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Oral examination:

Generation and analysis of transgenic mice
expressing CRE recombinase in defined interneurons

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Heidelberg, 17. September 2007

Angelika Vogt

Dedicated to my parents.

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Summary

GABAergic interneurons are the main source of inhibition in the central nervous system. In addition they play a crucial role during development since in a paradoxical fashion they are the origin of the first excitatory signals in the immature brain.

GABAergic interneurons comprise about 10 – 20% of the neuronal cell population and they can be divided into several subtypes. GABAergic interneuron classifications have been based on different criteria, including anatomical, neurochemical or physiological characteristics. Although the overall number of interneurons is small compared to that of principal cells, by virtue of their connectivity, interneurons are able to shape and regulate the activity of numerous principal cells and thus influence network activity.

To elucidate the role of interneurons during development and in the mature brain specific modifications of their molecular and physiological properties are required and can be achieved by selective ablation of distinct genes. An established and widely used technique is that of the CRE/loxP system. For ablation of a desired gene in a cell-type specific fashion the generation of mice expressing CRE recombinase in a subset of cells plus the generation of mice with a floxed allele are a prerequisite.

The aim of this study was the generation of mice with CRE expression in all GABAergic interneurons and of mice with restricted expression of CRE recombinase in a subset of GABAergic interneurons, the somatostatin-positive interneurons. To this end mice with CRE expression under the control of the GAD67 promoter ($GAD67^{CRE/+}$) - a common feature of almost all interneurons - and mice with CRE recombinase expression under the control of the somatostatin promoter ($SOM^{CRE/+}$) were generated. Immunohistochemical analysis of both $GAD67^{CRE/+}$ and $SOM^{CRE/+}$ mice provided evidence that CRE recombinase is functional *in vivo*. Co-localisation studies of CRE recombinase and endogenous GAD67 expression, demonstrated a 100% overlap. Double-labelling experiments of endogenous somatostatin and CRE recombinase demonstrated a good correspondence in the hippocampus but less so in other brain regions. No developmental or behavioural deficits were observed as a consequence of the genetic manipulations. Cell-type specific ablations of several genes of interest, e.g. trkB receptors in GABAergic interneurons, NR1 and GluR-A subunits in somatostatin-positive interneurons are currently being generated and will help provide more insights into the function of GABAergic interneurons during development and their involvement in specific network activity.

Zusammenfassung

GABAerge Interneurone stellen die Hauptquelle für Inhibition im zentralen Nervensystem dar. Sie spielen außerdem eine wichtige Rolle während der Entwicklung, da sie paradoxerweise im embryonalen Gehirn Ursprung der ersten exzitatorischen Signale sind.

GABAerge Interneurone umfassen 10 bis 20% der neuronalen Zellpopulation und können in verschiedene Subpopulationen eingeteilt werden. Die Klassifizierung von Interneuronen basiert auf der Einteilung nach anatomischen, neurochemischen und physiologischen Kriterien. Obwohl Interneurone eine vergleichsweise kleine Gruppe darstellen, haben sie auf Grund ihrer Verschaltung einen sehr großen modulierenden Einfluss auf Netzwerkaktivitäten indem sie die Erregbarkeit vieler Prinzipalzellen verändern.

Um mehr über die Rolle von Interneuronen im sich entwickelnden und im adulten Gehirn zu erfahren sind gezielte Veränderungen ihrer molekularen und physiologischen Eigenschaften notwendig. Diese Modifizierungen können durch selektives Abschalten einzelner Gene erreicht werden.

Eine etablierte Methode, um spezifisch Gene in bestimmten Zellpopulationen abzuschalten, ist das CRE/loxP System. Dieses System basiert auf der Kombination zweier transgener Mauslinien wovon eine CRE Rekombinase zelltypspezifisch exprimiert und die andere ein gefloxtes Allel besitzt.

Das Ziel dieser Studie war es zwei transgene Mäuse zu generieren, die CRE Rekombinase jeweils spezifisch unter der Kontrolle eines interneuronalen Promoters exprimieren: zum einen wurde der GAD67 Promoter verwendet, der ein gemeinsames Merkmal aller Interneurone darstellt ($GAD67^{CRE/+}$ - Maus) und zum anderen der Promoter des Somatostatin Gens, der spezifisch für eine bestimmte interneuronale Subpopulation ist ($SOM^{CRE/+}$ - Maus). Die immunhistochemische Analyse beider Mäuse bestätigte die Funktionalität der CRE Rekombinase *in vivo*. Zusätzlich wurde mit Kolo-kalisationsexperimenten überprüft, ob die Expression der CRE Rekombinase mit der des endogenen Gens, dessen Promoter verwendet wurde, übereinstimmt. Dies ergab im Fall der $GAD67^{CRE/+}$ - Maus eine 100% Übereinstimmung. $SOM^{CRE/+}$ - Mäuse wiesen eine gute Übereinstimmung im Hippocampus auf, jedoch nicht in kortikalen Gehirnregionen. Bei beiden Mäusen konnten keine entwicklungs- oder verhaltensspezifischen Auffälligkeiten auf Grund der genetischen Manipulationen festgestellt werden.

Verpaarungen der $GAD67^{CRE/+}$ - und der $SOM^{CRE/+}$ - Mäuse mit transgenen Mäusen, die ein gefloxtes Gen besitzen, werden zurzeit durchgeführt. Dadurch sollen unter anderem der trkB Rezeptor in GABAergen Interneuronen, sowie die NR1 und GluR-A Untereinheiten von NMDA- und AMPA Rezeptoren in Somatostatin positiven Interneuronen abgeschaltet werden. Die Analyse dieser zelltypspezifischen Knock-out Mäuse wird dazu beitragen die Funktion von GABAergen Interneuronen in Entwicklungs- und Netzwerkprozessen weiter aufzuklären.

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1. Introduction

The main functions of the mammalian nervous system are the integration of all external and internal afferent stimuli as well as the coordination and regulation of all processes of an organism. To fulfil these tasks, the basic units of the central nervous system, the neurons, are highly connected and build complex networks. These networks are based on a balance between two main groups of neurons that exhibit excitation and inhibition, respectively. Excitatory cells, often also referred to as principal cells constitute the majority of neurons in the brain. They use glutamate as a neurotransmitter. Inhibition is mediated by interneurons which represent 25% of all neurons in the neocortex and around 10% in the hippocampus (Aika Y et al., 1994; Woodson W et al., 1989; DeFelipe, 1993). Despite their small number, interneurons regulate and shape the activity of principal cells and thereby play an important role in network activity. Concerning their anatomical, physiological and molecular properties, principal cells form a homogenous group. On the other hand, interneurons are more heterogeneous and can be divided into several subgroups which cannot be clearly delimited from each other's. Still, they share some functional features that distinguish them from principal cells. First, almost all contain γ -aminobutyric acid (GABA) (Houser CR et al., 1983) as the primary inhibitory transmitter instead of glutamate (Baughman and Gilbert, 1981) and second, most interneurons have aspiny dendrites. However, regarding their morphological, molecular and electrophysiological properties GABAergic interneurons are quite diverse and in fact based on these criteria they can be divided in several subgroups. The following chapters describe these variations in more detail and refer exclusively to the interneurons of the hippocampus.

1.1. The hippocampus

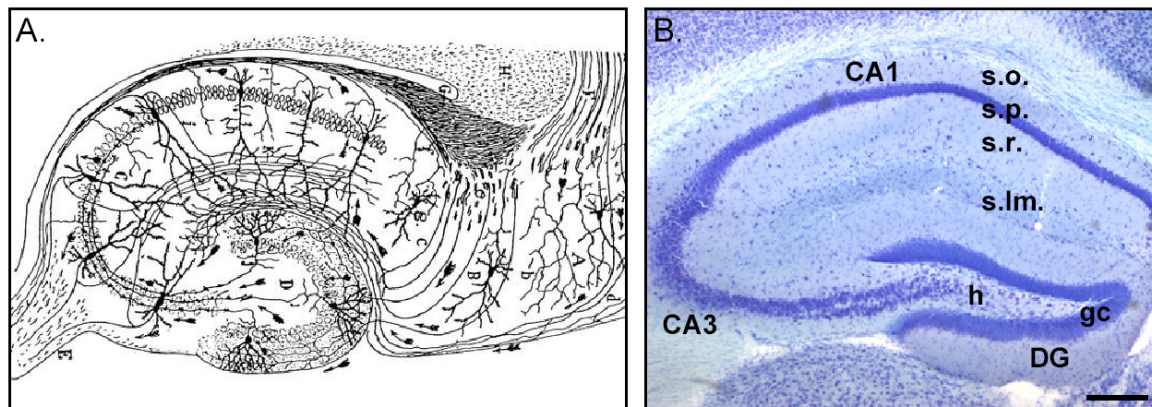


Fig. 1 *Anatomical structure of the hippocampus.* A: Drawing of the neural circuitry of a rodent hippocampus by S. Ramón y Cajal (Ramon y Cajal S., 1911). B: Nissl-stained section (coronal) of the hippocampus of an adult mouse. s. o.: Stratum oriens; s. p.: Stratum pyramidale; s. r.: Stratum radiatum; s. lm.: Stratum lacunosum-moleculare; h: Hilus; DG: Dentate gyrus; GC: Granule cell layer. Scale bar: 250 μ m.

The hippocampal formation is a structure located in the medial temporal lobe and belongs to the limbic system. Its shape resembles that of a sea horse which is reflected in the Greek name (hippo = horse, kampos = sea monster). The hippocampal formation consists of three distinct areas: the dentate gyrus, the cornu ammonis fields CA1 to CA3 and the subiculum (Fig. 1). The hippocampus proper (dentate gyrus and CA1 - CA3) is a highly laminar structure. The dentate gyrus is formed by the granule cell layer, which is the principal cell layer, the molecular layer above and the polymorphic zone (hilus) below. The CA1 - CA3 field is built of the stratum oriens followed by stratum pyramidale (principal cell layer), stratum radiatum and stratum lacunosum-moleculare (Lorente de No R., 1934; Ramon y Cajal S., 1893). The main excitatory pathway of the hippocampus is the so called trisynaptic circuit (Fig. 2) (Andersen et al., 1971; Ramon y Cajal S., 1911). The granule cells of the dentate gyrus are the first step in that loop. They receive input via the perforant path from layer II of the entorhinal cortex. The mossy fibers (granule cell axon bundle) project to the principal cell dendrites of the CA3 region where the information is further conveyed to the CA1 principal cells via the Schaffer collaterals (Schaffer K., 1892). CA1 principal cells represent the major output region of the hippocampus and they send their projections to the subiculum and back to the deep layers of the entorhinal cortex. From the entorhinal cortex the information is further projected to different cortical areas including the one from where the information originated.

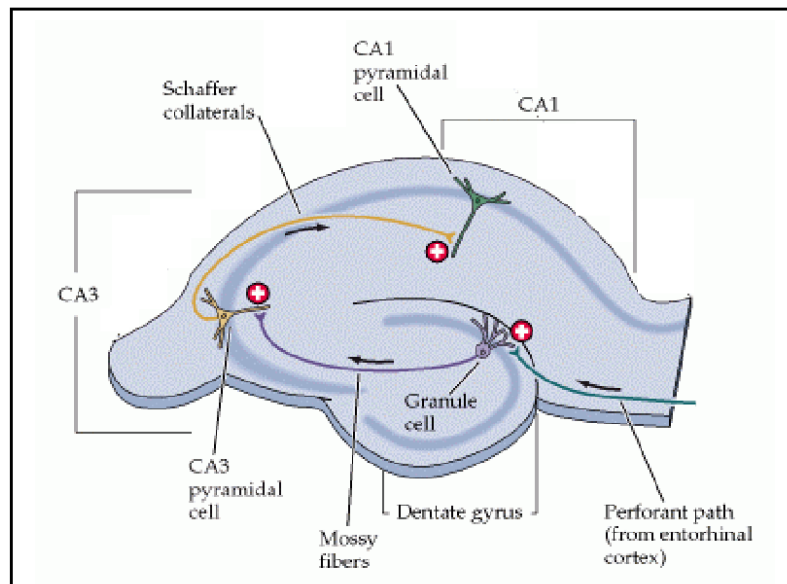


Fig. 2 *Scheme of the trisynaptic circuit of the hippocampus.* The major excitatory connections are shown: Granule cells receive input from the entorhinal cortex via the perforant path and send it via the mossy fibers to CA3 pyramidal cells which in turn mediate the input to CA1 pyramidal cells via the Schaffer collaterals. Figure (modified) from (Purves et al., 2001)

1.2. Classification of the different interneuronal subtypes of the hippocampus

Different attempts were made to divide the different interneuronal types into specific subgroups. Classifications were performed based on anatomical, neurochemical or physiological criteria. But there was increasing evidence that there was not always a direct correspondence when the above-mentioned classification criteria were applied. Thus, an anatomically homogenous class of interneurons may give rise to several subgroups if neurochemical or physiological parameters are also applied. However, keeping in mind that there are always some exceptions regarding the classification of interneuronal subpopulations, it still is useful to subdivide interneurons into several groups.

