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Oral-examination: **.....**

Different Genetic Ways for Conditional Gene Regulation in the Mouse Brain

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Hiermit erkläre ich, daß ich die vorliegende Dissertation selbst verfaßt und mich dabei keiner anderen Mittel als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Des Weiteren erkläre ich, daß ich an keiner anderen Stelle ein Prüfungsverfahren beantragt oder die Dissertation in dieser oder einer anderen Form bereits anderweitig als Prüfungsarbeit verwendet oder einer anderen Fakultät als Dissertation vorgelegt habe.

Heidelberg, 17. March 2005 Peixin Zhu

My parents and Liya

My Parents: ‘All the small little things make you different!
When everyone else is not doing the small things, then you should do it!’

Dr. Xiaoping Yang: ‘When I do not know what to do or where to go,
I will just sit down and read books!’

Dr. Patrick K. Arthur: ‘Our aim in life is not to get ahead of others,
but to get further forward of ourselves, to overtake our today with our tomorrow!’;

‘Research is seeing what everyone sees, but think what nobody thinks!’

Dr. Mazahir T. Hasan: ‘a prepared mind and an unbiased scientific investigation is a prerequisite for new discoveries’

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Summary:

The conditional gene expression system controlled by the tetracycline-responsive trans-activator (tTA) in transgenic mice is an important and well characterized genetic tool. In my studies I investigated the function of the Tet-regulated genes in the brain when inserted into the chromosome and when applied as extra-chromosomal elements with recombinant Adeno-Associated Virus (rAAV).

First, the pool of tTA expressing mouse lines was increased by generating eight functional itTA-expressing *Tg*$^{Thy\text{-}1.2(xx)\text{-}itTAnls}$ transgenic mouse lines. Since for tTA expression the *Thy-1.2* promoter was used, which is known to be highly integration-site dependent, lines were obtained which all differed in tTA expression level, expression pattern and ontogenetic profile in the central nervous system (CNS). These mice are a valuable tool for studies on physiological functions of different neuron populations during various developmental stages.

Second, the effect of the chromosomal insertion site of Tet-responder genes on their expression was analyzed. In three different Tet-responder mouse lines, the precise integration sites and copy number of tet-responder genes were determined. No correlation between copy number of the Tet-responder gene and its expression was detected, supporting the finding that the chromosomal insertion site has strong effects on the expression of the transgene. Strong Tet-responder gene expression was observed in mice of the transgenic line SA87.5, which has three copies of a transgene inserted in the *Pik3c3* gene locus. By retargeting the same position of the *Pik3c3* gene locus in embryonic stem cells, only one copy of the transgene was introduced. However, due to the presence of the neo selection marker the single Tet-responsive transgene showed poor induction by tTA. After Cre-virus mediated neo removal, the expression of the Tet-responsive transgene was improved.

The limitations of chromosomally inserted Tet-regulated gene elements can be overcome by using the recombinant Adeno-Associated virus (rAAV) mediated gene delivery system to introduce the different elements of the Tet-system into the CNS. It is shown that both Tet-activators and Tet-responder genes are functionally delivered by rAAV vectors, and tissue specific, homogeneous, rapid and robust

expression of multiple proteins simultaneously controlled by tTA in rAAV infected CNS areas can be achieved.

Moreover these viruses were used to demonstrate that the transcriptionally inactive Tet-responder genes are often epigenetically silenced during development when inserted into the host chromosome, but remain inducible when introduced extra-chromosomally after development.

Zusammenfassung:

Das konditionale Genexpressionssystem basierend auf dem Tetracyclin gesteuerten Transaktivator (tetracycline-responsive trans-activator tTA) ist ein wichtiger und gut charakterisierter genetischer Schalter in transgenen Mäusen. In der vorliegenden Arbeit untersuchte ich die Funktion tTA regulierter Gene, die sowohl als chromosomale Integrate als auch in transienter Form durch rekombinante Adeno-assoziierte Viren (rAAV) im Maushirn zur Expression gebracht wurden.

Erstens wurde die Sammlung tTA exprimierender Mauslinien durch acht itTA exprimierende Mauslinien *Tg*$^{Thy\text{-}1.2(xx)\text{-}itTAnls}$ ergänzt. Da zur tTA Expression der *Thy-1.2* Promoter, dessen Aktivität durch die Integartionsstelle stark beeinflusst wird, benutzt wurde, unterscheiden sich diese Mauslinien in Expressionsstaerke, Expressionsmuster und im ontogenetischen Profil. Diese tTA Mauslinien stellen ein wertvolles Wekzeug für physiologische Studien der Funktionen unterschiedlicher Neuronenpopulationen während verschiedener Entwicklungsstadien im Maushirn dar.

Zweitens wurde der Einfluß der chromosomalen Integartionsstelle für tTA kontrollierte Gene untersucht. In drei verschiedenen Mauslinien mit TA abhängigen Genen wurde der genaue Integrationsort sowie die Anzahl von Kopien des Transgens bestimmt. Dabei konnte kein Zusammhang von Kopienzahl des Transgens und dessen Expressionsstärke nachgewiesen werden. Dies kann als Hinweis auf den starken Einfluß des Integartionsorts für die Expressionsstärke tTA abhängiger Gene interpretiert werden. Starke Expression eines tTA abhängigen Transgens wurde in der Mauslinie SA87.5 beobachtet. Diese Mauslinie enthält drei Kopien des Transgens im *Pik3c3* Genlokus. Die drei Transgenkopien wurden durch eine einzige Kopie an derselben Stelle des *Pik3c3* Genlokus mittels Gentargeting in embryonalen Stammzellen ersetzt. Das Transgen ließ sich jedoch

bei Anwesendheit des Neomycinselektionsmarkers nur schwach durch tTA induzieren. Nach Exzision des Selektionsmarkers mittels Cre produzierendem rAAV konnte die Induktion des Transgens durch tTA verbessert werden.

Die Nachteile von fest im Chromosom integrierten tTA regulierten Respondergenen können durch Einsatz von rAAV als Vehikel zur Transgenexpression im Maushirn überwunden werden. Neben den tTA abhaengigen Respondergenen kann auch tTA selbst erfogreich mittels rAAV im Maushirn exprimiert werden. Eine gewebespezifische, homogene, schnell anflutende tTA abhängige Expression von verschiedenen Genen ist so im Maushirn möglich. Durch Expression mittels rAAV blieben tTA abhaengige Transgene induzierbar, deren Expression bei chromosomaler Integartion durch epigenetische Effekte während der Ontogenese zum Erliegen kam.

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1: Introduction:

All biological systems are capable of responding to a vast number of environmental challenges and thus optimize their potential for survival and propagation. Depending on the environmental conditions, organisms either activate or inactivate molecular and cellular machinery for short-term or long-term adaptation and survival. For example, at the genetic level, gene promoters can become either activated or inactivated by modification of gene transcriptional units within seconds and gene expression can be modified either transiently or permanently. Molecular switches that can turn gene expression “on and off” endow animals the capability to respond and adapt to changes in environmental and cellular conditions. There are many naturally occurring inducible gene expression systems, some of which have been employed to artificially control gene expression in different animal models. Clearly, inducible control of gene expression by artificial means has the great advantage as it can provide vital information for correlating gene function(s) with phenotypes. Below, I will present a brief overview of different naturally occuring inducible systems, including the tetracycline inducible systems, and how they have been modified to create genetic circuits for controlling heterologous gene expression in living cells and intact animals. My active studies focused on the Tetracycline (Tet) inducible systems for reversible control of gene expression in neurons *in vivo.*

Different inducible systems

Heat shock inducible system

The heat shock induced gene regulation system has been exploited in different cell lines (Schweinfest *et al*, 1988) and also in transgenic mice (Kothary *et al*, 1989), however its applicability in mammalian systems have been quite limited. Heat shock protein genes, such as heat shock protein 70 (hsp70) in *D. melanogaster*, and hsp68 in *C. elegans*, contain promoters, which are activated by endogenous heat shock induced transcription factors.

*Heavy metal inducible syste*m

Heavy metal ions dependent inducible systems, such as one based on metalloproteins, are controlled by promoters responsive to heavy metal ions uptake in cells, particularly Cd^{2+} and Zn^{2+}. These systems show high basal promoter activity, modest level of induction and toxicity associated with administration of heavy metal (Filmus *et al*, 1992).

Interferon inducible system

Introduction of the interferon regulated inducible system was greeted with great excitement. Genes under the control of interferon-inducible regulatory element, such as the promoter of *Mx1*, could be rapidly activated by injection, double-stranded RNA, or virus in mice (Arnheiter *et al*, 1990, Kühn *et al*, 1995). However, limited induction level in tissues and individual mouse, and the fact that treatment with either interferon or dsRNA would have profound biological side effects limit its general use.

Steroid hormone inducible systems

Steroid hormone receptors have also proven as valuable inducible systems. Steroid hormones, like glucocorticoid, estrogen, and progesterone, upon binding their cognate receptors activate specific genes containing inducible promoters. The natural steroid hormone inducible systems have been harnessed to control expression of heterologous genes in mammalian systems but these systems have limitations to stringently control gene expression (Friedman *et al*, 1989, Braselmann *et al*, 1993, and Wang *et al*, 1994). Furthermore, hormones are endogenously produced and regulated in mammals, and the hormone-responsive elements used to control specific transgene expression would be efficiently regulated by endogenous regulatory factors, which respond to the endogenous hormonal pathway. That is why hormone receptor mutations are used with high affinity to synthetic ligands like Ru486 and tamoxifen are used. As an alternative, the insect steroid hormone, ecdysone can be used. It has no cross-reactivity with the mammalian systems, and it was applied in mammalian cells and transgenic mice (Yao *et al*, 1993; No *et al*, 1996, Albanese *et al*, 2000, Yu *et al*, 2000).

Lac operon based inducible systems

There are two well-studied binary regulated gene expression systems, lactose- and tetracycline-dependent gene expression systems, described in *E. coli*. The lactose-dependent system consists of two components: a lac repressor (lacR) and its binding DNA sequence-lac operator (lacO). The lac-dependent regulated gene expression are under tight control by lactose availability. In E. coli, transcription of the survival genes involved in the uptake and metabolism of lactose could be efficiently turned on and off. Lac repressor specifically binds to operator (lacO) placed in promoter only in the absence of lactose, thus preventing recruitment and formation of RNA polymerase transcription initiation complex by steric hinderance. Upon treatment with lactose or its derivative, isopropyl β-D-thiogalactopyranoside (IPTG), the lactose-bound Lac repressor detaches the operator and transcription complex is formed. Transgenic mice have been generated using the lacR-dependent gene expression system in mammalian models. For example, transgenic mouse systems were established with three tandem lac operators were integrated into the tyrosinease promoter (Caronin *et al*, 2001). The lac repressor repressed mouse tyrosinase promoter activity and upon IPTG treatment gene expression was re-activated. One major problem with the lacR inducible systems is that IPTG does not readily cross the blood-brain-barrier (BBB). For stringent regulation of gene expression in the mammailan brain, it is extremely important that the inducers cross the BBB and for this reason most inducible systems discussed have limitations when used in the brain compared to peripheral tissues.

Tetracycline operon based inducible systems

The tetracycline-controlled transactivator (tTA) was originally designed as a fusion transcriptional factor of Tet repressor (tetR) from the Tn10 tetracycline resistance operon of E. coli and the C-terminal transcriptional activating domain of VP16, which is essential for the transcription of immediate early viral genes (Gossen *et al*, 1992) (Figure 1A). The tTA-responsive promoter (Ptet) is composed of tetracycline operator sequences, (tetO), usually heptamerized (tetO)7, followed

by a minimal RNA polymerase II transcription initiation site derived from the human cytomegalovirus immediate-early (IE) promoter (PhCMV).

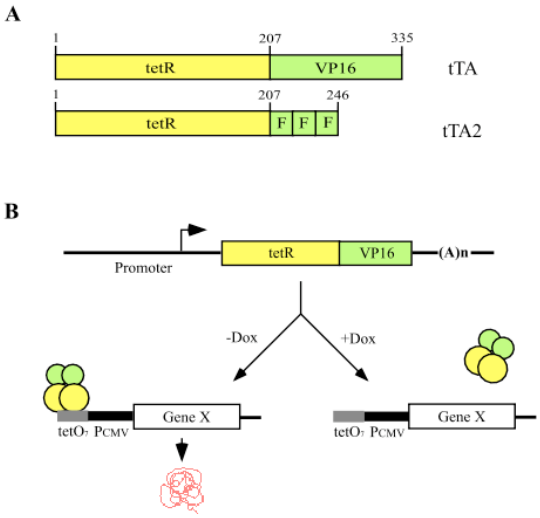


Figure1. The tetracycline-controlled transactivators (tTA) and schematic outline of tTA regulatory system. A: The 207 aa tet Repressor (tetR) and the 128 aa VP16 domain. In tTA2, 3 F domains was fused. **B**: tTA binds in absence of Dox to tetO7, but not in presence of Dox.

They are used to drive the downstream gene of interest. The regulatory TetR domain of tTA specifically binds to tetracycline operator sequences (tetO) in the absence of tetracycline or one of its many derivative, such as doxycycline (Dox), and thus activates gene transcription (Baron U. *et al*, 2000). Gene expression can be efficiently turned-off by Dox administration (Figure 1B). By symmetrically flanking two CMV minimal promoters with tetO sequences, bi-directional Tet promoters have been engineered (P_{tet}bi) allowing simultaneous regulation of two different transgenes (Baron *et al*, 1995).

Employment of Tet-systems in transgenic mouse models

Tetracycline-dependent gene regulatory system is by far the most versatile and widely applicable system for gene expression in all cell types in different experimental animal models, such as *D. melanogaster* (Girard, *et al*, 1998), plants (Weinmann *et al*, 1994; Zeidler *et al*, 1996) and mice (Schoenig *et al*, 2004). As an inducible gene expression system, it fulfills the following key requirements for a stringent gene regulatory circuit: low basal activity of Tet promoters, high binding affinity of Dox to tTA and also tTA to tetOs. Efficient gene induction is achievable with high dynamic range without obvious physiological side-effects on experimental organisms. The tTA-dependent gene expression system can be efficiently

achieved in different tissues and organs of transgenic mice and regulated gene expression can be achieved by either oral or intraperitoneal Dox administration. Particularly interesting is that the Tet system could be also applied in the nervous system of transgenic mice by expressing tTA driven by neuronal specific promoters (Mayford, 1996). The power of genetic-switch has created much excitement in neuroscience research especially to study protein functions in memory and learning and human-like neurological diseases in experimental animals. The fact that the physiological protein function(s) can be switched “on and off” at will in the same individual animals provide researchers with a unique opportunity to experimentally test whether diseases might be genetically curable in the future. For instance, transgenic mouse models have been generated to evaluate the function(s) of key proteins, Prion disease (Prusiner 1997; Tremblay *et al*, 1998) and Huntingtin Disease (Yamamoto *et al*, 2000) in neuronal plasticity, but also in learning and memory (Mayford *et al*, 1996; Mack *et al*, 2001).

Although tTA has been successfully expressed in the mammalian brain in a temporally and spatially restricted manner (Mayford *et al*, 1996), there is now an increased need to further restrict gene expression to specific cell types and subregions of the brain so that reversible gene expression in brain circuits can be further correlated with phenotypic changes, especially in relation to learning and memory. The other problem usually encountered when generating stable transgenic mouse lines is that the site of transgene integration in the mouse genome has effects on gene expression. We have observed that, compared to other tissues such as liver, tTA-dependent gene expression in the brain is not robust and it strongly depends on different Tet-responsive mouse lines, likely due to differences in site of integration in the genome. If tet-responsive genes are especially prone to epigenetic silencing, and especially when integrated in different arrangements in the genomes and as multi-copies, it would be very useful to genetically fingerprint each line in order to account for transgene arrangement as one of the variability factors. For these reasons, we undertook a systematic approach to address these issues.

First of all, *in vitro* and *in vivo* studies have been performed to improve the compatibility of the prokaryotic tTA with the mammalian transcriptional and trans-

lational systems. The tTA was humanized (itTAnls) by switching codon usage, elimination of potential splicing signal, avoidance of CpG sequence, and introduction of Kozak sequence, and also by increasing mRNA stability etc. (Zolotukhin *et al*, 1996; Cronin *et al*, 2001; Baron *et al*, 1997, Regier *et al*, 1993). In addition, the VP16 moiety in tTA was replaced by three minimal critical activating domains (F domain), which was shown to have higher intracellular tolerance and overexpression of transcription did not cause squelching effects (Gill and Ptashne 1988; Baron *et al*, 1997, Regier *et al*, 1993). Optimized tTA are highly proficient in inducing gene expression in cultured cells (Hasan, 2001; Kim, 2001) and in mice (Mayford *et al*, 1996, Malleret *et al*, 2001, Mansuy *et al*, 1998, Minichiello *et al*, 1999, Tsien *et al*, 1996; Chen *et al*, 1998; Tremblay *et al*, 1998; Jerecic *et al*, 1999). We used the *Thy-1.2* promoter for itTA expression in the mouse brain. The brain specific promoter fragments of the Thy-1.2 promoter were carefully mapped and investigated (Spanopoulou *et al*, 1988 and 1991;). And it is known that the temporal regulation of the Thy-1.2 promoter render transgenic mice the ability to express the transgene in developmental stage dependent manner (Campsall *et al*, 2002; Caroni, 1996; Feng *et al*, 2000;). Most importantly, in studies of many Thy-1.2-transgenic mouse models, transgenic mouse lines were established with transgenes integrated randomly in the genome and different lines showed brain-specific gene expression restricted, in some cases, to specific cortical layers and cell types (Ingraham *et al*, 1986; Kolsto, 1986; Kollias, 1987; Spanopoulou, 1988 and 1991; Caroni, 1996; Feng, 2000; Campsall, 2002;).

Second, several Tet-responder transgenic mouse lines as the other part of the binary tTA-dependent gene expression system were investigated. In these lines, the Tet-responsive DNA constructs are integrated randomly, both in copy-numbers and in integration loci, into the mouse genome. The Tet-responsive constructs contain P_{tet}-bi to drive the expression of reporter genes bi-directionally under tTA control. To investigate the integration dependence, we determined the copy number and genomic locus of the transgene integration in four lines, which show stable responder gene expression. The SA87,5 ($Tg^{nlacZTETO7GFPGluR-A(SA)}$) line was shown to have the strongest responder gene expression, and it has only 3 copies of transgene in the *Pik3c3* gene locus. Thus, we generated single-copy

tet-responsive cassette transgenic mice (Pik3c3-A1) by ES cell targeting at the *Pik3c3* locus. To prove the locus-dependent transgene expression, Pik3c3-A1 and SA87,5 were bred with KT1 mice to show that tTA dependent transgene expression is strongly dependent both on integration site and copy-numbers of the tet--responsive cassettes.

Third, recombinant Adeno-Associated Virus (rAAV) carrying tet-responsive mini-gene cassettes can escape epigenetic silencing programs and achieve rapid and robust tTA-dependent gene expression. The mosaic expression patterns of reporter gene observed in ThyF64 ($Tg^{Thy\text{-}1.2(64)\text{-}itTAnlsRsp}$) mice is supporting the hypothesis that it is due to the epigenetic silencing effects of integrated tet-responders. And also previous studies showed that CMV-derived transgene, retro-viral sequences (Flavell et al, 1994; Rossignol and Faugeron, 1994) and stably integrated tet-responsive elements containing NEO gene (Shin et al, 1996) are strongly influenced by epigenetic silencing programs. To overcome epigenetic effects in neurons, we employed rAAV, a human parvovirus with a single strand DNA (ssDNA) of about 4,68 kb, to show that non-integrated Tet-responsive DNA constructs can escape epigenetic silencing. rAAV with its dsDNA epigenetic genome favors tandem array linear status in the host cells for active long-term transcription, and it rarely integrates into the host genome (Schnepp *et al*, 2003). rAAV containing tet-responder mini-gene (rAAV-6p-minibiCre-Venus) was delivered into tTA-expressing mouse brains. Robust fluorescent reporter detected in selected brain regions of KT1($Tg^{CamKII\text{-}tTA}$) and in Thy1.2 ($Tg^{Thy\text{-}1.2(xx)\text{-}itTAnlsRsp}$) mice can be visualized by two-photon microscope to reveal fine neuronal structures in rAAV infected cortical neurons. And the expression of reporter gene can be turned off and on by application or removal of Doxycycline in drinking water. More interestingly, we showed that in ThyF64 transgenic mouse brain, the rAAV-tet-responders mediate tTA-dependent gene expression, which represent tTA expression patterns of ThyF64. Thus, the combination of tTA-expressing transgenic mice and rAAV-tet-responder viruses can mediate rapid and robust gene expression in tissue- and/or cell-type-specific manner. Apparently, rAAV does not show pathological effects in rodents. It has high infection efficiency in dividing and non-dividing cells cross broad range of hosts and tissues (Samulski *et al*, 1999; Stil-

well *et al*, 2003). The added advantage of rAAV system is convenient and easy to use for gene delivery in cells (Clark *et al*, 1995; Conway *et al*, 1999; Feudner *et al*, 2001; Gao *et al*, 1998; Inoue and Russell, 1998; Liu *et al*, 1999; Matsushita *et al*, 1998; Qiao *et al*, 2002a, 2002b; Sollerbrant *et al*, 2001; Xiao *et al*, 1998; Zhang *et al*, 1999). Furthermore, we also described that tTA-expressing rAAVs using cell-type specific promoters such as human synapsin 1 promoter (Kuegler et al, 2001) can achieve transgenic tTA-mice independent conditional gene expression. The co-infection of rAAV-tTA/rAAV-tet-responder viruses was shown to be a powerful tool of delivering a tTA-dependent gene regulation system in mouse and in rat brains.

Finally, rAAV expressing tTA injected into transgenic tet-responder mouse brains directly demonstrated epigenetic silencing effects on integrated tet-responsive gene cassettes. After detailed analysis of several tet-responder transgenic mouse lines, we introduced high tTA-expressing rAAV into four different transgenic tet-responder adult mouse brains. Indeed, integrated tet-responsive gene cassettes of most transgenic mouse lines did not show reporter gene expression in cortical neurons despite high cellular tTA levels. In contrast, transgenic tet-responder mice bred with ThyF64 and/or KT1 mice, which have constitutive tTA activity through all developmental stages, did show strong reporter gene expression in the adult mouse brains. In addition, transgenic tet-responder mice: $Tg^{LuciferaseTETO7Camgroo2}$ (MTH-CG19) (Hasan *et al*, 2004), infected with tTA-expressing rAAV did show robust reporter gene expression in cortical neurons. These demonstrated that integrated tet-responsive gene cassettes are actively silenced, if there was no active transcription through early developmental stages, in cortical neurons of the adult mouse brains. The exception of transgenic MTH-CG19 responder mice showed the integration locus dependency of epigenetic silencing, which could be employed in generation of transgenic tet-responder mouse lines by targeting the same integration locus, or by using BAC technology to keep the genomic environments of the permissive loci.

Thus, transgenic mouse technology using specific promoters (here mouse Thy-1.2 promoter) allows generation of tissue- and cell-type-specific tTA-expressing mouse lines, which, in combination of rAAV gene delivery systems,

achieve rapid and robust tTA-dependent gene expression. Furthermore, tTA-expressing rAAV and rAAV-tet-responder viruses can mediate strong and cell-type specific conditional gene expression both in mouse and in rat brains.

2.0 Results.

2.1 Generation of Thy-1.2-(nls)-itTA transgenic mouse lines

To increase the brain regions accessible for tTA regulated gene expression, we generated itTAnls expressing transgenic mice. The mouse *Thy-1.2* promoter (Caroni, 1996) was employed to drive the itTA expression. The itTAnls coding sequence was cloned into the Thy-1.2 expression cassette, which carries the endogenous 3'-untranslated regions and its polyadenylation site (Materials and Methods) (Figure 2a). After pronucleus injection, 14 founders were obtained. The tTA-expression was analyzed in offsprings, which contain the responder gene. Reporter β-galactosidase and/or GFP expressing founders were detected and analyzed further.

2.2 tTA expression of $Tg^{Thy\text{-}1.2(xx)itTAnls}$ founders were analyzed with indicator mice

tTA-reporter transgenic mouse lines were tTA dependent using different mice. Pattern and strength of responder gene expression were analyzed by immunohistochemistry and microscopy.

Offspring of different fourteen $Tg^{Thy\text{-}1.2(xx)itTAnls}$ transgenic founders were analyzed by examining the temporal and spatial expression pattern of lacZ reporter line $Tg^{GFPTETO7lacZ}$ (G3) (Krestel *et al*, 2001). To assess the transient activity of Thy-1.2 promoter driven itTAnls, the $Tg^{GFPTETO7lacZ}$ responder line carrying green fluorescent protein (GFP) and β-galactosidase in a bi-directional module was crossed to the $Tg^{Thy\text{-}1.2(xx)itTAnls}$ lines as an indicator mouse. *In vivo* strong GFP fluorescence was detected in brains of double transgenic offsprings from founder $Tg^{(Thy\text{-}1.2\text{-}itTAnls)10Rsp}$, $Tg^{(Thy\text{-}1.2\text{-}itTAnls)39Rsp}$, $Tg^{(Thy\text{-}1.2\text{-}itTAnls)64Rsp}$. The expression patterns of $Tg^{Thy\text{-}1.2(xx)itTAnls}$ mouse is different from mouse line to mouse line (Tabel 1 and Figure 2).

In lines $Tg^{Thy\text{-}1.2(09,\ 26,\ 39,\ 56,\ 64)itTAnls}$, we analyzed tTA expression by using anti-tTA antibody (tTA/rtTA specific rabbit polyclonal antibodies kindly provided by Dr. Beatrix Suess). For these lines we observed a nice immuno-histochemistry and –fluorescence staining, which could not be obtained for $Tg^{CaMKII\text{-}tTA}$ mice (data not shown).

Table 1:

Line Name	Responder	Reporter gene expression in the brain	Notes
Tg$^{Thy\text{-}1.2(09)}$	G3	Forebrain specific; cortex layer 5-6; hippocampus; Substantia nigra;	weak and homogenous expression.
Tg$^{Thy\text{-}1.2(10)}$	G3	Forebrain specific; somato-sensory cortex layer 5-6; hippocampus; Substantia nigra;	weak and sparse expression.
Tg$^{Thy\text{-}1.2(15)}$	G3	Very few positive cells in the forebrain.	-----
Tg$^{Thy\text{-}1.2(20)}$	G3	Forebrain specific; Cortex; CA1, Lateral amygdala;	cortical layer 5-6 in somatosensory cortex.
Tg$^{Thy\text{-}1.2(26)}$	G3	Forebrain specific; Cortex; Hippocampus; Olfactory bulb; Cerebellum;	few labeling in brain stem.
Tg$^{Thy\text{-}1.2(39)}$	G3	Forebrain specific; Cortex layer 2-3; Cortex layer 5-6; Hippocampal CA1; Substantia nigra;	sparse expression in cortical layer 5-6 and CA1.
Tg$^{Thy\text{-}1.2(56)}$	G3	cortex, DG, Thylamus, brain stem.	sparse labeling
Tg$^{Thy\text{-}1.2(64)}$	G3	Forebrain specific; Cortex layer 2-3; Cortex layer 5-6; Hippocampal CA1; Substantia nigra;	sparse expression, strong in cortical layer 5-6 and CA1.
Tg$^{Thy\text{-}1.2(66)}$	G3	Hippocampal CA1 specific expression;	sparse and strong expression.

Table 1. Transient activator tTA activity was assessed by crossing *Tg*$^{Thy\text{-}1.2(xx)\text{-}itTAnnls}$ and *Tg*^{GFPTETO7lacZ} transgenic lines. Reporter gene expression was observed in various brain regions with different intensity and diverse patterns.

In few cases, we observed that tTA positive cells in double-positive *Tg*$^{Thy\text{-}1.2(64)itTAnls}$/*Tg*^{GFPTETO7lacZ} mouse brain are negative for indicator gene expression, which might be due to the epigenetic silencing effects of stably-integrated Tet-responder transgene integration locus: *Tg*^{GFPTETO7lacZ} (Figure 10 and Figure 11). Thus we hypothesized that the efficacy of the Tet-responsive system might depend on both the tTA level and the epigenetic state of the responders. Later in this study, we developed viral mediated delivery of Tet-responsive systems to prove that stably-integrated Tet-responder transgenes are epigenetically silenced.

