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MOLECULAR MECHANISMS OF MEMORY AND SYNAPTIC PLASTICITY

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Presented by

KARSTEN BAUMGAERTEL

Diplombiologe, Ludwig-Maximilian University, Munich

born 13.09.1977

citizen of Germany

accepted by recommendation of

Prof. Isabelle Mansuy, examiner

Prof. Martin Schwab, co-examiner

Prof. Serge Laroche, co-examiner

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Summary

Memory makes us who we are and helps us to survive in our daily life. Pathologies of memory are therefore devastating conditions that are also difficult to treat. These pathologies usually take two extreme and opposite forms, severe deficits of memory on the one side and exceptionally salient traumatic memories on the other. What renders one memory trace particularly robust while another is only short-lived? Unraveling the cellular and molecular processes underlying different forms of memory will help us to answer this question and provide a starting point for developing clinical interventions.

At the cellular level, memory is based on synaptic plasticity, i.e. the ability of forming/retracting contacts between neurons and of existing contacts to change in strength. The present thesis summarizes data describing molecular mechanisms of synaptic plasticity in two different contexts.

We showed that modulating the activity of the neuronal, Ca^{2+} dependent protein phosphatase calcineurin (CN) in brain regions important for aversive memory is sufficient to alter the strength of this memory and its robustness to interference. Specifically, CN inhibition strengthened aversive memory while CN over-activation weakened it. In our experiments the involvement of CN was restricted to aversive associative memory and the same modulations of CN did not affect a non-aversive memory trace of another association. This is supported by the finding that the establishment of the aversive but not of the non-aversive memory trace naturally inhibited CN activity.

We extended these findings by demonstrating that CN determines aversive memory strength by limiting the transcriptional response to memory-related neuronal activity. Specifically, the level of a transcription factor known to be involved in the establishment of memory, Zif268, was increased after CN inhibition in a brain region important for aversive memory. To simulate this increase, we generated and characterized a new line of transgenic mice that allow the inducible and reversible over-expression of Zif268. Using these mice, we were able to mimic the effect of CN

inhibition on aversive memory and to thereby strengthen the link between CN and transcriptional regulation involving Zif268.

In collaboration with another laboratory, we also investigated the role of CN in ocular dominance shift. This form of developmental plasticity occurs after transient occlusion of one eye during a critical period. As a result, neurons in the visual cortex loose their binocular sensitivity and become selectively sensitive to only the spared eye. We showed that the ocular dominance shift is blocked if CN is over-activated in the visual cortex and thereby extended previous knowledge that CN functions as a constraint on different forms of neuronal plasticity.

For these studies, we employed advanced technology for transgenic mice that permit the inducible and reversible expression of a transgene in brain regions important for memory. As part of this thesis, we adapted and characterized a novel version of the reverse tetracycline transactivator system, rtTA2M2, in transgenic mice. Furthermore, in an attempt to construct an inducible gene knockout mouse with the possibility of a gene rescue, we created transgenic mice to co-express rtTA2M2 and the inducible Cre recombinase, CreER(T2). Using two parallel approaches we did not succeed in co-expressing these two factors. However, we successfully created transgenic mice expressing CreER(T2) in memory-related brain structures, that will allow ablation of target genes on demand.

We are confident that the knowledge gained as part of this thesis represents an important contribution to the understanding of the processes underlying memory. The newly generated transgenic mouse models will be extremely useful in future studies to extend this knowledge. Eventually, researchers hope to completely understand why and how memory traces are different and what governs their unique properties, including their salience. This knowledge will be crucial for the successful treatment of people suffering from pathologies of memory.

Zusammenfassung

Die Fähigkeit, Information zu speichern und sie wieder aufzurufen, macht uns zu dem Menschen, der wir heute sind. Jeden Tag verwahrt unser Gehirn Tausende von Informationen, ohne die wir den Alltag nicht bewältigen könnten. Fehlfunktionen unseres Erinnerungsvermögens sind somit ernstzunehmende Krankheiten, deren Behandlung sich bis heute ausgesprochen kompliziert gestaltet. Diese Fehlfunktionen treten in zwei gegensätzlichen Formen auf, als Erinnerungsschwäche oder als unerwünscht robuste Erinnerungen an traumatische Erlebnisse.
Welche Faktoren bestimmen die Stärke und Robustheit verschiedener Erinnerungen? Um diese Frage zu beantworten, müssen wir die zellulären und molekularen Grundlagen verschiedener Gedächtnisformen verstehen. Die vorliegende Arbeit leistet einen wichtigen Beitrag zur Klärung dieser essentiellen Fragestellung.

Auf der zellulären Ebene basiert das Erinnerungsvermögen auf dem Mechanismus der synaptischen Plastizität. Darunter versteht man Veränderungen der Verbindungen zwischen Nervenzellen, wobei diese unterbrochen, neu aufgebaut, verstärkt oder abgeschwächt werden können. Mit der vorliegenden Doktorarbeit haben wir das Verständnis der molekularen Grundlagen zweier Formen synaptischer Plastizität erweitert. Wir haben gezeigt, dass die Stärke einer aversiven Erinnerung allein durch Aktivitätsveränderungen der neuronalen, Kalzium-gesteuerten Protein Phosphatase Calcineurin (CN) in Gehirnregionen, in denen diese Erinnerung verarbeitet wird, gesteuert werden kann. Dabei führt die Inhibierung von CN zu einer robusteren Erinnerung, während die Aktivierung von CN die Erinnerung abschwächt. In unseren Experimenten wurde diese Abhängigkeit ausschliesslich bei aversiven Erinnerungen beobachtet. Eine nicht-aversive Erinnerung wurde durch eine Veränderung der CN Aktivität nicht beeinflusst. Dieser Zusammenhang wird durch unsere Beobachtung bestätigt, dass CN nur während der Etablierung aversiver, nicht aber nicht-aversiver Erinnerungen durch natürliche Mechanismen inhibiert wird.

Wir haben ausserdem gezeigt, dass CN die Stärke einer aversiven Erinnerung reguliert, indem sie die transkriptionelle Antwort auf neuronale Aktivitätsmuster hemmt. Dementsprechend wird die Expression des Transkriptionsfaktors Zif268 durch Inhibierung von CN verstärkt. Diese Verstärkung haben wir mit Hilfe von transgenen Mäusen simuliert, die eine induzierbare, reversible Überexpression von Zif268 ermöglichen. Zif268 Überexpression und CN Inhibierung zeigen vergleichbare Effekte auf aversive Erinnerungen, was klar auf eine funktionelle Verbindung zwischen CN und Zif268 hindeutet.

In einer Kollaboration mit einem amerikanischen Labor haben wir weiterhin die Rolle von CN bei der Verschiebung der Augendominanz im visuellen Kortex untersucht. Diese Form von Plastizität findet nur während eines kritischen Zeitfensters in der Frühentwicklung statt und wird im Experiment durch zeitlich begrenzten Verschluss eines Auges erreicht. Der Verschluss des Auges führt zu einer Veränderung des Aktivitätsverhaltens von Nervenzellen im visuellen Kortex. Die Nervenzellen werden selbst nach der Öffnung des verschlossenen Auges nur noch durch Anregung des anderen Auges statt durch beide angeregt. Wir haben gezeigt, dass diese Verschiebung durch die Aktivierung von CN verhindert wird und haben damit das Verständnis der Prozesse dieser Form der kortikalen Plastizität wesentlich erweitert.

Für unsere Untersuchungen haben wir neueste transgene Technologien in Mäusen eingesetzt, die eine induzierbare und reversible Expression verschiedener Transgene gestatten. Im Rahmen dieser Doktorarbeit haben wir transgene Mäuse mit der neuen Version des Tetrazyklin-gesteuerten Genregulationssystems rtTA2M2 generiert und die Eigenschaften des Systems beschrieben. Weiterhin haben wir ein Projekt durchgeführt, mit dem Ziel, in transgenen Mäusen ein induzierbares Ausschalten bestimmter Gene mit der gezielten Expression eines Ersatzgens zu vereinigen. Dafür wurden zwei verschiedene Ansätze angewandt, um rtTA2M2 und die induzierbare Cre Rekombinase, CreER(T2) simultan zu exprimieren. Während keiner der beiden Ansätze das erwünschte Ergebnis erzielte, ist es uns doch gelungen, eine transgene Maus zu züchten, die CreER(T2) in den wichtigsten Gehirnregionen für das

Erinnerungsvermögen exprimiert. In diesen Regionen ist mit Hilfe dieses Mausmodells ein gezieltes Ausschalten bestimmter Zielgene induzierbar.

Mit dieser Arbeit ist es uns gelungen, das Verständnis der Rolle von CN bei verschiedenen Formen der synaptischen Plastizität zu erweitern. Die Arbeit liefert somit einen weiteren, wichtigen Beitrag zum Verständnis der Regulierung der Stärke einer Erinnerung und von Erinnerungsprozessen allgemein. Die Mausmodelle, die im Rahmen dieser Arbeit geschaffen wurden, können zur Vertiefung und Erweiterung dieser Erkenntnisse in zukünftigen Forschungsarbeiten genutzt werden.

Ziel weiterführender Arbeiten muss es sein, vollständig zu verstehen, warum und wie sich verschiedene Typen von Erinnerungen unterscheiden und mit diesem Wissen die medizinische Behandlung von Fehlfunktionen des menschlichen Erinnerungsvermögens zu ermöglichen.

General Introduction

The Nervous System and the Environment

Survival in an ever-changing world greatly depends on the capacity to adapt to the conditions of the surrounding environment. This adaptation may arise over many generations by changes in gene pools that are driven by natural selection. However, increasing an individual's chances of survival and reproduction during its lifetime requires the ability of its nervous system to respond to environmental changes. This plasticity of the nervous system and its underlying mechanisms have been a major focus of research in neuroscience. Experiments at the cellular and molecular level have endeavored to unravel mechanisms for plasticity and stability, and to understand whether different forms of neural plasticity share common neural mechanisms. Experiments at the systemic level aimed to identify neural networks underlying different forms of plasticity, and to clarify in which way each brain structure contributes to cognitive processes such as learning and memory.

Memory makes us who we are and helps us survive in everyday life. Deficits in memory function are debilitating pathological conditions, which are to date very difficult to treat sufficiently and appropriately. Conversely, persistence of unwanted memories may be equally harmful. Negative events inappropriately recalled from the past can decrease quality of life markedly; in extreme cases, people cannot evade traumatic or aversive memories and suffer life-long from conditions like post-traumatic stress disorder. In order to treat these pathological conditions of memory, it is essential to understand the processes that underlie memory formation and convey memory establishment and persistence.

Over the past century research has focused on processes that are common to all forms of memory, independent of the memory content or the brain regions involved. This quest was driven by the ability to visualize cellular morphology in the brain and to

apply basic molecular techniques. For example, there is now substantial evidence that transcription and synthesis of new proteins is required for the formation of long-term memory, a finding that was first described in the 1960's (Bekinschtein et al., 2007; Bourtchouladze et al., 1998; Davis et al., 1976; Davis and Squire, 1984; Emptage and Carew, 1993; Flexner et al., 1962; Flexner et al., 1965; Flexner et al., 1964; Ghirardi et al., 1995; Grecksch and Matthies, 1980; Grecksch et al., 1980; Igaz et al., 2002; McGaugh, 1966; McGaugh, 2000; Meiri and Rosenblum, 1998; Montarolo et al., 1986; Rosenblum et al., 1993; Schafe and LeDoux, 2000; Scharf et al., 2002; Squire et al., 1976). However, studying the contribution of individual genes to specific memory functions and phases is difficult because the brain is a highly complex organ with distinct neuroanatomical structures, orchestrating virtually every function in the body. The potential for this kind of dissection is very recent, and has experienced a huge surge due to the availability of complete genomes and of new tools to study and dissect molecular events that underlie particular memory functions and phases. Recent technological advances are now enabling neuroscientist to address the contribution of single molecules to distinct memory forms with high temporal and spatial specificity (for review, refer to chapter 1).

Learning and Memory

Memory is a term used to describe a whole host of functions that are all involved in the storage of information. Memory traces may differ in terms of salience, persistence and robustness to interference, but mainly they diverge in their content. Along with the content, the regions required to process and store relevant information in the brain vary. There is also evidence that the main storage site of a given memory trace in the brain shifts with the age of the memory (Bontempi et al., 1999; Frankland et al., 2004a; Frankland et al., 2004b; Maviel et al., 2004). The temporal phases that constitute memory are shared by all memory traces (engrams) and are described briefly below.

- Acquisition: Acquisition is also referred to as “learning” or “encoding” and describes the initial formation of an engram. All engrams undergo acquisition.
- Consolidation: Consolidation is the transfer of an engram from short-term (STM) to long-term memory (LTM). Not all types of memory undergo consolidation. For instance, working memory is a form of transient memory.
- Retrieval: The presence of an engram cannot be determined unless it is retrieved. However, lack of retrieval is no proof for the absence of an engram, since it represents a process in itself that may be blocked or inhibited.
- Extinction: Extinction describes the gradual loss of the capacity of an engram to be recalled and to significantly affect behaviour. This is not based on a loss of the engram itself, but rather on the formation of a second relevant memory trace that becomes more salient and therefore dominates the first (Rescorla, 1967). Rather than a reversal of processes underlying the formation of the original engram, it therefore represents the formation of a second, independent engram with overlapping content. The presence of two different engrams is observed during spontaneous recovery. Here, an extinguished memory can recover over time and once again affect behavior. Furthermore, facilitated re-learning (so called savings) can occur. However, under some circumstances, e.g. if an engram was formed very recently, the processes underlying the formation of the original engram can be reversed (Mao et al., 2006).
- Reconsolidation: The term reconsolidation is used to describe the post-retrieval susceptibility of an engram to interference. After an engram is retrieved, it is reactivated and becomes labile and susceptible to interference by protein synthesis inhibitors. Recent evidence has shown that this susceptibility decreases with the age of memory (Eisenberg and Dudai, 2004; Eisenberg et al., 2003; Milekic and Alberini, 2002; Suzuki et al., 2004) and that the molecular requirements for reconsolidation change during memory maturation (Kemenes et al., 2006). Indeed, the molecular machinery engaged by recall varies with the age of the memory, a finding that is in agreement with assumptions central to the lingering consolidation hypothesis promoted by

Dudai and Eisenberg (Dudai and Eisenberg, 2004). According to this hypothesis, the post-retrieval susceptibility of a memory trace to interference can, and should, be viewed as a part of consolidation, which lingers but will eventually disappear. Reconsolidation is thus favoured for a young memory as opposed to an old and fully established memory. Not surprisingly, the underlying molecular mechanisms are different to extinction, suggesting that these two processes are independent of each other but can both be induced by memory retrieval (Suzuki et al., 2004).

- Memory Establishment: Memory establishment is a term to describe the combination of memory phases that determine the strength of a memory trace, its dominance and its control of behaviour. Of course, these parameters are highly dependent on processes occurring during acquisition and consolidation, but retrieval is also an important aspect of memory establishment.

Each memory phase is mediated by diverse molecular mechanisms and may recruit different signaling pathways and distinct cellular and molecular processes. To study the involvement of single molecules or whole signaling cascades in memory, it is imperative to temporally restrict any manipulation. Recent technologies address exactly this challenge and have been employed to study the involvement of single molecules in learning and memory (for review, refer to chapter 1). Further improvements to these approaches were developed as part of this PhD thesis (chapters 5 and 6).

Nervous System Plasticity

Plasticity in the nervous system primarily occurs at the cellular level and involves morphological and molecular changes. Neuronal connections may be either newly formed, retracted, and/or pre-existing ones modified. These processes take place under extreme circumstances, e.g. during development or after nervous system injury, but also on a daily basis during learning and memory to support activity-dependent

changes in the efficacy of synaptic transmission. During development, sensory experience shapes the maturation of neocortical circuits. In this PhD thesis, this form of neocortical plasticity was studied in a very traditional and popular model based on monocular deprivation during early postnatal life (chapter 3), which results in a shift in ocular dominance distribution of neurons in visual cortex (Gordon and Stryker, 1996; Wiesel and Hubel, 1963). Following monocular deprivation, visual acuity and contrast sensitivity for the deprived eye develop poorly and depth perception is lost. This heightened plasticity is restricted to a critical period (for mice P19 to P32, (Gordon and Stryker, 1996) during which the ocular dominance of cortical neurons is established and maintained by a process of activity-dependent competition between synapses serving both eyes (Wiesel and Hubel, 1965).

Plasticity subserving information storage may serve very different functions, such as spatial navigation, recognition or associations with previous harmful or beneficial experiences. In order to understand the mechanisms underlying learning and memory, processes that are common as well as those that are exclusive to specific forms of synaptic plasticity are of interest. In line with this, previous work was continued in this PhD thesis, providing new insights into the molecular mechanisms of different types of plasticity, including developmental and synaptic plasticity (chapters 2, 3 and 4).

Molecular Events Underlying Learning and Memory

Higher-order brain functions such as learning and memory simultaneously recruit several signaling cascades in different subcellular compartments with finely-tuned spatial and temporal components. *Figure 1* shows a simplified schematic overview of the key events of synaptic plasticity at the glutamatergic synapse, focusing on the postsynaptic compartment. Pre-synaptic components are also involved but will not be discussed here. In fact, to reduce complexity only the processes relevant to the data presented in this PhD thesis are depicted (for more information, refer to chapter 1).

The events of synaptic plasticity in the glutamatergic post-synapse may be divided into several stages that are dependent on each other. A key event in the initiation of these signaling cascades is a post-synaptic Ca^{2+} influx, which not only contributes to the depolarization of the membrane, but also acts as the main primary messenger in neurons (*Fig. 1*). The Ca^{2+} concentration crucially affects the balance between factors generally involved in strengthening (red, at high Ca^{2+} concentrations) and those in weakening (blue, at low Ca^{2+} concentrations) signal transmission between two neurons. Key mediators of changes in Ca^{2+} concentration are protein kinases (red) and protein phosphatases (blue). In the brain, a highly abundant and well-conserved phosphatase is calcineurin (CN, protein phosphatase 2B). It is the only Ca^{2+}-dependent protein phosphatase in the brain and it is strategically located at synaptic terminals to capture Ca^{2+} directly at the point of entry into the post-synapse (Coghlan et al., 1995; Colledge et al., 2000).

CN in Synaptic Plasticity and Learning and Memory

The formation long-term synaptic plasticity and memory requires Ca^{2+} influx through L-type voltage gated Ca^{2+} channels (L-VGCCs) and n-methyl d-aspartate NMDA receptors (Bauer et al., 2002). Because of its dependence on Ca^{2+} influx, CN was early considered an interesting candidate to study in the context of learning and memory. It is not only itself regulated by Ca^{2+} (Klee et al., 1979), but is also a key modulator of Ca^{2+} influx in response to neuronal activity. Thus, membrane-associated CN may shape Ca^{2+} influx directly by exerting dominant control on the activity of L-type voltage gated Ca^{2+} channels (L-VGCCs, *Fig. 1*) (Oliveria et al., 2007) or indirectly by maintaining α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors in a dephosphorylated state (*Fig. 1*) and thus altering neuronal excitability (Smith et al., 2006). Furthermore, CN may strongly modulate intracellular responses to Ca^{2+} influx. Thus, by modulating the generation of secondary messengers through the regulation of the type IX adenylyl cyclase, CN is able to determine the Ca^{2+}-dependent pool of cAMP (Antoni et al., 1998; Chan et al., 2005).

Moreover, CN is directly involved in intracellular signaling and controls and/or counteracts several of the kinases already implicated in neuronal plasticity, such as the calcium/calmodulin dependent-protein kinases, the mitogen-activated protein kinase (MAPK) or cAMP-dependent protein kinase (PKA), and other kinases as well as other phosphatases (Ahi et al., 2004). These enzymes are essential transducers of secondary messenger signals from the synapse to the nucleus and mediate persistent changes underlying the formation of memory by regulating gene transcription through transcription factors (TFs) like cAMP response element-binding protein (CREB), serum response factor (SRF) and Elk1 (*Fig.1*) (Ahi et al., 2004; Dash et al., 2005; Johnson et al., 1997). Finally, a small nuclear pool of CN may contribute to the regulation of transcription by direct interaction with TFs (Graef et al., 1999; Groth and Mermelstein, 2003; Shibasaki et al., 1996). CN may thus function as a major restraint on the efficacy of intracellular signaling and strongly shape transcriptional processes underlying long-term synaptic plasticity and memory.

In accordance with these molecular findings, the modulation of CN has severe effects on synaptic plasticity and memory:

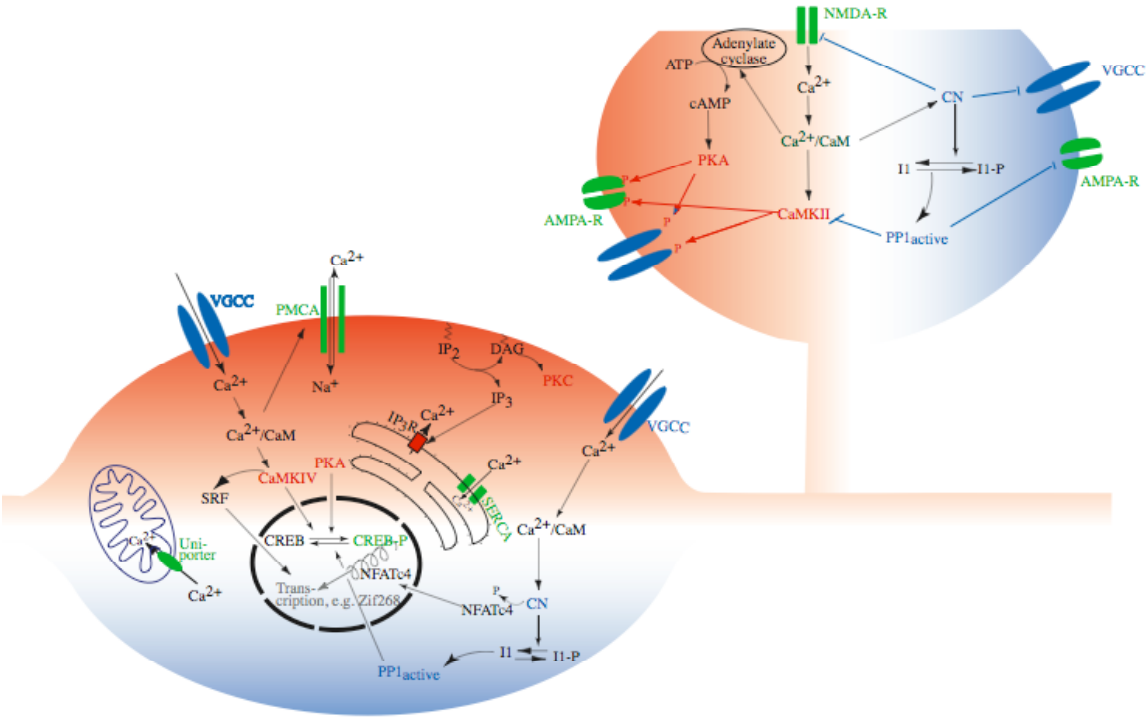
- Transgenic mice in which an active CN mutant is inducibly expressed in neurons of the forebrain display deficits in lasting forms of long-term potentiation (LTP, an artificial form of synaptic plasticity that is used as a model for processes underlying memory formation) and normal short-term memory but defective long-term memory in hippocampus-dependent memory tasks (Mansuy et al., 1998a; Mansuy et al., 1998b; Winder et al., 1998).
- Inducible genetic inhibition of CN in neurons of the forebrain facilitated long-term potentiation, *in vitro* and *in vivo*, impaired depotentiation (Jouvenceau et al., 2003), another artificial model of synaptic plasticity, enhanced learning and strengthened short- and long-term memory in several hippocampal-dependent spatial and nonspatial tasks in transgenic mice (Malleret et al., 2001).
- Forebrain neuron-specific knockout of the CN-B subunit selectively impaired long-term depression (LTD), another artificial model of synaptic plasticity,

shifted the induction threshold for LTD/LTP and interfered with working and episodic-like memory dependent on the hippocampus (Zeng et al., 2001).

- In mice lacking the predominant calcineurin isoform in the central nervous system, the Aα subunit, depotentiation was abolished completely whereas LTP was not affected (Zhuo et al., 1999).
- Antisense mediated knockdown of the CN-A subunit facilitates induction of LTP *in vivo* (Ikegami et al., 1996) and enhances memory in contextual fear conditioning (Ikegami and Inokuchi, 2000).

While these results nicely illustrate the importance of CN for synaptic plasticity and memory, they also raise important questions about the mechanisms involved. These are likely to include the regulation of *de novo* gene transcription, since this is required for persistent synaptic changes and long-term memory (Bailey et al., 1999) and may be modulated by CN as described above. Of particular interest are immediate early genes (IEGs), which provide the first transcriptional response within minutes after neuronal activity and represent key effectors of cytoplasmic signaling cascades. Interestingly, the expression of some IEGs is subject to CN-dependent regulation (Enslen and Soderling, 1994).

Figure 1 (next page). A simplified depiction of molecular events at a postsynaptic neuron in response to Ca^{2+} influx. Ca^{2+} enters the cell via the NMDA (N-methyl-d-aspartate) receptors and voltage gated Ca^{2+} channels in response to glu*tam*ate binding and/or depolarization. However, it can also be released from intracellular stores via IP_3 (inositol 1,4,5-trisphosphate) signaling. Cytoplasmic Ca^{2+} is then either directly bound by enzymes or via Ca^{2+} binding proteins such as calmodulin, which in turn bind and activate diverse enzymes including protein kinases and phosphatases. Depending on the Ca^{2+} concentration, kinases or phosphatases are preferentially activated. Kinases are generally involved in responses to strengthen connectivity between neurons and phosphatases in weakening. These signals may either feed directly back to receptors at the membrane via post-translational modification, or are conveyed to the nucleus to regulate gene transcription. One gene target of these signaling cascades is the immediate early gene Zif268, which can be regulated by the serum response factor, SRF, cAMP reponse element binding protein, CREB, and several others.



Immediate Early Genes

Rapid activation of genes encoding transcription factors is thought to play a key role in stimulus-induced neuronal plasticity. These transcription factors may indeed function as key players that orchestrate the response to a given stimulus by activating or repressing whole molecular programs within a neuron. The family of immediate early genes (IEGs) displays a rapid, protein synthesis-independent up-regulation of their mRNA, and a subsequent increase in protein, in response to a stimulus, including the kind that induces memory formation and/or synaptic plasticity. Functionally, the IEG family is very heterogeneous, but a large class constitutes transcription factors, including Fos, Jun and the Egr family, which carry Zinc finger motifs for DNA binding. Several IEGs, including a prominent member of the Egr (early growth response) family, Zif268 (zinc finger binding protein clone 268), have been implicated in synaptic plasticity and memory, but in most instances, detailed or mechanistic data is missing. Zif268 has received considerable attention in the scientific community (2379 citations when Pubmed is searched for Zif268 or its

acronyms Egr-1, Krox24, NGFI-A, Tis8, ZENK) since its first description (Milbrandt, 1987).

Zif268 in Synaptic Plasticity and Learning and Memory

Soon after the discovery of *Zif268*, which was, in fact, the first described immediate early gene, researchers realized the potential importance of the capacity to initiate response programs rapidly. On one hand, *Zif268* has been used as a marker for detecting previous neuronal activity in an effort to identify the brain regions recruited during different behavioural tasks (Chaudhuri et al., 1995; Dardou et al., 2006; Fujisaki et al., 2004; Guzowski, 2002; Jarvis et al., 1997; Lin et al., 1997; Mello and Clayton, 1995; Okuno and Miyashita, 1996; Thomas et al., 2002; Weitemier and Ryabinin, 2004). On the other hand, scientists studying learning and memory and synaptic plasticity soon addressed *Zif268's* involvement in memory-related neuronal activation. This was based on the idea that *Zif268* expression may represent a marker of synaptic plasticity rather than general neuronal activity, differentiating it from other IEGs like c-Fos (Rosen et al., 1998). Some of the evidence supporting this notion is reviewed below:

- Zif268 induction is associated with long-term potentiation in hippocampal granule cells (Cole et al., 1989; French et al., 2001; Wisden et al., 1990). Similar induction in CA1 pyramidal cells is controversial (French et al., 2001; Roberts et al., 1996).
- *Zif268* mRNA is markedly up-regulated throughout cortical areas following the induction of *in vivo* long-term potentiation (LTP) by BLA stimulation, including the insular cortex when tested 1hr after tetanic stimulation (Jones et al., 1999).
- Following behavioral training of a learning task (two-way active avoidance response) *Zif268* expression was induced in the rat hippocampus as well as the visual cortex (Nikolaev et al., 1992).

- *Zif268* mRNA levels are rapidly and transiently increased in the dorsal hippocampus by spatial water task training. RNA levels in hippocampus and entorhinal cortex decreased with extended training, but remained elevated above control levels (Guzowski et al., 2001). Furthermore, baseline *Zif268* expression negatively correlates with performance on the Morris water maze task. Indeed, chronic physical activity improved performance in this task and decreased basal levels of Zif268 (Fordyce et al., 1994).
- *Zif268* expression increased in the lateral nucleus of the amygdala following contextual fear conditioning (Malkani and Rosen, 2000a; Rosen et al., 1998) while levels in the hippocampus and the cortex remained constant for at least one hr after conditioning (Malkani and Rosen, 2000a). The anxiolytic diazepam blocked the fear-conditioning-induced increase in *Zif268* expression in the LA (Malkani and Rosen, 2000a).
- During the retrieval of contextual (but not cued) fear associations, increased expression of *Zif268* was observed within CA1 neurons, but not dentate gyrus neurons. In contrast, *Zif268* expression was increased within neurons of the amygdala (lateral, basal, and central nuclei) during the retrieval of both contextual and cued fear memories. The selective increase in hippocampal CA1 Zif268 expression seen after contextual fear memory retrieval was limited to the retrieval of recent (24hr) but not older (28 d) memories (Hall et al., 2001). Additionally, *Zif268* expression was increased in specific regions of the rat frontal cortex and nucleus accumbens following fear memory retrieval. The increased expression of *Zif268* in neurons in the core of the nucleus accumbens was common to the retrieval of contextual and discrete cued fear associations, while it was restricted to retrieval of contextual but not cued fear memories in neurons of the nucleus accumbens shell and the anterior cingulate cortex (Thomas et al., 2002).
- Expression of *Zif268* was increased in the temporal cortex of monkeys during visual paired associate learning (Okuno and Miyashita, 1996). Specific expression of *Zif268* was demonstrated in area 36 of the perirhinal cortex

during the learning of a new set of paired associates in the same task (Miyashita et al., 1998; Tokuyama et al., 2002).

- Retrieval of contextual fear memory caused an increase in Zif268 protein level of 200% in the CA1 and 50% in the CA3 region of the hippocampus and dentate gyrus (Lee et al., 2004).
- In eyeblink classical conditioning, Zif268 protein expression was rapidly increased during the early stages of conditioning and remained elevated during the later stages. Furthermore, expression of Zif268 protein required NMDA receptor activation as it was blocked by bath application of AP-5 (Mokin and Keifer, 2005).
- Significant increases in *Zif268* expression were observed 30min after memory retrieval in the taste potentiated odor aversion learning task (TPOA) in basolateral amygdala, insular cortex and hippocampus (Dardou et al., 2006).
- Furthermore, the pattern of *Zif268* expression in developing cat visual cortex was described to coincide with (a) the development of visual cortical connectivity, (b) the development of orientation selective receptive field properties, and (c) the level of visual cortical plasticity (Kaczmarek and Chaudhuri, 1997; Kaplan et al., 1995) in temporal and laminar distribution. These observations suggest an involvement of *Zif268* expression in these processes.
- In the visual cortex, expression of *Zif268* mRNA increased dramatically after eye opening, systemic injection of kainate, or 30min photo-stimulation after a brief (5 d) period of dark adaptation (Mataga et al., 2001).

This evidence suggests a functional link between *Zif268* regulation and processes underlying synaptic plasticity, and memory formation and its maintenance (Abel and Lattal, 2001). To generate a direct link supporting this notion, different approaches were selected to alter Zif268 levels and study the effect on learning and memory.

- Two different knock-in approaches have been used to achieve a functional knock-out of *Zif268*: in one variant, several stop codons were introduced into

the *Zif268* ORF, resulting in expression of a truncated Zif268 protein (KI-STOP, (Lee et al., 1995)); in the second variant, a LacZ containing cassette was inserted into the 5' untranslated region of Zif268 resulting in β-Galactosidase expression and a frame shift for the translation of *Zif268* mRNA (KI-LacZ, (Topilko et al., 1998)). Upon first examination both *Zif268* homozygous mutant mice were sterile and had a reduced body size (Lee et al., 1995; Topilko et al., 1998). First experiments with *Zif268* KO mice examined activity-dependent sensory plasticity. Monocular deprivation during the developmental critical period [postnatal day 24 (P24)-P34] had similar consequences in KI-STOP and WT mice. Visual responses developed normally. Moreover, a similarly significant shift of responsiveness in favor of the open eye was produced in both KI-STOP and WT mice by either brief (4 d) or long-term (>2 weeks) occlusion of one eye. There was no apparent compensation in mRNA or protein expression among egr2, egr3, or c-fos expression in the visual cortex of *Zif268* KO mice (Mataga et al., 2001). Concurrently, a thorough analysis of the KI-LacZ mice was published showing a severely impaired memory consolidation in several tasks (Bozon et al., 2003; Jones et al., 2001). However, this effect could be overcome by a more intensive training protocol, at least in the water maze (Jones et al., 2001) and a novel object recognition task (Bozon et al., 2003). Further investigation showed that *Zif268* is also required for reconsolidation of acquired information in a novel object recognition task (Bozon et al., 2003). In the same mice, late phase LTP in the dentate gyrus was impaired (Jones et al., 2001), suggesting that maintenance rather than establishment of LTP is affected, nicely fitting the behavioural data. In independent work, mainly looking at the role of Zif268 in the immune system or hormone secretion, it was shown that other members of the Egr family, like Egr2 (Li and Liu, 2006) and Egr4 (Tourtellotte et al., 2000), have similar properties to Zif268 and may compensate for the loss of Zif268 (Li and Liu, 2006, Tourtellotte, 2000 #105). To avoid this, several studies have been conducted on double KO mice, *Zif268*

and *Egr2* (Li and Liu, 2006), *Zif268* and *Egr4* (Tourtellotte et al., 2000), *Zif268* and *NGFI-B* (Kilduff et al., 1998). An inducible approach was required to reduce the possibility for compensation. The project presented here employs an inducible approach based on transgenic mice to circumvent problems of compensation and unspecific effects, to strengthen the link between Zif268 and memory, and to analyze Zif268's role in different phases of memory.

- Very recently an approach providing temporal and spatial control was employed based on antisense oligodesoxynucleotides (ODNs) against *Zif268*. ODNs can bind to a specific target mRNA and inhibit translation. It is a timed approach that mediates spatial control through stereotactic injection. The efficacy of the translational block depends on the kinetics of uptake, binding and clearance of the ODNs. Using this approach, Zif268 protein was reduced by 66% in the CA1 subfield of the hippocampus, while other hippocampal subfields were not affected (Lee et al., 2004). This Zif268 knock-down blocked reconsolidation but not consolidation in a contextual fear conditioning task (Lee et al., 2004). Interestingly, in a parallel approach, the reduction in Zif268 protein in the amygdala using ODNs interfered with consolidation of fear memory (Malkani et al., 2004), suggesting that the presence of Zif268 in the amygdala is required for consolidation while Zif268 in CA1 is required for reconsolidation.

While these results nicely illustrate the importance of Zif268 in different phases of memory, the differences in technique also raise important questions.

First of all, there is the question of the relative contributions of the constitutive and the stimulus-induced Zif268. Interruption of Zif268 on the genomic level ablates both constitutive and induced Zif268 expression, while an ODN approach will merely inhibit the latter by blocking translation of existing or newly transcribed mRNA. While most IEGs display very low constitutive levels, and are strongly up-regulated in response to an appropriate stimulation, *Zif268* in comparison has relatively high

constitutive levels. *Zif268* can therefore be regulated on two levels: the constitutive expression and/or the stimulus-induced expression may be altered. The two need to be viewed separately to examine the specific function of each. While data obtained from the ODN approach suggests that it is mainly the inducible, timed *Zif268* expression that is involved in memory processes, there is also some correlative evidence for a significant involvement of constitutive *Zif268* expression. Very recently, a late, BDNF-dependent phase of memory consolidation was described (Bekinschtein et al., 2007) that serves to increase persistence of a memory trace, and that is followed by a persistent increase in Zif268. If BDNF induction during this phase is blocked, a memory trace is rendered less persistent and this increase is no longer observed. Furthermore, environmental enrichment (EE) has been shown to enhance performance in hippocampal-dependent memory tasks in mice (Parsons and Spear, 1972), and to cause a marked increase in constitutive *Zif268* expression in the mouse hippocampus (Toscano et al., 2006). Interestingly, EE is able to improve memory performance (Duffy et al., 2001; Gardner et al., 1975; Williams et al., 2001) and to rescue the phenotype of some gene KOs, e.g. NMDA R1 subunit (Rampon et al., 2000), which has a defect in activity-related Ca^{2+} influx that is necessary for *Zif268* induction. Further, indirect evidence supporting this view comes from studies of age-dependent *Zif268* expression. For instance, it has been reported that *Zif268* expression mirrors the critical period in cat visual cortex (Kaplan et al., 1995), consequently the adult visual cortex has lower constitutive levels of Zif268 (Kaplan et al., 1996), correlating with lower plasticity in this region. Yau et al. showed a positive correlation between spatial learning and the constitutive expression of *Zif268* mRNA, selectively in CA1 pyramidal neurons (Yau et al., 1996). Here, the *Zif268* mRNA level decreased with age in CA1, CA2 and neocortex (Yau et al., 1996). Interestingly, in middle-age and aged mice, EE can rescue performance deficits observed in a memory test, such as the Morris water maze (Bennett et al., 2006; Frick and Fernandez, 2003; Frick et al., 2003). Addressing the following issues will solidify the role of Zif268 in memory: (a) to examine the significance of the constitutive expression of *Zif268* (b) to verify the significance of bi-directional regulation of Zif268 levels, and (c) to show a more

specific involvement of Zif268 in memory processes than by disrupting them through Zif268 ablation.

Overview

During my thesis, I have examined the contributions of the protein phosphatase CN and the IEG Zif268 to different forms of synaptic plasticity by using transgenic mice to achieve inducible, reversible and spatially restricted changes in the level and/or activity of these factors. In collaboration with my co-workers, I have employed novel transgenic technologies (chapter 5) to show that CN is a strong determinant of the strength of aversive memories (chapter 2) and that it underlies very specific forms of plasticity such as ocular dominance plasticity (chapter 3). I have further generated and characterized transgenic mice that permit the transient increase in the level of Zif268 in forebrain neurons (chapter 4). Using these mice, I have demonstrated that the level of Zif268 is a key restraint during memory formation, even in very robust forms of memory, by showing for the first time that an increase in Zif268 enhances memory performance (chapter 4). Finally, I have conducted projects to advance technologies for inducible transgenesis (for review, refer to chapter 1) and developed new system for this (chapters 5 and 6).

The implications of my work on CN and Zif268 are discussed in the final section of this thesis, which concludes with an outlook on the next steps in this research.

Chapter 1: Conditional Transgenesis and Recombination to Study the Molecular Mechanisms of Brain Plasticity and Memory[1]

KARSTEN BAUMGÄRTEL, CRISTINA FERNANDEZ, TORBJÖRN JOHANSSON AND ISABELLE M. MANSUY

Brain Research Institute
Medical Faculty of the University Zürich and
Department of Biology of the Swiss Federal Institute of Technology
Winterthurerstrasse 190, CH-8057 Zürich

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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.

[1] In this review, I coordinated the writing process and contributed the introduction, the discussion and section 3.

1 Why Employ Conditional Transgenesis or Recombination in Memory Studies?

Cognitive functions are highly complex processes which 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.

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 by 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., 1996a), 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., 1996b) or striatal (Kellendonk et al., 1996) neurons (see sub-chapter "NMDAR-dependent processes"). However to date, the choice of promoter sequences truly selective for brain sub-regions or nuclei is still slim and for instance, 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 sub-chapter "Nuclear events and transcriptional regulation by CREB"). Such method is nonetheless more invasive and depends on the availability of thus far rare ligand-dependent molecules with appropriate pharmacokinetic properties.

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 *knock out* 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) which transactivation activity can be controlled by *doxycycline* (administered in the 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 sub-chapter "Intracellular signaling").

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*.

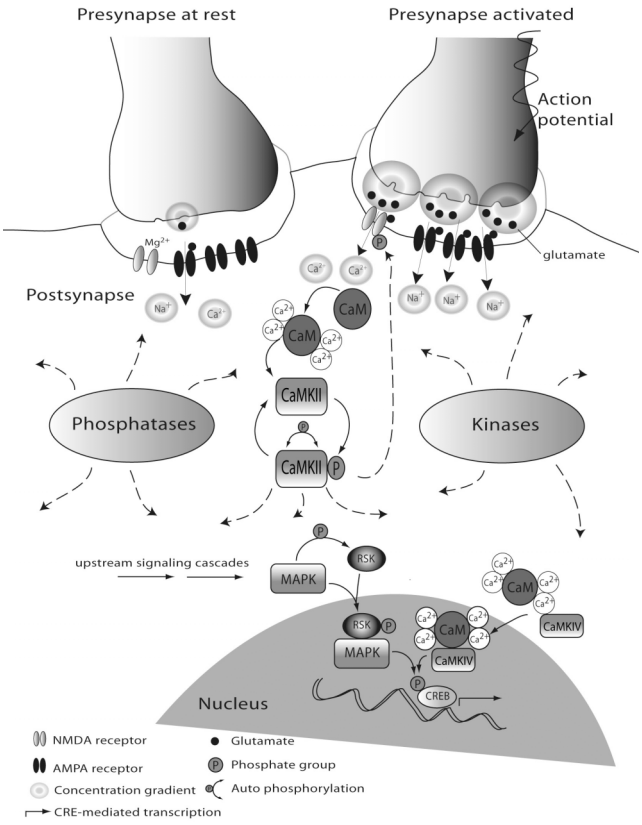


Figure 1. Major molecular components of signaling cascades during synaptic transmission.

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.

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. Further as part of the cascades relaying NMDAR-mediated signal, 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 an 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 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[2] and is now widely accepted as one of the pre-requisite 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

[2] The Organization of Behavior: A Neuropsychological Theory, Wiley-Interscience, New York, 1949.

and recombination 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 *knock out* in the mid-90s, 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 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 *knock out* 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., 1996b).

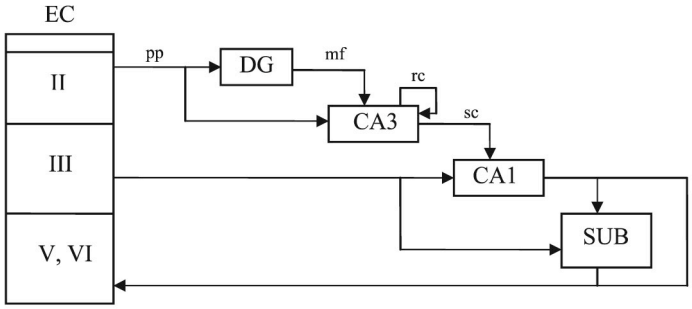


Figure 2. Main connections of the hippocampal/cortical circuit.
EC: entorhinal cortex, DG: dentate gyrus, SUB: subiculum, pp: perforant pathway, mf: mossy fibers, rc: recurrent collateral axons of CA3 pyramidal neurons, sc: Schaffer collateral axons.

In rodents and human, CA1 area is part of a tri-synaptic loop that together with the dentate gyrus, CA3 area and subiculum, constitutes the hippocampal formation. The hippoccampal 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 to 22 flanked by loxP sites) and expressing the CA1-specific recombining Cre were obtained. Unlike plain knock-out animals, these conditional knock-out 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). Non-spatial 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, non-hippocampal-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 (allow 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 knock-out 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 knock-out animals (Tsien et al., 1996b). Strikingly, when the rescue was suppressed only after learning (for one or two 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 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 four 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 (Cui et al., 2005; Cui et al., 2004; Shimizu et al., 2000).

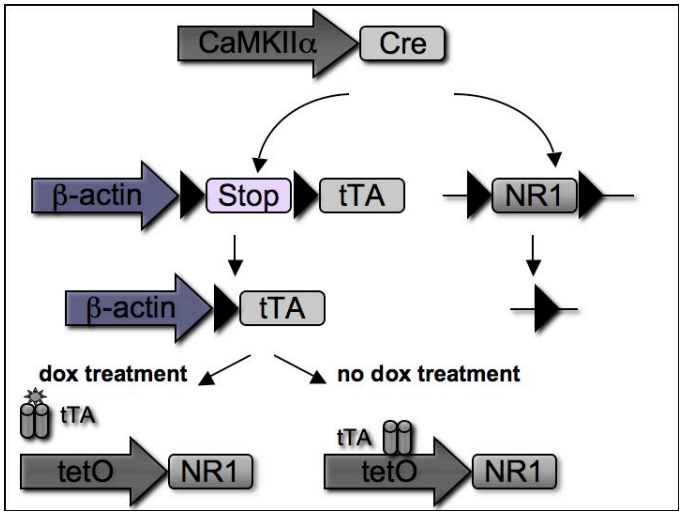


Figure 3. Strategy to obtain an inducible, reversible and CA1-specific NR1 knockout in the mouse.
Cre expression under the control of the CaMKIIα promoter leads to simultaneous expression of tTA, after excision of a stop cassette 5` to the tTA transgene by Cre, and inactivation of the endogenous NR1 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).

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 knock-out model was ideal to test this possibility (Cui et al., 2004). After NR1 inactivation (for 1 month) by *dox* treatment six months after learning (when consolidation is generally complete), the content of contextual and cued fear memory, both long-lasting forms of memory, was examined (Fanselow and Kim, 1992; Kim et al., 1992; 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 seven 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-cues conditions meaning that retrieval occured 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 five weeks after birth. The loss of NR1 severely impaired LTP at CA3 synapses but not in other hippocampal synapses. On 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 that NMDAR is required in these neurons 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 post-synaptic density (PSD), an electron-dense structure directly apposed to postsynaptic 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, 1994; Lisman and Goldring, 1988; Lisman and McIntyre, 2001; Lisman and Zhabotinsky, 2001; Pettit et al., 1994). CaMKII is in part de-activated by dephosphorylation by PP1, which 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 *knock out*. The resulting CaMKIIα deficiency diminished NMDAR-dependent LTP in hippocampus area CA1 (Silva et al., 1992b; Silva et al., 1992c). This defect was however not observed in heterozygous mice expressing half the amount of CaMKIIα, indicating that partial CaMKII activity is sufficient for proper LTP. The effect of CaMKIIα deficiency was moreover found to depend on the genetic make-up of the animal as LTP was not altered in another line of *knock out* mice with a different genetic background carrying the same null allele (Hinds et al., 1998). This may be due to compensatory mechanisms such as the recruitment of CaMKIIβ (Elgersma et al., 2002) activated to counter-act 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 (Frankland et al., 2001; Kirkwood et al., 1997). 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 100Hz bursts of 4 stimuli, 200ms 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 wildtype 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 Figure 1) with a sub-threshold dose of the NMDAR antagonist CPP, LTP was impaired similarly to that in homozygous mice (Ohno et al., 2001; Ohno et al., 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 thus 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 get 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 wildtype 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 towards 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 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 counter-balance 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 which does not affect ATP binding but renders this kinase mutant sensitive to low doses of a designed inhibitor 1-naphtylmethyl-PP1 (NM-PP1, IC50 = 32nM). When expressed in forebrain neurons under the control of the CaMKIIα promoter, CaMKIIα-F89G increased Ca^{2+}-dependent CaMKII activity by 2.6-fold and Ca^{2+}-independent activity by 2-fold 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 *knock out*, mild or high over-expression by transgenesis interferes with spatial and associative learning and memory. Thus, null, CaMKII-T286A or CaMKII-Asp286 mutant animals are not able to learn spatial information on the water maze (Giese et al., 1998; Mayford et al., 1996a; Silva et al., 1992a) or on the Barnes maze (Mayford et al., 1996b). 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., 2004b; Frankland et al., 2001). 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 (5mg/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 over-expression 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 expression 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* (Genoux et al., 2002; Malleret et al., 2001). LTP was also less prone to reversal by depotentiation ((Jouvenceau et al., 2003), unpublished observation for PP1 inhibition), 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 hippocampal area CA1 (Mansuy et al., 1998a; 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 on the water maze, and improved memory for objects on an object recognition test (Genoux et al., 2002; Malleret et al., 2001). 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 on the water maze (Mansuy et al., 1998a). 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), the 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 consolidation, 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 of phosphatases, synaptic efficacy and performance are respectively strengthened or weakened. But if shifted excessively or unduly in either one direction, it impairs these processes. This may explain why memory is impaired in mice expressing high levels of CaMKII-Asp286, or in *knock out* 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 investigate signaling processes in the nucleus downstream of the NMDAR and intracytoplasmic kinases/phosphatases 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 what 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 gets 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*).