1.2.1. Anatomical features of interneurons and the functional implications

GABAergic cells are also referred to as local-circuit interneurons since most often their comparatively short axons regulate the excitability of nearby principal cells. This contrasts with the axonal pattern of many principal cells that frequently project to distant brain regions. However, there are exceptions also to this rule and GABAergic interneurons with long projecting axons have been described. Retrograde tracing studies identified interneurons in the hilus that participate in the commissural pathway of the hippocampus (Seress L and Ribak CE, 1983; Ribak et al., 1986) and long-range GABAergic interneurons of the hippocamposeptal pathway (Alonso A and Köhler C, 1982). The latter are neurons in stratum oriens and stratum pyramidale of the CA3 region and the hilus that target interneurons in the medial septum (Rose and Schubert, 1977).

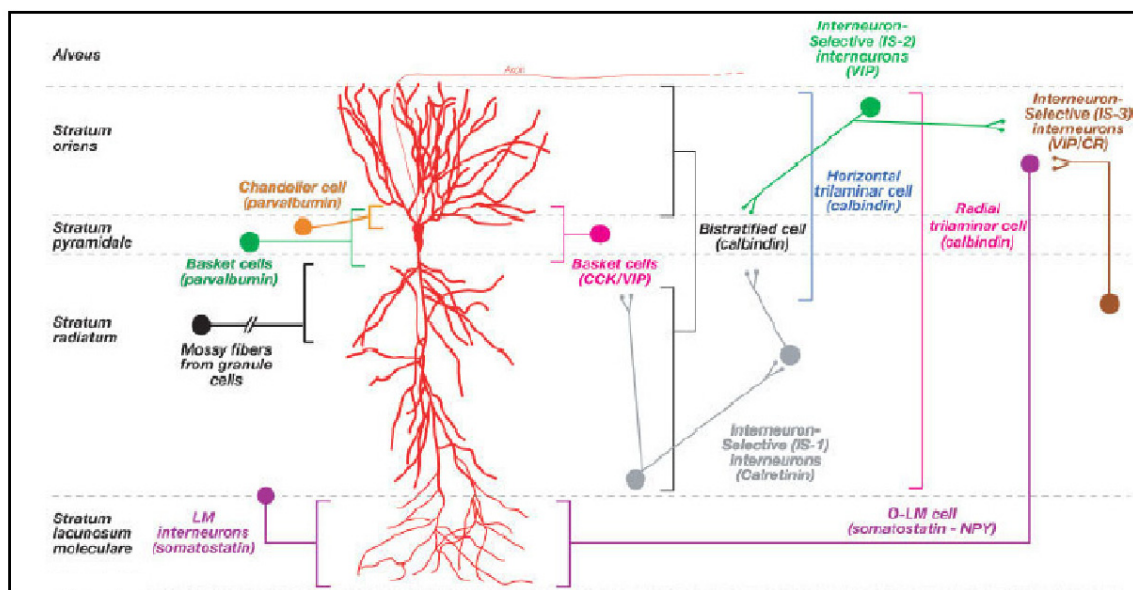


Fig. 3 *Afferences of GABAergic interneurons on pyramidal cells.* The coloured circles represent different interneuronal subtypes and the corresponding lines mark the domain of the pyramidal cell that gets innervated. Below each interneuronal subtype, the neurochemical content is indicated. Figure from (Danglot et al., 2004).

Since structure and function are closely linked, the morphology of interneurons provides important information about their role in neuronal networks. The laminar distribution of the axonal arborization of an interneuron demonstrates the locations of its postsynaptic target domains on the principal cell (Fig. 3). The three major types of interneurons in the hippocampus are axo-axonic (chandelier) (Kosaka,

1983;Somogyi P et al., 1985;Somogyi, 1977), basket and stratum oriens-lacunosum-moleculare (O-LM) cells (Lorente de No R., 1934;Ramon y Cajal S., 1893). The somata of axo-axonic cells are located in or near the principal cell layer and the dendritic tree spans all layers whereas the axon terminates in the principal cell layer and innervates the axon initial segment of principal cells (Kosaka, 1983). The group of basket cells is more heterogenous and named after the basket-like structure that their axons form around principal cell somata. This structure arises from convergent innervation of multiple basket cells, that preferentially synapse onto the somata and proximal dendrites of principal cells (Gulyás AI et al., 1993). Axons of O-LM cells, in contrast, target to distal dendrites of principal cells in stratum lacunosum-moleculare whereas their dendrites arborise either horizontally in stratum oriens (CA1) or span all layers except stratum lacunosum-moleculare (CA3). There are, however, some O-LM cells that exhibit long-distance projections to the medial septum (Toth et al., 1993).

In fact, the defined targeting of cellular compartments directly translates into differential functional effects of postsynaptic electrogenesis. For example, synapses of basket or axo-axonic cells terminate on the perisomatic region of principal cells. Hence, inhibition mediated by these cells will suppress repetitive generation of sodium-dependent action potentials and thereby directly control the output of principal cells. Synapses of O-LM cells on the other hand, target principal cell dendrites and will suppress calcium-dependent action potentials thus modulating the input from other cells (Miles et al., 1996).

Of particular note are GABAergic interneurons that appear to be interneuron selective. Anatomical studies of Ascády et al. and Gulyás et al. identified three different types of interneuron-selective inhibitory cells (IS-1, -2, -3) based on their connectivity and expression of neurochemical markers (Acsady et al., 1996b;Acsady et al., 1996a;Gulyas et al., 1996;Hajos N et al., 1996).

Another hallmark of interneuron connectivity is the presence of gap junctions that underlie the electrical coupling between GABAergic interneuronal subtypes. In most studies however it has been convincingly demonstrated that gap junction coupling of interneurons occurs by and large between interneurons of the same subtype (Venance et al., 2000), although exceptions to this rule have also been reported (Dr. A. Caputi, personal communication). Functional studies at the network level provided evidence that gap junction coupling stabilises robust synchronous rhythmic activity of

defined frequencies (γ) in the hippocampus (Hormuzdi et al., 2001; Buhl et al., 2003).

1.2.2. Distinction of interneurons according to their neurochemical content and functional implications

The most obvious common characteristic of GABAergic interneurons is the use of GABA as the only neurotransmitter (Storm-Mathisen et al., 1983), although co-release of GABA with other transmitters has also been reported in several systems such as retina (Huang S. and Moody S.A., 1998) and spinal cord (Jonas et al., 1998). Additionally, it has been reported that a small group of interneurons expresses glycine (Danglot et al., 2004) as an inhibitory transmitter as well as another group that expresses acetylcholine (Frotscher M et al., 1986). Furthermore, GABAergic interneurons also co-express the GABA synthesizing enzymes glutamic acid decarboxylase 65 (GAD65) and glutamic acid decarboxylase 67 (GAD67) (Ribak, 1978) that seem to subserve different roles which are not completely clarified so far.

After the identification of GABA as the main inhibitory transmitter in the forebrain, the almost exclusive presence of certain calcium-binding proteins and neuropeptides in GABAergic interneurons was reported in numerous studies (Fig. 3) (Celio, 1986; Kosaka et al., 1987; Baimbridge and Miller, 1982) (Jacobowitz DM and Winsky L, 1991). In fact, these proteins were frequently used particularly in anatomical studies to characterise and classify GABAergic interneuron subtypes. The most common neurochemical markers that have been extensively used are neuropeptides such as somatostatin (SOM) or cholecystikinin (CCK) and the calcium-binding proteins parvalbumin (PV), calbindin (CB) and calretinin (CR).

Dependent on the subregion of the hippocampus, PV-expressing interneurons constitute a large fraction of GABAergic interneurons that is about 20 to 29% (Freund and Buzsaki G, 1996; Jacobowitz DM and Winsky L, 1991). The GABA content in this cell population is low compared to that of other interneurons. This may be due to the high spontaneous firing rate of PV-positive interneurons leading to extensive transmitter release (Aika Y et al., 1994; Kosaka et al., 1987). PV-positive interneurons subserve perisomatic inhibition of principal cells and GABAergic interneurons and can be classified into basket and axo-axonic cells (Katsumaru H., 1988). It should be pointed out however, that some basket cells express the neuropeptides CCK or VIP rather than PV (Acsady et al., 1996b; Gulyás AI et al., 1991).

In contrast to the selective expression of PV in a subgroup of GABAergic interneurons, the expression of the calcium-binding protein CB is somewhat less specific. In addition to its presence in certain GABAergic interneurons it is also present in hippocampal principal cells such as granule cells of the dentate gyrus and some pyramidal cells of the CA1 region (Baimbridge and Miller, 1982). CB-expressing interneurons in the hippocampus constitute 10 -12% of all GABAergic cells and they preferentially innervate principal cell dendrites.

Another major calcium-binding protein is CR (Jacobowitz DM and Winsky L, 1991). If additional morphological features are taken into account, CR-positive interneurons can be further subdivided into spiny neurons that innervate dendrites of principal cells and aspiny neurons that innervate other GABAergic interneurons.

In addition to being useful interneuronal marker for anatomical studies, the functional role of calcium-binding proteins has been demonstrated by several electrophysiological studies (Blatow et al., 2003;Rozov et al., 2001;Katz and Miledi, 1968;Caillard et al., 2000;Neher, 1998). They bind calcium ions in the pre-synaptic terminal and hence modulate action potential dependent vesicular transmitter release. The kinetics of calcium binding and release from these cellular calcium buffers - slow kinetics for PV and fast for CB and CR - differentially effect transmitter release and hence short-term modifications such as facilitation and depression (Burnashev and Rozov, 2005).

The major neuropeptides subserving as neurochemical markers of GABAergic subclasses are cholecystokinin (CCK), vasoactive intestinal peptide (VIP), neuropeptideY (NPY) and somatostatin (SOM) which are either co-released with GABA or serve as modulators of GABA release. SOM, being important for the study described later, is expressed in a large number of GABAergic cells (12.5 – 16%) across all subregions of the hippocampus (Kosaka T. et al., 1988).

1.2.3. Electrophysiological characteristics of interneurons

GABAergic interneurons can be characterised based on their firing pattern and the shape of their action potentials that differ from that of principal cells. Upon current injection the latter exhibit either a regular spiking or an intrinsically bursting pattern that is often associated with accommodation during sustained stimulation (Madison and Nicoll, 1984;Stafstrom et al., 1984). In contrast, interneurons exhibit a variety of firing patterns and action potential characteristics. The molecular basis underlying

specific firing patterns of interneurons is the differential expression of voltage-gated ion channels (Zhang and McBain, 1995b; Zhang and McBain, 1995a). For instance, fast-spiking PV-positive cells, express Kv3 channels that possess certain properties such as fast deactivation, high activation threshold and lack of inactivation. These characteristics are crucial for the typical fast (up to 200 Hz), non-accommodating, non-attenuating spiking pattern (Baranauskas et al., 2003; Du et al., 1996). Most hippocampal CB-positive interneurons fire at lower frequency and the irregular spiking pattern is further characterised by a long interval between the initial action potential burst followed by more regular action potential spiking (Fuchs et al., 2007). O-LM cells on the other hand – that are mainly SOM-expressing – can be identified by a ‘sag’ (H-current) in response to hyperpolarizing current injection (McCormick and Pape, 1990; Sik et al., 1994; McBain et al., 1994) and depolarising current injection results in a regular train of action potentials that decrease in amplitude and slightly also in frequency.