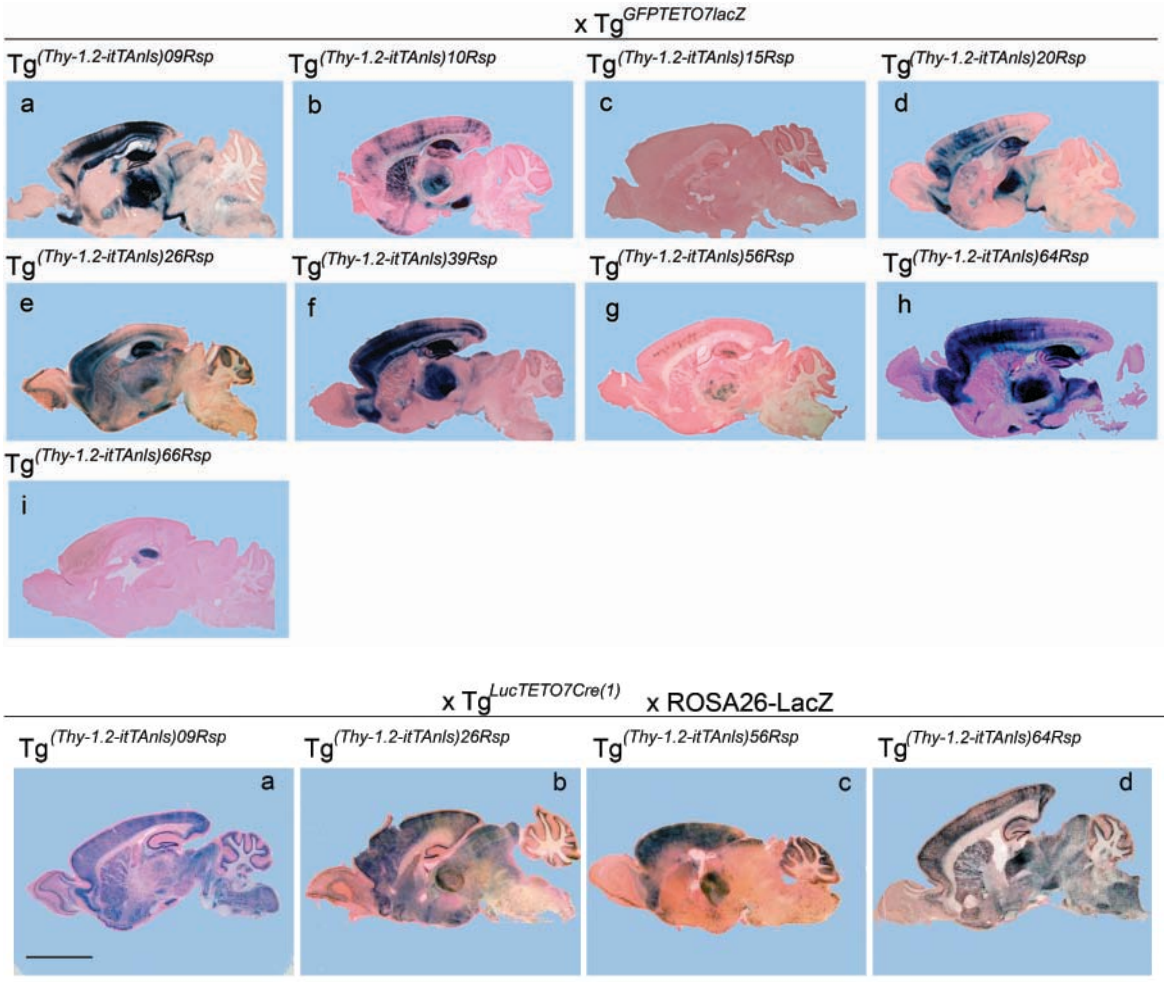


Figure 2. Strong and sparse transient expression of β-gal reporter was visualized by enzymatic reations. In *Tg*$^{Thy\text{-}1.2(xx)\text{-}itTAnls}$ transgenic mouse brains, expression in hippocampus is restricted in CA1 and very few neurons in DG, while in the cortex, the expression is restricted in layer 5-6 (**a**, **b**, **d**, **e** and **f**). *Tg*$^{Thy\text{-}1.2(15)\text{-}itTAnls}$ /*Tg*^{GFPTETO7lacZ} mice show only very few cells in somatosensory cortex (**c**). *Tg*$^{Thy\text{-}1.2(66)\text{-}itTAnls}$ /*Tg*^{GFPTETO7lacZ} mice show strong and highly restricted transgene expression in CA1 region (**g**). Ontogenic expression profiles of *Tg*$^{Thy\text{-}1.2(xx)\text{-}itTAnls}$ were obtained to show activator activity through early developmental stages (**j**, **k**, **l** and **m**). *Tg*$^{Thy\text{-}1.2(09)\text{-}itTAnls}$ /*Tg*^{LucTETO7Cre}/ Rosa26-STOP-lacZ mouse showed homogenous reporter expression in the whole brain, while the other three lines showed whole brain expression with only little or no expression in olfactory bulb. Scale bar: 0.5 mm.

To obtain the ontogenic expression profiles of the *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$ at different developmental stages, *Tg*$^{Thy\text{-}1.2(xx)\text{-}itTAnls}$ line were analyzed by *Tg*^{GFPTETO7lacZ} reporter lines' β-galactosidase activity. The β-galactosidase activity was detected widely in principal neurons of the forebrain, which show different expression patterns during development (Figure 2). *Tg*$^{Thy\text{-}1.2(09)\text{-}itTAnls}$ showed age-dependent tTA-

activity in the mouse forebrain (Figure 3), and its ontogenic tTA-profile was observed homogeneously in the whole brain (Figure 2 **j**). Tg$^{Thy\text{-}1.2(26)\text{-}itTAnls}$ also showed broad ontogenic tTA-activity in the early developmental stages, but there was no activity in the brain stem (Figure 2 **k**). Tg$^{Thy\text{-}1.2(64)\text{-}itTAnls}$ showed overall tTA-activity in the early developmental periods, while olfactory bulb showed no tTA-activity (Figure 2 **m**). In contrast, Tg$^{Thy\text{-}1.2(56)\text{-}itTAnls}$ only showed tTA activity in the cortex, thalamus and cerebellum (Figure 2 **l**).

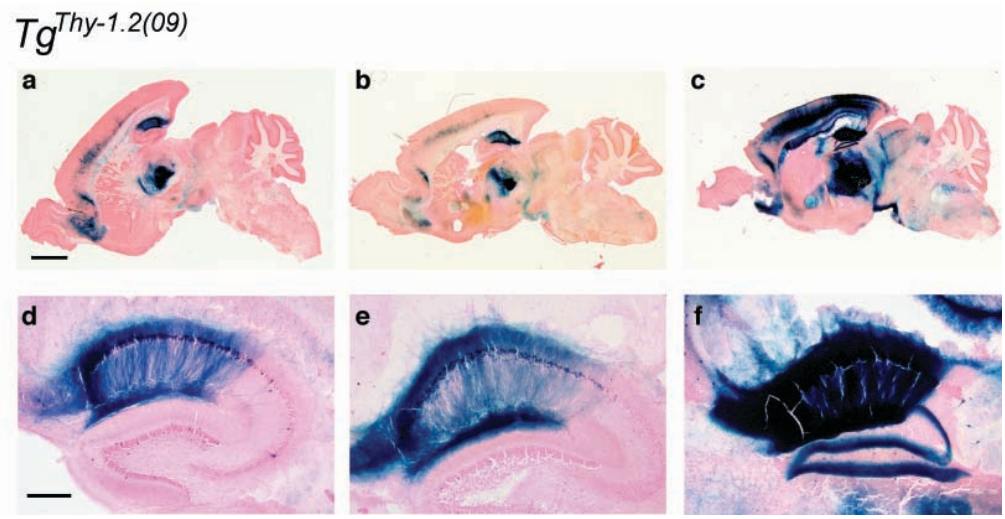


Figure 3. Developmental stage-dependent expression of *Tg*$^{Thy\text{-}1.2(09)\text{-}itTAnnls}$ transgenic mouse line. *Tg*$^{Thy\text{-}1.2(15)\text{-}itTAnnls}$ /*Tg*^{GFPTETO7lacZ} mice were analyzed at P28, P42 and P60. the expression of indicator genes in the brain, both the intensity and the area of expression, is increasing **(a-c)**, in hippocampus CA1 region, the expression is sparse and strong **(d-f)**. Scale bars are: 0.5 mm for **a-c**; 0.2 mm for **d-f**;

2.3 Copy-number and integrants pattern of *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$ founders were analyzed by Southern blotting

From seven out of fourteen independent *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$ transgenic mouse lines, we analyzed the transgene copy-numbers. From different mouse lines (*Tg*$^{Thy\text{-}1.2(09)}$, *Tg*$^{Thy\text{-}1.2(10)}$, *Tg*$^{Thy\text{-}1.2(15)}$, *Tg*$^{Thy\text{-}1.2(26)}$, *Tg*$^{Thy\text{-}1.2(39)}$, *Tg*$^{Thy\text{-}1.2(64)}$, *Tg*$^{Thy\text{-}1.2(66)}$) purified liver genomic DNA was digested and analysed by Southern blotting using PCR-amplified DNA probe specific for the endogenous mouse *Thy-1.2* gene and for the transgene (Figure 4).

Table 2:

Line name	Transgene Arrangements	Copy-number	tTA protein level
ThyF09	Tandem	2	low
ThyF10	Tandem	1	low
ThyF15	Tandem	3	low
ThyF26	Tandem	3	high
ThyF39	Complex	3	high
ThyF64	Complex	5	high
ThyF66	Complex	5	low

Table 2. Copy numbers of different $Tg^{Thy\text{-}1.2(xx)\text{-}itTAnnls}$ founders was quantitatively analyzed by Southern-Blot.

These results show that mouse lines $Tg^{Thy\text{-}1.2(09)}$, $Tg^{Thy\text{-}1.2(10)}$, $Tg^{Thy\text{-}1.2(15)}$ and $Tg^{Thy\text{-}1.2(26)}$ have simple tandem integrants patterns, while the expressed tTA protein levels are low in $Tg^{Thy\text{-}1.2(09)}$, $Tg^{Thy\text{-}1.2(10)}$, $Tg^{Thy\text{-}1.2(15)}$ mouse brains, but high in $Tg^{Thy\text{-}1.2(26)}$ mouse brain. The other lines, $Tg^{Thy\text{-}1.2(39)}$, $Tg^{Thy\text{-}1.2(64)}$, $Tg^{Thy\text{-}1.2(66)}$ show relatively complex transgene arrangements with high tTA levels in $Tg^{Thy\text{-}1.2(39)}$, $Tg^{Thy\text{-}1.2(64)}$ mice, but low in $Tg^{Thy\text{-}1.2(66)}$ mice. It also shows that these $Tg^{Thy\text{-}1.2(xx)itTAnls}$ founders carry relatively low copy-numbers of transgenes (Table 2).

2.4 Transgenes show mosaic and low-level expression in indicator mouse lines

The mosaic expression pattern and the low expression level of reporter gene observed in tetracycline-responsive gene expression system had not been fully understood. Transgenic $Tg^{CamKII\alpha\text{-}tTA}$ and $Tg^{CN\text{-}itTAnls}$ mouse lines show undetectable tTA expression level in mouse brains analyzed by specific antibodies, which are generated against the N-terminal of the tTA molecule (Hasan, *et al*, 2006).

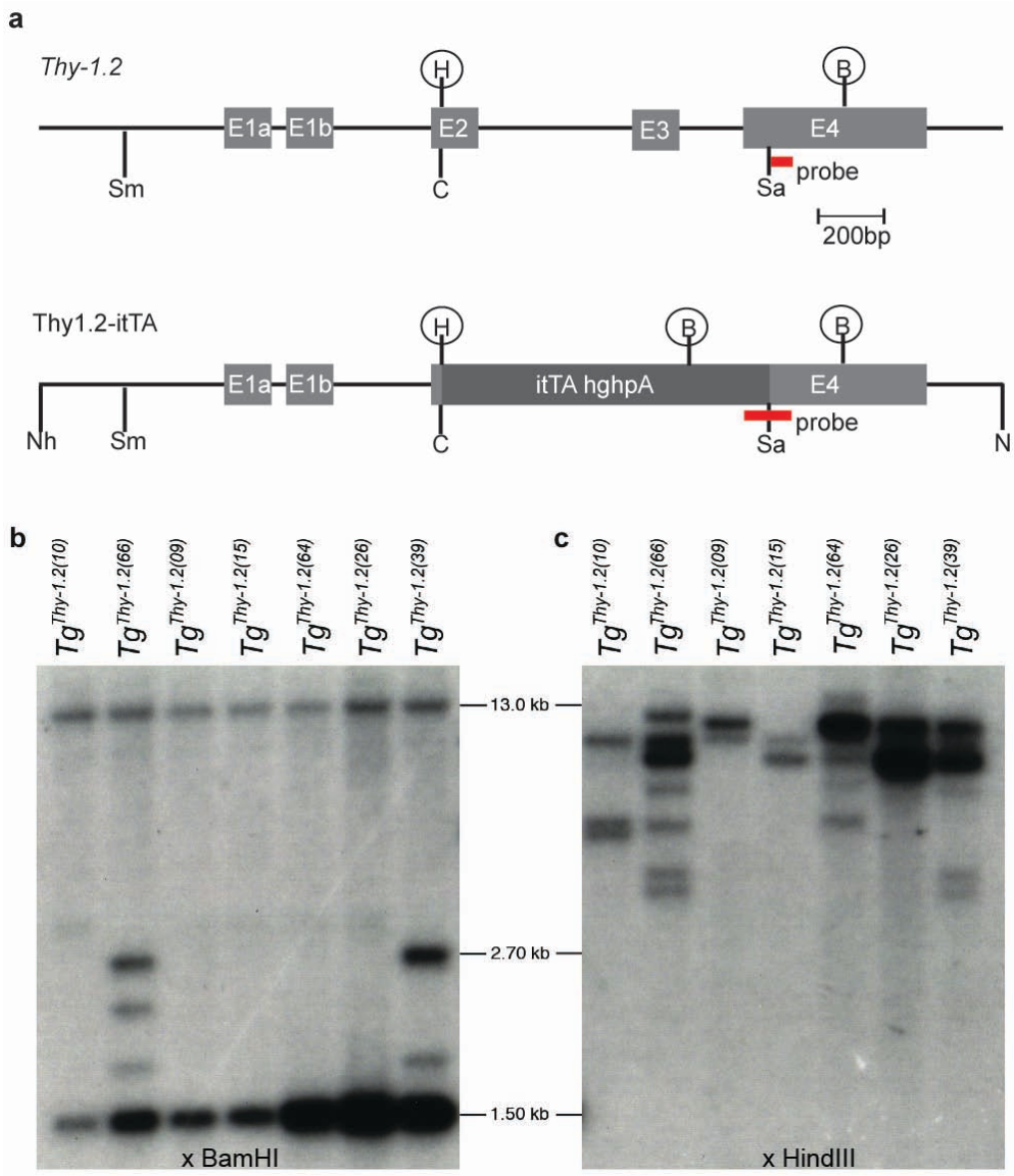


Figure 4. a: Schematic view of Thy-1.2-itTAnls minigene. Wild-type *Thy-1.2* gene was digested with BamHI and HindIII, respectively for Southern blot analysis; Minigene Thy1.2-itTA was digested with BamHI and HindIII for inner- and outter-probe pattern, respectively; **b-c**: **Southern blot analysis of *Tg*$^{Thy\text{-}1.2(xx)\text{-}itTAnls}$ founders.** Inner- (BamHI) and Outter-Probe (HindIII) pattern were probed in **b** and **c**, respectively, with PCR generated probe shown as red bar. B: BamHI; C: ClaI; H: HindIII; N: NotI; Nh: NheI; Sa: SalI; Sm: SmaI;

It had been disappointing that by immunohistochemistry we could not co-localize the tTA protein molecules and the reporter fluorescent proteins due to the undetectable tTA protein levels in neurons, from which we would have been able to understand the mosaic expression patterns. However, the generation of

Tg$^{Thy\text{-}1.2(xx)itTAnls}$ transgenic mice provide some insight into mosaic expression patterns seen in tTA system. The *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$ transgenic mice were demonstrated to have faithfully detectable itTAnls expression in neurons.

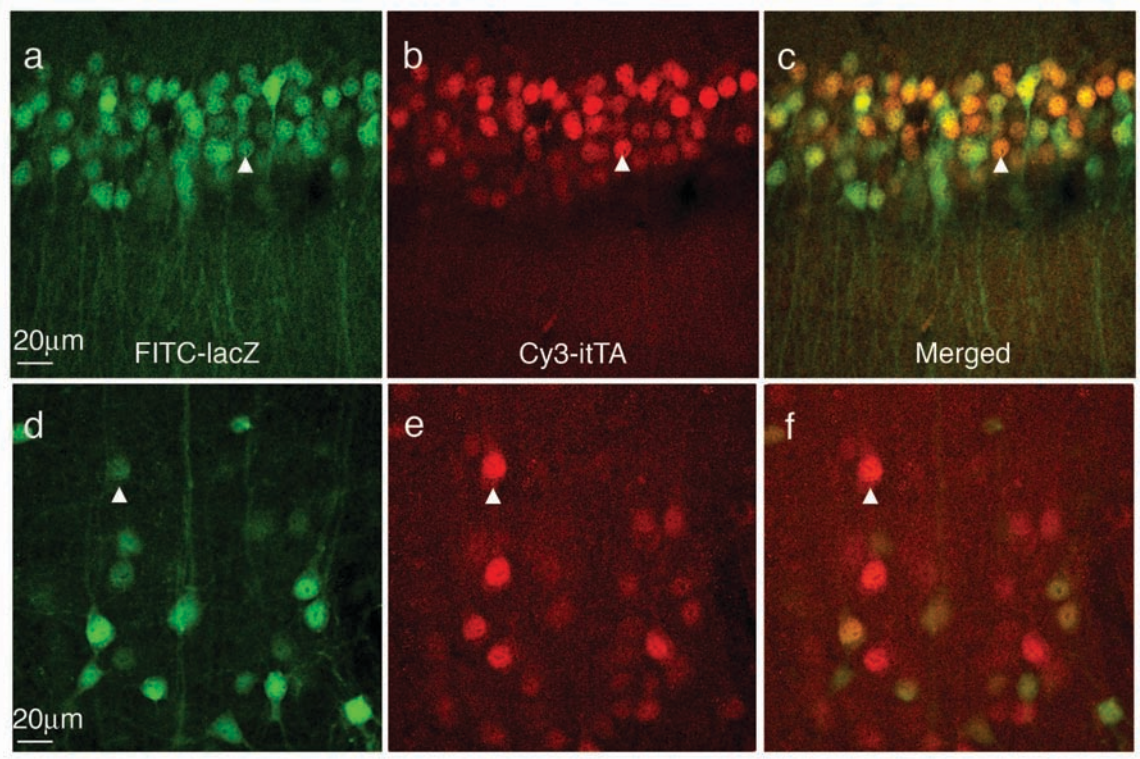


Figure 5: *Tg*$^{Thy\text{-}1.2(64)\text{-}itTAnnls}$ */Tg*^{GFPTETO7lacZ} **mouse brain was analyzed by immunohistochemistry using FITC-anti-β-galactosidase (a); Cy3-anti-tTA (b).** Hippocampus CA1 neurons were labeled (**a** and **b**) and colocalized (**c**); Cortical neurons were labeled (**d** and **e**) and colocalized (**f**); to show the overlapping expression of β-galactosidase indicator and tTA protein in cortex and in hippocampus. Arrowheads show some of the cells with relatively low β-galactosidase expression despite high levels of tTA.

In Tg$^{(Thy\text{-}1.2\text{-}itTAnls)64Rsp}$ and *Tg*^{GFPTETO7lacZ} double positive transgenic mice, we detected the co-localization of tTA and GFP by immunohistochemistry using poly-clonal rabbit anti-tTA and mono-clonal mouse anti-GFP antibodies, respectively. In most tTA expressing cells, reporter gene expression could be detected. However in few exceptions in cortex and hippocampal neuron populations, despite the presence of immuno-detectable tTA, the *Tg*^{GFPTETO7lacZ} Tet-responder integrant could not be efficiently activated. This might be the reason of mosaic expression patterns (Figure 5). These results show that the presence of high level of tTA is

not always sufficient for transgene reporter activation, suggesting that mosaic expression in the Tet system is caused by variable accessibility of the Tet-responsive transgene integrant in nerve cells.

2.4.1 Transgene Copy-Number and Pattern analysis by Southern-Blotting

We selected three different responder lines with stable by mosaic expression of the transgenic line: *Tg*$^{lacZTETO7gfpGluR\text{-}A}$, *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$ and *Tg*^{GFPTETO7lacZ}. We find that *Tg*^{GFPTETO7lacZ} trangenic locus is low in copy number (3-4 copies) and of a simple integration pattern (Figure 6A).

The Southern blot analysis showed that *Tg*$^{lacZTETO7gfpGluR\text{-}A}$ locus also has the feature of low-copy number (8-10 copies) and again a simple transgene integration pattern (Figure 6B).

The Southern blot analysis showed that the ^{GFP}GluR-A(SA) transgene integration locus has low-copy number (2-3 copies) and the multi copies were arranged simply in tandem repeats pattern (Figure 6C).

2.4.2 Copy-Number analysis by Quantitative RT-PCR and Sequencing

Real-time (RT) PCR could quantitate the relative amount of a given template. We used Real-Time PCR to confirm the copy-number determination from Southern blots. For mouse lines: *Tg*^{GFPTETO7lacZ}; *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$; *Tg*$^{lacZTETO7gfpGluR\text{-}A}$, we designed specific primers, and FITC- or Fam-labeled probes. Wildtype allele *Gria1* was used as internal control. The results showed that *Tg*^{GFPTETO7lacZ} has 4 copies; *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$ has 2 copies and *Tg*$^{lacZTETO7gfpGluR\text{-}A}$ has 10 copies of transgene (Figure 7a). Next we used the Chromatograph file in sequencing reactions, as an alternative method, to determine the copy number of *Tg*$^{lacZTETO7gfpGluR\text{-}A}$ and *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$ transgenic mice. Transgenic constructs of *Tg*$^{lacZTETO7gfpGluR\text{-}A}$ and *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$ transgenic mice are originally derived from rat cDNA library (Mack *et al*, 2001). Thus the Single Nucleotide Polymorphisms (SNPs) in rat and mouse GluR-A coding sequences were used to calculate the copy-number (Figure 7b).

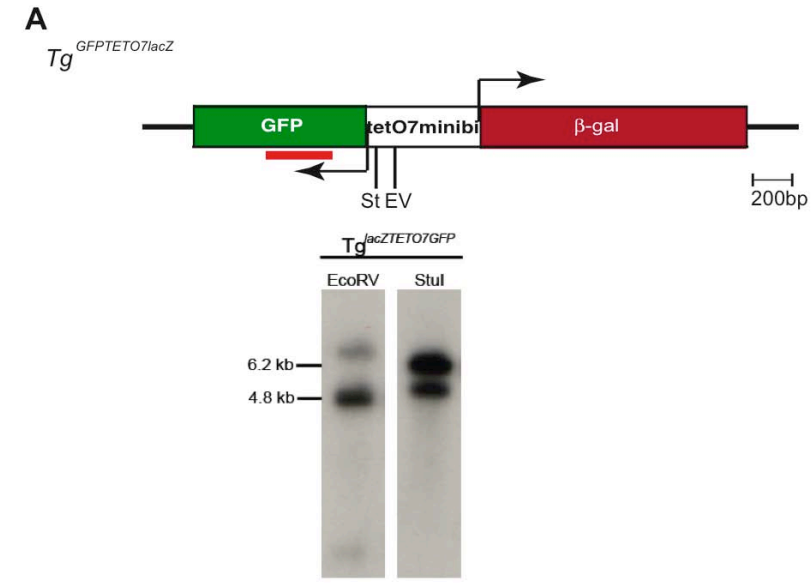
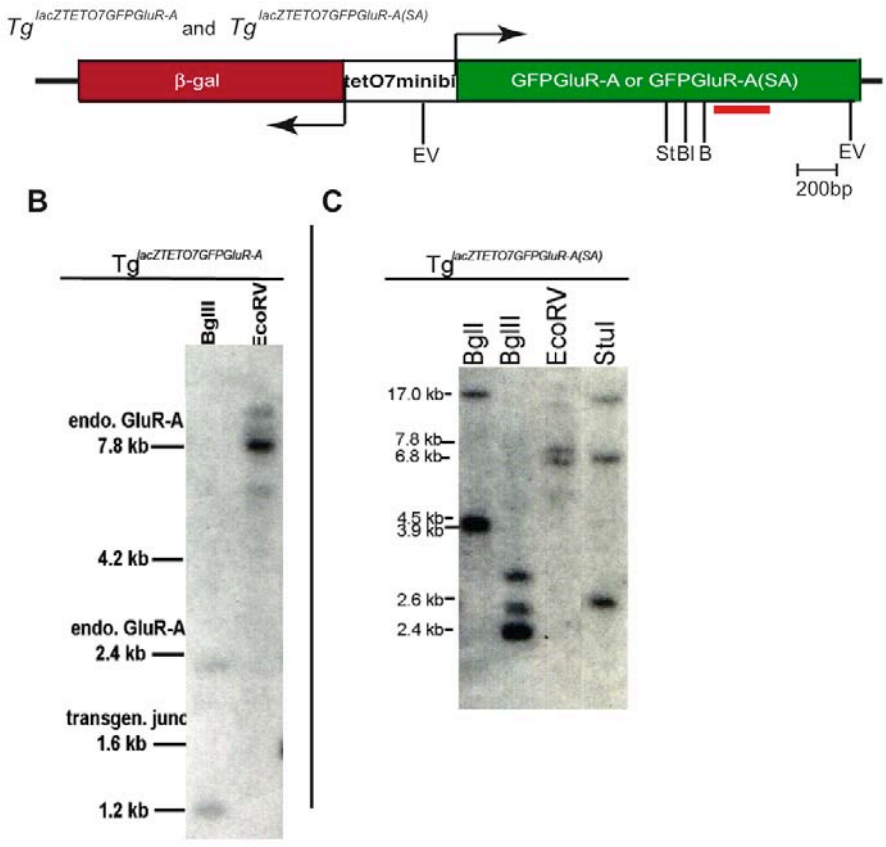


Figure 6. Schematic views of the mini-gene constructs from *G3, A1.1* and *SA87.5* transgenic mouse lines and Southern blot analysis. Southern blot analysis was performed using PCR amplied probes (red bar). **A:** Integrant in G3 mice were analyzed by EcoRV and StuI, which show tandem arrangement of 3-4 copies of transgene;



B: Tg*lacZTETO7GFPGluR-A* mouse was analyzed by BglII and EcoRV to show tandem array of 8-10 copies of transgene; **C:** Four restriction enzymes: BglI, BglII, EcoRV and StuI, were used to analyze Tg*lacZTETO7GFPGluR-A(SA)* mouse DNA. Pattern shows that 2-3 copies of transgene were arranged in tandem in the integration locus; B: BglI; Bl: BglII; EV: EcoRV; St: StuI;

These results showed that the copy number of the transgenic mouse lines detected by RT-PCR and by sequencing reactions, fits well in certain range compared to the Sounther-Blots analysis. Therefore, due to fast, easy and cheap performance, sequencing reactions mentioned here should be considered significantly as alternative means to detect transgene copy-numbers. However, the sensitivity of sequencing chromatograph shows major limitations in analyzing high copy-numbered transgenic animals.

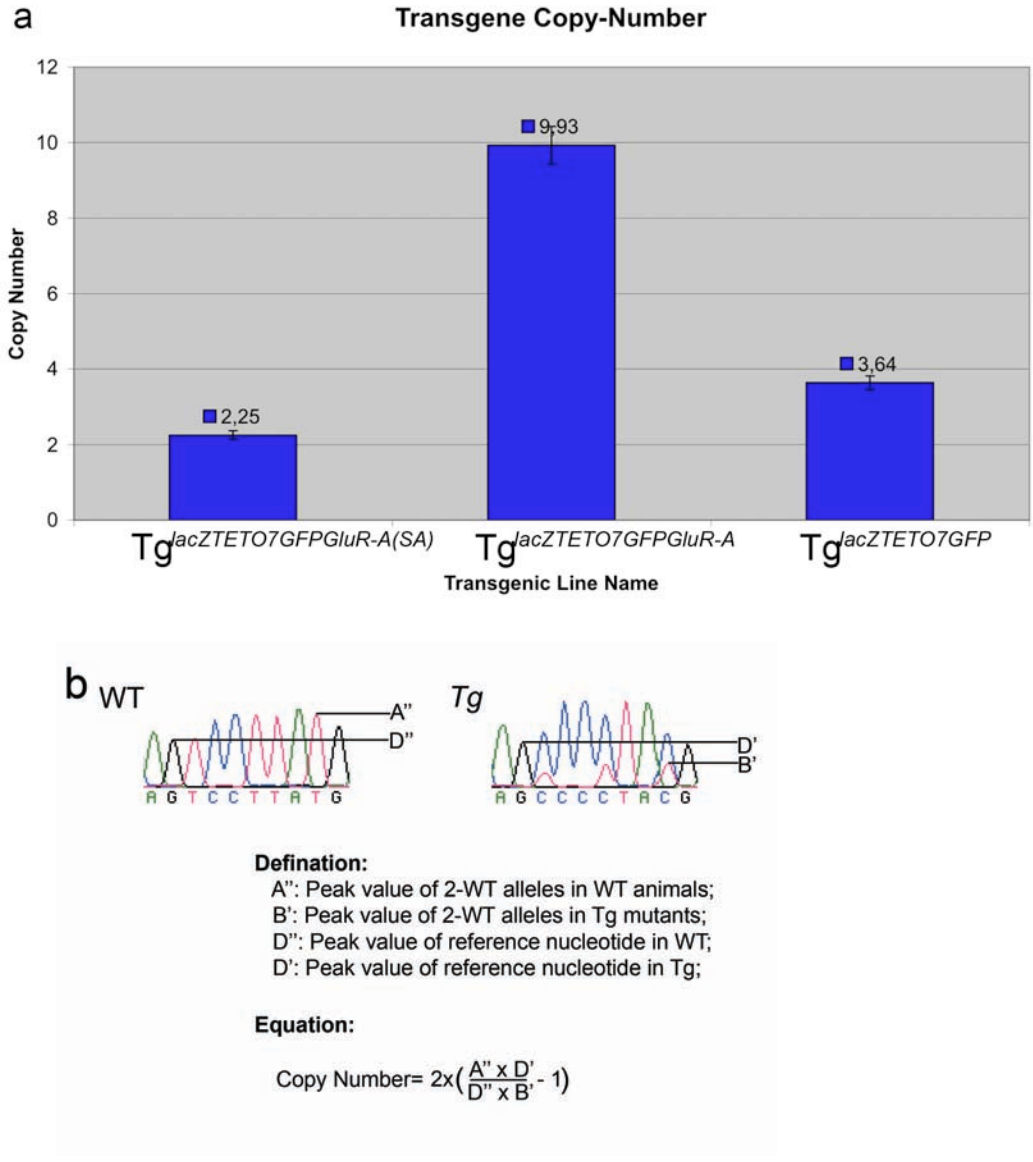


Figure 7: Transgene copy numbers were analyzed by Real-Time PCR and quantitative chromatography sequencing. a: Specific Real-Time PCR analysis show that *Tg*$^{lacZTETO7GFPGluR\text{-}A(SA87.5)}$, *Tg*$^{lacZTETO7GFPGluR\text{-}A}$ and *Tg*^{GFPTETO7LacZ} lines has 2, 10 and 4 copies of transgene constructs, respectively. **b:** The chromatograph shows that duplex of traces were compared and analyzed using equation shown beneath it, from which the copy numbers were calcu-

lated. Results are *Tg*$^{lacZTETO7GFPGluR-A(SA87.5)}$ has about 2 copies and *Tg*$^{lacZTETO7GFPGluR-A}$ has 7 copies.

2.4.3 Transgene Integration Sites detected by PCR-Mediated Genome Walking.

For the detection of precise transgene integration site in the genome, the targeted ROSA26 (ROSA-lacZ) line mouse liver DNA was used as a positive control for establishing the methods. The ROSA26LacZ heterozygous mice contain one allele of reporter gene and one wildtype ROSA26 allele. ROSA26lacZ mice livers were isolated and purified as described (Materials and Methods). Genomic DNA was digested using specific blunt-end restriction enzymes: *EcoRV*; *StuI*; *MscI* etc. Next the GenomeWalker libraries were constructed. The GenomeWalking PCRs were set up according to manufactor's instructions with slight modification (Materials and Methods) using ROSA26LacZ-Transgene-Locus-specific primers: rosa15 and rosa16. The amplicons of PCR products were analyzed on 1.0% EtBr/Gel. Many test runs were performed to establish the proper thermo-cycles for Touch-Down PCR programs. Amplified fragments were gel-purified and subjected to sequencing. To illustrate the genomic loci sequences, which could be used to identify the genomic loci of transgene integration by blasting the public genome Databases: NCBI, UCSC and Celera System. After analyzing the sequencing trace data using SeqMan (LaserGene V6, USA) program, the ROSA26 locus could be identically matched in all public genome Databases mentioned above.