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., 1995; Yin et al., 1994). 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 knock-out (Hummler et al., 1994). 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, LTM (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. Further, unspecific behavioral abnormalities such as thigmotaxis in the

watermaze (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 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 (Broadbent et al., 2004; Moser and Moser, 1998). When tested for spatial learning and memory on 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 of 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 (αCREB^{S133A}) (Gonzalez and Montminy, 1989) that competes with endogenous CREB. αCREB^{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; Feil et al., 1996; Logie and Stewart, 1995) to respond only to the synthetic ligand 4-hydroxy-*tamoxifen* and not to endogenous estrogen (Kida et al., 2002). Mice expressing αCREB^{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 αCREB^{S133A} to the nucleus and disruption of CREB-mediated transcription (within 6 hours). 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 αCREB^{S133A} by *tamoxifen* injection before training did not impair short-term contextual or cued fear memory when tested 2 hours after training. However, it impaired both types of memory after 24h, confirming that CREB-mediated transcription is required for LTM but not STM. The defect was not due to a failure in retrieval since αCREB^{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, αCREB^{S133A} was activated 18 hours later then the animals were re-exposed to the context or the tone (alone) 24 hours later. Re-exposure is meant to reactivate memory for context or tone, a process known to make memory traces transiently instable 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 (*Table 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 by the establishment of new promoters for instance.

The limitations and shortcomings of classical transgenesis will also have to be improved. For instance, 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 (Garrick et al., 1998; Henikoff, 1998; Martin and Whitelaw, 1996).

Promoter	Specificity	Characterization	Use 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., 2001) (Shimizu et al., 2000) (Yu 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)
PrP (inducible-ERT)	Brain, retina, hippocampus cerebellum	(Weber et al., 2001)	-
Thy-1	CNS and PNS (cortex, cerebellum, spinal cord, retina, dorsal root ganglion)	(Campsall et al., 2002)	-
NSE	Embryo: forebrain, midbrain, hindbrain flexure. Adult: cortex, cerebellum, hippocampus, septum	(Cinato et al., 2001)	-
Syn-1	Neuron specific (brain and spinal cord)	(Zhu et al., 2001)	-

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

Promotor	Specificity	Characterization	Use 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., 1996a)	(Mayford et al., 1996a) (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)	-

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

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 8.5kb 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., 1996a). However, when combined with tTA or Cre, it was found to be sometimes active during embryogenesis, leading to perinatal lethality as when used 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., 1996a) or in constrast only in CA1 neurons (with Cre, (Tsien et al., 1996a; Tsien et al., 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 (Giraldo and Montoliu, 2001). They require chromosome-type vectors like bacterial, plasmid or yeast artificial chromosomes (BACs, PACs, and YACs) with large cloning capacity (up to 1Mb). 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. Further, 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 170kb of non-coding genomic DNA of the CaMKIIα gene,

consisting of approximately 50kb of genomic sequence upstream of the ATG and 110kb 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 8.5kb promoter, indicating that a shorter fragment in this case was sufficient (Mayford et al., 1996a). 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 (Korets-Smith et al., 2004; Misawa et al., 2003). *Knock ins* help prevent expression variability linked to random integration and selects for active euchromatin ensuring efficient expression. However, *knock-ins* generally yield few positive animals and heterozygozity at the target locus may perturb the full function of the targeted gene. This may however be alleviated by simultaneous expression of a transgene and the endogenous gene using internal ribosomal entry sequences (IRES) (Funfschilling et al., 2004; Gorski et al., 2002; Lindeberg et al., 2004; Michael et al., 1999).

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. Further, 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., 2005a). Another study combined rtTA with 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).

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., 1996a)	(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., 1998b) (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 et al., 2004) (Saura et al., 2004) (Knuesel et al., 2005) (Saura et al., 2005)

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

Similar to transgenesis, gene targeting has been made conditional by combination with specific promoters and regulated systems. The first example of an inducible *knock out* 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 (Guo et al., 2005; Radomska et al., 2002; Saam and Gordon, 1999; Schonig et al., 2002; Yu et al., 2005a) 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* (Brocard et al., 1997; Casanova et al., 2002; Danielian et al., 1998; Feil et al., 1996; Guo et al., 2002; Hayashi and McMahon, 2002; Kellendonk et al., 1996; Metzger et al., 1995; Schwenk et al., 1998; 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 (Leone et al., 2003; Weber et al., 2001) but its tremendous potential has not yet been exploited for studies of cognitive functions.

Alternative approaches to gene over-expression 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 (Jager et al., 2004; Rossler et al., 2002) 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 extremely tight 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.

Chapter 2: The Protein Phosphatase Calcineurin Regulates the Establishment and the Dominance of Associative Memory Traces[3]

KARSTEN BAUMGÄRTEL[1], DAVID GENOUX[2], HANS WELZL[3], KYOKO KOSHIBU[1], CÉLINE MAMIE[1] AND ISABELLE M. MANSUY[1].

[1]*Brain Research Institute, Medical Faculty of the University of Zürich and Department of Biology, Swiss Federal Institute of Technology, Winterthurerstrasse 190, 8057 Zürich, Switzerland*

[2]*Department of Physiology, Faculty of Medical and Health Sciences, University of Auckland, 85 Park Road, Auckland, New Zealand*

[3]*Division of Neuroanatomy and Behavior, Institute of Anatomy, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland*

Abstract: The protein phosphatase calcineurin (CN) is a potent negative regulator of neuronal signaling known to constrain learning and memory. Here, we demonstrate that CN controls memory strength through mechanisms involving the immediate early gene Zif268. Using inducible transgenesis in mice, we show that strongly aversive forms of associative memory are strengthened and more resistant to extinction when CN is inhibited during memory establishment, but are weakened and more susceptible to extinction when CN is overactivated. This CN-dependent control operates during memory establishment but not extinction because modulating CN activity only during extinction has no effect. Consistently, memory establishment correlates with inhibition of endogenous CN in amygdala. CN acts through Zif268 since mimicking Zif268 upregulation, induced by CN inhibition or by the establishment of aversive memory, selectively during memory establishment similarly strengthens memory. These findings reveal that memory strength is determined by CN and Zif268-dependent mechanisms.

[3] In this study, I performed all experiments, but the ones presented in *Fig. 2a* and *Sup. Fig. 2*, which were conducted by Dr. Genoux. I was supported in the behavioral experiments by Dr. Welzl, Dr. Kyoko Koshibu and Céline Mamie. I wrote the complete manuscript with the help of Dr. Isabelle Mansuy.

Introduction

Our past experiences and the memory of these experiences are major factors that influence our behavior. Among the multiple traces of past experiences stored in memory, the strongest generally dominate and are recalled first, they then determine behavior even if negatively charged i.e. traumatic memory. Recall of strongly aversive or traumatic memory traces often leads to inadequate behavior, for instance inappropriate avoidance responses or excessive fear (Dudai, 2002; Eisenberg et al., 2003). Identifying the molecules that determine the strength and the salience of memory traces is critical for better understanding the mechanisms of pathological forms of memory and their control over behavior but also, the mechanisms of memory formation in general. To fulfill such functions, these molecules are expected to act as switches that rapidly capture the incoming information, which in many forms of traumatic memory is acquired instantly, but at the same time, be able to strongly and persistently modulate neuronal signaling. Ser/Thr protein phosphatases are potential candidates for fulfilling such functions because they are strong regulators of signaling cascades in neuronal cells that are strategically positioned near the NMDA receptor (Coghlan et al., 1995; Colledge et al., 2000) and are rapidly activated following Ca^{2+} influx.

The Ser/Thr protein phosphatase calcineurin (CN or PP2B) is of particular interest because it is the only Ca^{2+}-dependent protein phosphatase in the brain and highly abundant in neuronal cells. CN plays a major role in cognitive functions and as shown to negatively regulate several forms of learning and memory including working memory, memory for objects, and spatial navigation (Ikegami and Inokuchi, 2000; Malleret et al., 2001; Mansuy et al., 1998a; Mansuy et al., 1998b; Runyan et al., 2005).

For persistent memory, neuronal signaling is known to regulate *de novo* gene transcription (Korzus, 2003). Transcription may be influenced by CN through

modulation of signaling cascades or through direct regulation of transcription factors (TFs) (Groth et al., 2003). This includes TFs of the family of the immediate early genes (IEGs) (Enslen and Soderling, 1994; Schaefer et al., 1998; Shin et al., 2001) that are rapidly activated after neuronal activity, but have persistent effects and often act as master switches of whole transcription programs. Zif268 (zinc finger binding protein clone 268) is an IEG that is upregulated after CN inhibition ((Enslen and Soderling, 1994); *Fig. 5*), and that has been strongly linked to processes underlying learning and memory (Bozon et al., 2003; Jones et al., 2001; Lee et al., 2004; Malkani et al., 2004), making it a likely target of CN-dependent signaling.

To examine the potential involvement of CN in the mechanisms that control memory strength, we tested whether modulating its activity in the adult brain alters the establishment and the resistance to interference of associative memory, and whether this involves Zif268. We used conditioned taste aversion (CTA) in mice, which is a form of strongly aversive memory acquired in one-trial by associating a novel taste (conditioned stimulus (CS)) with a visceral malaise (unconditioned stimulus (US)). After learning, the association CS-US induces a strong avoidance response to saccharin, that can persist over several months (*Supplementary Fig. 1*) or years in mammals (Rescorla, 1967; Welzl et al., 2001). This aversive memory trace can be extinguished by re-learning a new non-aversive trace through repeated exposure to saccharin no longer paired with malaise (CS-no US) (Myers and Davis, 2007; Rescorla, 1967; Welzl et al., 2001). The non-aversive trace is more difficult to acquire because it is non-aversive by nature and characteristically weaker, thus initially strongly dominated by the aversive trace (Berman and Dudai, 2001; Berman et al., 2003). Nonetheless during extinction training, the non-aversive trace is progressively reinforced until gaining sufficient strength to dominate the aversive trace. The respective degree of dominance of each memory trace during training determines the animal's behavior towards saccharin, ranging from strong aversion to marked preference (Berman and Dudai, 2001; Berman et al., 2003). Here, we demonstrate that aversive memory can be strengthened or weakened by a decrease or increase in CN

activity during memory establishment and that this modulation determines the course of CTA extinction, even though extinction *per se* is not regulated by CN. We further demonstrate that CN-dependent mechanisms controlling memory strength involve the immediate early gene (IEG) Zif268, which overexpression mimics the effect of CN inhibition on CTA memory. These results overall provide new evidence for the existence of CN-dependent mechanisms that control Zif268 expression and determine the strength of aversive forms of associative memory. Our results further demonstrate that the establishment and extinction of aversive memory are distinct in that memory establishment depends on CN and Zif268 while extinction does not.

Methods

Animals

Adult transgenic mice and control littermates (3-6 months old) backcrossed to C57Bl/6J for at least 4 generations, and C57BLJOlaHsd wild-type mice (3 months old, Harlan, Netherlands) were used. Mice were maintained in standard conditions under a reversed light cycle (dark phase 7am-7pm). Doxycycline (West-ward Pharmaceuticals Corp.) -supplemented food (6mg/100g wet food) was administered to transgenic mice and control littermates daily at least 6 days before and throughout experimentation. All experiments were performed in accordance with guidelines and regulations of the cantonal veterinary office, Zürich.

Generation of Zif268 overexpressing mice

A 1744bp fragment bearing the complete coding sequence of Zif268 was excised from the vector pCMVsport6-Zif268 by AvaI/HpaI digest, blunt-ended using T4-Polymerase (New England Biolabs) and inserted into the EcoRV site of pNN265. From this, a NotI fragment was excised (3190bp) and introduced into the NotI site of pBI-G (Clontech). SalI/AseI digest resulted in an 8381bp fragment that was microinjected into fertilized oocytes derived from gamete donors of a mixed C57Bl/6J

and DBA2 F1 background. Founder mice were crossed with C57Bl/6J mice and their offspring to CaMKIIα-rtTA2 mice (Michalon et al., 2005) (chapter 5) to generate Zif268 over-expressing mice.

CTA test

Singly-housed mice were first habituated to get their daily water ration from two bottles presented for 20-min once or twice/day over 4 days. On conditioning day, they received saccharin (0.5%) instead of water for 20-min, then an i.p. injection of LiCl (0.14M, 2% BW) 40-min later. CTA was tested two days after conditioning, unless stated otherwise, by presentation of one bottle of saccharin and one bottle of water (choice test). CTA extinction was induced by successive daily choice tests (up to 10). Non-conditioned mice were injected saline instead of LiCl.

In CTA experiments with Zif268 mutants, the interval between conditioning and the onset of extinction training was 10 days for both on and off/on groups. Additionally, a 10-day interval was introduced after extinction day 5 to allow for establishment of the non-aversive memory trace.

Cued fear conditioning test

For cued fear conditioning, automated cubicles (Coulbourn Instruments, Allentown) and analysis software (Freezeframe, Coulbourn Instruments, Allentown) were used. Habituation, conditioning and test sessions were performed during the second half of the dark phase. Mice were taken to the behavioral rooms in their home cages at least 30min before each session. The three habituation sessions consisted of transport to the behavioral rooms and experiment-related handling on two consecutive days. One day after habituation, mice were conditioned in a 4-min session starting with 1min rest, followed by three 30-s tone presentations (2800Hz). Tone presentations were divided by 30s and were accompanied by an electric foot shock (0.3mA) during the last second. All mice jumped in reaction to each shock. Tests for cued memory were performed 1, 4, 7, 10, 13, and 16 days after conditioning by 2mins in a novel environment, the second accompanied by the tone. Freezing was determined using 0.75s bout length and expressed as a percentage of time of tone presentation.

CN activity assay

Amygdala and insular cortex were isolated and processed using the BIOMOL Quantizyme^{TM} Assay Kit (AK-816) with modifications including centrifugations at 16,000g and use of P_i-bind resin (Innova Biosciences) for desalting. The first pellet was resuspended in lysis buffer containing 1.2% NP40 (Fluka) to obtain the crude membrane fraction. For cytoplasmic and membrane fractions, 0.7µg and 1.4µg respectively of total protein (as determined by Bradford assay) were used. PSD95 and integral membrane proteins (GluR1, mGluR5) but no nuclear markers (Histone 2A, histone deacetylase 1, Zif268, CREB) were detected in this fraction by Western blotting.

Western blotting

Proteins from preparation for CN assay (20µg for cytoplasmic, 30µg for membrane fraction) were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Blocking and antibody dilution were performed in blocking buffer for near infrared detection (Rockland). Total CN protein was revealed using anti-CN-A (Abcam, final dilution 1:5,000) or anti-CN-B (Upstate, final dilution 1:1,000) antibodies followed by IRDye700-conjugated goat anti-rabbit IgG (Rockland, final dilution 1:5,000) and normalized to β-actin (Upstate, final dilution 1:3,000).

For nuclear enrichment, isolated amygdala were homogenized in lysis buffer (0.32M sucrose, 10mM HEPES, 1mM $MgCl_2$, 0.5mM $CaCl_2$ 5mM EDTA, protease inhibitor cocktail (Sigma), pH7.4), using teflon homogenators (Wheaton). After 10-min centrifugation at 1,000rpm the pellet was resuspended in lysis buffer without sucrose but with 2% SDS. Of this fraction, 50µg protein of each sample was resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Biorad). Blocking was performed in 4% milk powder/1x TBS-T and antibody dilution in blocking buffer for near infrared detection (Rockland). Total Zif268 protein was revealed using an anti-Egr1 (cell signaling technology, final dilution 1:1,000) antibody followed by IRDye700-conjugated goat anti-rabbit IgG (Rockland, final dilution 1:5,000) and normalized to histone 1 (Abcam, final dilution 1:1,000) followed by IRDye800-

conjugated goat anti-mouse IgG (Rockland, final dilution 1:5,000). Visualization and quantification were performed using the Odyssey Infrared Imaging system (LI-COR Biosciences).

Quantitative RT-PCR

RNA was extracted from amygdala using the Nucleospin® RNA II Kit (Macherey-Nagel). An additional DNA digest was performed to eliminate all contaminating genomic DNA (Ambion). Reverse transcription was conducted on 100ng RNA using the SuperScript™ First Strand Synthesis system (Invitrogen). Quantitative PCR was run on an 7500 real time PCR system (Applied Biosystems) using TaqMan© kits Mm00656724_m1 for Zif268, Mm00476361_m1 for NFκB, 4352664-0510003 for β-actin, and 4352662-0509004 for GapDH (Applied Biosystems). Results are expressed as percent of control average.

β-Galactosidase staining

Parasagittal and coronal brain sections of 14µm thickness were processed according to a previously described protocol (Michalon et al., 2005).

Immunostaining

For immunofluorescence mice were perfused, brains isolated and cryosectioned coronally at 20µm. Sections were permeabilized for 30min in 0.4% Triton X100 (Sigma) in 1x PBS. Unspecific binding was blocked for 1hr in 10% fetal calf serum (GibcoBRL), 0.5% TNB (Perkin Elmer), 0.2% Triton X100 in 1x PBS. All antibodies were diluted in 5% fetal calf serum, 0.05% Triton X100. Primary antibody was applied for 8hr at 4°C at following dilutions: 1:200 αZif268 (Rbb, cell signaling technology, Danvers, USA), 1:200 αβ-Galactosidase (Rbb, Molecular Probes; Mouse, Promega), 1:400 αNeuN (Mouse, Abcam), 1:400 αGFAP (Rbb, DAKO). After 3 washes in 1x PBS, secondary antibody was applied at following dilutions: 1:1000 αRbb-Cy5 (Jackson Immunoresearch), 1:1000 αMouse-Cy3 (Jackson Immunoresearch). Images were taken on a confocal microscope (Leica) and processed using Imaris Software (Bitplane AG).

Results

CN activity determines the strength of aversive memory

CN activity was inducibly decreased or increased in transgenic mice by expression of either an autoinhibitory domain of CN (Malleret et al., 2001) or an active form of CNAα (Mansuy et al., 1998b) selectively in forebrain neurons using the reverse tetracycline-controlled transactivators (rtTA or rtTA2) (Mansuy et al., 1998b; Michalon et al., 2005). CN inhibition (-35-45% (Malleret et al., 2001)) or overactivation (+50-75% (Mansuy et al., 1998b)) in adult forebrain neurons did not alter the animals' natural preference for saccharin as shown by similar saccharin intake in mutant and control mice during conditioning (mean saccharin intake, CN inhibitor mutants: 100 ± 6% of control; active CN mutant: 103 ± 5% of control; Fig. 1a).

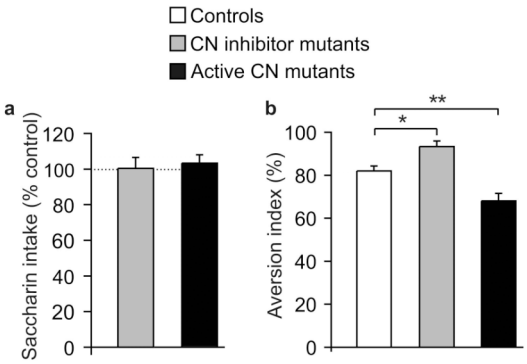


Figure 1. Aversive memory is enhanced by CN inhibition and diminished by CN overactivation. **(a)** Natural preference for saccharin is not altered by CN inhibition or overactivation as shown by a similar ratio of relative saccharin intake in control and mutant mice expressing a CN inhibitor (n = 15, gray bar; controls, n = 14) or active CN (n = 16, black bar; controls, n = 21) over several daily presentations of water and saccharin. **(b)** Aversion for saccharin is significantly higher in mutant mice expressing a CN inhibitor (n = 6) and lower in mutant mice expressing active CN (n = 18) than in control mice (pooled, n = 31), suggesting stronger and weaker aversive memory trace respectively. Error bars represent s.e.m., *P < 0.05, **P < 0.005 in this figure and following ones.

Two days after conditioning, mutant and control mice from both transgenic lines strongly avoided saccharin, but in the mutant mice, avoidance was increased or decreased depending on whether CN was inhibited or overactivated. Thus, aversion to saccharin was significantly increased by CN inhibition (mutants: 93 ± 3%; controls: 82 ± 2%, Fisher's PLSD, P < 0.05), but it was reduced by CN overactivation

(mutants: 68 ± 4%; controls: 82 ± 2%, Fisher's PLSD, $P < 0.001$) (ANOVA, main effect of genotype: $F_{2,53} = 11.20$; $P < 0.0001$, *Fig. 1b*), indicating that the level of CN activity at the time of establishment of aversive memory modulates the strength of this memory.

CN inhibition or overactivation during the establishment of memory modulates its resistance to extinction

We next examined whether the resistance of aversive memory to interference is also altered by a change in CN activity and for this, we assessed extinction of CTA memory. CTA extinction was induced by presenting both saccharin and water to the animals daily, and measuring their aversion to saccharin across time. While extinction training resulted in a gradual decline in aversion to saccharin in control mice (53 ± 14% decline within 7 days, ANOVA; day 1 versus day 7: $F_{1,16} = 8.31$; $P < 0.02$; *Fig. 2a*), it induced only a minor decline in mutant mice expressing a CN inhibitor (12 ± 4% decline within 7 days, ANOVA; day 1 versus day 7: $F_{1,10} = 5.82$; $P < 0.04$; *Fig. 2a*). In mutant mice, memory for the aversive trace was significantly more persistent than in control littermates (main effect of genotype on decline: $F_{1,13} = 5.2$; $P < 0.05$), and continued to dominate behavior by inducing an aversion to saccharin even after 7 days of extinction training (repeated measures ANOVA, day by genotype interaction: $F_{1,78} = 4.05$; $p < 0.002$; *Fig. 2a*). This effect was specific to CN and did not involve PP1, another Ser/Thr protein phosphatase partly regulated by CN and implicated in memory (for review see (Mansuy and Shenolikar, 2006; Munton et al., 2004), since PP1 inhibition in adult forebrain (-45-65% (Genoux et al., 2002) during and after conditioning did not alter the establishment or the extinction of CTA (*Supplementary Fig. 2*).

To examine the persistence of the effect of CN inhibition on the dominance of the aversive memory trace, training was stopped for 1 month then resumed. After this delay, aversion to saccharin was still dominant in mutant mice expressing a CN inhibitor (aversion on day 40: 87 ± 4%, ANOVA; day 7 versus day 40: $F_{1,10} = 0.24$; P

< 0.6), while it was only moderately dominant in control mice (52 ± 12%, ANOVA; day 7 versus day 40: $F_{1,16} = 0.17$; $P > 0.6$; main effect of genotype on day 40: $F_{1,13} = 5.28$, $P < 0.04$; *Fig. 2a*), consistent with the hypothesis of an initially more aversive trace in the mutant mice. However, following two additional days of extinction training, CTA rapidly extinguished in the mutant animals (51 ± 16% decline within 3 days; repeated measures ANOVA, main effect of day: $F_{5,10} = 9.68$, $P < 0.005$) and was similar to CTA in control animals (aversion on day 42: mutants, 45 ± 16%; controls 25 ± 13%, ANOVA; main effect of group: $P > 0.3$), indicating that CN inhibition did not permanently block extinction training but rather shifted memory trace dominance in favor of the aversive trace.

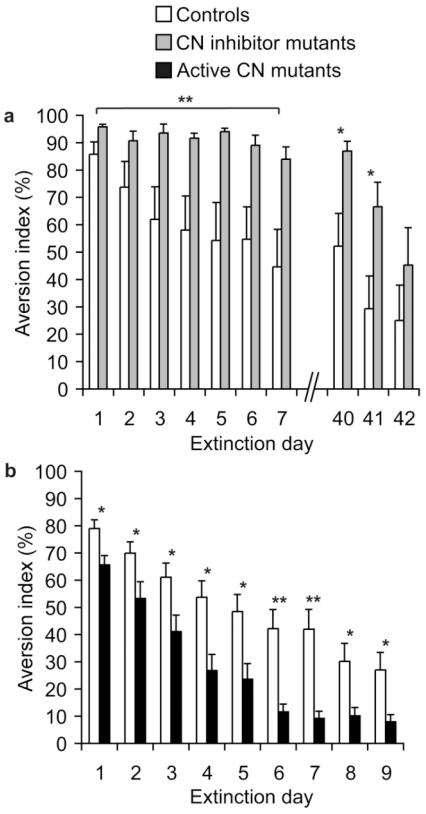


Figure 2. CN inhibition renders CTA memory more, CN overactivation less resistant to extinction **(a)** CTA extinction in mice expressing a CN inhibitor. During the first 7 days of extinction training, aversion to saccharin (aversion index defined as volume of water intake over total liquid intake) remains high in mutant mice expressing a CN inhibitor (n = 6) while aversion gradually declines in control littermates (n = 9). Aversion is still prominent in the mutant mice after a 33-day delay (double bars breaking x-axis) but is rapidly extinguished after two days of extinction training (day 40-42). **(b)** CTA extinction in mice expressing active CN. CN overactivation in mutant mice (n = 16) results in a lower initial aversion index and faster return to baseline than in control littermates (n = 21).

Consistent with these findings, CN overactivation was found to facilitate extinction training in mutant mice (decline within 7 days: mutants, 87 ± 4%; controls, 47 ± 10%; ANOVA, main effect of genotype on decline: $F_{1,35} = 11.59$; $P < 0.002$; overall extinction, repeated measures ANOVA, day by genotype interaction: $F_{1,175} = 2.63$: $P < 0.05$; *Supplementary Fig. 3*). In these mice, extinction was complete after 7 days of

training and saccharin intake was comparable in conditioned mutants (9 ± 3%) and non-conditioned animals (2 ± 1%) on that day and following days ($P > 0.1$; *Fig. 2b*).

Importantly, a change in CN activity did not only affect aversive memory but also modulated fear memory, another form of associative memory. Thus, CN inhibition was found to delay the extinction of memory for a paired tone-footshock while CN overactivation facilitated extinction (repeated measures ANOVA, main effect of genotype: $F_{2,22} = 8.47$; $P < 0.002$; *Supplementary Fig. 4*). These results confirm that CN is a critical regulator of associative forms of memory.

CN is not involved in the formation of the non-aversive memory trace

If CN has a general function in memory formation, it is expected to not only modulate the aversive trace but also the non-aversive trace acquired during extinction training. To test this possibility, we examined whether the process of extinction itself can be altered by CN inhibition or overactivation. We took advantage of the inducibility of transgene expression in our mutant mice and induced CN inhibition or overactivation only after conditioning. In the absence of transgene expression during conditioning, the aversion to saccharin was similar in mutant mice and control littermates when tested after conditioning (CN inhibitor mutants 73 ± 7% versus controls 69 ± 6%, ANOVA; main effect of genotype: $F_{1,22} = 0.14$; $P > 0.7$; active CN mutants 87 ± 4% versus controls 82 ± 5%, ANOVA; main effect of genotype: $F_{1,15} = 0.37$; $P > 0.5$; *Fig. 3*), indicating a comparable level of learning. Furthermore, induction of transgene expression after conditioning did not alter the extinction of the aversive trace as indicated by a similar decline in aversion in mutant mice expressing either a CN inhibitor or an active CN and control littermates (decline within 6 days for CN inhibitor mice: mutants, 91 ± 3%; controls, 84 ± 7%, ANOVA; main effect of genotype on decline: $F_{1,22} = 0.52$; $P > 0.4$; decline within 6 days for active CN mice: mutants, 56 ± 13%; controls, 44 ± 10%, ANOVA; main effect of genotype on decline: $F_{1,15} = 0.58$; $P > 0.4$; *Fig. 3*). These results suggest that the process of extinction *per se* is not regulated by CN.

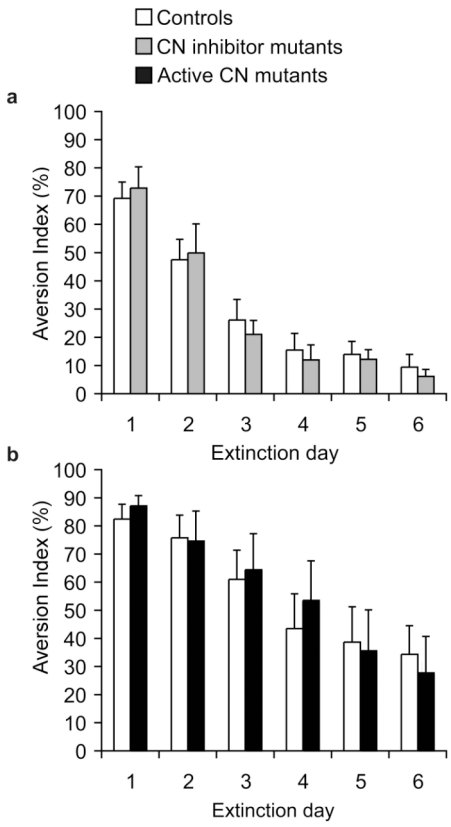


Figure 3. CN inhibition or overactivation after conditioning does not modify CTA extinction. Similar CTA extinction in **(a)** mice expressing a CN inhibitor (n = 8) and control littermates (n = 16), or **(b)** mice expressing an active CN (n = 6) and control littermates (n = 11).

CN activity is inhibited during CTA establishment

To confirm the physiological relevance of CN in CTA, we next examined whether CN can be naturally regulated by the establishment of CTA. C57BL/6J wildtype mice were subjected to CTA conditioning, followed by extinction training or not, then CN activity and protein level were measured in the amygdala and insular cortex, two brain areas involved in CTA (Bahar et al., 2004; Bahar et al., 2003; Rosenblum et al., 1993). CN activity was significantly reduced (-26%) selectively in the amygdala by conditioning (conditioning/no extinction, 46 ± 4% versus no conditioning, 62 ± 4%; ANOVA, main effect of group: $F_{2,21} = 10.59$; $P < 0.001$; Fischer's PLSD, $P < 0.01$; *Fig. 4a*) but it was not changed in the insular cortex (conditioning/no extinction, 55 ± 3% versus no conditioning, 57 ± 3%; *Fig. 4b*). The decrease in CN activity was specific to the membrane fraction where CN is the most concentrated through binding to anchoring proteins (Dodge and Scott, 2003), and was not seen in the cytoplasmic fraction in the amygdala (conditioning/no extinction, 50 ± 1% versus no conditioning, 50 ± 1%; *Fig. 4a*) or insular cortex (conditioning/no extinction, 52 ± 1% versus no

conditioning, 50 ± 1%; *Fig. 4b*). CN activity was no further decreased by extinction training (amygdala membrane fraction: conditioning/no extinction, 46 ± 4% versus conditioning/extinction, 42 ± 1%; Fischer's PLSD, P > 0.4; *Fig. 4a*), indicating a persistent effect of conditioning on CN. The decrease in CN activity did not result from decreased CN expression since the level of CN catalytic A (CN-A, alpha and beta isoforms) or regulatory B (CN-B) subunits was similar before and after conditioning whether followed by extinction training or not (*Fig. 4c*), suggesting the possibility that CN inhibition may result from the action of endogenous inhibitors (Liu, 2003) or other regulatory mechanisms (Wang et al., 1996). These results therefore provide direct evidence that CN is naturally down-regulated by conditioning.

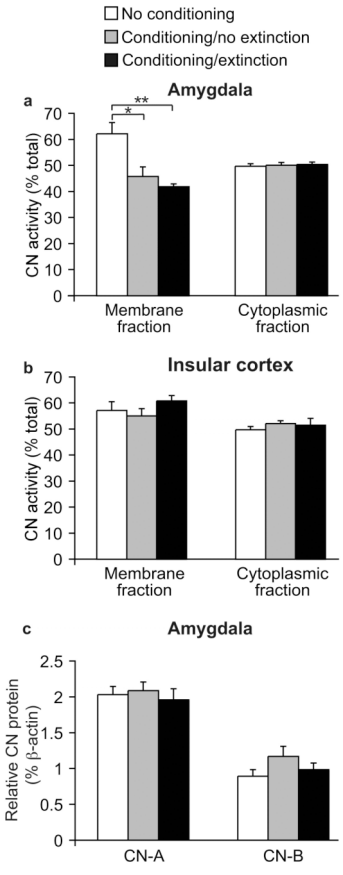


Figure 4. Conditioning in the CTA test decreases CN activity selectively in the amygdala. (**a**) Relative CN activity expressed as percentage of total Ser/Thr protein phosphatase activity in membrane and cytoplasmic fractions of the amygdala 40-min after a choice trial on extinction day 2. CN activity is significantly reduced in the membrane fraction in conditioned animals whether subjected to extinction training (conditioning/extinction, 42 ± 1%, n = 8 versus no conditioning, 62 ± 4%, n = 8) or not (conditioning/no extinction, 46 ± 4%, n = 8, versus no conditioning, 62 ± 4%, n = 8). Cytoplasmic CN activity is unchanged. (**b**) Same as a) for insular cortex. CN activity is not altered by conditioning or extinction training in membrane or cytoplasmic fractions. (**c**) Quantitation of CN in membrane and cytoplasmic fractions of the amygdala by Western blotting. The level of total CN-A or CN-B protein (normalized to β-actin) is not changed by conditioning or extinction training.

Zif268 is increased in CN inhibitor mutants and during memory establishment

We hypothesized that the persistent effect of CN inhibition on aversive memory can only be mediated through changes on the transcriptional level and therefore investigated TFs that may be modulated by CN inhibition. Zif268 is an IEG TF that is required for fear memory formation in the amygdala (Malkani et al., 2004) and that correlates with the persistence of memory (Bekinschtein et al., 2007). Interestingly, Zif268 mRNA was increased 1.8 fold in the amygdala of CN inhibitor mutants (CN inhibitor mutants 100 ± 14% versus controls 180 ± 20%; ANOVA, main effect of genotype: $F_{1,6}$ = 10.35; P < 0.02; *Fig. 5a*). In contrast, the level of another transcription factor implicated in learning and memory, NFκB, was only slightly changed (CN inhibitor mutants 100 ± 1% versus controls 116 ± 8%; ANOVA, main effect of genotype: $F_{1,6}$ = 4.11; P > 0.05; *Fig. 5b*).

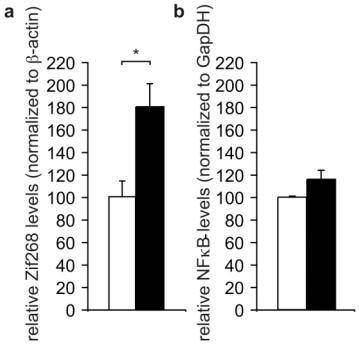


Figure 5. Zif268 levels are increased by CN inhibition. Zif268 **(a)** but not NFκB **(b)** levels are increased in the amygdala of CN inhibitor mice (n = 4) as compared to control littermates (n = 4).

Zif268 overexpression mimics CN inhibition in memory establishment

To test this hypothesis, we mimicked increased Zif268 levels by inducibly overexpressing Zif268 in transgenic mice. In these mice, the β-Galactosidase reporter is co-expressed with a Zif268 transgene and is detected in forebrain neurons (*Fig. 6a, c*). Correspondingly, Zif268 levels are increased on the mRNA (amygdala: mutants 1.6 ± 0.4, n = 6; controls 1.0 ± 0.1, n = 6; ANOVA; main effect of genotype: $F_{1,10}$ = 1.22; P = 0.29; cortex: mutants 2.1 ± 1.1, n = 6; controls 1.0 ± 0.4, n = 6; $F_{1,10}$ = 1.75; P = 0.22; *Fig. 6b*) and protein level (cortex: mutants 14.4 ± 1.1; controls, 11.2 ± 0.9; n = 6; ANOVA, main effect of genoytpe: $F_{1,10}$ = 4.98; P < 0.05; *Supplementary Fig. 5*),

exemplified for the neocortex. When memory performance of the Zif268 mutants was tested in the CTA task, conditioning resulted in a similar initial aversion to saccharin in Zif268 mutants (91 ± 2%) and control littermates (84 ± 4%) when first tested 10 days after conditioning (ANOVA, main effect of genotype: $F_{1,12}$ =1.92; P > 0.1; *Fig. 6c*). However, this may be due to a ceiling effect, since aversive memory is initially extremely robust in both groups, but more persistent in Zif268 mutants during subsequent extinction training (repeated measures ANOVA, main effect of genotype: $F_{1,12}$ = 11.61; P < 0.01; main effect of group by extinction day interaction: $F_{9,108}$ = 2.72; P < 0.01; *Fig. 6c*). Indeed, five daily extinction trials result in a slower decline in aversion (main effect of genotype on decline: $F_{1,12}$ = 10.48; P < 0.01) in Zif268 mutants (49 ± 10% decline within 5 days; ANOVA, day 1 versus day 5: $F_{1,10}$ = 22.58; P < 0.001; *Fig. 6c*) than in control littermates (83 ± 5% decline within 5 days; ANOVA, day 1 versus day 5: $F_{1,14}$ = 112.18; P < 0.0001). After an interval of 10 days, that allowed for the establishment of the non-aversive memory trace, aversive memory was still more pronounced in Zif268 mutants (57 ± 9%) than in control littermates (21 ± 7%; ANOVA, main effect of genotype on extinction day 15: $F_{1,12}$ = 10.39; P < 0.01; *Fig. 6c*). After 3 additional extinction trials however, aversive memory is extinguished in the Zif268 mutants (aversion index on extinction day 18: 22 ± 10%) and is no longer different from controls (aversion index on extinction day 18: 14 ± 7%; ANOVA, main effect of genotype on extinction day 18: $F_{1,12}$ = 0.44; P > 0.5; *Fig. 6c*).

Such delay in CTA extinction may be explained either by enhanced establishment of aversive memory, which would further support the link between CN and Zif268, or by inhibition of the establishment of the non-aversive memory. Therefore, we examined whether the course of extinction itself is altered by Zif268 overexpression and performed the experiment with Zif268 overexpression after conditioning. In the first test ten days after CTA conditioning, aversion to saccharin was comparable between Zif268 mutants (81 ± 5%) and control littermates (72 ± 6%; ANOVA, main effect of genotype: $F_{1,16}$ = 1.18; P > 0.2; *Fig. 6d*). Similarly, repeated presentation of extinction trials resulted in an equivalent course of extinction in Zif268 mutants and control

littermates (repeated measures ANOVA, main effect of genotype: $F_{1,16} = 0.01$; $P > 0.9$; day by genotype interaction: $F_{9,144} = 1.06$; $P > 0.3$; *Fig. 6d*). Zif268 overexpression therefore selectively strengthens the aversive memory trace, while it does not affect the non-aversive trace. Additionally, Zif268 is not involved in the retrieval of CTA memory under these circumstances, as it has been proposed for other forms of memory (Hall et al., 2001).

We conclude that Zif268, like CN, is a strong determinant of the strength of the aversive memory acquired in the CTA task. Likewise, both Zif268 and CN are not involved in extinction of this memory. These findings in Zif268 overexpressing mice further support the notion that Zif268 is a major mediator of the CN-dependent regulation of memory strength described herein. Altogether, the present data highlight the essential role of CN in the establishment and persistence of aversive associative memory. They reveal that the level of CN activity during memory establishment determines the strength of a memory trace and in turn, its degree of dominance. The results further suggest that this may recruit a Zif268 dependent mechanism, since Zif268 mRNA is increased by CN inhibition, Zif268 is physiologically increased during memory establishment, and explicitly increasing Zif268 levels mimics the effect of CN inhibition in CTA.

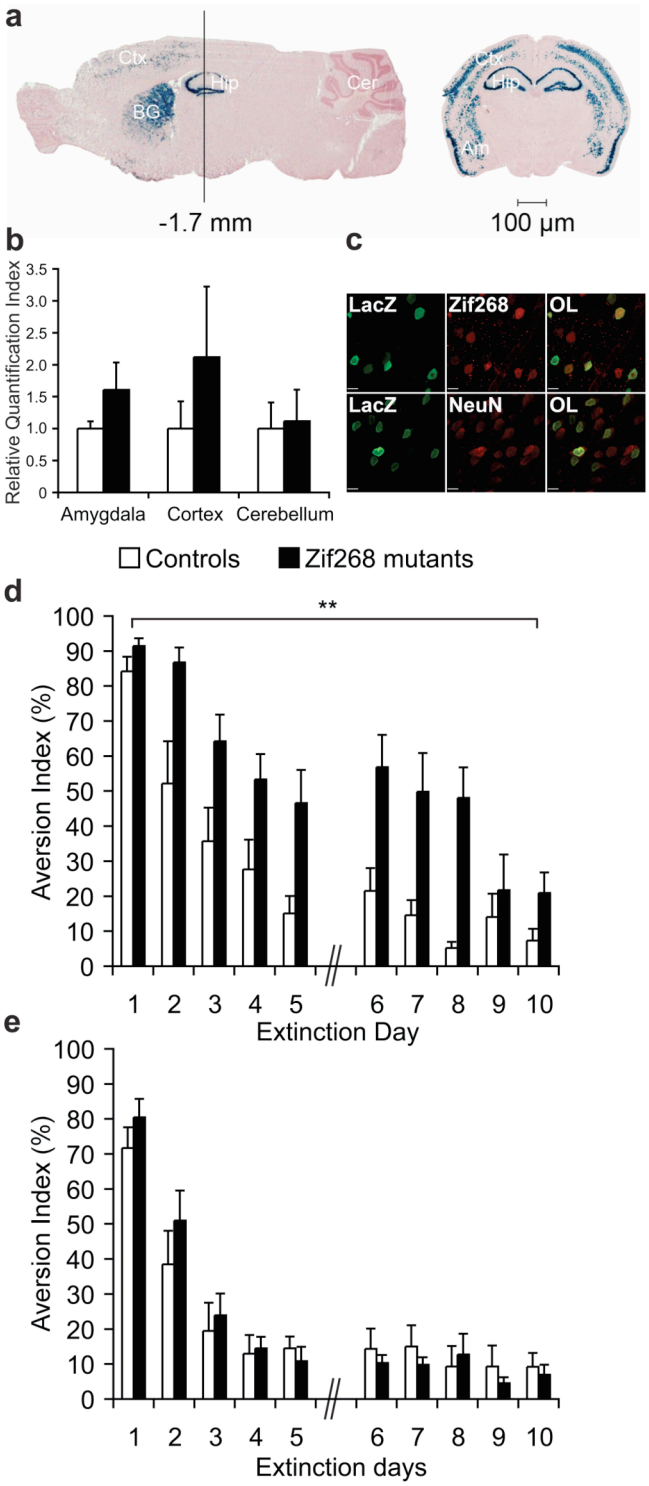


Figure 6. Zif268 overexpression mimics CN inhibition in CTA extinction. (**a**) In Zif268 mutants, the reporter β-Galactosidase is specifically expressed in forebrain, including neocortex (Ctx), hippocampus (Hip), amygdala (AM), and basal ganglia (BG), shown on a parasagittal section and an exemplary coronal section at bregma -1.7mm. (**b**) Zif268 mRNA levels are increased in amygdala and cortex of Zif268 mutants (n = 6, black bar; controls: n = 6, white bar), shown here in neocortex. (**c**) Co-immunostaining of β-galactosidase (LacZ) with Zif268 (top) and NeuN (bottom). (**d**) CTA extinction in Zif268 mutants. During the first 5 days of extinction training, aversion to saccharin remains high in Zif268 mutants (n = 6, black bars) while aversion gradually declines in control littermates (n = 8, white bars). Aversion is still prominent in Zif268 mutants after a 10-day delay (double bars breaking x-axis), but is extinguished after three days of extinction training (days 15-18). (**e**) Zif268 overexpression after conditioning does not modify CTA extinction (mutants, n = 8, black bars; controls, n = 10, white bars).

Discussion

This study investigated the role of CN in the establishment of associative forms of memory. It demonstrates that the strength of aversive or fear memory, and its resistance to interference are determined by the level of CN activity during the establishment of memory. The degree of dominance of a formed memory trace, whether aversive or fear memory trace, is shown to be shifted bidirectionally by a moderate decrease (-35-45%) or increase (+50-75%) in CN activity at the time of conditioning. Further, conditioning to an aversive stimulus is shown to be itself associated with physiological CN inhibition selectively in the amygdala. The strong and opposite effect of a change in CN activity on associative memory highlights the importance of a proper fine-tuning of CN during learning and the central role of CN in the mechanisms of associative memory formation. The importance of such fine-tuning may explain why a drastic perturbation of CN activity by, for instance, genetic knock-out, was found to perturb rather than improve memory in mice deficient for CNA or B subunit (Miyakawa et al., 2003; Zeng et al., 2001), further illustrating that a large deficiency or excess in CN is detrimental to memory formation. The present results also suggest that CN may provide an immediate and potent molecular switch during learning that determines the quality and persistence of memory. This switch may derive from the strategic positioning of CN in the postsynaptic density upstream of major signaling cascades, and its association with several neurotransmitter receptors at the membrane that may allow it to rapidly capture incoming signals (Colledge et al., 2000; Groth et al., 2003; Yakel, 1997). Here, CN may function as a major restraint on the efficacy of signaling to the nucleus that mediates persistent changes underlying the formation of long-term memory. In support of this, the immediate early gene Zif268 is increased in CN inhibitor mutants in the amygdala. Furthermore, CTA conditioning results in a dramatic and persistent increase of Zif268 in the amygdala of wildtype mice. Accordingly, inducible overexpression of Zif268 affects CTA memory similarly to CN inhibition, i.e. the strength of aversive memory is increased, while CTA extinction is unaffected. These data provide a compelling

link between CN, the induction of the IEG Zif268 and gene regulation, and thereby present a molecular pathway for the regulation of aversive memory strength. This may indeed involve direct interaction between CN and TFs that regulate Zif268 expression since CN has been reported to translocate and persist in the nucleus in a complex with nuclear factor of activated T-cells (NFAT (Shibasaki et al., 1996); *Fig. 7*). Additionally, CN in proximity to synaptic sites may activate adenylate cyclases (AC), which regulate CREB phophorylation via cAMP-dependent protein kinase (PKA), mitogen-activated protein kinase (MAPK) and mitogen- and stress-activated protein kinase 1 (Msk1) (Sindreu et al., 2007). MAPK may further induce nuclear translocation of extracellular signal-regulated kinase (ERK), where ERK activates CREB and Elk-1 (Paul et al., 2003). Persistent ERK signaling is known to be required for expression of IEGs (Bito et al., 1996) and was shown be limited by the CN target striatal enriched tyrosine phosphatase (STEP) (Paul et al., 2003).

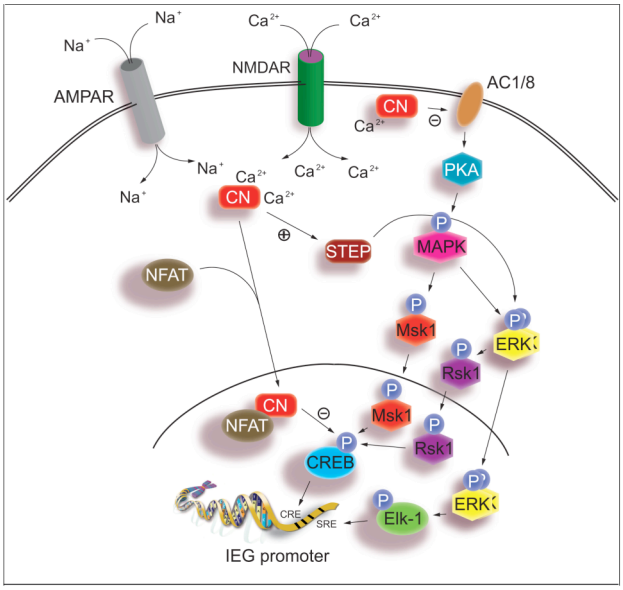


Figure 7. Known signal transduction pathways by which calcium influx regulates gene transcription through CN-dependent mechanisms. The expression of the IEG Zif268 may be coupled to CN by several biochemical routes: (1) CN may directly act on TFs that regulate Zif268 expression; (2) CN functions via the striatal-enriched phophatase (STEP), which negatively controls extracellular signal-regulated kinase (Erk); (3) CN inhibits adenylate cyclases 1/8 and thereby reduces the phosphorylation and nuclear translocation of cAMP-dependent mitogen- and stress-activated protein kinase (Msk1) and of Erk. These cytoplasmic signaling cascades concatenate on the TFs camp response element binding protein (CREB) and the ternary complex factor (TCF), that regulate Zif268 expression via serum response elements (SRE) or cAMP responsive elements (CRE) in the Zif268 promoter.