GABAergic interneurons differ from principal cells also in respect to their excitability (McCormick et al., 1985) and the expression of ligand-gated ion channels. Thus, there is a vast body of literature showing the differential subunit expression of several receptors (Monyer et al., 1994; Geiger et al., 1995). One of the best studied with several techniques, including *in situ* hybridisation, immunohistochemistry (Catania et al., 1995; Catania et al., 1998), single cell-PCR combined with electrophysiology (Geiger et al., 1995) are α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPA) that underlie the mediation of fast excitatory neurotransmission in the brain (Keinanen et al., 1990; Sato et al., 1993). AMPARs are multimeric assemblies of different subunits (GluR-1-4; GluR-A-D) that form tetramers, and depending on the subunit composition the channel properties and hence excitatory transmission varies. Most interneurons, in contrast to principal cells, possess AMPARs that do not contain the GluR-B (GluR-2) subunit or only in small amounts. The unique AMPAR expression profile in interneurons translates into channels with high calcium permeability and fast kinetic properties (Jonas et al., 1994; Seeburg, 1993). The slow component of excitatory synaptic transmission is mediated by the activation of N-methyl-D-aspartate receptors (NMDAR). These are composed of heterodimers comprising NR1 and NR2 (NR2A-D) subunits (Monyer et al., 1992; Moriyoshi et al., 1991; Monyer et al., 1994) which also appear to be differentially

expressed in interneurons and principal cells (Dr. J. von Engelhardt, personal communication, ongoing work in the lab).

1.3. Distinct functions of interneurons in the developing and mature brain

1.3.1. Origin of interneurons and their role during development

There is an increasing body of evidence indicating that a specific GABAergic interneuron subtype is determined by the site and time of birth. It is of note that the generation and migration of GABAergic interneurons is distinct from that of principal cells (Parnavelas, 2000). Thus, neural precursor cells of glutamatergic principal cells arise from the ventricular zone of the dorsal telencephalon and migrate radially to their destination (Mione et al., 1997; Rakic P, 1990; Tan et al., 1998). In contrast, GABAergic interneurons originate from distinct regions of the subpallium of the telencephalon (Fig. 4) depending on the interneuronal subtype (Anderson et al., 1997; De Carlos et al., 1996; O'Rourke et al., 1995).

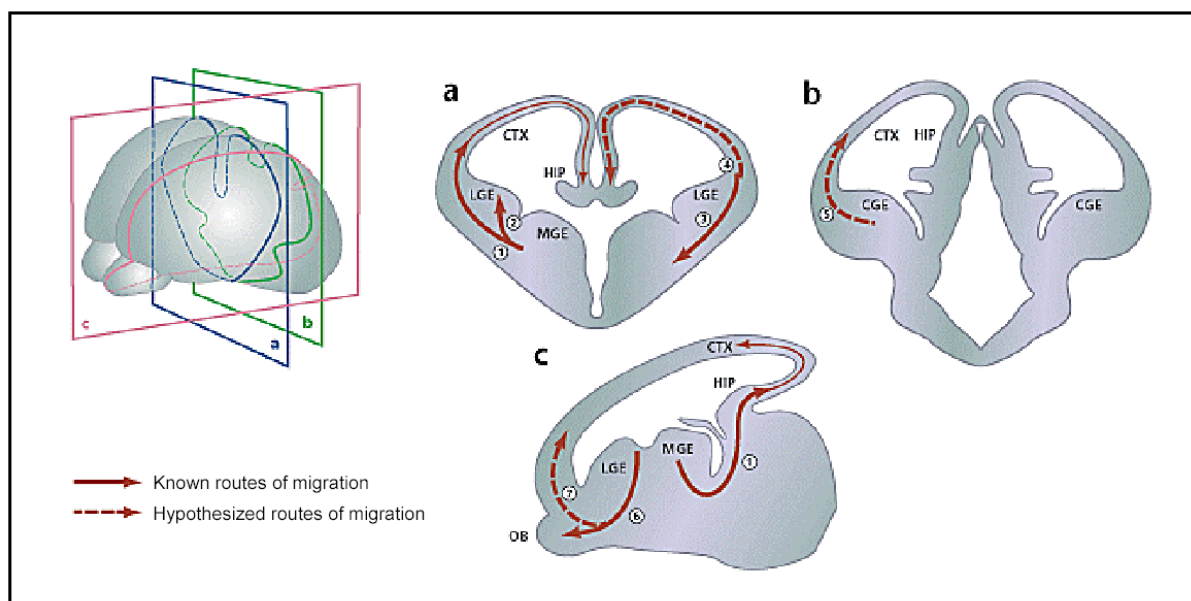


Fig. 4 Routes of tangential migration of interneurons from the subpallidum to the neocortex and the hippocampus. Coronal (a, b) and sagittal sections (c) of the embryonic telencephalon are illustrated as well as the known (solid arrow) and hypothesized (dashed arrow) routes of migration. Hippocampal interneurons arise from the medial (MGE) and the lateral ganglionic eminence (LGE). Figure (modified) from (Corbin et al., 2001).

Thus, *in utero* fate mapping studies revealed that PV- and SOM-positive interneurons are generated in the medial ganglionic eminence (Wichterle et al., 2001; Nery et al., 2002) whereas CR-positive cells originate from the caudal ganglionic eminence (Sussel et al., 1999). They migrate tangentially from their place of birth to their final

position (O'Rourke et al., 1992). Birthdating studies showed that the hippocampal primordium starts to be colonised by GABAergic interneurons at embryonic day 15 (Manent et al., 2006). In addition, these studies revealed that the migration of principal cells is dependent on GABA signalling and vice versa.

GABAergic interneurons play a major role in brain development since they are the initial source of excitation in the immature brain. During embryonic and early postnatal development the neurotransmitter GABA is excitatory. This paradox can be explained by the initial absence of the K^+ - Cl^- coupled co-transporter KCC2 (Rivera et al., 1999; Ganguly et al., 2001) and hence the high intracellular chloride concentration in immature interneurons (Ben Ari et al., 1989). Although, the excitatory action of GABA is thought to influence cell proliferation by acting on DNA synthesis (LoTurco et al., 1995) and to regulate maturation of GABAergic innervation pattern through neurotrophic mechanism during development (Spoerri PE, 1988) and adult neurogenesis (Ge et al., 2006).

1.3.2. Interneurons and their role in hippocampal network activity

In the mature brain interneurons shape the activity of principal cells via feedback or feedforward inhibition by hyperpolarising the membrane potential in the perisomatic or dendritic region of principal cells. Thereby they decrease the firing probability of targeted principal cells. During feedforward inhibition interneurons receive activation through extra- and intrahippocampal inputs and reduce in parallel the firing rate of principal cells (Fig. 5A) (Buzsaki, 1984; Lacaille et al., 1987). Whereas feedback inhibition is mediated by principal cells of the same hippocampal region through recurrent axon collaterals that excite a group of interneurons which then again inhibits the principal cells (Fig. 5B). Examples for these feedback mediating cells are basket and O-LM cells (Blasco-Ibáñez JM and Freund TF, 1995; Lacaille et al., 1987). By exerting their inhibition, interneurons are responsible for the generation and maintenance of network oscillations in populations of synaptically connected principal cells. Oscillations do not encode information but give a spatial and temporal structure to synchronous activity of different groups of principal cells. Oscillations can be measured in the hippocampus in different frequency bands and can be associated with distinct behavioural states in an animal: theta activity (4 - 12 Hz) is detectable during explorative behaviour (Vanderwolf CH., 1969), gamma oscillations (30 -80 Hz) are associated with higher cognitive functions (Gray and Singer, 1989; Buzsaki and

Eidelberg, 1983;Bragin et al., 1995) and sharp wave ripples and high-frequency oscillations (140 - 200 Hz) are observed during slow-wave sleep, awake immobility and consumatory behaviour (Buzsaki and Eidelberg, 1983;Buzsaki, 1986).

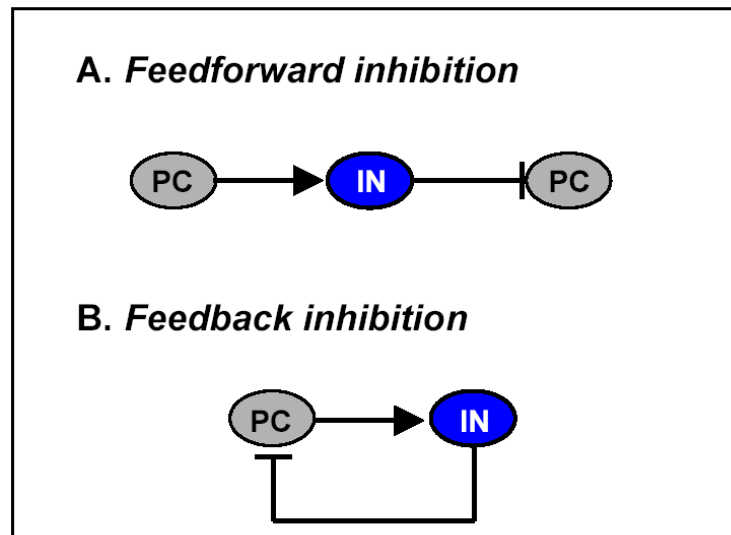


Fig. 5 Principles of feedforward and feedback inhibition. A: Feedforward inhibition. A principal cell excites an interneuron which then reduces the firing rate of another principal cell. B: Feedback inhibition. A principal cell mediates excitation onto an interneuron which in turn inhibits again the principal cell.

Numerous studies have indicated that fast-spiking PV-positive interneurons that perisomatically innervate principal cells play an important role in the generation and maintenance of gamma oscillations. In contrast, it is thought that interneurons involved in the generation of theta rhythms preferably innervate the distal dendrites of principal cells and are slow-spiking. These interneurons become rhythmically excited either by cholinergic and/or GABAergic input from the septum or by intrinsic oscillatory properties of hippocampal interneurons and principal cells (Buzsaki and Eidelberg, 1983). Furthermore, distinct interneuronal subtypes fire phase-locked to theta (and sharp wave ripple) oscillations (Fig. 6). In combination with the innervation of different domains of principal cells interneurons are thereby able to coordinate the activity of principal cells in a temporally distinct and brain-state-dependent manner (Klausberger et al., 2003).

The role of specific GABAergic interneurons in oscillations in defined frequency ranges has been shown in a number of electrophysiological studies *in vitro* and *in vivo*, however, a causal involvement can only be hypothesised (Klausberger et al., 2004; Gloveli et al., 2005; Whittington et al., 1995; Whittington and Traub, 2003). Their

direct involvement could be tested in genetically manipulated animals in which key molecules were turned off in selective cell populations. The ablation of the major neuronal gap junction protein Cx36 and hence, the gap junction-mediated electrical signalling in Cx36 knock-out mice, resulted in the reduced power of gamma oscillations whilst rhythms in other frequency ranges were not altered (Buhl et al., 2003; Hormuzdi et al., 2001). Other genetic models that gave insight into the function of particular interneuronal subtypes were based on genetic modifications that lead to reduced excitability and hence insufficient recruitment of GABAergic interneurons that in turn resulted in reduced gamma oscillations (Fuchs et al., 2001; Fuchs et al., 2007).

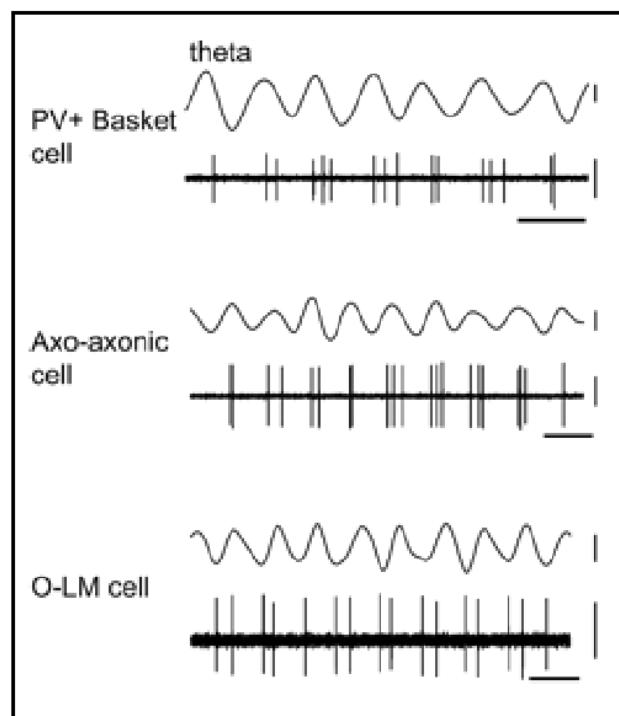


Fig. 6 *Phase-locked firing of distinct interneurons during theta oscillations.* The firing of interneurons depends on the subtype. Basket cells fire preferably at the descending phase of the theta wave in contrast to axo-axonic cells that fire around the peak or O-LM cells that discharge at the trough. Figure (modified) from (Somogyi and Klausberger, 2005).

