonegawa laboratory next focused on area CA3 of the hippocampus, an area that is one of the main input sites for area CA1. To restrict the loss of the NMDAr to area CA3, Nakazawa and colleagues (2002) utilized a transgenic mouse line in which Cre expression was driven by the KA1 receptor (Fig. 1.1A). Expression from this promoter is specific for area CA3 and the DG in adult mice (Kask *et al.*, 2000). In mice that were homozygous for the floxed NR1 subunit and positive for the Cre transgene, loss of the NR1 subunit was restricted to area CA3 of the hippocampus (Nakazawa *et al.*, 2002, 2003). Electrophysiological studies on these mice revealed that loss of the NR1 subunit in area CA3 impairs LTP in the recurrent collaterals (input from CA3 neurons to other CA3 neurons which might serve as an auto associative network) (Fig. 1.1F). As a consequence of the loss of NMDAr in area CA3, mice had deficits in a process called pattern completion (the retrieval of memories when only a fraction of the visual cues are presented) (Fig. 1.1G and H), and for memory acquisition of a one-time experience (Nakazawa *et al.*, 2003). In line with these observations, Nakazawa and colleagues (2003) showed that place fields in these mice were significantly

larger (e.g., less specific for a spatial location) in mutant mice as compared with control mice. The DG is the third major region of the hippocampus that is part of the tri-synaptic circuit together with area CA1 and CA3. Making use of the proopiomelanocortin (PomC) promoter which is activated in the DG (Fig. 1.1A), the Tonegawa laboratory was able to investigate the role of DG NMDARs in perforant path LTP (Fig. 1.1I). McHugh *et al.* (2007) found that mice without NMDARs in the DG could not distinguish between two relatively similar contexts. Thus, they concluded that NMDARs in the DG are required to distinguish relatively similar environments, a process referred to as pattern separation (Fig. 1.1K). Together these studies showed that the same gene (NR1) was required for different aspects of memory formation in different subregions of the brain, an important conceptual advance made possible by the spatially restricted Cre/LoxP deletion system.

In addition to these studies focusing on the role of the NMDAR in memory storage, numerous other researchers used the Cre/LoxP system to study neurobiological processes. One of the ways that this system has been used is to ablate specific groups of neurons by crossing a Cre-expressing line with a second line that carries the loxP-STOP-loxP-IRES-diphtheria toxin fragment A (DTA). Cre expression mediates the excision of the floxed STOP codon leading to the expression of DTA resulting in cell death (Brockschneider *et al.*, 2004). Numerous mouse lines with floxed alleles as well as mutants that express Cre recombinase under control of a wide variety of promoters are becoming available (See for instance:

<http://www.mshri.on.ca/nagy/Cre-pub.html>, <http://www.jax.org>, GENSAT

<http://www.gensat.org>, and the Allen Brain Atlas <http://www.brain-map.org>). The

combination of distinct Cre expression and floxed alleles allows researchers to generate unique mouse models to study the role of specific genes in a distinct subset of cells.

III. THE USE OF DOMINANT NEGATIVE INHIBITORS TO MANIPULATE GENE FUNCTION

As mentioned in the previous paragraphs, compensatory mechanisms hampered the interpretation of early mutant mouse models. Pharmacological inhibition of the activity of the cAMP-dependent PKA impaired LTP in hippocampal slices and suggested a role for PKA in memory storage (Frey *et al.*, 1993; Huang and Kandel, 1994). However, analyses of knockout mice lacking the RI β isoform of PKA showed no deficits in hippocampal LTP, but rather impairments in long-term depression (the weakening of neuronal connections) and a compensatory upregulation of the RI isoform (Brandon *et al.*, 1995). Thus, while the pharmacological approaches indicated an important role for PKA in memory storage, genetic studies failed to confirm the pharmacological observations.

A novel approach to determine the role of PKA in memory storage was taken by Abel *et al.* (1997). They generated a dominant negative form of the regulatory subunit of PKA referred to as R(AB). This was an inhibitor of both types of PKA catalytic subunits due to mutations in the two cAMP binding sites on the regulatory subunit (Clegg *et al.*, 1987; Ginty *et al.*, 1993). The advantage of using a dominant negative approach is that it is effective when multiple genes encode the same enzyme (as is the case for PKA) or when many homologues of the same gene exist (e.g., the coactivators CBP and P300, Vo and Goodman, 2001). Because the authors used the α -CaMKII promoter to drive expression of the transgene, the expression of the transgene was restricted to postnatal fore-brain neurons (Fig. 1.2A) thereby avoiding developmental side effects or compensatory mechanisms. Using mice expressing R(AB) in forebrain neurons, Abel *et al.* (1997) examined the role of PKA in LTP in area CA1 of the hippocampus. They found that reduction of PKA activity impaired LTP (Fig. 1.2B). The deficit in LTP was paralleled by behavioral deficits in spatial memory and in long-term, but not short-term memory for contextual fear conditioning (Fig. 1.2C), thus proving that PKA plays a critical role in the consolidation of long-term memory. In a later study, Rotenberg and

colleagues (2000) showed that suppression of PKA activity in forebrain neurons impaired long-term place cell stability (Fig. 1.2D), an effect that could account for the memory deficits seen in these mice (Abel *et al.*, 1997).

IV. CONDITIONAL MANIPULATION OF GENE FUNCTION

A. The tTA system

Memory has several stages including acquisition, consolidation, and retrieval. These processes require the transient activation of specific cellular signaling pathways in particular brain regions. Previously formed memories can be modified further through reconsolidation and performance can change during extinction trials while the original memory remains intact (Abel and Lattal, 2001). To determine the role of specific genes in these different stages of memory, genetic-based systems were needed with a higher temporal resolution. To this end, the laboratory of Hermann Bujard developed a 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 antibiotic. By fusing this promoter with the activation domain of virion protein 16 (VP16) of the herpes simplex virus *tetR* was converted to a transcriptional activator in eukaryotic cells. The product of this fusion protein, called the tetracycline-regulated transactivator (*tTA*), activates the transcription of transgenes containing the tetracycline operator (*tetO*). Importantly, the binding *tTA* to the *tetO* promoter was suppressed in presence of tetracycline or doxycycline (dox).