The mechanisms by which CN is inhibited during memory establishment are not known but may recruit multiple factors and processes including redox inactivation (Wang et al., 1996), inhibition by endogenous inhibitors such as the CN binding protein cabin/cain, the calcineurin homolog protein (CHP), the dual regulators modulatory calcineurin-interacting proteins (MCIPs), or anchoring proteins such as A kinase anchoring proteins (AKAPs) (Liu, 2003).

The present findings further reveal that the mechanisms of establishment and extinction of aversive memory are somewhat different, in that they do not share dependence on CN and Zif268. While the establishment of CTA (acquisition and early phases of consolidation) appears to involve both, extinction *per se* does not. This novel finding is an important step in the understanding of the mechanisms of memory formation because it newly indicates that extinction, although known to be a form of re-learning, can be mechanistically different from initial learning. At the molecular level, this may result from the fact that CN, which inhibition appears to be required for optimal learning, is strongly and persistently repressed by conditioning (at least in the amygdala) and therefore, is no longer subject to regulation during subsequent learning. Similarly, Zif268 is upregulated for at least 24hrs in response to conditioning. This "locked" status may be restricted to CTA conditioning since CN activity was reported to be modifiable one day after conditioning upon recall on the fear-potentiated startle or fear conditioning tasks (Cannich et al., 2004; Lin et al., 2003a; Lin et al., 2003b).
Alternatively, the involvement of the amygdala may be transient and restricted to the formation of a memory trace as shown in fear memory for the lateral and basal amygdala (LBA) (Wilensky et al., 1999). Extinction of CTA memory may thus involve other brain regions, such as the prefrontal cortex, which is indispensable for extinction of fear memory (Quirk et al., 2006).

Such distinction between the establishment and the extinction of memory with CTA was possible because this test has unique properties that make it suitable to

distinguish these processes. It was previously used in the rat to study memory dominance (Eisenberg et al., 2003). CTA is acquired in a single trial, thus has a simple and clear acquisition phase. This is of major importance to study the effect of our manipulation on this specific phase, which is difficult with incremental learning because it has more complex processing that recruits several brain areas successively (Cammarota et al., 2005). CTA is also extremely robust and reliable but nonetheless, can be progressively extinguished in a controlled and quantifiable manner. The natural stimuli used to induce CTA (taste and digestive malaise) also make it ethologically relevant and close to several forms of traumatic memory in human.

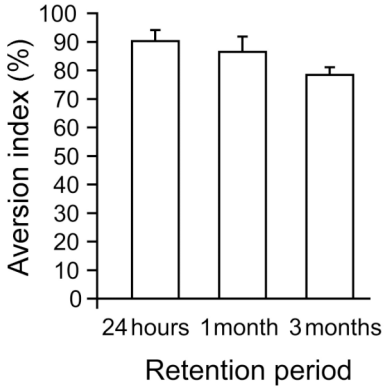
Overall, the present results significantly extend previous findings showing that CN is a molecular constraint on hippocampal-dependent and -independent learning and memory such as spatial working and reference memory or object recognition (Malleret et al., 2001; Mansuy et al., 1998a; Mansuy et al., 1998b; Runyan et al., 2005), by revealing that CN further critically gates highly salient forms of associative memory such as aversive and fear memory. They further demonstrate that this is achieved through a differential regulation of the level of Zif268, a transcription factor known to be involved in long-term memory (Jones et al., 2001).

The findings also strongly support previous evidence that inhibition of another protein phosphatase, PP1, in forebrain is required for efficient memory acquisition (Genoux et al., 2002). Together, these findings firmly establish Ser/Thr protein phosphatases as crucial molecular constraints on memory in the adult brain. The present data finally highlight a potential new target for the development of therapeutic treatment against brain pathologies such as intrusive memory recall in aversive-, fear- or anxiety-associated disorders or post-traumatic stress disorder (PTSD).

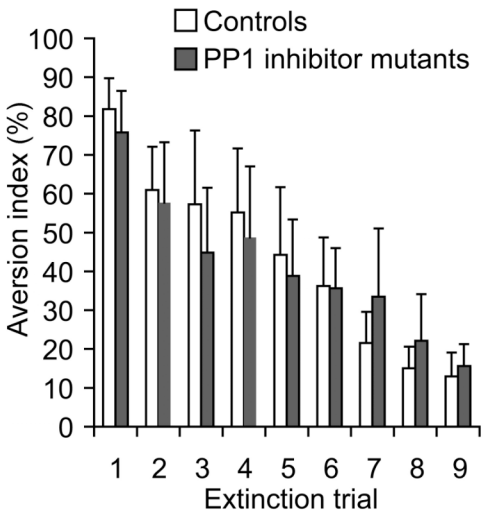
Acknowledgements

We thank Holger Russig, Fritjof Helmchen and Björn Kampa for advice with statistical analyses, Tim Bliss, Serge Laroche, and Dietmar Kuhl for discussion about IEGs, Ursula Haditsch and Lubka Spassova for technical help, Thomas Rülicke and Pawel Pelzar for microinjection of the transgene, and Gregor Fischer for help with animal maintenance. Mouse Zif268 cDNA was generously provided by Dr. A. Sesay and Dr. T. Bliss, the pBI-G vector by Dr. Philipp Berger and Dr. Hermann Bujard. This project was funded by the Swiss Federal Institute of Technology, the University of Zürich, the National Center of Competence in Research "Neural Plasticity and Repair", the Swiss National Science Foundation, the Human Frontier Science Program, the Slack-Gyr Foundation and EMBO Young Investigator Program.

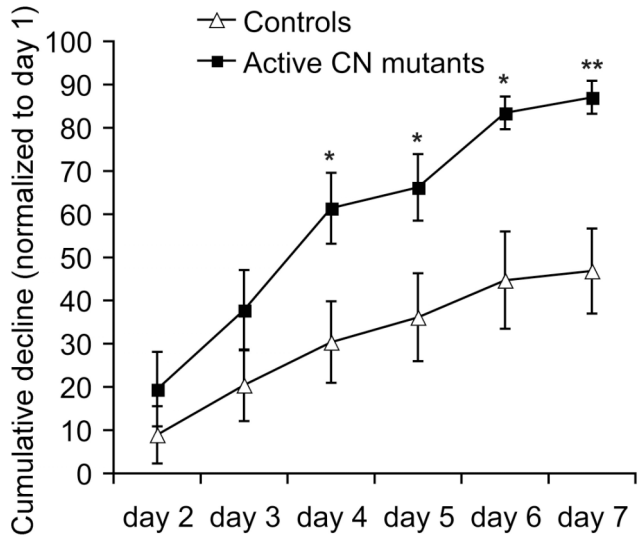
Supplementary Information



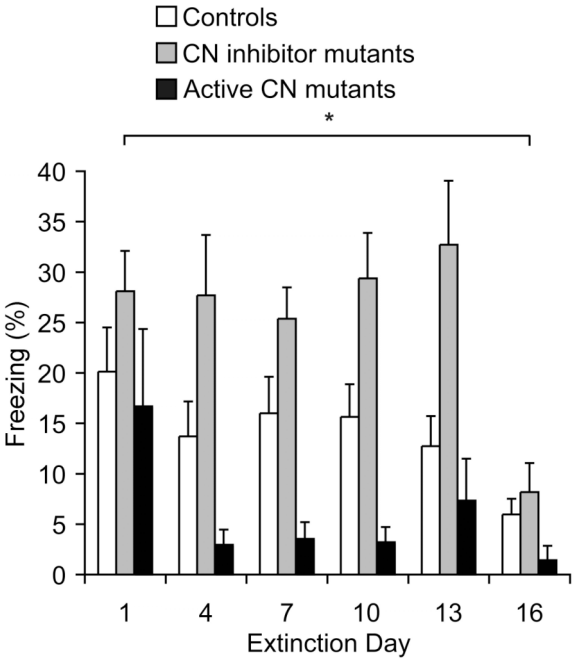
Supplementary Figure 1. CTA memory persists for several months in adult C57Bl/6J mice. One-trial conditioning to saccharin paired with malaise induces a robust CTA as seen by a high aversion index 24 hours after conditioning (90% ± 4%, n = 4). CTA does not significantly decline after 1 month (86% ± 5%, n = 8) or 3 months (78% ± 3%, n = 8; repeated measures ANOVA; main effect of day: $F_{3,6} = 1.84$, $P > 0.2$).



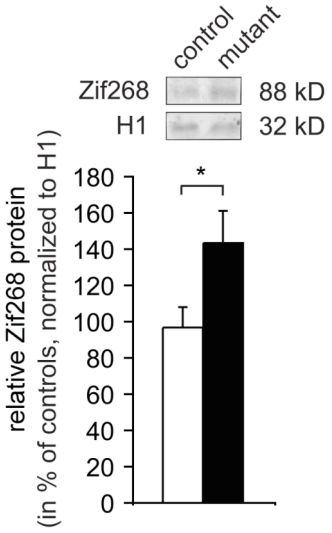
Supplementary Figure 2. PP1 inhibition does not alter the establishment or the extinction of CTA. Similar aversion index in transgenic mice expressing an inhibitor of PP1 (Genoux et al., 2002) (75.79% ± 10.71, n = 6, dark gray bars) and control littermates (81.78% ± 7.97, n = 5, white bars) 3hrs after conditioning (extinction trial 1). Additional extinction trials 15, 24, 39, 48, 63, 72, 87 and 96hrs following conditioning result in comparable decline in aversion index in PP1 inhibitor mutant mice (69.59 ± 14.68%) and control littermates (84.16 ± 7.58%, ANOVA; main effect of group: $F_{1,9} = 0.68$; $P > 0.4$).



Supplementary Figure 3. CN activation during and after conditioning accelerates CTA extinction. Active CN mutant mice (n = 16, black squares) show an overall higher decline in aversion index than control littermates (n = 21, white triangles). Decline (%) is normalized to aversion index at day 1.



Supplementary Figure 4. Extinction of cued fear conditioning is delayed by CN inhibition and favored by CN overactivation. Memory of a tone-shock association is similar in control mice (time spent freezing: 20 ± 4%, n = 14, white bars) and mice expressing a CN inhibitor (28 ± 4%, n = 7, gray bars) or a CN active mutant (17 ± 8%, n = 4, black bars) 1 day after conditioning (extinction day 1; ANOVA, main effect of genotype: $F_{2,22}$ = 0.96, p > 0.3). However, during four subsequent tone presentations (extinction days 4, 7, 10, 13), while freezing gradually declines in response to the tone in control mice, it remains high in CN inhibitor mutants but rapidly declines in CN active mutants (repeated measure ANOVA, main effect of genotype: $F_{2,22}$ = 8.47; p < 0.002). During the sixth tone presentation (extinction day 16), cued fear conditioning is fully extinguished in all groups (controls: 6 ± 2%; CN inhibitor mutants: 8 ± 3%, active CN mutants: 1 ± 1%, ANOVA, main effect of group: $F_{2,22}$ = 1.56; P > 0.2).



Supplementary Figure 5. In Zif268 mutants, the increase of Zif268 in neocortex is also reflected on the protein level as analyzed by semiquantitative immunoblotting (normalized to histone 1, inlay, mutants: n = 6, black bar; controls: n = 4, white bar).

Chapter 3: Reversible Blockade of Experience-Dependent Plasticity by Calcineurin in the Mouse Visual Cortex[4]

YUPENG YANG[1], QUENTIN S. FISCHER[1], YING ZHANG[2], KARSTEN BAUMGÄRTEL[3], ISABELLE M. MANSUY[3] AND NIGEL W. DAW[1]

[1]*Department of Ophthalmology and Visual Science, Yale University School of Medicine, New Haven, Connecticut 06520 USA*

[2]*Department of Chemical Engineering, University of Connecticut, Storrs, CT 06269 USA*

[3]*Brain Research Institute, Medical Faculty of the University of Zürich and Swiss Federal Institute of Technology, 8057 Zürich, Switzerland*

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Abstract: Numerous protein kinases have been implicated in visual cortex plasticity but the role of Ser/Thr protein phosphatases has not yet been established. Calcineurin, the only known Ca^{2+}/calmodulin-activated protein phosphatase in the brain, has been identified as a molecular constraint on synaptic plasticity in the hippocampus and memory. Using transgenic mice overexpressing calcineurin inducibly in forebrain neurons, we now provide evidence that calcineurin is also involved in ocular dominance plasticity. A transient increase in calcineurin activity is found to prevent the shift of responsiveness in the visual cortex following monocular deprivation, and this effect is reversible. These results imply that the balance between protein kinases and phosphatases is critical for visual cortex plasticity.

[4] In this study, I performed the immunoblot analysis and provided *Fig. 1b* and *1c*.

Introduction

Brief monocular deprivation (MD) during the critical period of development dramatically alters neuronal responsiveness to subsequent stimulation of deprived and non-deprived eyes in the visual cortex. This experience-dependent phenomenon is called ocular dominance (OD) plasticity. Calcium influx through NMDA receptors is believed to be one of the initial steps of the mechanism of OD plasticity (Daw et al., 1999; Roberts et al., 1998), followed by the activation of protein kinases and phosphatases. The function of protein kinases in OD plasticity has been studied extensively (Beaver et al., 2001; Di Cristo et al., 2001; Taha et al., 2002). However the contribution of protein phosphatases, known to be involved in hippocampal synaptic plasticity and memory (Mansuy, 2003; Winder and Sweatt, 2001), has not been investigated.

Calcineurin is a Ser/Thr protein phosphatase, highly sensitive to Ca^{2+} (K_d = 0.1-1 nM) and the only phosphatase activated by Ca^{2+}/calmodulin (Klee et al., 1979). It is selectively enriched in pyramidal cells of the central nervous system (Goto et al., 1993a; Goto et al., 1993b). In primary visual cortex, its expression and laminar distribution are developmentally regulated and follow the inside-out pattern of cortical maturation (Goto et al., 1993b). Calcineurin can regulate a wide array of substrates involved in brain plasticity by direct dephosphorylation or through activation of the downstream protein phosphatase 1 (PP1). Calcineurin and PP1 dephosphorylate specific sites on NMDA and AMPA receptors, thereby contributing to the mechanisms of long-term potentiation (LTP), long-term depression (LTD), and depotentiation (Ehlers, 2000; Genoux et al., 2002; Lee et al., 2000; Lieberman and Mody, 1994; Morishita et al., 2001; Tong et al., 1995; Winder and Sweatt, 2001). The pharmacological blockade of calcineurin impairs LTD (Torii et al., 1995) and enhances the induction of LTP (Funauchi et al., 1994) in visual cortex. Mechanistically, it may serve in part to antagonize the cAMP-dependent protein kinase A (PKA) by downregulating molecular substrates activated by PKA (Funauchi

et al., 1994; Mansuy, 2003). It may also control PKA activity itself by inhibiting specific isoforms of the cAMP-producing adenylyl cyclase (AC9) (Antoni et al., 1998). In neurons, calcineurin and PKA are active simultaneously and their concerted action is facilitated by A-kinase-anchoring proteins (AKAP) (Coghlan et al., 1995; Oliveria et al., 2003) through concomitant binding. Interestingly, previous results have demonstrated that type II PKA (Fischer et al., 2004; Rao et al., 2004) and AKAP150 (Fischer et al., *Soc. Neurosci. Abs.* **29**:37.9, 2003) are necessary for OD plasticity. In addition to PKA, calcineurin/PP1 controls other OD plasticity-related substrates that include autophosphorylated Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Taha et al., 2002), a kinase with a similar pattern of mRNA and protein expression as calcineurin in cortical and hippocampal structures (Goto et al., 1993a; Goto et al., 1994), and the cAMP-response element binding protein (CREB) transcription factor (Bito et al., 1996; Mower et al., 2002; Pham et al., 1999). In the visual cortex, CREB-mediated transcription is increased after MD (Pham et al., 1999), and blocking CREB activation prevents the loss of responses to the deprived eye (Mower et al., 2002). Overall, these findings suggest the involvement of calcineurin in OD plasticity.

To test this hypothesis, we took advantage of a line of transgenic mice expressing an active form of calcineurin inducibly and reversibly in the brain with the tetracycline-controlled transactivator (tTA) system (Mansuy et al., 1998a), and examined OD plasticity in these mutant mice. We observed that an excess of calcineurin activity in the visual cortex during the critical period impairs OD plasticity in a reversible fashion. These findings indicate that calcineurin is critical for OD plasticity and support the model that calcineurin negatively regulates various forms of brain plasticity.

Methods

All procedures used in this study were approved by the Animal Care and Use Committee at Yale University and conform to the guidelines of the National Institutes of Health and The Society for Neuroscience.

Generation and maintenance of *TetO*-CN279 transgenic mice

TetO-CN279 transgenic mice (*tetO* promoter–ΔCaM-AI transgene) were generated by microinjection of a linear DNA construct into fertilized 1-cell eggs as previously described[19]. The founder mouse was backcrossed to C57BL6/J mice for 9-10 generations to generate heterozygous offspring then crossed to heterozygous CaMKIIα promoter-tTA mice to generate *TetO*-CN279 mice. Genotyping was performed by PCR on tail DNA. Double mutants *TetO*-CN279 (CNO) and wild-type (WT) littermates were used. The animals were maintained in the facility according to standard protocols. CNO and WT littermates were fed with normal food or with food supplemented with 2mg/kg of *doxycycline* (Research Diets Inc).

In Vivo Electrophysiology

Electrophysiological recordings were performed under nembutal/chlorprothixene (50mg/kg, i.p.; Abott Laboratories/10mg/kg, i.m.; Sigma) anesthesia using standard procedures (Fischer et al., 2004). Atropine (20mg/kg s.c., Optopics) was injected to reduce secretions and parasympathetic effects of anesthetic agents, and dexamethasone (4mg/kg s.c., American Reagent Laboratories) was administered to reduce cerebral edema. Mice were placed in a stereotaxic device, and a tracheal tube and i.p. cannula were inserted. A craniotomy was made over the right visual cortex, and agar was applied to enhance recording stability and prevent desiccation. Eyelids were removed from both eyes, and corneas were protected thereafter by frequent application of silicon oil. Body temperature was maintained at 37°C by a homeostatically-controlled heating pad. Heart rate and respiration were monitored continuously.

4-6 cells (>90 microns apart) through the full thickness of the cortex were evaluated in each of 4 to 6 penetrations spaced evenly (at least 200 microns apart) crossing the binocular region (azimuth <25 degrees) of area 17 to avoid sampling bias. Cells were assigned to OD categories according to the 7-category scheme of Hubel and Wiesel. OD histograms were constructed and WOD scores were calculated for each mouse with the formula: WOD = (1/6G2+2/6G3+3/6G4+4/6G5+5/6G6+G7)/N, where Gi is the number of cells in OD groups, and N is the total number of cells. Normal mice have an average WOD of about 0.28 that is, dominated by the contralateral eye. Response quality was assessed by rating the level of visually driven and spontaneous activity, each on a three-point scale (1 = low to 3 = high). For all measures, data was expressed as mean ± SEM.

Monocular deprivation

Lid suture of the left eye was performed under 1-2% halothane anesthesia on postnatal day 24 (P24) or P33 for all mice receiving deprivation. Lid margins were trimmed and lids sutured together using 6-0 silk. Experiments were performed blind to genotype and drug treatment.

Phosphatase assay

Animals were killed by decapitation after anesthesia with halothane. Phosphatase assays were performed using an assay kit (Calbiochem). Pooled binocular visual cortices (L2-4mm, P0-2mm) were homogenized and centrifuged. After desalting to remove free phosphates, supernatants were diluted in 50mM Tris (PH7.5), 1mM DTT, 100μM EDTA, 100μM EGTA, 0.2% NP-40 and incubated at 30°C for 30min in reaction buffer. Okadaic acid (1μM) was added to inhibit PP1 and PP2A activity. PKA regulatory subunit type II was used as substrate (Ase-Leu-Asp-Val-Pro-Ile-Pro-Arg-Phe-Asp-Arg-Arg-Val-pSer-Val-Ala-Ala-Glu). Calcineurin activity was expressed in nmol Pi released/min/mg protein. The protein concentration was measured using a Biorad protein assay. All measures were performed in triplicate.

Immunoblotting

Homogenates from cortex were subjected to sucrose gradient centrifugation and cytoplasmic, nuclear and crude synaptosomal fractions were collected. Samples were separated by 10% SDS-PAGE then transferred to nitrocellulose membranes. After 1hr blocking (2% goat serum) at room temperature, membranes were incubated with 1:6,000 anti-calcineurin antibody (Chemicon, ab1695) and 1:3,000 anti-β actin antibody (Sigma, A-5316) for 1hr, washed, then further incubated in 1:6,000 anti-rabbit antibody (Upstate Biotechnology 12-348) and 1:3,000 anti-mouse HRP-conjugated secondary antibody (Upstate Biotechnology 12-349). Horseradish peroxidase was detected by adding 300µl chemiluminescence reagent (Perkin Elmer Western Lightning™) and exposing membranes to Kodak MR films. Quantification was conducted using the Image software. Immunoblots were prepared in duplicate or triplicates and results were averaged.

Results

Calcineurin Activity in the Visual Cortex

To confirm that calcineurin activity is increased in primary visual cortex (V1) of the calcineurin overexpressing (CNO) mice, we performed phosphatase assays on extracts from binocular V1 at the peak of the critical period (P28-P29). Assays showed a 48% ± 7% increase in calcineurin activity in CNO mice compared to wild type (WT) littermates (*Fig. 1a*). This increase in calcineurin activity was lower than that observed in the adult hippocampus (Mansuy et al., 1998a) (112% ± 9%), most likely due to a different efficiency and time course of transgene expression in these structures.

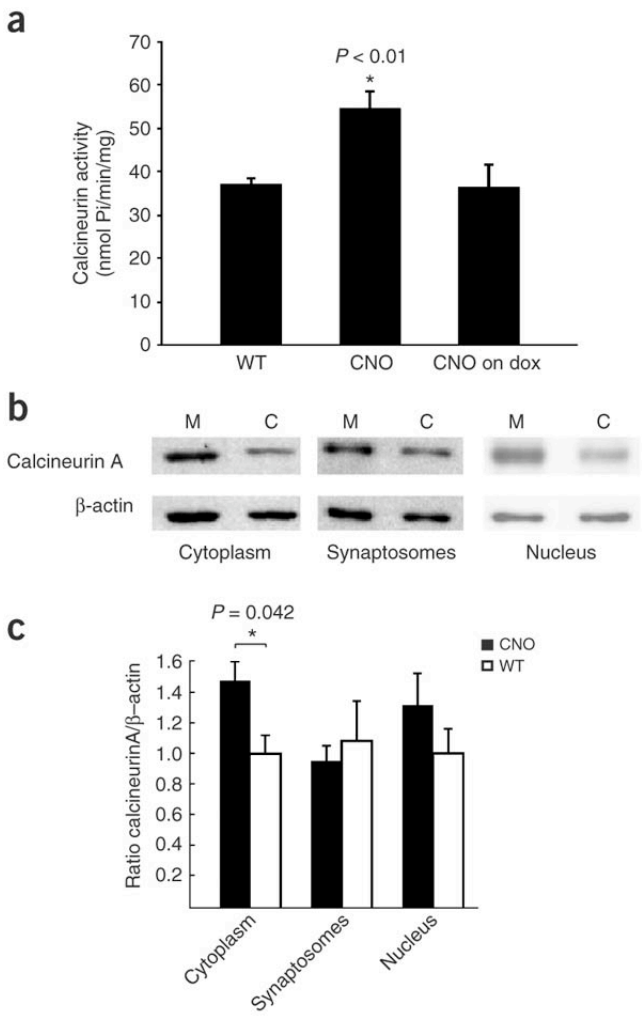


Figure 1. Calcineurin activity is increased in the visual cortex of CNO mice

(a) Enzyme activity in extracts from visual cortex of P28-29 CNO and WT mice as measured by the dephosphorylation rate of PKA RII phosphopeptide in the presence of okadaic acid (PP1/PP2A inhibitor). Wild type (WT): 36.8 ± 1.8 nmol Pi/min/mg, n = 4; CNO mutants: 54.5 ± 4.0 nmol Pi/min/mg, n = 3, $p < 0.01$; CNO on *doxycycline*: 36.2 ± 5.4 nmol Pi/min/mg, n = 4, $p > 0.05$. *Doxycycline* was administered at least 7 days before tissue collection. (b) Representative examples of bands observed on immunoblots in three homogenate fractions from mouse cortex using an anti-calcineurin A antibody (M = CNO mutants, n = 5; C = WT, n = 3), (c) Bar graphs showing relative calcineurin content normalized to β-actin (43kD). Calcineurin protein is significantly enriched in cytoplasmic fraction but not in synaptosomal or nuclear fractions.

Like in hippocampus, calcineurin over-expression in the visual cortex could be suppressed by *doxycycline* treatment, reflecting the full reversibility of the genetic manipulation (*Fig. 1a*). Analysis of the subcellular localization of calcineurin over-expression found a significant increase in the cytoplasmic (46%) but not in synaptosomal or nuclear fractions in the mutant mice (*Fig. 1b* and *1c*). This indicates that the calcineurin transgene concentrates in the cytoplasm in resting conditions.

Impaired OD Plasticity in CNO Mutants

Four days of MD during the critical period is sufficient to induce a robust and saturating OD shift to the open eye (Gordon and Stryker, 1996). In WT littermates, neurons in the binocular zone of V1 were predominantly driven by the contralateral eye as shown in *Fig. 2a*. After MD of the contralateral eye, the OD distribution clearly moved to the right (*Fig. 2b*), indicating a shift of the balance of inputs to the open ipsilateral eye. Longer periods of deprivation did not induce any further OD shift (t-test, 4-5d vs. 6-7d, p = 0.58). In non-deprived CNO mice, cortical neurons showed similar OD distribution as WT littermates (*Fig. 2c*). However after MD, no significant shift was observed in CNO mice (*Fig. 2d*), indicating impaired OD plasticity. OD distribution was still biased to the deprived contralateral eye even after 7-8 days of deprivation (2 of 7 mice, *Fig. 4f*), suggesting that the impairment in OD plasticity was not due to a reduced sensitivity of the mutant mice to MD. Furthermore, the impairment was not due to a delay in the onset of the critical period since 4-5 days MD starting at P33-34 (after the typical critical period (Gordon and Stryker, 1996)) did not induce any apparent OD shift in CNO or WT mice (*Fig. 3*).

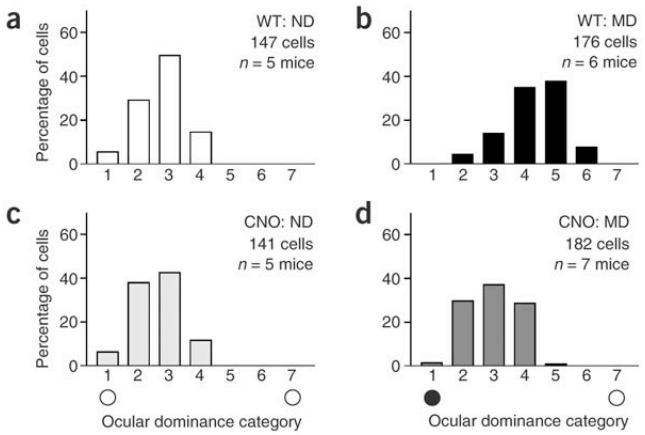


Figure 2. OD plasticity is impaired by calcineurin over-expression.
(a) OD distribution in non-deprived (ND) WT mice. (b) After monocular deprivation (MD) initiated on P24, OD distribution shifts to the right in WT mice. (c) Without MD, CNO mice show a similar OD distribution as WT mice. (d) After MD, CNO mice show no OD shift indicating impaired plasticity.

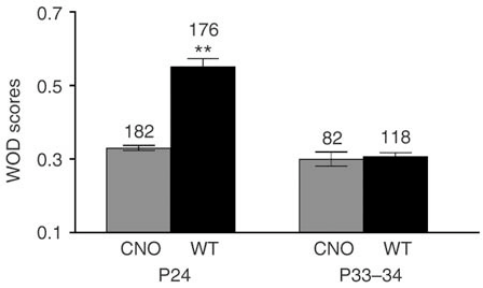


Figure 3. The impairment is not due to a delayed plasticity in CNO mutant mice.
Columns show the effect of MD initiated during (P24, left) or after (P33-P34, right) the critical period on WOD scores in CNO (n = 7 mice and 3 mice respectively) and WT mice (n = 6 mice and 4 mice respectively). The number of cells is labeled above the column. **: $p < 0.001$, t-test.

The Impairment in Plasticity Can Be Rescued

To further determine if the impairment in plasticity was a direct consequence of the increased calcineurin activity, we suppressed calcineurin over-expression with *doxycycline* starting 7 days before MD and examined whether this restored OD plasticity (*Fig. 4a*). In WT controls, OD distribution with or without *doxycycline* treatment was similar in non-deprived and deprived mice (*Fig. 4b, 4c* cf. *Fig. 2a, 2b*). However in mutants, *doxycycline* treatment prior to and during MD (leading to transgene suppression) restored normal plasticity and induced a robust OD shift (*Fig. 4e*), similar to that in WT littermates. The rescue was not due to a non-specific effect of *doxycycline* since *doxycycline* itself had no effect on OD plasticity in WT mice (*Fig. 4d,* see also *Fig. 4b* and *4c*).

Fig. 4f shows the summary of weighted ocular dominance (WOD) scores for individual animals. Without MD (ND, open symbols), all mice had similar low WOD scores whether treated or not treated with *doxycycline* (WT, 0.29 ± 0.02; WT with *doxycycline*, 0.27 ± 0.01; CNO, 0.27 ± 0.02; CNO with *doxycycline*, 0.29 ± 0.01, t-test, $p = 0.38, 0.88, 0.85$, respectively) as in previous studies (Fischer et al., 2004; Gordon and Stryker, 1996; Rao et al., 2004), indicating that calcineurin over-expression or *doxycycline* treatment had no effect on OD distribution in non-deprived animals. Following MD (solid symbols), both WT groups showed a significant increase in WOD scores (with *doxycycline* = 0.55 ± 0.02, without *doxycycline* = 0.55 ± 0.02). CNO mice without *doxycycline* had only a small increase in WOD scores (0.33 ± 0.01; $p = 0.05$ relative to non-deprived WT baseline), which was significantly lower than in deprived WT littermates (t-test, $p < 0.0001$). However, with both *doxycycline* and MD treatment, WOD scores in CNO mice were similar to those in

WT littermates (0.56 ± 0.01, t-test, p = 0.62) but significantly different from those in CNO mice without *doxycycline* (t-test, p < 0.0001), indicating a specific effect of calcineurin over-expression that could be abolished by transgene suppression.

Calcineurin Acts During the Critical Period But Does Not Alter Development

Calcineurin protein is not detectable until P4 while its mRNA is found as early as P1 in rat brain (Polli et al., 1991). To exclude the possibility that an increase in calcineurin activity due to transgene expression during early brain development (before the critical period) alters MD-induced plasticity, we suppressed calcineurin over-expression at birth. Suppression was maintained either through development, or only until one week prior to MD such as to elevate calcineurin activity selectively during the critical period (Robertson et al., 2002). In CNO mice with endogenous levels of calcineurin (due to permanent transgene suppression), the OD distribution after MD shifted to the open eye, similarly to that in WT littermates (*Fig. 5a*). However, following restoration of transgene expression, no OD shift was observed in CNO mice after MD (*Fig. 5b*, left; also see *Supplementary Fig. 1*). WOD values in both CNO groups were significantly different (*Fig. 5c*, *Dox* On/On, 0.56 ± 0.03; *Dox* On/Off, 0.35 ± 0.01; t-test, p < 0.001), indicating that an over-expression of calcineurin during the critical period is sufficient to impair OD plasticity.

Figure 4 (below left). Plasticity is rescued by transgene suppression in CNO mutant mice.
(a) Suppression of calcineurin over-expression by *doxycycline* treatment starting on P17. (b) *Doxycycline*-treated WT littermates without deprivation. (c) *Doxycycline*-treated WT littermates subjected to MD. Both groups show an OD distribution similar to non-treated WT mice (*Fig. 2a* and *2b*). (d) Without MD, *doxycycline* itself has no effect on OD distribution in CNO mice, identical to *Fig. 2c*. (e) After MD, *doxycycline*-treated CNO mice show a dramatic shift to the open eye, similar to that in WT. (f) Weighted ocular dominance (WOD) scores for individual animals. In ND groups (open symbols), WOD scores for WT (○, 0.29 ± 0.02, n = 5 mice) and CNO mice (△, 0.27 ± 0.02, n = 5 mice) are similar (p = 0.38). After MD (closed symbols), WT mice show a robust plasticity and increased WOD scores (●, 0.55 ± 0.02, n = 6 mice), while CNO mice show impaired plasticity (▲, 0.33 ± 0.01, n = 7 mice), not significantly different from WT-ND animals (p = 0.05, t-test). After *doxycycline* treatment (black bar above), ND WT and CNO animals (WT, □, 0.27 ± 0.01, n = 3 mice; CNO, ▽, 0.29 ± 0.01, n = 5 mice) show WOD scores similar to non-treated ND groups (p = 0.88 and 0.85 respectively). After MD, *doxycycline* treatment in CNO animals rescued the impaired plasticity and led to an OD shift (▼, 0.56 ± 0.01, n = 6 mice) similar to that in WT littermates (■, 0.55 ± 0.02, n

= 4 mice; $p < 0.001$). Each symbol represents the WOD score for a single mouse. Mean values are shown by horizontal bars with numbers.

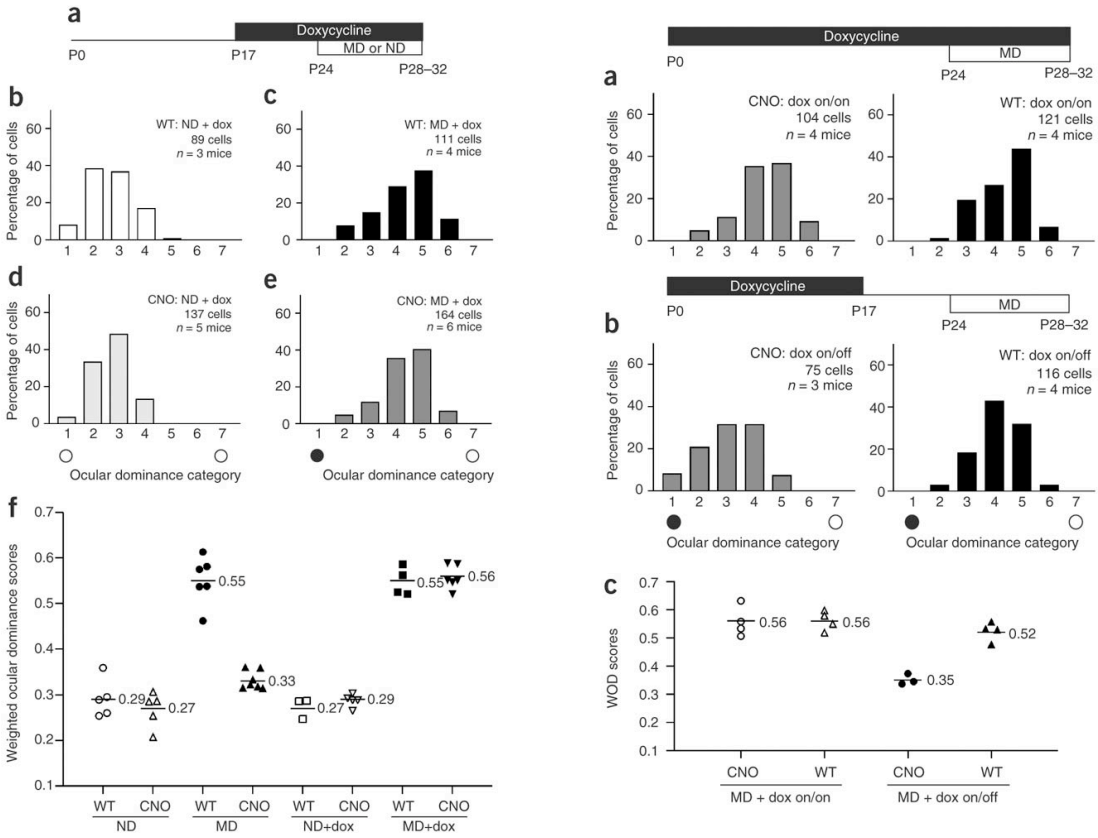


Figure 5 (top right). Calcineurin over-expression only during the critical period impairs OD plasticity. (a) *Doxycycline* was administered to CNO and WT mice from P0 to the date of recording (*Dox* On/On). MD induced a similar OD shift in CNO mutants (left) and WT littermates (right). (b) *Doxycycline* was removed one week prior to MD (P17, *Dox* On/Off) such as to turn calcineurin over-expression back on. Impaired OD shift was observed in CNO mice (left) while plasticity was normal in WT mice (right). (c) WOD scores for individual animals. In *Dox* On/On group, MD induces high WOD scores in both CNO and WT mice (CNO, ○, 0.56 ± 0.03; WT, △, 0.56 ± 0.02). In contrast, CNO mice in MD+*Dox* On/off group (●, 0.35 ± 0.01) have significantly lower WOD scores than WT littermates (▲, 0.52 ± 0.02, $p < 0.001$, t-test). Each symbol represents the WOD score for a single mouse. Mean values are shown by horizontal bars with numbers.

Further, WOD scores in On/Off *doxycycline* conditions were similar to those in non-treated conditions for both CNO and WT groups (cf. *Fig. 5b* vs. *Fig. 2b* and *2d*; $p = 0.37$ for WT, $p = 0.19$ for mutants), suggesting that the impairment in OD shift in the mutant mice was not due to any developmental anomaly induced before the critical period. Consistently, the retinotopic map of V1 (*Fig. 6a*), the size of receptive fields (*Fig. 6b*), response strength, and signal to noise ratio (*Fig. 6c*) were normal in CNO animals.

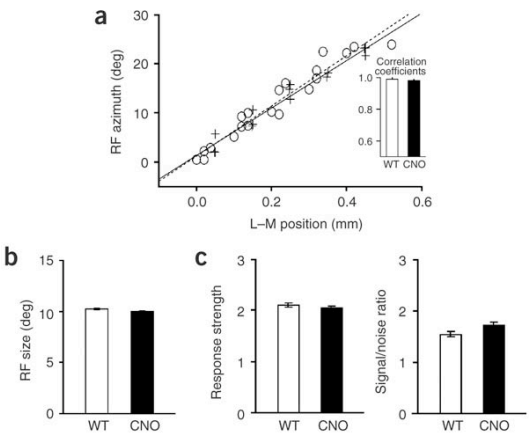


Figure 6. Response properties are normal in CNO mutant mice.

(a) Retinotopic mapping in WT mice (○, n = 15 units in 3 mice) is similar to that in CNO animals (+, n = 20 units in 4 mice). Each symbol represents the average receptive field azimuth of a single penetration. Inset: Correlation coefficients for three WT and four CNO regressions (p = 0.5, t test). (b) Size of receptive field (WT, n = 118 cells in 4 mice; CNO, n = 128 cells in 5 mice) is unchanged (p = 0.2, t-test). (c) Response strength (left panel) and ratio of signal and noise (right panel) are similar in WT and CNO animals (WT, n = 118 cells; CNO, n = 128 cells, p > 0.01 for both, t-test).

Plasticity Can Also Be Rescued in Adult CNO Mice

To examine whether calcineurin over-expression may alter the closing of the critical period plasticity and if so, whether plasticity could be rescued in adulthood, we started *doxycycline* treatment at P38 and performed MD one week later (P45). 4-5 days deprivation did not induce any shift in WT mice (*Fig. 7*, top). However in CNO mice, a low but significant shift was observed (*Fig. 7*, middle; WOD = 0.43 ± 0.06, p = 0.003), suggesting that calcineurin over-expression interfered with the closing of the critical period and thereby extended the critical period.

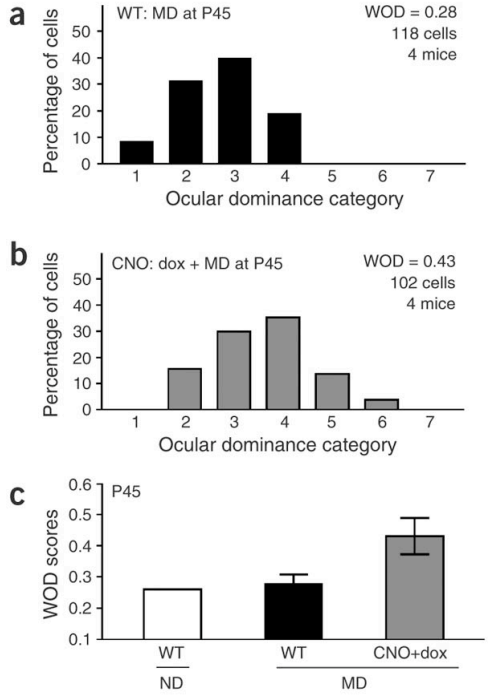


Figure 7. Restoring normal calcineurin activity induces OD plasticity in adult mice.

Top, brief MD does not induce an OD shift in adult WT mice (P45). Middle, *doxycycline* treatment at P38 (7 days before MD) results in an OD shift in CNO mice ($p < 0.0001$, χ^2 test). Bottom, average WOD score for adult WT mice (P45) is not altered by MD (WOD = 0.28 ± 0.03 compared to WOD = 0.26 according to Gordon and Stryker, 1998). Restoration of normal calcineurin at P38 in CNO mice induces a significant increase in WOD scores after MD (WT, WOD = 0.28 ± 0.03; CNO, WOD = 0.43 ± 0.06, $p < 0.01$, t-test).

Discussion

OD plasticity is a form of sensory experience-dependent plasticity that has been extensively studied, but the molecular mechanisms remain poorly understood. This study provides evidence that the protein phosphatase calcineurin is involved in OD plasticity by showing that an excess of calcineurin blocks the shift in OD normally induced by monocular deprivation. The impairment was induced by a transient increase in calcineurin activity during the critical period and could be rescued by restoring normal calcineurin activity, owing to the inducibility and reversibility of the genetic manipulation. Our results are consistent with previous studies showing that downregulation of protein kinase activity impairs OD plasticity (Beaver et al., 2001; Di Cristo et al., 2001; Taha et al., 2002) and emphasize the importance of the interplay between protein kinases and phosphatases in this form of plasticity.

Another interesting finding is that plasticity can be restored partly in adulthood in CNO mice. One possible explanation is that over-expression of calcineurin downregulates GABA inhibition and mimics the effect seen in GABA deficient mice. Compromised GABAergic transmission in GAD65 knockout mice impairs OD plasticity through a delay of the critical period onset. Once the onset is restored by 2 days of benzodiazepine treatment, OD plasticity becomes normal even if GABA transmission was most likely still deficient at the time of deprivation since the treatment was stopped 1 day before monocular deprivation. This contrasts with our result that OD plasticity is impaired by an increase in calcineurin activity only after the onset of the critical period (see *Supplementary Fig. 1* and *Fig. 5*). Therefore, calcineurin may regulate OD plasticity through mechanisms other than GABA inhibition. This is also supported by the fact that calcineurin is essentially overexpressed in cytoplasmic fractions and not at synapses and is therefore more likely to perturb intracellular signaling pathways than membrane receptors. Another possibility is that plasticity also lies dormant in all plasticity-impaired mutant models, such as PKA RIIβ knockout, CaMKII knockout and tPA knockout (Mataga et al., Soc. Neurosci. Abs. 28:131.5, 2002). It will be of great interest to examine whether OD plasticity can be rescued in these mice.

As calcineurin is associated with PKA through anchoring proteins, it is likely to affect similar substrates during OD. One of these substrates may be inhibitor-1 (I-1), a PP1 inhibitor activated by phosphorylation by PKA and blocked by dephosphorylation by

calcineurin. Once calcineurin is stimulated by Ca^{2+} influx through NMDA receptors, it can activate PP1 via relief of I-1-mediated inhibition. Calcineurin can also stimulate the striatal-enriched protein tyrosine phosphatase (STEP), another protein phosphatase that is inactive in basal conditions (Paul et al., 2003). A potential molecular cascade involved in OD plasticity may thus engage calcineurin and PKA in a gate that controls PP1 activity and in turn, the autophosphorylation of CaMKII (Giese et al., 1998; Taha et al., 2002), a critical player in OD plasticity. Calcineurin and PKA may also gate ERK pathway by modulating ERK activity through direct phosphorylation/dephosphorylation (Cancedda et al., 2003), or by interfering with its translocation through STEP (Paul et al., 2003). An ultimate component of the cascade may implicate CREB and CREB-dependent gene expression in the nucleus, as OD plasticity requires CREB (Mower et al., 2002) activation and protein synthesis (Taha and Stryker, 2002). Control of CREB-mediated transcription depends on CREB phosphorylation/dephosphorylation at Ser-133 by protein kinases including PKA, CaMKIV, and MAPK, and the protein phosphatase PP1 possibly after activation by calcineurin (Bito et al., 1996; Groth et al., 2003). Calcineurin also modulates intracellular Ca^{2+} by reducing NMDA receptor current decay time and regulating intracellular Ca^{2+} release (Cameron et al., 1995), thereby controlling other Ca^{2+}-dependent enzymes. Thus, calcineurin may be involved in the control of signaling from the synapse to the nucleus during ODP. This is consistent with the fact that in our mutants calcineurin is essentially over-expressed in the cytoplasmic fractions and not at synapses and is therefore more likely to perturb intracellular signaling pathways than membrane receptors. Recent work suggests that ODP induction and expression machinery may be different. Benzodiazepines restore the onset of critical period, which is altered in $GAD65^{-/-}$ mice, but appear not to intervene in the expression of ODP (Iwai et al., 2003). Further, $GABA_A$ $\alpha 1$ knock-in mice show normal expression of ODP, although the transition to the precocious critical period by benzodiazepines is impaired (Fagiolini et al., 2004) because benzodiazepines act through this subunit of GABA receptors. Thus, GABAergic inhibition seems to be uniquely responsible for the induction machinery. In out mutant mice, however, ODP is impaired by an increase in calcineurin activity with an intact onset of the critical period (*Supplementary Fig. 1*, *Fig. 5*). Therefore, calcineurin may regulate ODP through mechanisms other than GABA inhibition during the critical period. Our results also show that calcineurin over-expression may disturb the closure of the critical period.

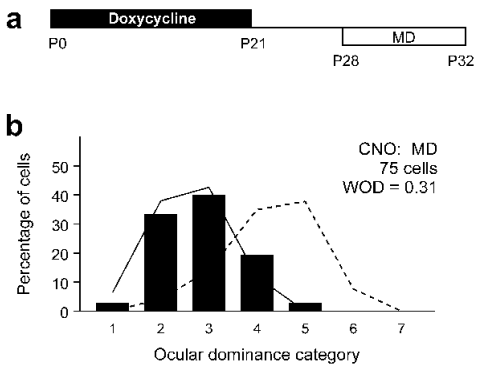
The mechanisms of closure are not well understood but may involve the maturation of extracellular matrix (Berardi et al., 2004) and intracortical myelination. Recent experiments reveal that ODP persists into adulthood in mice deficient for myelin-associated protein Nogo-A or its receptor (A.W. McGee, Y.Y., N.W.D. and S.S. Strittmatter, unpublished data). Thus, it could be of interest to examine whether the synthesis of these molecules is altered in CNO mice, as well as in other kinase-deficient mice.

Short deprivation episodes during the critical period can induce structural changes in the brain. For instance, spine motility is significantly increased after MD (Oray et al., 2004). In the hippocampus, calcineurin is known to induce a shrinkage of dendritic spines, possibly by dephosphorylating cofilin and perturbing cofilin-actin polymerization (Zhou et al., 2004). Together with PKA, it is also believed to control neurite extension and axonal regeneration (Fansa et al., 2000; Lautermilch and Spitzer, 2000). It is therefore possible that calcineurin over-expression in our model impairs visual cortex plasticity by interfering with structural changes necessary for such plasticity. Further investigations would be necessary to examine this point.

OD plasticity has been suggested to share mechanisms with LTD in the visual cortex (Heynen et al., 2003). However, previous work showed that calcineurin inhibition by FK506 impairs LTD in visual cortex (Torii et al., 1995) and calcineurin activity increases during LTD *in vivo.* Calcineurin over-expression in our mutant mice did not change LTD in layer II/III in visual cortex (data not shown), consistent with its reported lack of effect on LTD in the hippocampus in the same mice (Winder and Sweatt, 2001). This suggests that LTD and OD plasticity may not be directly related or have a complex relationship as suggested by several others studies (Hensch et al., 1998; Rao et al., 2004; Renger et al., 2002), which may be explained by the existence of several forms of LTD in different cortical layers (review see Daw et al, 2004).