1.4. Influencing the interneuronal communication by genetic manipulations

The elucidation of the role of distinct GABAergic interneurons at a cellular and network level during development and in the adult requires genetic alterations that are specific for the given cell population. It is possible to specifically change the excitability of defined GABAergic interneurons or to ablate key molecules that might affect crucial functions. The effect can be subsequently studied *in vitro* (e.g. cell culture, acute slice preparation) and/or *in vivo* (electrophysiology, behaviour). Currently, the most frequently used approach to ablate genes in specific subsets of cells is the CRE/loxP system (Kwan, 2002; Nagy, 2000).

1.4.1 CRE/loxP system

Recombination occurs in all organisms and is promoted by recombinases, specific enzymes that catalyse cleavage and religation of the modified DNA. The physiological function of these enzymes is to repair DNA if necessary and to provide a way of changing the genomic arrangement thereby creating genetic diversity.

The finding that some bacteria and yeast recombinases are able to also catalyse excision, integration, inversion and translocation of DNA in eukaryotic systems, opened up new possibilities of genetic engineering. The two recombinases that are commonly used for this purpose are a recombinase derived from the P1 bacteriophage (CRE) (Abremski and Hoess, 1984; Hamilton and Abremski, 1984) and one from *Saccharomyces cerevisiae* (flp) (Broach et al., 1982; Dymecki, 1996). Both belong to the integrase family of site-specific recombinases.

The molecular mechanism of the recombination event will be explained exemplifying the CRE recombinase. CRE recombinase is a 38 kDa protein that recognizes a 34 bp consensus sequence, called loxP site, consisting of two palindromic 13 bp flanking sequences separated by an 8 bp asymmetric core spacer sequence, that determines the orientation of the recognition site (Fig. 7A). For CRE recombinase-mediated recombination to occur two loxP sites are required. A CRE recombinase molecule binds to each palindromic half of two loxP site and then CRE recombinase forms a tetramer that brings the two loxP sites together. Thus allowing recombination within the two spacer areas of the loxP sites (Voziyanov Y et al., 1999). After the recombination event one loxP site remains consisting of the other palindromic halves

of both former loxP sites, respectively. Depending on the orientation of the recognition sites, cis or trans, a floxed DNA fragment can either be excised or inverted (Fig. 7B).

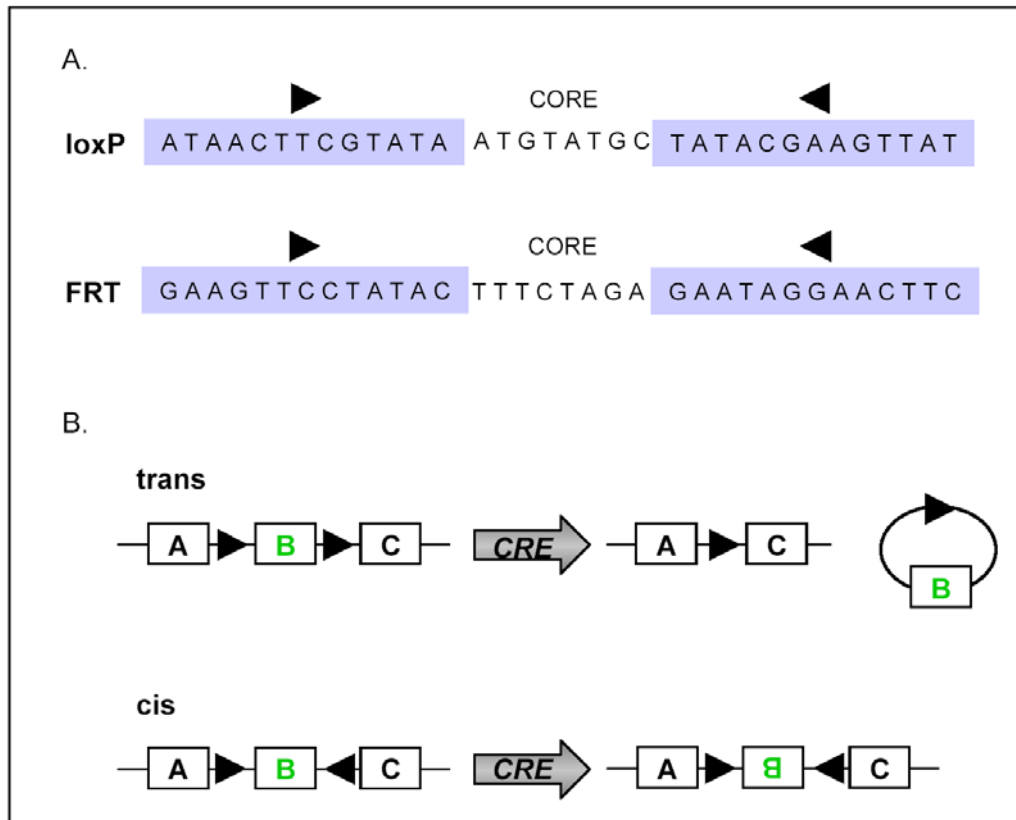


Fig. 7 Scheme of the Cre/loxP system. A: Structure and sequence of the loxP and FRT recombinase recognition site. They consist of two 13 bp inverted repeats and an 8 bp core sequence that defines the orientation of the site (indicated by arrowheads). B: Scheme of CRE recombinase mediated trans and cis recombination between two recognition sites (orientation is indicated by the arrowheads) and the resulting products. Adapted from (Kwan, 2002; Nagy, 2000).

Initially many studies resorted to the CRE/loxP system because it provided the higher recombination efficiency compared to the FLP/frt system. The reduced recombination efficacy of the FLP recombinase is due to the decrease of its enzymatic stability at 37°C (Buchholz et al., 1996). However, some modifications have recently improved the thermo-stability of FLP (FLPe) and offered an alternative to the CRE/loxP system which is equally powerful (Rodriguez et al., 2000).

The Cre/loxP system aiming at targeted gene disruption in specific cell populations requires the combined use of homologous recombination in mouse embryonic stem cell and transgenic techniques. To this end two mouse lines need to be generated: one carrying the floxed gene of interest and the other expressing CRE recombinase

under the control of a subtype-specific promoter. The latter can be achieved either by transgenic techniques or by homologous recombination in embryonic stem cells.

The most common way to use the CRE/loxP system to ablate gene functions are either to create a mouse line that expresses the CRE recombinase under a cell-type specific promoter and cross it with a transgenic mouse line that expresses the floxed gene of interest or vice versa (Fig. 8) (Gu et al., 1993).

One of the first approaches using CRE recombinase-mediated activation of a targeted gene in mice was performed by Lakso *et al.* (Lakso et al., 1992). They generated mice carrying a lens-specific α A-crystallin promoter separated from a SV40 tumor antigen gene sequence by a floxed stop-cassette. These mice were crossed with mice expressing CRE recombinase under distinct promoters and in the progeny the excision of the stop-cassette resulted in the formation of lens tumors.

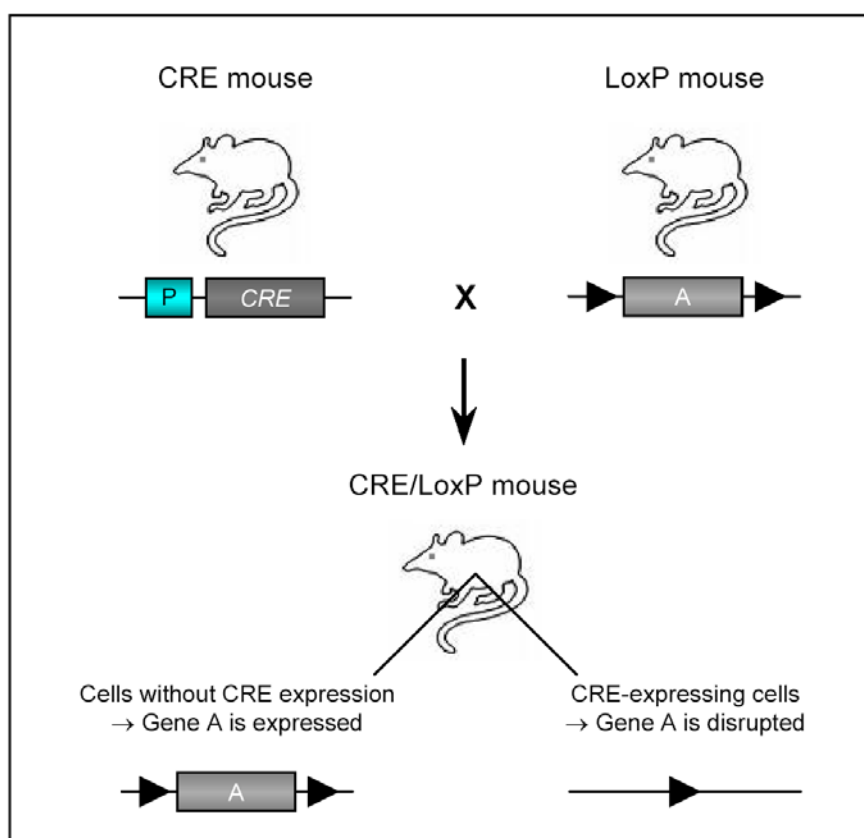


Fig. 8 Scheme illustrating cell-type specific gene ablation using the CRE/loxP system. The 'loxP mouse' (right) possesses a floxed targeted gene (loxP sites are indicated by black arrowheads) and the CRE mouse drives CRE recombinase under a cell-type specific promoter (P). Mice from the two lines are intercrossed and in the progeny gene function is disrupted in cells that express Cre recombinase but not in cells lacking CRE recombinase expression.

To monitor the expression pattern of CRE recombinase and simultaneously test the functionality of the recombinase, CRE recombinase-expressing mice are bred with reporter mice. A commonly used reporter line is ROSA26 (R26R, (Soriano, 1999)) in which the modified locus guarantees the ubiquitous expression during development and in the adult mouse. Soriano and colleagues identified the ROSA26 locus by random retroviral gene trapping in embryonic stem cells (Zambrowicz et al., 1997). In R26R mice a modified lacZ cassette that is normally not transcribed is inserted in the ROSA26 locus. The cassette contains a splice acceptor site (SA) as in the original gene-trap allele and a NEO expression cassette flanked by loxP sites followed by the lacZ gene. Upon CRE recombinase-mediated removal of the NEO cassette the lacZ gene is transcribed and its product β -galactosidase can be visualised by immunohistochemical methods (Fig. 9).

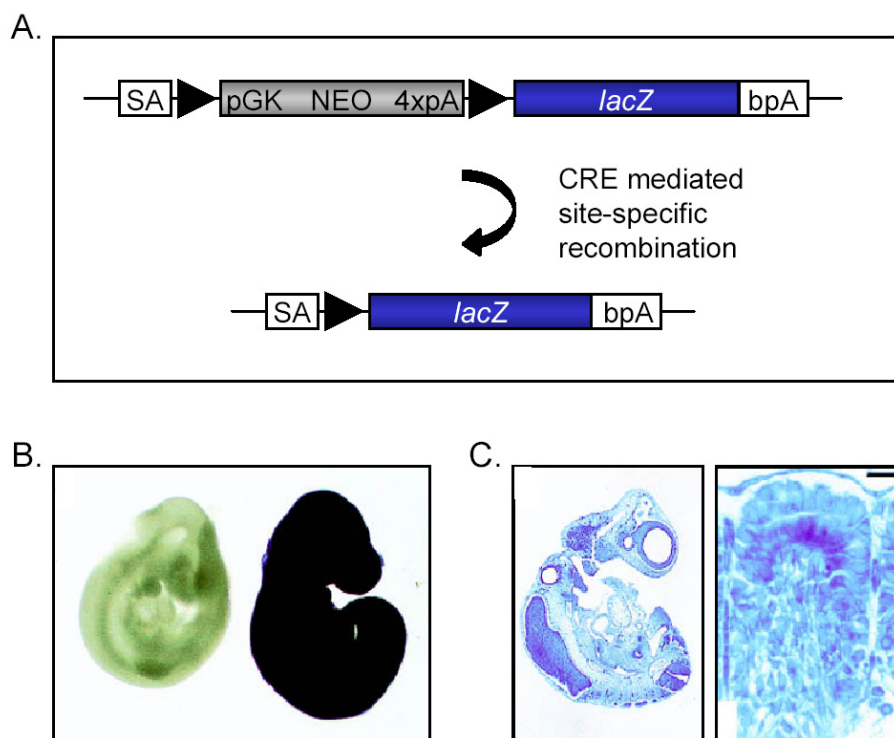


Fig. 9 *ROSA26 CRE reporter mouse line (R26R)*. A: Targeted ROSA26 locus with an expression cassette consisting of a splice acceptor sequence (SA) upstream of the floxed NEO cassette and a lacZ gene followed by a polyadenylation sequence (bpA) downstream of the NEO cassette. After CRE mediated excision of the NEO cassette, the ROSA26 promoter drives the lacZ gene expression. Arrowheads indicate loxP sites. B: LacZ expression after CRE recombinase mediated recombination. X-Gal whole mount staining of E9 R26R (left) and R26R/R26Cre compound heterozygous embryos (right). C: X-Gal staining of a sagittal section of an E9 R26R/R26Cre compound heterozygous embryo (left) and higher magnification of the somites of that embryo (right). Adapted from (Soriano, 1999).