The laboratory of Eric Kandel used this newly developed *tTA* system to generate mice in which a transgene could be “conditionally” expressed in the mouse brain. The use of this system had the major advantage that transgene expression could be suppressed by administration of dox in the food or drinking water of animals. To study the role of α -CaMKII in learning and memory, they conditionally expressed a constitutive active form of α -CaMKII (Mayford *et al.*, 1996). They first developed a mutant mouse that expressed *tTA* under control of the α -CaMKII-promoter. This mutant was crossed to a second mutant in which the *tetO* promoter regulated expression of a mutant form of α -CaMKII that was calcium-independent, due to point mutation of a critical residue, Asp286. In bi-transgenic offspring, expression of the constitutive active form of CaMKII resulted in the loss of hippocampal LTP induced by 10 Hz stimulation, as well as the loss of spatial memory. Importantly, delivery of dox resulted in the suppression of transgene expression, leading to the restoration of normal LTP and memory (Mayford *et al.*, 1996). Thus, this pivotal study showed that the loss of LTP and spatial memory was due to adult-specific expression of a transgene and not to developmental side effects. In the years thereafter, the *tTA* system has been used by many other laboratories to conditionally manipulate molecular signaling pathways in specific tissues or cell types.

Conditional genetic systems opened a new era of research that was not possible using constitutive transgenic or knockout approaches. Investigators could now establish the role of specific transgenes in memory retrieval, reconsolidation as well as memory extinction. For instance, the Abel laboratory used this conditional strategy to determine the role of PKA in the extinction of previously acquired contextual fear memories. For this purpose, they generated bi-transgenic mice in which R(AB) expression was controlled by the *tTA* system (Fig. 1.2E), thus enabling them to suppress R(AB) expression under dox conditions (Fig. 1.2F). During development and continuing through training in the contextual fear conditioning paradigm, mice were fed dox to suppress the expression of the R(AB) transgene. After training, the animals were removed from dox, initiating the expression of the PKA inhibitor. After 4 weeks, the rate of extinction of the previously acquired contextual fear memory was assessed. The researchers found that reducing PKA activity selectively during the extinction phase slowed extinction, suggesting that PKA functions as a constraint on the erasure (erasure or formation

of extinction memory) of previously acquired fear memories (Isiegas *et al.*, 2006). Using the same conditional approach, a later study by Havekes *et al.* (2008) demonstrated that conditional suppression of forebrain calcineurin (also known as protein phosphatase 2B) facilitated the extinction of contextual fear memories. This was of particular interest because PKA and calcineurin target similar substrates and function antagonistically. Thus, the conditional tTA system has allowed analysis of gene function during distinct types of memory formation.

A second major use of this conditional system has been to manipulate gene function selectively during development, while leaving it undisturbed during adulthood (by putting the mice on dox starting from 2 months after birth). In a recent paper by Kelly and colleagues (2008), overexpression of the G-protein subunit *Gas*, a protein subunit that stimulates adenylyl cyclase activity is genetically linked to schizophrenia (Avissar *et al.*, 2001; Memo *et al.*, 1983; Minoretti *et al.*, 2006), selectively during development induced several schizophrenia-related phenotypes. They found impairments in spatial learning and memory formation in adult mice using the Morris water maze when the transgene was only expressed during development. In addition, they found that the ventricles of the mice were enlarged, while the ventral and dorsal striatum size were reduced as a consequence of overexpression of the *Gas* subunit selectively during development. These experiments, along with others that examined anxiety-related behaviors (Gross *et al.*, 2002), demonstrate that certain endophenotypes of psychiatric disorders are likely the result of alterations in the function of neuronal circuits that occur during development.

In addition to the use of the tTA system to study the role of specific genes in neurons, the laboratory of Philip Haydon has used this system to determine the role of astrocytes in synaptic plasticity, modulation of sleep homeostasis, and the cognitive consequences of sleep loss. The laboratory first generated a transgenic mouse line in which tTA was driven by the astrocyte-specific GFAP to obtain astrocyte-specific expression of tTA (Pascual *et al.*, 2005). Next, they generated a transgenic mouse that expressed the cytosolic portion of the *N*-ethylmaleimide-sensitive factor attachment protein (SNARE) domain of synaptobrevin 2, under control of the tetO promoter. A previous study by the same laboratory showed that overexpression of this portion of the SNARE protein blocks gliotransmission, another example of using a dominant negative approach (Zhang *et al.*, 2004). By breeding the GFAP-tTA line and tetO-SNARE line they were able to produce double transgenic mice with vesicular transmission blocked selectively in astrocytes. They found that gliotransmission critically regulated synaptic plasticity and had a previously unsuspected role in sleep regulation. Inhibition of gliotransmission attenuated the accumulation of sleep pressure, the drive to go to sleep, and prevented cognitive deficits associated with sleep loss, thus providing evidence that astrocyte signaling regulates the relationship between sleep and memory formation (Halassa *et al.*, 2009).

B. A novel form of the tTA system: The rtTA system

Although the tTA system has great advantages in comparison with constitutive transgenic systems, this system requires that mice be kept on dox during breeding and at least the first weeks after birth to suppress transgene expression during development. To avoid this long period of dox exposure which could lead to developmental side effects and create an imbalance of the intestinal flora resulting in diarrhea and in a smaller number of animals, a reversed version of tTA was developed by the Kandel group called rtTA. In case of rtTA, dox treatment results in activation of a tetO-driven transgene, rather than its suppression (1998). Using this novel system, mice had to be kept on dox for a minimum of 1 week prior to the start and during the course of an experiment. Using this modified form of tTA, Mansuy and colleagues (1998) showed that forebrain-specific overexpression of the catalytic subunit of calcineurin, the calcium-dependent protein phosphatase calcineurin, impairs the formation of spatial memories

and an intermediate form of LTP. In agreement with this finding, Malleret and colleagues (2001) found that conditional overexpression of an inhibitor of calcineurin facilitates memory formation and enhanced LTP. This phenotype was reversed when animals were taken of dox. In a more recent study, the laboratory of Isabelle Mansuy elucidated that conditional inhibition of a downstream target of calcineurin, the protein phosphatase 1 (PP1) enhances the formation of spatial memories using various learning tasks as well as LTP and that this enhancement could be reversed by taking the animals of dox (Genoux *et al.*, 2002).