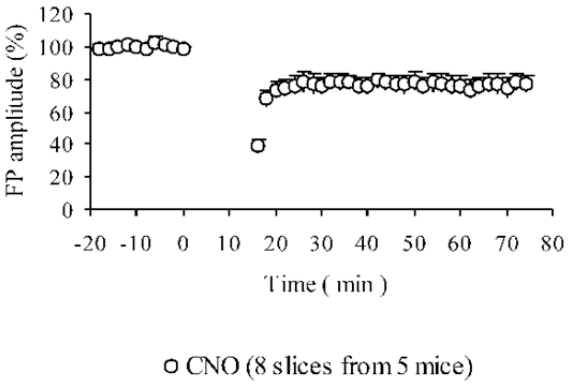
Finally, we did not observe any OD plasticity in adult WT animals (*Fig. 3*) like several other studies (Gordon and Stryker, 1996; Taha et al., 2002). This contrasts, however, with recent reports describing the existence of a form of visual plasticity measured by visually-evoked potentials in adult animals (Pham et al., 2004; Sawtell et al., 2003). This adult plasticity is strongly suppressed by nembutal (Pham et al., 2004) and can therefore not be observed in our experimental conditions.

Supplementary Material



Supplementary Fig. 1. Lack of ocular dominance plasticity with an intact onset of the critical period in CNO mice. (a) Calcineurin transgene is suppressed by *doxycycline* treatment from birth to P21 after the typical onset of the critical period (P19, Gordon and Stryker, 1996). The animals are monocularly deprived at P28, and recordings are performed four days later. (b) Columns showing ocular dominance distribution after monocular deprivation in CNO mice, which is identical to that in non−deprived wild−type mice (solid line), but significantly different from that in deprived wild−type mice (dash line, $P < 0.0001$, Ï‡ 2 test), indicating the impaired ocular dominance plasticity.

Supplementary Fig. 2. LTD is normal in visual cortex in CNO mice. LTD was induced by 15-min 1-Hz presynaptic stimulation paired with postsynaptic depolarization in CNO mice (77 ± 5%, n = 8; $P < 0.01$, compare to the baseline), which was identical to that observed in wild type animals (74.7 ± 3.1%).



Acknowledgments

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Chapter 4: The Immediate Early Gene Zif268 Regulates the Mechanisms of Aversive Memory

KARSTEN BAUMGÄRTEL[1], HANS WELZL[2], AND ISABELLE M. MANSUY[1]

[1] *Brain Research Institute, Medical Faculty of the University of Zürich and Swiss Federal Institute of Technology, 8057 Zürich, Switzerland*

[2] *Institute of Anatomy, University of Zürich, 8057 Zürich, Switzerland*

Unpublished.

Abstract. Classically, the term "memory disorder" is synonymous with the term "dementia disorder". It refers to pathological conditions of deteriorating memory performance that affect an estimated 4.5 million Americans. However, post-traumatic stress disorder (PTSD), a form of anxiety disorder, may also be viewed as a memory disorder. This condition is usually triggered by past aversive and traumatic experiences and affects about 7.7 million American adults. Its development strongly depends on the genetic load of each individual and the presence of susceptibility genes. To better understand whether and how genetic factors affecting memory performance contribute to this predisposition, we are generating transgenic mouse models that allow the inducible, non-invasive over-expression or inhibition of candidate genes in the brain.

Zif268 (or Egr-1, Krox24, NGFI-A, Tis8, ZENK) is a ubiquitous immediate early transcription factor that functions as coordinator of cellular responses to diverse types of stimuli. *Knock-out* and *Knock-down* approaches have shown that the complete or partial absence of *Zif268* results in disruption of memory consolidation and reconsolidation. Here, we show that the acute over-expression of *Zif268* in excitatory neurons of the forebrain is sufficient to enhance the consolidation of aversive memory traces, making it more resilient to extinction or interference. However, it does not alter the extinction of memory traces *per se*. Further analysis of the mechanisms underlying this selective enhancement may help understand the predisposition and pathogenesis of memory disorders, including PTSD.

1. Introduction

1.1. The Immediate Early Gene Zif268

Zif268 (Egr-1, Tis-8, NGFI-A, Krox-24, ZENK) was first identified in 1987 by Milbrandt *et al.* in PC12 cells after induction by nerve growth factor (NGF) which elicits their differentiation into neuron-like cells (Milbrandt, 1987). Approximately at the same time it was described by Sukhatme and colleagues using a differential screening strategy with serum-stimulated fibroblasts in the presence of cycloheximide (Sukhatme et al., 1987) and by Lau and Nathans in 3T3 cells where it is also induced by serum and growth factors (Lau and Nathans, 1987). Egr-1 has since been found to be induced in a wide variety of cells, by a long list of stimuli (Gashler and Sukhatme, 1995). Its acronyms are a result of the parallel discovery of this gene and its product: Egr-1 – early growth response gene 1, Tis-8 – tetradecanoyl phorbol acetate-induced sequence 8, NGFI-A – nerve growth factor-induced gene A, Krox-24 - gene containing sequences homologous to the *Drosophila* Kr finger probe, ZENK – an acronym of the previous four names.

1.1.1. Zif268 Protein

Zif268 mRNA carries two different initiation sites that yield either an 82 or 88kD protein (Lemaire et al., 1990), but no functional difference between these two isoforms has been described so far. Zif268 protein is short-lived, extensively phosphorylated and glycosylated at serine residues (Lemaire et al., 1990), and localises to the nucleus. Its crystal structure was resolved very early and showed that Zif268 binds to the major groove of B-DNA (Pavletich and Pabo, 1991). Sequence specificity of this binding is mediated by three zinc finger domains found in the 3' exon (Changelian et al., 1989; Tsai-Morris et al., 1988)(*Zif268* only contains 2 exons), each containing an α-helix and an antiparallel β-sheet held together by a zinc ion and a set of hydrophobic residues. These zinc finger domains are highly conserved between different Egr proteins and homologues from different species emphasizing the importance of sequence specificity of this binding (Crosby et al., 1991; Drummond et al., 1994). The zinc finger domains also contain the nuclear localization signal for Zif268 (Matheny et al., 1994).

1.1.2. Zif268 induction

Zif268 expression, DNA binding and transactivation activity are highly regulated and thereby provide several levels of control of Zif268-mediated transcription. The murine as well as the human *Zif268* promoters (Sakamoto et al., 1991; Schwachtgen et al., 2000) contain five SRE elements. Two kinds of transcription factors are required for SRE mediated activity: (a) the serum response factor (SRF) and, (b) the ternary complex factor (TCF). The TCF includes the transcription factors Elk1, Sap1 and Sap2 that must both contact DNA and bind to SRF to exhibit biological activity. In the c-*fos* promoter, it was shown that the TCF interacts with the SRE only when SRF is already bound, thereby forming a ternary complex composed of an SRF dimer and a single molecule of TCF (Mueller and Nordheim, 1991; Shaw, 1992; Treisman et al., 1992).

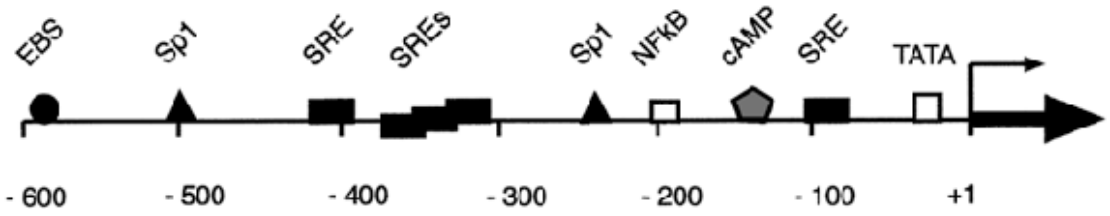

Figure 1. Regulatory region of the human *Zif268* gene. EBS = Egr binding site, Sp1 = Specificity protein 1 element sites, SRE = steroid response element, NFκB = nuclear factor kappa B, cAMP = cyclic adenosin monophosphate, TATA = binding and assembly site for general transcriptional machinery. Taken from (Thiel and Cibelli, 2002).

Elk1 is phosphorylated by the c-Jun N-terminal protein kinase (JNK) and extracellular signal-regulated protein kinase (ERK), leading to enhanced DNA-binding activity, ternary complex formation, and SRE-mediated transcription (Whitmarsh et al., 1995). Accordingly, expression of a constitutively active mitogen-activated protein kinase kinase, the kinase responsible for the phosphorylation and activation of ERK, strongly stimulates *Zif268* promoter activity (Kaufmann et al., 2001; Kaufmann and Thiel, 2001). Both SRF and ERK have been shown to play prominent roles in use-dependent modification of synaptic strength in the adult brain (Martin et al., 1997; Ramanan et al., 2005). Indeed, in PC12 cells *Zif268* expression in response to NGF is mediated via the SRE-1, SRE-2 and an AP-1-like site (DeFranco et al., 1993). In other immortalized cells, 3T3-F442A cells, Zif268 expression is up-regulated after growth hormone treatment in dependence of its SRE sites (Hodge et al., 1998). In B-cells of

the immune system, two of the SREs appear to be the dominant regulators of *Zif268* transcription, and the activation of one SRE is sufficient for maximal expression (McMahon and Monroe, 1995). However, in neurons SRF binding to SRE sites in the *Zif268* promoter appears to be constitutive and the role of SRF in synaptic plasticity may instead be conveyed by its regulation of *c-fos* and *junB* expression (Lindecke et al., 2006).

Zif268 can additionally auto-regulate its own transcription by binding to the Egr binding site in its promoter with high affinity (Sakamoto et al., 1991). As a result, Zif268 inhibits transcription of its own gene (Cao et al., 1993). Other features of its 5' region include two CRE-like (cAMP responsive element) elements, four Sp1 (Specificity protein 1 element) sites (Tsai-Morris et al., 1988) (compared with two in humans, (Christy et al., 1988; DeFranco et al., 1993; Sakamoto et al., 1991; Schwachtgen et al., 2000)), and two AP-1 (Activator protein 1 element) sites (Tsai-Morris et al., 1988) (*Fig. 1*). In glioblastoma cells, activation of adenylate cyclases by forskolin does not stimulate *Zif268* promoter activity (Meyer et al., 2002) indicating that the cyclic AMP response element (CRE) in the *Zif268* promoter may not function as a cAMP-inducible enhancer element in this context. In contrast, CRE as well as SRE activation has been shown to induce *Zif268* expression in response to neuronal activity (Davis et al., 2000). Nothing is known so far about the role of the Sp1 and AP-1 sites in *Zif268* gene regulation.

Zif268 expression is not only induced by countless chemical factors and drugs (Bhat et al., 1992a; Bhat et al., 1992b; Brinton et al., 1998; Dahmen et al., 1997; Derkinderen et al., 2003; Dickey et al., 2004; Herdegen and Leah, 1998; Malkani and Rosen, 2000a; Malkani and Rosen, 2000b; Moratalla et al., 1992), but also by neurotransmitter-receptor stimulation and depolarization (Herdegen et al., 1993; Schulte et al., 2006), as well as peripheral sensory stimulation (Bisler et al., 2002; Patra et al., 2004; Staiger et al., 2000) and hypoxia (Yan et al., 1999). It was later shown that an increase in *Zif268* expression in response to stimulation is mediated by Ca^{2+} influx into the neuron, either through NDMA receptors (Bading et al., 1995; Condorelli et al., 1994; Malkani and Rosen, 2001; Massieu et al., 1992; Szekely et al., 1990; Vaccarino et al., 1992) or voltage sensitive calcium channels (Murphy et al., 1991). Furthermore, paradigms to induce activity-dependent changes in synaptic strength yield robust *Zif268* expression (Cole et al., 1989; Wisden et al., 1990).

After artificial stimulation, *Zif268* mRNA reaches detectable levels within 30min whereas the nuclear protein signal reaches its peak within 2hrs. This was confirmed in natural forms of *in vivo*-stimulation (Zangenehpour and Chaudhuri, 2002). In the absence of any stimulation Zif268 levels drop drastically, suggesting that constant neuronal activity maintains it at high levels (Kaczmarek and Chaudhuri, 1997; Worley et al., 1991). Accordingly, reduction of neuronal activity by preventing stimulus detection, markedly decreased Zif268 levels in corresponding brain regions (Filipkowski et al., 2001). In rat brain, this "constitutive" expression of the mRNA of *Zif268* occurs primarily in a subset of excitatory neurons (Chaudhuri et al., 1995) and is found in the neocortex, primary olfactory and entorhinal cortices, amygdaloid nuclei, nucleus accumbens, striatum, cerebellar cortex and the hippocampus (Davis et al., 2003b). "Constitutive" neocortical *Zif268* expression has been shown to depend on noradrenaline-mediated tone coming from the locus coeruleus (Bhat et al., 1992b; Cirelli et al., 1996).

1.1.3. Zif268 transactivation

Zif268 mediates transcriptional activation by its N-terminal (Gashler et al., 1993). However, the mechanism is not yet established. The transcriptional activating domain may interact with components of the basal transcription machinery, recruit transcriptional co-activators (Silverman et al., 1998b), or increase gene expression via both mechanisms. In addition to the activating effect of the N-terminal, the amino acids 281-314 display repressive effects (Molnar et al., 1994).

In search for the activation domain of Zif268, mutant forms were generated with deletions in various locations throughout the protein. Mutants that lacked a short region to the N-terminal side of the zinc-finger domains, the repression domain R1, were more efficient in driving expression of a reporter gene than the wild-type forms (Gashler et al., 1993). This 34bp stretch, used as "bait" in a yeast two hybrid screen, was shown to interact with two novel (NGFI-A binding) proteins, named NAB1 and NAB2. These two proteins not only repress transactivation by all R1 domain containing Egr family members, i.e. Zif268, Krox20 and Egr3 (Russo et al., 1995; Sevetson et al., 2000; Svaren et al., 1996), but can repress expression if targeted to any constitutive promoters (Swirnoff et al., 1998; Thiel et al., 2000). The NABs are so-called late genes and require protein translation for their induction, suggesting that they may provide a negative-feedback mechanism for Zif268-mediated transcription

(Svaren et al., 1996). Indeed, Zif268 itself may in part regulate expression of NAB2 providing an additional negative feedback loop of Zif268 induced transcription activation (Kumbrink et al., 2005). However, NABs have also been reported to have opposite effects. NAB-activated promoters such as LHβ contain EGR consensus sites that are fewer in number and lower in binding affinity than those found at NAB-repressed promoters such as basic fibroblast growth factor (Sevetson et al., 2000). Further co-activators of transactivation are the androgen receptor (Yang and Abdulkadir, 2003), which are abundant in memory related brain regions such as the CA1 region of the hippocampus (Clancy et al., 1992; Clancy et al., 1994; Kerr et al., 1995; Sar et al., 1990) and have been implicated with learning and memory function (Goto et al., 2005; Jones and Watson, 2005; Naghdi et al., 2005; Naghdi et al., 2001; Pouliot et al., 1996; Rizk et al., 2005).

Zif268 specifically binds to the consensus sequence GCG(G/T)GGGCG but not the characteristic Sp1 elements (Al-Sarraj et al., 2005; Cao et al., 1990; Christy et al., 1988), which is very similar. However, studies with recombinant proteins indicate that Zif268 can displace pre-bound stimulating protein 1, Sp1 (35 to 40% homology in zinc finger domains) from zinc finger protein binding regions (ZIP) (Huang et al., 1997b; Khachigian et al., 1996; Khachigian et al., 1995; Skerka et al., 1995), implying binding motifs that are common to both. Indeed, the two transcription factors were shown to compete for binding sites *in vivo* (Baek et al., 2004; Cao et al., 1993; Davis et al., 2003a; Kumbrink et al., 2005; Thottassery et al., 1999).

1.1.4. Posttranslational Modifications on Zif268

A very poorly characterized aspect of Zif268-regulation is post-translational modification. Although Zif268 is highly glycosylated and phosphorylated, nothing known about the function of glycosylation, and there are very few insights as to the role of phosphorylation. The latter may influence DNA binding and transcriptional activities of Zif268, but the evidence is conflicting (Huang et al., 1994; Jain et al., 1996; Szeberenyi, 1998; Yamaguchi et al., 2006). However, phosphorylation has been known to create binding sites for other proteins and may allow the high-affinity binding of Zif268 to co-activators. Zif268 phosphorylation may indeed mimic CREB phosphorylation (cAMP responsive element binding protein), where it does not change the DNA binding affinity of CREB but rather its association with CREB-binding protein (CBP) and p300 (Chrivia et al., 1993; Kwok et al., 1994).

Interestingly, CBP and p300 have also been reported to interact with Zif268 to modulate gene transcription (Mouillet et al., 2004; Silverman et al., 1998b). Zif268 phosphorylation by casein kinase II has been suggested (Jain et al., 1996), but this does not always appear to be the case (Yamaguchi et al., 2006).

1.1.5. Targets of Zif268 Transactivation

Gene regulated	Reference	Gene regulated	Reference
adenosine deaminase	(Ackerman et al., 1991)	4E-BP1 (down)	(Rolli-Derkinderen et al., 2003)
synapsin I	(Thiel et al., 1994)	PSEN-2	(Renbaum et al., 2003)
IL2	(Skerka et al., 1995)	Nag1	(Baek et al., 2004)
synapsin II	(Petersohn et al., 1995)	p300	(Yu et al., 2004)
ID1	(Tournay and Benezra, 1996)	CBP	(Yu et al., 2004)
TGFβ	(Liu et al., 1996; Svaren et al., 2000)	Arc/Arg3.1	(Li et al., 2005)
PC2	(Jansen et al., 1997)	NCX1	(Wang et al., 2005)
Lh beta (down)	(Sevetson et al., 2000; Wolfe and Call, 1999)	Psmb9	(James et al., 2006)
FGF2	(Jin et al., 2000)	SGK	(James et al., 2006)3}
Egr3	(Ehrengruber et al., 2000)	Tap1	(James et al., 2006)3}
NAB2	(Ehrengruber et al., 2000)	ATF3	(Yamaguchi et al., 2006)
IGFII	(Abdulkadir et al., 2001)	tyrosine hydroxylase	(Stefano et al., 2006)
p53	(Krones-Herzig et al., 2003)	insulin	(Eto et al., 2006)
ABCA2	(Davis et al., 2003b)	EGFR	(Chen et al., 2006)
TOE-1	(De Belle et al., 2003)	PDX-1	(Eto et al., 2007)

Table 1. A list of genes known to be regulated by the IEG Zif268 and the respective references.

A partial list of recently published targets of Zif268 is shown in *Table 1*. These target genes were described in diverse kinds of organs and cell types, emphasizing the broad involvement of Zif268 in many different functions. Accordingly, Zif268 has been studied in cancer progression since *Zif268* expression is often altered in tumors (Abdulkadir et al., 2001; Calogero et al., 2001; Eid et al., 1998; Huang et al., 1997a; Levin et al., 1994; Pipaon et al., 2004; Svaren et al., 2000; Thigpen et al., 1996). In fact, counteracting this change in Zif268 level can either inhibit (Calogero et al., 2001; Huang et al., 1997a; Levin et al., 1995) or promote (Abdulkadir et al., 2001; Baron et al., 2003; Svaren et al., 2000; Virolle et al., 2003) cancer progression, depending on the cellular context. Furthermore, Zif268 appears to be strongly linked to processes underlying the formation of addiction and addictive behavior (Hellemans et al., 2006; Lee et al., 2005; Lee et al., 2006a; Thomas et al., 2003; Valjent et al.,

2006). Finally, Zif268's involvement in synaptic plasticity and learning and memory has received a lot of attention and is detailed next.

1.3. Project Outline

In this project, the primary aim was to over-express Zif268 and examine the effect on different phases of memory. For this, we employed a system based on conditional transgenesis (chapter 5) that combines features such as brain- and cell-type specificity, inducibility, reversibility and non-invasiveness. We generated new transgenic mouse lines, verified and characterized the parameters of expression using molecular techniques and extended previous knowledge of the role of Zif268 in memory. Further experiments using this novel Zif268-mutant model will allow us to study the molecular consequences of altered Zif268 expression on different types and phases of memory, and synaptic plasticity.

2. Methods

2.1. Media for Bacteria

LB (LUBIA BERTANI) MEDIUM	
Bacto tryptone (AppliChem, Gatersleben, Germany)	10g/L
Bacto yeast extract (AppliChem, Gatersleben. Germany)	5g/L
NaCl (Merck, Darmstadt, Germany)	170mM

Autoclave before use.

NZYM MEDIUM	
NZ amine (AppliChem, Gatersleben, Germany)	10g/L
NaCl (Merck, Darmstadt, Germany)	85mM
Bacto yeast extract (AppliChem, Gatersleben, Germany)	5g/L
$MgSO_4.7H_20$ (Sigma, Munich, Germany)	25mM

Adjust to pH7 with 5N NaOH (Merck, Darmstadt, Germany). Autoclave before use.

SOC MEDIUM	
Bacto tryptone (AppliChem, Gatersleben, Germany)	20.0g/L
NaCl (Merck, Darmstadt, Germany)	0.4g/L
Bacto yeast extract (AppliChem, Gatersleben, Germany)	5.0g/L
$MgSO_4.7H_20$ (Sigma, Munich, Germany)	10.0mM
$MgCl_2$ (Fluka, Buchs, Switzerland)	10.0mM
KCl (Sigma, Munich, Germany)	2.5mM

Adjust to pH7 with 5N NaOH (Merck, Darmstadt, Germany). Autoclave and add sterile glucose to a final concentration of 20mM before use.

2.2. Enzymatic Methods

2.2.1. Restriction Enzyme Digests

Plasmid digest for preparative and analytical digests were conducted according to the supplier's recommendation (NEB, Beverly, USA; Promega, Madison, USA; Invitrogen, Carlsbad, USA). For standard digests, 10x the theoretical amount of restriction enzymes was used to ensure complete digestion. Efficiency of digestion was verified by agarose gel electrophoresis.

2.2.2. T4-Polymerase to generate blunt ends

The T4-Polymerase is used to fill up 5'overhangs and thus allow blunt end cloning. The reaction was conducted according to the protocol provided by the suppliers (NEB, Beverly, USA).

2.2.3. Dephosphorylation of 5'-Ends

This methods serves to prevent re-ligation of a linearized plasmid. The 5' phosphate is removed by the alkaline phosphatase, thereby ensuring that ligation may only occur if the phosphate group is donated by the insert. The dephosphorylation was conducted according to the supplier's recommendations (NEB, Beverly, USA).

2.2.4. Ligation

Ligations were performed over night at 16°C using the T4 ligase (NEB, Beverly, USA) according to the supplier's recommendations. If large DNA fragments were ligated, 15% PEG-6,000 (Fluka, Buchs, Switzerland) was added to the reaction mixture. Usually, two different insert to vector ratios were chosen for the ligation, usually between 1:1 to 10:1 in bp terms. As a re-ligation control, no insert was added to the ligation mixture.

2.2.5. Reverse Transcription

Reverse transcription was performed to generate cDNA for TaqMan® quantitative PCR analysis. The reaction was conducted with the SuperScript™ First Strand Synthesis system according to the suppliers recommendations (Invitrogen, Carlsbad, USA), using a 1:1 mixture of oligo$(dT)_{16-18}$ primers and random hexamers.

2.2.7. Purification of DNA and RNA

2.2.7.1. Gel Extraction

Gel extraction was conducted using the QIEX II gel extraction kit (Qiagen, Hilden, Germany) based on adsorption Chromatography according to the supplier's recommendations.

2.2.7.2. Purification in Ethanol using High Salt Concentrations

For additional purification of DNA, ethanol precipitation could be conducted in the presence of high salt concentrations. For this, 0.1 volumes of 3M sodium acetate (pH5.2, Sigma, Munich, Germany) was added to 1 volume of DNA solution and mixed. Subsequently, 10 volumes of ice-cold ethanol was added, mixed and kept at -20°C for 30min. The DNA was then pelleted by centrifugation at 13,000rpm on a cooled table-top centrifuge, the pellet was dried for 10min at RT and resuspended in 10mM Tris (Sigma, Munich, Germany), 0.1mM Na_2.EDTA (pH8.3; Sigma, Munich, Germany).

2.2.7.3. RNA Extraction from tissue

RNA was extracted from different brain regions using the adsorption Chromatography Macherey Nagel Nucleospin RNA II Kit, according to the supplier's recommendations (Macherey Nagel, Oensingen, Switzerland). In some cases, large pieces of tissue (> 30mg) were used and the DNA digest was extended to 30min with additional DNase. Any remaining con*tam*inating genomic DNA was detected using the CycD1 PCR (2.2.9) and was removed using a further DNase step (Ambion, Austin, USA).

2.2.8. Lysis of Tail Biopsies

2.2.8.1. Proteinase K Digest

Proteinase K stock (10mg/ml in 40mM Tris-HCl; Sigma, Munich, Germany; -20°C) was diluted 1:100 in tail lysis buffer (4°C). Tail biopsies were lysed in 200µl of this preparation at 55°C overnight on a shaker and the enzyme was subsequently inactivated by 20min at 90°C.

TAIL LYSIS BUFFER	
KCl (Sigma, Munich, Germany)	50.0mM
gelatine (Sigma, Munich, Germany)	0.1g/L
Tris (Sigma, Munich, Germany)	10.0mM
$MgCl_2$ (Sigma, Munich, Germany)	2.0mM
Nonidet P40 (Fluka, Buchs, Switzerland)	7.5mM
Tween 20 (Sigma, Munich, Germany)	3.5mM

Add ddH_2O to 500ml. Store at 4°C.

2.2.8.2. Alkaline Lysis

Genomic DNA preparation from tail clipping (or ear clipping) was conducted according to the protocol by Truett et al., 2000. Briefly, biopsies were lysed in alkaline buffer at 95°C for 10min, subsequently cooled to 4°C and neutralized with acidic neutralization buffer (Truett et al., 2000).

ALKALINE LYSIS REAGENT	
NaOH (Merck, Darmstadt, Germany)	25.0mM
Na_2.EDTA (Fluka, Buchs, Switzerland)	0.2mM

Set precisely to pH12 with 5N NaOH.

NEUTRALIZING REAGENT	
Tris (Sigma, Munich, Germany)	40.0mM

Set precisely to pH5 with 5N HCl.

2.2.9. Polymerase Chain Reaction for Genotyping

The polymerase chain reaction (PCR) was performed to genotype our transgenic mice. All genotyping PCRs were always performed on a PCR machine Eppendorf Mastercycler gradient (Eppendorf, Eppendorf, Germany) with

i) a water control containing all the mix components except the sample (genomic DNA) to exclude contaminating byproducts,

ii) a negative control containing genomic DNA of a mouse not carrying the transgene,

iii) a positive control containing genomic DNA of a mouse carrying the transgene.

PCR products were loaded on al 2% agarose gel/0.5x TBE gel (2.4.1.). The gel was run at 200V for 15-20min and PCR products were visualized by UV light.

All primers were ordered from Microsynth, Balgach, Switzerland.

Constituents (stock concentration)	rtTA2	hrGFP	LacZ	CycD1
Forward primer (10 µM)	0.2	0.2	0.2	1.0
Backward primer (10 µM)	0.2	0.2	0.2	1.0
dNTPs (10 mM)	0.2	0.2	0.2	1.0
$MgCl_2$ (25 mM)	1.0	-	1.0	2.1
10 x Taq polymerase buffer	2.1	2.1	2.1	2.1
DMSO	-	-	-	1.0
Taq polymerase (5 units/µl)	0.1	0.1	0.1	0.1
Gel loading buffer	4.0	4.0	4.0	4.0
ddH_2O	12.2	13.2	12.2	7.7
Genomic DNA	1.0	1.0	1.0	1.0
Total	21	21	21	21

Table 2. A list of chemicals and the amount required of each for the detection of the four different transgenes by PCR (volumes in µl).

	rtTA2	hrGFP	LacZ	CycD1
Forward primer	5' tgc ctt tct ctc cac agg tgt gc 3'	5' atg gtg agc aag cag atc ct 3'	5' atg tcc tcg ggg tac ttg gt 3'	5' acc agc tcc tgt gct gcg aa 3'
Backward primer	5' agc agg cag cat atc aag gtc 3'	5' atg tcc tcg ggg tac ttg gt 3'	5' gcc tcc agt aca gcg cgg ctg 3'	5' acc gag tcc tag caa cgc ac 3'
Annealing temperature (°C)	62	62	62	53
Product size (bp)	775	248	409	300

Table 3. Details of the PCR protocol for the detection of four different transgenes.

2.3. Microbiological Methods

2.3.1. Transformation of Competent Cells

DNA vectors or ligations were transformed either in Sure2 supercompetent® (Qiagen, Hilden, Germany) or Top10® (Biorad, Richmond, USA) cells according to the supplier's recommendations.

2.3.2. Preparing Plasmid for Analytical Purpose

For analytical purposes, DNA was extracted from small amounts of bacterial suspensions either by alkaline lysis (Birnboim and Doly, 1979), or using the Qiaprep® Spin Miniprep Kit (Qiagen, Hilden, Germany).

2.3.3. Preparing Plasmid for Preparative Purpose

For plasmid preparations of greater quantity and purity, the QiaPrep® Plasmid Purification Kit (Qiagen, Hilden, Germany) was used according to supplier's recommendations. For transfection and microinjection, the Endofree® Plasmid Purification Kit (Qiagen, Hilden, Germany) was used to remove endotoxins. The final construct was excised from the vector backbone, the desired band was visualized and excised from an agarose gel (2.4.1.), and extracted from the gel by overnight electroelution using the Elutrap® device (Schleicher & Schuell, Dassel, Germany). The DNA in the resulting solution was precipitated by Ethanol precipitation in the presence of salts (2.2.7.2.), washed twice with 70% Ethanol and dried at RT. The pellet was taken up in 30µl TE buffer (10mM Tris pH8.0, 1mM EDTA) dissolved at 37°C for 10min and overnight at 4°C before freezing.

2.4. Further Methods involving Nucleic Acids

2.4.1. DNA Electrophoresis using Agarose Gels

5x DNA LOADING BUFFER	
sucrose (Sigma, Munich, Germany)	1.3M
cresol red (Sigma, Munich, Germany)	1mM

Stored at 4° C.

0.5x TBE BUFFER	
Trizma base (Sigma, Munich, Germany)	45mM
boric acid (Fluka, Buchs, Switzerland)	45mM
Na_2.EDTA (Fluka, Buchs, Switzerland)	1mM

x% AGAROSE GEL	
Agarose (Peqlab, Erlangen, Germany)	X% (w/v)
0.5x TBE Buffer	Desired v

Heat in microwave until all agarose is dissolved. Cool down to 55°C while shaking.

Ethidium bromide (Sigma, Munich, Germany)	1.25µM

2.5. Protein Methods

2.5.1. Bradford Assay for Measuring Protein Concentration

For determining protein concentration a kit based on the Malachi green assay (Biorad, Richmond, USA) was used in conjunction with a protein standard curve of bovine serum albumin (BSA, Sigma, Munich, Germany).

BSA stock: 0.2mg/ml in 0.2M NaOH (Merck, Darmstadt, Germany)

PROTEIN AMOUNT	BSA STOCK	0.2M NaOH
10 µg	10 µl	40 µl
20 µg	20 µl	30 µl
30 µg	30 µl	20 µl
40 µg	40 µl	10 µl
50 µg	50 µl	0 µl

Sample preparation

1. Dilute samples with 0.2M NaOH to a final volume of 50µl.
2. Add 750µl of ddH_2O.
3. Add 200µl of dye reagent.
4. Mix.
5. Measure absorbance at 595nm (Genequant pro, GE Healthcare, Otelfingen, Switzerland) within the next hr.

2.5.2. SDS PAGE and Immunoblotting

ELECTROPHORESIS BUFFER	
Tris (Sigma, Munich, Germany)	25.0mM
Glycine (Sigma, Munich, Germany)	250.0mM
SDS (Biorad, Richmond, USA)	3.5mM

Adjust to pH8.3 with 5N HCl.

LOADING BUFFER	
Tris (Sigma, Munich, Germany)	50.0mM
SDS (Biorad, Richmond, USA)	70.0mM
Bromophenol blue (Sigma, Munich, Germany)	1.5mM
Glycerol (Sigma, Munich, Germany)	1.1M

Adjust to pH6.8 with 5N NaOH. Store as aliquots at –20°C.
Add to 1.4M β-mercapto-ethanol (Sigma, Munich, Germany) before use.

TRANSFER BUFFER	
Tris (Sigma, Munich, Germany)	50mM
Glycine (Sigma, Munich, Germany)	40mM
SDS (Biorad, Richmond, USA)	1.25mM

Adjust to pH8.3 with HCl. Dilute to 1x with 700ml ddH_2O + **200ml Methanol**.

TBS-T	
Tris (Sigma, Munich, Germany)	10mM
NaCl (Merck, Darmstadt, Germany)	150mM
Tween-20 (Sigma, Munich, Germany)	400µM

Adjust to pH8.3 with 5N NaOH. For the blocking buffer, 2% (w/v) milk powder was dissolved in 1x TBS-T.

2.5.2.1. Gel Preparation

1. Glass plates were wiped with EtOH and dried with Kim wipes (Kimberley-Clark, Reigate, UK).
2. Plates and spacers were assembled.
3. The plates were sealed with an agarose gel.
4. The separating gel was poured and covered with isopropanol (Fluka, Buchs, Switzerland) or isobutanol (Fluka, Buchs, Switzerland). Allowed >30min for polymerization.
5. The isopropanol was rinsed away with ddH2O, the glass plates dried with Kim wipes (Kimberley-Clark, Reigate, UK).
6. The stacking gel was poured, the comb placed immediately and polymerization was allowed for >30min.
7. The gels could be stored wet with combs at 4°C.

Volume for 3 mini-gels 100 x 105 x 1mm: separating gel 7ml; stacking gel 2ml.

SEPARATING GEL	
Acrylamide (Biorad, Richmond, USA)	400mM
N'-N' methylane bis-acrylamide (Biorad, Richmond, USA)	20mM
Tris (Sigma, Munich, Germany)	375mM
SDS (Biorad, Richmond, USA)	3.5mM
Ammonium Persulfate (Sigma, Munich, Germany)	4.5mM
TEMED (Sigma, Munich, Germany)	2.0mM

Adjust to pH8.8 with 5N NaOH.

STACKING GEL	
Acrylamide (Biorad, Richmond, USA)	200mM
N'-N' methylane bis-acrylamide (Biorad, Richmond, USA)	10mM
Tris (Sigma, Munich, Germany)	125mM
SDS (Biorad, Richmond, USA)	3.5mM
Ammonium Persulfate (Sigma, Munich, Germany)	4.5mM
TEMED (Sigma, Munich, Germany)	6.5mM

Adjust to pH6.8 with 5N NaOH.

2.5.2.2. Gel Electrophoresis

1. Samples were prepared on ice in 0.5ml tubes; 4x loading dye with 10% β-mercapto-ethanol (Sigma, Munich, Germany) was added to a final concentration of 1x.
2. Proteins in sample were denatured at 95°C for 3min.
3. Samples were stored on ice and spun down briefly before loading.
4. The gels were run at 70V for 1hr (time necessary to go through stacking gel), then accelerated to 150V for 2hr.

2.5.2.3. Transfer

1. Gel and nitrocellulose membrane were bathed in transfer buffer for 15min.
2. The transfer sandwich was prepared in a large shallow tray filled with cold transfer buffer accordingly: Cathode to the anode:
   sponges / filter papers (Whatman, Brentford, UK)/ gel / membrane (Immun-blot PVDF membrane, Bio-Rad, Richmond, USA) / filter paper / sponges
3. The transfer was run at 150mA for 3hr.

2.5.2.4. Antibody Incubations

1. To avoid unspecific binding of the antibodies (Ab) to the membrane, the membrane was blocked in 2% milk powder/TBS-T (pH8.0) for at least 1hr at RT or at 4°C with gentle shaking.
2. The membrane was incubated with primary Ab at specific concentrations in 2% blocking buffer for 1hr at RT.
3. The membrane was rinsed 4x 15min in a large volume of TBS-T at RT.
4. The membrane was incubated with secondary Ab linked to an infrared dye diluted 1/5,000 in 2%milk powder/TBS-T for 6hr at 4°C.
5. The membrane was rinsed 4x 15min in a large volume of TBS-T at RT.
6. For detection, the membrane was exposed on the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, USA).

2.5.2. Immunofluorescence

For immunofluorescence mice were perfused (2.7.3.), brains isolated and cryosectioned coronally (2.7.4.) Slides with sections were frozen and thawed in a dry chamber at RT. Antibody staining was conducted in a humid chamber at RT (unless stated otherwise) as follows:

1. Permeabilization: 30min in 0.4% Triton X100 (Sigma, Munich, Germany) in 1x PBS.
2. Quenching of endogenous peroxidases: 15min in 3% H_2O_2 in 1x PBS
3. Washed 3x in 1x PBS for 5min.
4. Blocking: 1hr in 10% fetal calf serum (GibcoBRL, Paisley, UK), 0.5% TNB (Perkin Elmer, Waltham, USA), 0.2% Triton X100 in 1x PBS.
5. 1° Ab incubation: Ab dilution in 5% fetal calf serum, 0.05% Triton X100 over night (approx 13hr) at 4°C.
   Antibody dilutions
   1:200 αZif268 (Rbb, cell signaling technology, Danvers, USA)
   1:200 αβ-Galactosidase (Rbb, Molecular Probes, Eugene, USA; Mouse, Promega, Madison, USA)
   1:400 αNeuN (Mouse, Abcam, Cambridge, UK)
   1:400 αGFAP (Rbb, DAKO, Heverlee, Belgium)
6. Washed 3x in 1x PBS for 5min.
7. 2° Ab incubation: Ab dilution in 5% fetal calf serum, 0.05% Triton X100 for 1hr at RT.
   Antibody dilutions
   1:1,000 αRbb-Cy5 (Jackson Immunoresearch, Newmarket, UK)
   1:1,000 αMouse-Cy3 (Jackson Immunoresearch, Newmarket, UK)
8. Washed 3x in 1x PBS for 5min.
9. Sections were coverslipped (Menzel, Braunschweig, Germany) in MOWIOL mounting medium and stored at 4°C.

Images were taken on a confocal microscope (Leica, Wetzlar, Germany) and processed using the Imaris Software (Bitplane AG, Zürich, Switzerland).

MOWIOL MEDIUM	
MOWIOL (Calbiochem, Nottingham, UK)	120g/L
Glycerol (Sigma, Munich, Germany)	3.25M
0.2M Tris pH8.3 (Sigma, Munich, Germany)	120mM
DABCO (Sigma, Munich, Germany)	10mM

1. Mix for 4hr and let stand for further 2hr at RT.
2. Heat at 50°C in water bath for 10min.
3. Centrifuge at 5,000g for 15min.
4. Aliquot and keep at -20°C.
5. For coverslipping, warm up to max. 37°C.

2.6. Cell Culture Methods

2.6.1. HELA Cells in Culture

Cells were cultivated in DMEM medium (GibcoBRL, Basel, Switzerland) with 10% fetal goat serum (GibcoBRL, Basel, Switzerland) and antibiotics (GibcoBRL, Basel, Switzerland) at 37°C and 10% humidity.

2.6.2. Transfection of HELA Cells

HELA cells were split to obtain 30% confluence the morning of the transfection. In the afternoon 2µg of DNA in TE (10mM Tris, 1mM Na_2.EDTA, pH7.4) was filled up to 100µl with DMEM and 8µl SuperFect® was added (Qiagen, Hilden, Germany). While this reaction mixture was incubated at RT for 10min the cells were washed in PBS and the reaction mixture was subsequently added with 1ml of full medium. Cells were put in the incubator overnight. The medium was changed the next day and cells were fixed 48hr after transfection. SuperFect® Reagent consists of activated-dendrimer molecules with a defined spherical architecture. Branches radiate from a central core and terminate at charged amino groups, which can then interact with negatively charged phosphate groups of nucleic acids. SuperFect® Reagent assembles DNA into compact structures that bind to the cell surface and are taken into the cell by non-specific endocytosis. The reagent buffers the pH of the endosome, leading to pH inhibition of endosomal nucleases, which ensures stability of SuperFect®–DNA complexes.

2.6.3. Immunostaining of HELA Cells

After the medium was washed off, cells were fixed in 3ml medium plus 1ml 8% PFA/PBS for 1hr at RT. They were subsequently washed in cold 2x PBS and were kept at 4°C until immunostaining. For this, unspecific antibody binding was blocked by incubation in blocking buffer (10% normal goat serum, 0.02% saponin in PBS) for 1hr. The cells were incubated with the primary antibody mixture containing 1:400 rabbit polyclonal α Zif268 antibody (Rbb; Santa Cruz Biotechnology, Santa Cruz, USA) and 1:200 mouse monoclonal α β–galactosidase antibody (Mouse; Promega, Madison, USA) in blocking buffer for 2hr at RT. This was followed by three washes in PBS, a second blocking step for 30min and incubation with the following secondary antibody mixture, 1:250 α mouse-FITC conjugate (Jackson Immunoresearch, Newmarket, UK) and 1:500 α Rbb-Cy3 conjugate (Jackson Immunoresearch, Newmarket, UK) in blocking buffer for 1.5hr. A further three

washes in PBS were followed by the embedding in MOWIOL mounting medium for fluorescent microscopy. The edges were covered with nailpolish to prevent the samples drying out.

2.7. Mice

A service laboratory at the University of Zürich conducted the pronuclear injection of the DNA construct into fertilized oocytes to create pseudo-founders. Both male and female mice used as gamete donors for transgenesis were of a mixed C57Bl/6J and DBA2 F1 background. This background was used for hybrid vigor, which is the phenomenon that hybrid strains are better for the generation of transgnic mouse lines for three reasons:

a) higher reproductive performance

b) easier to superovulate

c) higher quality embryos with higher survival rates after microinjection

2.7.1. Housing

Mice were kept in a room with controlled temperature and humidy level, under inverted 12-hr light/dark cycle, lights on at 7pm. All mice were allowed access to food and water *ad libitum*, unless they were in experiment. All tests and trainings were conducted at the same time of day, between 10am and 3pm. Facility, experimenters and behavioral experiments were in accordance with guidelines and regulations of the cantonal veterinary office, Zürich.

2.7.2. Euthanasia

Mice were euthanized by several means. For tissue collection, mice were killed by neck dislocation and decapitation. For perfusion, mice were given a lethal dose of 100mg/ml Sodium-Pentobarbital (1:4 in ddH_2O, Eutha®77, Essex Animal Health, Friesoythe, Germany), i.p. 200µl/20g body weight (BW).

2.7.3. Perfusion

The preparation for perfusion was only commenced when anasthesized mice showed a complete lack of reflexes. The thorax was subsequently opened and a canula introduced into the left ventricle of the heart. The right atrium was opened with a cut and perfusion started by pumping physical saline at 8ml/min for 2min. Without introducing air bubbles into the tubing, the ice-cold fixative (4% PFA in 0.1M PB pH7.4) was next pumped at 8ml/min for 4min, followed by 26min at 4ml/min. The

brain was extracted and kept in sucrose (20% in PBS) at 4°C until sunk to the bottom of the container.

PHYSICAL SALINE	
NaCl (Merck, Darmstadt, Germany)	150mM
KCl (Sigma, Munich, Germany)	0.3mM

4% PFA FOR PERFUSION	
Na_2HPO_4 (Sigma, Munich, Germany)	35mM
Paraformaldehyde (Sigma, Munich, Germany)	1.3M
NaH_2PO_4 (Sigma, Munich, Germany)	23mM

For each mouse approximately 145ml of fixative are required.

2.7.4. Cryosectioning the Brain

Brains for cryosectioning were either fresh and processed for β-Gal staining, or perfused and used for immunostaining. In the first case sections were made at -16°C chamber temperature, -14°C knife temperature and cut at 14µm. In the second case, the respective parameters were -22°C, -20°C, 20µm on a Micron cryostat (Micron Instruments, San Marcos, USA). Sections were taken up on microscope slides (Menzel, Braunschweig, Germany) and stored at -20°C.

2.7.5. Staining for β-Galactosidase Activity

The β-galactosidase activity can be detected by a reaction with the substrate X-Gal (5-Brom-4-chlor-3-in*dox*yl-β-D-galactopyranosid) that is converted to galactose and a blue, hydrophobic Indigo-stain. The procedure is carried out in three steps.

1) Brain dissection and freezing. As described above in 2.
2) Cryostat sections. As described above in.
3) β-galactosidase staining.

100mM $NaPO_4$ BUFFER	
monobasic $NaPO_4$ (NaH_2PO_4) (Sigma, Munich, Germany)	30mM
dibasic $NaPO_4$ (Na_2HPO_4) (Sigma, Munich, Germany)	70mM

Adjust to pH7.2 with pH paper.

LacZ FIXATION BUFFER	
Glutaraldehyde (Sigma, Munich, Germany)	0.14mM
EGTA (Sigma, Munich, Germany)	5mM
$MgCl_2$ (Fluka, Buchs, Switzerland)	2mM
$NaPO_4$	100mM

Adjust to pH7.2 with pH paper.

LacZ WASH BUFFER	
$MgCl_2$ (Fluka, Buchs, Switzerland)	2.0mM
Na deoxycholate (Sigma, Munich, Germany)	0.2mM
NP40 (Fluka, Buchs, Switzerland)	0.3mM
$NaPO_4$	100mM

Adjust to pH7.2 with pH paper.

LacZ STAIN	
Xgal (dissolve first in 2ml DMSO) (AppliChem, Gatersleben, Germany	0.5mM
K-ferrOcyanide $K_4Fe(CN)_6 .3H_2O$ (Sigma, Munich, Germany)	5.0mM
K-ferrIcyanide $K_3Fe(CN)_6$ (Sigma, Munich, Germany)	5.0mM

Prepare solution in 1x wash buffer. Filter-sterilize LacZ stain before use to prevent crystal formation. Possible to keep frozen, protected from light, and filter again before use.

Sections on slides were fixed for 10min in ice-cold LacZ fixation buffer. Subsequently, they were washed three times for 5min in LacZ wash buffer and stained overnight at 37°C, protected from light. When the staining was completed, three 5min rinses in PBS followed. The sections were counterstained with acidified hematoxylin (add 4% fuming acetic acid in stain, washing baths and dehydration baths), coverslipped (Menzel, Braunschweig, Germany) in DPX mounting medium (TAAB laboratory equipment, Calleva Park, UK) and stored at RT.

2.8. TaqMan© qRT-PCR

TaqMan© Gene Expression Assays are built on 5′ nuclease chemistry. Each assay consists of two unlabeled PCR primers and a FAM™ dye-labeled TaqMan© MGB (minor groove binder) probe. The PCR reaction exploits the 5′ nuclease activity of the DNA polymerase system to cleave a TaqMan© probe during PCR. The TaqMan© probe contains a reporter dye at the 5′ end of the probe and a quencher dye at the 3′ end of the probe. When the probe is intact, the proximity of the reporter dye to the

quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer. The 5′–3′ nucleolytic activity of the DNA polymerase system cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3′ end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. Figure X provides an overview of the TaqMan© kits used for quantification in this work. Figure X shows the forklike-structure-dependent, polymerization-associated 5′–3′ nuclease activity of the DNA polymerase system during PCR.

Target Gene	AB code	Supplier
Zif268	Mm00656724_m1	Applied Biosystems
NFκB	Mm00476361_m1	Applied Biosystems
β-actin	4352664-0510003	Applied Biosystems
GapDH	4352662-0509004	Applied Biosystems

Table 4. TaqMan© probes used for qRT-PCR.

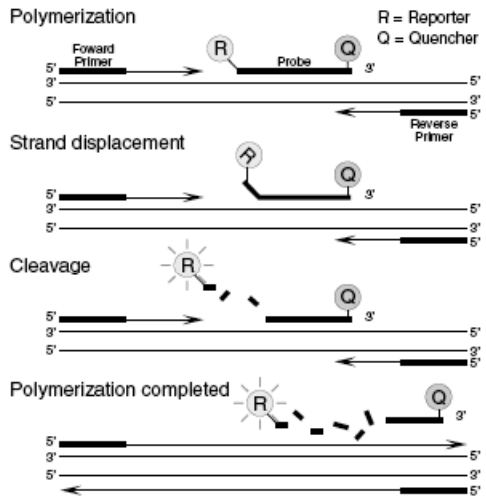

Figure 2. The principle of TaqMan© gene expression systems (taken from Applied Biosystems, www.appliedbiosystems.com).

2.9. Behavioural Analysis

2.9.1. Home Cage Activity

To assess the effect of Zif268 over-expression on home cage activity an activoscope© setup was used. Briefly, 26 mice were single caged for at least two weeks before measurement. Activity recordings were initiated, and followed three days later by *dox* administration to 16 mice, 7 mutant and 9 littermate controls. During the next nine

days, home cage activity was screened for any changes due to induction of Zif268 over-expression or *dox* feeding.