Transgenic *Tg*^{GFPTETO7lacZ}, *Tg*$^{lacZTETO7gfpGluR-A}$ and *Tg*$^{lacZTETO7gfpGluR-A(SA)}$ positive mice were then analyzed similarly by Genome Walking using beta-globin polyA specific primers (G03/G04 and G08/G09) and human growth hormone polyA specific primers (H03/H04). The precise location of the integration site was mapped in Celera (ABI, USA) and public genomic Database.

2.4.4 Transgene Integration Sites were confirmed

Several well studied transgenic mice models were analyzed by GenomeWalking, and the according transgene integration sites were matched to the public genome Databases. To confirm the results, specific primers flanking the genomic sequences of the integration site were designed. The FP- and RP-primers com-

bined with transgene-specific primers used in detecting the integration sequences were subjected in PCR reactions. Amplification products were purified and sequenced, and analyzed using SeqMan (LaserGene V6, USA) program with matching Reference Sequences. All detected transgene integration sites could be confirmed by PCR and sequencing, and the predicted cutting pattern of Southern blot data were also matched to band-pattern in most of negatives shown in Southern blot analysis.

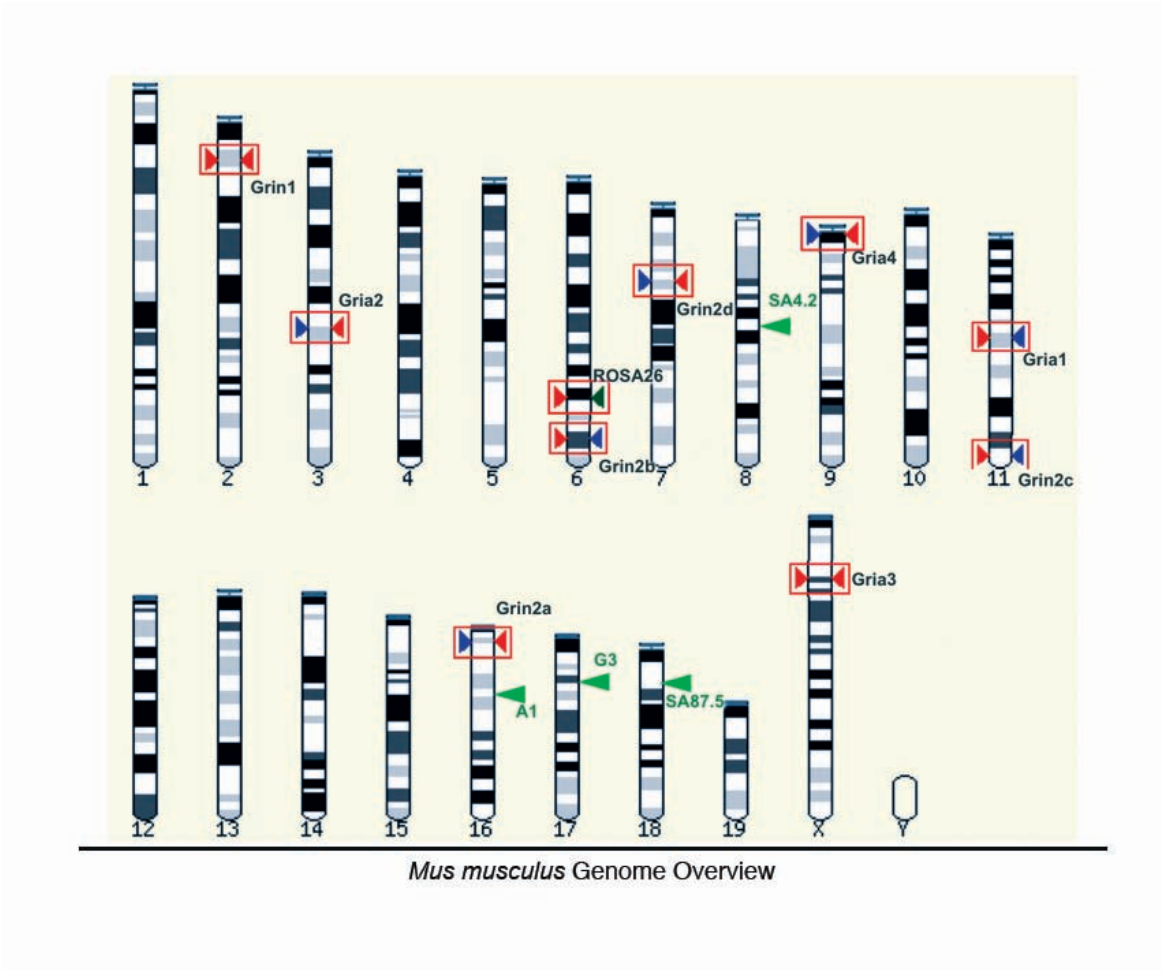


Figure 8. Genome overview of the integration sites for transgenes: *Tg*$^{lacZTETO7GFPGluR-A}$ (A1), *Tg*^{GFPTETO7LacZ} (G3) and *Tg*$^{lacZTETO7GFPGluR-A(SA87.5)}$ (SA87.5), *Tg*$^{lacZTETO7GFPGluR-A(SA4.2)}$ (SA4.2) transgene integration sites with other highlighted gene loci. Important genes for the lab are given as reference: NR1: NMDA Receptor subunit 1; NR2A, NR2B, NR2C and NR2D: NMDA Receptor subunit 2 A, B, C and D; GluR-A, -B, -C and -D: glutamate AMPA Receptor A, B, C and D; ROSA26: Gt(ROSA)26Sor locus;

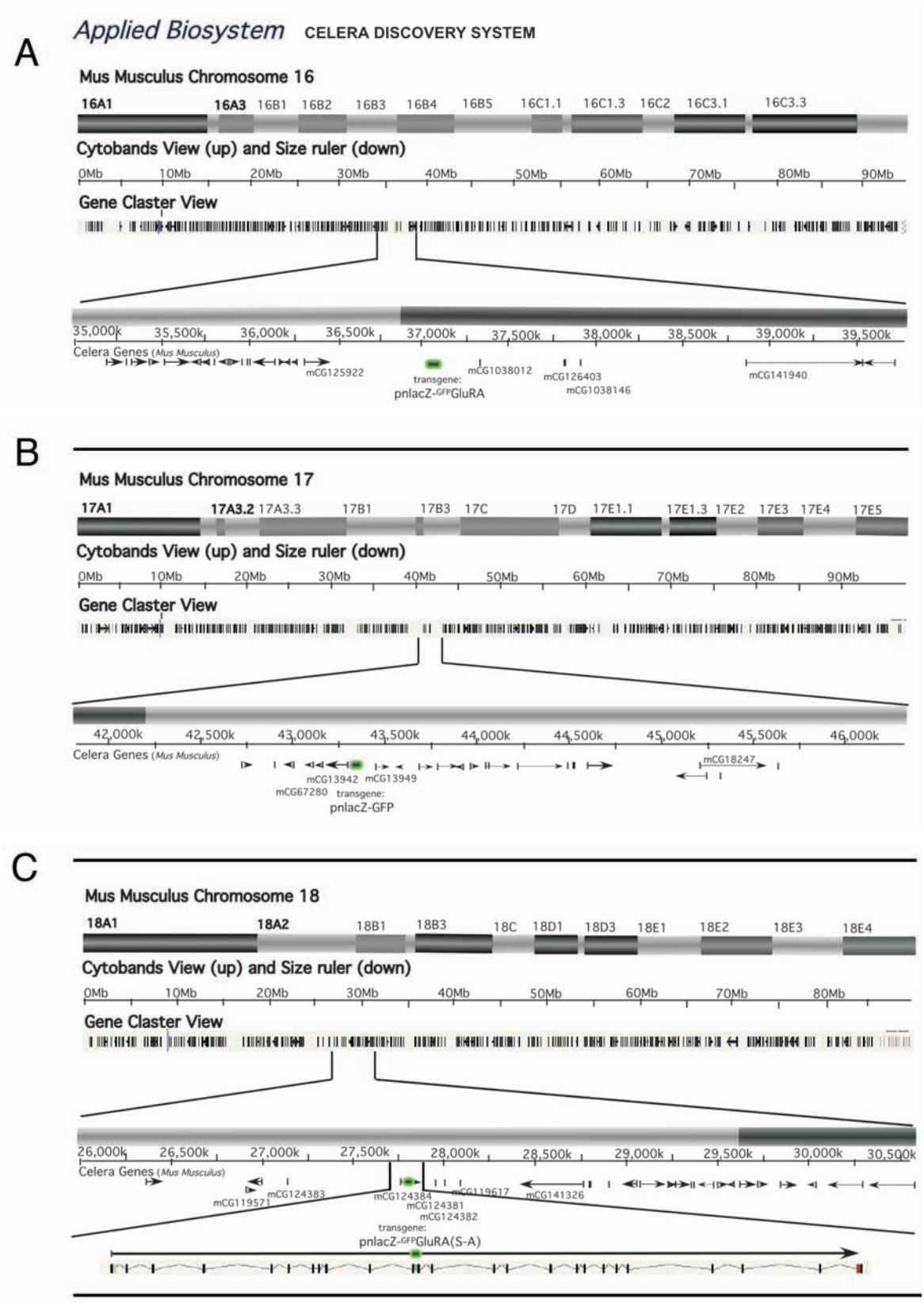


Figure 9: Genome location of *Tg*$^{lacZTETO7GFPGluR-A}$, *Tg*^{GFPTETO7LacZ} *and Tg*$^{lacZTETO7GFPGluR-A(SA87.5)}$ transgene integration loci (*Applied Biosystem* Celera Discovery System). A: *Tg*$^{lacZTETO7GFPGluR-A}$ transgene was integrated into Chromosome 16 at 37,106,406 bp position, near

gene mCG1038012 (Immunoglobulin superfamily, member 11) and mCG1038012 (Pseudo gene predicted by computer software, no transcripts or protein were detected so far)**; B:** *Tg*^{GFPTETO7LacZ} transgene was incorporated into Chromosome 17 at 43,287,531 bp, near gene mCG13942 (CD2 associated protein) and mCG13949 (Tumor necrosis factor superfamily, member 21)**; C:** *Tg*$^{lacZTETO7GFPGluR-A(SA87.5)}$ transgene was inserted into gene mCG124384 (RIKEN cDNA 5330434F23 gene. PI3 kinase family member), on Chromosome 18 at 27,772,916 bp position;

The results show that *Tg*^{GFPTETO7lacZ} transgene locus is at position: 43,287,531 bp on Chromosome 17 (Figure 8 and Figure 9B); *Tg*$^{lacZTETO7gfpGluR-A}$ transgene locus was found on Chromosome 16 at 37,106,406 bp (Figure 8 and Figure 9A); *Tg*$^{lacZTETO7gfpGluR-A(SA)}$ transgene locus position is on Chromosome 18 at 27,772,916 bp (Figure 8 and Figure 9C). The exact positions of the integration loci are given according to the scaling in *Applied Biosystem* Celera Discovery System (year 2003-2005).

2.4.5 Homozygosity of the Transgenic Mouse Lines.

To discover the potential importance of the integrated gene loci and to reduce the workload of keeping the transgenic mouse lines, transgene homozygosity identification was carried out using integration sites flanking FP- and RP-primers. For *Tg*^{GFPTETO7lacZ} (G3) line, specially designed transgene integration site specific primers were used to genotype the offsprings (Figure 10A and 10B). Three homozygous out of 18 offsprings were confirmed to be *Tg*^{GFPTETO7lacZ} (G3) homozygous (Figure 10C and 10D). However, the homozygous *Tg*^{GFPTETO7lacZ} animals die between P15 and P42. The reason for death is unclear, since the transgene cassettes integrated into a 50 kb long stretch non-coding genomic DNA. Whether the expression of the neighbouring loci: are affected remains to be worked out.

For *Tg*$^{lacZTETO7gfpGluR-A}$ line, the transgene integrated into a long non-coding genome region: Chromosome 16, position 37 mega bp. Though the integration happened in non-coding region, we could not manage to get any homozygous offsprings from *Tg*$^{lacZTETO7gfpGluR-A}$ line. For line *Tg*$^{lacZTETO7gfpGluR-A(SA)}$, PCR reactions with junction primers recognizing both the transgene- and wildtype allele genomic sequence, revealed no rearrangement in the wildtype allele, although about 100-200 nucleotide inverted short copy from the genomic sequence at the junction of transgene-genome boundaries could be detected.

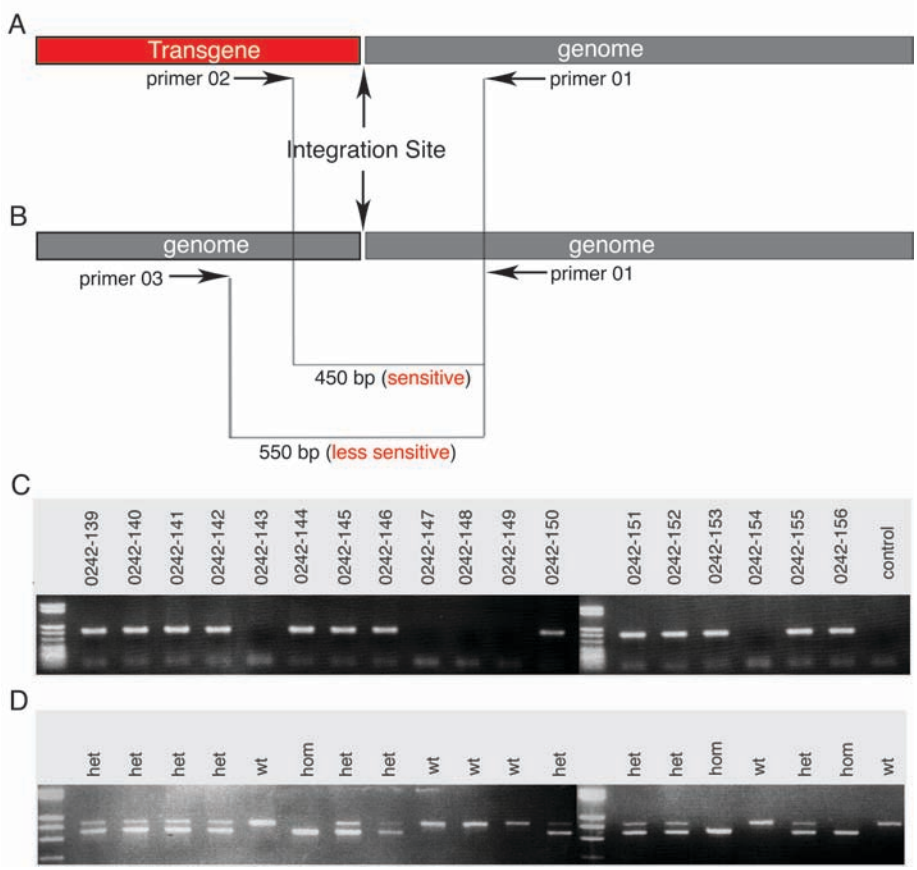


Figure 10. Specifically designed primers could efficiently detect homozygous *Tg*^{GFPTETO7LacZ} transgenic mice. A: Schematic transgene allele shows integration junction and the flanking primers: primer 01: genome specific primer; primer 02: transgene specific primer. **B**: Schematic wildtype allele shows two genome specific primers flanked integration site. **C**: Genotyping PCRs using normal transgene specific primers detect either transgenic (positive) or wildtype (negative) indivaduals. **D**: Complex homozygousity-genotyping PCRs using primer 01, 02 & 03 detect wildtype, hetrozygous and homozygous *Tg*^{GFPTETO7LacZ} individuals.

2.5 Deliver new Tet-responsive cassette into transgene integration site of *Tg*$^{lacZTETO7gfpGluR-A(SA)}$

After the *Tg*^{GFPTETO7lacZ}, *Tg*$^{lacZTETO7gfpGluR-A}$ and *Tg*$^{lacZTETO7gfpGluR-A(SA)}$ transgene integration loci were detected by GenomeWalking and confirmed, we decided to target the locus with new but a sequence comparable Tet-responder cassette to analyze if retargeting with a different responder cassette gives comparable results. Among the analyzed transgenic mouse lines, *Tg*$^{lacZTETO7gfpGluR-A(SA)}$ transgenic mice showed the highest level of responder gene expression induced by *Tg*$^{CamKIIα\text{-}tTA}$ mice, and low-copy number of transgenes were integrated in simple tandem repeats pattern. Therefore, the *Tg*$^{lacZTETO7gfpGluR-A(SA)}$ locus might be a

good candidate for generating transgenic mice by locus specific single-copy Knock-In experiments.

2.5.1 *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$-Transgene-Integration-Locus is gene: *Pik3c3*

As mentioned above, *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$ transgene was integrated into Chromosome 18 at position : 27,772,196. It is inserted in the 11th intron of gene *Pik3c3*, which belongs to PI3 kinase family, subfamily 9. The function of the protein is not defined up to now. However, till now we could not obtain any *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$ homozygous transgenic mice, which might indicate the important function of Pik3c3 during the early stage of development. Though the protein function was not well investigated, the mRNA expression was investigated in details in different tissues and through the developmental stages (Figure 11; GNF database), which indicates that the *Pik3c3* gene locus is active (see below), therefore should be accessible in most adult mice brain regions.

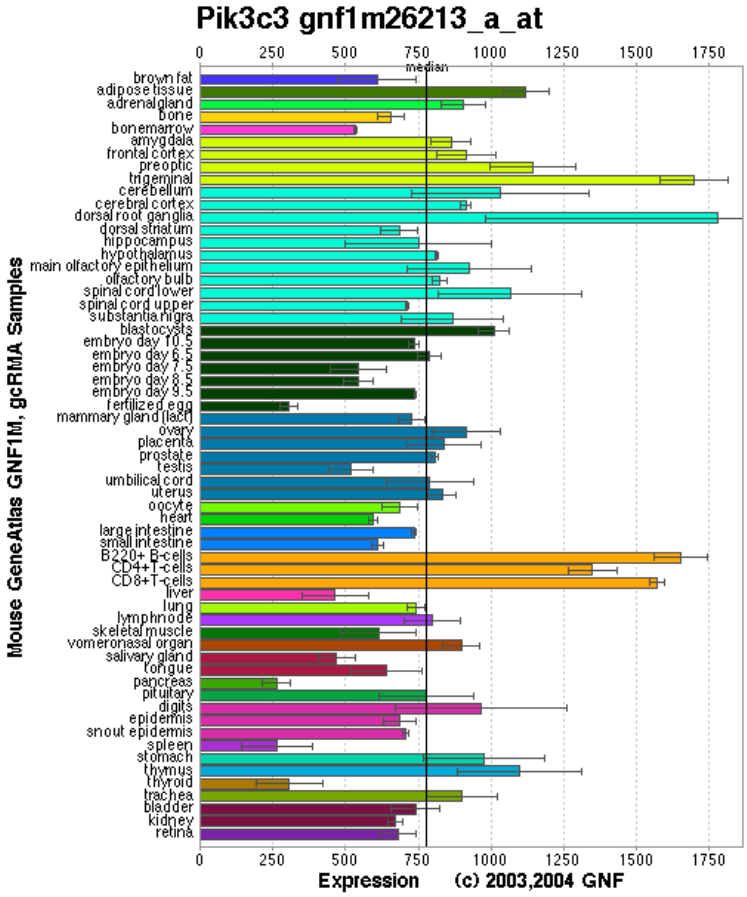


Figure 11: *Pik3c3* gene expression atlas (adapted from GNF Mouse Gene Atlas library). Relative expression intensity of mRNA detected in various tissue extracts. Here shows that gene *Pik3c3* is actively expressed in all samples, with brain subregions and B-cells outstanding.

2.5.2 Expression Analysis of Endogenous *Pik3c3* gene by *in situ* Hybridization

The GNF data base has shown that the mRNA of Pik3c3 is expressed in various regions of adult mouse brain. However, the data base does not indicates the anatomical details, which might be important information for brain-specific transgenic mouse model generation. Thus, *in situ* hybridization was carried out to illustrate the endogenous expression profile of *Pik3c3* mRNA. Oligonucleotide probes were designed specifically binding to exon 11, exon 14 and the junction of exon 14 and 15, respectively. And NMDA Receptor 1 specific Oligonucleotide probe was used as positive control for the hybridization procedure (Figure 12A). *In situ* hybridization using exon 14 specific probes showed that the mRNA of gene *Pik3c3* is expressed in olfactory bulb and most brain regions, including hippocampus, cortex and cerebellum (Figure 12B). These results demonstrate that the *Pik3c3* locus is active in various tissues, therefore it might be accessible for tTA trans-activation in the adult mouse brain. The results also fit to the the GNF data showing the mRNA expressed in most stages of mouse development and regions of the mouse brain.

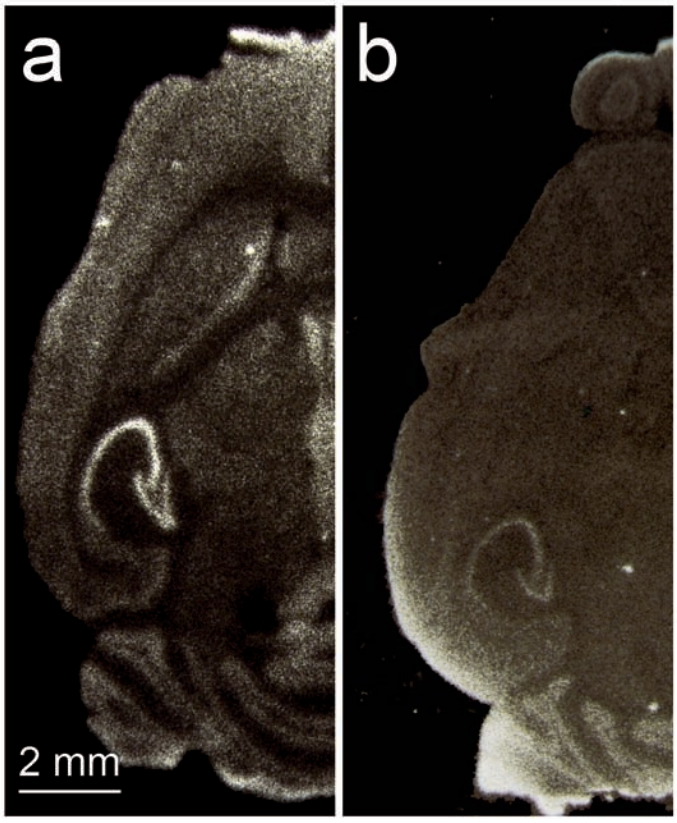


Figure 12: Horizontal *in situ* hybridization was performed to show the anatomical expression atlas of *Pik3c3* gene. A: Positive control was performed in parallel by NMDA Receptor 1 oligonucleotide probe. **B:** Oligonucleotide probe against exon 14 was shown here, which illustrates the expression of *Pik3c3* in hippocampus, cerebellum and cortex.

2.6 Targeting constructs were made for *Pik3c3* locus

Next we carried out experiments to target the locus with new transgene constructs by homologous recombination in ES cells. First, specific primers flanking the locus of 1.0 kb genomic sequence were designed and PCRs were performed to amplify the genomic sequence from both C57Bl6 wildtype mice and SV129/K1 ES cell. The PCR products were subjected to sequencing reactions to determine the Single-Nucleotide-Polymorphism (SNP). The trace data shows that there is no SNPs detected in 1.0 kb of genomic sequence, neither among 3 individual C57Bl6 wildtype mice, nor between C57Bl6 and SV129/K1 ES cell genome. So the Pik3c3 locus will be a perfect locus for ES targeting in SV129/K1 ES cell line using PCR amplified homologous arms.

Second, the BAC (bacterial artificial chromosome) clone: RP24-185E11, containing the *Pik3c3* locus genomic sequence was ordered from BACPAC company (USA). By PCR reactions and sequencing, using Pik3c3-locus-specific genomic primers, the identity of the BAC was verified .

Third, the homologous arms of 6.7kb-longarm and 2.2kb-shortarm used for targeting vector were obtained from PCR reactions using the BAC as template sequence. A multiple cloning site was introduced into the vector containing two homologous arms. Before further processes, the homologous arms cloned into pLongShortArmsTK were sequenced and analyzed by specific restriction enzymes. Sequencing reactions were performed and sequencing results showed that there are no mutations from either the PCR reactions, or cloning procedures.

Fourth, a tet-reporter-transgene-cassette was cloned into the targeting vector between the short- and long-arm. The tet-reporter transgene cassette was comparable: pnlacZ-tetO7-^{GFP}GluR-A(flip) to the transgenic insertion in *Tg$^{lacZTETO7gfpGluR-A(SA)}$* and had only 3 nucleotides differences. Both cassettes express reporter genes under the control of bi-directional CMV minimal promoters. The reporter β-Galactosidase, and ^{GFP}GluR-A as wild-type protein (new cassette) or as C-terminal mutant (*Tg$^{lacZTETO7gfpGluR-A(SA)}$* line cassette).

After the modification of the backbone sequence, plasmid pnlacZ-^{GFP}GluR-A(i) was cut by *Hind III* and *Ase I,* which released the nlacZ-^{GFP}GluR-A(i) fragment. The nlacZ-^{GFP}GluR-A(i) DNA fragment was cloned between PacI and AscI

sites of the modified pBackbone (Figure 13a). After that, the nlacZ-^{GFP}GluR-A(i) fragment was released by *PacI* and *AscI*, and cloned into linearized, PacI, AscI digested pLongShortArmsTK, which carries the homologous arms for targeting to generated the targeting vector (Pik3c3-A1.1) (Figure 13b).

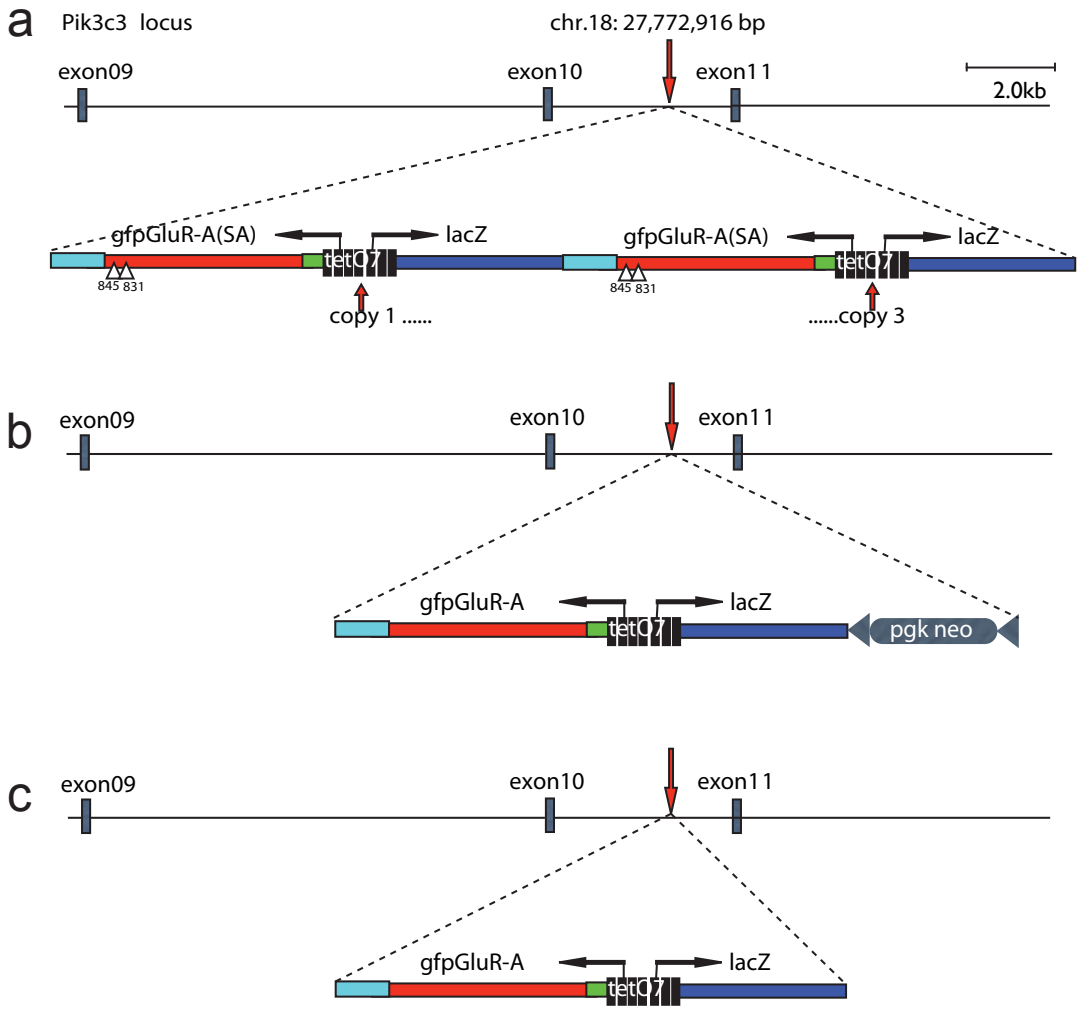


Figure 13: Schematic views are shown to illustrate the high homology of two constructs: Minigene pLacZ-tetO7-GFPGluR-A(S843A & S831A) of transgenic SA87.5 mice was shown in (**a**). Pik3c3-A1 targeting construct: pLacZ-tetO7-^{GFP}GluR-A with neo-selcetion marker (**b**) was delivered into *Pik3c3* locus. The new construct: pLacZ-tetO7-^{GFP}GluR-A has only 3 nucleatides difference in the C-terminal of GluR-A coding region after neo removal (**c**).

2.7 Embryonic Stem Cell Targeting

2.7.1 PCR Selection of neo-Resistant ES-Colonies

The targeting construct: Pik3c3-A1.1 was linearized by NotI and electroporated into ES cells (Materials and Methods). After 8 days of neomycin selection, the

neo-resistant colonies were isolated and expended. Small amount of DNA was extracted. Before PCR analysis, the quality of the genomic DNA was controled on 1.0% agarose gel (Figure 14). According to the targeting strategy, PCR primers were designed flanking the short-arm of the targeted locus (Primer FP-SA-400 and RP-SA-400). Long-template PCR were performed using these primers to identify the targeted ES clones by showing the expected PCR amplicons with 2.6 kb in size (Figure 14). From 250 ES colonies analyzed, twelve PCR-positive colonies: clone C-G1, clone D-D3 for example were further expended and DNA was extracted and purified for Southern blot analysis (Figure 15).