V. PHARMACOGENETIC APPROACHES

A. Tamoxifen-controlled gene manipulation

The application of the Cre/loxP system in combination with promoters that are expressed selectively during adulthood allowed researchers to knockout genes during adulthood avoiding developmental side effects or even during specific stages of memory, but the temporal resolution was still relatively poor. To improve this temporal resolution, Cre was fused to the mutated ligand-binding domain of the human estrogen receptor (ER) (Feil *et al.*, 1997; Metzger *et al.*, 1995a,b). As a consequence of the mutation, the receptor does not bind endogenous estradiol, but is highly sensitive to tamoxifen. Under conditions without tamoxifen, the fusion product (referred to as CreERT2) is trapped in the cytoplasm by binding to heat shock proteins (Brocard *et al.*, 1997). By injections of tamoxifen, the fusion product is released from the complex and translocates to the nucleus and catalyzes the recombination of loxP sites resulting in deletion of the flanked gene (Erdmann *et al.*, 2007; Metzger *et al.*, 1995b). The use of CreERT2 has two substantial advantages: (1) gene manipulation can be restricted to distinct populations of cells via the local delivery of tamoxifen and (2) application of tamoxifen causes Cre-mediated gene excision on the order of days (Brocard *et al.*, 1997). Imayoshi *et al.* (2008) used this system to study the role of continuous neurogenesis in the structural and functional integrity of the adult forebrain. They generated transgenic mice in which the nestin promoter was used to drive CreERT2 expression in neural stemcells (Imayoshi *et al.*, 2006) and crossed this transgenic line with a second transgenic line in which carried the loxP-STOP-loxP-IRES-DTA, driven by the neuron-specific *enolase2* gene. Importantly, tamoxifen treatment in bi-transgenic adult mice led to the deletion of the STOP codon in DTA cassette in neuronal stem cells. As a result, DTA was expressed as soon as cells started to differentiate into neurons, thereby killing them. This resulted in impaired neurogenesis and the specific loss of granule cells in the olfactory bulb and DG of the hippocampus. Thus, rather than deleting a gene, in this study the Cre/loxP system was used to cause expression of a gene as opposed to knocking one out. Behavioral screening of transgenic mice that received tamoxifen indicated that loss of these granule cells resulted in impairments in spatial memories (as indicated by deficits in the retention test of the spatial version of the Morris water maze and Barnes maze) and in contextual memories (Imayoshi *et al.*, 2008). These studies by Imayoshi *et al.* (2008) demonstrated that adult neurogenesis is required for the modulation and refinement of the existing circuits in the DG important for hippocampus-dependent memory formation.

The tamoxifen-inducible regulation conferred by fusion of the ERT domain onto a protein of interest has also been applied to tightly control the time of transcription factor activation during memory formation. To temporally manipulate functioning of the cAMP-response element-binding protein (CREB, a transcription factor), Kida and co-workers (2002) fused an α -CREB isoform with a mutation in the S133 site (a form of CREB that cannot be activated by phosphorylation of the S133 site) to a ligand-binding domain of the mutant human ER. In the absence of tamoxifen, the fusion protein is inactive (Feil *et al.*, 1996). However, administration of tamoxifen activates the inducible CREB-repressor fusion protein (CREBIR), allowing it to compete with endogenous CREB and disrupt cAMP-responsive element (CRE)-mediated transcription (Kida *et al.*, 2002). Using this inducible and reversible CREB repressor system, they showed that CREB is essential for the consolidation of long-term conditioned fear

memories. Administration of tamoxifen 6 or 12 h, but not 24 h or 30 min before fear conditioning training impaired memory formation indicated by reduced freezing levels during the 24 h retention test. Importantly, the finding that CREBIR mice administered tamoxifen more than 12 h prior to fear conditioning showed no deficit in subsequent freezing during the 24 h retention test demonstrated that the impairment produced by tamoxifen in CREBIR mice is behaviorally reversible.

Using a similar approach, Li *et al.* (2007) generated an inducible and reversible mutant mouse model to study the role of the “Disrupted-in-schizophrenia 1” (DISC1) gene, which is thought of playing a pivotal role in the development of schizophrenia pathogenesis. To do this, they generated mice expressing DISC1-cc, a dominant negative form of the DISC1 protein, under control of the α -CaMKII promoter, which lead to expression of the transgene selectively in forebrain neurons. The DISC1-cc protein was fused to the mutant ER, which can be activated by tamoxifen. Without the presence of tamoxifen, the DISC1-cc transgene is sequestered into inactive complexes by heat shock chaperone proteins. When tamoxifen is present, DISC1-cc is released from the ligand-binding domain and competes with wild-type DISC1 protein for the binding of Nudel and Lis1, genes that regulate several aspects of brain development. However, the effect of tamoxifen is transient: as soon as tamoxifen is metabolized DISC1 signaling returns to normal. This inducible and reversible transgenic system enabled Li and co-workers to study the impact of disruption of DISC1 function on specific time points during brain development. Their study shows that induction of a mutant DISC1 protein early in postnatal development is sufficient to impair spatial working memory and other depressive like traits that parallel changes associated with schizophrenia-related DISC1 sequence variations in humans. Thus, with these studies, Kida *et al.* (2002) and Li *et al.* (2007) demonstrated the power of using tamoxifen-inducible transgenic systems to study complex cognitive processes.

B. Pharmacogenetic regulation of neuronal excitability

In recent years, new technologies have been developed to control the activity of genetically restricted neural populations with high temporal resolution. One of the pharmacogenetic approaches used to suppress neuronal activity, through the enhancement of chloride currents, is the expression of the Ivermectin-gated chloride channels from *C. elegans*. Lerchner and colleagues (2007) first coexpressed the two subunits (GluCl α and GluCl β) that together form the Ivermectin-gated chloride channel unilaterally in the striatum of rats using viral injection. Next, they tested whether unilateral activation of the chloride channels in the striatum using Ivermectin treatment perturbs of rotational behavior induced by amphetamine. As expected, mice in which the chloride channels were activated in one hemisphere showed a marked increase in the net number of rotations towards the injection side as a consequence of the imbalance of neuronal activity between the striatal regions in the two hemispheres. Wild-type animals did not show any preference for the direction of the rotations. After 4 days, the effect of Ivermectin was fully reversed. Thus, with this study the authors show that Ivermectin-gated chloride channels can be used to temporally inactivate a restricted population of cells over a relatively short time period.

An alternative approach to inhibit neuronal activity was developed by Tan *et al.* (2006). They used the *Drosophila* allatostatin receptor to reduce neuronal activity in a specific population of cells. Binding of allatostatin to its receptor results in the opening of the G-protein coupled inward rectifier potassium channel (GIRK) leading to hyperpolarization of the cell. Gosgnach *et al.* (2006) used the allatostatin receptor to temporally block the activity of V1 motor neurons to define the function of these neurons in motor behavior. They found that V1 motor neurons regulate the speed of vertebrate locomotor movements.

A third approach to inhibit neuronal function is via manipulation of GABAA receptor activity. GABAA receptors can be positively modulated by the drug zolpidem. Application of this drug results in the facilitation of GABA receptor-mediated transmission, inhibiting excitatory responses (thereby reducing neuronal activity). Wulff and colleagues (2007) manipulated the sensitivity of the GABAA receptor by first generating a line of mice that expressed a mutated gamma2 subunit of the GABAA receptor that is insensitive to zolpidem. Next, they reintroduced the wild-type subunit in a genetically restricted group of neurons by using Cre recombinase to swap the wild-type subunit in place of the mutant allele to restore zolpidem sensitivity. Bath application of zolpidem in cerebellar slices of mice expressing the wild-type receptor selectively in Purkinje cells resulted in the facilitation of inhibitory postsynaptic currents in cerebellar Purkinje cells. Intraperitoneal injections of zolpidem in these mutant mice resulted in impairments in motor coordination as measured using the rotarod task (Wulff *et al.*, 2007).