2.9.2. Conditioned taste aversion (CTA)

Singly-housed mice first habituated to receive their daily water ration from two bottles presented for 20-min once/day over 4 days. On conditioning day, mice received saccharin (0.5%) instead of water for 20-min then an i.p. injection of LiCl (0.14M, 2% BW) 40-min later. CTA was tested eight days after conditioning by presenting the animals one bottle of saccharin and one bottle of water (choice test). CTA extinction was then induced by 5 successive daily choice tests. To evaluate whether there may be any differences in spontaneous recovery between Zif268 overexpressors and control littermates, a 10-day interval was introduced after extinction day 5. Subsequently, extinction training was continued for another 5 days. Conditioned aversion to saccharin is evaluated by an aversion index defined as the amount of water consumed over total liquid (water and saccharin) consumed measured by weight. Non-conditioned mice were injected saline instead of LiCl. Mice were either given *dox*-spiked food for 8 days before conditioning or immediately after conditioning for 8 days before onset of extinction training and throughout the experiment.

3. Results

3.1. Generation of Transgenic Mice

During the first phase of this project, the constructs for the transgenic mouse lines were generated. Two parallel approaches were employed to generate rtTA2M2-responsive animals, that permit inducible co-expression of Zif268 and a reporter gene under the control of the bidirectional tetracycline transactivator-responsive *bitetO* promoter (Baron et al., 1995). Constructs containing the *bitetO*-promoter flanked by the Zif268 CDS and a reporter gene were created. As reporters, either **(a)** the lacZ gene coding for the β-galactosidase (*Fig. 3* and *4*), or **(b)** an enhanced version of the green fluorescent protein, hrGFP, were employed (*Fig. 3* and *5*). β-Galactosidase staining is a sensitive and convenient method for detecting gene expression. GFP monitoring on the other hand allows easy detection of gene expression and does not require additional staining steps for visualizing. It is therefore faster than the β-galactosidase method.

All cDNAs were flanked with synthetic regulatory sequences to improve *in vivo*-transcription and translation levels, as well as mRNA stability. These sequence contains a 230-bp hybrid intron that contains an adenovirus splice donor and an immunoglobulin G splice acceptor, and an SV40 polyadenylation signal (Choi et al., 1991). All DNA constructs were microinjected into fertilized oocytes by Thomas Rülicke, University Hospital Zürich. Both male and female mice used as gamete donors were of a mixed C57Bl/6J and DBA2 F1 background for hybrid vigor. Transgene integration into the genome of each founder was tested by transgene specific PCR. Transgene transmission was assessed by crossing the positive founders with C57Bl/6J mice.

Figure 3. The DNA constructs created to generate transgenic mice in this project.

3.1.1. Generation of *bitetO*-Zif268/LacZ transgenic mice

Mouse Zif268 cDNA was generously provided by Dr. A. Sesay and Dr. T. Bliss. The pBI-G vector was generously provided by Dr. Philipp Berger and Dr. Hermann Bujard. Integration of the *bitetO*-Zif268/LacZ trangene occurred in 12 founders (out of 80 pseudofounders). However, only 8 of these transmitted the transgene to the F1 generation (*Table 5*), giving rise to 8 independent *bitetO*-Zif268/LacZ lines, named ZifZ1KB, ZifZ2KB, ZifZ3KB, ZifK4KB, ZifZ5KB, ZifZ6KB, ZifZ7KB and ZifZ9KB.

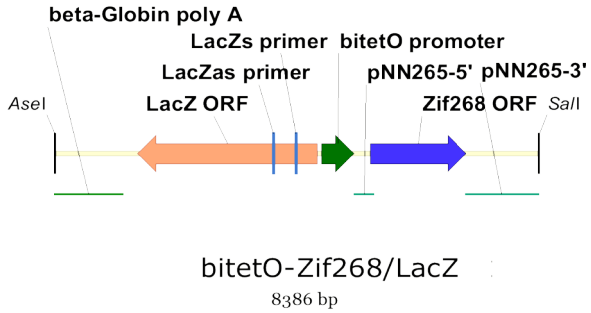

Figure 4. ***bitetO*-Zif268/LacZ**: A 1744bp fragment was excised from the vector pCMVsport6 Zif268 by AvaI/HpaI digest, blunt-ended and inserted into the EcoRV site of pNN265. From this, a NotI fragment was excised (3190bp) and introduced into the *bitetO*-LacZ containing vector pBI-G (Clontech) at the NotI site. A SalI/AseI digest resulted in an 8381bp fragment that was microinjected.

3.1.2. Generation of *bitetO*-Zif268/hrGFP transgenic mice

Mouse hrGFP (Stratagene) was the kind gift of Dr. J. Sitz. Integration of the *bitetO*-Zif268/hrGFP trangene occurred in 12 founders (out of 83 pseudofounde However, only 7 of these transmitted the transgene to the F1 generation (*Table 5*), giving rise to 7 independent *bitetO*-Zif268/hrGFP lines, numbered 1239, 1241, 1242, 1243, 1244, 1245 and 1246.

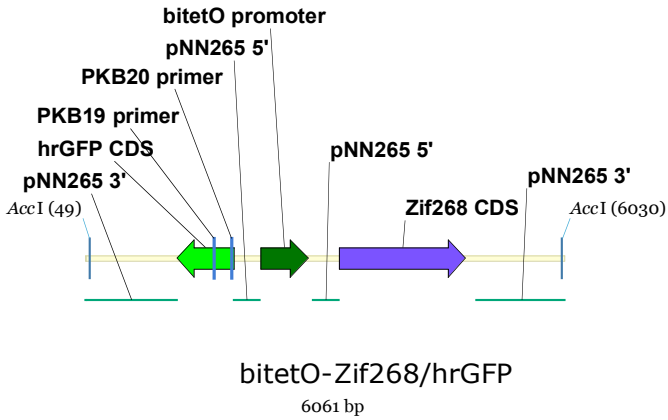

Figure 5. ***bitetO*-Zif268/hrGFP**: A 732bp fragment containing the hrGFP cDNA was excised from the phrGFP vector using XhoI and KpnI, blunt-ended and ligated into the EcoRV site of pNN265. The *bitetO*-promoter (620bp) was excised from the pBI3 vector using XbaI, blunted and introduced into the blunted SacI site of pNN265 Zif268. Subsequently, a fragment of hrGFP and the flanking regulatory sequences (NotI, blunted, 2179bp) was introduced into the blunted SalI site of *bitetO*-Zif268. Excision with AccI yielded a construct of 5981bp for microinjection.

Constructs	F0/pseudo-founders	Lines trans.	Inducer Line	Expression
lacZ – bitetO-promoter – Zif268	12/80	8	CaMKII-rtTA2	6 lines with inducible expression.
hrGFP – bitetO-promoter – Zif268	12/83	7	CaMKII-rtTA2	No expression of hrGFP in any line.

Table 5: A summary of statistics regarding the generation of the transgenic mouse lines.

3.2. Screening for Functionality of the Constructs

3.2.1. *In vitro* Screening

To test the functionality of the rtTA2M2 responsive constructs, *bitetO*-Zif268/LacZ and *bitetO*-Zif268/hrGFP, *in vitro* screening was performed. For this, we took advantage of a stably transfected human cervical cancer cell line (HeLa), that constitutively expresses the original tetracycline regulated transactivator (tTA) (Philipp Berger). The tTA-system activates transcription in the absence of *dox* and is therefore switched off by *dox* administration. Co-expression of Zif268 and the reporter genes, **(a)** LacZ and **(b)** hrGFP, and reversibility by *doxycycline* administration were verified by immunostaining.

(a) All cells expressing Zif268 also expressed β-galactosidase. Moreover, this expression was completely suppressed by *dox* administration. No systematic quantification was conducted but staining intensity for Zif268 and β-galactosidase appeared to correlate by eye in different cells, suggesting that the strength Zif268- and β-galactosidase expression are comparable (*Fig. 6*).

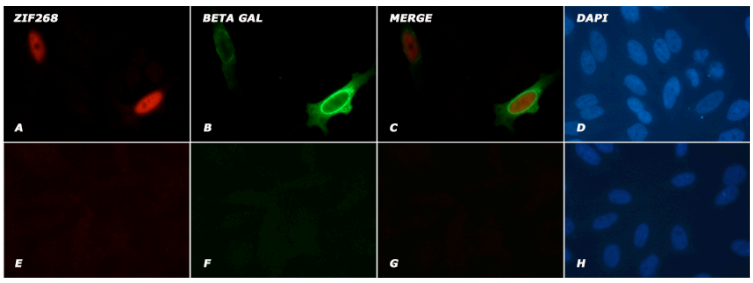

Figure 6. Immunostaining of Zif268 and β-galactosidase in tTA-expressing HeLa cells transiently transfected with the *bitetO*-Zif268/lacZ construct. A to D shows immunostained cells that express Zif268 and β-galactosidase, E to H show the effect of 24hrs of *dox* treatment to surpress transgene induction.

(b) Contrary to the *bitetO*-Zif268/LacZ construct, not every cell that stained for Zif268, also showed an hrGFP signal. Moreover, *dox* administration did not result in a complete ablation of signal. Zif268 immunostaining and hrGFP fluorescence could not be suppressed completely by *dox* administration suggesting some autonomous, tTA independent activity of the *bitetO*-promoter in this context (*Fig. 7*). This may

indeed disqualify the hrGFP reporter as a reliable marker. Since the aim was to generate a mutant mouse over-expressing Zif268, this construct was nevertheless used for microinjection.

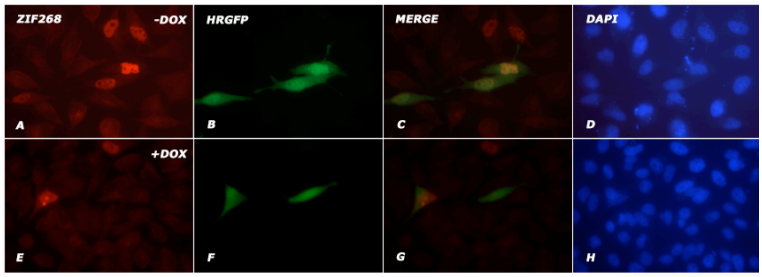

Figure 7. Immunostaining of Zif268 of tTA-expressing HeLa cells transiently transfected with the *bitetO*-Zif268/hrGFP construct. For hrGFP, innate fluorescence was observed. A to D show transfected cells expressing Zif268 and hrGFP, while E to H show the effect of 24hrs of *dox* treatment to surpress transgene induction.

3.2.2. *In vivo* Screening

Out of a total of 24 founder lines, 15 transmitted the transgene to the F1 generation and were examined for transgene expression. For this, *bitetO*-Zif268/LacZ and *bitetO*-Zif268/hrGFP lines were screened by crossing them with tetracycline responsive transactivator expressing lines. Initially, a CaMKIIα promoter rtTA line was used, however, a newly generated CaMKIIα promoter rtTA2M2 line was employed when it became available. A *dox*-inducible signal was found in 6 *bitetO*-Zif268/LacZ lines. However, all lines displayed a slight leakage of varying degrees in mice carrying both transgenes without *doxycycline* administration. The following section describes the *dox*ycyline-induced expression of β-Galactosidase and the leakage signal for each line.

3.2.3. Expression pattern of CaMKIIα promoter rtTA2 *bitetO*-Zif268/LacZ lines

3.2.3.1. bitetO-Zif268/LacZ1

Broad expression of β-galactosidase due to leakage was found in neocortex and caudate putamen of mice carrying the *bitetO*-Zif268/LacZ transgene only. Due to this extensive leakage expression, line one was abandoned and inducibility was not examined (data not shown).

3.2.3.2. bitetO-Zif268/LacZ2

Dox administration induced strong expression of β-galactosidase in the hippocampus, CA2, caudate putamen, amygdala and piriform cortex. Much weaker expression was seen in individual cells of the cortex and the olfactory bulb. However, in mice bearing

the *bitetO*-Zif268/LacZ transgene strong leakage was found in the habenular nuclei and the line was abandoned (data not shown).

3.2.3.3. bitetO-Zif268/LacZ3

In line three *dox* administration induced weak β-galactosidase expression, restricted to neocortex, several cells in the hippocampus, medial caudate putamen and amygdala (*Fig. 8A, B*). Leakage expression was restricted and only found in several cells in the rostral neocortex (data not shown).

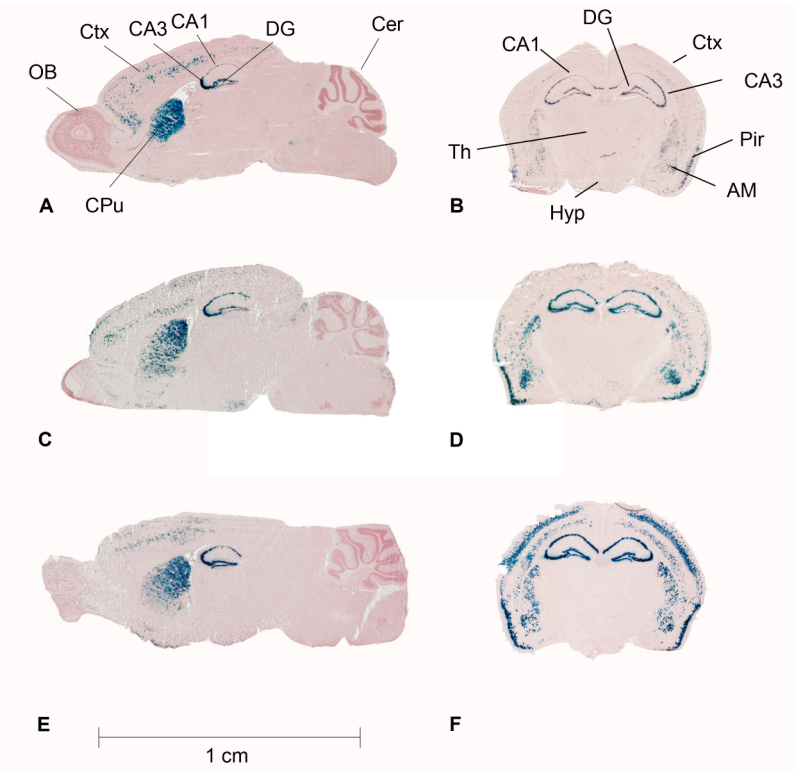

Figure 8. Sagittal and coronal brain sections of *dox*-induced expression of β-galactosidase in CaMKIIα promoter rtTA2 x *bitetO*-Zif268/LacZ mice. (A) Sagittal section of *bitetO*-Zif268/LacZ3 line. (B) Respective coronal section of at approx bregma -1.7mm. (C) Sagittal section of *bitetO*-Zif268/LacZ4 line. (D) Respective coronal section of at approx bregma -1.7mm. (E) Sagittal section of *bitetO*-Zif268/LacZ6 line. (F) Respective coronal section of at approx bregma -1.7mm. (blue signal = X-Gal stain).

3.2.3.4. bitetO-Zif268/LacZ4

Dox administration resulted in a very strong and broad expression of β-galactosidase, largely excluding the medial part of the neocortex, but prominent in all subfields of the hippocampus, amygdala, piriform cortex, anterior neocortex and medial caudate putamen (*Fig. 8C, D*). Slight expression due to leakage was found in individual cells of the medial part of the neocortex (typically 5-10 cells per 14µm section, data not shown).

3.2.3.5. bitetO-Zif268/LacZ6

Dox administration resulted in extremely strong β-galactosidase expression in this line. This was found in the hippocampus sparing CA2, neocortex, piriform cortex, amygdala and the caudate putamen. Additionally, weak expression was observed in the olfactory bulb and the habenular nuclei (*Fig. 8E, F*). Expression due to leakage

was restricted to some cells of the neocortex and very rare in hippocampus (< 1 cell per 14µm section; data not shown).

3.2.3.6. bitetO-Zif268/LacZ9

Very strong *dox*-induced β-galactosidase expression was found in the hippocampus, the caudate putamen, neocortex and septum (data not shown). However, expression due to leakage was especially strong in the cerebellum, dentate gyrus, VMTh and olfactory bulb (data not shown) and the line was therefore discarded.

3.3. Molecular Characterization of Zif268 Over-expression

3.3.1. Co-localization of the Zif268 Transgene and the β-Galactosidase Reporter

Ideally, co-localization of the β-Galactosidase reporter and the Zif268 transgene in the same cells would be shown to verify the reliability of the reporter. However, Zif268 is an IEG with comparably high constitutive protein levels, especially in the neocortex (Bhat and Baraban, 1992; Worley et al., 1991). Since endogenous and transgenic Zif268 mRNA and protein are identical, it is not possible to distinguish the two. This is true for most brain regions, except for those areas with very low endogenous levels. Therefore, in order to have an indication of co-localization, β-galactosidase-expressing cells where examined for high signal levels of Zif268 in co-immunostainings. In most tissues, β-galactosidase-expressing cells also displayed high levels of Zif268 expression. Interestingly, this was not as reliable in the hippocampus, which fits with the quantification results in hippocampus (3.3.3.). Therefore, the expression pattern observed in the β-galactosidase stainings (*Fig. 8*) is a useful but not ideal indication of the pattern of Zif268 over-expression.

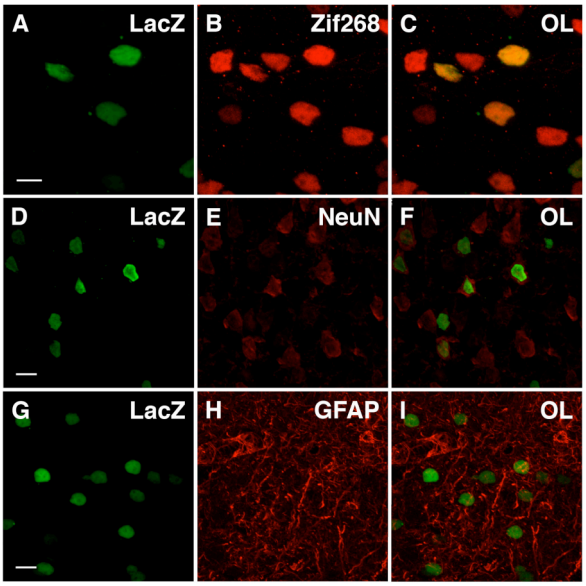

Figure 9. β-Galactosidase expressing cells in the neocortex of Zif268 mutant mice are neurons that also express high levels of Zif268. Double immunostaining showing the localization of β-Gal (A, B, G) and Zif268 (B), NeuN (E) and GFAP (H) in the neocortex and the overlayed images (C, F, I) (Scale bar 10µm, OL = overlay).

3.3.2. Neuronal Expression of the β-Galactosidase Reporter

To verify neuron-specificity of transgene expression, co-immunostainings were performed using antibodies for a specific neuronal marker protein (neurofilament, NeuN) in combination with a β-galactosidase antibody. Since NeuN protein is found in the cytoplasm, while the β-galactosidase protein is nuclear, owing to a nuclear localization signal, co-expression of the two was evaluated based on proximity. *Fig. 9F* (cortex) and *10F* (CA3 subregion of the hippocampus) show that β-galactosidase is always localized in proximity to NeuN. Additionally, co-immunostainings of β-galactosidase with an astrocytic marker protein (glial fabrilliary acidic protein, GFAP) were performed (*Fig. 9I and 10I*). This protein localizes in the cytoplasm of astrocytes (Ludwin et al., 1976) and the staining shows the processes of these cells. Clearly β-galactosidase - and GFAP-expressing cells are localized in a mutually exclusive pool of cells, suggesting that β-galactosidase is not found in astrocytes.

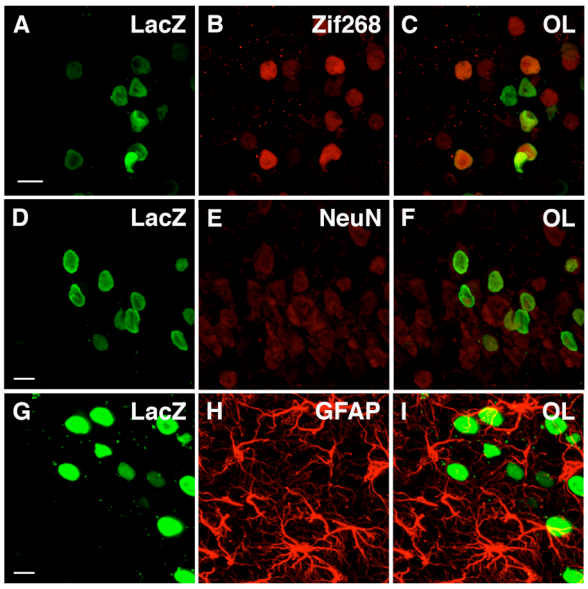

Figure 10. β-Galactosidase expressing cells in the CA3 region of the hippocampus of Zif268 mutant mice are neurons that also express high levels of Zif268. Double immunostaining showing the localization of β-Gal (A, B, G) and Zif268 (B), NeuN (E) and GFAP (H) in the CA3 region of the hippocampus and the overlayed images (C, F, I) (Scale bar 10µm, OL = overlay).

3.3.3. Quantification of Zif268 Over-expression

To determine Zif268 over-expression in transgenic mice, quantitative reverse transcription–polymerase chain reaction (qRT-PCR) was conducted on RNA extracted from different forebrain regions and the cerebellum as a negative control. Threshold detection levels in the TaqMan© qRT-PCR reflect total mRNA levels of Zif268 and were normalized to an endogenous control (β-actin, GapDH). In Zif268 mutants, higher levels of Zif268 were detected in amygdala (controls: 1.0 ± 0.1, n = 6; mutants: 1.6 ± 0.4, n = 8; ANOVA; main effect of genotype: $F_{1,12} = 1.32$, $p > 0.2$; *Fig. 11*) and cortex (controls: 1.0 ± 0.4, n = 6; mutants: 2.1 ± 1.1, n = 6; ANOVA; main effect of genoytpe: $F_{1,10} = 1.75$, $p > 0.2$; *Fig. 11*) however this was not significant due to high variability in the Zif268 mutant group. Interestingly, levels of Zif268 in the hippocampus were unchanged (controls: 1.0 ± 0.2, n = 6; mutants: 0.9 ± 0.2, n = 8; ANOVA; main effect of genotype: $F_{1,12} = 1.97e^{-3}$, $p > 0.9$; *Fig. 11*) and Zif268 levels in cerebellum (controls: 1.0 ± 0.4, n = 6; mutants: 1.1 ± 0.5, n = 8; ANOVA; main effect of genotype: $F_{1,12} = 0.14$, $p > 0.7$; *Fig. 11*) were also comparable to control littermates, as expected from the β-galactosidase expression pattern. To verify these findings on the protein level, immunoblots on nuclear enriched fractions were conducted. As a loading control, histone was detected, quantified and used for normalizing Zif268 levels. In cortex, Zif268 was 1.4 x as abundant in Zif268 mutant than in control littermates (ANOVA, main effect of genoytpe: $F_{1,10} = 4.98$; $P < 0.05$; *Fig. 12*). In the other brain regions, Zif268 protein levels were too low to be visualized by immunoblotting.

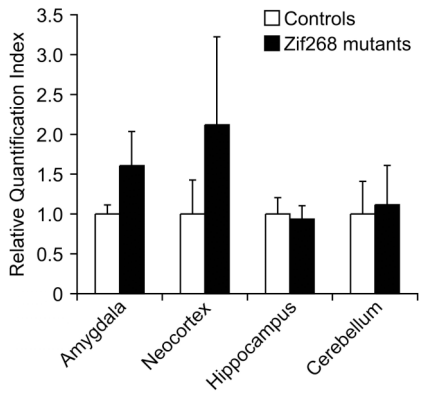

Figure 11. Zif268 mRNA levels are increased in amygdala and neocortex, but not in hippocampus and cerebellum.

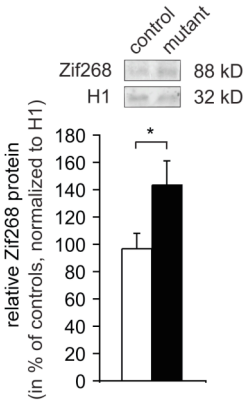

Figure 12: Zif268 protein levels are increased in neocortex of Zif268 mutant mice.

3.4. Behavioral Characterization of Zif268 Mutant Mice

3.4.1. Home Cage Activity

To exclude effects of increased Zif268 levels on spontaneous activity or locomotor behavior, we measured home cage activity in singly housed mice before, and during, induction of Zif268 over-expression using the ActivoScope© detection system (New Behavior, Zürich, Switzerland). For this, four groups were tested, both Zif268 mutants and control littermates were either administered *dox* or not. While there was no differences in activity during light phase between the groups (main effect of group on activity during the light phase: $F_{3,22} = 0.63$; $p > 0.6$; data not shown), during the dark phase Zif268 mutants that received *dox* in the course of the recordings (n = 6) differed from those that did not (n = 5; *Fig. 13a*; repeated measures ANOVA, main effect of *dox* treatment: $F_{1,9} = 6.1$, $p < 0.04$). This difference in home cage activity was mainly due to a marked difference in the second half of the dark phase (*Fig. 13e*; repeated measure ANOVA, hr by treatment interaction: $F_{22,198} = 4.54$, $p < 0.0001$). However, this difference in home cage activity was not explained by either *dox* feeding or Zif268 over-expression, since no correlation between days and *dox* administration was observed (repeated measures ANOVA, day by treatment interaction: $F_{9,81} = 1.481$, $p > 0.1$). Additionally, there is no activity difference between day 1 (before *dox* is first administered) and day 10 (when Zif268 over-expression has reached the maximum) in Zif268 mutants on *dox* (*Fig. 13a*; Fischer's PLSD: $p > 0.6$). This data supports the notion that Zif268 over-expression has no effect on activity, which is confirmed by the finding that home cage activity is not different in *dox* administered Zif268 mutants and *dox* administered controls (n = 10) (*Fig. 13c*; repeated measures ANOVA, main effect of genotype: $F_{1,14} = 2.16$, $p > 0.1$; day by genotype interaction: $F_{9,126} = 0.63$, $p > 0.7$).

In controls on the other hand, *dox* administration resulted in an increase of activity that persisted until the end of the recording period (*Fig. 13b*; controls – *dox*: n = 5; controls + *dox*: n = 10; repeated measures ANOVA, day by treatment interaction: $F_{9,117} = 1.96$, $p > 0.05$). Thus *dox* administration *per se* can result in changes in home cage activity and this lends further credence to the practice of using a *dox* administered control group in behavioral testing.

In summary, Zif268 over-expression in forebrain neurons does not alter home cage activity *per se*. However, *dox* administration can affect this behavior and should therefore be considered before behavioral testing. Finally, home cage activity can differ between groups independent of genotype and treatment, and therefore needs to be checked before any behavioral testing that may hinge on activity.

Since the Zif268 over-expressing group (on *dox*) displays lower home cage activity and this is unique to the individuals within this group, a task that is independent of home cage activity was selected to examine learning and memory in this cohort, the CTA test.

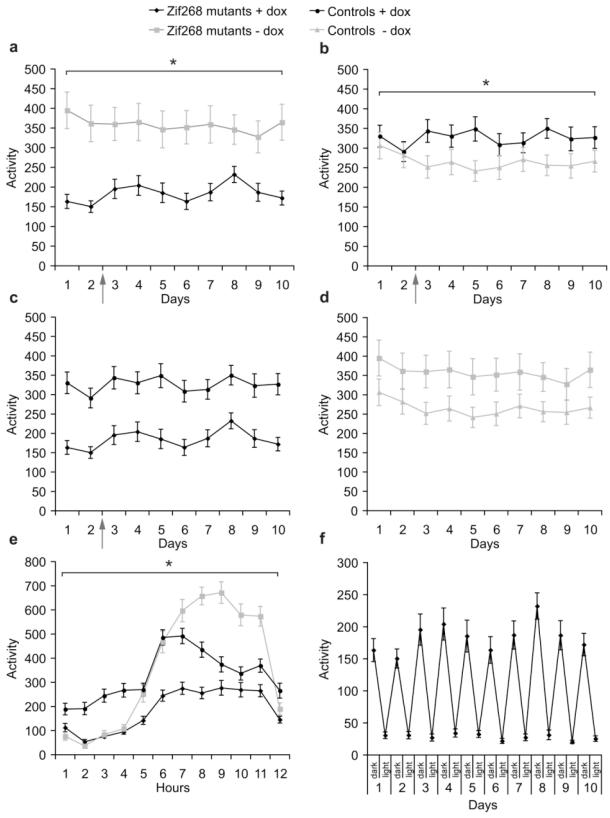

Figure 13. Home cage activity before, and during, *dox* administration in Zif268 mutants and littermate controls. (**a**) Zif268 mutants with or without *dox* treatment. Induction of Zif268 over-expression did not result in a change in home cage activity in Zif268 mutants, however the two groups differed inherently. (**b**) In control mice, *dox* administration significantly affected home cage activity. (**c**) Activity is insignificantly lower in the group of *dox*-treated Zif268 mutants than in the *dox* treated littermate controls. (**d**) Untreated Zif268 mutants and littermate controls have the same home cage activity (grey arrow – onset of *dox* administration). (**e**) The difference between Zif268 mutants with and those without *dox* is attributed to decreased activity in the second half of the dark phase. (**f**) Light-dark cycle in Zif268 mutants on *dox* is normal.

3.4.2. Conditioned Taste Aversion (CTA)

To verify findings from published experiments on Zif268 KO mice showing an involvement of Zif268 in memory consolidation, and to examine whether the over-expression of Zif268 may have the opposite effect, we conducted CTA with Zif268 mutant mice. In CTA, Zif268 KO mice were impaired in long-term retention (Jones et al., 2001).

In the first experiment, Zif268 over-expression was induced before the onset of the experiment and maintained throughout. Zif268 mutants (n = 6) and the littermate controls (n = 8) did not differ in: (a) weight (Zif268 mutants: 40.32 ± 1.73; controls: 39.00 ± 2.29; $F_{1,12} = 0.19$, $p > 0.6$; *Fig. 14a*), (b) liquid intake during habituation (average liquid intake in Zif268 mutants: 0.73 ± 0.05; in controls: 0.86 ± 0.11; $F_{1,12} = 1.22$, $p > 0.2$; *Fig. 14b*), or (c) saccharin preference (saccharin intake during conditioning normalized to the water intake of the fourth habituation trial) (Zif268 Mutants: 1.32 ± 0.12; controls: 1.27 ± 0.06; $F_{1,12} = 0.18$, $p > 0.6$; *Fig. 14c*). Conditioning resulted in similar initial aversion index in transgenic mice over-expressing Zif268 during conditioning and extinction (91 ± 2%, n = 6, black bars) and control littermates (84 ± 4%, n = 8, white bars) when first tested 10 days after conditioning (ANOVA, main effect of genotype: $F_{1,12} = 1.92$, $p > 0.1$; *Fig. 14a*). Additional extinction trials 11, 12, 13, 14, 25, 26, 27, 28, 29 days following conditioning result in a decline in aversion index in control littermates, that is delayed in Zif268 mutant mice (repeated measures ANOVA, main effect of group: $F_{1,12} = 11.61$, $P < 0.01$; main effect of group by extinction day interaction: $F_{9,108} = 2.72$, $P < 0.01$; * $P < 0.05$, ** $P < 0.01$; *Fig. 14a*).

A 10-day interval in extinction training was introduced between days 14 and 25 to examine any change in spontaneous recovery due to Zif268 over-expression, during this time mice only encountered water. Spontaneous recovery did not differ between Zif268 mutants (aversion index on extinction day 14: 46.61 ± 9.43, extinction day 25: 56.85 ± 9.23; ANOVA, main effect of extinction: $F_{1,10} = 0.6$, $p > 0.4$; *Fig. 14a*) and controls (aversion index on extinction day 14: 15.07 ± 5.01 extinction day 25: 21.49 ± 6.54; ANOVA, main effect of extinction day: $F_{1,14} = 0.61$, $p > 0.4$; mean spontaneous recovery in Zif268 mutants: 10.24 ± 7.03; controls: 6.40 ± 3.04; ANOVA, main effect of treatment: $F_{1,12} = 0.3$, $p > 0.5$; *Fig. 14a*)

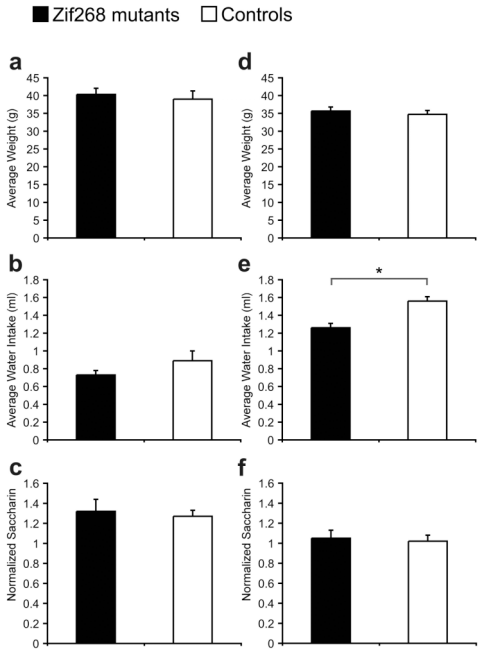

Figure 14. Average weight, water intake and saccharin preference of Zif268 mutants and littermate controls. Neither mutant mice over-expressing Zif268 before nor after CTA conditioning any differences in average weight (**a, e**), or saccharin preferences (as determined by the saccharin solution intake during conditioning normalized to the water intake on habituation day 4; **c, f**). However, while mutants over-expressing Zif268 before conditioning did not differ in water intake during habituation (**b**), mutants over-expressing Zif268 after CTA conditioning did (**e**).

In the second CTA experiment, transgene expression was induced after conditioning and before the onset of extinction training to exclude the possibility that Zif268 over-expression affects CTA extinction *per se*. In this experiment, no difference was seen between the Zif268 mutants and controls in weight (Zif268 mutants: 35.67 ± 1.12, n = 8; controls: 34.72 ± 1.11, n = 10; main effect of genotype: $F_{1,16} = 0.36$, $p > 0.5$; *Fig. 14d*). However, there was a difference in liquid intake during habituation (average liquid intake in Zif268 mutants: 1.26 ± 0.05, n = 8, in controls: 1.56 ± 0.05, n = 10; main effect of genotype: $F_{1,16} = 17.77$, $p < 0.001$; *Fig. 14e*), which was however not seen during extinction training (average liquid consumption during extinction training Zif268 mutants: 1.63 ± 0.12, controls: 1.60 ± 0.12; main effect of genotype: $F_{1,16} = 0.03$, $p > 0.8$; repeated measures ANOVA, total daily liquid consumption by group interaction: $F_{9,144} = 1.25$, $p > 0.2$; data not shown). This difference in liquid intake did not affect preference for saccharin (saccharin intake during conditioning normalized to the water intake of the fourth habituation trial) (Zif268 mutants: 1.05 ± 0.08, n = 8; controls: 1.02 ± 0.06, n = 10; main effect of genotype: $F_{1,16} = 0.09$, $p > 0.7$; *Fig. 14f*). Similar aversion index in transgenic mice overexpressing Zif268 only during extinction (81 ± 5%, n = 8, black bars) and control littermates (72 ± 6%, n = 10, white bars) 10 days after conditioning (ANOVA, main effect of genotype: $F_{1,16} = 1.18$, $p > 0.2$; *Fig. 15b*). Additional extinction trials 11, 12, 13, 14, 25, 26, 27, 28, 29 days

following conditioning resulted in comparable decline in the aversion index of Zif268 mutant mice and control littermates (repeated measures ANOVA, main effect of genotype: $F_{1,16} = 0.01$, $P > 0.9$; day by genotype interaction: $F_{9,144} = 1.06$, $P > 0.3$; *Fig. 15b*).

As observed in the previous experiment, there was no significant spontaneous recovery between extinction days 14 and 25. (Zif268 mutants - aversion index on extinction day 14: 10.84 ± 4.05, extinction day 25: 10.28 ± 2.29; ANOVA, main effect of extinction: $F_{1,14} = 0.01$, $p > 0.9$; controls - aversion index on extinction day 14: 14.52 ± 3.30, extinction day 25: 14.36 ± 5.74; ANOVA, main effect of extinction day: $F_{1,18} = 5.44e^{-4}$, $p > 0.9$). Therefore, spontaneous recovery between Zif268 mutants (-0.56 ± 3.02) and controls (-0.16 ± 3.49) was not different (ANOVA, main effect of treatment: $F_{1,16} = 0.01$, $p > 0.9$).

Additionally, there was further no correlation between aversion index on extinction day 11 and spontaneous recovery from day 14 to 25 in any of the groups (data not shown).

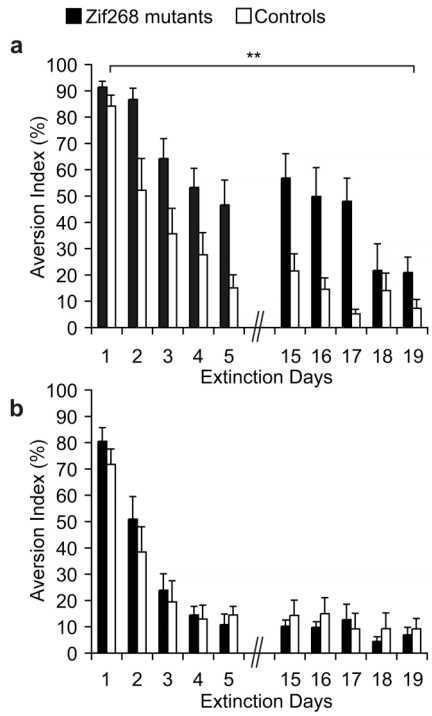

Figure 15. (**a**) Effect of Zif268 over-expression on CTA conditioning and extinction. (**b**) Effect of Zif268 over-expression on CTA extinction *per se*. While a strong effect in consolidation was observed, where the aversive memory trace is strengthened and extinction is delayed (**a**), there is no effect on processes instigated by retrieval, namely extinction and reconsolidation. Furthermore, retrieval is also not affected since Zif268 mutants display similar aversion in the first choice trial.

4. Discussion

The aim of this project was to generate a transgenic mouse that would allow the investigation of the consequences of the inducible, neuronal over-expression of the transcription factor Zif268 on higher cognitive function, particularly learning and memory and the underlying processes of synaptic plasticity. To achieve this, we took advantage of a recently generated transgenic mouse carrying the novel *doxycycline*-inducible transcriptional activator system, rtTA2-M2 (Michalon et al., 2005). In this line, rtTA2 is expressed in neurons of the forebrain. rtTA2 can only bind to its recognition sequences in a corresponding promoter and drive expression of a gene of interest if *doxycycline* or tetracycline is present. As part of this project, a second transgenic mouse line was created that compiles this rtTA2 responsive promoter to drive the simultaneous expression of both, a Zif268 transgene and a reporter. We generated two constructs for microinjection, either carrying the reporter β-Galactosidase, or hrGFP. Ultimately, three Zif268 overexpressing mouse lines, combining the desired characteristics of brain region-specificity, inducibility, tightness and reversibility were identified. The characterization of the effects of Zif268 modulation will contribute to a better understanding of memory related processes and of the involvement of immediate early genes in these processes.

4.1. In vitro Testing

In vitro screening may identify problems with a given transgene, e.g. 3' untranslated regions can contain degradation signals or cryptic splicing sites that may result in truncated or non-functional mRNAs of transgenes. Furthermore, *in vitro* screening may serve to ensure tightness, inducibility and co-expression of a reporter and the gene of interest, in our case Zif268. HELA cells, stably expressing the *doxycycline* responsive tetOFF factor (tTA), were transiently transfected with the *bitetO*-Zif268/LacZ or *bitetO*-Zif268/hrGFP constructs. While reliable co-expression and reversibility by *dox* administration was observed for the construct carrying LacZ, this was not the case for the hrGFP-construct. This cannot be easily explained but several likely explanations may be carefully offered: (a) Errors in the design or sequence of the construct cannot be absolutely excluded, since the final construct was not sequenced before injection. Rather only the sequence around ligation sites used for the generation was verified. (b) For the generation of the *bitetO*-Zif268/hrGFP

construct, the *bitetO*-promoter was taken from the original vector and introduced into a different vector context. This may have caused a change in responsiveness to tTA, possibly due to a secondary structure of the new vector, such as supercoiling. (c) Often either Zif268 or hrGFP expression was detected in our transfected cells, which may suggest that tTA has not bound to all recognition sites in the *bitetO*-promoter. This may be due to sterical reasons that affect accessibility of elements of the promoter region to tTA. The accessibility may also explain the incomplete reversibility observed with the *bitetO*-Zif268/hrGFP construct since some tTA may get trapped close to the promoter and continue to activate expression. Stable integration of the construct into the genome would actually solve these problems. Since this occurs after microinjection the construct was nevertheless used to generate transgenic mice. Moreover, while co-expression of the reporter with the Zif268 transgene were a valuable tool for the characterization of Zif268 over-expression, we aimed to over-express Zif268 in these mice. By using the *bitetO*-Zif268/LacZ as well as the *bitetO*-Zif268/hrGFP constructs, we increased our chances of realizing this goal.

4.2. Screening of the Novel Transgenic Mouse Lines

4.2.1. Breeding Success

As part of this project, 24 transgenic mouse lines were generated with the help of Thomas Rülicke at the central biological laboratory (BZL), carrying either the *bitetO*-Zif268/LacZ or the *bitetO*-Zif268/hrGFP construct. In the first step, all founder mice were bred with C57Bl6J wildtype mice. Several breedings with founders did not give offspring, which may be due to infertility in the founder mouse, incompatible or infertile breeding partners, but also because some of the mice were already middle-age at the onset of the breeding. This was inevitable because available space in the animal facilities was limited and thus breedings cages were initiated sequentially for these mouse lines. For the last lines, breeding cages were only initiated by 9 months of age of the founder. Although mice should still be fertile at this age, breeding success may be limited. Apart from age, an unfavorable genetic background may contribute to infertility. However, the fertilized eggs that were microinjected were derived from a mixed C57Bl6J and DBA2 breeding, which should display good reproductive performance. The contribution of each background may nevertheless differ between founder mice and may therefore affect their mating success by

determining litter size and number of litters. Since our constructs carry inducible transgenes there should be no concern about embryonic lethality of expression of the transgene. However, since these transgenes are randomly integrated, there is a remote probability that a transgene is integrated into a crucial gene for development, interrupts expression of this gene and thus causes embryonic lethality.

4.2.2. Transgene Transmission

In a further selection step transgene transmission to the F1 generation was tested. Of the 18 founder mice that gave offspring, 15 transmitted the transgene to the next generation. Lack of transmission is a frequent and easily explained phenomenon. Here, the timing of transgene integration into the genome after microinjection is critical. Random integration of DNA into the genome usually occurs at double strand breaks (Brinster et al., 1985) in the S phase of the cell cycle (Sonoda 2001, Peterson 2001). Therefore, it may also take place during later cell division, where only a subset of cells in the organism carry the transgene (Palmiter et al., 1983; Wilkie et al., 1986), a phenomenon called genetic mosaicism. In some cases this does not include germ cells, making transgene transmission impossible. A second, however less likely cause, may be a secondary loss or rearrangement of the transgene in the progenitors of the germ line (Brinster and Palmiter, 1984; Palmiter et al., 1982; Palmiter et al., 1983; Shani, 1986). This may also occur in somatic cells by homologous recombination during early cell divisions in the blastocyst (Wilkie et al., 1991) and may be more likely with multiple copies of the transgene (Scrable and Stambrook, 1999).

4.2.3. Functionality of Transgene

Mouse lines that carry and transmit the transgene to the F1 generation were subsequently tested for the functionality of the transgene. A transgene may not be functional for several reasons:

4.2.3.1. Insufficient Expression of the Transgene

4.2.3.1.1. Poor design

Poor design of a construct may significantly affect efficient transgene expression. For instance, it was shown that prokaryotic (Chada et al., 1985a; Chada et al., 1985b; Hammer et al., 1985; Krumlauf et al., 1985; Townes et al., 1985) or viral (Jahner et al., 1982) sequences may inhibit expression of a transgene. It was demonstrated that efficient expression of a cDNA requires splicing (Brinster et al., 1988) and a polyadenylation signal at the 3' end. To ensure accurate and efficient translation the

start codon ATG should be preceeded by a Kozak sequence (Kozak, 1987). For tissue-specific expression it is better to include all naturally occurring intron elements that are involved in mRNA splicing and expression (Choi et al., 1991; Palmiter et al., 1991) since regulatory elements may also reside in intron sequences, usually in the first intron in fact (Beermann et al., 1990; Ganss et al., 1994; Tanaka et al., 1990). Finally, codon usage needs to be optimized and adjusted to the target organism, since it may affect expression strength. This and all other points were taken into account by using pre-existing and established components to generate the constructs (regulatory sequences suggested by Choi *et al.*, 1991).

4.2.3.1.2. Position Effects and Epigenetic Mechanisms

The transgene is influenced by the genomic sequences flanking the integration site, called position effects (Overbeek et al., 1986; Palmiter et al., 1982). In extreme cases the integration event may occur in heterochromatin, constituting regions of packed chromatin matrix in the caryoplasm of a cell. Here, the DNA is found in hyperspiraled form bound to histones and other proteins in a double strand. Any genes in heterochromatin cannot be expressed unless the heterochromatin is changed into the loose conformation euchromatin where the DNA can be cleaved into single strands. Heterochromatin is mainly found in proximity to the centromers of each chromosome, where very few genes are located. But silencing of transgenes near telomeres has also been reported (Pedram et al., 2006).On the other hand, flanking sequences may contain regulatory elements of neighboring genes that act as enhancers on the transgene promoter, and consequently cause ectopic expression of the transgene. Lastly, secondary methylation may silence transgene expression (Palmiter et al., 1982), this is thought to frequently occur at high copy numbers (Garrick et al., 1998).

4.2.4. Incomplete Integration or Expression of the Transgene

4.2.4.1. Incomplete Integration of the Transgene

The construct is cleaved during the preparation for microinjection and is subsequently only partially integrated. Great care was taken during the preparation of the constructs and the final preparation of each linearized construct was checked by restriction enzyme and agarose gel analysis before microinjection. However, the founders were not checked for complete integration of the construct, which can be done using southern blot. This was mainly due to time constraints in the face of large numbers of generated transgenic mouse lines. Instead, genotyping PCRs were used to detect parts

of the CreER(T2) and rtTA2 coding sequences in the genomic DNA of the founder mice.

4.2.4.2. Incomplete Expression of the Transgene

A sequence error in the transgene can result in a stop codon or render the mRNA unstable, thereby preventing efficient expression of the transgene. While the ligation site in all constructs generated in this project were checked by sequencing before microinjection, the constructs were not sequenced completely. Thus, errors in transgenes cannot be entirely excluded but are highly unlikely, since they would have been caused by random mutations occuring during the generation of the constructs.

For screening, mouse lines carrying the *bitetO*-Zif268/LacZ or *bitetO*-Zif268/hrGFP were initially crossed to CaMKIIα promoter-rtTA mice. Once CaMKIIα promoter-rtTA2 mice were generated in our lab and were shown to have greater transactivation capacity, screenings were continued with these. None of the lines generated from the *bitetO*-Zif268/hrGFP construct displayed any GFP fluorescence, though several methods of tissue preparation were used to prevent possible decomposition of the fluorescent protein. The sensitivity of GFP detection is much lower than that of other reporters, e.g. β-galactosidase, since no staining is applied that provides amplification. If the expression of hrGFP was simply too low to be visualized directly, the functionality of the system may have been verified by immunostaining or RT-PCR. However, the need for such procedure would defeat the purpose of this reporter, to follow transgene expression *in vivo* and/or without time-consuming detection methods. In general, the use of GFP as a reporter may indeed only be recommended with strong promoters, like the R26 promoter (Kisseberth et al., 1999). Since reliable β-galactosidase expressing lines were identified from the *bitetO*-Zif268/LacZ pseudo-founders, all lines carrying the *bitetO*-Zif268/hrGFP construct were discarded. Interestingly, several *bitetO*-Zif268/LacZ lines were identified that exhibited large leakage of transgene expression. This may be explained by position effects that depend on the chromosomal environment around the integration site of the transgene. Strong cis or trans acting elements may drive expression of the transgenes in the absence of any transactivator. In particular, β-galactosidase expression in cerebellum in line *bitetO*-Zif268/LacZ9 has to be caused by these position effects since the

CaMKIIα promoter used to express rtTA2 is not active in cerebellum. In other lines, this explanation is further supported by the fact that β-galactosidase expression is detected even in individuals carrying only the *bitetO*-Zif268/LacZ construct.