The following two paragraphs will describe the characteristics and expression pattern of the two genes - GAD67 and somatostatin - that were chosen in this study to generate cell-type specific CRE recombinase expressing mice.

1.4.2. GAD67- a common feature of interneurons

A general feature that applies to almost all interneurons is the expression of GAD67, together with GAD65, one of the major GABA synthesising enzymes (Erlander et al., 1991). GAD67 is responsible for 90% of the synthesis of GABA (Asada et al., 1997; Condie et al., 1997).

The murine GAD67 gene is localised on chromosome 2 (Brilliant et al., 1990) and comprises a gene with an approximate size of 70 kb. Out of these 70 kb, 42 kb encode the 18 exons and at least 20 kb account for the 5' regulatory elements (Szabo G et al., 1996). The translational start codon is situated on exon 2. The 5' flanking region of the GAD67 gene (Fig. 10) consists of three transcriptional initiation sites with its promoters P1-P3 as well as neuron-specific silencer and region-specific enhancer elements (Katarova et al., 1998; Erlander and Tobin, 1992; Szabo G et al., 1996). Different combinations of the promoters and the regulatory elements lead to the expression of three distinct GAD67 transcripts in a developmental and tissue-specific manner. GAD67 expression, for instance, in the cerebellum, which develops postnatally, requires the most upstream promoter P3 (Katarova et al., 1998).

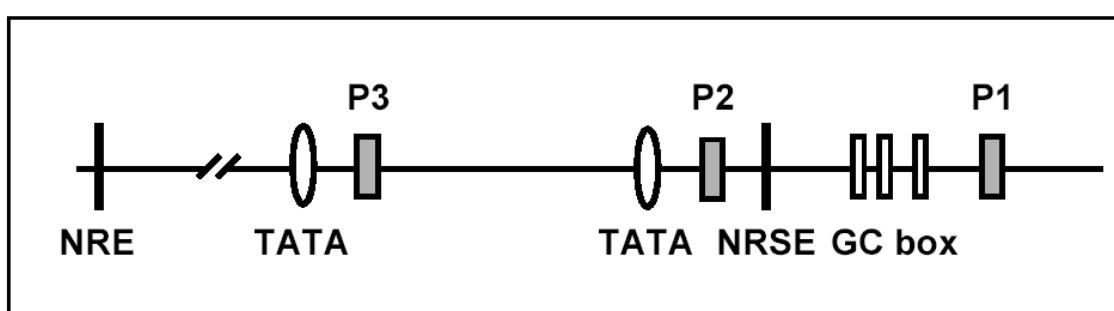


Fig. 10 Schematic representation of the promoter region of the GAD67 gene. The grey boxes indicate the start sites of transcription under the control of the corresponding promoters: the proximal housekeeping promoter (P1) composed of the GC box, two distal conventional (TATA) promoters (P2, P3). In addition, a neuron-restrictive silencer element (NRSE) and a negative regulatory element (NRE) are shown.

Furthermore, studies from Szabo *et al.* showed that the GAD67 gene is subjected to post-transcriptional modification during development which is the alternative

bicistronic splicing of exon 7 to obtain GAD25 (leader peptide) and the embryonic active form GAD44 (Szabo et al., 1994).

Concerning the mRNA expression, the two embryonic forms of GAD67 (GAD25, GAD44) and GAD67 can be detected as early as embryonic day 10.5 in the mouse brain (Katarova Z et al., 2000). Immunohistochemical staining for GAD67 demonstrates that it is present both in the cell body and proximal dendrites as well as in axon terminals (Fig. 11) (Esclapez et al., 1994; Kaufmann DL et al., 1991).

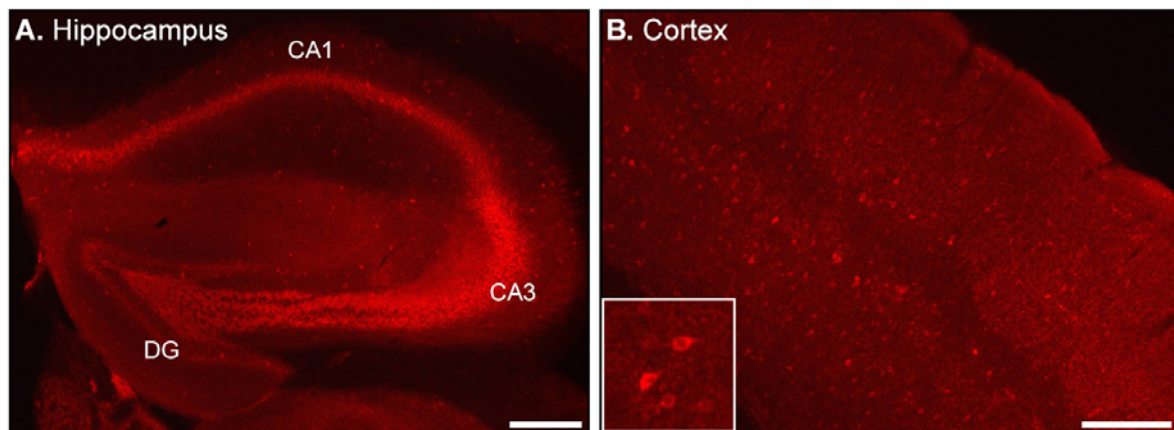


Fig. 11 *GAD67 protein expression in the adult mouse brain.* Immunohistochemical staining of coronal sections of an adult mouse with an anti-GAD67 antibody (red signal). A: GAD67 expression pattern in the hippocampus. B: Distribution of GAD67 expression in the cerebral cortex. Inset illustrates a higher magnification of cortical cells. Hi: Hippocampus; DG: Dentate gyrus. Scale bars: 250 μ m.

Until now, several transgenic mice using the GAD67 gene have been generated. Reporter lines expressing EGFP under the GAD67 promoter helped to further characterise GABAergic projection neurons in the neocortex (Tomioka et al., 2005). Furthermore, they helped to identify factors modulating the migration of hippocampal interneurons during development (Manent et al., 2006). Knock-out approaches showed that the absence of GAD67 leads to neonatal lethality due to cleft palate and a reduced GABA content but major structural defects in the brain were not detectable (Asada et al., 1997; Condie et al., 1997). Mice heterozygous for the modified GAD67 allele were viable. Hence the GAD67 promoter is a powerful tool to drive CRE recombinase expression selectively in this cell population. These mice can be used for subsequent gene deletion that will affect all GABAergic interneurons of the nervous system.

1.4.3. Somatostatin

Somatostatin was initially described as a regulatory hormone of the hypothalamus (Brazeau et al., 1973) but later additional physiological functions were revealed. It is expressed in a subset of GABAergic interneurons (Freund and Buzsaki G, 1996) and serves as a neurotransmitter as well as neuromodulator (Patel, 1999). In the hippocampus it has been shown that somatostatin presynaptically inhibits excitatory transmission by reducing glutamate release. The postsynaptic inhibitory effects are dependent on the reduction of voltage-activated Ca^{2+} currents (Vilchis et al., 2002; Wang et al., 2002; Wang et al., 1990) and the potentiation of voltage-dependent outward K^+ currents (Moore et al., 1988; Watson and Pittman, 1988). Both, the pre- and the postsynaptic effects are mediated by somatostatin receptors that comprise five subtypes and belong to the G-protein-coupled receptor family (Hoyer et al., 1995). However, also excitation of principal cells of the subiculum and the CA1 region mediated by somatostatin were demonstrated (Dood and Kelly, 1978; Vilchis et al., 2002; Wang et al., 2002; Greene and Mason, 1996).

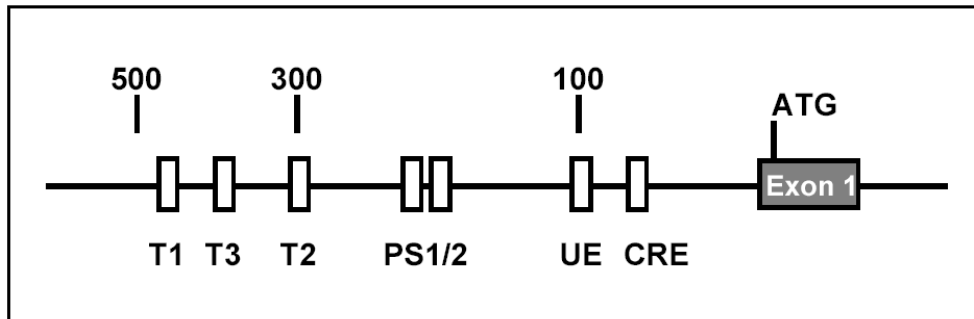


Fig. 12 The 5' flanking region of the somatostatin gene and its regulatory elements. The main regulatory elements are the cAMP responsive element (CRE), an upstream enhancer element (UE), two proximal silencers 1 and 2 and the tissue-specific enhancer (T1 – T3). The numbers indicate the nucleotides relative to the transcriptional start codon (ATG). Figure adapted from (Burbach, 2002; Schwartz and Vallejo, 1998)a).

The somatostatin gene, located on chromosome 16 of the mouse (Lalley PA et al., 1987; Montminy et al., 1984), consists of two exons with the translational start codon located on exon 1 (Fuhrmann et al., 1990). Including the 5' and 3' untranslated regions the somatostatin gene covers 2.5 kb. The promoter region (Fig. 12) possesses a cAMP-response element (Montminy et al., 1986; Montminy and Bilezikjian, 1987) and an upstream enhancer element that binds homeodomain proteins (Vallejo et al., 1992a). The latter is responsible for high expression levels in

the pancreas and seems to function as a repressor in neural cells (Schwartz and Vallejo, 1998).

The somatostatin biosynthesis consists of the production of precursor molecules (prosomatostatin) followed by post-translational cleavage into several peptides (Funckes et al., 1983; Goodman et al., 1983). The two main bioactive forms are somatostatin-14 (Brazeau et al., 1973) and somatostatin-28 (Pradayrol et al., 1980) which are expressed in the brain and the guts, respectively .

Somatostatin mRNA can be already detected at mouse embryonic day (E) E7.5 in the foregut (Gittes and Rutter, 1992). In the brain, somatostatin mRNA is detectable in several regions at E14, the mRNA levels increase in the first three postnatal weeks and then decrease to the adult expression level (Bendotti C. et al., 1990). The expression is also ontogenetically regulated, since expression in the cerebellum is upregulated during early postnatal development and is negligible in the adult.

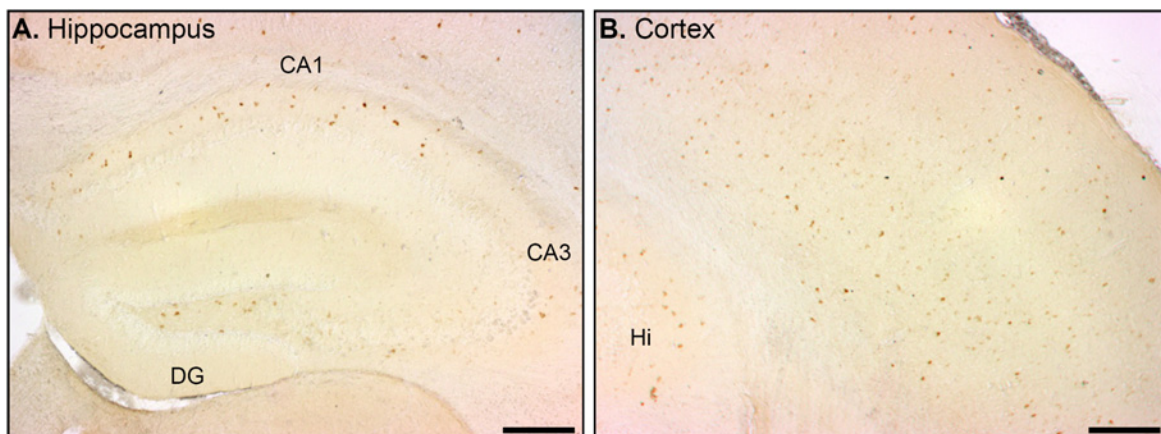


Fig. 13 *Expression of somatostatin in the adult mouse brain.* Somatostatin-immunostaining of coronal sections of an adult mouse brain (brown signal). A: Expression pattern of somatostatin in the hippocampus. B: Somatostatin-positive cells in the cerebral cortex. Hi: Hippocampus; DG: Dentate gyrus. Scale bars: 250 μ m.