An alternative approach to the methods described above is the application of molecules for inactivation of synaptic transmission (MISTs). MISTs are modified presynaptic proteins that interfere with the synaptic vesicle cycle when cross-linked by small molecule “dimerizers” (Karpova *et al.*, 2005). Karpova and colleagues (2005) showed that MISTs based on VAMP2/Synaptobrevin and Synaptophysin could rapidly (e.g., within 10 min) and reversibly inhibit synaptic transmission. As a proof of principle, they expressed the MISTs selectively in Purkinje neurons. Mice were acquainted with the rotarod task in the absence of dimerizer. Next, they injected dimerizer in the lateral ventricle during or following the acquisition of the task. Rotarod performance was significantly impaired in the presence of the dimerizer. The effect was fully reversed 36 h after the injection. Thus, with this study, they show that MISTs can be successfully used to specifically perturb the function of specific neuronal circuits *in vivo* with temporal precision in the order of hours.

In summary, numerous new sophisticated methods are emerging that permit temporary reduction in the activity of a genetically restricted group of cells and demonstrate that acute silencing of selected populations of neurons can be used to elucidate their function in well-defined behaviors. However, these techniques cannot be used to elucidate the function of specific cellular signaling pathways in learning, memory formation, and its retrieval, since these methods regulate neuronal activity rather than manipulating a specific intracellular signaling pathway. Thus, specific pharmacogenetic approaches are needed that rapidly modulate specific intracellular signaling processes within subsets of neurons.

C. Targeting of intracellular signaling pathways using pharmacogenetic approaches

Biochemical processes within neurons are activated with a time course of minutes to hours during memory storage. Further, distinct biochemical processes underlie each of the different stages of memory including acquisition, consolidation, and retrieval (Abel and Lattal, 2001). Many studies have tried to unravel the temporal dynamics of distinct cellular signaling pathways using pharmacological approaches. Although these approaches allow for fast manipulation of cellular signaling pathways, they are not specific for distinct cell types. To circumvent this problem, novel tools have been generated that combine the cell type and regional specificity possible with transgenic techniques together with the high temporal resolution needed to activate specific molecular pathways in distinct groups of cells.

The Abel laboratory developed such a tool to transiently manipulate the cAMP pathway specifically in neurons by taking advantage of heterologous G-protein-coupled receptors found in invertebrates (Isiegas *et al.*, 2008). They expressed a *Gas*-coupled *Aplysia* octopamine receptor (Ap oa1) under control of the tTA system. Activation of the receptor leads to the activation of the Gs-signaling pathway, resulting in a rapid and transient increase in levels of the intracellular second messenger cAMP specifically in forebrain neurons due to the use of

α -CaMKII promoter to drive tTA expression (Fig. 1.3A). The use of this Ap oal had two major advantages. (1) Octopamine is present only at trace levels in the mammalian central nervous system; thus, the Ap oal is only active when exogenous octopamine is supplied. (2) Octopamine does not act on other receptors in the mammalian central nervous system; thus, expression of the Ap oal is required for an effect of exogenous octopamine on Gas signaling. LTP is facilitated in hippocampal slices from Ap oal mice after treatment with octopamine (Fig. 1.3B), and transient activation of the Ap oal *in vivo* facilitates the formation of long-term memories for contextual fear (Fig. 1.3C), and also facilitated the retrieval of previously formed memories (Fig. 1.3D). Overall, this study demonstrated the usefulness of using the octopamine receptors to manipulate specific intracellular pathways in a conditional and spatially restricted manner.

Besides the use of heterologous octopamine receptors to examine the role of distinct second messenger pathways in the mammalian system, other genetically engineered G-protein coupled receptors have been developed to combine genetic with pharmacological approaches. For example, Sweger and colleagues (2007) developed a transgenic mouse that expresses a genetically engineered receptor that is activated solely by a synthetic ligand (receptor activated solely by a synthetic ligand, RASSL). The ro1 receptor is a κ -opiod receptor (KOR) modified by replacing its second extracellular loop with the second extracellular loop of the δ -opiod receptor, such that receptor activation causes reduced cAMP-levels via the Gi-pathway. As a consequence of this modification, the affinity of this receptor for its endogenous ligands is reduced, while it maintains the ability to bind the synthetic ligand spiradoline. By conditionally expressing this receptor in astrocytes using the tTA system cells on a KOR knockout background, the authors were able to manipulate Gi-signaling specifically in glial cells while measuring changes in neuronal excitability. They found that expression of the modified KOR selectively in astrocytes induces the development of hydrocephalus (the abnormal accumulation of cerebrospinal fluid in the brain). This study shows the potency of using RASSLs that can be activated solely by synthetic drugs. Indeed, many new RASSLs have recently been developed that target specific second messenger systems *in vivo* (Conklin *et al.*, 2008), allowing for the manipulation of other G protein-coupled receptor pathways in addition to the Gi-signaling pathway.

The Tsien laboratory recently examined the role of α -CaMKII via pharmacogenetic manipulation (Cao *et al.*, 2008; Wang *et al.*, 2008). First, the authors generated mice that expressed a constitutively active form of α -CaMKII in forebrain neurons (using the α -CaMKII promoter). As a result, the overall activity of α -CaMKII is elevated in forebrain neurons, but the activity of this mutant form of α -CaMKII is inhibited by a small inhibitor molecule called NM-PP1 that does not affect any other proteins in the brain. Using this system, the Tsien laboratory could manipulate α -CaMKII activity within the range of minutes by simply injecting mice with NM-PP1 allowing them to explore the role of α -CaMKII activity levels during distinct phases of memory and LTP. They found that the initial 10 min of memory formation and LTP are sensitive to inducible genetic downregulation of α -CaMKII activity, suggesting that molecular dynamics of α -CaMKII play an important role in the representation of short-term memory during this critical time window (Wang *et al.*, 2008).