4.3. Rederivation of Lines 4 and 6

For further experimentation the *bitetO*-Zif268/LacZ lines 4 and 6 were rederived using the embryo transfer technique performed by Pawel Pelzar at the BZL, Zürich. SPF and OHB breedings were subsequently initiated and functionality of the transgene was reconfirmed.

4.4. Molecular Characterization of Zif268 Overexpressing Mice

As part of the molecular characterization, co-expression of Zif268 and β-galactosidase was examined using immunostaining. Contrary to expectation not all neurons expressing β-galactosidase also exhibited high levels of Zif268. This was particularly evident in the hippocampus and may have several reasons:

a) The level of β-galactosidase expression was extremely high and the transactivation as well as the transcription machinery were monopolized for β-galactosidase expression.

b) While translation of β-galactosidase mRNA is constitutive, translation of Zif268 mRNA underlies regulatory steps, which are usually induced by some sort of stimulus. In the absence of this stimulus translation only occurs at very low rates.

c) The stability and kinetics of production or degradation of β-galactosidase and Zif268 differ, both for mRNA and protein. In fact, the profound stability of the β-galactosidase protein has been previously described (Strange, 1966) and it may therefore accumulate while Zif268 protein is quickly degraded (Chaudhary et al., 1996). However, it is also possible that secondary regulation systems on the genomic or mRNA level are involved, e.g. micro RNA – specific gene silencing of mRNA targets that is only beginning to be understood in the scientific community.

d) In some brain regions the endogenous level of Zif268 is very high (e.g. cortex, CA1, (Igaz et al., 2002; Taubenfeld et al., 1999)) and may therefore render any increase due to transgene expression undetectable. In

the case of the hippocampus, there are high levels of Zif268 in CA1, but very low levels in dentate and CA3.

In a second step, the extent of Zif268 over-expression was determined at the mRNA level using qRT-PCR. Contrary to β-galactosidase expression, Zif268 does not seem to over-expressed in the hippocampus of the mutant mice. This may be explained by the points b)-d) above. Additionally, dilution effects may occur when quantifying Zif268 mRNA in whole hippocampus since levels of endogenous Zif268 are high in CA1, but very low in dentate and CA3. On the other hand, high constitutive levels of endogenous Zif268 may point to mechanisms stabilizing the mRNA and protein, and would thus permit more efficient Zif268 over-expression.

In other brain regions, for instance amygdala, over-expression was high but not significantly different between controls and mutants due to a large variability in the mutant mice. Since quantification results were re-confirmed in two independent experiments, using β-actin as endogenous controls in the one case and GAPDH in the other, any contribution due to variability in the endogenous control can be excluded. It was therefore concluded that a difference in efficacy of transgene induction in mutant mice may be the cause of this variability. This difference may result from variations in *dox* consumption, epigenetic differences between individuals affecting either rtTA2 or Zif268-transgene expression, as well as differences due to menstrual cycle, since female mice were used for this quantification. However, variability in males and females in the behavioral tests did not differ (data not shown), suggesting that this explanation may not hold true. Another likely explanation for the high variability lies in the genetic background of the mice used for the study, which were only a F2 generation. For microinjection, fertilized oocytes from DBA:C57J/Bl6 breedings were used for hybrid vigor. However, DBA mice, which have poor learning performance on hippocampal-specific tasks compared to C57 mice (Fordyce and Wehner, 1993; Paylor et al., 1994; Wehner et al., 1990), also display lower basal levels of Zif268 mRNA in the hippocampus (Fordyce et al., 1994). Depending on their genetic makeup, the constitutive level of Zif268 may therefore differ significantly.

To confirm and extend the over-expression of Zif268 to the protein level, immunoblots were performed for neocortex and amygdala. In these immunoblots, Zif268 protein was readily detectable in homogenates from neocortex, but not from

amygdala. This is due to low levels of Zif268 in amygdala. Endogenous mRNA levels are 4x lower than in neocortex according to the qRT-PCR analysis. Furthermore, transgene expression was more prominent and widespread in neocortex than in amygdala.

4.5. Behavioral Characterization of Zif268 Overexpressing Mice

4.5.1. ActivoScope© Recordings

In the control experiment, the effects of *dox* administration and Zif268 over-expression on home cage activity were examined using an automatic ActivoScope© set-up. Importantly, there was no effect of Zif268 over-expression on home cage activity. However, it is of interest to note that *dox* administration *per se* may actually affect home cage activity. In controls, *dox* administration increased home cage activity significantly while it had no effect in Zif268 mutants. The experiment demonstrated that groups of mice may inherently differ in home cage activity, and that this is independent of genotype, age and other external factors. These differences should be considered when tests are to be conducted that assume similar activity. Since the Zif268 mutants that were *dox*-treated during activity measurements exhibited lower home cage activity as compared to their littermate controls, a memory task was selected that was independent of activity, CTA.

4.5.2. Conditioned Taste Aversion (CTA)

Apart from its independence of activity, there were further characteristics of the CTA task that recommended its use. In CTA, a very robust long-lasting memory trace is created that is difficult to extinguish, requiring numerous extinction trials. It is therefore an ideal test of memory trace strength and the competition between an aversive trace acquired during conditioning and a non-aversive trace acquired during extinction training. The mechanisms, including the brain regions required, are well-described, hippocampal-independent, and the measure of memory is extremely objective (saccharin relative to total liquid consumption).

Interestingly, there is a marked difference in average liquid intake during habituation between the groups that were *dox* administered before conditioning as compared to after conditioning. This is due to the high liquid content in the food used for *dox* administration and does not affect the preference for saccharin during conditioning or during subsequent extinction trials.

While there is an increase in aversion index (spontaneous recovery) in the group receiving *dox* before conditioning this is not observed in the group receiving *dox* after conditioning. This is probably explained by the already advanced extinction in the latter group, mice hardly avoid saccharin anymore at extinction day 5, and spontaneous recovery therefore does not occur. However we cannot exclude that extending the interval between choice trials 5 and 6 to several weeks may indeed yield some significant recovery, which would reflect the natural strength and persistence of the aversive memory trace over the non-aversive memory trace.
A further peculiarity is that mice encountering *dox* before conditioning displayed a slightly more robust aversive memory than those encountering *dox* after conditioning (repeated measures ANOVA on course of extinction: controls before versus controls after: $F_{9,144} = 1.54$, $p > 0.1$). Furthermore, initial memory retention as measured in the first extinction trial is not significantly, but obviously different (controls before: 84.17 ± 4.18, controls after: 71.71 ± 5.87; ANOVA of aversion index on extinction day1 $F_{1,16} = 2.71$, $p > 0.1$). This may actually be due to interference of the novel taste of *dox*-spiked food with the CS-US association. However, the course of extinction is significantly different between the two groups of Zif268 mutants (repeated measures ANOVA, extinction day by group interaction: $F_{9,108} = 3.83$, $p < 0.001$) while it is not different for controls (repeated measures ANOVA, extinction day by group interaction: $F_{9,144} = 1.54$, $p > 0.1$), suggesting that the difference observed is actually due to Zif268 over-expression and not to the timing of *dox* administration.
From the data gathered in this project several new insights into the role of Zif268 in memory can be drawn. We have shown that increasing constitutive Zif268 levels during and after conditioning enhances CTA memory strength and robustness to interference. In the field there is little work on the role of the constitutive level of Zif268, which is quite high for an IEG, and its stimulus-induced level and the contribution of each to plasticity related events. This may however be due to a larger pool of Zif268 that is available for recruitment and can only fulfill its function in concert with other factors that are specifically induced during acquisition and consolidation of a task.

The results from the CTA experiment presented here confirm a long line of evidence that suggests a role of Zif268 in memory consolidation (Bekinschtein et al., 2007; Jones et al., 2001). In several memory tasks, Zif268 *knock out* (KO) mice exhibit very

poor learning and memory performance (Jones et al., 2001). For consolidation in contextual fear conditioning, a task that requires both the hippocampus and the amygdala, Zif268 is required in the amygdala (Malkani et al., 2004), but not in the hippocampus, at least in the early phase of consolidation (Lee et al., 2004). A very recent study has provided an interesting addition to this topic by describing a late BDNF-dependent component of consolidation, that functions to render a memory trace more stable and persistent (Bekinschtein et al., 2007). Blockade of this phase by BDNF *knock down* or protein synthesis inhibition only affects late long-term memory only (> 7 days), while leaving early long-term memory two days after acquisition intact. The study further demonstrated that Zif268 is persistently increased in the hippocampus in a BDNF-dependent manner after this phase, suggesting a role of hippocampal Zif268 in rendering a memory trace persistent.

The finding by Bekinschtein *et al.* provides molecular evidence for observations in lesion experiments, showing that the hippocampus appears to have a temporary role in memory storage. Recurrent rounds of consolidation-like events take place for maintenance of the memory trace in the hippocampus (Anagnostaras et al., 1999; Brown, 2002; Scoville and Milner, 1957; Squire, 1987; Zola-Morgan and Squire, 1990) that may serve memory transfer to the neocortex, the region of permanent memory storage (Bontempi et al., 1999; Burwell et al., 2004; Frankland et al., 2004a; Frankland et al., 2004b; Maviel et al., 2004). Zif268 over-expression may therefore actually enhance information processing within the hippocampus as well as its transfer to the neocortex. Thus, the enhancement of aversive memory described in this chapter, may actually have two causes: early on in memory consolidation, the increased Zif268 levels in the amygdala contribute, while later Zif268 in the hippocampus comes into play. However, to confirm this hypothesis, Zif268 mutants need to be tested in other memory tasks, preferably contextual fear conditiong.

Interestingly, we observe no effect if expression of the Zif268 transgene is only expressed after conditioning. The discrepancy between these finding and those previously published, which implicate Zif268 in extinction (Herry and Mons, 2004), reconsolidation (Bozon et al., 2003; Lee et al., 2004), or retrieval (Weitemier and Ryabinin, 2004) can be explained in several ways:

First of all, it is possible that Zif268 over-expression is not achieved in the brain regions required for these processes. Effects of Zif268 ablation or down-regulation on

reconsolidation were demonstrated in amygdala- and hippocampal-dependent tasks. While Zif268 in the amygdala was required for consolidation of the original a fear memory trace (Malkani et al., 2004), the CA3 regions hippocampus was implicated in reconsolidation (Lee et al., 2004). In line with this, retrieval in fear conditioning causes an increase in Zif268 in the dentate gyrus (Weitemier and Ryabinin, 2004, Lee, 2004 #89) and CA3 regions of the hippocampus (Lee et al., 2004). In Zif268 mutant mice, Zif268 levels are increased in the amygdala, but curiously this is not observed in the hippocampus. This may indeed explain the divergence in terms of reconsolidation. Extinction of fear conditioning on the other hand, may be mediated by an increase of Zif268 in the medial prefrontal cortex (Herry and Mons, 2004). While we did not specifically quantify Zif268 over-expression in pre-frontal cortex, LacZ expression is weak in this area and concomitant Zif268 may therefore not be sufficient to affect extinction in our mice.

Another possible explanation can be found in the research of Yadim Dudai and Christina Alberini. They demonstrated that reconsolidation is not a universal phenomenon, but rather there are boundary conditions that determine whether retrieval instigates reconsolidation or extinction. These include, among others, the age of the memory (10 days for CTA, 24hr in many other tests, (Eisenberg and Dudai, 2004; Eisenberg et al., 2003; Milekic and Alberini, 2002; Suzuki et al., 2004), the intensity of previous training (Morris et al., 2006) and the intensity of the retrieval session (Pedreira and Maldonado, 2003; Power et al., 2006). It is likely that the 20-min choice trial presented in the CTA represents an intensive retrieval session that is thought to mainly induce extinction (Pedreira and Maldonado, 2003; Power et al., 2006). The slow progress of CTA extinction observed in wildtype mice would therefore not be explained by a competition between reconsolidation and extinction, but rather by the extraordinary strength of the aversive memory trace. To verify this hypothesis, Zif268 levels would have to be quantified in different brain region after retrieval trials of different intensities. An increase of Zif268 levels in the prefrontal cortex may only become detectable after more intensive retrieval sessions.
In another line of reasoning, it is feasible that Zif268, like protein kinase A (Kemenes et al., 2006), may indeed only play a role in retrieval-induced reactivation if it occurs several hours or days after conditioning. In the case of reports on fear conditioning (Lee et al., 2004) and novel object recognition tasks (Bozon et al., 2003), the delay

between conditioning and retrieval in both studies amounted to 24hrs. In the CTA experiments presented here, the first retrieval session was conducted 10 days after conditioning, which may explain the lack of effect of Zif268 over-expression on reconsolidation.

Zif268 is an immediate early gene and its expression is as such dramatically increased after neuronal activity. Antisense oligonucleotide approaches that transiently block translation of Zif268 mRNA (Lee et al., 2004; Malkani et al., 2004), preferably target the activity-dependent induction of Zif268. However, Zif268 over-expression in the mutant mice presented here was constitutive, as detected on the mRNA and protein level, and therefore independent of neuronal activity. Since this over-expression led to an enhancement of memory strength, we can therefore conclude that the constitutive Zif268 level represents an important parameter in memory formation. This fits with results showing that constitutive Zif268 levels are increased after environmental enrichment (Toscano et al., 2006), an intervention that persistently improves memory performance in laboratory rats and mice (Parsons and Spear, 1972). A general increase of Zif268 may indeed enlarge the pool of available transcription factor that can be recruited after memory-related neuronal activity. This implies that post-transcriptional or -translational events may further regulate Zif268 transactivation activity. Alternatively, additional factors may be required for Zif268 transactivation to occur, which may be provided by other transcription factors (Mouillet et al., 2004 and others; Silverman et al., 1998a), or by transcriptional co-activators (Silverman et al., 1998b). Alternatively, Zif268-regulated genes may mediate this memory improvement by rendering neurons more plastic or lowering the threshold for plasticity related changes to take place. To gain insights into this issue, we are currently conducting proteomics analysis on the Zif268 mutant mice in an effort to identify regulated target genes.

4.6. Outlook

In this research, we have gathered evidence to show that increasing constitutive levels indeed enhances memory performance. We therefore suggest that the constitutive Zif268 level is a critical determinant of long-term memory strength and that most likely depends on the late BDNF-dependent phase of consolidation described by Bekinschtein et al. Further experiments using the Zif268 mutants are underway to

strengthen this hypothesis and specifically address questions left unanswered about the involvement of Zif268 in other memory phases, e.g. reconsolidation. For this, inhibitory avoidance (IA) will be employed because it allows to easily address the issue of reconsolidation and it provides an unbiased measure of memory (latency to leave a platform). Furthermore, IA is a hippocampus-dependent task that, like CTA, also has a strong emotional component and therefore shares its dependence on the amygdala with CTA (Izquierdo et al., 1997). In two independent approaches, a lack of Zif268 has been described to inhibit reconsolidation. In contrast, over-expressing Zif268 should result in an enhancement of reconsolidation, which has only been reported for two molecules so far (Lee et al., 2006b; Tronson et al., 2006).

Chapter 5: Inducible and Neuron-Specific Gene Expression in the Adult Mouse Brain with the rtTA2s-M2 System[5]

AUBIN MICHALON, KYOKO KOSHIBU, KARSTEN BAUMGÄRTEL, DOMINIQUE HAINGOTIANA SPIRIG, ISABELLE M. MANSUY

Brain Research Institute
Medical Faculty of the University Zürich and
Department of Biology of the Swiss Federal Institute of Technology
Winterthurerstrasse 190, CH-8057 Zürich

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Abstract: To achieve inducible and reversible gene expression in the adult mouse brain, we exploited an improved version of the tetracycline-controlled transactivator-based system (rtTA2^S-M2, rtTA2 thereafter) and combined it with the forebrain-specific CaMKIIα promoter. Several independent lines of transgenic mice carrying the CaMKIIα promoter-rtTA2 gene were generated and examined for the anatomical profile, *doxycycline*(*dox*)-dependence, time course and reversibility of gene expression using several lacZ reporter lines. In two independent rtTA2-expressing lines, *dox*-treatment in the diet induced lacZ reporter expression in neurons of several forebrain structures including cortex, striatum, hippocampus, amygdala and olfactory bulb. Gene expression was dose-dependent and was fully reversible. Further, a similar pattern of expression was obtained in three independent reporter lines, indicating the consistency of gene expression. Transgene expression could also be activated in the developing brain (P0) by *dox*-treatment of gestating females. These new rtTA2-expressing mice allowing inducible and reversible gene expression in the adult or developing forebrain represent useful models for future genetic studies of brain functions.

[5] In this study, I provided *Fig. 4*.

1. Introduction and Results

Cellular functions recruit multiple specific proteins, which functions often depend on tissue, cell-type or developmental stage. To study these functions *in vivo*, genetic technologies such as transgenesis and gene knock-out were developed in mice for overexpressing, or partially or completely eliminating selected proteins. To be specific, these manipulations ought to be spatially- and temporally-controlled for the activity of the protein of interest to be modulated only in selected cells and over a restricted time window. Spatial control is generally achieved by using tissue- or cell-specific promoters, and temporal control with ligand-dependent expression systems. The tetracycline-controlled transactivator (tTA)-dependent systems (Gossen and Bujard, 1992) have been widely used for inducible gene expression both *in vitro* and *in vivo*. tTA (also called Tet-Off) is a fusion protein between the tetracycline repressor from *Tn10 tetracycline* resistance operon of *E. coli* and the C-terminal domain of the transcription factor from *Herpes Simplex Virus* (HSV) VP16. The resulting hybrid transcriptional activator can trigger expression from a cognate promoter made of minimal promoter sequences placed downstream from seven repeats of the tetracycline operator (*tetO*). It is constitutively active but its activity can be blocked by tetracycline or analogs such as *doxycycline* (*dox*) or minocycline. The reverse tTA (rtTA or Tet-On), obtained by random mutagenesis of tTA, has opposite features. It is constitutively silent but needs *dox* to bind *tetO* sequences and induce gene transcription (Gossen and Bujard, 1995; Orth et al., 2000).

Both tTA and rtTA factors have been extensively used in transgenic mouse models to achieve *dox*-controlled gene expression (Morozov et al., 2003; Yamamoto et al., 2001; Yamamoto et al., 2000). Although very useful, these systems have several limitations that make their use sometimes difficult *in vivo*. When coupled to promoters active during early development, the tTA system may need to be turned off by *dox* treatment to avoid potential detrimental effects of early transgene expression on general developmental processes. After prolonged *dox* treatment, transgene induction has a slow kinetic due to poor *dox* clearance from the body and may not be fully reactivated ((Bejar et al., 2002) and own observation). The rtTA system is better in this respect because its kinetic of induction can be faster and transgene expression can be detected in the brain within several days after oral *dox* treatment (Mansuy et

al., 1998b) or several hours when *dox* is injected stereotactically (own observation). However, rtTA is often less efficient than tTA and leads to lower levels of transgene expression. It also requires higher doses of *dox* for induction and was sometimes reported to show *dox*-independent expression *in vitro* (Gimenez et al., 2004; Urlinger et al., 2000).

To improve the use of *dox*-dependent systems for *in vitro* and *in vivo* applications, Urlinger *et al.* recently developed new versions of the rtTA factor with increased mRNA stability, optimized codon-use for mammals, lower background activity and higher affinity for *dox* (Urlinger et al., 2000). We took advantage of one of these factors, rtTA2^S-M2 (rtTA2 hereafter) and adapted it to the adult mouse brain by combination with the forebrain-specific CaMKIIα promoter (Mayford et al., 1996a). Several independent lines of CaMKIIα promoter-rtTA2 were generated and crossed with mice carrying a *tetO*- or *bitetO*-lacZ reporter gene to evaluate the *dox*-dependent, inducibility and reversibility of gene expression (*Fig. 1a*).

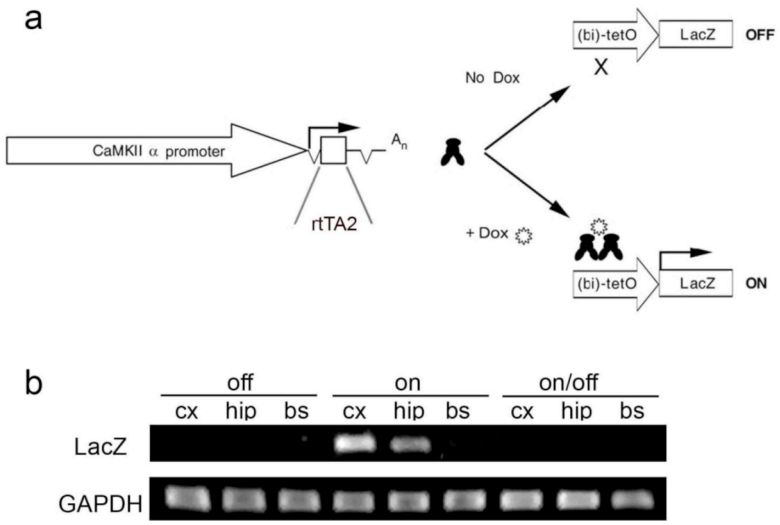


Figure 1. (**a**) Principle of *dox*-controlled rtTA2 expression system. A first transgene carries the CaMKIIα promoter driving the expression of rtTA2^S-M2 (rtTA2) in forebrain neurons. A second independent transgene carries tet operator sequences (*tetO* or *bitetO*) and a lacZ reporter gene encoding β-galactosidase. The *bitetO* promoter is a bidirectional promoter allowing simultaneous expression of two genes. When bound to *dox*, rtTA2 switches to an active conformation, which favors binding to *tetO* sequences (Orth et al., 2000) and induction of gene expression (ON state). In the absence of *dox*, rtTA2 does not bind to *tetO* and the transgene is not expressed (OFF state). (**b**) Inducibility and reversibility of *dox*-dependent gene expression (line 898 used as representative). Double transgenic mice (rtTA2 line 898/*tetO*-lacZ) were fed 6mg/g *dox* in food for 8 days (on). For some animals, *dox* was withdrawn and regular food was provided for three weeks (on/off). Double mutant mice not treated with *dox* (off) are negative controls. LacZ expression was examined by RT-PCR in cortex (cx), hippocampus (hip), and brainstem (bs) with glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) as positive control.

In two lines of rtTA2-expressing animals (894 and 898), forebrain-specific expression of lacZ reporter gene was induced by *dox* treatment (6mg/g *dox* for minimum 6 days) but none in the absence of *dox* (off), indicating full *dox*-dependence of transgene expression (*Fig. 1b*, not shown for 894). LacZ gene expression could be suppressed in both cortex and hippocampus after *dox* removal (on/off), showing full reversibility of transgene expression. When induced in the adult brain, β-galactosidase activity was detected in various cortical regions, including hippocampus, striatum, septum, olfactory bulb, and amygdala but not in thalamus, brainstem or cerebellum, consistent with the endogenous pattern of CaMKIIα expression (Burgin et al., 1990; Herms et al., 1993; Sola et al., 1999; Zou et al., 2002) (*Fig. 2*). The expression pattern was reproducible across animals and was similar in 894 and 898 lines (not shown for 894). Forebrain-specificity was also consistently observed in two other reporter lines carrying a bidirectional *tetO* promoter (*bitetO*) fused to lacZ gene (Baron et al., 1995). When crossed with rtTA2-expressing mice (line 898), these animals showed β-galactosidase expression in most forebrain structures (*Fig. 3* and *4*). The signal was however stronger and denser than in the *tetO* reporter line. For instance, the vast majority of pyramidal cells in hippocampus area CA1, CA2, CA3 and dentate gyrus expressed lacZ in both *bitetO* reporter lines, contrasting with the mosaic expression observed in the *tetO*-lacZ line. Similar heterogeneity in lacZ expression pattern and intensity was observed in cortex, striatum and amygdala depending on the reporter lines.

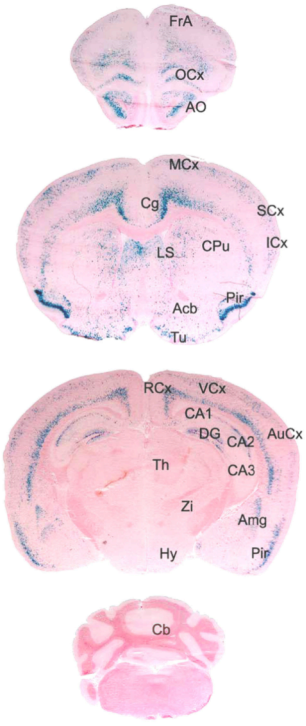
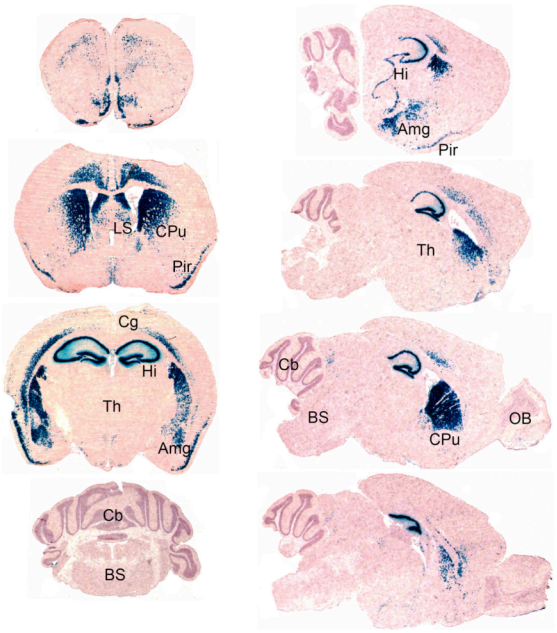


Figure 2. *Dox*-dependent gene expression with the rtTA2 system. Double transgenic adult mice carrying the CaMKIIα promoter-rtTA2 (line 898) and *tetO*-lacZ transgenes were fed 6mg/g *dox* for 11 days. β-galactosidase activity was detected in forebrain structures, including nucleus accumbens (Acb), amygdala (Amg), anterior olfactory nuclei (AO), auditory cortex (AuCx), cornu ammonis regions 1,2,3 (CA1,2,3), cingulate cortex (Cg), caudate pu*tam*en (CPu), dentate gyrus (DG), frontal association cortex (FrA), insular cortex (ICx), lateral septum (LS), motor cortex (MCx), orbital cortex (OCx), piriform cortex (Pir), retrosplenial cortex (RCx), somatosensory cortex (SCx), olfactory tubercles (Tu), visual cortex (VCx). There was no expression in midbrain or hindbrain structures such as the cerebellum (Cb), hypothalamus (Hy), thalamus (Th), zona incerta (Zi), or brain stem (Bs).

Figure 3. Expression pattern in a *bitetO* promoter-lacZ reporter line (*bitetO*-lacZ line 1). (a) Coronal and (b) sagittal brain sections from adult double transgenic mice carrying CaMKIIα promoter-rtTA2 (line 898) and *bitetO*-lacZ transgene (line 1) fed *dox* (6mg/g in food). β-galactosidase expression is restricted to forebrain structures including the amygdala (Amg), hippocampus (Hi), cingulate cortex (Cg), caudate pu*tam*en (CPu), lateral septum (LS), and piriform cortex (Pir). There was no expression in the olfactory bulb (Ob), thalamus (Th), brainstem (Bs) or cerebellum (Cb).



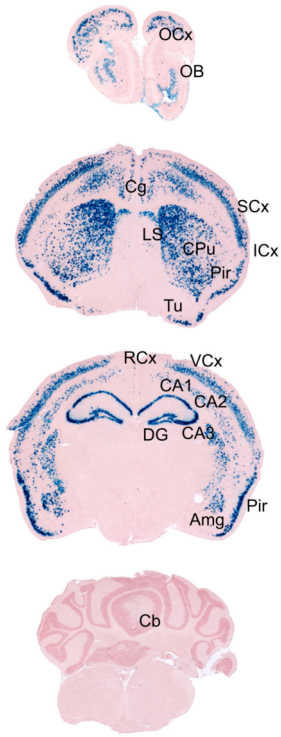


Figure 4. Expression pattern in a second *bitetO* promoter-lacZ reporter line (*bitetO*-lacZ line 2). (a) Coronal brain sections from adult double transgenic mice carrying CaMKIIα promoter-rtTA2 (line 898) and *bitetO*-lacZ transgene (line 2) fed *dox* (6mg/g in food). β-galactosidase expression was observed in the amygdala (Amg), cornu ammonis regions 1,2,3 (CA1,2,3), cingulate cortex (Cg), caudate pu*tam*en (CPu), dentate gyrus (DG), insular cortex (ICx), lateral septum (LS), olfactory bulb (OB), orbital cortex (OCx), piriform cortex (Pir), retrosplenial cortex (RCx), somatosensory cortex (SCx), olfactory tubercles (Tu), visual cortex (VCx). There was no expression in midbrain or hindbrain structures such as the cerebellum (Cb), hypothalamus, thalamus, or brain stem.

A great advantage of the CaMKIIα promoter is its neuronal specificity (Mayford et al., 1996a). To evaluate whether this feature was maintained in our rtTA2-expressing mice (line 898), we performed double immunostaining using antibodies against β-galactosidase and the neuron-specific marker NeuN. Co-staining revealed that all cells expressing lacZ in cortex, hippocampus, amygdala and other brain structures are positive for NeuN, confirming the neuronal specificity of gene expression (*Fig. 5*). We also examined the dose-dependence and time course of gene expression. Administration of increasing doses of *dox* showed that 6mg/g *dox* (in wet food, corresponding to 3.47 ± 1.17mg *dox*/g body weight/day) induced the strongest lacZ expression while 3 or 1mg/g (corresponding to 1.89 ± 0.4 or 0.53 ± 0.07mg *dox*/g

body weight/day respectively) induced a more restricted expression, essentially excluding cortical structures (*Fig. 6a*). Likewise, *dox* administered in drinking water at 3.5 or 2mg/ml induced only partial lacZ gene expression but no expression at a lower concentration (0.05mg/ml) (*Fig. 6b*). These results indicate that gene expression depends on the dose of *dox* and that full expression requires 6mg/g *dox* in food. The restricted expression obtained with lower doses is notable and may be useful to spatially limit the effect of a transgene. In terms of time course, a gradual increase in lacZ expression was observed after 4, 6, or 8 days of *dox* treatment (6mg/g wet food). β-galactosidase activity was first detected after 4 days (no signal after 2 days) but was less dense and intense than after 6 or 8 days (*Fig. 6c*). Thus overall, the level of lacZ expression correlated with the amount and duration of *dox* treatment without any remarkable change in expression pattern.

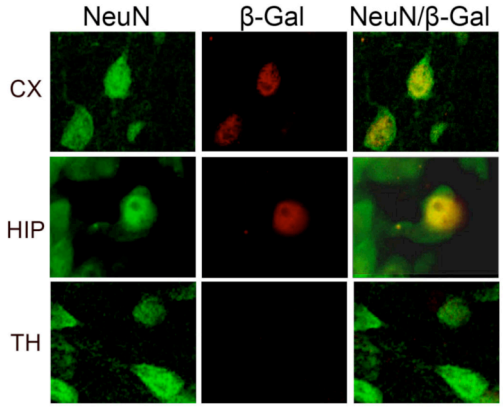


Figure 5. Neuronal-specificity of gene expression with CaMKIIα promoter-rtTA2 mice. Colocalization of the neuron-specific marker NeuN (green, left panel) and β-galactosidase (red, middle panel) in neurons of cortex and hippocampus as indicated by the yellow signal (right panel) in double transgenic mice (line 898/*tetO*-lacZ). Neurons in thalamus do not express lacZ (no β-galactosidase signal).

Because inducible expression systems can be useful to developmental studies, we examined whether gene expression could be induced during embryogenesis in our rtTA2-expressing animals (line 898). We tested an early time point of induction by administering *dox* to dams through gestation and examined β-galactosidase expression in the brain of newborn pups. Expression was detected in the olfactory bulb, cortex, striatum and hippocampus and in small areas of midbrain and hindbrain (*Fig. 7*). Such early expression is consistent with previous data showing prenatal transgene expression using the same CaMKIIα promoter (Krestel et al., 2001; Yamamoto et al., 2000). Prenatal activation is specific to this promoter fragment but does not reflect endogenous CaMKIIα expression, which is essentially postnatal in the mouse and rat brain (Burgin et al., 1990; Herms et al., 1993; Sola et al., 1999; Zou et al., 2002).

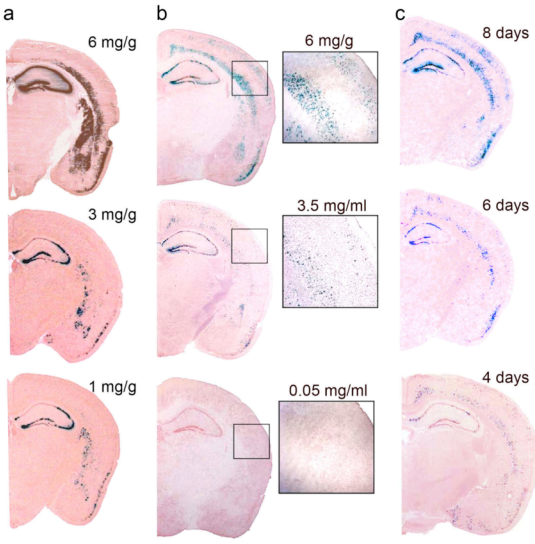


Figure 6. Dose-dependent gene expression with the rtTA2 system using *bitetO*-lacZ (a) or *tetO*-lacZ line (b). (a) Double transgenic mice (line 898/*bitetO*-lacZ line 1) received 6mg/g, 3mg/g, or 1mg/g *dox* in food for 18-20 days. (b) Double transgenic mice (line 898/*tetO*-LacZ) received 6mg/g *dox* in food, or 3.5mg/ml or 0.05mg/ml *dox* in drinking water for one month. Long treatment was chosen to avoid interference between *dox* dosage and induction kinetic. The strongest expression was obtained with 6mg/g *dox* in food, while no expression was induced at 0.05mg/ml in drinking water. (c) Time course of induction of *dox*-dependent gene expression. Double transgenic mice (line 898/*tetO*-lacZ) were fed 6mg/g *dox* food for 2, 4, 6 or 8 days. A minimum of four days of *dox* treatment was required to induce lacZ expression and accumulation of β-galactosidase to a detectable level. The highest expression was detected after 8-day of *dox* treatment.

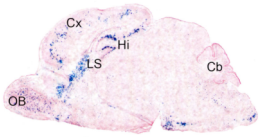


Figure 7. β-galactosidase activity in newborn pups (line 898/*tetO*-lacZ) after *dox* treatment of gestating dams. Forebrain structures showed β-galactosidase expression, including cortex (Cx), hippocampus (Hi), lateral septum (LS) and olfactory bulb (Ob). β-galactosidase activity was quasi absent from midbrain and cerebellum (Cb), but ectopic expression was detected in brain stem (Bs).

In summary, we have generated two new lines of transgenic mice expressing rtTA2 under the control of the CaMKIIα promoter that allow inducible and reversible *dox*-dependent gene expression in the adult brain. With these mice, gene expression can be induced in a dose- and time-dependent fashion in neurons of most forebrain structures including cortex, hippocampus, amygdala and striatum. Expression is overall consistent in several independent rtTA2-responsive lines whether carrying a *tetO* or *bitetO* promoter. The slight variations in the pattern and level of expression most likely result from different integration site, copy number or post-integration modifications of the *tetO*-carrying transgene such as recombination or epigenetic changes (Mayford et al., 1996a; Robertson et al., 2002). In this respect, the lower lacZ expression observed in the *tetO*-lacZ reporter line reflects a general reduction in transactivation efficiency that may result from extensive back-crossing of the line since its creation.

It should be noted that some properties of rtTA2 reported *in vitro* were not replicated *in vivo*. The rtTA2 factor was shown to require 10 times less *dox* than rtTA for full transactivation (0.1 versus 1µg *dox*/ml) in stably transfected cells (Urlinger et al.,

2000). *In vivo* however, we observed that both tTA and rtTA2 factors required a similar dose of *dox* to achieve comparable kinetics of gene induction. This discrepancy suggests that the sensitivity of rtTA and rtTA2 for *dox* may be similar *in vivo* (unlike *in vitro)* and in turn, that rtTA2 is less effective *in vivo* than *in vitro* due to unknown mechanisms. In the future, the design of more efficient drugs may help enhance the efficacy of these expression systems. For instance, 4-epi*doxycycline* (4-ED), a hepatic metabolite of *dox* without antimicrobial activity or side effect on intestinal flora, was recently shown to be an efficient alternative to *dox* for regulating transgene expression in mice (Eger et al., 2004). A new induction system using photolabile-caged *dox* was also developed for cell-specific delivery and local gene expression that may be useful *in vivo* (Cambridge et al., 2006). Alternatively, more sensitive transactivators may be created in the future.

2. Materials and Methods

2.1. Generation of CaMKIIα promoter-rtTA2 transgenic mice

rtTA2^{s}-M2 coding sequence was flanked with hybrid regulatory sequences and fused to 8.5 kb fragment of the CaMKIIα gene. To create this construct, the rtTA2s-M2 gene was first excised from the pUHD-rtTA2-MT2 plasmid with EcoR1 and BamH1 (Urlinger et al., 2000) and blunt-ended. This fragment was inserted into the EcoRV site of the pNN265 plasmid carrying 5' and 3' regulatory sequences with hybrid introns favoring transgene expression (Choi et al., 1991) and a polyadenylation signal from SV40. This construct was removed by Not1 excision and inserted downstream of the CaMKIIα promoter at a Not1 site of the pMM403 plasmid (Mayford et al., 1996a). The full construct was excised from the vector with Sfi1, isolated from the vector backbone and purified by electro-elution followed by ethanol precipitation. DNA was microinjected in pronuclei of hybrid B6D2F1 eggs. Resulting founders were backcrossed to C57/Bl6J mice and animals from the F1 generation were crossed with mice heterozygous for *tetO*-LacZ (Mayford et al., 1996a) or *bitetO*-LacZ reporter gene. Animals carrying both transgenes and control littermates carrying no transgene or either one of the two transgenes were used (3-8 month old).

2.2. Mouse housing

Mice were housed under constant temperature, humidity and inverted light-dark cycle of 12hr (dark 9:30am-9:30pm). Housing and maintenance conditions were according to guidelines established by the Federation of Swiss Cantonal Veterinary Officers.

2.3. Genotyping

Mice were marked by ear punching and genotyped by PCR on tail DNA prepared with the HOTSHOT method (Truett et al., 2000). For rtTA2, primers F1 (5'-TGCCTTTCTCTCCACAGGTGTCC-3') and rtTA2-260R (5'-GAGAGCACAGCGGAATGAC-3') were used. For lacZ, primers lacZ-s (5'-CCCATTACGGCTAATCCGCCG-3') and lacZ-as (5'-GCCTCCAGTACAGCGCGGCTG-3') were used. The PCR reaction contained 2.5mM $MgCl_2$ and run at 94°C for 30s, 62°C for 40s, and 72°C for 1min for 35 cycles.

2.4. Doxycycline administration

Dox (West-ward Pharmaceutical) was mixed in soft mouse chow (soften in sterile water, 50/50 w/v) or drinking solution (Sigma, 5% sucrose). Medicated food was prepared daily and given at the beginning of the dark phase (morning) to minimize light exposure. *Dox* solutions were provided *ad libitum* in opaque bottles and renewed once (2mg/ml and 0.05μg/ml) or twice (3.5mg/ml) a week.

2.5. β-galactosidase staining

Sixteen micrometer fresh brain sections were fixed for 10-min in ice-cold 100mM phosphate buffer pH7.2 containing 0.2% glutaraldehyde, rinsed 3 times and incubated overnight at 37°C in 5mM potassium ferrocyanide, 5mM potassium ferrocyanate, 2mM MgCl2, 1mM X-Gal, 0.01% sodium deoxycholate, and 0.02% NP40 in 100mM phosphate buffer pH7.2. Sections were counterstained with acidified hematoxylin, dehydrated, then mounted with Neo-mount (Merck) or DPX (Taab).

2.6. RT-PCR

Total RNA was isolated with TRI Reagent® (Research Center, Inc.) according to the manufacturer's protocol. Four microliters Polyacryl Carrier (Research Center, Inc.) were added to each sample to enhance RNA yield. Total RNA was treated with 3 to 4 units of RQ1 RNase free DNase (Promega) in 1× RQ1 DNase Reaction Buffer (Promega) for 2 to 3 times to remove potential DNA contamination. The reaction was terminated by adding 2mM RQ1 DNase Stop Solution (Promega). Total RNA was purified by precipitation using sodium acetate and ethanol and stored at -80°C. Reverse transcription of mRNA to cDNA was conducted using the Enhanced Avian HS RT-PCR kit (Sigma), according to the manufacturer's instructions. Approximately 1μg of total RNA was used in each reaction. The cDNA samples were then tested for the presence of lacZ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene used as a positive control, using lacZ forward (5'-CCCATTACGGCTAATCCGCCG-3') and reverse (5'-GCCTCCAGTACAGCGCGGCTG-3') primers, and GAPDH forward (5'CACTGAGCATCTCCCTCACA3') and reverse (5'GTGGGTGCAGCGAACTTTAT3') primers. The PCR reaction contained 2.5mM

(for lacZ) or 1.5mM (for GAPDH) $MgCl_2$ and reacted at 94°C for 30s, 62°C for 40s, and 72°C for 1min for 35 cycles (lacZ) or 94°C for 30s, 56°C for 45s, and 72°C for 45s for 40 cycles (GAPDH). PCR products were run on 2.5% agarose gel in 0.5x TBE with ethidium bromide and visualized by UV.

2.7. Immunohistochemistry

Mice were perfusion-fixed in 4% formaldehyde with 2.4mg/ml sodium periodate and 0.1M lysine and post-fixed in the same fixative for 1hr at 4°C. The fixed brains were cut at 50-micrometer thickness using a Leica VT1000s vibratome. Free-floating sections were washed with 0.3% TritonX-100 in phosphate buffered saline (PBS) then three times in PBS alone. Sections were blocked in 4% dry milk in PBS at room temperature for 1hr and incubated in primary antibody for 2 days at 4°C and secondary antibody for 1.5hrs. All antibodies were diluted in 1% BSA in PBS using the following dilutions: 1:200 mouse anti-NeuN (Abcam), 1:300 rabbit anti-β-galactosidase (Molecular Probes), 1:500 FITC goat anti-mouse secondary antibody (Abcam), 1:500 Cy3 goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc). Images were digitized using a DMRE confocal microscope and a TCS SP2 scan head (Leica).

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Chapter 6: Combined Approach for Inducible Gene Recombination and Expression

KARSTEN BAUMGÄRTEL, AUBIN MICHALON, URSULA HADITSCH AND ISABELLE M. MANSUY

Brain Research Institute
Medical Faculty of the University Zürich and
Department of Biology of the Swiss Federal Institute of Technology
Winterthurerstrasse 190, CH-8057 Zürich

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Introduction

The use of conditional transgenesis to study learning and memory and synaptic plasticity is thoroughly introduced in chapter 4. It includes an outline of the specific requirements in this field of research, an exposé over novel technical developments and their application, as well as an outlook towards future directions of this field in sight of emerging areas of interest. Therefore only a brief introduction to directly relevant literature is presented here, that focuses on transgenic technologies that are used in this project.

The advent of molecular biology in the second half of the last century gave rise to a growing surge to assign functions to specific genes and their products. This surge was accelerated by the availability of complete genomes in the last decade and the possible relevance to disease. At the same time, novel and ingenuous tools to manipulate genes and their products have continuously emerged. Initially, this innovation was restricted to specific pharmacological agents that either inhibit or activate protein targets. However, the application of these agents is limited by their innate chemical properties, including sensitivity, kinetics of action and clearance, tissue and plasma membrane penetration and solubility. Furthermore, for many target proteins no compounds with the required specificity and properties have been identified thus far. While this drug development continues, a different approach using methods of genetic manipulation has emerged, that overcomes these limitations. in their earliest form, these manipulations were restricted to the constitutive and ubiquitous ablation of single genes. The study of such "gene ***k****nock* ***o****uts* (KO)" has fundamentally contributed to today's broad understanding of molecular mechanisms underlying phenomena such as synaptic plasticity. However, the KO approach lacked inducibility, reversibility and spatial control. Accordingly, developmental effects of a gene KO were often debated and in some cases embryonic lethality made functional studies in adults impossible (e.g. (Ernfors et al., 1994; Knuesel et al., 2005; Wang et al., 1995)). Additionally, spatial specificity became an issue, especially for genes that fulfill diverse functions in distinct organs or tissues.

In response to these concrete requirements, several gene regulation systems were taken from bacteria, phages or plants and adapted for research use in different animal

models. Among them, and certainly the most prominent for studies in mice, are the tetracycline-controlled transactivator (tTA) and the Cre recombinase (Cre) systems.

The Tetracycline-controlled Transactivator System

The tTA system was first developed for cell-culture and later in transgenic mice by Gossen and Bujard (Gossen and Bujard, 1992). In its original form, it consists of the *Escherichia coli* tetracycline repressor (TetR) protein fused to the carboxy-moiety of a VP16 domain therefore enabling transcriptional transactivation. tTA binds and activates a hybrid promoter that contains the minimal CMV promoter downstream of the cognate tTA binding site, an array of seven tetracycline operator sequences (*tetO* promotor). This activation is prevented by administration of *tetracycline* or its analogue *doxycycline* (*dox*, a classical antibiotic widely used in medical treatment). They form a complex with tTA and thereby block binding to the *tetO* promotor. Since continuous *dox* treatment is required to repress tTA activity, this system is not suitable for all studies. One solution to this problem lies in rendering tTA expression subject to Cre activity. After excision of a floxed stop codon upstream of the coding sequence of tTA, the latter is expressed and in turn activates expression of a transgene (Shimizu et al., 2000). The alternative and more proficient solution was the engineering of the reverse tetracycline-controlled transactivator (rtTA) system by random mutagenesis of the tTA gene (Gossen and Bujard, 1995). In contrast to tTA, rtTA requires *dox* to binding to the *tetO* promoter and to thereby activate transgene expression. This eliminated the need for long-term *dox* exposure to repress transgene expression. The rtTA system was further improved from the tTA system with regard to induction kinetics, as *dox* treatment can rapidly induce transgene expression, in some cases even within an hour *in vitro* (Hasan et al., 2001). However, in some instances rtTA retained some affinity for *tetO* sequences in the absence of *dox*, and the *tetO* promoter showed autonomous rtTA independent activity, rendering the system slightly leaky (Zhu et al., 2002). To improve tightness, the expression of rtTA, like tTA in the example above, was made subject to a recombination event removing a stop cassette to induce rtTA expression (Belteki et al., 2005). An additional disadvantage of the rtTA system is the need for high level of *dox* to activate transcription in some cases (Gimenez et al., 2004; Imhof et al., 2000; Keyvani et al., 1999).

Therefore, in rtTA2, a novel version of rtTA, *dox*-affinity was improved by site-directed mutagenesis of tTA, and stability of mRNA was increased by optimized codon-usage for mammals (Urlinger et al., 2000). Transgenic mice bearing the rtTA2M2 variant under control of the CaMKIIα promoter were generated in our laboratory (Michalon et al., 2005) (*Fig. 1*).

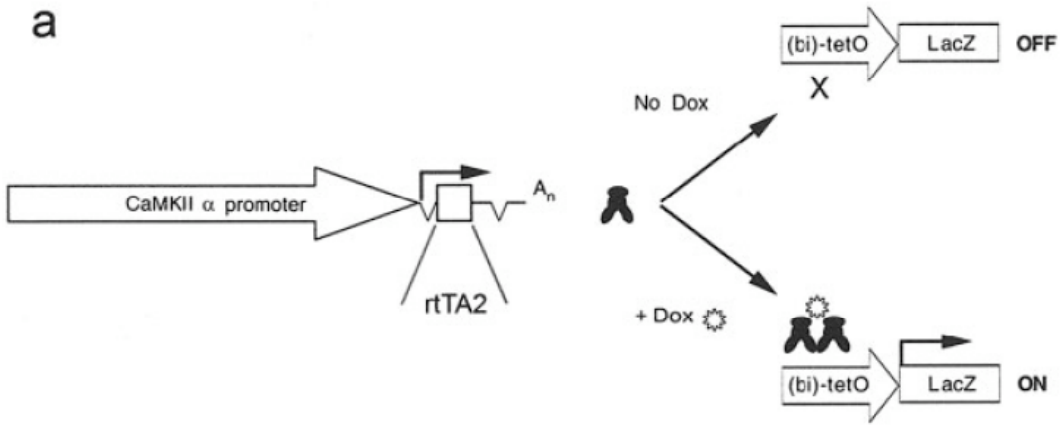

Figure 1. Doxycycline-controlled transactivation: rtTA2 can only bind to its promoter and drive expression of a transgene in the presence of *dox*ycyline (taken from Michalon *et al.*, 2005).