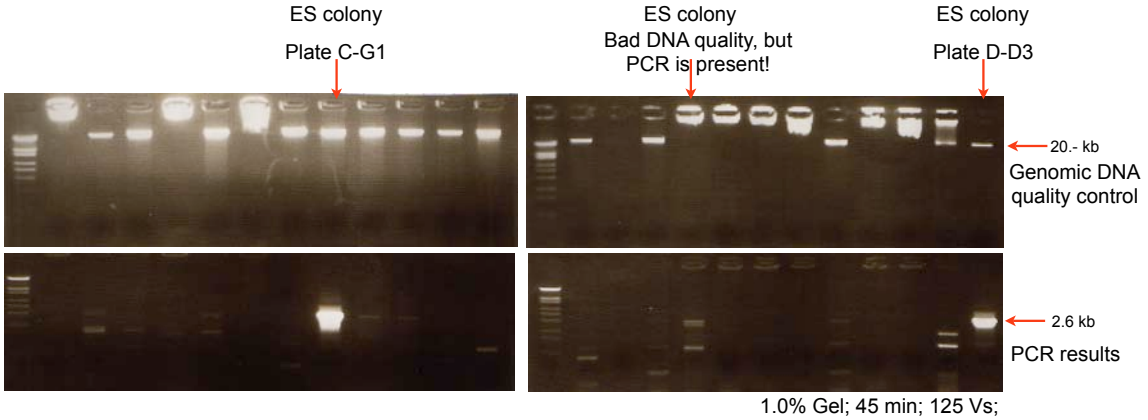


Figure 14: Genomic DNA extracted from different ES clones was checked on gel and PCR identified correctly targeted clones.

2.7.2 Southern-Blotting Analysis of PCR-Positive Colonies

Since PCR analysis of the ES colonies could only give one hint that the *Pik3c3* locus was correctly targeted. Southern blot analysis was carried out on the genomic DNA of PCR-positive colonies to confirm there is only one correctly targeted transgene copy. The genomic DNAs were digested using BglII and AflII restriction enzymes (Figure 15a). Southern blot probe was located between primer pair neo04 and neo05 on the junction of the loxP-pgkneo-loxP and 2.0kb short arm (Figure 15a). The specific size and pattern of the Southern blot showed the correctly targeted ES colonies, which might be ready for generation of mice by Blastocyte ES cell injection (Figure 15b).

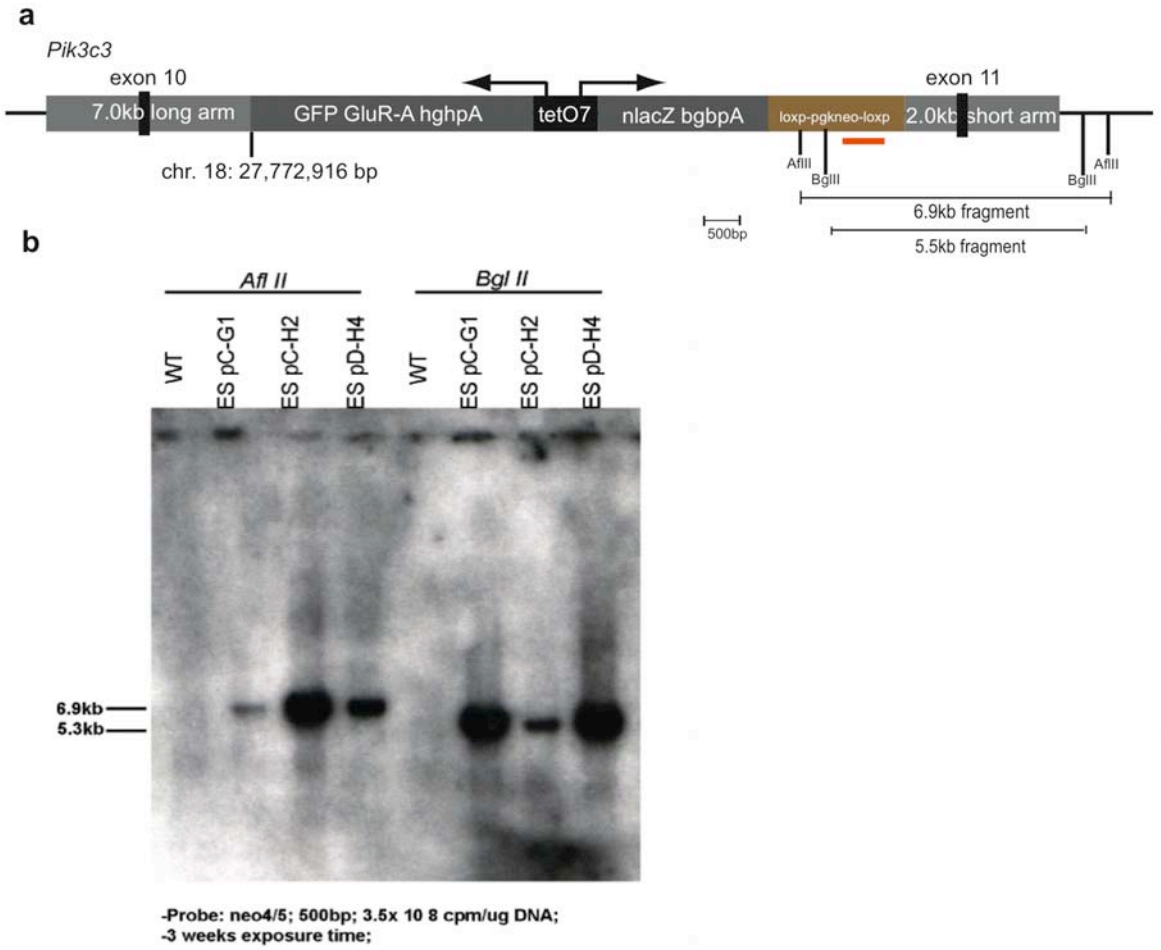


Figure 15: Targeting constructs were designed with Long- and Short-homologous arms, and Southern-Blot analysis was performed to verify the PCR selected ES clones. Schematic view of the targeted allele was shown in (**a**) with Afl II and Bgl II (both are external probe-pattern) located beneath showing the Southern blots band size. Southern blot negative was developed after 3 weeks exposure, showing specific bands at 6.9 kb and 5.3 kb for Afl II and Bgl II, respectively (**b**).

2.7.3 Cloning of AAV-itTAnls virus for fast application of itTAnls *in vitro*

Before the blastocyst injection of correctly targeted ES colonies, it is important to know if the positive ES clones could function *in vitro* as tTA reporter transgene, which had been targeted into Pik3c3 locus of the positive ES colony. For this purpose, we developed recombinant Adeno Associated Viruses (rAAV), which were constructed to express improved tTA (itTAnls) described previously. The itTAnls was cloned into pAAV-MCS (Figure 19) to get rAAV-cmv-itTAnls. The rAAV-cmv-itTAnls virus was examined and titrated in HEK293FT cell culture before applications. rAAV-cmv-itTAnls virus (5 μl) was added into the ES cell culture with gentle

shaking. After 3 days incubation, the ES cell culture was washed twice with 1x PBS buffer and fixed by 4% PFA solution for 10 min at RT. After washing with 1x PBS, enzymatic staining was performed using X-gal solution at RT overnight (Figure 20). It showed that the targeted ES colony could be induced by tTA *in vitro*. This demonstrated the tetracycline-responsive gene construct was functional. Then, the targeted ES colony was prepared as described in Materials and Methods for blastocytes injection to generate mouse line, named Pik3c3-A1. Fourty injections were performed to generate Pik3c3-A1 mice. Four high chimeric mice were born and the targeted locus was transmitted in 2 founders. The mice were identified by coat color and genotyping PCR using transgene- and integration-locus-specific primers, respectively.

2.8 Analysis of Pik3c3-A1 mice with transgenic tTA mouse lines

Next we asked whether we could obtain the same expression pattern and intensity of the targeted mice using GFPGluR-A when compared to the transgenic line *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$. For this, Pik3c3-A1 mice were crossed to *Tg*$^{CamKII\alpha\text{-}tTA}$ and/or *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$ founders and compared with *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$/*Tg*$^{CamKII\alpha\text{-}tTA}$ (or *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$) double positive mice.

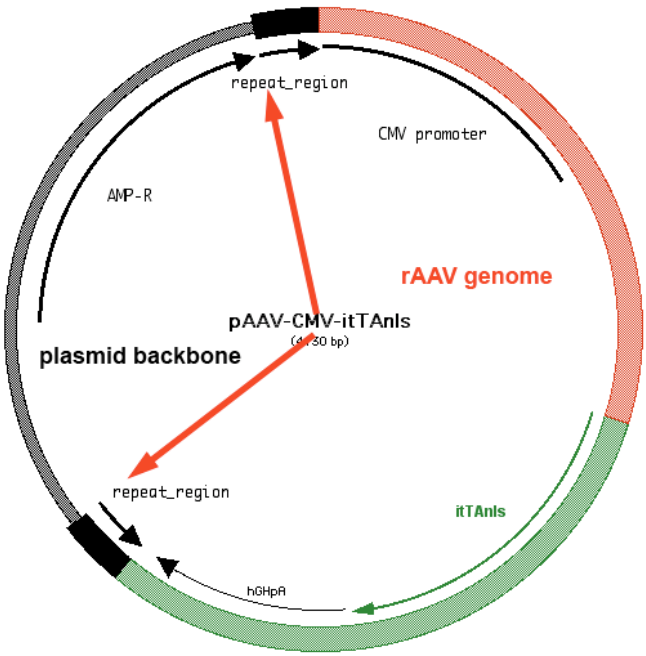


Figure 16: rAAV-CMV-itTAnls was cloned. nlstTA was liberated cloned into pAAV-MCS vector to generate recombinant AAV 'gutless' constructs for virus production.

2.8.1 Analysis of Pik3c3-A1 mice bred with *Tg*$^{CamKII\alpha\text{-}tTA}$ (8.5kbCaMKII-tTA) transgenic mice

After one generation of back-crossing to C57Bl6, the positive Pik3c3-A1 mice were bred with *Tg*$^{CamKII\alpha\text{-}tTA}$. Double positive mice were sacrificed and brains were analyzed by immunohistochemistry. The brain slices were stained against reporter gene products by polyclonal rabbit anti β-galactosidase using DAB staining methods. It was, however, disappointing that only small fraction of striatum cells were positive (Figure 17 A 1-5), which indicated the low expression of transgene under *Tg*$^{CamKII\alpha\text{-}tTA}$ induction. There were no signals visible in other brain regions. The results showed that Pik3c3-A1/*Tg*$^{CamKII\alpha\text{-}tTA}$ carrying neo-selection marker gene did not inherit the expression pattern and intensity of *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$/ *Tg*$^{CamKII\alpha\text{-}tTA}$ mice.

Thus, the presence of the Neo selection marker might inhibit the tTA-dependent gene regulation. We supposed that the neo selection marker hinders the expression of nearby promoters due to epigenetic silencing of the P_{tet}-bi transgene cassettes. Removal of the neo-selection marker is therefore necessary for a site by site comparison.

2.8.2 Reporter gene expression pattern of Pik3c3-A1 mice showed no significant difference when bred with *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$ transgenic mice compared with *Tg*$^{CamKII\alpha\text{-}tTA}$ mice

Furthermore, we would like to know whether extremely high level of nuclear localized tTA would rescue the expression levels and patterns in Pik3c3-A1 mice. Pik3c3-A1 mice were bred with *Tg*$^{(Thy\text{-}1.2\text{-}itTAnls)64Rsp}$, one of the highest tTA-expressing founders. Pik3c3-A1/*Tg*$^{(Thy\text{-}1.2\text{-}itTAnls)64Rsp}$ double-positive mouse brains were analyzed. The results were comparable to Pik3c3-A1/*Tg*$^{CamKII\alpha\text{-}tTA}$ reporter gene expression, that is, no enzymatic β-gal activity was observed in cortical neurons (Figure 17 B 1-5). Therefore, the results further support the hypothesis of epigentic silencing of Pik3c3-A1-neo transgene cassette due to the presence of the neo-selection marker gene. Despite of high cellular tTA levels in cortical neurons, in the presence of neo gene, Pik3c3-A1 did not show detectable reporter gene expression in cortical- and hippocampal neurons.

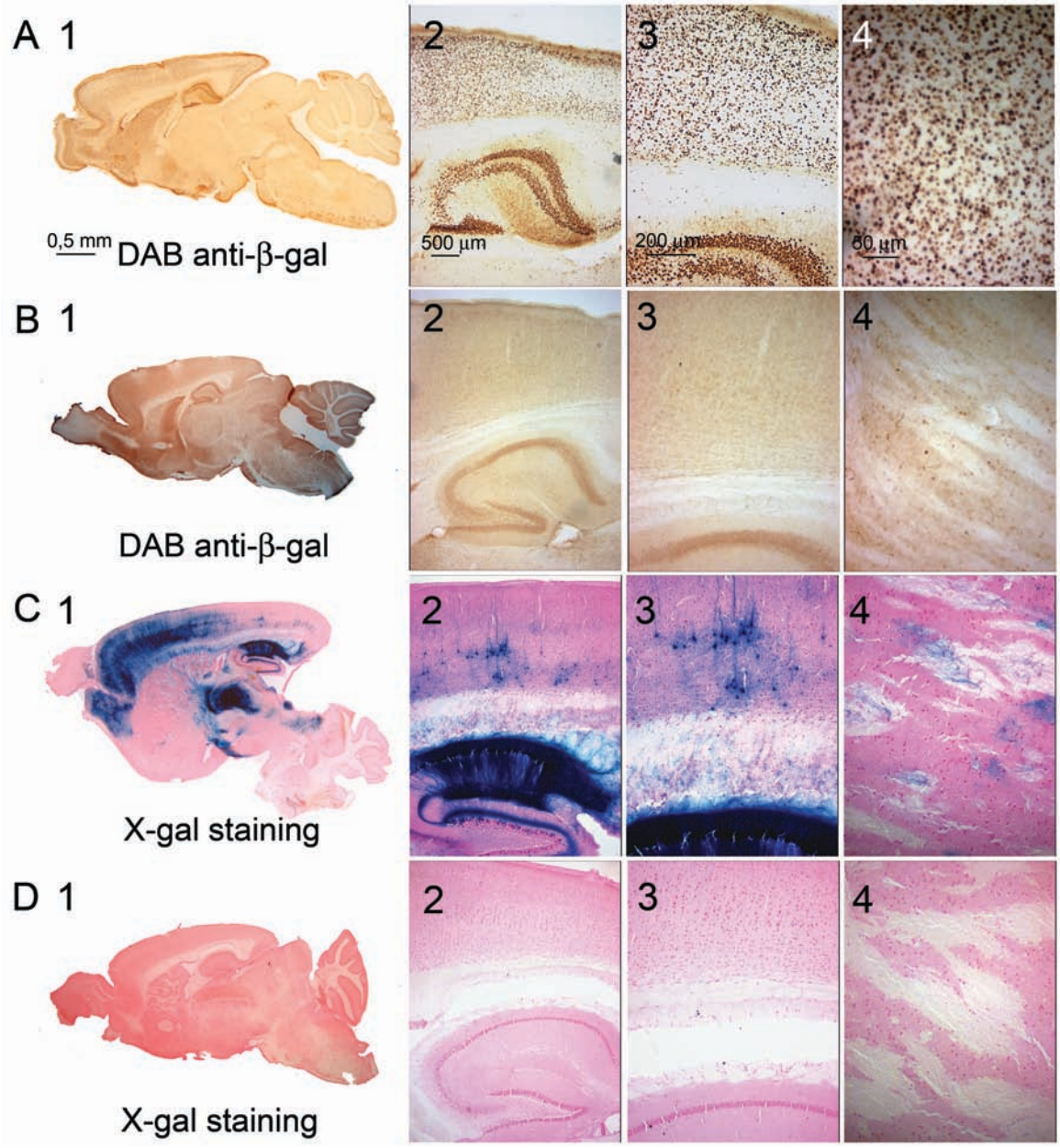


Figure 17. Immunohistochemstry and enzymatic activity analysis of *Tg*$^{CamKII\text{-}tTA}$/SA87.5, *Tg*$^{CamKII\text{-}tTA}$/Pik3c3-A1, ThyF64/G3 and ThyF64/Pik3c3-A1 double positive mouse brains. Tg$^{CamKII\text{-}tTA}$/SA87.5 mice were analyzed as positive control for nucleus localized β-gal using poly-clonal β-gal antibody immunostainings, many β-gal positive cells were detected in cortex, CA1 and striatum (**A 1-4**); Sparse β-gal positive neurons were observed only in srtiatum of double positive Tg$^{CamKII\text{-}tTA}$/Pik3c3-A1 mouse brain (**B 1-4**) by immunochemical stainings. ThyF64/G3 double positive mice were analyzed as positive control for β-gal enzymatic activity stainings, many β-gal positive neurons were detected in cortex, CA1(**C 1-4**). No β-gal enzymatic activity was observed for overnight stainings (**D 1-4**) in double positive ThyF64/Pik3c3-A1 mouse brain.

2.8.3 Removal of neomycin selection marker gene from Pik3c3-A1 using rAAV-6p-minibiCre-Venus reprogramed epigenetic silencing in *Pik3c3* locus

Next we examined if removal of neo-selection marker gene would reprogram the epigenetic silencing effects in Pik3c3 locus and therefore show enhanced reporter gene expression. To remove the neo marker, we applied rAAV-6p-minibiCre-Venus virus injection into double positive Pik3c3-A1 / *Tg*$^{(Thy\text{-}1.2\text{-}itTAnls)64Rsp}$ mouse brains. tTA expressed from Thy-1.2-itTAnls minigenes in double positive *Tg*$^{(Thy\text{-}1.2\text{-}itTAnls)64Rsp}$/ ROSA26-nlacZ (lacZ indicator mouse) was shown to be able to induce Cre recombinase mediated gene recombination *in vivo* within 10 days (data not shown). Virus rAAV-6p-minibiCre-Venus infected Pik3c3-A1 / *Tg*$^{(Thy\text{-}1.2\text{-}itTAnls)64Rsp}$ mice were analyzed 14 days post-infection. Improvement of gene expression was shown by reporter β-gal activity, which was detected specifically in infected cortical neurons (Figure 18 a-c), but not on the contralateral side of the brain (Figure 18 d-f).

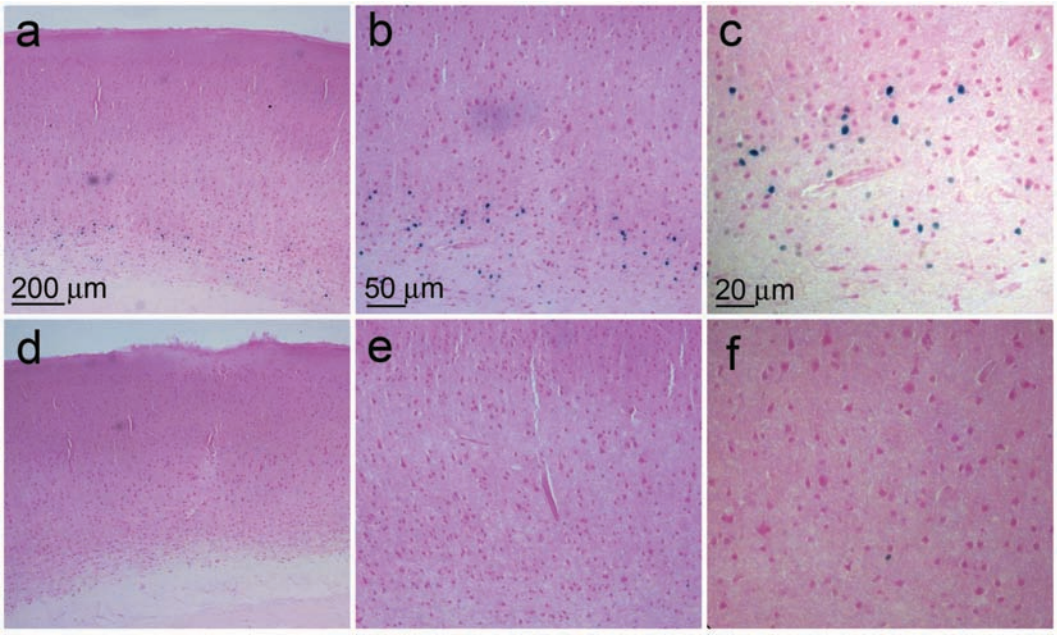


Figure 18. Pik3c3-A1/ThyF64 double positive mice showed improvement of reporter gene expression after neo selection marker gene removal. a-c: In rAAV-6p-minibiCre-Venus infected cortical neurons of double positive Pik3c3-A1/ThyF64 mouse brain, reporter β-galactosidase expression from targeted nlacZ-GFPGluR-A tet-responsive construct was detected; but absent in the uninfected contralateral side (**d-f**).

More interestingly, due to the fact that ThyF64 express tTA specifically in layer 5-6 cortical neurons in somotasensory cortex, the β-gal reporter was detected only in layer 5-6 cortical neurons in somotasensory cortex. Thus, by delivering rAAV-6p-minibiCre-Venus virus into double positive Pik3c3-A1/ThyF64 transgenic mouse brains, we showed Pik3c3-A1 is inducible after neo marker removal.

2.9 Working with recombinant Adeno associated viruses (rAAV)

The mosaic expression pattern observed in all tTA-controlled transgenes, regardless of CaMKIIα or Thy-1.2 promoter, low or high tTA levels in the brain, is supporting the hypothesis of epigenetic silencing of stably-integrated tetracycline-responsive transgenes. The silencing might happen to the integrated transgenes by chromatin reorganizations or by other mechanisms. To answer this question, we used rAAV as Tet-responsive gene delivery system. rAAV had been shown as powerful gene delivering system both in primates and rodents. rAAVs infect a broad range of host cells with high rates and also mediate high levels and long-term expression of virus delivered genes. rAAV has been shown to have no neurovirulence observed relative to high-dose (>10^7 particles) and long-term (2.5-8 months) infection of rAAV. And most importantly, there have been shown as episomal DNA virus, which forms multicopy concatemers, and rarely integrates into the host genome incurring cellular toxicity. Thus, if episomal tet-responder can be activated by tTA, but not the transgenic tet-responders, this could be the ultimate proof that tet-responder transgenes are silenced in absence of transcription activity.

2.9.1 Construction of recombinant Adeno associated viruses

Several tTA and/or rtTAnls expressing rAAV viruses were cloned and tested in dissociated neuronal cultures. First, pAAV-CMV-itTAnls and pAAV-CMV-nlsrtTAnls are made for generation of rAAVs, which express itTAnls or rtTAnls driven by CMV-promoter (Figure 19 a). *In vitro* culture tests showed us that the CMV-promoter could lead relative strong expression of tTA/rtTAnls in cultured non-neuronal cells within 3-10 days post-infection. We further cloned pAAV-hSyn-itTAnls and pAAV-hSyn-nlsrtTAnls plasmids (Figure 19 b) for production of rAAV

expressing tTA driven by human synapsin 1 promoter, which was shown to have strong promoter activity specifically in neurons (Kuegler, 2003). Furthermore, we also developed mini-tet-responder rAAVs. P_{tet}-bi CMV promoters were used to co-express Venus and Cre-recombinase in rAAV (rAAV-6p-minibiCre-Venus) (Figure 19 c). The rAAV-hSyn-itTAnls, rAAV-hSyn-nlsrtTAnls and rAAV-6p-minibiCre-Venus were produced, purified and tested both *in vitro* and *in vivo*. The results described below will show that rAAV-hSyn-itTAnls, rAAV-hSyn-nlsrtTAnls and rAAV-6p-minibiCre-Venus viruses are very efficient and powerful tools in delivering Tet-responsive reporter gene expression systems in the CNS of rodents brains, independent of any transgenic mouse models.

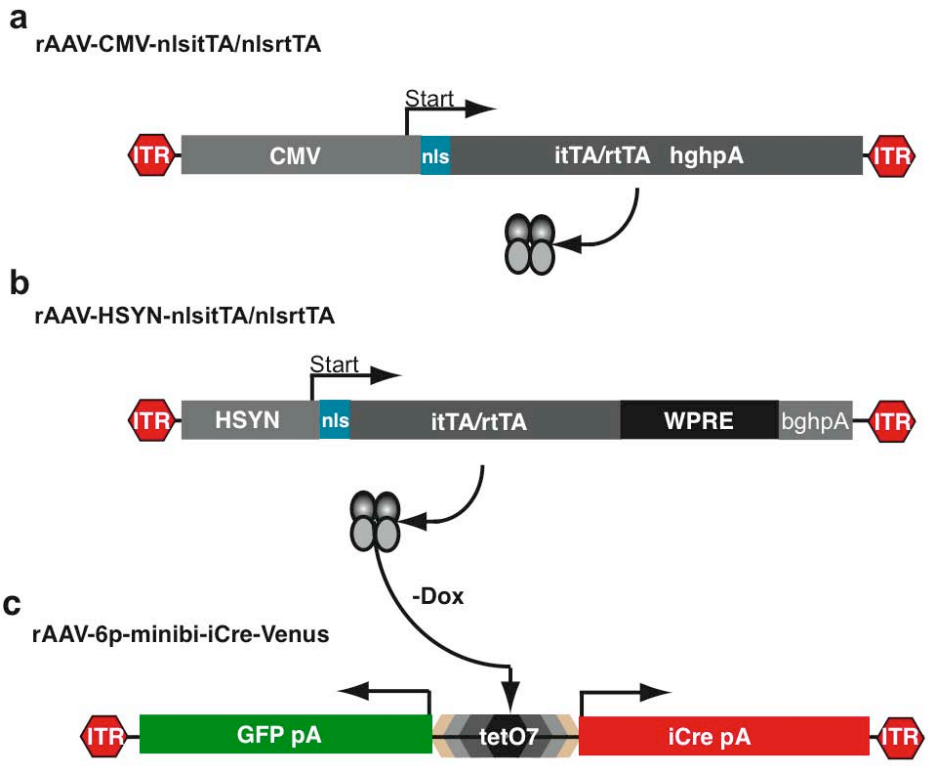


Figure 19: Schematic view of three rAAV constructs. a: CMV promoter was used to drive itTAnls or rtTAnls expression in rAAV-CMV-itTAnls/rtTAnls viruses. b: Human Synapsin1 promoter drives itTAnls or rtTAnls expression in rAAV-hSyn-itTAnls/rtTAnls viruses. c: Ptetbi CMV minimal promoters were used to simultaneously expression Venus and Cre-recombinase in rAAV-6p-minibiCre-Venus virus.

2.9.2 Cross-dressing and purification of rAAV

There are many advantages of using recombinant AAV as gene delivery system in rodent brain, one of them is the purity of the rAAV will not cause any immune response in long term application of the virus (Kuegler, 2003). The most favored rAAV purification method is Heparin-Affinity binding column (Amersham, USA), which has extremely high binding efficiency to serial type 2 rAAV virus capsid. rAAV serial type 2 surface antigen was known to mediate moderate infection in brain compared to other serial types, such as serial type 1, 3 and/or 5. To archive high-titer stock preparation and to reach the maximum infection efficacy in brain tissue, two Helper plasmids, serial type 1 and 2, were co-transfected with rAAV genome vector in HEK293 cells (Invitrogene, USA). Three different mixing ratios of serial type 1 and 2 Helper plasmids were prepared and used to produce rAAV-CMV-Venus, which was shown to have strong expression of fluorescent Venus protein in HEK293 cells. After harvesting and treatment of the crude virus stock as described in Materials and Methods, they were loaded onto pre-casted Amersham Heparin-5ml-column. The purified rAAV-CMV-Venus cross-dressed with three different Dp1:Dp2 ratios were titrated on monolayer HEK293 cells. The Heparin-5ml-column binding efficiency of cross-dresssed rAAV viruses were calculated and compared with previous publications, which showed the same results of about 30 times virus enrichment (10 ml concentrated into 0.3 ml). To further address the infection efficacy of different Dp1:Dp2 mixing ratio, we produced and purified three different cross-dressed rAAV-hSyn-eGFP viruses, and they were tested both *in vitro* in primary neuron cultures and *in vivo* in mouse brain. The results of rAAV-hSyn-eGFP infection in mouse brain demonstrate that rAAV achieves the most efficient infection in brain when cross-dressed with 3:1 of Dp1:Dp2 mixing ratio. The production and purification of mouse brain applicable rAAV viruses are the preliminary steps for any further studies using rAAV in mouse brain. The successful establishments of this method brought us the possibility of introducing Tetracycline-Responsive gene expression system into rAAV.

2.9.3 Titration of rAAV infectious particles

According to cross-dressing tests, all our rAAV viruses were prepared in huge amounts using slightly modified purification methods (see Materials and Methods), and the viruses were aliquoted and stored for later use. Infectious particles of all the virus stocks were titrated in HEK293 cells, primary neuron cultures and in mouse brain, according to the property of the promoter used in rAAV vector, respectively. Pictures covering 0.48 mm^2 of 3-6 Days-Post-Infection (DPI) culture cells were taken, and the total cell number in the picture field is set to 'N_A', the fluorescent cell number is set to 'M_A', the picture area is 'A' and the culture dish area is 'S', the applied virus volume is 'V', which was normalized to one micro litter. The infectious particle number was calculated using equation shown in Figure 20. In addition to infectious particle titration, Dot-Blot- and Real-Time-PCR-Analysis were performed to confirm the results (data not shown). For application of rAAV in mouse brain, we adjust the rAAV titer to about 1.0 x 10^7 particle/μl according to previous studies of rAAV immunological consequences (Kuegler *et al*, 2001).

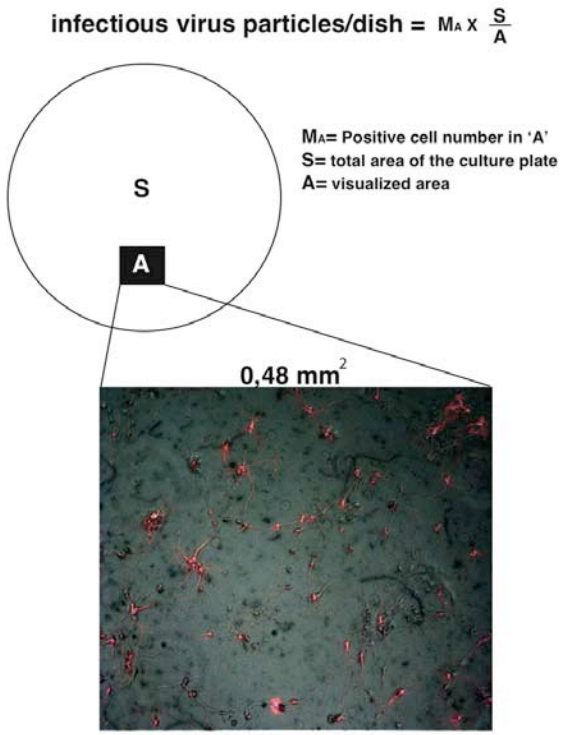


Figure 20: Determination of infectious rAAV particles in cell culture (here in dissociated primary neuronal culutre; rAAV-hSyn-itTAnls and rAAV-6p-minibiCre-tdTomato were titrated). Calculation equation is illustrated, with defined factors: M_A; S; A.