Approaches to genetically modify the expression of somatostatin include the complete knock-out of somatostatin itself (sst-KO) (Buckmaster PS et al., 2002; Cammalleri et al., 2006) and the somatostatin receptor 2 (sst2-KO) (Dutar et al., 2002). No obvious physiological or anatomical defects of the brain or other organs could be detected. The sst-KO showed elevated plasma growth hormone levels and significant impairment concerning motor learning (Zeyda et al., 2001). The sst2-KO mice exhibited a slightly increased short-term potentiation and improved spatial discrimination learning (Dutar et al., 2002). The absence of more noticeable

deficits in sst-KO mice is surprising considering the many important physiological functions of somatostatin and might be explained at least in part by compensatory mechanisms. However, the lack of major alterations in sst-KO mice can be considered an advantage if one plans to use the somatostatin promoter to drive CRE recombinase expression. Moreover, in the planned genetic alteration only one somatostatin allele will be modified by the knock-in of CRE recombinase that will not influence the viability of the transgenic mice.

1.5. Aim of the study

Despite the fact that GABAergic interneurons only comprise about one fifth of all neurons in the brain they have a great influence on developmental processes as well as network activity in the mature brain. During development of the brain they are able to modulate cell proliferation, migration of neurons as well as differentiation and synaptogenesis. In the adult brain interneurons inhibit principal cell activity and thus are responsible for shaping and maintenance of network oscillations.

To gain more insight into the function of interneurons and their molecular components in the above-mentioned processes, celltype-specific ablation of genes of interest is a powerful tool. An approach to perform such experiments is to apply the CRE/loxP system that is based on CRE recombinase-mediated site-specific recombination of loxP-sites that flank the gene of interest. Two promoters were chosen to drive CRE recombinase expression, the GAD67 promoter that ensures expression in all GABAergic interneurons and the somatostatin promoter, that limits the manipulation to a specific interneuronal subpopulation.

The aim of this study was to generate and analyse two mouse lines expressing CRE recombinase under the control of the GAD67 and somatostatin promoter, respectively. These transgenic mice were generated via a knock-in approach that was performed by homologous recombination in embryonic stem cells. After breeding CRE-expressing mice with ROSA26 reporter mice, the pattern of CRE recombinase expression and the functionality of the recombinase was verified using immunohistochemical methods. Ablation of the trkB receptor in all GAD67-positive cells as well as of the NR1 subunit of NMDAR and the GluR-A subunit of AMPAR in somatostatin-expressing cells are currently in progress.

2. Materials and Methods

2.1. Materials

2.1.1. List of oligonucleotides

All oligonucleotides were synthesised by Invitrogen GmbH (Karlsruhe, Germany).

2.2.1.1. Oligonucleotides for cloning the SOM/CRE-targeting construct

5' Rt for	ATAAGAATGCGGCCGCCTGCTGTTCCCGAGTAGGTG
5' Rt rev	GGGTTCGAACCTCCTATCTCTCCTACTGG
3' Rt for	CCCAAGCTTTCTCGTTTATGTTCCAATGT
3' Rt rev	CCTGATCAATAAGTGAGGCGAGCGAGAT
5' Tg for	GGGGTACCGTGCAGGCTTTCTTTTTC
5' Tg rev	GGACATGTTCCCTTGCCTCAGG
3' Tg for	GAAGATCTCTGTCCTGCCGTCTCCA
3' Tg rev	TATCAAATCGCCGGCGAGGGGACCCTAACTTCAA

2.1.1.2. Oligonucleotides for sequencing

T3	AATTAACCCTCACTAAAGGG
T7	TAATACGACTCACTATAGGG
SOMs7586	AAGTCTCACTCGCCATACAA
NEO 1as	CCCGCTTCAGTGACAACGTCGAGCACA
NEO 3as	CTTGAGCCTGGCGAACAGTTCGGC
NEO Rayb	GCGATGCCTGCTTGCCGAATA
Cre1s	CGCCTGCTGGAAGATGGCGATTAG
rspcre-2	AGGCTAAGTGCCTTCTCTACAC
Cre2as	CATTGGTCCAGCCACCAGCTTGCA
Cre3s	GCCATCTGCCACCAGCCAGCTATC
Cre3as	TCAGTGCGTTCGAACGCTAGAGCC
Cre5up	GGCAAATTTTGGTGTACGGTC

2.1.1.3. Oligonucleotides for genotyping

Ye-GAD5'	CCGGCGCCCCTTAGCTGTGAG
5Creup2	GGTTCTTGCGAACCTCATCACTCG
rspneo4	GGCTATTCGGCTATGACTGGGC
rspneo5	GGGTAGCCAACGCTATGTCCTG
flp1	CACTGATATTGTAAGTAGTTT
flp2	CTAGTGCGAAGTAGTGATCAGG
rspcre1	ACCAGGTTTCGTTCACTCATGG
rspcre2	AGGCTAAGTGCCTTCTCTACAC
LacZ7	CCCATTACGGTCAATCCGCCG
LacZ8	GCCTCCAGTCAGCGCGGCTG
NR1-1	AGGGGAGGCAACACTGTGGAC
NR1-2	CTGGGACTCAGCTGTGCTGG
MM60	CACTCACAGCAATGAAGCAGGAC
3int3	CTGCCTGGGTAAAGTGACTTGG

2.1.1.4. Oligonucleotides for generating probes for Southern blot analysis

5'probe II for	CTTAGTAGCCCAACACCCAC
5'probe II rev	TTTGTTCTTGCTGTGTCCTC
3'probe for	GAGCGTCTGAAAAGTTAGCA
3'probe rev	CCTGTTCTCCAGCAATAAT

2.1.1.5. Oligonucleotides for *in situ* hybridisation

SOM oligo1 AGCCAAGCTGGAGCGCGGTGGGTCAGTCTAGTCGCAGGTCCTCAG
SOM oligo2 TGGGGCAAATCCTCGGGCTCCAGGGCATCATTCTGTCTGGTGG
SOM oligo3 AAGTTGAGCATCGGGGGCCAGGAGTTAAGGAAGAGATATGGGGTT

2.1.2. Bacterial strains

DH5 α : Stratagene

2.1.3. Cell lines

Feeder cells: Primary mouse fibroblasts from E13.5 NMRI embryos.

R1 ES cells: Pluripotent mouse embryonic stem cells (passage number 11) derived from the inner cell mass of 129/SV x 129/SVJ- mouse blastocysts (Nagy et al., 1993).

E14 ES cells: Provided by Prof. P. Offermanns, Institute of Pharmacology, University of Heidelberg, Germany.

2.1.4. Mouse strains

C57Bl/6 (from Charles River, Germany)

ROSA26 (Soriano, 1999)

Flp deleter (Rodriguez et al., 2000)

NR1^{2lox} (Shimshek et al., 2006)

GluRA^{neo} (Zamanillo et al., 1999)

2.1.5. Antibodies

Primary antibodies and used dilutions:

mouse anti- β -actin (Sigma)	1:20000
mouse anti-Calbindin (Swant)	1:5000
mouse anti-GAD67 (Chemicon)	1:3000
rabbit anti-GAD67 (Chemicon)	1:1000
rabbit anti- β -galactosidase (Cappel)	1:3000
mouse anti-Parvalbumin (Sigma)	1:3000
rat anti-somatostatin (Chemicon)	1:1000

Secondary antibodies and used dilutions:

Cy TM 3-conjugated AffiniPure goat anti-mouse/rabbit/rat antibodies (Dianova)	1:1000
AlexaFluor®488/555 (Invitrogen)	1:1000
Biotinylated anti-mouse/rabbit/rat IgG (H+L) (Vector Laboratories)	1:400
Peroxidase anti-mouse/rabbit IgG (H+L) (Vector Laboratories)	1:20000

2.1. Methods

2.2.1. Molecularbiological methods

Standard molecularbiological methods were performed as described in Sambrook et al. (Sambrook J. et al., 1989). The isolation and purification of DNA were performed with the Plasmid Mini/Midi-Kit, the PCR Purification Kit and the Gel Extraction Kit from Qiagen (Hilden, Germany) according to the manual provided by the manufacturer.

2.2.2. Sequencing

The sequencing of the cloned constructs was performed by Horst Grosskurth at the MPI for Medical Research (Heidelberg, Germany). It was carried out following the 'Dye Terminator Cycle Sequencing' protocol (96°C, 15 s; 55°C, 15 s; 60°C, 4 min; 25 cycles) from ABI.

2.2.3. *In situ* hybridisation with radioactive-labelled probes

Mice were anaesthetised with isoflurane, brains were removed from the skull, frozen immediately, cut on a microtom-cryostat (14 µm) and thaw-mounted to poly-L-lysine-coated slides. Then sections were fixed with 4% paraformaldehyde in phosphate buffer pH 7.4, dehydrated in ethanol and stored in 100% ethanol at 4°C.

In situ hybridisation experiments with ³⁵S-labeled oligonucleotide probes were performed as previously described in Wisden et al. (Wisden W and Morris BJ, 2002). Briefly, the oligonucleotides were 3' end labelled using terminal deoxynucleotidyl transferase and [α -³⁵S]dATP. Sections were hybridised overnight at 42°C in 50% formamide, 0.6 M NaCl, 0.06 M sodium citrate (4x SSC), 10% dextrane sulfate with 1 pg/µl probe. Non-specific labelling of the sections was assessed by hybridising labelled oligonucleotide in the presence of 100-fold excess of unlabelled oligonucleotide. Subsequently, the sections were washed in 1x SSC at 55°C for half an hour and dehydrated in ethanol. Exposure time to Kodak XAR-5 film was 14 days. Three oligonucleotides specific for the somatostatin gene were chosen and gave identical results. The nomenclature used for the analysis of the *in situ* hybridisation study follows that of Altman and Bayer, Franklin and Paxinos (Altman J. and Bayer S.A., 1995; Franklin K.B.J. and Paxinos G., 1997; Paxinos G. et al., 1994).

2.2.4. X-Gal staining

5-Bromo-4-chloro-3-indolyl- β -D-galactosidase (X-Gal) staining was performed as previously described (Bonnerot and Nicolas JF, 1993). Briefly, mice were anaesthetised by intra-peritoneal injection of ketamine hydrochloride and xylazine hydrochloride, and perfused through the ascending aorta with 4% paraformaldehyde in phosphate buffer pH 7.4 (PBS). Brains were removed from the skull, postfixed for two hours at 4°C in 4% PFA and stored in 1× PBS at 4°C until use. Brains were blocked in 4% agar (1× PBS), cut at a vibratome (50 μ m; Leica VT1000S) and the sections were stored in 1× PBS at 4°C. Embryos were fixed in 4% paraformaldehyde in phosphate buffer pH 7.4 (PBS) for 1 hour at 4°C, washed in 1× PBS and equilibrated in 30 % sucrose in 1× PBS overnight at 4°C. Embryos were punctured at the neck to allow better penetration of the paraformaldehyde and sucrose solutions. Subsequently, 18 μ m cryostat sections were prepared.

For the X-Gal staining both the free floating sections as well as the cryostat sections were washed three times with 1× PBS and transferred to the staining solution (5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 2 mg/ml X-Gal in dimethylformamide/1×PBS). The sections were incubated at 37°C in the dark for 10 to 15 minutes. Subsequently, they were washed three times for 5 minutes with 1× PBS. Sections were either mounted on slides and counter-stained with Neutral Red solution (Sigma) or further processed for immunohistochemical staining experiments. For imaging, an Olympus BX51 microscope that was connected to a camera system from INTAS was used. The applied software was MagnaFire 2.1C. The nomenclature used for the analysis follows that of Altman and Bayer, Franklin and Paxinos (Altman J. and Bayer S.A., 1995; Franklin K.B.J. and Paxinos G., 1997; Paxinos G. et al., 1994).

2.2.5. Immunohistochemistry

Mice were anaesthetised by intra-peritoneal injection of ketamine hydrochloride and xylazine hydrochloride, and perfused through the ascending aorta with 4% paraformaldehyde in phosphate buffer pH 7.4. Brains were removed from the skull, postfixed for two hours at 4°C in 4% PFA and stored in 1× PBS at 4°C until use.