VI. OPTOGENETIC APPROACHES

Great progress has been made in understanding how neural network activity underlies the formation of memories. Many of the previously discussed approaches allow the study of involvement of activity or signaling cascades on a timescale of minutes to hours. However, neuronal and network activity works on a millisecond timescale. Thus, several research laboratories started to search for ways to manipulate neuronal activity with a time-scale of milliseconds. One successful approach used to manipulate the activity of groups of cells with

the desired time-scale is the use of caged neurotransmitters that can be released upon stimulation with light (a process called photo-uncaging). For example, Tanaka *et al.* (2008) demonstrated the necessity of protein synthesis and brain derived neurotrophic factor for spine enlargement of CA1 neurons, a process thought to underlie the strengthening of synapses, by combining two-photon photo stimulation to locally uncage glutamate in combination with synaptic stimulation. Harvey and Svoboda used two-photon uncaging of glutamate and synaptic stimulation to show that after LTP-induction in a specific spine, weak stimulation can induce strong LTP in neighboring spines (Harvey and Svoboda, 2007). The disadvantage of this approach is that it lacks cell type specificity and cannot be used *in vivo* to stimulate cells in deeper layers of the brain.

The discovery of two light-sensitive rhodopsin channels in the unicellular green alga *Chlamydomonas reinhardtii* (Nagel *et al.*, 2002, 2003; Sineshcikov *et al.*, 2002) allowed for novel ways to manipulate neural function within a time-scale of milliseconds. Channelrhodopsin-1 is a proton-gated (H^+) channel (Nagel *et al.*, 2002), whereas Channelrhodopsin-2 (ChR2) is a light-gated cation channel (Nagel *et al.*, 2003). Initial *in vitro* studies demonstrated the feasibility of using ChR2 to manipulate neural activity using light. Using lenti-viral gene delivery to express ChR2 in mammalian neurons, Boyden *et al.* (2005) showed that with a series of brief pulses of light, ChR2 could reliably mediate trains of spikes or synaptic events including excitatory and inhibitory synaptic transmission with millisecond-timescale temporal resolution. Adamantidis and colleagues (2007) applied this optogenetic tool *in vivo* to manipulate the activity of hypocretin-expressing neurons in the lateral hypothalamus (de Lecea *et al.*, 1998; Peyron *et al.*, 1998) to examine the causal relation between activity of this distinct group of neurons and arousal stability. They specifically targeted hypocretin-neurons, using a lentivirus that drives the expression of a ChR2 construct under control of the hypocretin promoter (Fig. 1.4A), in mice chronically implanted with electroencephalographic (EEG) and electromyographic (EMG) electrodes (Fig. 1.4B) to measure brain and muscle activity, respectively, to monitor the sleep/wake state of mice. A 200- μ m optical fiber was used to deliver laser light into the lateral hypothalamus of freely moving mice. They first investigated whether light stimulation facilitated protein expression of the immediate early gene FOS. They found that 10 s of 20 Hz delivered once per minute over 10 min increased the number of Fos expressing Hcrt-neurons from 25 to 65%, elegantly demonstrating that stimulation of ChR2 channels with light *in vivo* facilitates neuronal activity. Next, Adamantidis *et al.* (2007) stimulated the hypocretin-neurons that expressed the ChR2 channels with light during distinct phases of sleep (as defined by the EEG recordings). They determined the latency between the end of the photo-stimulation and the next transition to wakefulness. They found that 10 s of light stimulation with a frequency of 5–20 Hz or continuous light markedly reduced the latency to waking up from slow wave sleep (Fig. 1.4C). Similarly, photo-stimulation with a frequency of 5–30 Hz shortened the latency to waking from rapid eye movement sleep (a sleep state that is characterized by high frequency activity waves in the brain) in ChR2 expressing mice (Fig. 1.4C). Thus, using this sophisticated optogenetic approach, Adamantidis *et al.* (2007) were able to establish a causal relationship between frequency-dependent activity of a genetically defined neural cell type and specific mammalian behavior central to clinical conditions and neurobehavioral physiology. This pioneering study paved the road for future studies to determine how specific activity patterns of a genetically defined group of neural cells underlie the formation and storage of long-term memories.

VII. VISUALIZATION OF BRAIN STRUCTURE AND FUNCTION

During the twentieth century numerous new molecular techniques and approaches became available to study the role of specific genes in behavior *in vivo*. The biochemical and genetic approaches developed were groundbreaking and pushed the neuroscience field rapidly forward. Yet, there was a lack of tools that allowed for the visualization of intra- and extra-cellular

processes, including protein–protein interactions. A major step towards the development of such a tool was made by Osamu Shimomura, Martin Chalfie, and Roger Tsien by the discovery and development of the green fluorescent protein (GFP) for which they were awarded the Nobel Prize in 2008. The GFP gene was cloned and described in 1992 (Prasher *et al.*, 1992). Many researchers attempted to heterologously express GFP in various model systems without success. Martin Chalfie discovered that the original cDNA isolated by Prasher contained an inhibitory sequence preventing GFP from being expressed. By deleting the inhibitory sequence, Chalfie succeeded in expressing GFP in various model organisms including *C. elegans* leading to a publication in science in 1994 (Chalfie *et al.*, 1994). In the years thereafter, many new forms GFP were developed including various spectral variants (Heim and Tsien, 1996; Heim *et al.*, 1994, 1995; Zacharias and Tsien, 2006). In the paragraphs below, we will describe some of the most recently developed tools to visualize the processes underlying memory storage and synaptic plasticity.

A. The TetTag system

A very popular technique to determine these activity profiles of neuronal populations is to examine the expression of immediate early genes like *c-fos* using immunohistochemistry. Immunohistochemistry was first described in 1941 by Coons *et al.* (1941) (for review see LeDoux, 2000; Ramos-Vara, 2005) and relies on the binding of specific antibodies to antigens of interest. The antigen–antibody binding is visualized by histochemical reactions or fluorochromes. The development of phospho-specific antibodies that bind specifically to the activated form of a protein increased the potency of this technique. One of the most frequently used phospho-sites to map neuronal activity is the serine 133 residue of the CREB, a transcription factor that is crucial for memory consolidation. Upon phosphorylation of this site, the transcription factor CREB initiates gene transcription of CREB target genes (Mayr and Montminy, 2001). By monitoring CREB phosphorylation using immunohistochemistry, researchers have been able to monitor when and where CREB activity occurs during after learning or memory retrieval. The disadvantage of this technique is that you can only assay the activity patterns at one particular time point, making it impossible to determine the activity profiles of the same cells at different times during learning, memory formation, and its retrieval. Alternative approaches including fMRI and small animal PET currently do not yet have the desired resolution to look at individual neurons. To circumvent these problems, a novel transgenic system (called the TetTag system) was developed recently by the Mayford laboratory that allows persistent tagging of neurons activated during a time window enabling them to determine whether this subset of neurons that were activated during learning were reactivated during retrieval (Reijmers *et al.*, 2007). This system is based on the combination of two novel transgenes. First, they generated a transgenic mouse expressing tTA under control of the promoter of the immediate early gene *c-fos*. Using this transgenic line, tTA is transiently expressed in those cells in which the *c-fos* promoter is activated (Fig. 1.5A). The second part of the TetTag system consists of a transgenic mouse containing a LacZ reporter and a dox-insensitive form of tTA (tTA*) both under control of a bi-directional tetO promoter. Double transgenic mice, referred to as TetTag mice, are raised on dox. As a consequence of dox treatment, neurons do not express tTA* and tau-LacZ (Fig. 1.5A, left yellow block). The time window for tagging is opened by taking the mice of dox which will initiate the activation of the tetO promoter in those neurons in which the *c-fos* promoter is activated, resulting in the expression of tTA* and tau-LacZ in this specific population of neurons (Fig. 1.5A, white panel). This initial expression of tTA* generates a transcriptional feedback loop that maintains its own activity. By putting the mice back on dox further tagging of neurons is prevented (Fig. 1.5A, right yellow panel). Using this system, Reijmers *et al.* were able to show that neurons activated and tagged during learning are reactivated during retrieval suggesting that these neurons may encode memory itself (Fig. 1.5B–F). The TetTag system will allow this possibility to be further