2.10 Combination of transgenic mouse models with recombinant Adeno associated viruses (rAAV)

rAAV has a very small genome size, which dramatically limits the size of the delivered transgene. That means the bulk DNA fragments rendering the promoter cell-type- and or tissue-specificity would not fit into the limiting space of rAAV genome. However, the intrinsic property of the trans-activation systems used for inducible transgene expression brings us alternative ways to expand the usage of rAAV in combination with transgenic mouse technologies.

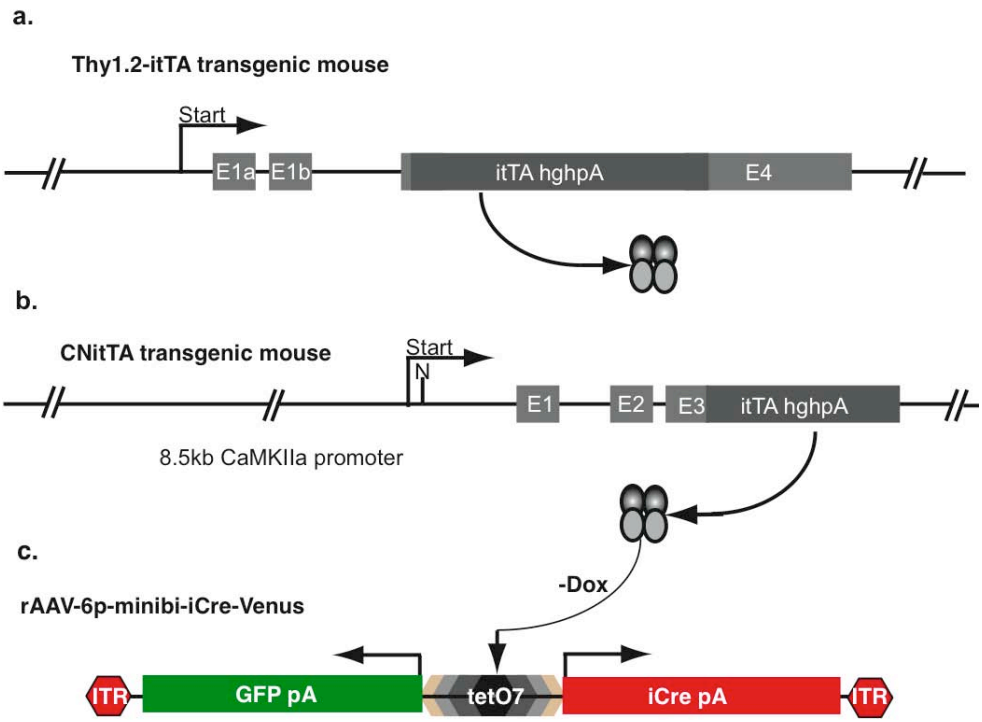


Figure 21: Schematic view of two alternative combinations of transgenic mouse models and rAAV transgene delivery systems. Transgenic *Tg*$^{Thy1.2(xx)\text{-}itTAnls}$ (**a**) and *Tg*$^{CN(xx)\text{-}itTAnls}$ (**b**) mice drives genetically coded tTA expression in the mouse brain. Ptetbi CMV minimal promoters were used to simultaneously expression Venus and Cre-recombinase in rAAV-6p-minibiCre-Venus virus (**c**).

We have generated tTA-expression trangenic mouse lines, such as: *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$ lines (Figure 21 a) (this thesis); CNitTAnls lines (Kim, 2002); Tg$^{CaMKII\text{-}tTA}$ (Figure 21 b) (Mayford *et al*, 1996), which show specific promoter driven expression of tTA in restricted patterns. Thus, gene expression mediated

by rAAV delivered into the transgenic tTA-expressing mice will be restricted in cell-type- or brain subregion-specific manner. This will increase the accuracy of rAAV application. Furthermore, many well characterized Tet-responsive transgenic mouse models are available in the lab, such as: *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$; *Tg*$^{lacZTETO7gfpGluR\text{-}A}$; *Tg*^{GFPTETO7lacZ}; MH-CG07; etc. (Figure 22). These transgenic mice carry Tet-responsive gene cassettes (6-11 kb), which would not be possible to put into the rAAV genome. By delivery of rAAV-tTA expressing viruses, we would be able to express multiple relatively large genes in rAAV invaded neuron populations.

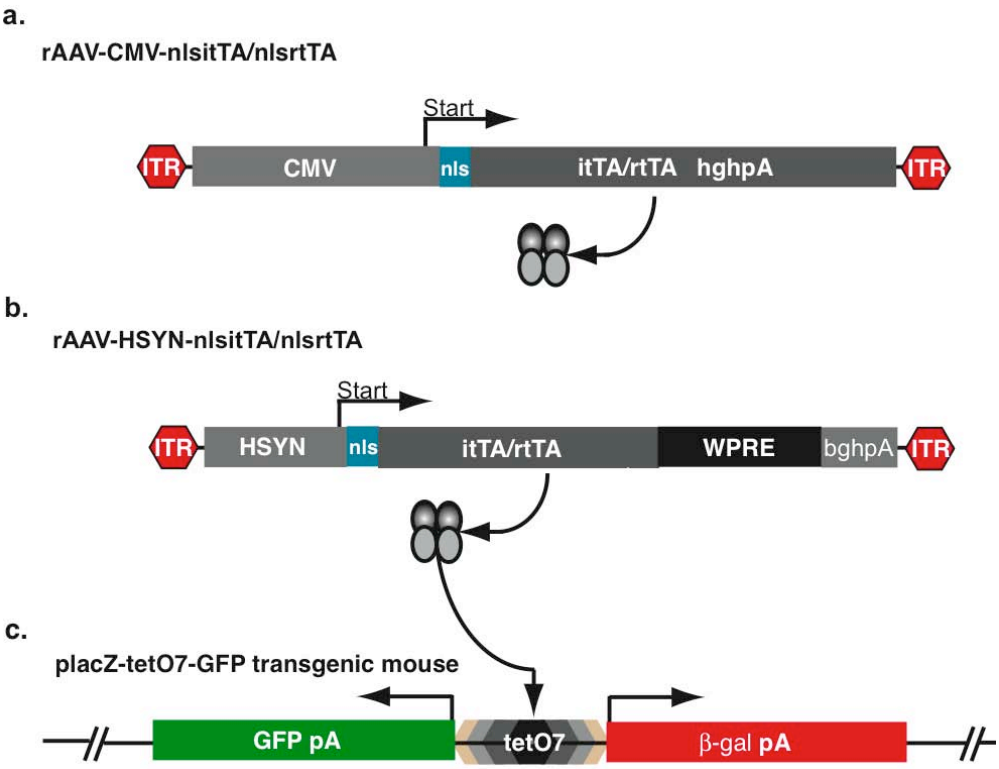


Figure 22. a: CMV promoter was used to drive itTAnls or rtTAnls expression in rAAV-CMV-itTAnls/rtTAnls viruses. **b**: Human Synapsin1 promoter drives itTAnls or rtTAnls expression in rAAV-hSyn-itTAnls/rtTAnls viruses. **c**: Transgenic *Tg*^{GFPTETO7LacZ} mice were used to indicate the rAAV delivered tTA induction in the mouse brain.

Here, we managed by a combination of rAAV, rAAV co-infection and transgenic mouse lines, to transfer efficient tet regulated gene expression system into the CNS of rodents.

2.10.1 Deliver rAAV-hSyn-itTAnls into transgenic Tet-responsive mouse brains

First, we asked whether we could induce transgene expression in Tet-responder mice by delivering tTA-expressing rAAV viruses. Since the rAAV-hSyn-itTAnls/ nlsrtTAnls viruses were generated and tested as described above, through Stereotaxic injection, the rAAV-hSyn-itTAnls viruses were delivered into selected brain areas of various Tet-responder mouse brains: *Tg*^{GFPTETO7lacZ}; *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$; *Tg*$^{lacZTETO7gfpGluR\text{-}A}$, MTH-IP1 and MTH-Cg2-7. Brain slices were analyzed by immunohistochemistry using specific antibodies against GFP and tTA. Among the several tetracycline-responsive transgenic lines and MTH-Cg2-7, only very few cortical and hippocampal neurons showed detectable expression of reporter gene (GFP or related proteins) after rAAV-hSyn-itTAnls infection (Figure 23 a-x). These results are best explained by epigenetic gene silencing of the transgenes. Our data do not exclude that there might be some transgene integration loci still permissive for trans-activation by tTA. However, our data suggest that the mosaic reporter gene expression patterns might be due to the transgene locus, which happened to be specifically silenced by epigenetic silencing programs in adult mouse brain.

2.10.2 Epigenetic silencing programs in CA1 and cortical neurons could be significantly reversed by long-term itTAnls antagonizing efficacy.

The permissive loci such as Tg^{LuciferaseTETO7InversePericam} (MH-IP1) and Tg$^{Luciferase\text{-}TETO7Camgroo2(07)}$ (MTH-Cg2-07) still showed mosaic responder gene expression in cortical neurons and in CA1 pyramidal cells, after short-term (14 days) rAAV-tTA viruses infection. We hypothesized that high itTAnls cellular leves will be able to significantly de-silence some populations of neurons in long-term infection. To test the idea, rAAV-hSyn-itTAnls virus was delivered into two groups of MH-IP1 mouse brains. One group of rAAV-hSyn-itTAnls infected mice were analyzed at 14 DPI. The results showed weak reporter gene expression in some cortical- and CA1-neurons (Figure 24 d-f). However, the other group was analyzed at 75 DPI, which showed significantly increased reporter expression intensity, and also in significantly increase neuronal populations in cortical- and CA1-neurons (Figure 24 a-

c). Thus, rAAV could mediate long-term tTA-dependent gene expression in the mouse brain, and by delivering rAAV-hSyn-itTAnls, we demonstrated that the epigenetic silencing programs in certain neuronal populations could be reversed.

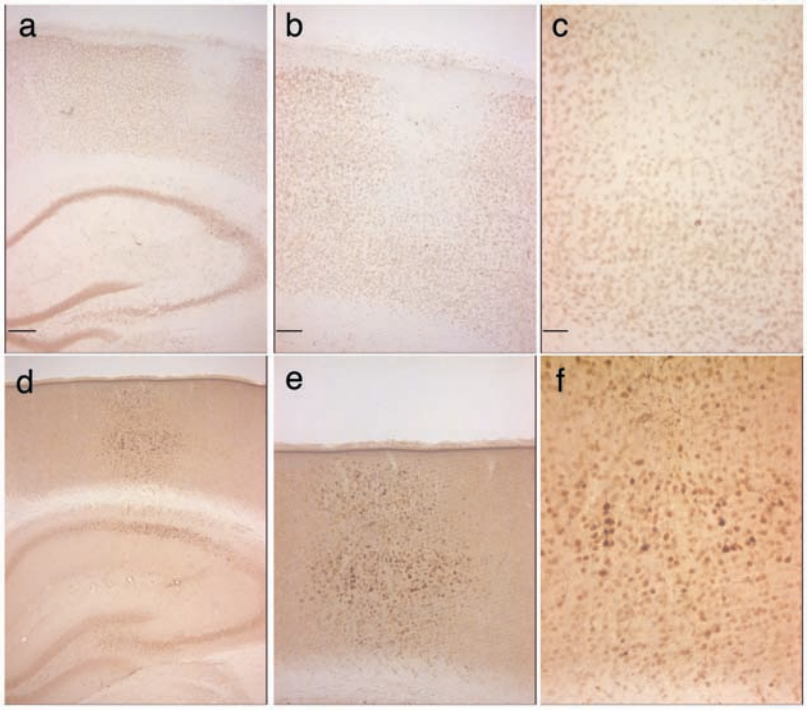
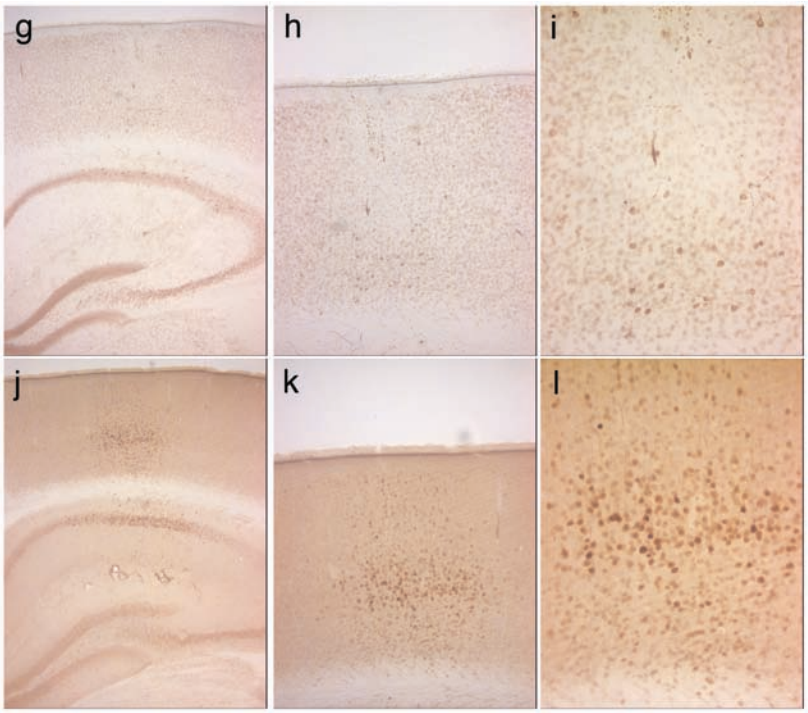
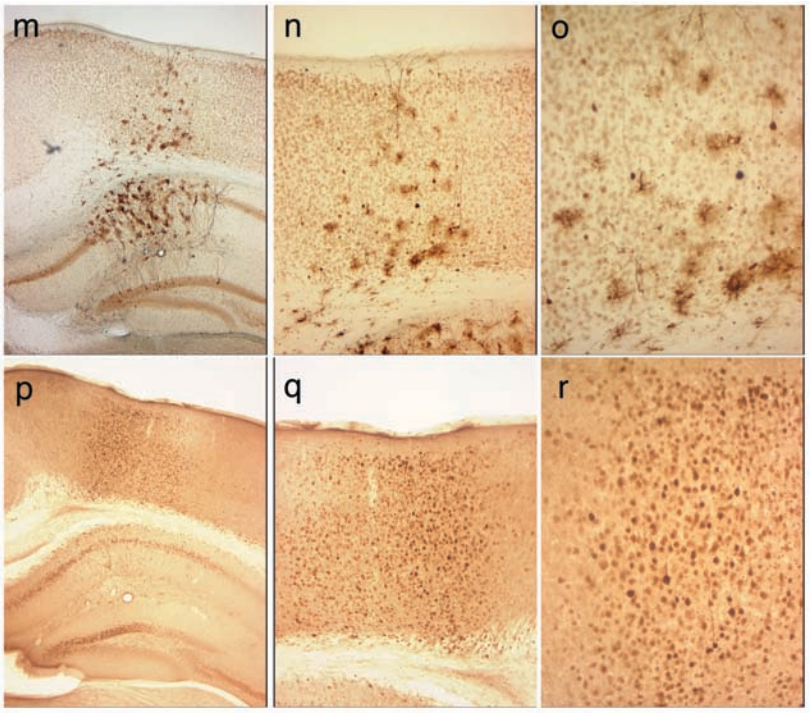


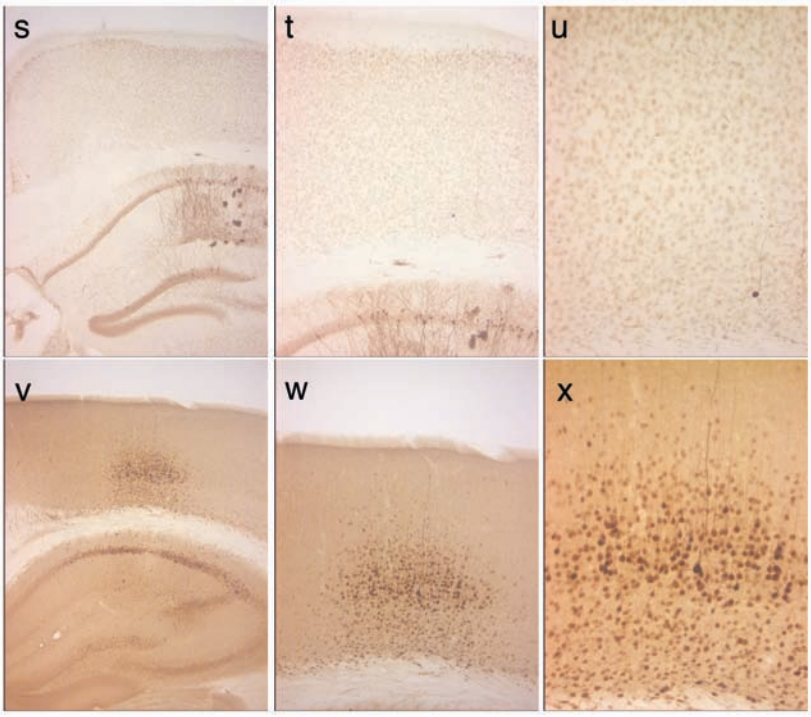
Figure 23. High concentration of transactivator could not activate transgene expression in adult transgenic reporter mouse brains. No ^{GFP}GluR-A expression was detected in cortex (**c**). Very few positive neurons were stained in CA1 and CA2 regions (**a** and **b**). Strong tTA signals were detected in the same infected brain regions (**d**-**f**).



Very low ^{GFP}GluR-A(SA) expression was detected in the cortex (**g** and **i**), and only very few positive neurons in CA1&2 regions (**g** and **h**); but strong tTA signals were detected in corresponding brain regions (**j**-**l**).



Tg^{GFPTETO7LacZ} mice showed low GFP expression in the cortex (**m** and **o**), and only very few positive neurons in CA1 region (**m** and **n**); while strong tTA was present in the same rAAV invaded brain regions (**p**-**r**).



MTH-Cg2-7 mice showed low GFP expression in the cortex (**s** and **u**), and also only very few positive neurons in CA1 region (**s** and **t**); while strong tTA were present in the same rAAV infected brain regions (**v**-**x**).

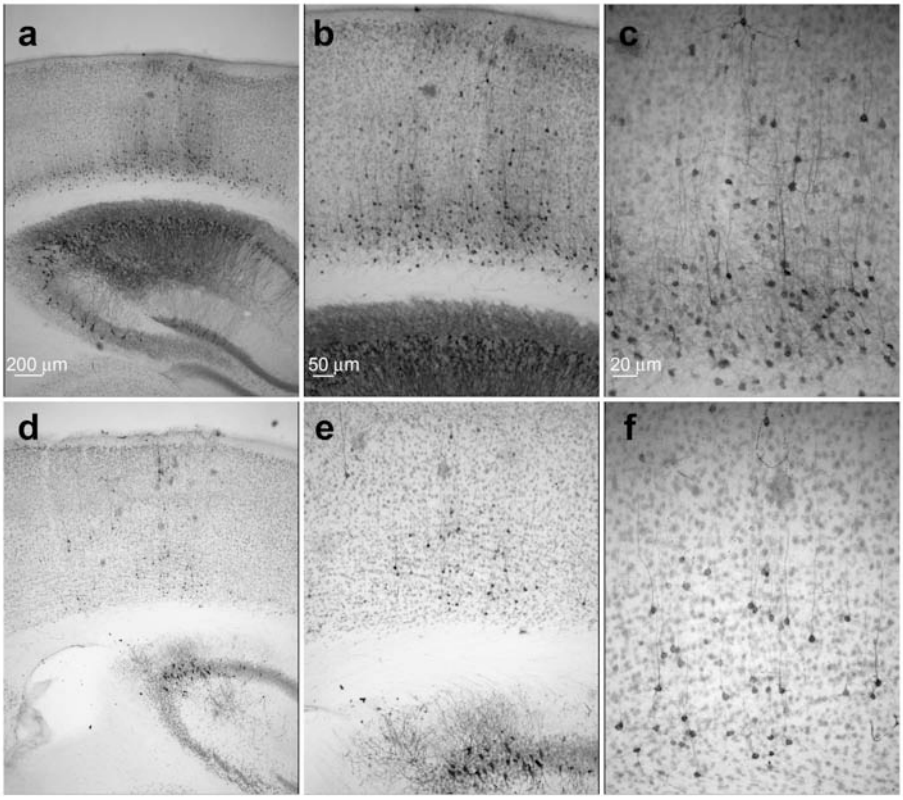


Figure 24. Epigentic silencing effects in cortical neurons of MT-IP01 transgenic mice can be released by high cellular itTAnls. After 14 days of infection, only small numbers of reporter positive neurons were detected in cortex and CA1 regions (**d**-**e**) of first group MT-IP01 mouse brains**;** At 75 days post infection, significantly increased number of reporter positive neurons were detected in cortex and CA regions (**a**-**c**).

2.10.3 Deliver rAAV-Tet-responsive viruses into transgenic tTA-expressing mouse brains

We have shown that rAAV-delivered focally elevated transactivator concentration in transgenic Tet-responder mouse brains could not potentiate transgene expression. Thus we suppose that the tet-responder transgene itself and its copy-number determine the expression limits. To test this hypothesis, we developed rAAV-Tet-responsive viruses, which harbor P_{tet}-bi driven dual expression cassettes. rAAV-6P-minibi-iCre-Venus recombinant AAV vector was used, which expresses improved Cre recombinase (Shimshek, 2001) and Venus driven by bi-directional CMV minimal promoters. After delivery of rAAV-Tet-responsive viruses into existing tTA-expression transgenic mouse brains, the invaded neuron population will be transduced with multi-copies of rAAV Tet-responder genes. Transgenic

tTA mouse lines such as *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$, CaMKII-tTA (*Tg*$^{CamKII\alpha\text{-}tTA}$), CaMKII-rtTAnls (KM2) and 8.5kbCaMKII-NR2C-itTAnls (*Tg*$^{CN(12)\text{-}itTAnls}$) were studied for activation of rAAV delivered genes. Tg$^{(Thy\text{-}1.2\text{-}itTAnls)64Rsp}$ mice exhibited strong intrinsic Venus signals after infection of rAAV-6p-minibiCre-Venus (Figure 25 a-c). The immnofluorescent stainings against Venus and Cre-recombinase showed that rAAV-Tet-responder viruses could efficiently and simultaneously express both reporter genes, which could be completely abolished upon oral Doxycyline administration (Figure 25 d-k).

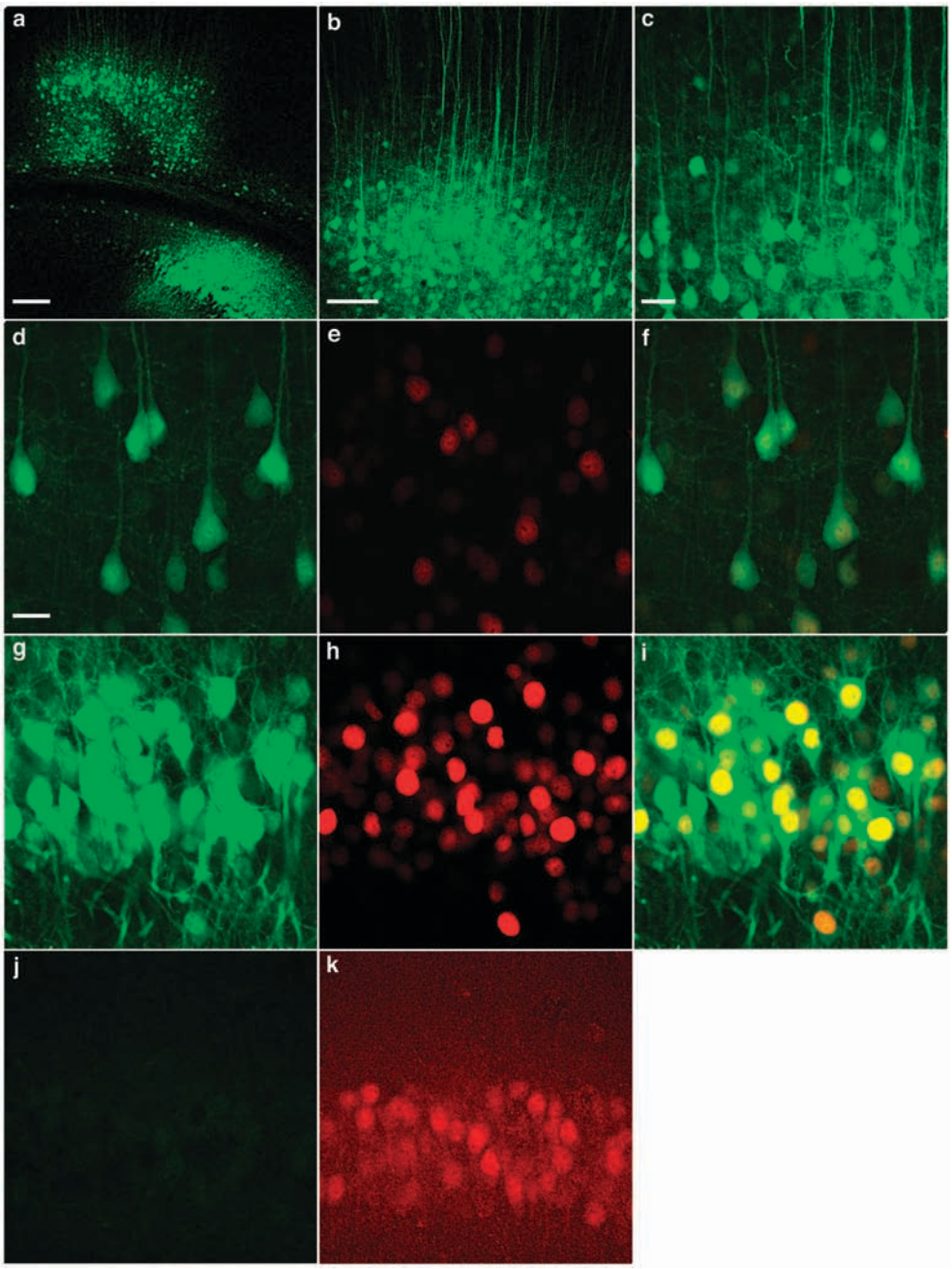


Figure 25. Transgenic tTA mice could achieve different induction efficacy of rAAV-delivered tet-responsive transgene expression in the rAAV invaded brain regions. rAAV-6p-minibiCre-Venus was delivered into *Tg*$^{Thy\text{-}1.2(64)\text{-}itTAnls}$ mouse brain. Strong intrinsic Venus signals were observed (**a-c**); immunofluorescent stainings showed that rAAV delivered P_{tetbi} could efficiently mediate dual protein expression in the cortex (**d-f**) and in the hippocampus (**g-i**); the reporter gene expression was shown under the tight control of Doxycycline (**j-k**).

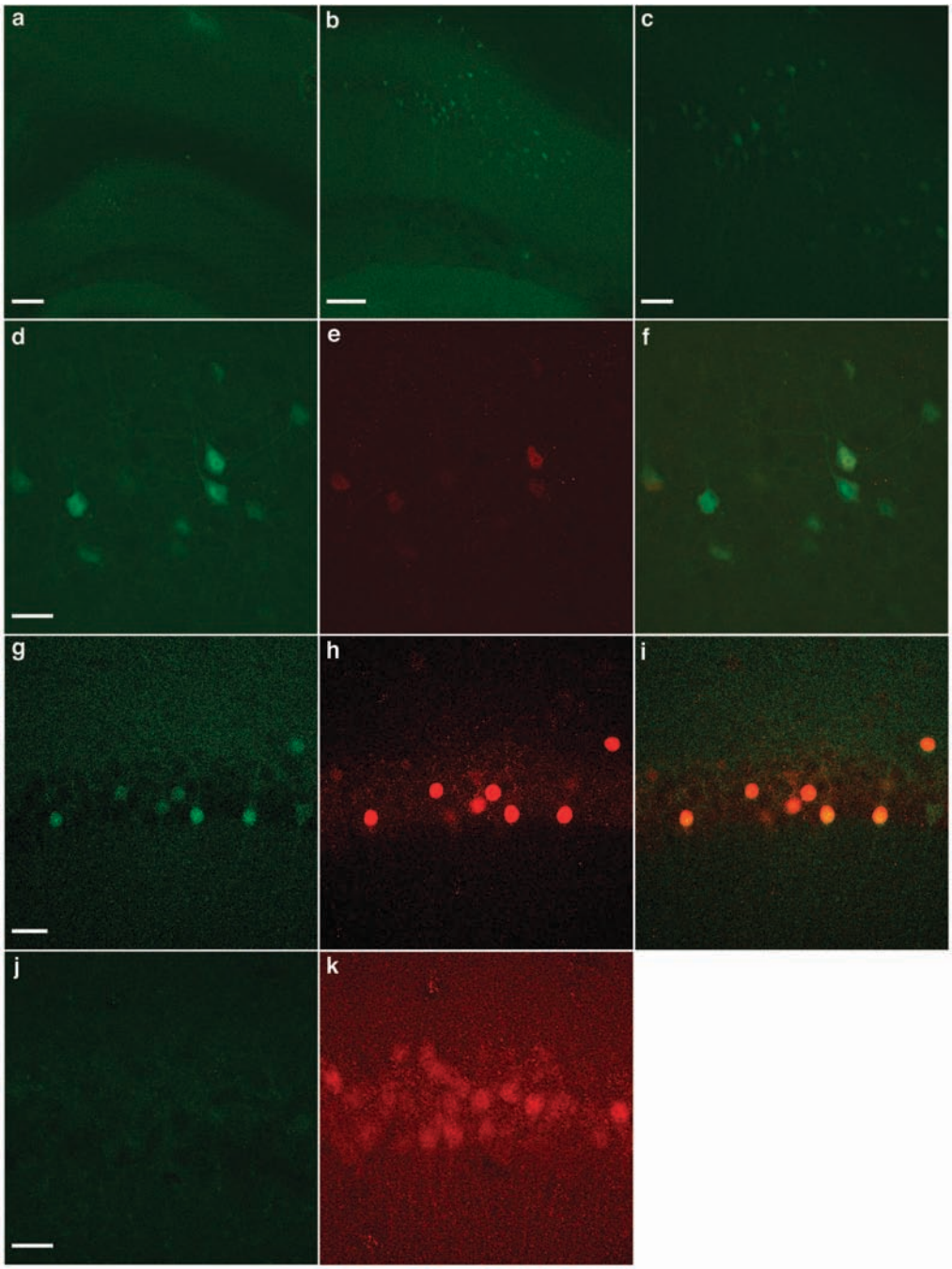


Figure 26: Weak intrinsic Venus signals were observed, when rAAV-6p-minibiCre-Venus was delivered into *Tg*$^{CamKII\text{-}tTA}$ mouse brain (a-c); immunofluorescent stainings showed that

rAAV delivered P_{tetbi} could also mediate dual protein expression in the cortex (**d-f**) and in the hippocampus (**g-i**); the reporter gene expression was also shown under the tight control of Doxycycline (**j-k**).