The Cre Recombinase System

The Cre recombinase gene is taken from the bacteriophage P1 and encodes a 38kD recombinase of the Type I topoisomerase family. It efficiently catalyzes site-specific recombination at 34bp cognate sequences, termed loxP sites, in the absence of any additional cofactor. This system was adapted to animal models to achieve spatial specificity and cell-type selectivity of gene ablation through the use of tissue-specific promoters to drive Cre expression. These promoters not only conveyed spatial specificity but recombination followed the time window of promoter activity. Different Cre mouse lines could thus be employed to achieve the desired specificity of the KO. However, the practicability of this approach is restricted, since the availability of suitable promoters to drive Cre expression is limited. This is mainly due to multiple time windows of promoter activity, so that recombination would frequently occur at an undesirable time point, for instance during development.

Therefore, the tTA or rtTA systems were used in combination with an (r)tTA-responsive promoter to drive expression of Cre. This allowed timing of the induction of Cre recombinase and of recombination (Guo et al., 2005; Lindeberg et al., 2002; Radomska et al., 2002; Saam and Gordon, 1999; Schonig et al., 2002; Utomo et al.,

1999; Yu et al., 2005b). However, this requires complicated and extensive breeding programs since the final mouse line carries at least three different transgenes. In a more elegant approach to resolve this lack of temporal control, the activity of the Cre recombinase was rendered inducible by fusing it to the ligand-binding domain (LBD) of the human estrogen receptor (ER). To avoid the interference of endogenous ligands, point mutations known to impair estradiol-, but not *tamoxifen*-(*tam* - a synthetic ER antagonist) binding were introduced (Brocard et al., 1998; Danielian et al., 1998; Feil et al., 1996; Guo et al., 2002; Hayashi and McMahon, 2002; Indra et al., 1999; Kellendonk et al., 1996; Littlewood et al., 1995; Metzger and Feil, 1999; Schwenk et al., 1998). The resulting CreER(T2) fusion gene then allowed inducible, tissue specific gene ablation (*Fig. 2*), thus permitting to circumvent any promoter activity during development or at undesirable time points. However, its irreversibility is a major limitation of this system. In this project, we therefore generated novel transgenic mice to overcome this limitation.

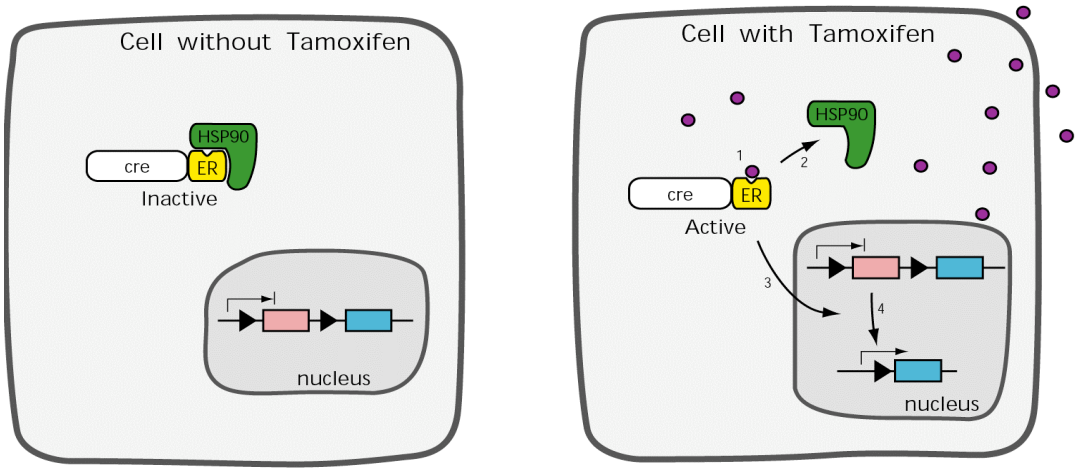

Figure 2. *Tamoxifen*-controlled recombinase: The fusion protein CreER(T2) is blocked from translocation to the nucleus by estrogen receptor (ER)-mediated binding of heat shock proteins (HSPs). *Tamoxifen* (purple circles) binds to ER (1) and displaces the HSPs (2), allowing CreER(T2) to enter the nucleus (3) and to catalyze recombination (4) between two loxP sites (black triangles)(taken from Leone *et al.*, 2003).

Project Outline

The aim of this project was to generate a transgenic mouse line that permits the independent induction of either an over-expression, an ablation or a rescue (in a *knock out* background) of a gene of interest in the brain regions implicated in learning and memory. For this, we combined the CreER(T2) and the rtTA2 systems and put them

under the control of the forebrain neuron-specific calcium/calmodulin-dependent kinase IIα (CaMKIIα) promoter (*Fig. 1*). The CaMKIIα promoter is active in forebrain neurons, specifically in hippocampus, cortex and amygdala (Erondu and Kennedy, 1985; Mayford et al., 1996a; Miller and Kennedy, 1985), and would therefore restrict all manipulations to these areas of the brain known to be critical for learning and memory (Fries et al., 2003). Co-expression of CreER(T2) and rtTA2 allows the independent induction of recombination by *tam* injection and of transgene expression by *dox* administration in the same neurons of the forebrain (*Fig. 3*). This approach provides both spatial and temporal specificity of a bi-directional manipulation to study the contribution of candidate genes to cognitive functions such as learning and memory.

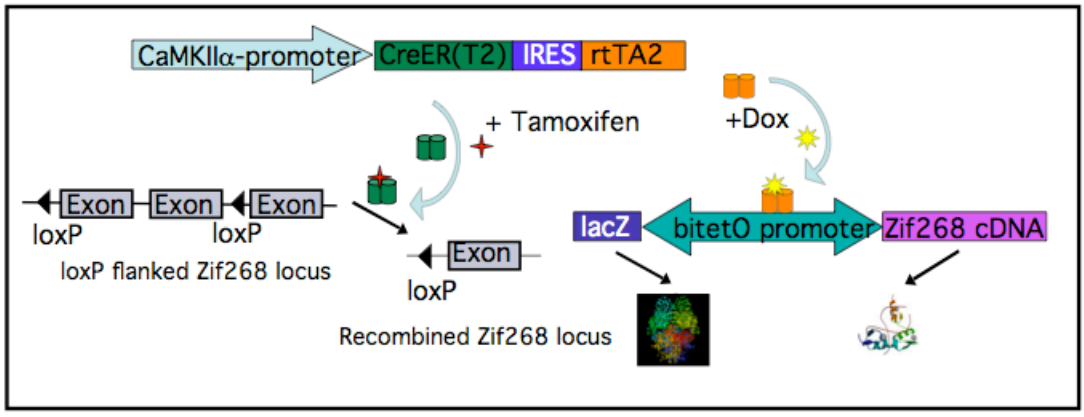

Figure 3. Scheme of gene recombination/expression/rescue made possible with combined transgenes exemplified on Zif268. CreER(T2) and rtTA2M2 are co-expressed in forebrain neurons owing to an IRES sequence and the CaMKIIα promoter. Recombination and inactivation of Zif268 gene by CreER(T2) is controlled by *4-hydroxy-tamoxifen*. Zif268 deficiency can be rescued by activation of an rtTA2-dependent Zif268 transgene with *doxycycline*. Furthermore, in the absence of *tamoxifen*, Zif268 can be over-expressed by *doxycycline* administration.

Methods

2.1. Media for Bacteria

LB (LUBIA BERTANI) MEDIUM	
Bacto tryptone (AppliChem, Gatersleben, Germany)	10g/L
Bacto yeast extract (AppliChem, Gatersleben. Germany)	5g/L
NaCl (Merck, Darmstadt, Germany)	170mM

Autoclave before use.

NZYM MEDIUM	
NZ amine (AppliChem, Gatersleben, Germany)	10g/L
NaCl (Merck, Darmstadt, Germany)	85mM
Bacto yeast extract (AppliChem, Gatersleben, Germany)	5g/L
$MgSO_4.7H_20$ (Sigma, Munich, Germany)	25mM

Adjust to pH7 with 5N NaOH (Merck, Darmstadt, Germany). Autoclave before use.

SOC MEDIUM	
Bacto tryptone (AppliChem, Gatersleben, Germany)	20.0g/L
NaCl (Merck, Darmstadt, Germany)	0.4g/L
Bacto yeast extract (AppliChem, Gatersleben, Germany)	5.0g/L
$MgSO_4.7H_20$ (Sigma, Munich, Germany)	10.0mM
$MgCl_2$ (Fluka, Buchs, Switzerland)	10.0mM
KCl (Sigma, Munich, Germany)	2.5mM

Adjust to pH7 with 5N NaOH (Merck, Darmstadt, Germany). Autoclave and add sterile glucose to a final concentration of 20mM before use.

2.2. Enzymatic Methods

2.2.1. Restriction Enzyme Digests

Plasmid digest for preparative and analytical digests were conducted according to the supplier's recommendation (NEB, Beverly, USA; Promega, Madison, USA; Invitrogen, Carlsbad, USA). For standard digests, 10x the theoretical amount of restriction enzymes was used to ensure complete digestion. Entirety of digest was verified by agarose gel electrophoresis.

2.2.2. Blunting using T4-Polymerase

The T4-Polymerase is used to fill up 5'overhangs and thus allow blunt end cloning. The reaction was conducted according to the protocol provided by the suppliers (NEB, Beverly, USA).

2.2.3. Dephosphorylation of 5'-Ends

This methods serves to prevent re-ligation of a linearized plasmid. The 5' phosphate is removed by the alkaline phosphatase, thereby ensuring that ligation may only occur if the phosphate group is donated by the insert. The dephosphorylation was conducted according to the supplier's recommendations (NEB, Beverly, USA).

2.2.4. Ligation

Ligations were performed over night at 16°C using the T4 ligase (NEB, Beverly, USA) according to the supplier's recommendations. If large DNA fragments were ligated, 15% PEG-6000 (Fluka, Buchs, Switzerland) was added to the reaction mixture. Two different insert to vector ratios were chosen for the ligation, usually between 1:1 to 10:1 in bp terms. As a re-ligation control, no insert was added to the ligation mixture.

2.2.5. Reverse Transcription

Reverse transcription was performed to generate cDNA to detect transgene expression. The reaction was conducted with the SuperScript^{TM} First Strand Synthesis system according to the suppliers recommendations (Invitrogen, Carlsbad, USA), using a 1:1 mixture of oligo$(dT)_{16\text{-}18}$ primers and random hexamers.

2.2.7. Purification of DNA and RNA

2.2.7.1. Gel Extraction

Gel extraction was conducted using the QIEX II gel extraction kit (Qiagen, Hilden, Germany) based on adsorption Chromatography according to the supplier's recommendations.

2.2.7.2. Purification in Ethanol using High Salt Concentrations

For additional purification of DNA, ethanol precipitation could be conducted in the presence of high salt concentrations. For this, 0.1 volumes of 3M sodium acetate (pH5.2, Sigma, Munich, Germany) were added to 1 volume of DNA solution and mixed. Subsequently, 10 volumes of ice-cold ethanol were added, mixed and kept at -20°C for 30min. The DNA was then pelleted by centrifugation at 13,000rpm on a cooled table-top centrifuge, the pellet was dried for 10min at RT and resuspended in 10mM Tris (Sigma, Munich, Germany), 0.1mM Na_2.EDTA (pH8.3; Sigma, Munich, Germany).

2.2.7.3. RNA Extraction from tissue

RNA was extracted from different brain regions using the adsorption Chromatography Macherey Nagel Nucleospin RNA II Kit, according to the supplier's recommendations (Macherey Nagel, Oensingen, Switzerland). In some cases, large pieces of tissue (>30mg) were used and the DNA digest was extended to 30min with additional DNase. Any remaining contaminating genomic DNA was detected using the CycD1 PCR (2.2.9.) and was removed using a further DNase step (Ambion, Austin, USA).

2.2.8. Biopsy Lysis

2.2.8.1. Proteinase K Digest

Proteinase K stock (10mg/ml in 40mM Tris-HCl; Sigma, Munich, Germany; -20°C) was diluted 1:100 in tail lysis buffer (4°C). Tail biopsies were lysed in 200µl of this preparation at 55°C overnight on a shaker and the enzyme was subsequently inactivated by 20min at 90°C.

TAIL LYSIS BUFFER	
KCl (Sigma, Munich, Germany)	50.0mM
gelatine (Sigma, Munich, Germany)	0.1g/L
Tris (Sigma, Munich, Germany)	10.0mM
$MgCl_2$ (Sigma, Munich, Germany)	2.0mM
Nonidet P40 (Fluka, Buchs, Switzerland)	7.5mM
Tween 20 (Sigma, Munich, Germany)	3.5mM

Store at 4°C.

2.2.8.2. Alkaline Lysis

Genomic DNA preparation from tail clipping (or ear clipping) was conducted according to the protocol by Truett *et al*. Briefly, biopsies were lysed in alkaline buffer at 95°C for 10min, subsequently cooled to 4°C and neutralized with acidic neutralization buffer (Truett et al., 2000).

ALKALINE LYSIS REAGENT	
NaOH (Merck, Darmstadt, Germany)	25.0mM
Na_2.EDTA (Fluka, Buchs, Switzerland)	0.2mM

Set precisely to pH12 with 5N NaOH.

NEUTRALIZING REAGENT	
Tris (Sigma, Munich, Germany)	40.0mM

Set precisely to pH5 with 5N HCl.

2.2.9. Polymerase chain reaction for Genotyping

The polymerase chain reaction was performed to determine whether mice carried a transgene. All PCRs were performed on the Mastercycler gradient PCR machine (Eppendorf, Eppendorf, Germany) with

i) a water control containing all the mix components but sample (genomic DNA) to exclude con*tam*ination of the components,

ii) a negative control containing genomic DNA from a mouse not carrying the transgene,

iii) a positive control containing genomic DNA from a mouse carrying the transgene.

PCR products were loaded on a 2% agarose gel/0.5x TBE gel (2.4.1). The gel was run at 200V for 15-20min and PCR products were visualized by UV light. All primers were ordered from Microsynth, Balgach, Switzerland.

Constituents (stock concentration)	rtTA2	Cre	R26RΔ	LacZ	CycD1
Forward primer (10 µM)	0.2	1.0	0.5	0.2	1.0
Backward primer (10 µM)	0.2	1.0	0.5	0.2	1.0
dNTPs (10 mM)	0.2	0.6	0.6	0.2	1.0
$MgCl_2$ (25 mM)	1.0	1.8	-	1.0	2.1
10 x Taq polymerase buffer	2.1	3.0	3.0	2.1	2.1
Gelatine (0.2%)/DMSO	-	0.2	-	-	1.0
Taq polymerase (5 units/µl)	0.1	0.2	0.2	0.1	0.1
Gel loading buffer	4.0	4.0	4.0	4.0	4.0
ddH_2O	12.2	17.2	20.4	12.2	7.7
Genomic DNA	1.0	1.0	0.8	1.0	1.0
Total	21	30	30	21	21

Table 1. A list of chemicals and the amount required of each for the detection of the four different transgenes by PCR (volumes in µl).

	rtTA2	Cre	R26RΔ	LacZ	CycD1
Forward primer	5' cgg aac aac gcc aag tca tt 3'	5' acc agg ttc gtt cac tca tgg 3'	5' aaa gtc gct ctg agt tgt tat 3'	5' atg tcc tcg ggg tac ttg gt 3'	5' acc agc tcc tgt gct gcg aa 3'
Backward primer	5' agc agg cag cat atc aag gtc 3'	5' agg cta agt gcc ttc tct aca c 3'	5' gtt ttc cca gtc acg acg ttg 3'	5' gcc tcc agt aca gcg cgg ctg 3'	5' acc gag tcc tag caa cgc ac 3'
Annealing temperature (°C)	62	54	58	62	53
Product size (bp)	467	216	569	409	300

Table 2. Details of the PCR protocol for the detection of four different transgenes.

2.2.10. Polymerase chain reaction for Detection of Transcripts

The polymerase chain reaction on complementary DNA (cDNA) was performed to determine whether mice express a transgene. For this, 1µl cDNA (2.2.5.) was introduced into any one of the PCR reactions below. After 30 cycles, 10µl of the reaction were loaded and run on an agarose gel, and amplificants visualized (2.4.1).

Constituents (stock concentration)	rtTA2 (intron)	rtTA2 (CDS)	Cre
Forward primer (10 µM)	0.2	0.2	1.0
Backward primer (10 µM)	0.2	0.2	1.0
dNTPs (10 mM)	0.2	0.2	0.6
$MgCl_2$ (25 mM)	1.0	1.0	1.8
10 x Taq polymerase buffer	2.1	2.1	3.0
Gelatine (0.2%)/DMSO	-	-	0.2
Taq polymerase (5 units/µl)	0.1	0.1	0.2
Gel loading buffer	4.0	4.0	4.0
ddH_2O	12.2	12.2	17.2
cDNA	1.0	1.0	1.0
Total	21	21	30

Table 3. A list of chemicals and the amount required of each for the detection of the four different transcripts by RT-PCR (volumes in µl).

	rtTA2 (intron)	rtTA2 (CDS)	Cre
Forward primer	5' cga ttc tag aat tcg ctg tct gc 3'	5' cgg aac aac gcc aag tca tt 3'	5' cc agg ttc gtt cac tca tgg 3'
Backward primer	5' gag agc aca gcg gaa tga c 3'	5' agc agg cag cat atc aag gtc 3'	5' agg cta agt gcc ttc tct aca 3'
Annealing temperature (°C)	62	62	54
Product size (bp)	594/364	467	216

Table 4. Details of the PCR protocol for the detection of four different transcripts.

2.3. Microbiological Methods

2.3.1. Transformation of Competent Cells

2.3.1.1. Chemically Competent Cells

DNA vectors or ligations were transformed either in Sure2 supercompetent® (Qiagen, Hilden, Germany) or Top10® (Biorad, Richmond, Germany) cells according to the supplier's recommendations.

2.3.1.2. Electrocompetent Cells

Transformation of the CaMKIIα promoter CreER(T2)IRESrtTA2 construct after ligation was not successful using chemical transformation. Therefore, electro-compentent cells were selected (EP Max® – Biorad, Richmond, USA) and 25µl of cells were transformed with 2µl of the ligation mixture at 1.5kV, 200Ohm and 25µF.

2.3.2. Preparing Plasmid for Analytical Purpose

For analytical purposes, DNA was extracted from small amounts of bacterial suspensions either by alkaline lysis (Birnboim and Doly, 1979), or using the Qiaprep® Spin Miniprep Kit (Qiagen, Hilden, Germany).

2.3.3. Preparing Plasmid for Preparative Purpose

For plasmid preparations of greater quantity and purity, the QiaPrep® Plasmid Purification Kit (Qiagen, Hilden, Germany) was used according to supplier's recommendations. For transfection and microinjection, the Endofree® Plasmid Purification Kit (Qiagen, Hilden, Germany) was used to eliminate endotoxin con*tam*inations. Final constructs were excised from the vector backbone, visualized on an agarose gel (2.4.1.), and extracted from the gel by over-night electro-elution using the Elutrap® device (Schleicher & Schuell, Dassel, Germany). The DNA in the resulting solution was precipitated by ethanol precipitation in the presence of salts (2.2.7.2.), washed twice with 70% Ethanol and dried at RT. The pellet was taken up in

30µl TE buffer (10mM Tris pH8.0, 1mM EDTA; Sigma Munich, Germany) dissolved at 37°C for 10min and overnight at 4°C before freezing.

2.4. Further Methods involving Nucleic Acids

2.4.1. DNA Electrophoresis using Agarose Gels

5x DNA LOADING BUFFER	
sucrose (Sigma, Munich, Germany)	1.3M
cresol red (Sigma, Munich, Germany)	1mM

Stored at 4° C.

0.5x TBE BUFFER	
Trizma base (Sigma, Munich, Germany)	45mM
boric acid (Fluka, Buchs, Switzerland)	45mM
Na_2.EDTA (Fluka, Buchs, Switzerland)	1mM

x% AGAROSE GEL	
Agarose (Peqlab, Erlangen, Germany)	x% (w/v)
0.5x TBE Buffer	Desired v

Heat in microwave until all agarose is dissolved. Cool down to 55°C while shaking.

Ethidium bromide (Sigma, Munich, Germany)	1.25µM

2.5. Protein Methods

2.5.1. Bradford Assay for Measuring Protein Concentration

For determining protein concentration a kit based on the Malachi green assay (Biorad, Richmond, USA) was used in conjunction with a protein standard curve of bovine serum albumin (BSA, Sigma, Munich, Germany).

BSA stock: 0.2mg/ml in 0.2M NaOH (Merck, Darmstadt, Germany)

PROTEIN AMOUNT	BSA STOCK	0.2M NaOH
10 µg	10 µl	40 µl
20 µg	20 µl	30 µl
30 µg	30 µl	20 µl
40 µg	40 µl	10 µl
50 µg	50 µl	0 µl

Sample preparation

1. Dilute samples with 0.2M NaOH to a final volume of 50µl.
2. Add 750µl of ddH_2O.
3. Add 200µl of dye reagent.

4. Mix.
5. Measure absorbance at 595nm (Genequant pro, GE Healthcare, Otelfingen, Switzerland) within the next hour.

2.5.2. SDS PAGE and Immunoblotting

ELECTROPHORESIS BUFFER	
Tris (Sigma, Munich, Germany)	25.0mM
Glycine (Sigma, Munich, Germany)	250.0mM
SDS (Biorad, Richmond, USA)	3.5mM

Adjust to pH8.3 with 5N HCl.

LOADING BUFFER	
Tris (Sigma, Munich, Germany)	50.0mM
SDS (Biorad, Richmond, USA)	70.0mM
Bromophenol blue (Sigma, Munich, Germany)	1.5mM
Glycerol (Sigma, Munich, Germany)	1.1M

Adjust to pH6.8 with 5N NaOH. Store as aliquots at –20°C.
Add to 1.4M β-mercapto-ethanol (Sigma, Munich, Germany) before use.

TRANSFER BUFFER	
Tris (Sigma, Munich, Germany)	50mM
Glycine (Sigma, Munich, Germany)	40mM
SDS (Biorad, Richmond, USA)	1.25mM

Adjust to pH8.3 with HCl. Dilute to 1x with 700ml ddH_2O + **200ml Methanol**.

TBS-T	
Tris (Sigma, Munich, Germany)	10mM
NaCl (Merck, Darmstadt, Germany)	150mM
Tween-20 (Sigma, Munich, Germany)	400µM

Adjust to pH8.3 with 5N NaOH. For the blocking buffer, 2% (w/v) milk powder was dissolved in 1X TBS-T.

2.5.5.1. Gel Preparation

1. Glass plates were wiped with EtOH and dried with Kim wipes (Kimberley-Clark, Reigate, UK).
2. Plates and spacers were assembled.
3. The plates were sealed with an agarose gel.
4. The separating gel was poured and covered with isopropanol (Fluka, Buchs, Switzerland) or isobutanol (Fluka, Buchs, Switzerland). Allowed >30min for polymerization.
5. The isopropanol was rinsed away with ddH2O, the glass plates dried with Kim wipes (Kimberley-Clark, Reigate, UK).
6. The stacking gel was poured, the comb placed immediately and polymerization was allowed for >30min.

7. The gels could be stored wet with combs at 4°C.

Volume for 1 large gel 155 x 160 x 2mm: 37.5ml separating gel; 12ml stacking gel

SEPARATING GEL	
Acrylamide (Biorad, Richmond, USA)	400mM
N'-N' methylane bis-acrylamide (Biorad, Richmond, USA)	20mM
Tris (Sigma, Munich, Germany)	375mM
SDS (Biorad, Richmond, USA)	3.5mM
Ammonium Persulfate (Sigma, Munich, Germany)	4.5mM
TEMED (Sigma, Munich, Germany)	2.0mM

Adjust to pH8.8 with 5N NaOH. Use 7ml per gel.

STACKING GEL	
Acrylamide (Biorad, Richmond, USA)	200mM
N'-N' methylane bis-acrylamide (Biorad, Richmond, USA)	10mM
Tris (Sigma, Munich, Germany)	125mM
SDS (Biorad, Richmond, USA)	3.5mM
Ammonium Persulfate (Sigma, Munich, Germany)	4.5mM
TEMED (Sigma, Munich, Germany)	6.5mM

Adjust to pH6.8 with 5N NaOH. Use 2ml per gel.

2.5.2.3 Gel Electrophoresis

1. Samples were prepared on ice in 0.5ml tubes; 4x loading dye with 10%β-mercapto-ethanol (Sigma, Munich, Germany) was added to a final concentration of 1x.
2. Proteins in sample were denatured at 95°C for 3min.
3. Samples were stored on ice and spun down briefly before loading.
4. Gels were run at 70V for 1hr (time necessary to go through stacking gel), then accelerated to 200V for 5-6hr.

2.5.2.4. Transfer

1. Gel and nitrocellulose membrane were bathed in transfer buffer for 15min.
2. The transfer sandwich was prepared in a large shallow tray filled with cold transfer buffer accordingly: Cathode to the anode:
   sponges / filter papers (Whatmann, Brentford, UK)/ gel / membrane (Immun-blot PVDF membrane, Bio-Rad, Richmond, USA) / filter paper / sponges
3. The transfer was run at 150mA over night (approx. 15hr).

2.5.2.5. Antibody Incubations

1. To avoid unspecific binding of antibodies (Ab) to the membranes, membranes were blocked in 2% milk powder/TBS-T (pH8.0) for at least 1hr at RT or at 4°C with gentle shaking.
2. Membranes were incubated with primary Ab at specific concentrations in 2% blocking buffer for 1hr at RT.
3. Membranes were rinsed 4x 15min in a large volume of TBS-T at RT.
4. Membranes were incubated with secondary Ab linked to an infrared dye diluted 1/5,000 in 2%milk powder/TBS-T for 6hr at 4°C.

5. Membranes were rinsed 4x 15min in a large volume of TBS-T at RT.
6. For detection, membranes were exposed on the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, USA).

2.6 Mice

A service laboratory at the University of Zürich conducted the pronuclear injection of the DNA construct into fertilized oocytes to create pseudo-founders. Both male and female mice used as gamete donors for transgenesis were of a mixed C57Bl/6J and DBA2 F1 background. This background was used for hybrid vigor, which is the phenomenon that hybrid strains are better for the generation of transgnic mouse lines for three reasons:

a) higher reproductive performance,

b) easier to superovulate,

c) higher quality embryos with higher survival rates after microinjection.

2.6.1 Housing

Mice were kept in a room with controlled temperature and humidity level, under inverted 12-hour light/dark cycle, lights on at 7pm. All mice were allowed access to food and water *ad libitum*, unless they were in experiment. All tests and training were conducted at the same time of day, between 10am and 3pm. Facility, experimenters and behavioral experiments were in accordance with guidelines and regulations of the cantonal veterinary office, Zürich.

2.6.2. Euthanasia

For tissue collection, mice were euthanized by neck dislocation and decapitation.

2.6.3. Cryosectioning the Brain

Brains for cryosectioning were fresh frozen and processed for β-Gal staining. Sections were cut at 14µm at -16°C chamber temperature, -14°C knife temperature on a Micron cryostat (Micron Instruments, San Marcos, USA). Sections were taken up on microscope slides (Menzel, Braunschweig, Germany) and stored at -20°C.

2.6.4. Staining for β-Galactosidase Activity

The β-galactosidase activity can be detected by a reaction with the substrate X-Gal (5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid) that is converted to galactose and a blue, hydrophobic Indigo-stain. The procedure is carried out in three steps.

1. Brain dissection and freezing. As described above in 2.

2. Cryostat sections. As described above in.
3. β-galactosidase staining.

100mM $NaPO_4$ BUFFER	
monobasic $NaPO_4$ (NaH_2PO_4) (Sigma, Munich, Germany)	30mM
dibasic $NaPO_4$ (Na_2HPO_4) (Sigma, Munich, Germany)	70mM

Adjust to pH7.2 with pH paper.

LacZ FIXATION BUFFER	
Glutaraldehyde (Sigma, Munich, Germany)	0.14mM
EGTA (Sigma, Munich, Germany)	5.00mM
$MgCl_2$ (Fluka, Buchs, Switzerland)	2.00mM
$NaPO_4$	100.00mM

Adjust to pH7.2 with pH paper.

LacZ WASH BUFFER	
$MgCl_2$ (Fluka, Buchs, Switzerland)	2.0mM
Na deoxycholate (Sigma, Munich, Germany)	0.2mM
NP40 (Fluka, Buchs, Switzerland)	0.3mM
$NaPO_4$	100mM

Adjust to pH7.2 with pH paper.

LacZ STAIN	
Xgal (dissolve first in 2ml DMSO) (AppliChem, Gatersleben, Germany	0.5mM
K-ferrOcyanide $K_4Fe(CN)_6$.$3H_2O$ (Sigma, Munich, Germany)	5.0mM
K-ferrIcyanide $K_3Fe(CN)_6$ (Sigma, Munich, Germany)	5.0mM

Prepare solution in 1x wash buffer. Filter-sterilize LacZ stain before use to prevent crystal formation. Possible to keep frozen, protected from light, and filter again before use.

Sections on slides were fixed for 10min in ice-cold LacZ fixation buffer. Subsequently, they were washed 3 times for 5min in LacZ wash buffer and stained overnight at 37°C, protected from light. When the staining was completed, three 5min rinses in PBS followed. The sections were counterstained with acidified hematoxylin (add 4% fuming acetic acid in stain, washing baths and dehydration baths), coverslipped (Menzel, Braunschweig, Germany) in DPX mounting medium (TAAB laboratory equipment, Calleva Park, UK) and stored at RT.

2.6.5. *Tamoxifen* Injections

Tamoxifen (*tam*) is a non-steroidal estrogen antagonist used in the treatment of advanced breast cancer in women, because it blocks the effects of estrogen on breast

tissue, but mimics the action of estrogen on the bones and uterus. *Tam* was purchased from Sigma (Munich, Germany) and kept at 4°C at all times. 10mg of *tam* were given into 100µl of pure Ethanol, 900µl of sunflower oil was added and the solution mixed over night on a shaker at RT. Each day each mouse received either one or two injections of 100µl/1mg *tam*, for 5 or 10 consecutive days.

Recombination was allowed to take place and β-galactosidase levels to accumulate for either 1 or 4 weeks before mice were sacrificed, brains isolated and processed further.

Results

Generation of Transgenic Mice

In the first phase of this project, the DNA constructs for the transgenic mouse lines were generated. To attain a transgenic mouse line expressing the *tamoxifen*-inducible Cre recombinase, CreER(T2) and the novel reverse tetracycline-controlled transactivator, rtTA2M2 in the same forebrain neurons simultaneously, two parallel approaches were employed: **(a)** In one approach, a mixture of two different DNA constructs, CaMKIIα promoter-CreER(T2) and CaMKIIα promoter-rtTA2, was microinjected (*Fig. 4* and *5*). **(b)** In the second approach, a polycistron was created under control of a single CaMKIIα promoter element using the human FGF2 internal ribosomal entry site (IRES) sequence. This strategy resulted in the CaMKIIα promoter-CreER(T2)-IRES-rtTA2M2 construct (*Fig. 4* and *6*).

All cDNAs were flanked with synthetic regulatory sequences improving in vivo transcription and translation levels, as well as mRNA stability. These sequences boast a 230-bp hybrid intron that contains an adenovirus splice donor and an immunoglobulin G splice acceptor, as well as a SV40 polyadenylation signal (Choi et al., 1991). All DNA constructs were microinjected into fertilized oocytes by Thomas Rülicke, University Hospital Zürich. Both male and female mice used as gamete donors were of a mixed C57Bl/6J and DBA2 F1 background for hybrid vigor. Transgene integration into the genome of each founder was tested by transgene specific PCR. Transgene transmission was tested in offspring from breedings of positive founders with C57Bl/6J mice.

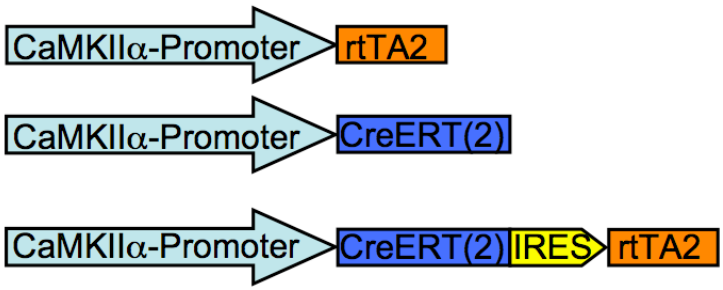

Figure 4. The DNA constructs created to generate transgenic mice in line with this project.

1(a) Generation of CaMKIIα-CreER(T2)/CaMKIIα-rtTA2M2 mice

The constructs were generously provided by Dr. Ursula Haditsch and Dr. Aubin Michalon. Several copies of a transgene will integrate into the genome at a single integration locus. Statistically, this will result in co-integration of the two transgenes (Brinster et al., 1985). Since locus and promoter elements are the same, simultaneous expression of CreER(T2) and rtTA2M2 should occur. Integration of the CaMKIIα-CreER(T2)/CaMKIIα-rtTA2M2 constructs occurred in 8 founders (out of 54 pseudofounders). However, only 7 of these transmitted the transgene to the F1 generation, giving rise to 7 independent lines, named DOP1KB, DOP2KB, DOP3KB, DOP4KB, DOP5KB, DOP6KB and DOP8KB.

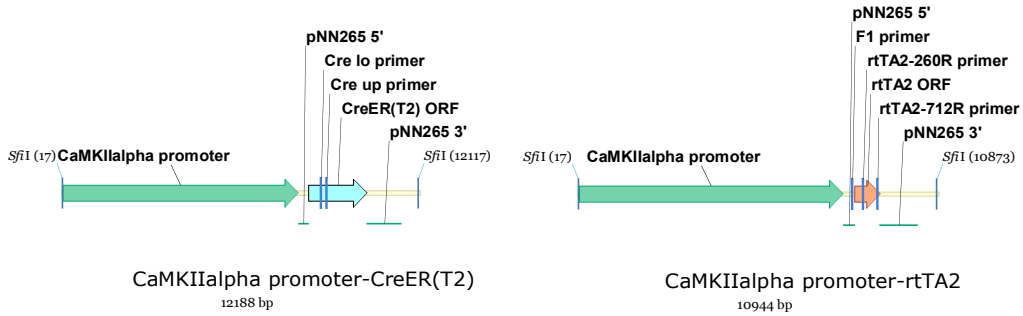

Figure 5. **CaMKIIα promoter- CreER(T2)/CaMKIIα promoter- rtTA2M2**: Sfi1 digests yielded a 12100bp (left) and a 10856bp (right) fragment for microinjection.

1(b) Generation of CaMKIIα promoter-CreER(T2)-IRES-rtTA2M2 mice

The hFGF2 IRES is reported to increase specificity of neuronal expression(Creancier et al., 2000) and its cDNA was a generous gift from Prof. A.C. Prats. CreER(T2) was generously provided by Dr. Philipp Berger and Dr. Hermann Bujard. Integration of the CaMKIIα promoter-CreER(T2)-IRES-rtTA2M2 constructs occurred in 12 founders (out of 55 pseudofounders). However, only 7 of these transmitted the transgene to the F1 generation, giving rise to 7 independent lines, named IRSrtTA21294, IRSrtTA21295, IRSrtTA21299, IRSrtTA21300, IRSrtTA21301, IRSrtTA21302 and IRSrtTA21303.

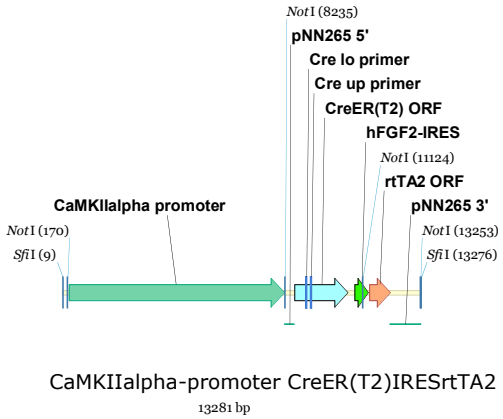

Figure 6. **CaMKIIα promoter-CreER(T2)-IRES-rtTA2M2**: The CreER(T2) cDNA was excised from pBSII SK+ using SpeI/KpnI (blunt) yielding a 2253bp fragment carrying the cDNA. This was inserted into the blunted BamHI site of pNN265 rtTA2M2 (provided by Aubin Michalon). The FGF2 IRES (514bp) was excised from pCRFL by NarI/SpeI, blunted and inserted into the blunt SalI site of pNN265 CreER(T2) rtTA2M2. The resultant construct was excised by NotI to create a 5022bp fragment, which was inserted into the NotI site of pMM403. SfiI was used produce the resultant fragment (13049bp) for microinjection.

Constructs	F0/pseudo-founders	Lines trans.	Reporter lines	Expression
CaMKIIα-promoter rtTA2 + Coinjection! CaMKIIα-promoter CreER(T2)	8/54	7	tetO-LacZ, bitetO-NIPP1/LacZ ROSA26R	Anterior DG for rtTA2 in one line out of 5
CaMKIIα-promoter CreER(T2) IRES rtTA2	12/55	7	bitetO-Zif/LacZ6	None of the lines positive for rtTA2 dependent transactivation, three express CreER(T2).

Table 5. A summary of the statistics on the generation of the transgenic mouse lines.

Screening for Functionality of the Constructs

In vivo Screening

Out of a total of 20 founder lines, 14 transmitted the transgene to the F1 generation and were screened for transgene expression and functionality. This was done by crossing the F1 generation with several different established rtTA2-responsive reporter lines for the rtTA2 components. Results are summarized in *Table 3*. Briefly, a *tetO*-LacZ line (Mayford et al., 1996a) was initially used in the DOPxKB lines, however this line turned out to be a weak reporter in our hands. Therefore, we switched to the newly generated *bitetO*-Zif268/LacZ6 (chapter 3) and *bitetO*-NIPP1/LacZ lines (Kyoko Koshibu). Of 7 DOPxKB lines only one displayed expression (DOP2KB), however this was restricted to single cells in the anterior dentate gyrus (data not shown). Several of these lines (DOP2KB, DOP3KB, DOP4KB, DOP5KB) were also crossed with the R26R Cre recombinase reporter line (Soriano et al., 1999). In this reporter, a floxed stop codon and a LacZ gene have been inserted 3' of the ubiquitous ROSA promoter (*Fig. 7*). The stop codon is excised and β-galactosidase expressed upon Cre mediated recombination. This was detected using

β-galactosidase stainings on brain slices and a PCR protocol specific for the recombined ROSA26R locus on genomic DNA preparations (kindly provided by Daniel Metzger). No recombination-induced lacZ expression was observed in any of the lines (data not shown). On a genomic DNA level, recombination was detected in control mice (Leone et al., 2003) but in none of the DOPxKB x R26R transgenic mice (*Fig. 9*).

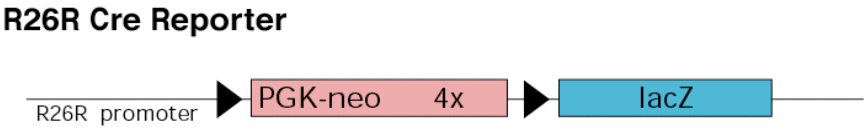

Figure 7. Transgene of the Cre reporter line R26R: The ubiquitous R26R promoter cannot induce lacZ expression unless a stop cassette containing the PGK-neo selection cassette is removed first by Cre-loxP mediated recombination. (black triangles – loxP sites).

To test whether this lack of signal may by explained by insufficient activation of CreER(T2) after *tamoxifen* injection or by lack of CreER(T2) expression, we performed western blot analysis on several DOP lines (DOP2KB, DOP5KB, DOP6KB, DOP8KB), where no CreER(T2) protein could be detected (example in *Fig. 9*). All DOP lines were therefore discarded.

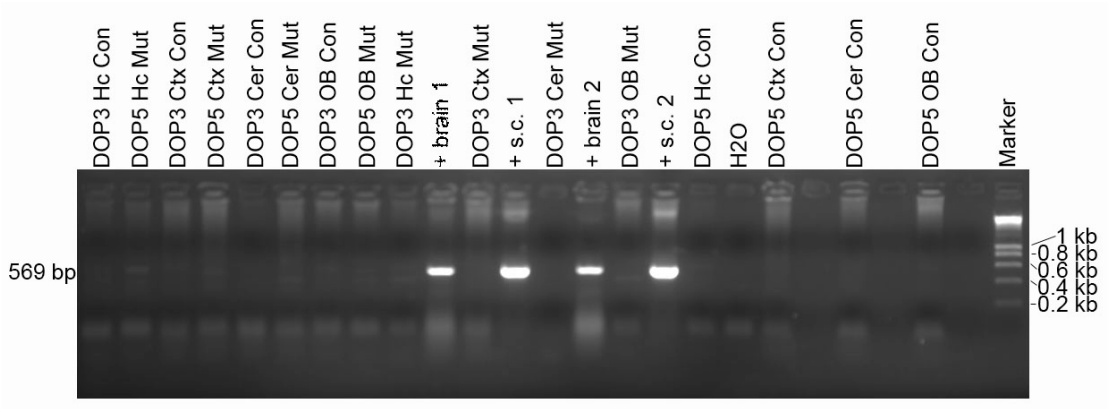

Figure 8. PCR evaluation of recombination at the ROSA26 locus. The PCR product for the delta (recombined) locus (569bp) at the ROSA26 locus is only found in the positive controls from brain and spinal cord (s.c.), but not in genomic DNA isolated from hippocampus (Hc), neocortex (Ctx), cerebellum (Cer) or olfactory bulb (OB) of DOP3 x R26R and DOP5 x R26R mutant (Mut) or control (Con) mice treated with *tamoxifen*.

Of the 12 CaMKIIα promoter CreER(T2)-IRES-rtTA2M2 only 7 gave offspring and transmitted the transgene to the F1 generation. These were crossed with *bitetO*-Zif268/LacZ6 mice to test rtTA2 dependent transgene expression. No β-galactosidase expression was detected in any of the 7 lines. Since this is easiest explained by a lack of rtTA2 expression, rtTA2 mRNA was detected using RT-PCR. In a first step, the

reliability of the positive control and the cDNA preparation was examined. For this, specific primers for the CaMKIIα promoter rtTA2M2 line were used, that allow to distinguish amplificants from genomic and cDNA, since the forward primer is located just 5' of an intron in the 5' regulatory sequences of the transgene. Amplificant from cDNA were observed in all forebrain structures, but very little in the cerebellum (*Fig. 9a*). No band corresponding to amplificants from genomic DNA, and no amplificant in CaMKIIα promoter CreER(T2)-IRES-rtTA2M2 or wildtype mice was detected (*Fig. 9a*). In a second step, rtTA2 expression was tested in CaMKIIα promoter CreER(T2)-IRES-rtTA2M2 mice by using a PCR protocol specific for the rtTA2M2 coding sequence. The desired band was only seen in the positive controls, different forebrain regions of the CaMKIIα promoter rtTA2M2 mice, indicating that rtTA2 is not expressed at significant amounts from CaMKIIα promoter CreER(T2)-IRES-rtTA2M2 transgene (*Fig. 9b*). In a third PCR reaction, the expression of CreER(T2) was examined. While there was no amplificant for any brain regions of wildtype or CaMKIIα promoter rtTA2M2 mice, lines IRSrtTA21294 and 1301 express CreER(T2) in all forebrain regions that were tested. Interestingly, there also appears to be very low CreER(T2) expression in the cerebellum of line IRSrtTA21294, which is unexpected from the activity of the CaMKIIα promoter (Erondu and Kennedy, 1985) and reflects the sensitivity of the RT-PCR method.

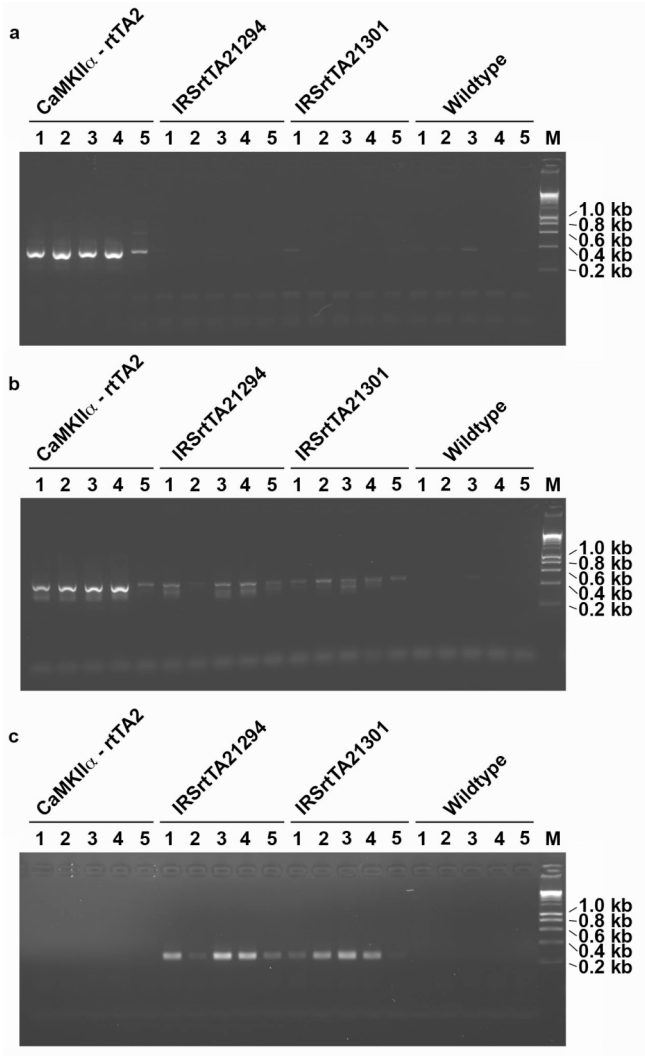

Figure 9 (previous page). CreER(T2) but not rtTA2 transcript is detected in lines IRSrtTA21294 and IRSrtTA21301. Specific PCR amplification for (**a**) rtTA2 (only cDNA from the CaMKIIα promoter rtTA2M2 line), (**b**) rtTA2 CDS, (**c**) CreER(T2) performed on cDNA of amygdala (**1**), basal ganglia (**2**), hippocampus (**3**), neocortex (**4**) and cerebellum (**5**) of CaMKIIα promoter rtTA2M2, IRSrtTA21294, IRSrtTA21301 and wildtype mice. (**a**) A 394bp fragment corresponding to spliced rtTA2 transcript is only amplified in brain regions of a CaMKIIα promoter rtTA2M2 mouse, since the forward primer is specific to the this transgene. No 594bp band corresponding to unspliced RNA or genomic DNA was detected. (**b**) In the same brain regions, rtTA2 is also detected using a reaction common to all rtTA2 (467bp). No fragment is amplified in the two IRSrtTA2 lines and the wildtype samples. (**c**) A 216bp fragment corresponding to CreER(T2) is detected in samples from IRSrtTA21294 and IRSrtTA21301, but not from CaMKIIα promoter rtTA2M2 and wildtype mice.

To verify these results, we performed western blot analysis to detect CreER(T2) protein. Lines 1294 and 1301 showed significant amounts of CreER(T2) expression in the hippocampus and the cortex, but none in the cerebellum (*Fig. 10*). Encouragingly, the level of CreER(T2) protein (hippocampus, 1294: 0.47, 1301: 0.58; cortex, 1294: 0.75, 1301: 0.67), as normalized to β-actin, was higher than what is observed in GFAP promoter CreER(T2) mice in cerebellum (0.45) (Hirrlinger et al., 2006), used as a positive control in these experiments (*Fig. 10*). However, this quantification was not pursued systematically.

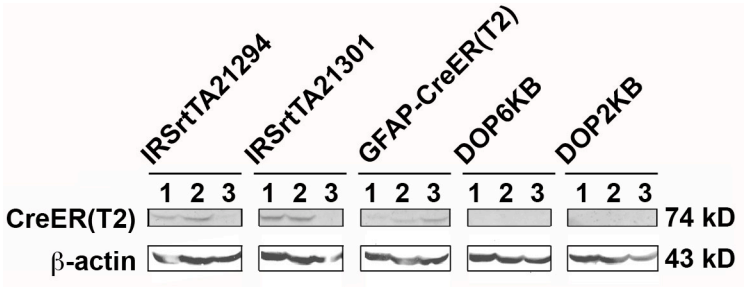

Figure 10. Western blot analysis of the expression of CreER(T2) in hippocampal, cortical and cerebellar homogenates from different DOP and CaMKIIα promoter CreER(T2)-IRES-rtTA2M2 transgenic mice. Lines 1294 and 1301 showed strong CreER(T2) expression in hippocampus (1, 4) and cortex (2, 3), but as expected none in cerebellum (3, 6), while the DOP lines, DOP2KB and DOP6KB, displayed no detectable CreER(T2) (6-9, 13-15). The GFAP promoter CreER(T2) positive control, showed the expected CreER(T2) in cerebellum (12) and less in hippocampus (10) and cortex (11).