Brains were blocked in 4% agar (dissolved in 1x PBS), cut on the vibratome (50 μ m; Leica VT1000S) and the sections were either stored in 1x PBS at 4°C or washed four times for 10 minutes in order to proceed with the immunohistochemical staining. Sections were incubated first in 10% sucrose/1xPBS for 15 minutes at room temperature and in 30% sucrose/1xPBS over night at 4°C. Permeabilisation of the sections was performed by repeated (4-5 times) freezing (over liquid nitrogen) and defrosting (55°C). To remove the sucrose four washing steps with 1x PBS (each 10 minutes) followed. Sections were incubated in 1% H₂O₂ in 1x PBS for 10 minutes and washed with 1x PBS four times for 10 minutes. For blocking unspecific binding sites, sections were incubated 45 minutes in 5% BSA/1x PBS at room temperature. Primary antibodies were diluted in 1x PBS and incubated for 24 hours at 4°C. The antibody dilutions were: rat anti-somatostatin (Chemicon) 1:1000, mouse anti-GAD67 (Chemicon) 1:3000, rabbit anti-GAD67 (Chemicon) 1:1000, rabbit anti- β -galactosidase (Cappel) 1:3000, mouse anti-Parvalbumin (Sigma) 1:3000, mouse anti-Calbindin (Swant) 1:5000. For double labelling experiments incubation with both primary antibodies were carried out at the same time. After washing three times with 1x PBS sections were incubated for two hours at room temperature with the secondary antibody for the DAB staining: biotinylated anti-rat, anti-rabbit or anti-mouse IgG (Vector Laboratories) 1:400 in 1x PBS. To enhance the staining reaction sections were incubated with an avidin-biotin complex (ABC Elite Kit, Vector Laboratories) for 90 minutes. Two washing steps with 1x PBS and two with 20 mM TRIS-HCl buffer pH 7.6 (TRIS), each for 10 minutes, followed. Sections were incubated in DAB solution (0.4 mg/ml, Sigma) for 20 minutes. The staining reaction was started by adding 0.01% H₂O₂ and stopped by transferring the sections to TRIS-buffer. Three washing steps with TRIS buffer followed. For developing a second primary antibody slices were washed additionally with 1x PBS and incubated with the fluorescent secondary antibody (CyTM3-conjugated AffiniPure goat anti-rabbit/mouse/rat antibodies (Dianova) 1:1000; AlexaFluor®488/555 (Invitrogen) 1:1000) at RT for two hours. Three final washing steps in TRIS buffer were performed before the sections were mounted with gelatine on microscopic glass slides. After the sections were dried, they were cover-slipped with Mowiol. For imaging an Olympus BX51 microscope that was connected to a camera system from INTAS was used. The applied software was MagnaFire 2.1C.

2.2.6. Mouse embryonic stem cell culture

Pluripotent embryonic stem cells (cell line R1) were cultured either on primary mouse fibroblasts (mitomycin C-treated, Sigma) or on gelatine-coated (0.1 %) culture dishes in ES cell medium (containing Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine (Gibco), 0.1 mM non-essential amino acids (Gibco), 1 mM sodiome pyruvate (Gibco), 10^{-4} M β -mercaptoethanol (Sigma), 20% fetal calf serum (PAN Biotech GmbH), 50 μ g/ml penicillin and streptomycin (Gibco) and 1000 U/ml leukaemia inhibitory factor (Gibco).

For transfection of the ES cells, plasmid DNA containing the targeting construct was linearised with NotI and purified with gelfiltration-columns (Chroma Spin TE-1000). The electroporation (BioRad Genpulsar, 500 μ FD, 240 V) of ES-cells suspension was performed with 30 μ g of linearised DNA. Transfected ES-cells were selected with G418 for 4 days with different concentrations: 1. and 2. day: 175 μ g/ml; 3. day: 280 μ g/ml; 4. day: 350 μ g/ml. Resistant clones were isolated and further expanded to extract genomic DNA to screen for positive clones by applying Southern blot analysis. Positive clones were expanded and frozen until the blastocyst-injection.

2.2.7. Generation and care of transgenic mice

Mouse blastocyst injection was performed by Frank Zimmermann (IBF, University of Heidelberg, Germany). For details of this procedure see Hogan et al. (Hogan B. et al., 1994). Animals are kept according to the animal welfare law (Regierungspräsidium Karlsruhe, Germany) in the animal facility of the University of Heidelberg (IBF).

2.2.8. Genotyping of transgenic mice by PCR analysis

Mouse tail biopsis were digested by incubating them for two hours at 55°C in TENS buffer (50 mM TRIS-HCl pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS) that contained proteinase K (1 mg/ml). After precipitation with one volume of isopropanol and washing with 70% ethanol, the genomic DNA was resuspended in 250 μ l 1x TE buffer (1 mM TRIS, 0.1 mM EDTA pH7.6) for one hour at 55°C. For PCR analysis 2 μ l of the dissolved genomic DNA was used in a total volume of 25 μ l reaction mix that contained PCR buffer (Invitrogen), 2 mM $MgCl_2$, dNTPs (0.2 mM per nucleotide),

specific oligonucleotides (0.4 μ M each), 0.5U Taq polymerase (Invitrogen) and sterile water. The conditions for the PCR dependent on the genotypes of the mice that had to be analysed and are listed below:

1. Initial denaturing step: 95°C, 3 minutes
2. Denaturing: 94°C, 30 seconds

Oligonucleotides	3. Annealing temperature, time	4. Elongation temperature, time	Size of PCR product
5'Tg for/5 Creup2	55°C, 30 seconds	72°C, 40 seconds	391 bp
YeGAD5'/5Creup2	53°C, 30 seconds	72°C, 40 seconds	400 bp
rspNEO4/rspNEO5	53°C, 30 seconds	72°C, 40 seconds	625 bp
rspcre1/rspcre2	53°C, 30 seconds	72°C, 40 seconds	227 bp
flp1/flp2	53°C, 30 seconds	72°C, 40 seconds	700 bp
LacZ7/LacZ8	53°C, 30 seconds	72°C, 40 seconds	410 bp
NR1-1/NR1-2	55°C, 30 seconds	72°C, 45 seconds	wt allele: 455 bp mutant allele : 532 bp
MM60/3int3	53°C, 20 seconds	72°C, 20 seconds	wt allele: 190 bp mutant allele: 250 bp

Steps 2 to 4 were repeated 34 times, followed by a final elongation step at 72°C for 5 minutes. The PCR product was determined by gel electrophoresis.

2.2.9. Behaviour

The behavioural studies were performed by Dr. Elke Fuchs (Department of Clinical Neurobiology, University of Heidelberg, Germany). GAD67^{CRE} mice and their wildtype littermates (age: 3-4 month, weight: 22-28 g) were maintained at the standard animal facilities in polypropylene macrocolon cages with food and water *ad libitum*. Light was on from 7 a.m. to 7 p.m. and the temperature and humidity were kept at 20 \pm 1 °C and 50 \pm 10%, respectively. Animal tests were approved by the Regierungspräsidium Karlsruhe (Germany). The testing person was not aware of the genotype of the animals. For the behavioural tests six GAD67^{CRE/+} and seven control mice were used.

Rotarod test:

Mice were put on an accelerating rotarod (TSE Systems, Bad Homburg, Germany, model V4.0) that was used with a starting speed of 4 rpm. The rotation speed increased from 4 to 40 rpm over a six minutes period. The test was performed on three consecutive days with three trials per mouse each day.

Open field test:

The open field is a grey PVC enclosed area (50 x 30 x 18 cm) that is divided into 10 x 10 cm squares. The mouse was placed into one corner facing the walls and its behaviour was observed for five minutes. The number of crossed squares and the number of rearings were recorded and analysed on two consecutive days.

Novel object recognition:

Mice were placed for five minutes into an open field arena with two different objects and the time spend with each object was measured. Following a five minutes break one object was exchanged and again the time spent with the objects was measured for five minutes. This test was repeated on three consecutive days.

2.2.10. Electrophysiology

The electrophysiological recordings were performed by Dr. Jakob von Engelhardt (Department of Clinical Neurobiology, University of Heidelberg, Germany). Hippocampal slices (250 μm) were prepared from brains of P14 or P28 old mice. Cells were visualized by infrared differential-contrast video microscopy and identified by their firing pattern upon current injection. Whole-cell voltage clamp recordings were performed in cells that were located in the stratum oriens (O-LM cells). AMPA/NMDA rasion measurements were performed as previously described (Fuchs et al., 2007). To test whether the somatostatin-positive cells of $\text{SOM}^{\text{CRE/+};\text{GA-/-}}$ mice still possess fast excitatory currents, voltage clamp recordings at -70 mV with extracellular stimulation were performed. GABA currents were blocked by bath application of 10 μM gabazine and the blockade of NMDA currents was performed by adding 1 mM magnesium to the extracellular solution and by holding the cells at a potential of -70 mV.

3. Results

In this study the CRE/loxP system (Kwan, 2002; Nagy, 2000) was applied to generate genetically modified mice with CRE recombinase expression in selected cell populations with the aim to subsequently ablate selected genes in these cells. Two mouse lines expressing CRE recombinase under a subtype-specific promoter were generated, one that expresses CRE recombinase under the control of the glutamic acid decarboxylase (67 kDa isoform) (GAD67) promoter and one that expresses CRE recombinase under the control of the somatostatin promoter. These mouse lines were established via a knock-in approach and were named GAD67^{CRE/+} and SOM^{CRE/+}. Subsequent to germline transmission of the targeting constructs, the transgenic mice were bred with flp-deleter mice (Rodriguez et al., 2000) to remove the NEO cassette and were backcrossed with C57Bl/6 mice to obtain a uniform genetic background. CRE recombinase functionality was tested by breeding transgenic mice with ROSA26 reporter mice (Soriano, 1999) followed by analysing sections obtained from progeny brains with immunohistochemical methods.

3.1. Generation and analysis of the GAD67^{CRE/+} mouse line

3.1.2. Cloning of the targeting construct and production of targeted ES cell clones

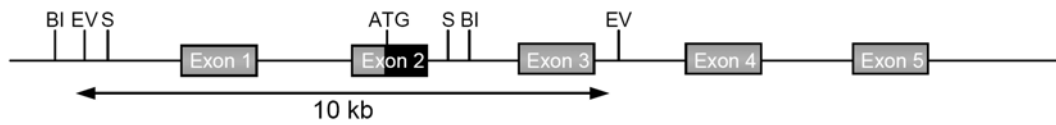
The GAD67 gene spans 16 exons and the translational startcodon is located on exon 2. To generate mice expressing CRE recombinase under the GAD67 promoter, the CRE recombinase cDNA was placed 30 bp upstream of the translational startcodon. Cloning of the targeting construct, homologous recombination in embryonic stem (ES) cells and screening for positive clones was performed by Dr. Irinel Coserea (Abbott, Ludwigshafen, Germany).

For the targeting construct pBluescript SK+ (pBS) was used as a backbone and consisted of a CRE recombinase cassette (pMC-CRE recombinase, (Gu et al., 1993)) followed by a NEO cassette containing the neomycin resistance gene (neomycin phosphotransferase, NEO) for selection in ES cell culture. Dr. Irinel Coserea modified the original vector with the NEO cassette (Cope et al., 2004) by removing the loxP site located at the 3' end such that the NEO cassette was flanked by two frt sites. At the 5' and 3' end of the targeting construct two recombination arms

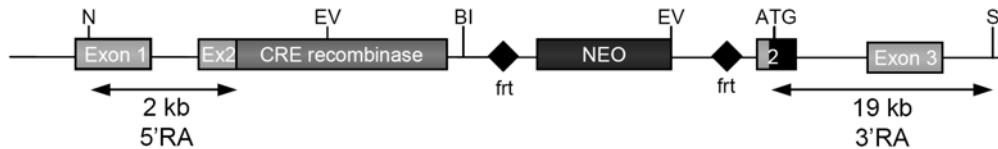
(RA) with a length of 270 and 250 bp, respectively, were located. These were used to introduce the targeting construct via homologous recombination in yeast (Storck et al., 1996) into the correct position in the YCplac22 shuttle vector. The vector contained a 21 kb NotI-Sall fragment of the murine GAD67 gene spanning parts of exon 1 up to intron 6 (for details see (Fuchs et al., 2001)). After homologous recombination in yeast the targeting construct possessed a 2 kb 5' and 19 kb 3' recombination arm for homologous recombination in ES cells. The correct assembly of the targeting vector was validated by enzymatic restriction analysis and sequencing (Fig. 14 B).