However, $Tg^{CamKII\alpha\text{-}tTA}$ mice, which express less tTA in the forebrain or restricted patterns showed only mild Venus signals at 7-12 DPI after delivery of rAAV-6p-minibiCre-Venus responder viruses (Figure 26 a-i). These results demonstrated that upon super-infection of rAAV-Tet-responsive viruses, neurons contain higher concentration of trans-activator-tTA could achieve higher and more efficient transgene induction under the tight control of Doxycyline (Figure 25 j-k and 26 j-k). Furthermore, these results show that the rAAV-mediated transgene expression in $Tg^{Thy\text{-}1.2(xx)itTAnls}$ mouse brains is specifically restricted in tTA-positive neurons, and therefore, a tTA independent expression of rAAV delivered tTA-responder genes is below our method of detection (data not shown). Several other bi-directional tetracycline-responsive rAAVs possessing different fluorescent dyes and/or functional proteins, provided similar results (data not shown). Thus the defined expression pattern and intensity of tTA expression in tTA-expressing transgenic mouse lines allow precise targeting of neurons in specific brain subregions. This efficient dual-transgene expression of genes is made possible by combination of transgenic tTA-expression mouse lines and rAAV-P_{tet}-bi-responder viruses such as rAAV-6p-minibiCre-Venus. Over-expression of multiple genes, such as fluorescent markers and and other cellular gene, is now readily achievable.

2.11 rAAV-hSyn-itTA / rAAV-6p-minibi-iCre-Venus could mediate strong inducible transgene expression *in vivo*, independent of transgenic tTA-expressing mouse lines

Combination of rAAV with transgenic mouse models is a powerful tool. However, for many applications *in vitro* and/or *in vivo*, it would be more convenient to avoid generation of transgenic mouse models for obvious reasons. Thus, we developed both tTA-expressing rAAV, and rAAV-tet-responders, which could mediate inducible dual-protein expression in neurons. For rAAV shows high coinfection efficiency in the mouse brain, co-delivery of multiple dyes or proteins is now possible. This is also important in many Real-Time monitoring studies. Here, we

showed the rAAV-Mediated-Tet-Responsive coinfection system, which could accomplish co-delivery of as many as 4 proteins simultaneously in neurons. And most importantly, the strong expression of the 4 proteins could be synchronized by tTA-dependent gene induction.

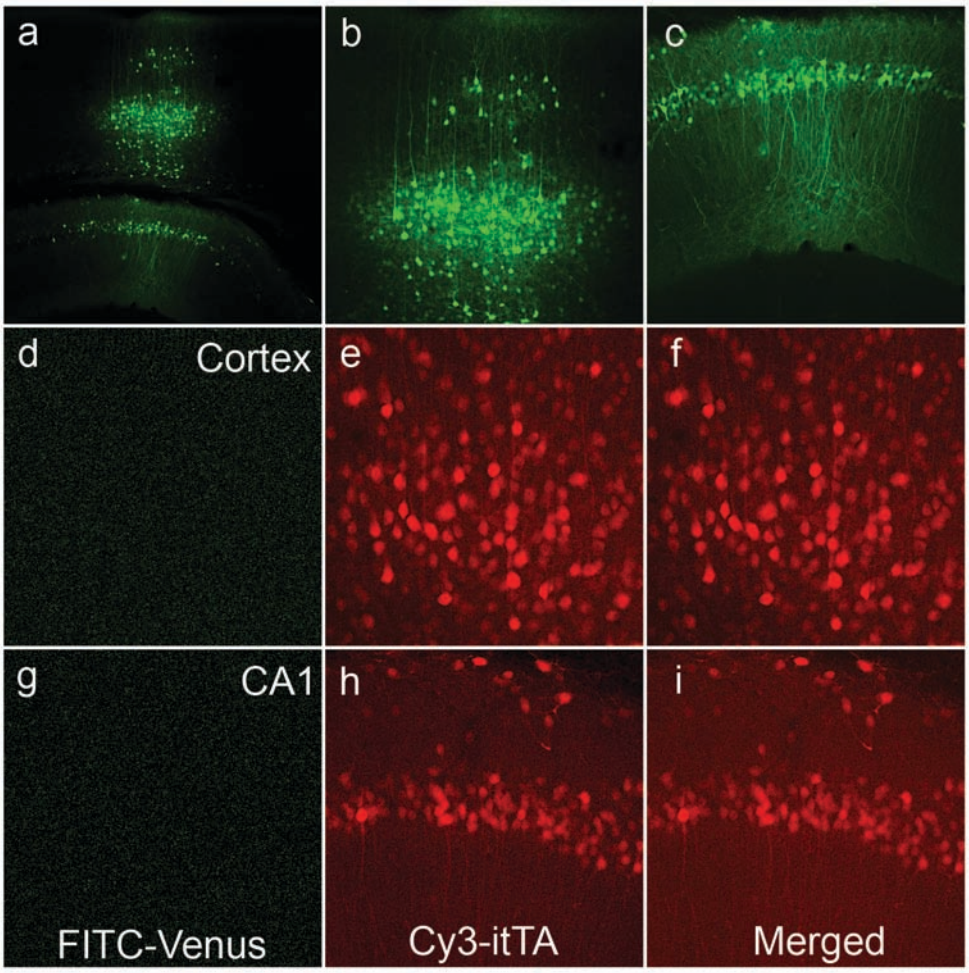


Figure 27. rAAV-hSyn-itTAnls / rAAV-6p-minibiCre-Venus coinfection mediated robust regulatory gene expression in selected wild-type mouse brain. a-c: Robust intrinsic Venus signal was observed in cortex and hippocampus with infected neurons labeled in their entirety; d-f: Gene expression in infected cortical neurons could be turned off by Dox treatment; g-i: rAAV-tTA/ rAAV-tet-responder mediated gene expression in CA1 neurons is under tight control of Doxycycline.

To show that the rAAV-hSyn-itTAnls / rAAV-tet-responder could mediate robust expression of Venus, we first did *in vitro* primary neuronal culture studies by co-infection of rAAV-hSyn-itTAnls / rAAV-tet-responder viruses. Robust fluorescence was observed five days after infection, and most importantly the reporter Venus

expression could be efficiently turned off by Dox administration within 7 days (data not shown). Thus it will become an important and powerful tool if the rAAV-hSyn-itTA / rAAV-tet-responder combination could maintain the same induction efficacy and could achieve the same strong induction level *in vivo* in rodents brain.

To prove that rAAV-hSyn-itTA / rAAV-minibi-Cre-Venus viruses could achieve robust tTA-dependent gene expression, the two viruses were mixed and injected into the wildtype mouse brains. The mice were kept 10 days before analysis. The mice were analyzed by immunohistochemistry and Confocal Microscopy (Figure 27). The results revealed that rAAV-hSyn-itTA / rAAV-P_{tet}-bi-genes could induce strong reporter gene expression. Most importantly, the reporter gene expression could be turned off efficiently by oral administration of Doxycycline in the drinking water (Figure 27). Therefore, the rAAV-hSyn-itTA / rAAV-P_{tet}-bi-genes system could be an alternative, efficient and economic tool for tet-regulated gene expression in the CNS of rodents.

2.12 Leakiness control experiments for rAAV-tet-responsive viruses demonstrates expression is under tight control of Doxycycline.

All important features of the rAAV-tet-responder and/or transgenic tet-responsive mouse models mentioned above are based on the fact that the bidirectional tetracycline responsive promoters are under tight control of tTA, which could be regulated by Doxycycline administration. To demonstrate this, we delivered rAAV-tet-responder into wild-type mouse brains with different concentration of infectious virus particles (1: 1; 1 : 10; 1 : 50 and 1 : 100 serial dilutions). No reporter could be detected either by immunohistochemistry or by fluorescence live imaging using GFP-specific antibodies in the infected mouse brain when the injected virus concentration is lower than 10^8 particles/ml (75 nl / injection site) (Figure 28). These results demonstrated the tightness of the tet-responsive bidirectional promoters, which could be combined with rtTA expressing rAAV virus to conditionally express toxins and dominant negative mutants in the CNS of rodents.

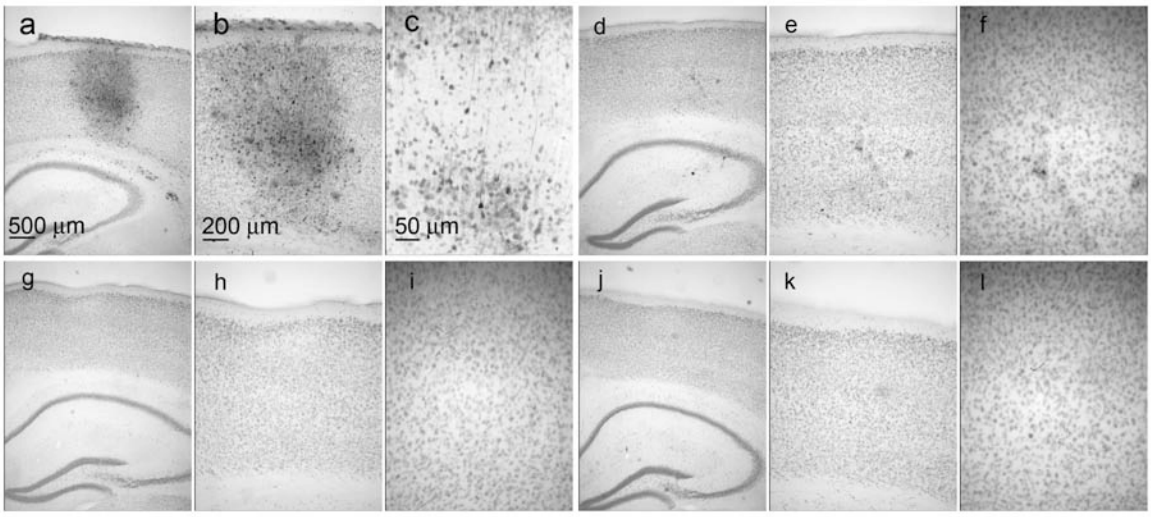


Figure 28. To test the leakiness of the episomal state tet-responsive constructs, serial dilutions of purified rAAV-6p-minibiCre-Venus were injected into mouse brains in cortical neurons. Mouse brains were analyzed by immunostainings using GFP-antibody. rAAV-6p-minibiCre-Venus (1:1; Volume ratio in 1xPBS) shows mild basal expression of Venus in cortex (**a**-**c**); Ten times dilution of rAAV-6p-minibiCre-Venus (1:10) shows nearly no expression of Venus (**d**-**f**); Fifty- (**g**-**i**) and hundred-times (**j**-**l**) dilutions of rAAV-6p-minibiCre-Venus show no detectable Venus signals.

2.13 Neuronal cells infected with rAAV in *Tg(Thy-1.2-itTAnls)64Rsp* mouse brain can be visualized *in vivo* by two-photon imaging

It has shown that rAAV delivered into *Tg(Thy-1.2-itTAnls)64Rsp* mouse brain could mediate intense fluorescent reporter gene expression in apical cortical neuronal populations. Here, we demonstrate that the intense fluorescent reporter gene products can be visualized *in vivo* by two-photon microscopy. *Tg(Thy-1.2-itTAnls)64Rsp* mice were infected with rAAV-6p-minibiCre-Venus virus. After 5-7 days, the infected *Tg(Thy-1.2-itTAnls)64Rsp* mice were anethetized and using the two-photon microscope rAAV infected neurons could be detected in different cortical layers with entire labeling in cell bodies, dendrites and spines (Figure 29 A). Microstructures of the infected neurons are clearly visible due to the intense and sparse labeling, which reflects the expression features of *Tg Thy-1.2(xx)itTAnls* transgenic mouse lines mentioned above (Figure 29 B). Furthermore, series of images were also collected either at frequence rates of 5-15 Hz, or at depths of 50 µm of interval with cooled-CCD camera.

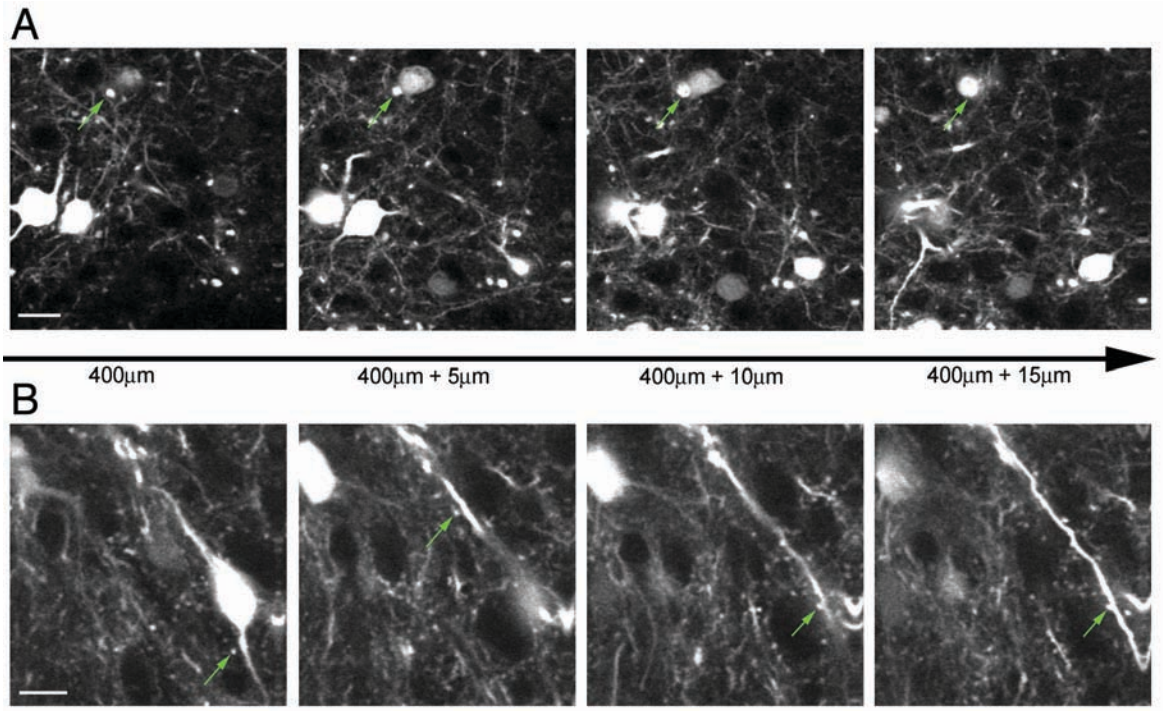


Figure 29. rAAV infected *Tg*$^{Thy\text{-}1.2(64)\text{-}itTAnls}$ mouse brain was visualized by Two-photon microscopy. A: *ex vivo* visualization of Mouse I, green arrow shows the axon of a cell; B: Mouse II was *in vivo* visualized to reaveal the fine structures of cortical neurons, green arrow shows the spines of rAAV infected neuron.

The serial pictures taken at different time and/or depth interval were transformed into movie and/or 3-Dimension formats to illustrate the micro-structure mobility upon certain stimuli, and to reveal the 3-D structure of infected neurons, respectively (data not shown). Thus, the combinatorial system of *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$ transgenic mice and the rAAV has been proven to be a powerful tool for *in vivo* live imaging of micro neuronal anatomical structures and/or study transgene functions in real-time in invaded cortical neuronal populations. (Done in collaboration with Dr. Hasan, MT and Dr. Denk, W., MPI for Med. Research, Heidelberg, Germany)

2.14 rAAV infected neurons have normal neuronal electrophysiology.

rAAV has been shown as an important and powerful alternative method for gene delivery in the rodents brain, however, the strong and intense reporter gene expression brought the concerns of changing the neuronal properties of the rAAV invaded neuron population. Therefore, we asked whether the multiple-rAAV-

invaded neurons have normal electrophysiological properties. Young wild-type mice (P20) were infected by rAAV-hSyn-itTAnls and rAAV-6p-minibiCre-Venus, rAAV-hSyn-itTAnls and rAAV-6p-minibiCre-tdTomato in hippocampus region, respectively.

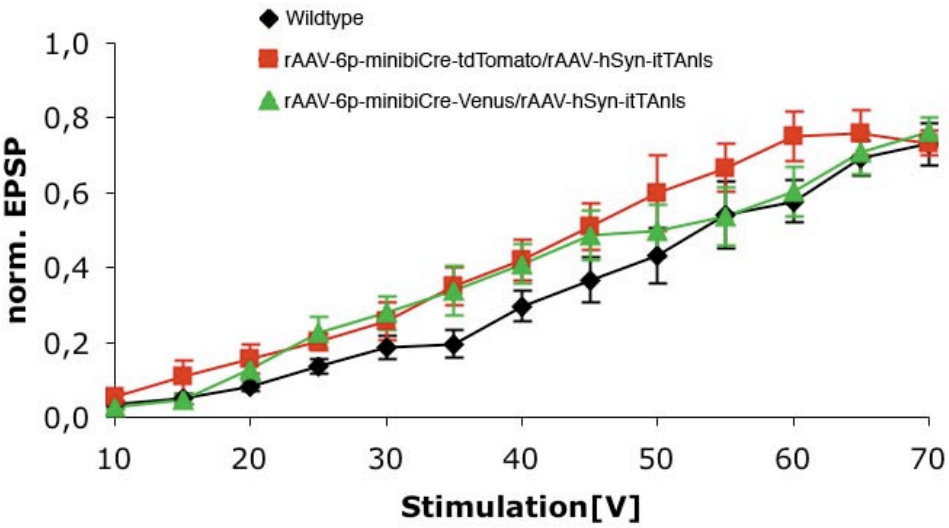


Figure 30. Electrophysiology revealed that rAAV invaded CA1 neurons have normal neuronal properties. rAAV-hSyn-itTAnls/rAAV-6p-minibiCre-Venus (Green) and rAAV-hSyn-itTAnls/ rAAV-6p-minibiCre-tdTomato (Red) infected hippocampal CA1 neurons were examined by studying the I/V curve relationship. Neighboring non-infected neurons were patched as wild-type control (Black). There is no significant difference among Green, Red and control observed for resting potential, peak amplitude, half width, peak threshold and rise time.

Seven days post-infection, the infected mice were sacrificed and Venus positive neurons (5-7) of acute brain slices were patched with neighboring black neurons (6-8) as wildtype controls to study the neuronal properties. Several basic parameters, such as resting potential, peak amplitudes and peak thresholds, etc. were recorded. Input-Output curves and statistical studies showed that the multiple-rAAV-invaded neurons have normal neuronal properties in terms of investigated parameters (Figure 30) (Done in collaboration with Sven Berberich and Georg Köhr; MPI for Med. Research, Heidelberg, Germany).

3. Discussion:

3.1 Generation of novel tTA-expressing mouse lines.

The increasing number of tTA-expressing transgenic mouse lines provide new opportunities to investigate the brain functions using reversible gene expression system. The experimental data provided by expanding pool of tTA-expressing mice will also further help to improve the tetracycline-responsive gene expression system in the mouse brain. Although *Tg*$^{CamKII\alpha\text{-}tTA}$ and several other transgenic tTA mouse lines were proven to be useful activator lines, they could not achieve over-expression of many Tet-responder lines. The moderate tTA expression was often not suitable for visualizing detailed neuronal sub-structures *in vivo*. As an important and promising tool, we showed that Tg$^{Thy\text{-}1.2(xx)itTAnls}$ transgenic mouse lines can be used for various applications of regulated gene expression in the mouse brain. *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$ transgenic mice exhibited robust tTA expression as did several other transgenes, which used the *Thy-1.2* promoter (Carroni, 1996; Feng, 2003).

In *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$/*Tg*^{GFPTETO7lacZ} double positive mice, the trans-activator (tTA) and the reporter (GFP or β-galactosidase) could be co-localized in the same neuron. These co-localization studies showed that regardless of tTA levels, the reporter can be expressed in high or low levels, and sometimes not at all. This shows that the efficiency of reporter gene expression strongly depends on the tet-responder transgene. This is supported by the finding that overall expression of reporter gene is not significantly potentiated, though quantitative analysis, such as Western-Blots, should be carefully performed to confirm this finding. In summary our results indicate that the induction efficacy of the transgenic Tet-responsive system might be highly dependent on three factors: first, the tTA concentration; second, the copy-numbers of the reporter genes; third, the accessibility of the Tet-responder locus.

As shown by the Southern-Blots analysis, the intensity of the transgene expression under certain promoters is not correlated with copy-numbers, and therefore depends on the chromosomal integration site. The analysis of Tet-responder integration sites in *Tg*^{GFPTETO7lacZ}, *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$ and *Tg*$^{lacZTETO7gfpGluR\text{-}A}$ transgenic mice showed that *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$ has the lowest transgene copy-

number among the three, but *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$ shows the strongest transgene expression in *Tg*$^{CamKII\alpha\text{-}tTA}$ breedings. Therefore, we studied the expression pattern and intensity of tet-responder genes targeted to the chromosomal insertion site of *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$.

3.2 Gene tartgeting transfer of single-copy transgene using detected locus.

By gene targeting, we generated a mouse line with one copy of lacZ-tetO7-GFPGluR-A (Pik3c3-A1.1) and compared to *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$, which carry three copies of a very similar transgene in the same locus. Upon induction with *Tg*$^{CamKII\alpha\text{-}tTA}$, *Tg*$^{lacZTETO7gfpGluR\text{-}A(SA)}$ mice showed robust lacZ and GFPGluR-A(SA) expression. However, in Pik3c3-A1.1/*Tg*$^{CamKII\alpha\text{-}tTA}$ mice, β-gal could hardly be detected. The reduced activation of the responder genes might reflect a copy number dependence. But it is also possible that the neo selection marker gene in the targeted locus is attenuating the expression of the tet-responder genes. The removal of the neo selection marker is necessary before conclusions can be drawn. Especially since it is known that single-copy integrant was shown to be successful (Bond *et al*, 2000; Stiw 1999), which also showed that the neo-gene had a negative effect on the expression level.

Next we performed *in vivo* experiments in the mouse brain to show the epigenetic silencing effects in cortical neurons can be reprogramed. In the presence of neo selection gene, double transgenic Pik3c3-A1/TgCaMKII-tTA and Pik3c3-A1/ThyF64 mice did not show detectable reporter gene expression in cortical neurons, in which active epigenetic silencing programs were shown by delivery of rAAV-6p-minibiCre-Venus virus into several tet-responder mouse lines. However, after removal of neo gene specifically in selected cortical neuron populations by rAAV-6p-minibiCre-Venus virus injection, we showed that tTA expressed from Thy-1.2-itTAnls minigene cassettes successfully induced tTA-dependent gene expression of targeted nlacZ-tetO7-GFPGluR-A responder cassette in *Pik3c3* locus. Therefore, consistent with previous in vitro studies mentioned above, we demonstrated that epigenetic silencing effects or promoter competition in cortical neurons of rodent brains can be reprogrammed.

3.3 Epigenetic silencing strongly influences the integrated Tet-responsive transgene cassettes in the mouse brain

It is well known that two independent founders carrying the same copy number of the same transgene constructs still show significant difference both in expression patterns and intensity. This reflects the profound effects of genomic loci. Research in *Drosophila* using Enhancer-Trap (Wilson, *et al*, 1990) and/or P transposon (Levis *et al*, 1985; Hazelrigg and Petersen, 1992; Zhang and Spradling, 1994;) demonstrated the position effects in invertebrates. Transgenic vertebrate model also showed that transgene suppression could be an intrinsic property evoked by repeated transgene constructs (Flavell, 1994; Rossignol and Faugeron, 1994;). This suggests that Histone H4 lysine residue deacetylation and coincident condensation of chromatin structures are underlying gene silencing of both transgene and virally transduced delivery of genes (Chen and Toweens, 2000). In our studies described before, by introducing tTA-expressing rAAV into several Tet-responder transgenic mouse lines, we demonstrated that epigenetic silencing effects strongly influence the expression of integrated tTA-dependent transgene expression. And also, double transgenic Pik3c3-A1/ $Tg^{CamKII\text{-}tTA}$ and Pik3c3-A1/ThyF64 mice failed to show reporter gene expression with the presence of neo-selection gene, which might hinder the gene expression by epigenetic silening in *Pik3c3* locus. But results also showed that CG19 locus is permissive to tTA transactivation in the adult mouse brain, while in all the others, including $Tg^{lacZTETO7gfpGluR\text{-}A(SA)}$ mice, no transgene expression was detectable (Figure 31) (Zhu, *et al*, 2006). This showed that there is rare permissive locus existing in *Mus musculus* genome, although most of the loci are vulnerable to gene silencing effects in adult mouse brain. Yet, the activation of the permissive locus of $Tg^{LuciferaseTETO7Camgroo2(19)}$ (MTH-CG19) needs relatively high cellular tTA concentration (this thesis and Zhu *et al*, 2006).

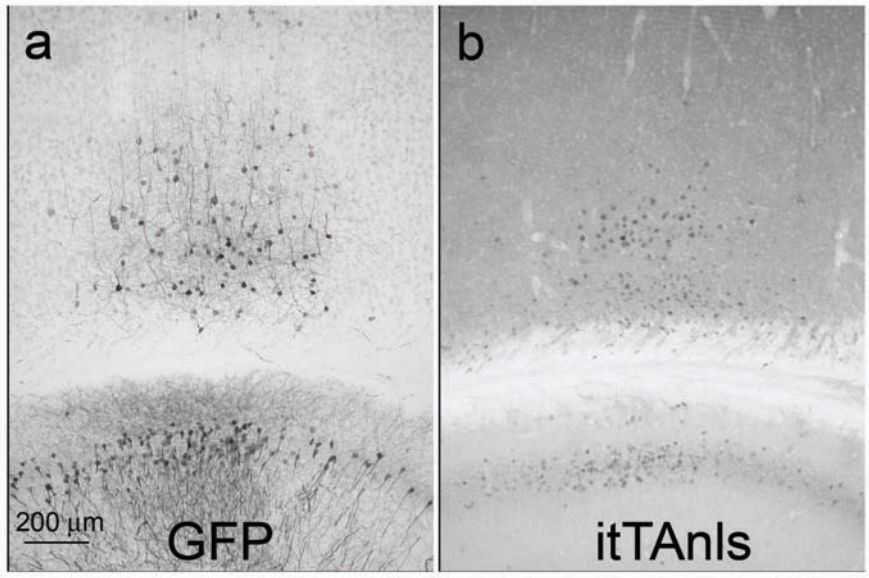


Figure 31: **High concentration of tTA delivered by rAAV-hSyn-itTA can induce reporter gene expression in MT-CG19 transgenic mouse brain.** Reporter gene: Camgroo2 was visualized by GFP antibody in cortical neurons and CA1 hippocampal neurons **(a);** High concentration of tTA was stained using tTA antibody in the same regions of neighboring slices **(b).**

Interestingly also, studies found that LCR-derived specific insulator sequences could increase the possibility of efficient transgene and retroviral expression, seemingly regardless of chromosomal integration sites (Rivella *et al*, 2000). Preliminary studies from our lab using insulator elements in tet-responder mice failed to improve the tet regulated gene expression. Different Human Insulator 40 flanked tet-responding transgenic mice bred with the same tTA-expressing mouse lines: $Tg^{CamKII\alpha\text{-}tTA}$ and/or $Tg^{Thy\text{-}1.2(xx)itTAnls}$ showed that the cellular expression is still strongly influenced by the chromosomal integration site (Krüth, U. unpublished data).

Thus it remains hard to achieve stable transgene expression in the mouse, especially in the CNS.

3.4 Episomal rAAVs could overcome the epigenetic silencing effects.

Work using retroviral vector transferred rtTAnls-mediated inducible transgene expression was successful in the CNS of rodents (Szulc *et al*, 2006). However, dual-inducible transgene over-expression using both tTA and rtTAnls was challenging. Here, we showed that we achieved fast, strong and dual-inducible trans-

gene expression using rAAV and the combination with retroviral vectors and transgenic mouse lines. Since we have showen that rAAV could maintain strong gene expression for at least 75 days in the mouse brain, focal expression using rAAV mediated gene transfer is no longer an issue. To investigate the roles of bigger brain regions, rAAV has to be delivered into selected brain regions by micro-injection into the P0-P2 young mice and/or rats brains (work in progress). Since rAAV-mediated transgene expression is stable for at least 6-8 months in the mouse brain (Kuegler *et al*, 2003), the P0-infected-mice will be expected to have large brain region infected when the animal is 4-8 weeks in age. In principle, this might achieve rAAV-tTA / rAAV-Tet-responders-mediated, rAAV/retroviral-vectors-mediated and rAAV / transgenic-mice-mediated dual-transgene expression in large brain regions.

However, the large region and long-term infection of rAAV and its combination retroviral vectors require extremely low neurovirulence effects in the rodents CNS to be used for physiological studies. This raises the question of how the virus titers could be increased, but without eliciting immune responses by activation of microglia cells in the CNS. We have managed the purification of high-titer rAAV stocks, which did not show any detectable neurovirulence effects within 6 weeks in the mouse brain (data not shown).

3.5 Combination of transgenic mice, retroviral vectors and rAAV systems

By injecting rAAV-6p-minibiCre-Venus into the brain of tTA expressing transgenic mice ($Tg^{(Thy\text{-}1.2\text{-}itTAnls)64Rsp}$), we observed a high co-localization rate of Venus and Cre, which indicates the efficient transactivation of rAAV-delivered genes in the adult mouse brain. More importantly, the rAAV-6p-miniCre-Venus expression was restricted only in $Tg^{(Thy\text{-}1.2\text{-}itTAnls)64Rsp}$ tTA-positive cells, and therefore strictly dependent on the $Tg^{(Thy\text{-}1.2\text{-}itTAnls)64Rsp}$ tTA-expression pattern. This combination could also be used for fast screening of the newly generated tTA expressing transgenic lines in desired brain regions.