tested by selective expression of tetO-regulated trans-genes in these specific neuronal populations after learning.

In a second study, the Mayford laboratory wanted to answer the question how new proteins, synthesized in the soma exert their effect on specific synapses involved in synaptic or behavioral plasticity (Matsuo *et al.*, 2008). To test the hypothesis that newly synthesized AMPA receptors are recruited to specific synapses, the Mayford laboratory first generated a mouse that expresses a GluR1 subunit, the main subunit of the AMPA receptor, fused to GFP under control of the tetO promoter and crossed this mouse line with the *c-fos*-tTA mouse line. This combination, referred to as GFP-GluR1^{c-fos}, allowed them to express the GluR1-GFP subunit in a dox regulated and neuronal activity dependent manner. Using this system, Matsuo *et al.* (2008) showed that learning induces the synthesis of GluR1 subunits that are recruited selectively to the mushroom shaped spines.

B. The Brainbow mouse

Santiago Ramón y Cajal pioneered the study of neurobiology by describing the neuroanatomical structure of neurons and their connections using Golgi's silver stain to label small numbers of neurons. Although this technique could be used to examine the connections of small groups of neurons, its usage was limited in the case of high density populations. To overcome this problem, a multicolor Golgi technique needed to be established that would allow researchers to map many neurons within a single brain section.

Livet *et al.* (2007) developed a new approach taking advantage of the Cre/LoxP system (Branda and Dymecki, 2004 see also Section II.A), that can alter gene expression by DNA insertion, deletion, or recombination. They first generated several constructs containing the DNA of different variants of fluorescent proteins which were available, including yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), red fluorescent protein (RFP), and orange fluorescent protein (OFP) (Shaner *et al.*, 2004). In these constructs, referred to as Brainbow 1.0 and 2.1, tamoxifen-induced Cre recombinase results in the excision and/or inversion of tandem DNA segments encoding the different fluorescent proteins due to the presence of incompatible loxP variants. Cells expressing the *Brainbow-2.1* construct in which Cre expression could mediate three different inversions and two excisions showed stochastic expression of all different forms of fluorescent protein upon the delivery of Cre (e.g., OFP, YFP, MFP, and RFP).

Next, they generated transgenic mouse lines in which the different Brainbow constructs were expressed under control of the regulatory elements of the *Thy1* gene, a promoter that drives high levels of expression in a variety of neurons (Caroni, 1997; Feng *et al.*, 2000). The founder mouse was mated with other transgenic lines expressing the tamoxifen-inducible Cre. Cre-mediated excision and/or inversion was achieved by giving pups a single injection of tamoxifen on the day of birth (P0). Inversion and excision recombination events led to several distinct colors due to stochastic recombination. Besides the traditional fluorescent colors, they also found the coexpression of multiple colors in individual cells. The presence of polychromatic cells was a consequence of the method used for the generation of the transgenic lines. Pronuclear injection of constructs can lead to tandem integration of multiple transgene copies (Palmiter and Brinster, 1986) and Cre mediates recombination in an independent manner in these distinct copies. This led to a mosaic color palette with up to 89 distinguishable colors in a transgenic mouse line expressing the *Brainbow-1.0* construct. The distinct color patterns of adjacent neurons allowed for visualization of cellular interactions on a large scale in regions of high neuronal density. Currently, new methods to section, image, and analyze entire mouse brains are being developed by the Jeff Lichtman and others in hopes of generating a complete map of all connections within the mouse brain.

VIII. THE CHALLENGES FACING GENETIC APPROACHES IN THE MOUSE

Over the past decade, many novel tools and methods have been generated to alter gene function *in vivo*. Although these techniques have been greatly refined to allow researchers to manipulate gene function in a spatially and temporally restricted manner, and thus avoid behavioral side effects unrelated to learning, caveats still exist that need to be considered when interpreting results using these techniques.

Leakiness of a transgene can occur due to loss of the specificity of the promoter depending on the site of integration resulting in a lack of temporal control over transgene expression. Furthermore, disruption of an unknown gene as a consequence of transgene insertion can lead to a phenotype that is partly or entirely due to loss of the unknown gene, rather than expression of the transgene itself. For that reason, multiple transgenic lines carrying the same construct should be tested. If the different mutants have the same phenotype, one can assume that the phenotype is a consequence of the gene manipulation and not the loss of an unknown gene. In case of the tTA system, this issue could be addressed by looking at the phenotype of mice in the presence of dox. Under dox conditions, transgene expression should be suppressed, thus the phenotype should be lost. If the phenotype is still present under dox conditions then this would argue that the phenotype is a consequence of the loss of an unknown gene due to the location where the transgene is inserted.

Certain promoters are more broadly expressed during development, but in a more restricted fashion during adulthood. Some studies have used these promoters to manipulate genes selectively in the regions in which the promoter is expressed during adulthood. For example, the KA1 promoter can be used to manipulate gene function specifically in hippocampus subregions (Nakashiba *et al.*, 2008; Nakazawa *et al.*, 2002, 2003), although the promoter is also activated more broadly during development (Kask *et al.*, 2000). Likewise, expression of the α -CaMKII promoter has been reported in a few studies to be restricted to area CA1 in 3-week old mice (Tsien *et al.*, 1996a), whereas expression is more widespread in adults (Nakazawa *et al.*, 2004). Thus, one should be careful to evaluate deletion patterns as a function of age, especially at the ages used for behavioral characterization.