Further experiments to verify the functionality of the IRSrtTA21294 and IRSrtTA21301 by examining the inducibility of the recombinase activity were initiated but not completed in the scope of this PhD thesis.

4. Discussion

The aim of this project was to generate a transgenic mouse that allows the inducible, bi-directional regulation of the level of a given target gene in neurons. This mouse line would co-express the *tamoxifen*-inducible Cre recombinase, CreER(T2), and the *doxycycline*-inducible transcriptional activator rtTA2. Two parallel approaches were designed to establish this line. The first approach was based on two separate constructs, each containing the CaMKIIα promoter to regulate expression of either CreER(T2) or rtTA2 (lines termed DOPxKB). These were co-injected in fertilized oocytes, resulting in co-integration of the constructs at the same locus. Since both the regulatory elements and the chromosomal environment coincide under these conditions, rtTA2 and CreER(T2) should be co-expressed (Jankowsky et al., 2001). The second approach based on the use of a polycistron, where CreER(T2) and rtTA2 are located on the same construct under control of the CaMKIIα promoter (lines termed IRSrtTA2xxxx).

Eight DOPxKB founders were generated, seven of which were crossed with rtTA2 responsive reporter lines. Line DOP7KB did not produce any offspring, which may have different reasons (detailed in section 4.1). Only lines DOP2KB and DOP6KB displayed rtTA2 dependent reporter expression, which was however very restricted (possible explanations in section 4.3).

Four of the DOPxKB lines were also crossed to the R26R line to examine CreER(T2) dependent recombination. Here, none of them displayed recombination induced reporter expression (refer to section 4.3.1.). Finally, a protocol was developed to detect CreER(T2) protein using immunoblotting. Applying this newly established protocol, none of the DOPxKB line showed CreER(T2) protein expression. Therefore, all DOPxKB lines were discarded. The lack of transgene expression may be explained in different ways (refer to section 4.3).

Twelve IRSrtTA2xxxx founders were generated, but transgenic offspring was obtained only for seven of these. The remaining five did either not reproduce at all (for possible explanations, refer to section 4.1.) or did not transmit their transgene (for possible explanations, refer to section 4.2.). Of these seven, none displayed rtTA2 expression or rtTA2 dependent reporter expression, while two expressed CreER(T2) at good amounts (for possible explanations, refer to section 4.3).

In the IRSrtTA2xxxx lines, CreER(T2) and rtTA2 are expressed under the same promoter via a polycistron, so they should be expressed simultaneously. However, rtTA2 mRNA is absent while CreER(T2) protein and mRNA are present in the forebrain of mice from lines IRSrtTA21294 and IRSrtTA21301 (*Fig. 9* and *10*). A possible explanation for this phenomenon could be the integration of an incomplete transgene. However, when the founders of these lines were checked for integration of the transgene, both CreER(T2) and rtTA2 coding sequences were detected using genotyping PCRs. Since only CreER(T2) is present on the mRNA level (*Fig. 9*), and CreER(T2) is just 3' of the CaMKIIα promoter in the construct (*Fig. 6*), the only conclusion states that the transgene is not completely transcribed. This is not a single incidence, since it has also been reported for other IRES sequences (Jankowsky et al., 2001). Incomplete transcription infers the lack of any 3' regulatory sequences on the resulting mRNA, which does not prevent efficient translation of CreER(T2) in this case (*Fig. 10*).

4.1. Breeding Success

As part of this project, 20 transgenic mouse lines carrying different constructs were generated in Zürich with the help of Thomas Rülicke at the central biological laboratory (BZL). In a first step, all founder mice were bred with C57Bl6J wildtype mice. Several breedings with founders did not give offspring, which may be due to infertility in the founder mouse, incompatible or infertile breeding partners, a stressful environment, but also because some of the mice were already middle-age at the onset of the breeding. This was inevitable because available space in the animal facilities was limited and thus breedings cages were initiated sequentially for these mouse lines. For the last lines, breeding cages were only initiated by 10 months of age of the founder. Although mice should still be fertile at this age, breeding success may be limited. Apart from age, an unfavorable genetic background may contribute to infertility. However, the fertilized eggs that were microinjected were derived from a mixed C57Bl6J and DBA2 breeding, which should display good reproductive performance.

The contribution of each background may nevertheless differ between founder mice and may therefore affect their mating success by determining litter size and number of litters. Since our constructs carry inducible transgenes there should be no concern about embryonic lethality of expression of the transgene. However, since these

transgenes are randomly integrated, there is a remote probability that a transgene is integrated into a crucial gene for development, interrupts expression of this gene and thus causes embryonic lethality.

4.2. Transgene Transmission

The C57Bl6J breeding served to assess transgene transmission to the F1 generation. Of the 16 founder mice that gave offspring, 14 transmitted the transgene to the next generation. Lack of transmission is a frequent and easily explained phenomenon. Here, the timing of transgene integration into the genome after microinjection is critical. Random integration of DNA into the genome usually occurs at double strand breaks (Brinster et al., 1985) in the S phase of the cell cycle (Sonoda et al., 2001). If this does not take place during the first but later cell divisions, only a subset of cells in the organism carry the transgene (Palmiter et al., 1983; Wilkie et al., 1986), a phenomenon called genetic mosaicism. In some cases this subset of cells does not contribute to the germ line, thus preventing transgene transmission. A second, however less likely, cause may be a secondary loss or rearrangement of the transgene in progenitors of the germ line (Brinster and Palmiter, 1984; Palmiter et al., 1982; Palmiter et al., 1983; Shani, 1986). This can occur by homologous recombination during early cell divisions in the blastocyst or in the germ line (Wilkie et al., 1991) and is more likely with multiple copies of the transgene (Scrable and Stambrook, 1999).

4.3. Functionality of Transgene

Mouse lines that carry and transmit their transgene(s) to the F1 generation were subsequently tested for the functionality of the transgene. A transgene may not be functional for several reasons:

4.3.1. Insufficient Expression of the Transgene

4.3.1.1. Poor design

Poor design of a construct may significantly affect efficient transgene expression. For instance, it was shown that prokaryotic (Chada et al., 1985a; Chada et al., 1985b; Hammer et al., 1985; Krumlauf et al., 1985; Townes et al., 1985) or viral (Jahner et al., 1982) sequences may inhibit expression of a transgene. It was demonstrated that efficient expression of a cDNA requires splicing (Brinster et al., 1988) and a polyadenylation signal at the 3' end. To ensure accurate and efficient translation the

start codon ATG should be preceeded by a Kozak sequence (Kozak, 1987). For tissue-specific expression it is better to include all naturally occurring intron elements that are involved in mRNA splicing and expression (Choi et al., 1991; Palmiter et al., 1991) since regulatory elements may also reside in intron sequences, usually in the first intron in fact (Beermann et al., 1990; Ganss et al., 1994; Tanaka et al., 1990). Finally, codon usage needs to be optimized and adjusted to the target organism, since it may affect expression strength. This and all other points were taken into account by using pre-existing and established components to generate the constructs (regulatory sequences suggested by Choi *et al.*, 1991).

4.3.1.2. Position Effects and Epigenetic Mechanisms

The transgene is influenced by the genomic sequences flanking the integration site, so called position effects (Overbeek et al., 1986; Palmiter et al., 1982; Reitman et al., 1995). In extreme cases the integration may occur in heterochromatin, where the DNA is found in hyperspiraled form bound to histones and other proteins in double strand. In heterochromatin, genes cannot be expressed unless it is turned into the loose conformation (euchromatin) where the DNA can be cleaved into single strands. On the other hand, flanking sequences may contain regulatory elements of neighbouring genes that act in the transgene promoter as an enhancer and result in ectopic expression of the transgene. Lastly, it may be secondary methylation or acetylation that silences transgene expression (McBurney et al., 2002; Mutskov and Felsenfeld, 2004; Palmiter et al., 1982). This is achieved by methylation and acetylation of histones and CpG islands in the promoter regions of the transgene and is known to be more likely with higher copy numbers (McBurney et al., 2002; Pena et al., 2004). High copy numbers have also been shown to favor formation of heterochromatin (Garrick et al., 1998). Furthermore, it was reported that lacZ sequences can have transcription-silencing effects when they are associated with regulatory sequences of ubiquitously expressed genes (Cohen-Tannoudji et al., 2000). This may actually explain the weak reporter expression observed in the *tetO*-LacZ line that was used to detect rtTA2 dependent expression in this project. It was originally described as a very strong reporter line (Furth et al., 1994), but in our hands it only exhibited weak β-galactosidase expression.

4.3.2. Incomplete Integration or Expression of the Transgene

4.3.2.1. Incomplete Integration of the Transgene

The construct is cleaved during the preparation for microinjection and is subsequently only partially integrated. Great care was taken during the preparation of the constructs and the final preparation of each linearized construct was checked by restriction enzyme and agarose gel analysis before microinjection. However, the founders were not checked for complete integration of the construct, which can be done using southern blot. This was mainly due to time constraints in the face of large numbers of generated transgenic mouse lines. Instead, genotyping PCRs were used to detect parts of the CreER(T2) and rtTA2 coding sequences in the genomic DNA of the founder mice.

4.3.2.2. Incomplete Expression of the Transgene

A sequence error in the transgene can result in a stop codon or render the mRNA unstable, thereby preventing efficient expression of the transgene. While the ligation site in all constructs generated in this project were checked by sequencing before microinjection, the constructs were not sequenced completely. Thus, errors in transgenes cannot be entirely excluded but are highly unlikely, since they would have been caused by random mutations that occurred during the generation of the constructs.

4.4. Inducible Recombination using CreER(T2)

At the beginning of this project, a reliable protocol to detect CreER(T2) expression was not available, so that this analysis had to be omitted. Instead, the induced activity of CreER(T2) was tested using a Cre reporter mouse line, R26R. In this line, recombination yields expression of the lacZ reporter gene. The quality of *tamoxifen* (*tam*) as well as the selected injection protocol may be critical. Two daily injections of 1mg on five consecutive days, or one daily injection of 1mg on ten consecutive days were given in our experiments. These two protocols were successfully employed to induce recombination in oligodendrocytes of the CNS (Leone et al., 2003). In fact, it appears that the extent of induction achieved by the two protocols was very similar (Leone et al., 2003). Moreover, *tam* treatment with 2mg/day rather than 1mg/day over five days yields little improvement in recombination efficiency (personal communication). Increasing *tam* dose is not a valid option, since there may be concerns about the effect of *tam* on memory. Reports from breast cancer patients have

shown that *tam* treatment can cause memory deficits (Bender et al., 2006), common clinical dose: 40mg/day periorally). In laboratory research, *tam* treatment (10mg/kg i.p.) has also been shown to affect memory and its retrieval in mice (Chen et al., 2002). For experimental use, low doses of *tam* should be applied, and sufficient temporal delay should be introduced between *tam* treatment and onset of testing, to ensure that there is no interference.

Another critical factor in the induction of CreER(T2) dependent recombination is the quality of *tam* (Sigma, Munich, Germany) that differs greatly between batches (personal communication). A proper batch has to be identified to exclude this source of error and continue the work on the IRSrtTA21294 and IRSrtTA21301 lines. These lines remain to be tested for the induction of Cre activity with *tam*. For this, tests are currently in progress to identify a functional batch of *tam*.

Additionally, a second Cre reporter line was attained (graciously provided by Dr. Metzger) that permits a more sensitive detection of recombination. In these "Z/AP" mice the lacZ reporter gene is expressed throughout all embryonic and adult stages, unless Cre-mediated excision occurs. Cre excision removes the lacZ gene, allowing expression of the second reporter, the human alkaline phosphatase gene (Lobe et al., 1999). "Z/AP" mice have been reported to carry a locus that permits a more sensitive detection of recombination than the R26R line used in the experiments presented here (Dubois et al., 2006). The "Z/AP" line is currently rederived at the central laboratory Zürich and will then be available to examine Cre mediated recombination in the lines IRSrtTA21294 and IRSrtTA21301.

4.5. Outlook

The aim of this project was to generate a mouse line that allows the concomitant expression of CreER(T2) and rtTA2 in forebrain neurons. Despite using two approaches we were unable to attain this line. However, we have been successful in generating a mouse line that expresses CreER(T2) and thereby should allow the inducible recombination in forebrain neurons. This system was reported before, however recombination efficiency was very low (Casanova et al., 2002), thus leaving room for improvement. The final characterization of the two CreER(T2) expressing mouse lines generated in this project will entail the breeding of these lines with the rederived Z/AP reporter mouse line, the induction of CreER(T2) using a good batch of *tamoxifen*, and the description of recombination efficiency and pattern.

Finally, the value of a line permitting the rescue of an inducible gene KO is illustrated using a specific example. In a study by Shimizu *et al.* molecular mechanisms of learning and memory were investigated by combining the tetracycline responsive transactivator system and the Cre recombinase to achieve ablation and inducible rescue of a gene of interest (Shimizu et al., 2000). To examine whether the NMDA receptor is necessary for the transfer of an engram from short- to long-term memory the study had to circumvent acquisition and retrieval, since the NMDA receptor is required for both (Heale and Harley, 1990; Staubli et al., 1989; Venable and Kelly, 1990). For this, it was necessary to inactivate the major NMDA receptor subunit, NR1, after training and to examine performance thereafter again in the presence of NR1. This was achieved with a combined conditional approach, and composed of a CaMKIIα promoter-Cre transgene, a Cre-dependent tTA gene placed under the control of a ubiquitous promoter, a tTA-dependent NR1 transgene fused to a *tetO* promoter, and an endogenous NR1 gene flanked by loxP sites. In the quadruple transgenic mouse, Cre was expressed in forebrain neurons under the CaMKIIα promoter and catalyzed recombination both at the endogenous NR1 locus and in the tTA transgene, thus inducing the simultaneous ablation of endogenous NR1 and the expression of a rescue NR1 transgene. The only remaining NR1 gene could therefore be controlled by *dox* treatment and NR1 deficiency was thus made reversible. The authors were able to show that the period during which the transfer from short- to long-term memory occurs can extend over several months, and that the NMDA receptor is required during the whole time window (Cui et al., 2004; Shimizu et al., 2000). Very recently, the feasibility of this approach was further proven in a study on serotonin receptors (Stark et al., 2007). While this approach represents a very elegant alternative to the the system presented in this chapter, it requires the generation of 4 different mouse lines that are crossed with each other to attain the final mouse. A less time consuming and more general approach to achieve the same result will certainly represent a valuable and interesting alternative and will continue to attract researchers' interest.

General Discussion

Memory is an essential function of everyday life. Loss of memory or uncontrolled recall of traumatic memories can severely affect quality of life. Understanding the mechanisms that control the persistence of memory and its robustness to interference, will provide valuable information for the development of the treatment of these pathological conditions.

Calcineurin (CN) is a Ca^{2+}-dependent Ser/Thr protein phophatase that has been implicated in synaptic plasticity and memory formation. The aim of this thesis was to extend the understanding of the involvement of CN in synaptic plasticity (a) and memory (b) in continuation of previous research in the laboratory, and to reveal underlying mechanisms (c). For this, new systems to induce transgenes *in vivo* were employed and further developed in mice (d).

(a) CN in neocortical plasticity

Previous research has shown that changing neuronal CN activity affects synaptic plasticity in the hippocampus (Heynen et al., 2003; Ikegami et al., 1996; Malleret et al., 2001; Mansuy et al., 1998a; Mansuy et al., 1998b; Winder et al., 1998). More specifically, CN inhibition facilitates long-term potentiation (LTP) (Ikegami et al., 1996; Malleret et al., 2001) and blocks long-term depression (LTD) (Heynen et al., 2003). These types of plasticity occur after artificial stimulation and are thought to simulate physiological processes underlying memory formation (Whitlock et al., 2006). LTP and LTD can also be induced in the visual cortex (Funauchi et al., 1994; Torii et al., 1995). Here, they may serve to mediate a dramatic form of experience-dependent neocortical plasticity, the ocular dominance shift (ODS) (Crozier et al., 2007; Heynen et al., 2003). ODS occurs after monocular deprivation (MD), i.e. when one eye is temporarily deprived of vision during early postnatal life. This intervention alters synaptic transmission in such a way that cortical neurons cease to respond to neuronal input coming from the deprived eye. In collaboration with a group of Nigel Daw at Yale University, we studied the effect of a transient increase in CN activity on neocortical reorganization after MD. We showed that increased activity of this protein phosphatase blocked ODS (chapter 2). Thus, fine regulation of CN activity is essential for this type of plasticity. However, LTD was not affected in the visual cortex by increased CN activity, at least in cortical layers II/III. This evidence

suggests that LTD and ocular dominance plasticity (ODP) are not directly related or that they have a complex relationship. Another explanation may be the existence of different forms of LTD and/or synaptic plasticity in distinct cortical layers. In summary, this result extended the role of CN from the cellular level of hippocampal plasticity to the functional level of neocortical plasticity and provides further support for a ubiquitous function of CN in processes of neuronal plasticity.

Our simplified model - depicted in *Figure 1* of the general introduction - suggests a delicate balance of protein phosphatases and protein kinases during plasticity related-synaptic activity. Our research showed for the first time that protein phosphatases are critically involved in ODP. Several other studies strongly indicated that many of the kinases included in our model also play an important role in ODP. In consistence with our model, inhibition of the protein kinases CaMKII (Taha et al., 2002) and cAMP dependent protein kinase (PKA) (Beaver et al., 2001), impaired ODS. Since tipping the phosphatase-kinase balance towards protein phophatases by up-regulation of CN or inhibition of kinases blocks ODP, we can conclude that phophatases act as molecular restraints on this form of plasticity.
The involvement of PKA suggests a role for adenylate cyclases in ODP. Since CN regulates Ca^{2+}-sensitive adenylate cyclases (Chan et al., 2005) that contribute to hippocampal memory by regulation of the cAMP response element-binding protein (CREB) (Sindreu et al., 2007), this could explain the effect of CN up-regulation and PKA inhibition on ODP. However, ODS was shown to be independent of Ca^{2+}-sensitive adenylate cyclases (Fischer et al., 2004). Furthermore, the activation of adenylate cyclases through metabotropic glutamate receptors (mGluRs) (Simonds, 1999) can be excluded since blocking mGluRs has no effect on ODP (Hensch and Stryker, 1996). Therefore, PKA must be regulated by alternative mechanisms and the involvement of CN in ODP is not explained by this pathway.

As CN is associated with PKA through anchoring proteins (Coghlan et al., 1995), it is possible that they share common substrates. In this context, the kinase Erk (extracellular signal-regulated kinase) is particularly interesting because it was shown to be indispensable for ODS (Di Cristo et al., 2001). PKA regulates Erk, activity by gating its nuclear translocation (Impey et al., 1998). CN on the other hand, determines the duration of Erk signaling through the striatal-enriched phosphatase (STEP) (Paul

et al., 2003). Erk in turn, indirectly activates the cAMP response element-binding protein (CREB) (Vanhoutte et al., 1999) that is crucial for ODP (Mower et al., 2002) and may thereby control ODP-related transcription. Indeed, the complete PKA-Erk-CREB pathway has been implicated in ODP (Cancedda et al., 2003). However, PKA and CN pathways may also converge on protein phosphatase 1 (PP1) through regulation of the PP1 specific inhibitor-1 (Bito et al., 1996)). PP1 may in turn directly or indirectly activate CREB and thereby contribute to transcriptional regulation of plasticity related genes (Genoux et al., 2002).

The gene targets of the transcription factor CREB in the context of ODP have remained elusive. While monocular deprivation results in a decrease in the mRNA of the immediate early genes (IEGs) Zif268, c-Fos, and BDNF in the visual cortex (Chaudhuri et al., 1995; Kaczmarek et al., 1999; Lein and Shatz, 2000), the absence of the IEG TF Zif268 does not affect ODS (Mataga et al., 2001). This clearly differentiates plasticity underlying ODS and aversive memory, which is strengthened by an increase in Zif268 (chapters 1 and 3) and impaired by its decrease (Jones et al., 2001; Malkani et al., 2004).

(b) CN – a ubiquitous modulator of memory?

Memory formation is based on synaptic plasticity in a similar way to the above described developmental plasticity during ODS (Whitlock et al., 2006). In support of this, our laboratory has shown that CN acts as a major restraint on learning and memory in hippocampus-dependent tasks (Malleret et al., 2001). However, memory is not a uniform process but a large array of distinct functions that all involve the storage of information. The type of information, the brain regions involved, and the processes underlying this storage may therefore differ greatly. CN was shown to play a key role in synaptic plasticity in different brain regions, including hippocampus (Heynen et al., 2003; Ikegami et al., 1996; Malleret et al., 2001; Mansuy et al., 1998a; Mansuy et al., 1998b; Winder et al., 1998), visual cortex (Funauchi et al., 1994; Torii et al., 1995) and the amygdala (Lin et al., 2003a). To conclude that the involvement of CN in synaptic plasticity in these brain regions is of functional importantance, it is necessary to confirm the role of CN in memory tasks that do not rely on the hippocampus.

Here, associative memories with aversive contents are of particular interest because they are relevant to human conditions of pervasive and traumatic memory, such as post-traumatic stress disorder (PTSD) and types of anxiety disorders. Aspects of these conditions may therefore be modeled with appropriate memory tests in the mouse. Using CTA, we studied the dominance of memory traces by analyzing their persistence and robustness to interference and implicated CN as a major determinant of these parameters (chapter 1). Interestingly, this role of CN in CTA was exclusive to the aversive memory. The non-aversive memory trace acquired during CTA extinction was unaffected by modulations of CN activity. This was confirmed for fear memories in a model for PTSD, the fear conditioning task (chapter 1). We further strengthened this link by showing that CN is physiologically regulated during the establishment of the aversive CTA memory (chapter 1).

However, this contradicts previous data from similar models. In another task of fear memory, the fear potentiated startle, Lin *et al.* have shown that CN acts as a main mediator of extinction (Lin et al., 2003b). This may be explained as follows.
Firstly, extinction of CTA, fear conditioning or fear potentiated startle use different experimental procedures. While extinction of CTA and fear conditioning is achieved with the same cue (saccharin, tone) as during conditioning, the extinction of fear-potentiated startle uses a different cue (light) than conditioning (tone/light) and thus introduces a discrepancy between conditioning and extinction, which may recruit CN. Furthermore, unlike CTA and fear conditioning, extinction of fear potentiated startle is preceded by a preextinction test that reactivates the conditioning memory trace. It is possible that preextinction training itself recruits CN activity that subsequently affects CTA extinction.

Secondly, distinct subnuclei of the amygdala are involved in CTA acquisition and extinction, i.e. inhibiting protein synthesis in the central amygdala selectively blocks acquisition, while inhibiting protein synthesis in the basolateral amygdala selectively blocks extinction (Bahar et al., 2003). In fear conditioning, a similar dissection during acquisition and extinction is observed (Wilensky et al., 2006). Moreover, CN level and activity are described to be oppositely regulated in basolateral (increase) and central amygdala (decrease) during fear memory extinction (Cannich et al., 2004). Lin et al. addressed the role of CN in the rat basolateral amygdala (Lin et al., 2003b), and

were thus likely to find an effect in extinction. In our experiments, we did not specifically target any subnucleus of the amygdala and therefore observed a net result. CN inhibition enhanced CTA memory, while CN over-activation weakened it. Accordingly, CN was decreased during memory formation.

Thirdly, CN activity may vary depending on the level of extinction achieved in the animals and on the delay after conditioning. Indeed, the interval between conditioning and extinction was recently shown to determine the type of processes recruited by extinction, at least for inhibitory avoidance (Milekic and Alberini, 2002) and fear conditioning (Lee et al., 2006b). Lin et al measured CN after a single extinction session that resulted in full extinction of fear potentiated startle (1 day after conditioning) while our measurements were done early in extinction training (3 days after conditioning) when the conditioning trace was still inducing a strong avoidance response. This may explain why a different effect on CN was observed.

CTA as a mouse model of traumatic memory in humans

In analogy to human conditions, aversive memory tasks in mice are usually based on very robust and salient forms of memory. The strongest of these may be acquired in conditioned taste aversion (CTA), a task extensively studied in this thesis. In this task, mice learn to associate a novel taste (saccharin, which is normally appetitive) with malaise, induced by LiCl injection. Mice that get sick after drinking saccharin will avoid it during later presentations. We have shown that the memory for this association persists for several months without significant memory decline and that it completely dominates behavior during this time (chapter 1). Indeed, to extinguish this avoidance behavior several consecutive presentations of saccharin are required that are not followed by malaise. In response to these presentations, mice form a second association - saccharin is safe – that competes with the aversive memory and affects behavior to the extend that it dominates (Rescorla, 1967). CTA may be deemed a suitable model for human pathologies of traumatic memory based on (a) the persistence of the aversive memory, (b) its continual control of behavior despite other relevant information, (c) its robustness to interference and (d) the one-trial learning character of CTA memory. This is especially relevant and significant since it is mainly the persistence of traumatic memory traces and its robustness to interference that is the target of clinical treatment of memory disorders like PTSD and anxiety disorders.

(c) CN dependent mechanism of determining memory strength

Based on the findings presented here and previously, we conclude that CN must be a key modulator of signaling cascades underlying memory-related plasticity. Since persistent forms of memory require *de novo* gene expression (Korzus, 2003), these signals need to be conveyed to the nucleus where they regulate transcription factors (TFs). In search of a possible mechanism of CN-dependent regulation of memory strength we discovered that mRNA levels of the IEG Zif268 are increased after CN inhibition.

To simulate this regulation, novel transgenic mice were generated that allowed the inducible forebrain-specific expression of a Zif268 transgene (chapter 3). We thoroughly characterized these mice (chapter 3) and subsequently showed that Zif268 over-expression indeed results in a similar phenotype as CN inhibition in CTA (chapters 1 and 3). This suggests that CN determines memory dominance by limiting the induction of memory-related genes like Zif268, which is a transcription factor that may function as a switch for whole transcriptional response programs (Pfenning et al., 2007). This limitation may be achieved in two ways that take place simultaneously. CN may act as an active component of the molecular cascade that mediates signals from the synapse to the nucleus, or CN limits this signaling by impinging on parts of the cascade.

During memory establishment CN may act in different compartments, including the nucleus. Indeed, cytoplasmic CN can translocate to the nucleus in a complex with the transcription factor NFAT (nuclear factor of activated T-cells (Shibasaki et al., 1996). Control of transcriptional processes can occur via direct regulation of TFs as described for NFAT (Shibasaki et al., 1996) and for myocyte enhance factor 2 (MEF2) (Mao and Wiedmann, 1999). Indeed, the CN-MEF2 pathway has recently been shown to restrict synapse number in an activity-dependent manner (Flavell et al., 2006).

CN can further restrict signaling from the synapse to the nucleus by acting right at the post-synaptic membrane, where these signaling cascades are initiated. Indeed, our observation that CN activity is specifically and physiologically decreased in a crude membrane fraction during the establishment of aversive memory (chapter 1) supports a function of CN here. Indeed, at the post-synaptic density CN is found in a complex

with the cAMP dependent protein kinase (PKA) and the A kinase anchor protein 79 (AKAP79) (Coghlan et al., 1995). CN and PKA fulfill opposite functions to coordinate signaling cascades that originate here (Scott, 1997). Via AKAP79, CN is associated with glutamate receptors and regulates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) (Smith et al., 2006), NMDA (Lieberman and Mody, 1994), and other receptor types (e.g. mGluR5 (Alagarsamy et al., 2005). It achieves this by shortening NMDA receptor opening time (Lieberman and Mody, 1994), by promoting AMPA receptor (GluR1) internalization (Smith et al., 2006) or by decreasing AMPA receptor activity in response to low concentration of Ca^{2+} (Morishita et al., 2005).

However, at the membrane CN may also act on the adenylate cyclases 1 and 8, which regulate PKA, a kinase that can acts as counterpart to CN (Smith et al., 2006), through catalyzing cAMP production. PKA may subsequently regulate mitogen-activated protein kinase (MAPK), which in turn controls the kinases Msk1 (mitogen- and stress-activated kinase 1) and Erk (extracellular signal-regulated kinase). This pathway was recently implicated in he establishment of fear memory (Sindreu et al., 2007).

Erk activation is also controlled by striatal-enriched phosphatase (STEP), a tyrosine phophatase that is itself regulated by CN and determines the duration of Erk signaling (Paul et al., 2003). Erk in turn can tranlocate to the nucleus where it phosphorylates and activates the TFs cAMP responsive element binding protein (CREB) and Elk-1 (Davis et al., 2000). Persistent Erk signaling is required for the induction of IEGs and of transcriptional events related to memory formation (Bito et al., 1996).

CN may further regulate the TF CREB through a PP1 (protein phosphatase 1)-dependent mechanism. Two PP1 binding proteins, inhibitor-1 (I-1) and DARPP32, are targets of CN and PKA and inhibit PP1 in their dephosphorylated state (Bito et al., 1996; Halpain et al., 1990). PP1 itself regulates phosphorylation and activity of the TF CREB in the nucleus (Genoux et al., 2002). However, we have gathered evidence that PP1 is not involved in CTA memory formation. Mutant mice with a decrease in PP1 activity perform normally in CTA (chapter 1, *Supplementary Fig. 2*) and there is no apparent regulation of cytoplasmic PP1 after CTA conditioning (personal observation).

Indeed, all of the above pathways converge on the transcription factors CREB and Elk1. CREB is absolutely necessary for memory formation and Ca^{2+} dependent gene regulation (Frank and Greenberg, 1994) via the cAMP responsive element CRE. Elk1 is part of the ternary complex factor and binds to steroid response elements SRE in the presence of the serum response factor (Whitmarsh et al., 1995). Both SRE and CRE are found in the Zif268 promoter and may therefore mediate the effect of CN activity on Zif268 expression.

However, CN may also mediate some of its effects by inducing structural changes. Together with PKA, it controls morphological changes like neurite extension and axonal regeneration (Fansa et al., 2000; Lautermilch and Spitzer, 2000). In the hippocampus, CN has been shown to mediate shrinkage of dendritic spines in response to low Ca^{2+} influx in a cofilin-dependent manner (Zhou et al., 2004). Furthermore, PKA (Liu et al., 2004) and CN (Wei et al., 2002) coordinately determine tau phosphorylation. However, here CN inhibition results in increased levels of phosphorylated tau that accumulates and may thereby impair memory performance (Chen, 2005; Yu et al., 2006). This may actually explain the memory deficits CN KO mice (Miyakawa et al., 2003; Zeng et al., 2001), which intuitively contradict our results. However, tau accumulates extensively because of chronic or persistent ablation of CN (Kayyali et al., 1997) and this may conceal any improvement of memory performance. Tau hyperphosphorylation through CN inhibition may also contribute to memory deficits in Alzheimer's disease (Liu et al., 2006) by promoting Aβ deposition (Pierrot et al., 2006).

Zif268 - a memory-specific gene?

In line with its nature as an immediate early gene, Zif268 is induced in several cell types by numerous stimuli. Importantly, Zif268 is also induced after the type of neuronal activity triggering events of synaptic plasticity (Cole et al., 1989; Wisden et al., 1990). While Zif268 was thereafter extensively used to detect plasticity related neuronal activity, induction of Zif268 was not always required for plasticity to occur (Mataga et al., 2001).

However, this is a singular observation and in most studies the site of Zif268 induction has coincided with the site of memory storage. After contextual fear conditioning for example, Zif268 is induced in the lateral nucleus of the amygdala,

but not in the hippocampus (Malkani and Rosen, 2000a; Rosen et al., 1998). Retrieval of contextual fear conditioning on the other hand, results in an increase of Zif268 mRNA in the CA1 region of the hippocampus and in several amygdala nuclei (Hall et al., 2001; Rosen et al., 1998). Accordingly, blocking Zif268 by stereotactic injection of antisense desoxynucleotides in the amygdala blocks fear memory formation (Malkani et al., 2004), while the same intervention in the hippocampus shows no effect (Lee et al., 2004). However, hippocampus-specific blockade of Zif268 induction after retrieval interferes with reconsolidation of fear memory (Lee et al., 2004), in line with its induction under these conditions. Unfortunately, the importance of Zif268 induction in the amgdala after retrieval was not examined in these studies.

Further strong evidence for a specific role of Zif268 in long-term memory comes from the analysis of the Zif268 KO mouse. While brain anatomy, short-term synaptic plasticity and short-term memory are completely normal in this mouse, it displays strong deficits in long-term memory (Jones et al., 2001). Interestingly, these deficits can be overcome by extensive training, suggesting that there are redundant pathways that may be partially able to compensate for a loss of Zif268 (Bozon et al., 2003). The pathways required for Zif268 induction have been thoroughly studied and exclusively include proteins that have themselves been implicated in the induction of memory related plasticity type of activity (Davis et al., 2000). In studies on the immune system and on hormone secretion, it was shown that other members of the Egr family, like Egr2 (Li and Liu, 2006) and Egr4 (Tourtellotte et al., 2000), may be involved. However, the compensatory processes have not been studied in detail so far.

The archetypical mechanism of plasticity may therefore involve Zif268 induction, but alternative or redundant mechanisms exist. In fact, it is likely that Zif268 induction occurs as part of a large program and that it may specifically mediate the persistence of memory storage. In support of this hypothesis, recent research has demonstrated an increase in Zif268 in the hippocampus 24hrs after memory acquisition on a hippocampus dependent memory task (Bekinschtein et al., 2007). Interestingly, this increase was induced by a BDNF-dependent late phase of consolidation that occurred 12hrs after acquisition. Blocking this phase by protein synthesis inhibitors or BDNF *knock down* not only reduced the persistence of memory, but also the increase in Zif268. Therefore, this phase does not seem to be required for memory formation *per*

se but determining its strength. This nicely fits our data in Zif268 over-expressing mice (chapter 1 and 3). We also confirmed Zif268 upregulation in the amygdala after CTA acquisition. Among the broad range of late-response genes that are targeted by Zif268 are proteasome related proteins (Conway et al., 2007; James et al., 2005; James et al., 2006). Interestingly, Zif268-binding to the promoters of these target genes inhibits their promoter activity and decreases expression of the target gene (Conway et al., 2007; James et al., 2006). Inhibition of the expression of proteasome subunits alongside with activation of the expression of other target genes may indeed be required for Zif268 to render a memory trace persistent. Memory requires proper functioning of the ubiquitin-proteasome system (Jiang et al., 1998; Lopez-Salon et al., 2001). In line with this, blocking the proteasome specifically diminished the late phase of long-term potentiation, a candidate model for plasticity underlying long-term memory (Fonseca et al., 2006). Intriguingly, simultaneous blockade of the proteasome and protein synthesis restored this form of plasticity (Fonseca et al., 2006), suggesting that a balance between protein synthesis and degradation is required for persistent forms of plasticity and memory. It is of great interesting to find out how the over-expression of Zif268 affects this balance in gene regulation. To address this, we are currently examining the processes underlying Zif268-mediated memory enhancement by looking at the genes and proteins that are regulated in different brain regions in Zif268 over-expressing mutants. Furthermore, we will examine memory performance in different memory tasks requiring distinct neuronal substrates to verify the ubiquity of this enhancement.

(d) The use of transgenic mice for the study of memory

To gain the knowledge presented in this thesis, transgenic mouse models were employed that are based on the reverse tetracycline responsive transactivators, rtTA and the new and improved rtTA2. Chapter 5 describes the adaptation of the rtTA2 system into transgenic mice. The system permits the inducible and reversible expression of a transgene through the simple administration of antibiotics in the diet (either tetracycline or *doxycycline*, chapter 5). Both tissue- and cell-type specificity is achieved by choice of the promoter that drives expression of the transactivator. The CaMKIIα promoter was used in all the projects presented here, because it allows neuronal expression (shown in chapters 3 and 5) that is specific to forebrain regions (demonstrated in chapters 1, 3, 5 and 6) implicated in learning and memory. As part

of this work, the rtTA(2) systems were used to (a) reduce enzyme activity by expressing a specific inhibitor of an enzyme (chapter 1), (b) increase total enzyme activity by over-expression of a constitutively active form of an enzyme (chapters 1 and 2), (c) increase transcriptional response through over-expression of a transcription factor of the family of the immediate early genes (chapters 1 and 3), and (d) to co-express a transgene and a reporter gene to easily examine expression pattern of a transgene.

This great degree of versatility of these systems can be extended further through the combination with RNAi to achieve inducible and reversible *knock down* of selective targets *in vivo* (Szulc et al., 2006). Reversal of a gene *knock out* was the aim of the project presented in chapter 6. For this, the rtTA2 system was combined with the inducible Cre recombinase (CreER(T2)) system. CreER(T2) permits gene ablation in a spatially restricted manner, and was further improved by adding temporal control through the fusion with an activator domain (chapters 4 and 6). Co-expression of CreER(T2) and rtTA2 would thus allow the rescue of a tissue- and cell-type specific knockout. Despite using two parallel approaches, we failed to realize this transgenic mouse line. However, we managed to generate two mouse lines that express the inducible Cre recombinase in forebrain neurons. The inducibility of Cre activity and the efficiency of recombination achieved on different loci will be characterized in continuation of this project. We hope that these new mouse lines will represent a great improvement over the existing line, which is known to induce mosaic recombination patterns (Casanova et al., 2002).

Outlook

Based on the data gathered in chapters 1 and 3, we have proposed a model (*Fig. 1*) that illustrates CN-dependent signaling cascades that may contribute to the dominance of aversive memories (chapter 1). This model was developed by consolidating our new data with already published work and is notably hypothetical. However, it specifies known molecular pathways and suggests interaction between them.

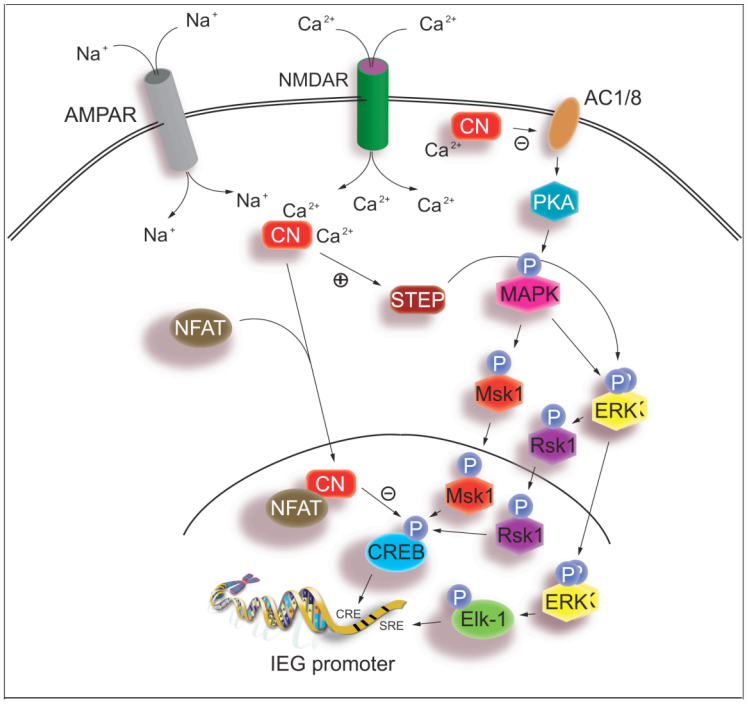

Figure 1. Known signal transduction pathways by which calcium influx regulates gene transcription through CN-dependent mechanisms. The expression of the IEG Zif268 may be coupled to CN by several biochemical routes: (1) CN may directly act on TFs that regulate Zif268 expression; (2) CN functions via the striatal-enriched phophatase (STEP), which negatively controls extracellular signal-regulated kinase (Erk); (3) CN inhibits adenylate cyclases 1/8 and thereby reduces the phosphorylation and nuclear translocation of cAMP-dependent mitogen- and stress-activated protein kinase (Msk1) and of Erk. These cytoplasmic signaling cascades concatenate on the TFs cAMP response element binding protein (CREB) and the ternary complex factor (TCF) Elk1, that regulate Zif268 expression via cAMP responsive elements (CRE) or serum response elements (SRE) in the Zif268 promoter.

These pathways, the interactions between them and their involvement in processes underlying the establishment of aversive memories need to be verified and studied in more detail. With CN and Zif268, we have already identified two key regulators of the strength of aversive memories, which have been implicated in other forms of memory before. In continuing experiments we are examining the target genes of Zif268 that mediate the formation of aversive memory. We are also testing the Zif268 mutant mice in different memory tasks to confirm the importance of Zif268 for memory formation requiring distinct brain regions. Additionally, we are conducting experiments to specifically examine the consequences of Zif268 over-expression on the reconsolidation, extinction and retrieval of aversive memory.

Further specifying the details of the processes underlying aversive memory processing will help to find suitable targets for treatment of pathological conditions of memory in humans. The knowledge may also help in devising remedies for memory deficits.

Eventually we will comprehend how information storage is achieved in the brain. We will understand the differences between distinct types of memory and we will be able to interfere with them selectively. This knowledge will aid in the development of treatments and preventional measures to reduce the severity and the incidence of memory pathologies. This should greatly improve quality of life of people affected by pathologies of memory.

Abbreviations

Ab - antibody
AMPA - α-Amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid
Arc - activity-regulated cytoskeleton
bp - basepair(s)
BSA - bovine serum albumin
BW - body weight
°C - degree Celsius
cAMP - Cyclic adenosine monophosphate (cAMP)
CNS - central nervous system
CRE - cAMP response element
CREB - cAMP response element binding protein
CTA - conditioned taste aversion
DABCO - 1,4-diazabicyclo[2.2.2]octane
DAG - diacylglycerol
DMSO - di-methyl sulfoxide
DNA - Deoxyribonucleic acid
dNTP - desoxyribonucleotides
dox – doxycycline
EDTA - ethylenediamine tetraacetic acid
e.g. - exempli gratia (latin), for example
EGR - early growth response
Erk - extracellular-signal regulated kinase
g - gram(s) OR Force in earth gravitation units (=9.8N)
HCl – hydrochloric acid
hr - hour
i.e. - id est (latin), that is (to say)
IEG - immediate early gene
i.m. - intra muscular (injection)
i.p. - intra peritoneal (injection)
IP_3 - inositol 1,4,5-trisphosphate
IRES - internal ribosomal entry site
KCl - potassium chloride
KI - Knock-in
KO - Knock-out
L - liter(s)
M – molar
MAPK - mitogen-activated protein kinase
min - minutes
ml - milliliter(s)
mM - milli molar
Msk1 - mitogen- and stress-activated protein kinase
NaCl - sodium chloride
NGFI-A - nerve growth factor induced protein A
NMDA - N-methyl-d-aspartate
ODN - oligodesoxynucleotides
PCR - polymerase chain reaction
PBS - phosphate buffered saline
PFA - paraformaldehyde
pH - pondus Hydrogenii (latin), literally weight of water
PKA - protein kinase A, also calles cAMP dependent kinase
Rbb - rabbit
RT - room temperature
s - seconds
s.c. - subcutaneously injected
SDS - sodium dodecyl sulfate
SRE - serum response element
SRF - serum response factor
tam - tamoxifen

TBE - Tris, Boric acid, EDTA
TEMED - short for N,N,N',N'-Tetramethylethylenediamine
TF - transcription factor
TOE-1 - target of Egr-1
Tris - short for Tris(hydroxymethyl)-aminomethan
V - Volts
WT- wildtype
Zif268 - zinc finger binding protein clone 268

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Curriculum Vitae - Resumé

Personal Details

Surname: Baumgärtel **First Name:** Karsten

Date of Birth: 13. September 1977 **Place of Birth:** Munich, Germany

Nationality: German

Education

2003-2007 PhD thesis in Neuroscience at the Brain Research Institute and Neuroscience Center Zürich, Federal Institute of Technology and University of Zürich, Switzerland
Supervised by Prof. Dr. Isabelle Mansuy: "Molecular Mechanisms of Memory and Synaptic Plasticity"

1997-2002 Degree in Biology at the Ludwig-Maximilians University, Munich Germany

2001-2002 Diploma thesis in the group of Prof. Dr. Beat Lutz "Molecular genetics of behaviour" at the Max-Planck-Institute of Psychiatry, Munich, Germany
Supervised by Dr. Jan Sitz: "Characterisation of Interaction Partners of the Kinase Dyrk1A"

1996-1997 Bachelor studies in "Biomedical Science" at the University of Technology, Sydney, Australia

1992-1995 High school, Sydney, Australia

1989-1992 Gymnasium, Munich, Germany

Scientific Achievements

Publications

- **Karsten Baumgartel**, David Genoux, Hans Welzl, Kyoko Koshibu, Céline Mamie, and Isabelle M. Mansuy. *The protein phosphatase calcineurin regulates the establishment and the dominance of associative memory traces*. Manuscript in Preparation.

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Oral Presentations

Jul 2006: "Control of Aversive Memories by Calcineurin" European Meeting of the Molecular and Cellular Cognition Society, Vienna, Austria

May 2006: "Control of Aversive Memories by Calcineurin" ETHZ bi-annual D-Biol Symposium, Davos, Switzerland

Feb 2006: "Studies of Molecular Constraints on Brain Plasticity and Memory" Benefri Course, Fribourg, Switzerland

Dec 2005: "Update on the Generation of Zif268 Mutant Mice" Summary and Progress report, HFSP Grant meeting, Cassis, France

Mar 2005: "Complementary Involvement of PP1 and Calcineurin in Associative Memory" First Swiss Meeting on the Cellular and Molecular Neurobiology of Mental Diseases, Giessbach, Switzerland

Oct 2004: "Complementary Involvement of PP1 and Calcineurin in Associative Memory" Data Blitz, ZNZ (Center for Neuroscience Zürich) Symposium, Zürich, Switzerland

Apr 2004: "Functional Analysis of Immediate Early Genes in LTP and memory" Summary and Progress report, HFSP Grant Meeting, Barcelona, Spain

Posters

Mar 2007 "Generation of a Pax2rtTA Mouse to Establish an Inducible Transgenic Wilms Tumor Model". Clinical Research Symposium, Zürich, Switzerland

Feb 2007: "Temporally and Spatially Restricted Gene Expression in the Adult Mouse Brain using Lentiviral Approaches". Joint NCCR (Neuroplasticity and Repair) and SSN (Swiss Society of Neuroscience) Meeting, Bern, Switzerland

Feb 2007: "The Immediate Early Gene Zif268 regulates the Mechanisms of Aversive Memory". Joint NCCR (Neuroplasticity and Repair) and SSN (Swiss Society of Neuroscience) Meeting, Bern, Switzerland

Oct 2006: "Involvement of Calcineurin in the Formation and Extinction of Associative Memory" ZNZ (Center for Neuroscience Zürich) Symposium, Zürich, Switzerland

Jul 2006: "Involvement of Calcineurin in the Formation and Extinction of Associative Memory" FENS Forum, Vienna, Austria

Jul 2006: "Involvement of Calcineurin in the Formation and Extinction of Associative Memory" European Meeting of the Molecular and Cellular Cognition Society, Vienna, Austria

May 2006: "Control of Aversive Memories by Calcineurin" ETHZ biannual D-Biol Symposium, Davos, Switzerland

Nov 2005: "Functional Analysis of the Immediate Early Gene Zif268 in Memory". Annual Meeting of the Molecular and Cellular Cognition Society. Washington D.C., USA

Oct 2005 “Complementary Involvement of Calcineurin and PP1 in Associative Memory”. ZNZ (Center for Neuroscience Zürich) Symposium, Zürich, Switzerland

Feb 2005: “Genetic systems for inducible and reversible gene recombination and expression in the mouse brain” NCCR (Neuroplasticity and Repair) Symposium, Kartause Ittingen, Switzerland

Feb 2005: “Functional Analysis of the Immediate Early Gene Zif268 in Memory”. Annual Meeting of the Swiss Society of Neuroscience, Zürich, Switzerland

Oct 2004: “Complementary Involvement of PP1 and Calcineurin in Associative Memory” ZNZ (Center for Neuroscience Zürich) Symposium, Zürich, Switzerland

May 2004: “Functional Analysis of the Immediate Early Gene Zif268 in Memory” ETHZ bi-annual D-Biol Symposium, Davos, Switzerland

Mar 2004: “Recombination and Gene Expression in Forebrain Neurons of the Mouse” NCCR (Neuroplasticity and Repair) Symposium, Constance, Germany

Sep 2003: “Functional Analysis of the Immediate Early Gene Zif268 in Memory” EMBO Young Investigator Program PhD Student course, Heidelberg, Germany