3.6 rAAVs can achieve transgenic mouse independent Tet-responsive gene expression

However, transgenic mice are an expensive and time-consuming method for most of the laboratories. Now, we have shown that rAAV are powerful tools for gene delivery in rodents brain. rAAV showed high coinfection efficiency and long-term non-toxic stable gene expression *in vivo*. Combining rAAV-tet-responder and rAAV-tet-activators make the rAAV a transgenic mouse independent gene delivery system. However, there is always shadow in the sun: the intrinsic property of the rAAV limits the packaging capacity of transgenes, the diffusion of the viral particles is too large (>1.5 mm in diameter), etc.. Different promoter driven tTA-expressing transgenic mouse models showed cell-type- and/or brain-suregion-specific expression of tTA, which is very important for inducible transgene expression in certain cell types or special brain regions. The small size of rAAV genome is not enough to package most of the promoter fragments used for specific expression, and also the small size of the rAAV particles renders huge diffusion diameters, which would cover more than one specific brain subregions. To overcome this issue, we also tested the combination of retroviral vectors, such as lenti-expression system, which has much bigger cloning capacity and would therefore compensate the rAAV system in delivering huge promoter DNA fragments. Lenti-CaMKII-tTA was made and injected together with rAAV-6p-minibiCre-Venus into the adult mouse brains. After 7-14 DPI, the mice were shown to have strong Venus expression in CaMKII-positive cells. Also other neuron specific promoters were cloned into lenti vectors and tested successfully (data not shown). These experiments demonstrated that the combination of retroviral vectors and rAAV could achieve cell-type-specific strong expression of reporter genes. This makes the rAAV-Tet-responder viruses a powerful tools in labeling, and expressing transgenes in desired neuron populations in the adult mouse brain.

3.7 Conclusions

We have generated novel tTA-expressing *Tg*$^{Thy\text{-}1.2(xx)itTAnls}$ transgenic mouse lines which show strong tTA-dependent expression of Tet-responsive genes in different neuronal populations and brain subregions, including cortical layer 5. These mouse lines, together with the previously described lines, would facilitate correlation of reversible gene expression in specific neuronal cells to phenotypes.

Clearly, stable transgenic lines are vital research tools as they also allow for reproducible spatiotemporal gene expression and thus are extremely useful for long-term biological studies, including learning and memory and diseases. The ability to utilize various cell type specific tTA-expressing mouse lines , including those generated by BAC, will facilitate targeted expression of any gene in specific cell types in selective brain subregions when combined with rAAV harboring Tet-responsive elements.

4. Materials and methods

4.1 Construction of Thy1.2-itTA Plasmids:

The Thy1.2-itTA plasmid was generated by genetically combining two plasmids: pBS.itTApA and pmThy1-PL (mouse Thy1-PolyLinker). Due to the intrinsically nature and sensitivity of the Thy1.2 expression cassette, only the congenital enzyme restriction sites were used. The 1.0 kb itTA was released from pBS.itTApA by ClaI/ SacII double digestion. And pmThy1-PL plasmids was linearized into two pieces by MluI, XbaI and ClaI. The DNA fragments were cleaned by Gel-Purification (QIAGEN, Germany) and ligated at 16°C (Ligase; ROCHE, Germany). After transformation and screening of the clones, the plasmids were verified by enzyme digestion and sequencing.

4.1.1 Generation of Thy1.2-itTA transgenic mice:

The pmThy1.2-itTA plasmids were prepared in large amount using HiSpeed Maxi-Prep. Kit (QIAGEN, Germany) and cleaned by Phenol/Chloroform Extraction. Then the DNA was fully digested by NdeI/NotI (Fermentas, Germany). The digested DNA was concentrated by Isopropanol precipitation and resolved in 1xTE buffer to 1.0 µg/µl. 10 µg of linear DNA of Thy1.2-itTA (8.5 kb) were separated by sucrose density gradient centrifugation in a Beckman L8-70M ultracentrifuge using an SW40T rotor (10 % - 40 % sucrose, 35,000 rpm, 16 h at 15 ℃). The 200 µl gradient fractions were checked on 1.0% agarose gel. Appropriate fractions containing the minigenes without vector backbone were purified by Amicon Micron 50 Filters (Millipore). Pronuclear-Injection was done by Zentral Labor im NeuheimFeld (ZTL), Heidelberg, Germany.

4.1.2 Genotyping of transgenic Mouse lines by tail-PCR

Mouse tails from (age P10-P14) different transgenic mouse lines were digested with Proteinase K (1 mg/ml) in TENS-Buffer (50 mM Tris- HCl pH=8.0, 100 mM EDTA, 100 mM NaCl, 1 % SDS) at 55 °C for 8-12 hours. After precipitation by 1 volume of isopropanol and washing with 70% ethanol, the genomic DNA was resolved in 100-300 µl water. Genotyping PCRs were setup in 25 µl reactions, containing PCR-buffer (GibcoBRL), 2 mM $MgCl_2$, dNTP-Mix (0.2 mM pro Nucleotide),

specific sense- and antisense-oligonucleotide (each 0.4 µM), 0.2-0.5 U *Taq*-Polymerase, ddH_2O and 1µl Template-DNA-solution (10-100 ng/µl). PCR programs were as following:

Thermo-Cycles	Temperature and Time
1cycles:	94°C 10 min
30 cycles:	94°C 15 sec
	55-58°C 15 sec
	72°C 15-50 sec
72°C for an additional 7 min after the final cycle.	

PCR amplicons were checked on 1.5-3.0% agarose gel. The results were evaluated according to special designs.

-- **A1.1:** VM4; GluRA-VM4 (Tg: 350 bp)

-- **G3:** lac3', lac5' (lac: 500 bp)

-- **GluR-A-1lox:** 1005; 3'intro3; 1Lox-PZ (wt: 180 bp; het: 180 bp and 250; hom: 250 bp)

-- **LC1:** rspCre1, rspCre2 (Cre: 200 bp)

-- **KT1:** tTA1; tTA4 (tTA: 350 bp)

-- **Pik3c3-A1:** FP-SA-400; Hgh04 (Tg: 550 bp)

-- **RLZ:** lac3', lac5' (lac: 500 bp)

-- **SA87.5:** FP-SA-400; Hgh04 (Tg: 700 bp)

-- **Thy1b:** Th1-tTA-FP; Thy1-tTA-RP (wt: 500; Tg: 300 bp)

4.2 X-gal staining for vibratome sections

Brains were removed and fixed for 2 h in 4 % Parformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4/2H_2O$, 1.4 mM KH_2PO_4). Afterwards the brains were rinsed wit PBS, embedded in 2.5% agarose (Seakem LE) in PBS and cut sagittally or coronally in 70-80 µm sections on a vibratome (Leica VT 1000S, Leica). The sections were incubated for 5-60 min at room temperature in X-Gal staining solution (5 mM $K_4Fe(CN)_6$, 5 mM $F_3Fe(CN)_6$, 2 mM $MgCl_2$, 2 mg/ml X-Gal in dimethylformamid/PBS). Sections were washed twice in PBS and once in 10 mM Tris/HCl, pH=7.6. Sections were immediately counter-stained with eosin (Sigma) for 1 min and rapidly and successively dehydrated in ethanol 70, 90, 99.5

% (v/v). The dry sections were dehydrated in Xylene and embedded in EuKitt (Kindler GmbH, Germany).

4.3 Immunocytochemistry (ICC)

Mice were anesthetized with lethal dose of halothane (Hoechst, Germany) and intracardially perfused with phosphate-buffered saline, pH=7.4 (PBS), at room temperature followed by ice-cold 4 % paraformaldehyde (PFA) in PBS. Brains were removed and post-fixed for 1 h in PBS containing 4 % PFA at 4℃. Afterwards the brains were rinsed with PBS and embedded in 2 % agarose in PBS and cut sagittally or coronally in 80 µm sections on a vibratome (Leica VT 1000S; Leica, Germany). For blocking of endogenous peroxidase, sections were incubated for 10 min in 0.5 % H_2O_2/PBS and washed twice for 10 min with PBS. Sections were permeabilized in Day 1 buffer (0.3 % Triton X-100, 1 % bovine serum albumine (BSA), 3 % normal goat serum in PBS) and incubated overnight in buffer 1 containing anti-β-galactosidase rabbit IgG (1:6000; ICN, Germany). The following day, sections were washed twice for 10 min in Day 2 buffer (0.1% Triton X-100 and 0.3 % BSA in PBS) and incubated for 2 h in Day 2 buffer supplemented with peroxidase-conjugated anti-rabbit IgG (1:600; Vector Laboratories, USA), washed 5 times, 10 min/time in Day 2 buffer , twice for 10 min in PBS. The staining reaction was developed using 20 mg Diaminobenzidine (DAB; Sigma) dissolved in 50 ml of 20 mM Tris/HCl pH7.6 and stopped in PBS. After a brief rinse sections were mounted Eukitt (Kindler GmbH, Germany).

4.4 Doxycycline Administration to mice

Doxycycline hydrochloride (Sigma) at a concentration of 50-2000 µg/ml, supplemented with 1-5 % sucrose, was dissolved in drinking water and provide to the parental mice in light-protected bottles. Animals were kept under doxycycline from P1 and until P21 (or P42). At P21 doxycycline was removed from the drinking water. For special applications in recombinant Adeno associated virus Chapter, the 1 mg / 100µl PBS C4-Doxycycline (Hasan, et al., 2006) was IP injected 2 hours, 4 hours, 8 hours, 24 hours and 72 hours before analysis, respectively.

4.5 Detection of transgene integration site in mouse genome

4.5.1 Preparation of genomic DNA from liver tissue

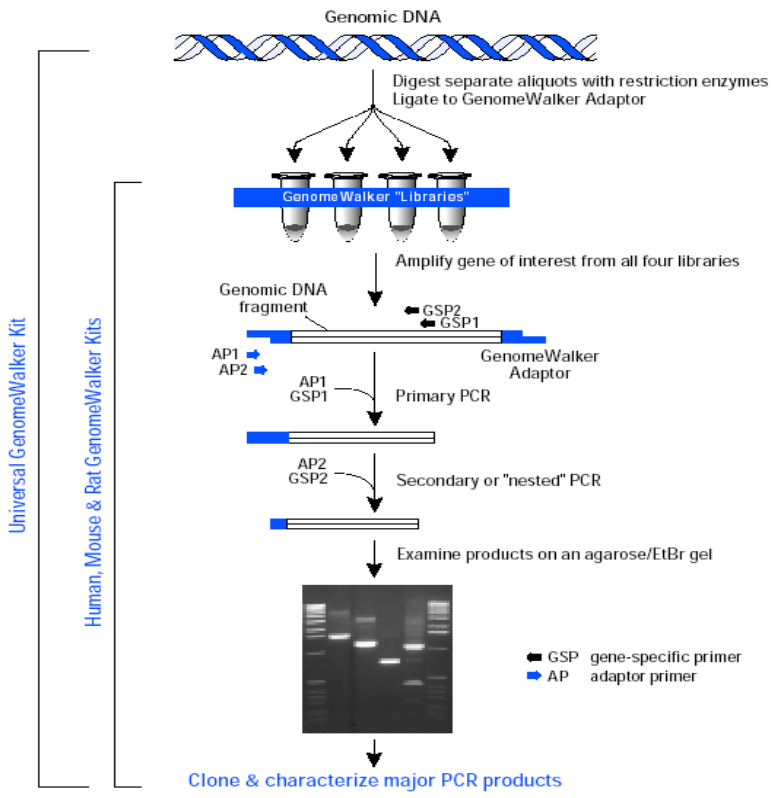
The correctly genotyped mouse was anesthetized by lethal dose of Isofloran (Sigma, Germany), then 10-20 grams of liver tissue was cut off and placed on ice-cold glass carrier. The tissue was then fragmented into small pieces by blade and transferred by blunt-tipped sterile plastic pipette tip into 15 ml Falcon-tube (Germany) filled with 8.0 ml TENS buffer and 1.0 ml of Proteinase K (CloneTech, Germany). Digest the tissue at 55°C with gentle shaking for 4-6 hours, and add another 1.0 ml Proteinase K, and further digest overnight. The next morning, the mixture was transferred into a new 50.0 ml Falcon tube, and the same volume of Phenol (Roti-Phenol, ROTH Corp.) was added, the 2-phase mixture was gently shaken for 10-30 min at room temperature (RT). Centrifugation was subjected to separate the 2 phases at 3000 rpm for 5 minutes at room temperature. The water phase was transferred into another new 50.0 ml Falcon tube. And the same volume of Chloroform (ROTH, Germany) was added and gently shaken for 5-10 minutes at room temperature. The mixture was spin down at 3000 rpm for 3 minutes at room temperature, and transfer the water phase to a new 50.0 ml Falcon tube. 2.5 volume of 100% ethanol was added and gently inverted 4-6 times, and spool the genomic DNA by fire-sealed glass capillary. The spooled genomic DNA was washed by 70% ethanol for 2-3 times and air-dried for 5-10 minutes at RT. To resolve the genomic DNA, 2.0 – 5.0 ml 1xTE (pH=7.8) buffer was added and incubated at 37°C for 1 hour. After the genomic DNA is fully resolved, add 100-300 µl of RNase-1 into the solution and let digest for 1-2 hours at 37°C to remove all the RNA.

Before embarking on library construction, the size and the purity of the genomic DNA were checked on 0.7-1.0 % agarose/EtBr gel. 1 µl of purified genomic DNA (about 0.1-0.2 µg/µl) and 1 µl of control genomic DNA (0.1 µg/µl) were loaded on 0.7% agarose/EtBr gel in 1xTBE buffer, along with 1.0 kb and 20.0 kb DNA-ladder size marker (NEB, Germany). Genomic DNA should be bigger than 50 kb with minimum smearing. And the quality was also checked by Dra I digestion: 5 µl purified genomic DNA; 16 units of Dra I restriction enzyme; 2 µl 10x restriction buffer for Dra I; 11.4 µl deionized water; a control digestion without Dra I

enzyme was also set-up. Reaction was mixed gently by inverting the tube. Incubate the reaction solution at 37°C overnight. The second morning, run 5 µl of each reaction on a 0.7% agarose/EtBr gel along with 0.2 µg of purified uncut genomic DNA as a control. A smear should be seen, indicating that the genomic DNA was digested well by the restriction enzyme.

For each library construction, 3-5 reactions were set up for the purified genomic DNA, and set up one Pvu II restriction enzyme digestion of human genomic DNA as a positive control. Label 3 to 5 1.5 ml Eppendorf tubes, and one positive control. The digestion was set up by mixing a Master Mix in a separate 1.5 ml tube: 2.5-5 µg of genomic DNA; 20 µl 10x restriction enzyme buffer; 10-20 units of restriction enzyme; fill with de-ionized water to 200 µl. Mix gently by inverting the tube. Incubate the reaction at 37°C for 2 hours and then shake the reaction at 750-1000 rpm for 20 sec, then add another 5-10 units of restriction enzyme, let digest overnight. The next morning, run 5 µl of each reaction on a 0.7% agarose/EtBr gel along with 0.2 µg of purified uncut genomic DNA as a control.

The digested genomic DNA must be purified by Phenol-Chloroform, so that a high efficient library could be achived. To each reaction tube, add an equal volume (95 µl here) of phenol, and vortex at low speed for 5-10 sec. Spin briefly to separate the aqueous and organic phases. Then use a pi-



pette to transfer the aqueous layer into a fresh 1.5 ml Eppendorf tube. Discard the organic phase properly into the chlorinated hazardous waste. To each tube, add an equal volume (95 µl here) of chloroform, vortex at low speed for 5-10 sec. Spin briefly to separate the aqueous and organic phases. And use a pipette to transfer the upper layer into a new 1.5 ml Eppendorf tube. Discard the organic layer properly into the chlorinated hazardous waste. 2 volumes of ice-cold 100% ethanol and 1/10 volume of 3 M NaOAc (pH=4.5) was added to each tube. Vortex at low speed for 5-10 sec. And centrifuge at 13,000 rpm for 20 minutes at 4°C. Decant supernatant and wash the pellet in 300 µl ice-cold 70% ethanol. And centrifuge at 13,000 rpm for 5 minutes at 4°C. Then the supernatant was decant and dry the pellet in speed-vacuum for 5 minutes at room temperature. The DNA pellet was resolved in 20 µl of 1xTE (10/0.1, pH=7.5), and vortex at low speed for 5-10 sec. Then spin down the liquid. Remove 1 µl out of each reaction tube, and load on a 0.7% agarose/EtBr gel to determine the approximate quantity of DNA after purification.

4.5.2 Generation of Ligation-Mediated Genomic-DNA-GenomeWalker®-Adaptor Libraries:

For each library construction, we set up 4-6 ligation reactions (3-5 digested genomic DNA, 1 positive ligation control). From each tube, transfer about 0.5 µg (volume should be less than 4 µl) of digested, purified DNA to a fresh 0.5 ml tube. To each, add the following: 1.9 µl GenomWalker® Adaptors (25µM); 1.6 µl 10x ligation buffer (Roche); 5 units T4 DNA ligase (Roche). Incubate the ligation reaction at 16°C on Thermo-Cycler (ABI 9700) overnight. And programmed the machine to stop the ligation reaction by heating the reaction up to 70°C for 5 minutes. To each tube, add 72 µl of 1xTE (10/0.1, pH=7.5), vortex at low speed for 5-10 sec. And spin down briefly.

4.5.3 PCR-based Genome Walking in Ligation-Mediated Genomic-DNA GenomeWalker®-Adaptor Libraries:

Prepare master mix as following (one reaction): 2.5 µl 10x Roche Expand Long Template PCR buffer1; 1 µl dNTP; 1 µl Adaptor Primer 1 (10 µM) (or **PCP1**: posi-

tive control primer 1); 5 units Roche Expand Long Template PCR Polymerase Mix; 1 µl Gene Specific Primer 1; 1 µl each genomic-adaptor DNA library (including the positive control and negative control (no DNA template)); 18.5 µl deionized water. After transfer of the reactions into each tube and mix gently by pipette up and down, place caps firmly on tubes, and briefly spin tubes in a micro-centrifuge. Commence cycling in the ABI DNA Thermo Cycler 9700 using the following 2-step cycle parameters:

Thermo-Cycles	Temperature and Time
-5 cycles:	94°C 25 sec
	72°C 3-5 min
-30 cycles:	94°C 25 sec
	67°C 3-5 min
-67°C for an additional 7 min after the final cycle.	

Secondary PCR was set-up by preparing master mix as following (one reaction): 2.5 µl 10x Roche Expand Long Template PCR buffer1; 1µl dNTP; 1µl Adaptor Primer 2 (10 µM) (or **PCP2**: positive control primer 2); 5 units Roche Expand Long Template PCR Polymerase Mix; 1µl Gene Specific Primer 2; 1µl each primary PCR reaction (including the positive control and negative control (no DNA template)); 18.5 µl deionized water. After transfer of the reactions into each tube and mix gently by pipette up and down, place caps firmly on tubes, and briefly spin tubes in a micro-centrifuge. Commence cycling in the ABI DNA Thermo Cycler 9700 using the following 2-step cycle parameters:

Thermo-Cycles	Temperature and Time
-5 cycles:	94°C 25 sec
	72°C 3-5 min + 5 sec
-30 cycles:	94°C 25 sec
	67°C 3-5 min + 5 sec
-67°C for an additional 7 min after the final cycle.	

Add 5 µl 6x loading buffer into 25 µl PCR reactions, and analyze the products on 1.5% agarose/EtBr gel, along with DNA 1.0 kb and 20.0 kb size markers. Normally 30 cycles are enough to have enough PCR products for sequencing after

agarose gel purification, but sometime 5 additional cycles are necessary to yield enough PCR DNA products. Positive PCR bands were Gel-purified using QIAquick Gel Extraction Kit as described below: Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose. Weigh the gel slice in an Eppendorf 1.5 ml tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl). For > 2% agarose gels, add 6 volumes of Buffer QG. The maximum of the gel slice per QIAquick column is 400 mg; for gel slices > 400 mg, and for more than 10 µg DNA use more than 1 QIAquick column. Incubate at 50°C for 10 min, with vortexing every 3 min during incubation. Gel slice have to be completely solubilized. For > 2% gels, we increased Buffer QG as mentioned above, and increase incubation time. After gel slice has dissolved completely, check the color of the mixture is yellow (similar to Buffer QG). If the color of the mixture is orange or violet, add 10 µl of 3 M NaAc, pH 5.0, and mix. Add 1 gel volume of isopropanol to the sample and mix. This step only slightly increases the yield of DNA fragments smaller than 500 bp or bigger than 4.0 kb. Transfer the mixture to a QIAquick spin column, which is placed on a provided 2 ml collection tube. And centrifuge for 1 min at 13,000 rpm. Discard the flow-through and place QIAquick column back in the same collection tube. To remove the contaminants, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min. DNA used for salt sensitive applications, such as direct sequencing, let the column stand 2-5 min after addition of Buffer PE, before centrifuging. Discard the flow-through and centrifuge the QIAquick column for an additional 2 min at 13,000 rpm. Place QIAquick column into a clean pre-labeled 1.5 ml Eppendorf tube. To elute DNA, add 20 µl of Buffer EB or H_2O to the center of the QIAquick membrane, let stand for less than 1 min, and then centrifuge the column for 1 min at 13,000 rpm.

The purified PCR amplicons were then subjected to sequencing reactions. Set up 20 µl sequencing reactions by combining the following reagents in a 0.5 ml PCR tube: 8 µl BigDye sequencing Mix (CloneTech, Germany); 5-10 µl eluted PCR products; 1 µl sequencing primer (10 µM); 6-1 µl deionized water. Mix by pipetting up and down several times. Then place caps firmly on the tubes. Com-

mence cycling in the ABI DNA Thermo Cycler 9700 using the following 3-step cycle parameters:

Thermo-Cycles	Temperature and Time
-1 cycles:	96°C 3 min
-5 cycles:	94°C 15 sec
	55°C 15 sec
	60°C 4 min
-60°C for an additional 7 min after the final cycle.	

Immediately before the PCR program finished, prepare and label the same number as set-up reactions of tubes on the SIDE of the tube. After the PCR is finished, transfer the PCR reactions into each labeled tube. To each tube, add 2.5 volumes of room temperature 100% ethanol, 1/10 volume of 3M NaOAc (pH=4.5). and vortex briefly at low speed. And centrifuge at 13,000 rpm for 20 minutes at room temperature. Decant supernatant and wash the pellet carefully in 300 µl room temperature 70% ethanol. And centrifuge at 13,000 rpm for another 5 minutes at room temperature. Discard supernatant and completely dry the pellet in speed-vacuum for 5 minutes at room temperature. Send the tube for sequencing. Sequencing data got at the second day and analyzed using LaserGene programs.

4.6 Southern-Blots analysis on Genomic DNA:

DNA from tissue (tail, liver or brain, etc.) was isolated and purified by proteinase K digestion and phenol-chloroform purification described above. Resolve the pellet DNA in water (or TE buffer), and measure the concentration in photometer at 600 nm using BioPhotometer (Eppendorf, Germany). All the DNA samples were adjusted to concentration of 0.5-1.0 µg/µl. DNA was then digested by properly selected restriction enzymes to generate DNA fragments, which will indicate the target bands hybridized by southern-probe(s). Depends on the positions of the probes, we performed two hybridization experiments on the digested DNAs: Inner-probe hybridization and Outter-probe hybridization.

Inner-probe hybridization: the restriction enzymes were chosen to cut within the known DNA sequences (or constructs) at least twice, and the probe was designed to hybridize onto one of the known DNA fragments so that they will generate at

least one DNA fragments band of known-size. The bands pattern of inner-probe hybridization will tell us the copy numbers of the known DNA sequences (or constructs), and tell us the full-digestion of the DNA samples. Outter-probe hybridization: the restriction enzymes were chosen to cut ONCE within the known sequences (or constructs), and cut also in the genomic sequence outside of the known sequences (or constructs). And this will generate one un-known size DNA fragment, and the probe was designed to hybridize onto the known region of the unknown DNA fragments. The bands pattern of outer-probe hybridization will tell us not only the copy numbers of the know DNA sequences (or constructs), but also the numbers of the loci, in which the known DNA sequences (or constructs) have been integrated (A.L. Joyner).

Separation of digested DNA fragments on agarose gel: 0.7-1.0% agarose gel (without Ethidium-Bromide(EB)) was used to separate the DNA fragments. After the gel was cool down, pull out the combs in 1x E-buffer in the gel-chamber. Load the DNA samples (at least 10 µg DNA with loading dye) following the DNA-size marker. Set up the running conditions: 25 volts; about 20 hrs; (or 90 volts; about 6 hrs; changing buffer every 2 hrs). After running, stain the gel in EB-solutions for 7-15 minutes, with gentle shaking. Take a picture with proper exposure time and magnification scale and the ruler should be included. Leave the EB-stained gel under UV light, expose for 10 minutes to generate DNA double-stand breaks. Put the gel into Denaturing-Buffer (1 M NaOH) for 30 minutes with gentle shaking, and then rinse the gel with Renaturing-Buffer once, and put into the Renaturing-Buffer for 30 minutes with gentle shaking. Place the gel on pre-soaked 3-4 layers of WHATMAN paper (Whatman, Germany), which was placed on a ‘Bridge’ over the 20 x SSC pool. Rinse the membrane (Cellulose porablot Nyamp (Macherey-Nage Nr. 741 200.)) first with Mili-pore water (Mili-pore, Germany), then with 10xSSC buffer. Place the membrane on the gel, and push out the air bubbles. Seal the sides with parafilm (or fresh folie). Add 4-layers of 10 x SSC pre-soaked WHATMAN paper on top of the membrane, and chase out the air bubbles. Add another 3-cm-thick dry WHATMAN paper on top and another 3 cm-thick dry paper towels. Place a weight of about 500 gram on top. Leave the blotting setup over-night (at least 16 hrs, not longer than 20 hrs.). The next morning disassemble the

blot tower, and wash the membrane in 5xSSC buffer twice for 10 minutes, and air-dry. Cross link the DNA onto the membrane by 2 times UV-light automatic cross-linking (Strategene).

The membrane was then Pre-hybridized. Quick-Hyb. Solution (QIAGEN) was used for hybridiztion. Gently shake the Quick-Hyb. Solution before use. For 250ml hybridization tube, 30 ml of hybridization solution (0.5% SDS of final concentration; 0.5 ml Salmon Sperm DNA; about 30 ml Quick Hyb. solution) was used. Put the pre-hybridization tube into the rotor-incubator before it got warmed-up to 68°C. Pre-hybridize the membrane in the solution for 1 hour. At the same time, label the probe in Radio-Active lab. PCR amplified probes were labeled using Radom Primed DNA labeling Kit (ROCHE). Pipette 100 ng DNA into a new screw-cap 1.5 ml tube, and add water to a final volume of 9 µl. Boil the DNA at 95°C for 10 min. After boiling. Put the DNA immediately on ice, and add the following solutions: 1 µl dATP; 1 µl dGTP; 1 µl dTTP; 2 µl Radom Primer and Buffers; 5 µl [P^{32}] dCTP; 1 µl Klenow Enzyme. Vortex the mixture and leave it at 37°C for 45 min. To stop the labeling reaction, put the reaction tube on ice and add 2 µl 0.2 M EDTA, and then add 80 µl 1xTE buffer, mix gently on ice. To purify the labeled probe, prepare a purification column (QIAGEN). Resuspend the column and remove the air bubbles, let the column drain at gravity for 10 min over a 2 ml tube. And centrifuge the column at 1000 rpm for 4 min to remove the solution completely. Place the column onto a new 1.5 ml Eppendorf-tube and apply the reaction mix into the middle of the column, and centrifuge at 1000 rpm for 4 min. Pipette 1 µl of the labeled DNA into a new 1.5 ml Eppendorf-tube, and determine the radio-activity. The probe's radio activity should be higher than $1x10^8$ cpm/g DNA.

Following pre-hybridization, hybridization of the membrane was performed. Mix the labeled probe DNA with 200 µl Salmon Sperm DNA, and boil the mixture at 95°C for 10 min in a screw-cap Eppendorf-tube. After boiling, take the pre-hybridization tube out of the incubator and pipette 500 µl solution into the probe, mix on ice and then transfer the probe into the hybridization tube. Shake the hybridization solution very well and put the hybridization tube back into the incubator. Hybridize the membrane for 1 hour at 68°C. After hybridization, wash the membrane with RT washing buffer (0.1 x SSC and 0.1 x SDS) briefly. Then add

20 ml washing buffer into the tube, wash the membrane twice with gentle rotating, each time 15 min. then wash the membrane once in 55°C washing buffer for 15 min. if necessary, wash the membrane once more at 55°C. Wrap the membrane into fresh folie, and put an illuminant ruler marker beside the sample loading slots. Expose the membrane in the cassette for 12 hours at -70°C, and develop the film (ECL, Kodak) in the next morning.

4.7 Real-time PCR analysis:

The real-time PCR system is based on the detection and quantitation of fluorescent probes , which bind specifically onto defined regions of templates sequences. The TaqMan probes (Heid, et al. 1996) and molecular beacons (and more recently, scorpions) use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target sequences in DNA samples. TaqMan probes are oligonucleotides, which are usually designed to be longer than the primers (20-30 bases long with a Tm value of 10 ℃ higher) . The probe oligonucleotides carry a fluorescent dye on the 5'-end, and a quenching dye (TAMRA) on the 3'-end. Laser scanning head of ABI 7000 system detects the accumulation of PCR products by analyzing the increase in fluorescence of the reporter dye. The TaqMan probes allows simultaneous detection of multiple DNA species (multiplexing) by use of 2 reporter dyes on 2 different probes. By multiplexing, both the target and endogenous control were amplified in single tube. But for our tests, the multiplexing did not work stably as we expected. So all runs were performed using simplex RT-PCR.

The threshold cycle or the C_T value is determined automaticly by the ABI 7000 system. For analysis of the RT-PCR resuslts, the most important parameter for quantitation is the C_T. Relative gene expression comparisons was carried out by using endogenous single copy genes: *ROSA26* and *GluR-A* genes to obtain stable and constant results. By using an endogenous control as an active reference, quantitation of the targets were normalized for differences in the amount of total genomic DNA input. Concerning the issue of the choice of a normalizer, work from Suzuki et al. (Bio Techniques 2000;29:332) were helpful. Two-step RT-PCR were used to perform all RT-PCR experiments. The PCR steps are as following: 94 °C

denature for 5 minutes; 94 °C denature for 10 seconds; 60 °C extension for 40 seconds.