As mentioned previously, fairly restricted expression of transgenes can be obtained using distinct promoters. For many brain regions however, no specific promoters are available or currently known. For example, no known promoters restrict gene expression to the amygdala. One way to spatially refine gene manipulation is by using two different promoters of which the expression pattern is different, but partly overlapping. As with many techniques that have been used in the study of mouse memory formation, inspiration can be found in work on *Drosophila* memory. For instance, Suster *et al.* (2004) used the combinatorial Gal4/Gal80 transgenic system to achieve ultra-precise transgene expression. The Gal4 transcriptional activator was expressed specifically in cholinergic neurons and an inhibitor of Gal4, Gal80, was expressed in a distinct but overlapping subset of neurons. This approach enabled them to reduce the expression of Gal4 from 200 to 20 neurons. Similar combinations could be made in mice by using one promoter to drive Cre expression and a second promoter to drive a construct that contains a floxed STOP codon prior to the transgene. Using this method, the STOP codon would be excised only in those regions that express both Cre and the floxed STOP-transgene constructs. This strategy will become even more enticing as a greater number of specific promoters and Cre-expressing lines become available (see for instance the GENSAT project).

The use of Cre/loxP recombination allows for more spatially restricted manipulation of genes bypassing lethal or severe developmental defects resulting from the loss of a gene in the entire organism. However, the use of Cre recombinase is not without any consequences. Several *in*

vitro studies have indicated that high levels of Cre expression alone can cause reduced growth, cytopathic effects, and even damage DNA (Loonstra *et al.*, 2001; Pfeifer *et al.*, 2001). More recently, a study by Forni and colleagues (2006) indicated that high levels of Cre expression *in vivo* in neuronal progenitor cells hampers brain development leading to microencephaly (the abnormal smallness of the head) and hydrocephaly (the accumulation of cerebrospinal fluid in the cavities and ventricles of the brain). These are clearly confounding factors that can result in learning-unrelated side effects.

An alternative method to obtain high spatial resolution is by using a virus to drive transgene expression. Rumpel and colleagues (2005) used a viral approach to examine the role of AMPA receptor trafficking in relation to learning and memory. They injected a vector that encodes the GluR1 subunit fused with GFP into the mouse amygdala. Their study showed that learning an association between a tone and the delivery of a foot shock drives GluR1-containing AMPA receptors into the synapses of a large fraction of postsynaptic neurons in the lateral amygdala. Importantly, this study indicated the potency of using viral transgene delivery to study the role of specific genes.

The tTA and rtTA system allows researchers to manipulate gene function in a conditional and reversible manner, but it should be noted that dox itself can have several side effects on behavior. Riond and Riviere (1988) showed that long-term administration of dox creates an imbalance of the intestinal flora, resulting in diarrhea and some cases even cause colitis (the inflammation of the colon). So it is important to include the relevant control group fed dox, but lacking the tetO-regulated transgene.

Another issue that makes the interpretation of gene function difficult is the confounding effect of genetic background on behavioral phenotypes (Bucan and Abel, 2002; Crawley *et al.*, 1997). The deletion of two isoforms of the transcription factor CREB may result in spatial learning and memory impairments in the Morris water maze on one strain of mice, but not another (Bourtchuladze *et al.*, 1994; Gass *et al.*, 1998; Graves *et al.*, 2002; Kogan *et al.*, 1997).

Also environmental influences can modify behavioral phenotypes. Crabbe *et al.* (1999) tested several frequently used mouse lines in three different laboratories at the exact same time of day. They subjected the different lines to several behavioral paradigms. Despite the exact same background and task parameters being used by access laboratories, behavioral findings varied significantly among the different laboratories involved.

One major challenge in the field of mammalian research is to determine which genes are really needed in which specific brain structure or system. Traditionally, a “necessity approach” is used: in which brain regions does the loss of a particular gene result in memory deficits? An alternative strategy that has frequently been used to determine the role of specific genes in memory formation is a so-called “sufficiency approach” examining in which brain region or regions rescue of the function of a specific gene is sufficient to rescue memory deficits. This approach has begun to be developed in *Drosophila*, but is largely absent from mouse experiments. By first knocking out *rutabaga* everywhere, and then selectively expressing the wild-type *rutabaga* adenylyl cyclase specifically in the kenyon cells of mushroom bodies of the *Drosophila* brain, Zars *et al.* (2000) showed that this restricted expression of the *rutabaga* adenylyl cyclase is sufficient for the formation of olfactory short-term memories. This is the sort of powerful demonstration of function and site of function that mouse behavioral genetics is currently lacking.

Despite the concerns and limitations, the temporal and spatial resolution achieved in genetic approaches to mouse memory over the past decade have been critical for refining our understanding of the molecular and cellular basis of memory. A major remaining challenge is

to refine these temporal and spatial resolutions. Combinatorial approaches using distinct, refined promoters to drive multiple components, such as combining promoter1-floxed Stop-tTA transgenes with promoter2-cre, provide one potential avenue for spatial refinement, as does a similar strategy using viral injection of Cre-expressing constructs in mice in which a broad promoter drives a construct in which a transgene is preceded by a STOP codon flanked with loxP sites.

The wealth of Cre lines being developed as part of the GENSAT BAC transgenic project (<http://www.gensat.org>) should soon provide an expanded repertoire of such region-specific Cre lines and the use of tamoxifen-inducible Cre for many of these BAC lines will expand their usefulness. This will be especially true if the GENSAT lines unearth more interesting activity-regulated promoters with more selective induction patterns than, for example, the immediate early gene *c-fos*. The temporal resolution achieved with light-activated channels is ideal, but this strategy only addresses cellular activity not biochemistry. The development of biochemical manipulations that could parallel this temporal precision would be a dramatic advance for the study of memory processes guided by biochemical cascades that function on timescales of seconds to minutes.

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Deletion of the NMDA receptor NR1 subunit in subregions of the hippocampus

Figure 1.1.

The deletion of NMDA receptor NR1 subunit in specific subregions of the hippocampus. (A) Loss of NMDA receptor function in specific hippocampal areas can be achieved by using specific promoters to drive Cre recombinase. (B) *In situ* hybridization of *NMDAR1* mRNA from wild types (left panels) and subregion specific NR1 knockout mice (right panels). (C) CA1-restricted deletion of the NR1 subunit impairs LTP in the Schaffer collateral pathway (wild-types open symbols, mutant closed circles), (D) CA1-specific knockout mice are impaired during training in the spatial version of the Morris water maze (wild-types open symbols, mutant closed circles), and (E) during the probe test (black bar indicates mutants). (F) CA3 specific deletion of the NR1 subunit blocks LTP at the recurrent commissural/associational synapses, (G) does not affect training in the spatial version of the Morris water maze, (H) but results in a reduced preference for the target quadrant during a probe trial with partial cues, indicating an impairment in pattern completion. (I) Perforant path LTP is lost as a consequence of ablation of the NR1 subunit in the dentate gyrus. (J) A design to test context discrimination in wild type and dentate gyrus-specific NMDA receptor knockout mice. For the

first 3 days of conditioning, mice visited only chamber A and each day received a single foot shock. Freezing was measured once in chamber A and once in chamber B over the subsequent 2 days. During days 6–17, mice visited each chamber daily (receiving a shock in one of the two), and freezing was assessed during the first 3 min in each chamber. (K) DG-restricted ablation of NMDA receptors results in impaired pattern separation indicated by a reduced discrimination ratio between a shocked context (context A) and a non-shocked context (context B). *Sources:* McHugh *et al.*, 2007; Nakazawa *et al.*, 2002; Tsien *et al.*, 1996b.