All TaqMan primers and probes were designed by following guidelines: 1: The Primer Express software designs primers with a melting temperature (Tm) of 58-60^0 C, and probes with a Tm value of 10^0 C higher. The Tm of both primers should be equal; 2. Primers should be 15-30 bases in length; 3. The G+C content should ideally be 30-80%. If a higher G+C content is unavoidable, the use of high annealing and melting temperatures, cosolvents such as glycerol, DMSO, or 7-deaza-dGTP may be necessary; 4. The run of an identical nucleotide should be avoided. This is especially true for G, where runs of four or more Gs is not allowed; 5. The total number of Gs and Cs in the last five nucleotides at the 3' end of the primer should not exceed two (the newer version of the software has an option to do this automatically). This helps to introduce relative instability to the 3' end of primers to reduce non-specific priming. The primer conditions are the same for SYBR Green assays; 6. Maximum amplicon size should not exceed 400 bp (ideally 50-150 bases). Smaller amplicons give more consistent results because PCR is more efficient and more tolerant of reaction conditions (the short length requirement has nothing to do with the efficiency of 5' nuclease activity); 7. The probes should not have runs of identical nucleotides (especially four or more consecutive Gs), G+C content should be 30-80%, there should be more Cs than Gs, and not a G at the 5' end. The higher number of Cs produces a higher DRn. The choice of probe should be made first; 8. To avoid false-positive results due to amplification of contaminating genomic DNA in the cDNA preparation, it is preferable to have primers spanning exon-exon junctions. This way, genomic DNA will not be amplified (the PDAR kit for human GAPDH amplification has such primers); 9. If a TaqMan probe is designed for allelic discrimination, the mismatching nucleotide (the polymorphic site) should be in the middle of the probe rather than at the ends; 10. Use primers that contain dA nucleotides near the 3' ends so that any primer-dimer generated is efficiently degraded by AmpErase UNG (P/N 402877). If primers cannot be selected with dA nucleotides near the ends, the use of primers with 3' terminal dU-nucleotides should be considered.

At the end of each reaction, the recorded fluorescence intensity is used for the following calculations: Rn^+ is the Rn value of a reaction containing all components, Rn^- is the Rn value of an unreacted sample (baseline value or the value detected in NTC). DRn is the difference between Rn^+ and Rn^-. It is an indicator of the magnitude of the signal generated by the PCR.

There are three methods to quantitate the amount of template: 1. Absolute standard method: In this method, a known amount of standard such as in vitro translated RNA (cRNA) is used, 2. Relative standard: Known amounts of the target nucleic acid are included in the assay design in each run, 3. Comparative C_T method: This method uses no known amount of standard but compares the relative amount of the target sequence to any of the reference values chosen and the result is given as relative to the reference value (such as the expression level of resting lymphocytes or a standard cell line).

4.8 Analysis of the endogenous gene of the transgene locus

4.8.1 Endogenous expression analyzed by in situ hybridization (ISH) with oligonucleotide probes

ISH to brain tissue slices with short (45-80 nucleotides) oligonucleotides was developed and optimized by Yong and his collaborators, Lewis, and others (Lewis et al., 1985, 1988; Morris et al., 1986; Yong et al., 1986a,b; Yong, 1989; reviewed, Wisden et al., 1991; Emson, 1993; Pratt and Kokaia, 1994). Radioactivity labeled oligonucleotide probes are popular in ISH and there are many protocol variations. Slide-mounted brain sections are cut from unfixed frozen tissue on a cryostat. Sections are then fixed with 4% Paraformaldehyde (PFA) and stored in ethanol at 4 °C until ISH will be performed. Synthetic oligonucleotides are radiolabeled (‘tailed’) using terminal deoxyribonucleotide transferase (Roche, Germany) and ‘hot’ deoxyadenosinetriphosphate ((^{35}S)dATP). The synthetic oligonucleotides is diluted to 0.3 pmol/µl before labeling. In our experiments, 0.3 pmol of oligonucleotide (50mers and 55mers) is labeled with 10 pmol (^{35}S)dATP (using a 30:1 molar ratio of isotope to oligonucleotide). This results in the addition of approximately 10-20 AMP residues to the 3’ end of the oligonucleotide (assessed by acrylamide gel analysis) (Wisden, W. and Morris, B. J. 2002). For 10 µl reaction: Mix 1 µl oli-

gonucleotide at concentration of 0.3 pmol/µl; 2 µl 5 x reaction buffer (Boehringer-Mannheim); 0.6 µl 25 mM $CoCl_2$ (Boehringer-Mannheim); 1.5 µl (^{35}S)dATP (1300 Ci/mmol, DuPont, NEN, NEG-034H); 5 µl sterile DEPC-treated H_2O; 1µl TdT at 25 U/µl (Boehringer-Mannheim). Incubate for 5-8 min at 37℃. The reaction was stopped by adding 40 µl TE buffer (10 mM Tris, 1mM EDTA). To remove the unincorporated nucleotides, the total 50 µl from step 3 is applied to a Qiaquick column (Qiagen, Germany). The column is spin down briefly, and the flow thru is discarded. Apply 50 µl of elution buffer (Qiagen, Germany) to the column and spin down shortly. 2 µl of the eluate is counted by using liquid scintillation counting. (the counts should be in the range 50,000 dpm/µl – 200,000 dpm/µl). After counting, 1 µl of 1 M DTT (dithiothreitol; 1 M stock. 3.09 g in 20 ml sterile H_2O) is added to the eluate to preserve the probes from oxidation. Labeled probes are diluted in hybridization buffer, and the probe-hybridization buffer mix is applied to the brain tissue slices, and hybridized overnight at 37℃. The next day excess probe is washed off, and after dehydation, the sections can be exposed to X-ray film (Kodak, USA).

4.8.2 Pik3c3 Gene Locus Targeting Vector Construction:

Overhang Primers were designed as following: to get >95% restriction enzyme digestion efficiency, the end of the overhang primers were designed according to NEB catalog. And the overhang nucleotides sequence is not longer than 40% length of the oligos used for PCR amplification, regardless of the overall annealing temperature. We used Long Range PCR amplification kit from ROCHE: Expand Long Template PCR System (ROCHE 1681 842). The 10.0kb Long Homologous Arm amplification was performed as following: Template DNA: BAC clone (RP24-185E11) purchased from BACPAC company. And the BAC clone was purified by NulearBond Maxi Purification Kit (NuclearBond). For PCR reaction, only tens of nanogram of BAC DNA was used to get specific amplification products. Primers were ordered from Thermo Corp. and primers were diluted in H_2O to 10 pmol/µl for PCR reactions.

Primer Name	Primer sequence
SA87.5-LongArm-5' end	AAGGAAGGCCGGCCAAAAGGTATTCTCCTTACGACTCTGTGCTTTCTGCTGCCAAG
SA87.5-LongArm-3' end:	CACTCAATTCTCATGCAGGACTTCCAGGCCTGAACCTTGTAAGAGTAAGAATCAAGGCGCGCCAA
SA87.5-ShortArm-5' end:	GACTAGTGGCCCTCGTGGCCGGCCAATTGGGCCTTGGCGCGCCTTAATTAAACAGCATGAGTACCGGCAGGGACACAGCAGAGTGTGTAAAGCAAGAATTGTACAT
SA87.5-ShortArm-3' end:	TTTTCCTTTTGCGGCCGCTTTGTTTAAACGGAGGGGAGGCCACGGGAGGAAGAGGGTTGGTTAT

PCR was done in Buffer 3 system (ROCHE, Expand Long Template PCR system), which was designed to amplify DNA fragments longer than 12.0kb. and the reaction condition is as following: 2.5 l 10mM dATP; 2.5 l 10mM dCTP; 2.5 l 10mM dGTP; 2.5 l 10mM dTTP; 1 l 10 mM downstream primer; 1 l 10 mM upstream primer; 10xPCR Buffer 3 with $MgCl_2$; 0.75 l Expand Long Template enzyme mix (supplied).

Thermal cycling program:

Thermo-Cycles	Temperature and Time
-1 cycles:	94°C 2 min
-5 cycles:	94°C 10 sec
	62°C 30 sec
	68°C 8 min + 5 sec
-30 cycles:	94°C 10 sec
	58°C 30 sec
	68°C 8 min + 5 sec
-68°C for an additional 7 min after the final cycle.	

After PCR reaction, the products were analyzed and purified by 0.7% agarose gel (without EtBr, but stained afterwards). Overhang-Primers: Long-arm 3' & 5' primers; Short-arm 3' & 5' primers introduced desired restriction enzyme cutting sites: *Hind III*; *Pac I*; *Afl II*; *Fse I*; *Asc I*; *Pme I*; etc.. PCR-amplified ShortArm was introduced into backbone plasmid: pHanno'sTK by *Hind III* & *Not I* sites (named pShortArmTK). Following that cloning, PCR-amplified-LongArm was cloned into *Hind III* & *Fse I* linearized pShortArmTK, resulting in plasmid pLongShortArmsTK,

which contains the Long- and Short-Arms, and also the Multiple Cloning Sites used to clone gene of interests. Before further processes, the homologous arms cloned into pLongShortArmsTK were sequenced and analyzed by specific restriction enzymes. Sequencing reactions were performed and sequencing results showed that there are no mutations from either the PCR reactions, or cloning procedures. To clone ^{GFP}GluR-A into the targeting constructs, plasmid pnlacZ-^{GFP}GluR-A(i) was modified to be compatible with the MCS in pLongShortArmsTK. The Backbone of pnlacZ-^{GFP}GluR-A(i) was amplified in two PCR reactions to introduce restriction enzyme sites. The first PCR amplified from *PshA I* & *Hind III* sites using 5'-End primer (A(i)Backbone01) and 3'-End primer (A(i)Backbone02), introducing *Asc I* and *Pac I* sites at the 5'-End and *Xba I*; *Fse I* sites at the 3'-End and destroying the *Pme I* site. The second PCR amplified from AseI-AseI-PshAI sites using 5'-End primer (A(i)Backbone03) and 3'-End primer (A(i) Backbone04), introducing *Xba I* site at the 5'-End and *Hind III*; *Ase I*; *Afl II*; *Spe I*; *Pac I*; *SgrA I*; sites at the 3'-End. PCR products were digested by *Xba I* and *Hind III*. The fragments were purified and ligated to generate a plasmid named pBackBone, which then was linearized by *Afl II* and *Pac I*, to accept the PCR-Amplified loxP-neo-loxP element of PGK-neo plasmid. The loxP-neo-loxP element was obtained by Overhang-Primers-Mediated (5'pgkneo & 3'pgkneo) PCR reactions, which introduced *SgrA I* and *Pac I* sites at the 5'-End; and *Afl II* site at the 3'-End. The loxP-neo-loxP element was digested by *Afl II* and *Pac I*, and cloned into plasmid pBackBone.

4.9 Targeting in Embryonic Stem Cells.

Plasmids were digested to linearize about 50 µg plasmids in 330 µl volume and then checked 5µl on 1.0% agarose gel. The rest was precipitated by 100% ethonal. The pellets were washed and resolved well in 80 µl sterile 1x PBS, then subjected on to Chroma spin 1000TE column for plasmid backbone elimination according to manufactural instruction. Change ES medium 2 hours prior to cell harvest, the passage number of ES cells was documented with date. Prepare 6 gelatine coated plates (10 ml) per electroporation. ES edium was removed completely, and washed twice with 5 ml pre-warmed 1x PBS. Add 1.5-2 ml Trypsin, gently

shake the plates and incubate 3-5 min. at 37 degree. Suspend cells well (with fire polished pasteur pipettes) in 1-2 ml ES medium on the culture dish. Transfer cells in 10 ml medium into 15ml Falcon tube and spin 3 min at 1000 rpm RT. Discard medium, and re-suspend cells first in 2 ml ES medium with fire polished pasteur pipettes, then dilute into 10 ml and plate on one plate. Let feeder cells settle for 15 min. at 37 ℃, then wash off ES cells with medium and transfer into 50 ml tube, pool cells from multi plates. Check 20 µl suspension under microscope. Count cells in 40-50 ml dilution, then spin down, and resolve in 1-1.3x 10^7/ml cold PBS. Put sterile cuvettes on ice, mix 700 µl cells with 100 µl DNA (20-30 µg), transfer into cuvette. Leave the cuvette 10 min. on ice.

After incubation on ice, turn on / off twice the device to unload condensor. Set the extender to 500 uF and Capacitance to 'Ext.'. Set Voltage to 0.24 kVolt. Dry cuvettes well before installing. Push both buttons until beep sounds, pay attention to the time constance. Double check parameters before putting cuvette for 10 min. on ice. Turn on/ off twice again when finished. Mix cells in cuvette and transfer into Falcon tube, add medium to 50 ml, plate out on 5 10 cm plates. Change medium after 24 hours. Start administration of G418: 250µg/ml final concentration, after another 24 hours.

4.9.1 Picking Resistant ES Colonies.

After selection in G418 containing medium for 8-12 days, cells on survival control plate should all be dead. Electroporation plates will have surviving colonies of ES cells which are 0.5-2mm in diameter. These clones are picked for propagation and analysis. Plate mitoC treated feeder cells to flat-bottomed 96-well plates on the evening before picking. One vial of 4Mio cells will suffice for two 96-well dishes. Use 200 µl medium/well. Change the medium on the clone plates and change the medium on the feeder cells to ES cell medium two hours before picking. Assemble following materials in the cell hood: Ppre-warmed PBS and PBS/PS (pen/strep, 1x); Pre-warmed ES cell medium in 15 cm culture plate; Round-bottomed 96-well dish with 50 µl trypsin/PBS (1:1) per well. Multi-tip pipettor (12-mer); Pipette tips (complete rows of 12). Wash gelatin plate containing clones 2x with PBS (after first wash, one can mark easily the position of clones on the bot-

tom of the plate if necessary) and add 5-8 mls PBS/PS to the plate. Pick the clones in to the 96-well plates on row after the other according to the following sceme. It's very helpful to find a personal rhythm and not to interrupt before you are finished. Get well organized before one starts. You may either pick in rows of twelve or in rows of eight depending on you speed. Keep in mind that the critical step is the trypsin reaction. his step should produce a single cell suspension by should not take for too long. Under the microscope, locate a clone; Lift the lid of the plate slightly and approach clone by eye with yellow tip of the pipettman; Under the microscope scrape gently to dislodge the clone from the plate; Suck the cells up into the tip, take 50 µl volume maximum, the less, the better; Eject the cells into one well of the 96-well plate containing 50 µl trypsin, pipette up and down; With a fresh tip, pick the second clone (orientation and clone number is easily maintained by relative position of pipette tip taken from rack and analogous position of well in 96-well plate); After picking on row (8 clones), pipette up and down the mixture in al wells simultaneously with the multi-tip pipettor; Leave these tips on the pipettor; Pick a second row of eight clones; Again pipette the cells in row one up and down with the multi-tip pepettor; Monitor dis-aggregation of ES cells u nder the microscope; Stop trypsin reaction by adding 100 µl ES cell medium (from a 15 cm culture dish) to all eight wells in row one with multi-pipettor (fresh tips), pipette up and down; Transfer approximately 2-3 drops to row one of 96-well feeder cell plate; Pool the remainder of each of the trypsin wells in row one into microfuge tubes; Pools of 4-12 are convenient, depending on number of clones to be picked and analyzed; Store the pools on lice until DNA preparation; Continue in this manner to pick clones. Analyze clones as quick as possible in order to minimized the time of growth in the 96-well plates. Propagate the positive clones step by step onto laarger plates and freeze as soon as possible; Change medium twice a day.

4.9.2 Genomic DNA Extraction from Resistant ES Colonies.

Expend the positive ES cells in 96-well plate for 2-4 days, then: add 90 µl lysis buffer containing 0.5 µl RNaseA solution, incubate the plate at 37 degree for 30 min. with gentle shaking, sealed with Parafilm. Take out the plate and let it cool

down to RoomTemperature. With multi-pipettor pipette up and down 10-12 times, then transfer the mixture into 8-mer PCR tubes, containing 30 µl Protein Precipitation Solution. Centrifuge for 10 min, 3000 rpm. About 100 µl Supernatant with multi-pipettor into new 8-mer PCR tubes, containing 100 µl Isopropanol (RT) and 10 ug Glycogen (2µl of 5 mg/ml solution), mix well. Centrifuge for 15 min at 4500 rpm. Discard supernatant one row after the other. 200 µl 70% EtOH to PCR tubes, and spin at 4500 rpm for 10 min, discard the supernatant. repeat this step once more. Dry the PCR tubes in SpeedVaccum for 10 min at 42 ℃. Disolve the pellets in 50 µl sterile H2O, and let it stand on the bench overnight, RT. Check the DNA quantity and quality on 1.0% agarose gel.

4.9.3 Preparation of ES Colonies for Blastocytes Injection.

After selection and identification of correctly targeted ES colonies, the ES cells were expanded and frozen at -70℃. Three to five days before injection, three 6 cm plates with Mito C-Feeder cells were prepared. One day after, the ES cells were recovered on 6 cm Feeder cell plates with three different concentrations, which will insure the correct growth rate of recovered ES colonies. On the injection day, the medium was changed 1-2 hrs before preparation. One to two hours later, the plate was washed twice with warm PBS, and added with 1 ml trypsin. Trypsinization reaction was monitored every minute under microscope. After 3-8 minutes, suspend the ES cells with polished plastic pipette carefully. The reaction was stopped by adding 1-2 ml medium. ES cells were well suspended in 8 ml medium using polished pipettes gently. ES cells were collected by spinning at 1000 rpm for 5 min at RT. Medium was discarded and ES cells were resuspended in 1 ml ES medium. ES cells were added 4 ml medium and put onto gelatin-coated 6 cm plate for 20-30 min incubation at 37 ℃. After 30 min incubation, the feeder cells were settled on the surface of the gelatin, but the ES cells were washed off by gently apply medium on the tilted plate. The washed off ES cells were transfered into a new gelatin-coated 6 cm plate. And the procedure was repeated 3-5 times, till most of the feeder cells were removed from the medium. Examine 15 µl ES cells containing medium under microscope. Collect all the ES cells by centrifugation at 1000 rpm for 5 min at RT. Resuspend the ES cells in 1ml

ES-Injection-medium (20 µl/ml ES-Medium 1M Hepes (final concentration 20 mM) + Pen/Strep) with polished pipette. ES cells were kept in ice-flake water bath and transferred to ZTL-Biotechnology Laboratory in Heidelberg immediately after preparation.

4.10 Western Blot analysis.

Pre-chill the homogenizing buffer (1:1=B1:B2, 1/10 Proteinase Inhibitor) on ice. Every step will be done in a 4°C cold room. Place the brain tissue into the homogenizer, and then add 100-300 times (weight of the tissue) homogenizing buffer into the homogenizer. Homogenize the brain up and down 4-6 times with 5-10 strokes each, at 250-500 rpm in a S874 Potter. After homogenizing, transfer the solution into a new labeled Eppendorf 1.5 ml tube. Incubate the homogenize on ice for 10 min. During incubation, wash the homogenizer with pre-chilled water, and dry it with fine tissue paper. After 10 min on-ice-incubation, centrifuge the homogenize at 13,000 rpm, 4°C for 10 min. Then transfer and aliquot (3-5 aliquots per sample) the supernatant into new labeled Eppendorf 1.5 ml tubes, freeze immediately in liquid Nitrogen and store the aliquots at –70°C.

Buffer Name	Chemical Contents	pH value
B1	25 mM HEPES	pH=7.4.
B2	25 mM HEPES buffer	pH=7.4
	300 mM NaCl	
	2% Triton X 100	
	Proteinase Inhibitor: 1 mini-tablets in 5 ml H_2O	

4.11 Recombinant Adeno Associated Virus (rAAV) as gene delivery system

4.11.1 rAAV Constructs

pAAV-CMV-nlsitTA was constructed by cloning HindIII and BamHI linearized nlstTA of p RK5-htTA+nls into pAAV-MC digested by HindIII and BglII. pAAV-CMV-nlsrtTA was made by combining HindIII and BamHI linearized nlsrtTA of pRK5-rtTA+nls into pAAV-MC digested by HindIII and BglII. pAAV-HSYN-nlsrtTA was made by three piece ligation: Fragment 1: BlpI and HindIII digested pRK5-

htTA+nls containing 3' end nlsrtTA sequence with polyA signal; Fragment 2: EcoRI and HindIII linearized pAAV-6pminibi-Sewb; Fragment 3: EcoRI and BlpI digested pBS.hM2+nls haboring 5' end nlsrtTA sequence. pAAV-HSYN-nlsitTA was produced by cloning EcoRI and HindIII digested pRK5-htTA+nls containing nlsitTA fragment into EcoRI and HindIII linearized pAAV-6p-minibi-Sewb.

4.11.2 rAAV production and purification:

After plasmids construction and amplification, rAAVs were produced by calcium-phosphate cotransfection of Helper plasmids pDp1, pDp2 and rAAV vector. From previous study and our own laboratory tests, cross-dressing of the rAAV by mixing of two Helper plasmids pDp1 and pDp2 (ratio: 3:1), would increase the purification efficiency by using precasted Heparin-Affinity Column (Amersham, Germany), and the cross-dressing will sustain the infection rate of rAAV in neurons. Cells were harvested 72 hours post-transfection. Cells were collected by centrifugation at 4000 rpm, 25℃. Cells were resuspended in Lysis Buffer. After 3 times freeze and thaw in dry-ice-ethonal bath, benzonase digestion was incubated at 37℃ for 45 mins. The crude lysate was centrifuged at 4000 rpm for 30 min at 25℃. Samples were filtered using low-protein binding filters: 0,45 µm and then 0,20 µm before loading onto heparin column. After filtration, samples were loaded onto heparin column. Flow rate was limited to 2 ml/min. After washing and elution, samples were subjected to concentrator (100kd, Millipore, Germany). Virus stocks were concentrated into 0,30 - 1,00 ml from 30,0 ml crude lysate collected from 20 times 10 cm plate ($3x10^6$ cells/plate). For in vivo applications, we adjust the infectious virus concentration to 10^7-10^8 particles/ml.

Buffer Name	Chemical Contents	pH value
Lysis Buffer	150mM NaCl; 50mM Tris; H2O;	pH=8.4.
Equilibration and Wash Buffer	1 mM MgCl2; 2,5 mM KCl; PBS;	pH=7.2
Elution Buffer	500 mM NaCl; PBS	pH=7.2

4.11.3 Stereotaxic Injection into the Mouse Brain:

Wild-type mice (C57/BL6J or NMRI, aged postnatal day P60) and tTA- expressin transgenic mice: $Tg^{CaMKII\text{-}tTA}$ (KT1) (P60); $Tg^{CaMKII\text{-}NR2C\text{-}itTAnls}$ (CN10 and CN12) (P60) and Thy1.2-itTA (P60); were anesthetized with i.p. injection of ketamine (100 mg/kg) / xylazine (10 mg/kg). Experimental animals were kept deeply anesthetized by monitoring pinch withdrawal, eyelid reflex and respiration rate. Heating block (Watlow) was utilized to maintain the experimental animals' body temperature. Experimental animals were subjected to Stereotaxic Injection using the SAS75 stereotaxic alignment system with combination of EM70G manipulator (Kopf Instruments, Germany). The three axes: X-axis; Y-axis and Z-axis, were referred to the medial-lateral axis; the anterior-posterior axis and the ventro-dorsal axis, respectively. One to three craniotomies (50-100 µm in diameter) were opened on the scull according to coordination map of rodents brain anatomy. Viral stock aliquots (75-100 nl) were delivered carefully using ultrathin (inner diameter 6-9 µm) glass pipette (Blaubrand intraMARK). All experiments were carried out according to the biosafety levels' guidelines, which are specified in the German GenTSV.

4.12 Confocol microscopy:

Confocal images were obtained using a Zeiss LSM5 PASCAL con-focal laser scanning microscope, with a 40x (1.3 NA) oil-immersion objective. Optical slices of all specimens were adjusted to 2.0-5.0 mm thick using the Reuse Function of Zeiss LSM5 PASCAL. The co-expressed reporter genes were stained using FITC- and/or Cy3, to counter stain each other. Images were processed through excitation of an argon laser (excitation, 488 nm; emission, BP505–530 nm emission filter) for intrinsic Venus signals, and a He-Ne laser (excitation, 543 nm; emission filter, LP560 nm) for Cy3-labeled signals. The two co-expressed reporter genes were localized in different compartments of the cells: itTA and Cre carry NLS (nuclear localization signal); but the Venus is soluble in the cytoplasma. Highly co-expression patterns were illustrated by merging images from both channels.

4.13 Live-Imaging using two-photon microscope:

$Tg^{Thy\text{-}1.2(64)\text{-}itTAnls}$ mice were infected with rAAV-6p-minibiCre-Venus virus as described in Materials and Methods. After 5-7 days, the infected $Tg^{Thy\text{-}1.2(64)\text{-}itTAnls}$ mice were anethetized and the head was fixed. Custom-designed head-plates were attached to the injection area after the skull thinned. Fluorescent signals were taken and analyzed using ImageJ (http://rsb.info.nih.gov/ij/) and IgorPro (Wavemetrics, Lake Oswego, Oregon, United States). Series of images were also collected either at frequence rates of 5-15 Hz, or at depths of 50 µm of interval with cooled-CCD camera (CoolSnapHQ; Photometrics, Huntington Beach, California, United States) attached on the custom-built upright fluorescence microscope with a 203, 0.95 NA water immersion objective (Olympus, Tokyo, Japan) and the corresponding filter sets (Chroma Technology, Rockingham, Vermont, United States): HQ495/30, Q520LP, and HQ545/50 for Venus. The serial pictures taken at different time and/or depth interval were transformed into movie and/or 3-Dimension formats to illustrate the micro-structure mobility upon certain stimuli, and to reveal the 3-D structure of infected neurons.

4.14 Material

4.14.1 Mouse lines:

C57BL/6: Fa. Charles River (Deutschland)

NMRI: Fa. Charles River (Deutschland)

Thy1.2-itTA: R. Sprengel

R26R: P. Soriano

G3: R. Sprengel

SA87.5: R. Sprengel

A1.1: R. Sprengel

Pik3c3-A1: R. Sprengel

4.14.2 Oligonucleotides Sequences (5'-3'):

rspCre1 ACCAGGTTCGTTCACTCATGG

rspCre2 AGGCTAAGTGCCTTCTCTACAC

Lac3' TTACCCGTAGGTAGTCACGCA

Lac5' TTACGATGCGCCCATCTACAC

tTA1: GTGATTAACAGCGCATTAGAGC

tTA4: GAAGGCTGGCTCTGCACCTTGGTG

FP-SA-400: TTCAAGTCTGGATTGCTCTCCTCTCATT

Hgh04: gtgatctacccaccttggcctc

A(i)Backbone01: cccaagcttgggtaaacgtcgacgaggggtggcatccctgtgacccctc cccagtgcctct

A(i)Backbone02:gctctagagcaaggccggccactggtcgagctgatacttcccgtccgcc aggggacat

A(i)Backbone03: cccaagcttggattaatcccttaagggactagtcccttaattaaggcacc ggtgaatcggccaacgcgcggggagaggcggtttgcgtattgggcgctc ttccgcttcct

A(i)Backbone04: gctctagagcgtcgagtgaagacgaaagggcctcgtgatac

5'pgkneo: gttggccgattcaccggtgccttaattaagggtgagttagctcactcattaggca ccccaggctttacactttatgctt

3'pgkneo: tttacccaagcttggattaatcccttaagggttacgccagctggcgaaaggggga tgtgctgcaaggcgattaagtt

4.14.3 Antibodies and their dilutions:

Rabbit polyclonal Anti-Cre (Cre Recombinase), PRB-106C, BabCo; IHC: 1:3000

Rabbit polyclonal Anti-tTA/(rtTA) (tetracycline-responsive transactivator; and reversed tetracycline-responsive transactivator), PRB-106C, BabCo; IHC: 1:3000

Peroxidase-conjugated Goat Anti-Rabbit IgG (H+L), PI-1000, Vector

Cy3 dye-conjugated Goat Anti-Mouse IgG (H+L), 115-075-146, Dianova

Cy3 dye-conjugated Goat Anti-Rabbit IgG (H+L), 111-075-144, Dianova

Fitc dye-conjugated Goat Anti-Mouse IgG (H+L), 115-095-146, Dianova

Fitc dye-conjugated Goat Anti-Rabbit IgG (H+L), 111-095-144, Dianova

4.14.4 Abbreviations:

α Alpha

α- Anti

AMPA L-a-Amino-3-hydroxy-5-methylisoxazol-4-propionacid

AMPAR AMPA-Receptor ect. -Receptors

ATP Adenosine-tri-Phosphate

β Beta-

bp Basepair

BSA Bovine Serum albumin

C- Carboxy-

CaMKII Calsium/Calmodulin-binding ProteinkinaseII

Ce Cerebellum

cm Centimeter

Cre Cre-Recombinase

CNS Central nervous system

DAB Diaminobenzidine-Hydrochloride

DNA Deoxyribonucleicacid

dNTP Deoxynucleoacidtriphosphate

EDTA Ethylendiamintetraacetate

et al. and others

g Gram

GFP green fluorescent Protein

GluR Glutamate-Receptor-Subunit

h Hour

H/E Hematoxylin/Eosin

HEPES N-(2-Hydroxyethyl)piperacin-N-ethansulfanacid

IHC Immunohistochemstry

i.p. intraperitonal

kbp Kilobasepair

kDa Kilodalton

kHz kiloherz

LTP Long Term Potentiation

m Milli

M Mol

µ Micro

mg Micro Gram
min Minute
mm Millimeter
mRNA Messenger-Ribonucleicacid
n nano
Neo Neomycin
NGS Normal Goat Serum
NMDA N-Methyl-D-Aspartate
NMDAR NMDA-Receptor
NEO Neomycinphosphotransferase
nt Nucleotide
NTP Nucleotide-tri-Phosphate
P Postnatal Days
pA Polyadenylation signal
pA Pico Ampere
PBS Phosphate Buffered Salt solution
PCR Polymerase Chain Reaction
pH potential Hydroxyl
PFA Paraformaldehyde
RNA Ribonukleoacids
rpm Rotate pro Minute
RT Room temperature
RT Real-Time
sec Second
SDS Sodiumdodecylsulfate
SSC Sodiumchloride-Sodiumcitrate-Solution
TE Tris/EDTA-Buffer
TENS Tris-EDTA-Sodiumchloride-SDS-Buffer
Tg Transgene
Tris Trishydroxymethylaminomethan
U Units
UTR Untranslated Region

V Volt

W Watt

X-Gal 5-Brom-4-Chlor-3-Indolyl-b-D-Galaktopyranoid

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