Figure 1.2.

Genetic demonstration of a role of PKA in memory formation and LTP. (A) A sagittal section of a transgenic mouse expressing R(AB) under control of the α -CaMKII promoter. The transgene is expressed throughout the hippocampus, cortex, olfactory bulb, amygdala, and striatum. (B) In two different lines of transgenic mice, L-LTP was reduced following four 100 Hz trains (1 s duration, 5 min apart) of stimulation. A stable level of robust potentiation was observed in wild-type controls (black circles), while a marked decline of potentiation back to near baseline values was measured in both lines of transgenic mice (open circles and squares). (C) R(AB) transgenics show a deficit in long-term, but not short-term memory for contextual fear conditioning. R(AB) mice and controls were given one CS/US pairing. No significant difference between mutants and controls was observed either immediately or 1 h after the CS/

US pairing. However, 24 h after the training session, freezing responses of both R(AB) transgenic lines were significantly reduced compared with wild-type mice. (D) Examples of place fields in wild type and R(AB) transgenic mice. In contrast to wild types, R(AB) transgenic mice have unstable place cells. (E) Schematic representation bitransgenic systems to induce R(AB). Two different transgenes are used: one expressing the tetracycline-responsive transactivator (tTA) under the control of the α CaMKII promoter (Mayford *et al.*, 1996) and another carrying the R(AB) cDNA under the control of tet operator sequences (tetO promoter). In mice bearing both transgenes, expression of R(AB) is suppressed when mice are fed with dox-containing chow. (F) Under doxycycline conditions, bitransgenic mice do not express R(AB). Bitransgenic mice raised on doxycycline and removed from doxycycline for 28 days showed a high level of R(AB) mRNA, as detected by *in situ* hybridization in hippocampus, cortex, striatum, and amygdala. (G) Under these conditions, transgenic reduction of PKA activity in forebrain neurons in the adult facilitates the extinction of contextual fear. Upper panel, both wild type and bitransgenic mice were raised on doxycycline to suppress R(AB) expression, fear conditioned, and removed from doxycycline 24 h later to permit R(AB) expression in the adulthood. After 28 days, mice were subjected to contextual extinction. Lower panel, R(AB) expressing mice and wild-type littermates were reexposed to the conditioning context in 24 min sessions for three consecutive days. Freezing is shown in blocks of 3 min for each 24 min daily session. Error bars indicate SEM. *Sources*: Abel *et al.*, 1997; Isiegas *et al.*, 2008.

Figure 1.3.

Conditional activation of the *Aplysia* octopamine receptor in forebrain neurons facilitates memory formation and LTP. (A) Activation of the *Aplysia* octopamine receptor by its natural ligand activates the Galphas signaling pathway to transiently increase cAMP. (B) Treatment of hippocampal slices from Ap *oa*₁ mice with octopamine induces long-lasting potentiation following stimulation that normally elicits transient potentiation. (C) Training and 24 h long-term memory test during contextual fear conditioning in Ap *oa*₁ mice and wild-type littermates injected intraperitoneally with octopamine 30 min before training. No differences in freezing behavior are observed between groups before and directly after the shock during training. In contrast, Ap *oa*₁ mice show a significant increase in freezing behavior when reexposed to the fear-conditioned context 24 h after training. (D) Intraperitoneal injections of octopamine 30 min prior to the retention test facilitates memory retrieval in Ap *oa*₁ mice expressing the *Aplysia* octopamine receptor. Source: Isiegas *et al.*, 2008.

Figure 1.4.

Optogenetic manipulation of the neural substrates of awakening. (A) A diagram of the construct used to drive ChR2 specifically in hypocretin-producing neurons. (B) Schematic of the behavioral setup used for *in vivo* deep-brain photostimulation in mice. Magnification (inset) shows the EEG/EMG connector used for sleep recording and the cannula guide used for lateral hypothalamus light delivery through an optical fiber. (C) Representative EEG/EMG recordings showing reduced time to awakening after a single bout of photostimulation (15 ms, 20 Hz, 10 s) of hypocretin neurons in mice expressing the ChR2. Light stimulations are represented by horizontal blue bars. Awakening events are indicated by vertical black arrows. *Source:* Adamantidis *et al.*, 2007.

Figure 1.5.

Tagging and visualization of previously activated neurons using the TetTag mouse. (A) TetTag mice are raised on food containing Dox (left yellow block). During this time, neuronal activation that leads to expression of tTA through Fos promoter activation will not trigger tagging, because doxycycline blocks activation of the tetO promoter. The time window for tagging is opened by switching mice to food without doxycycline (middle white block). Neuronal activation will now activate the transcriptional feedback loop and start expression of tau-LacZ. The time window is closed by putting mice back on doxycycline food (right yellow block) to block further feedback loop activation. However, neurons that were activated during the “no doxycycline” time window will continue to express tau-LacZ, because the feedback loop can maintain its own activation through the doxycycline-insensitive form of tTA. (B, left panel) A protocol was designed to detect repeated activation of neurons during learning and retrieval of conditioned fear. Learning takes place in the absence of doxycycline, when activation of neurons triggers long-lasting expression of tau-LacZ (LAC) and short-lasting expression of immediate-early genes like Zif/Egr (ZIF). Retrieval takes place 3 days after learning in the presence of doxycycline, preventing activation of the molecular feedback loop. Dissection of brains is done 1 h after retrieval, when LAC and ZIF can be used as indicators of learning-induced and retrieval-induced activation, respectively. (B, right panel) Example of LAC and ZIF expression in BLA neurons of a mouse that was subjected to the protocol described in (A). The yellow square in the left dark field picture marks the area shown in the three immunostaining pictures. Yellow arrows mark neurons that express both LAC and ZIF. (C) A diagram of the experimental design. (D) The FC group showed more freezing than the NS group during context retrieval. (E) The combined FC and FC-NR group had an increased number of LAC-positive neurons as compared with both the HC and NS groups. (F) The FC group had more LAC + ZIF neurons than the HC and FC-NR groups. *Source: Reijmers et al., 2007.*