After linearisation with NotI the targeting vector was electroporated into E14 embryonic stem cells (provided by Prof. P. Offermanns, Institute of Pharmacology, University of Heidelberg, Germany) that were selected by their resistance to G418. The screening for correctly targeted clones was performed by Southern blot analysis. For Southern blot analysis genomic DNA was restricted with EcoRV and hybridised with the 5' probe (Fig. 14, position of the probe) demonstrating the presence of a 10 kb wild-type fragment and a 4.2 kb fragment specific for the targeted allele. For hybridisation with the CRE- and NEO probe, genomic DNA was restricted with EcoRV and SacI, respectively, resulting in a 6 kb and 2.2 kb fragment that was recognised by the NEO- and the CRE probe, respectively (Fig. 14).

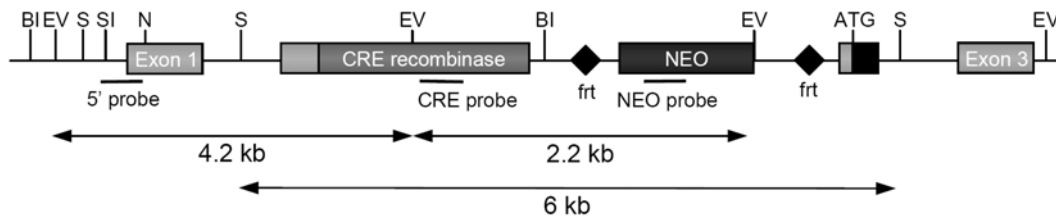
A. GAD67 locus



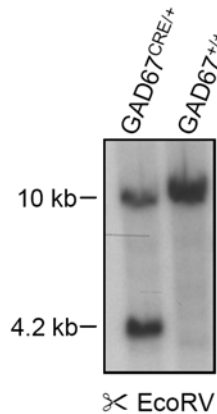
B. Targeting construct



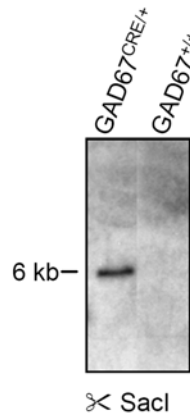
C. Targeted allele



D. 5' probe



E. NEO probe



F. CRE-probe

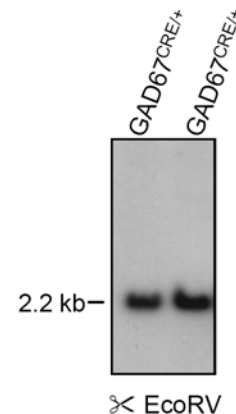


Fig. 14 Generation of $GAD67^{CRE/+}$ mice via a knock-in approach. A, B, C: Relevant features of the $GAD67$ wild-type ($GAD67^{+/+}$) allele, the targeting construct and the modified ($GAD67^{CRE/+}$) allele are illustrated. Start of the coding region in exon 1 is indicated by a black box, and the black diamonds stand for the two frt sites. Positions of relevant restriction endonuclease recognition sequences are indicated: BI: BamHI; E: EcoRV; N: NotI; S: SacI; SI: Sal I. D: Results of Southern blot analysis of EcoRV restricted genomic DNA obtained from $GAD67^{CRE/+}$ and $GAD67^{+/+}$ ES cells hybridised with the 5' probe demonstrating the presence of a 4.2 kb fragment specific for the modified allele (the 10 kb fragment derives from the wild-type allele). E: SacI restricted genomic DNA obtained from $GAD67^{CRE/+}$ and $GAD67^{+/+}$ ES cells was hybridised with the NEO probe in a Southern blot experiment showing a 6 kb fragment that indicates the presence of the NEO cassette. F: Southern Blot analysis of EcoRV restricted genomic DNA from $GAD67^{CRE/+}$ and $GAD67^{+/+}$ ES cells with the CRE probe revealed a 2.2 kb fragment corresponding to the CRE recombinase cassette.

3.1.3. Generation of the $GAD67^{CRE/+}$ mice

Two independent injections into C57Bl/6 blastocysts with ES cells derived from the same clone were performed by Frank Zimmermann (IBF, University of Heidelberg, Germany). 42 blastocysts were injected in the first round and implanted into four foster mothers. Five male and six female mice were born and the female offspring were eliminated. This is a common procedure since the used ES cells were derived from male embryos and thus a bias towards the male gender among chimeras was often observed that is reflected in phenotypic male chimera with a female genotype (Joyner A.L., 1993). The coat colour of the male progeny can be used to determine the level of chimerism since the coat colour of the mouse strain from which the ES cells were derived (agouti) and the one of the foster mother (black) differed. A higher contribution of ES cells to the germline will result in a higher proportion of agouti-coloured coat.

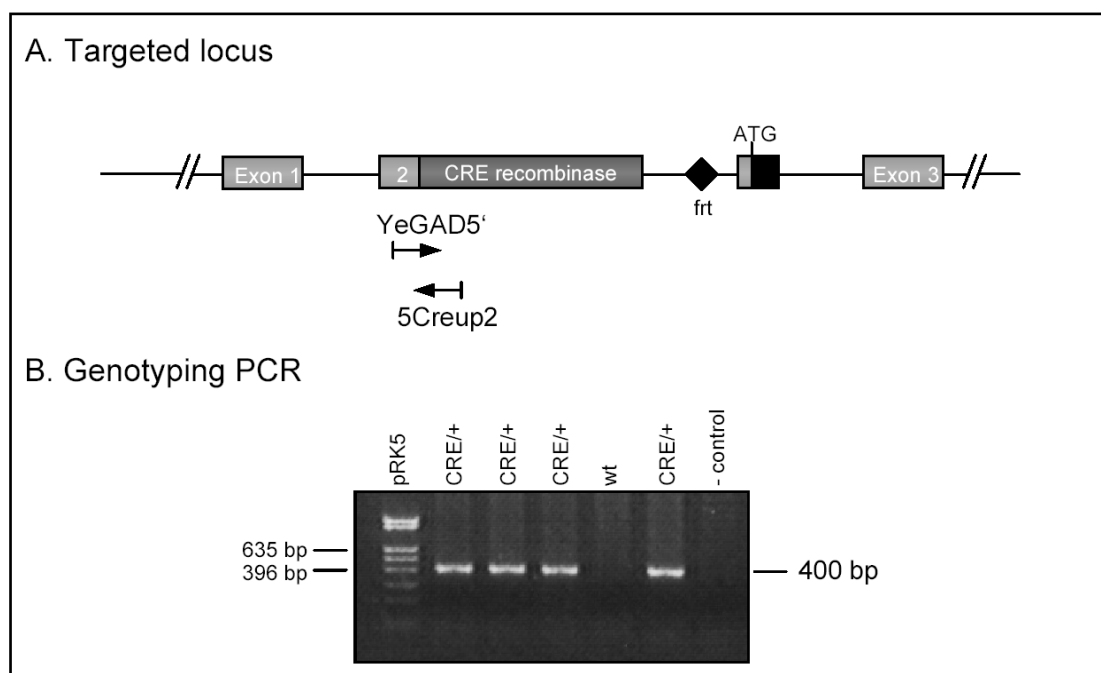


Fig. 15 Genotyping of $GAD67^{CRE/+}$ mice by PCR analysis. A: Overview of the targeted $GAD67$ locus and the position of the oligonucleotides (YeGAD5', 5Creup2) that were used for genotyping by PCR analysis. B: PCR amplification of a specific fragment (400 bp) for the targeted $GAD67$ allele from genomic tail DNA. Bp: Basepair; frt: Recognition site of flip recombinase; pRK5: DNA size marker; wt: Wild-type control.

The first injection gave rise only to middle and low chimeric male mice and hence a second injection was performed. The same ES cell clone was injected into 51 blastocysts and implanted into five foster mothers that gave birth to ten male and two

female mice. Out of the ten male mice eight were high and two were middle chimeric. From the first injection all five male mice were bred with wild-type C57Bl/6 to examine the offspring for germline transmission. As the offspring were born before the offspring of the second injection and germline transmission was successful, only two high chimeric males of the second injection were further bred. The progeny with agouti-coloured coat was genotyped by PCR analysis for the modified allele (Fig. 15) and positive animals were used to establish the mouse line. Mice were bred with flip-deleter mice (Rodriguez et al., 2000) that express flippase in all cells and removes frt flanked fragments - here the NEO cassette (Fig. 16 A). This step was performed to avoid a possible influence of the NEO cassette on the expression level of the CRE recombinase in knock-in mice. The removal of the NEO cassette as well as the germline transmission of the modified allele was controlled by PCR and Southern blot analysis (Fig. 16 B, C). Knock-in mice with successfully removed NEO cassette were bred with C57Bl/6 mice to obtain a uniform genetic background.

The inheritance of the modified allele was investigated by analysing the genotype and the gender from offspring derived from 21 breedings. 46 % (\pm 11.9 SEM) of the progeny (n= 147) possessed the allele with CRE recombinase and thereof 29 % were female and 20 % male. These results indicated that the inheritance of the altered allele followed the Mendelian ratio.

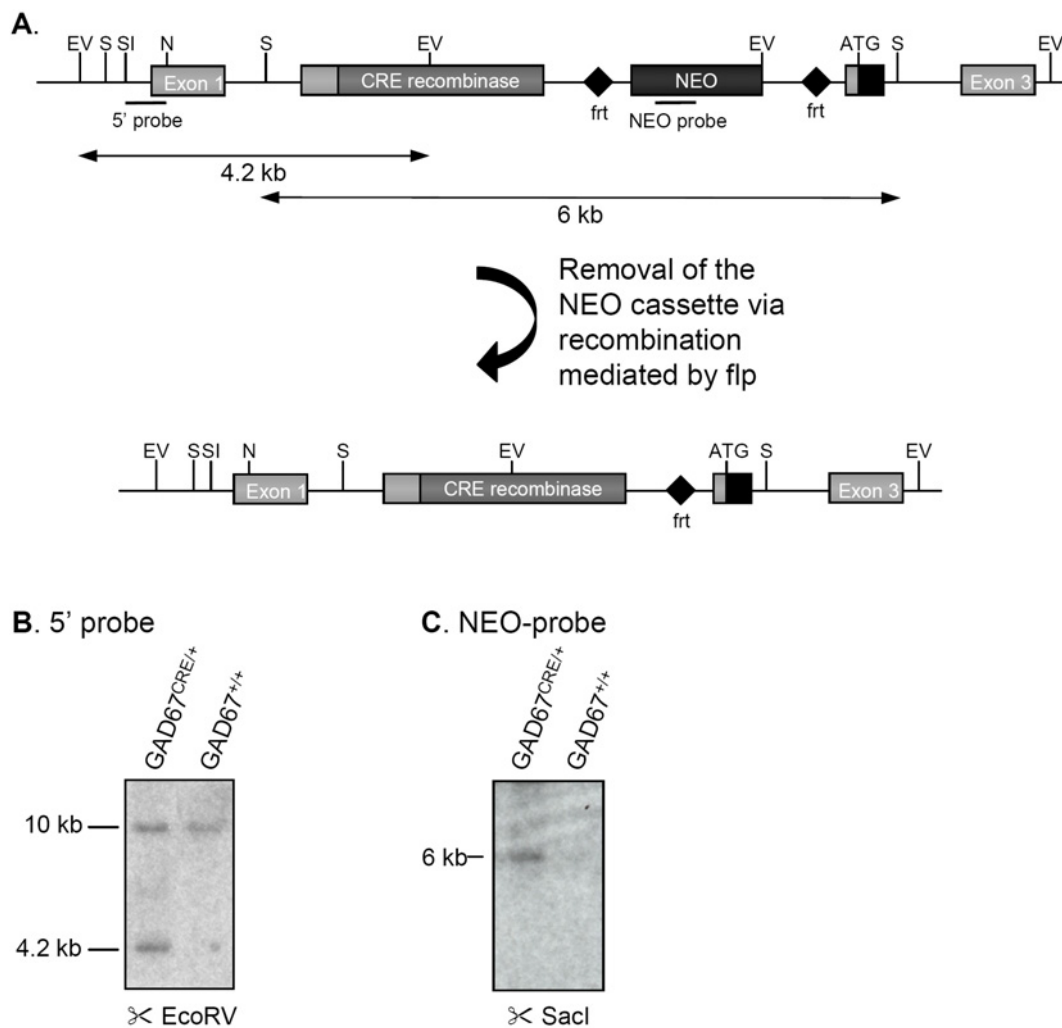


Fig. 16 Removal of the NEO cassette and confirmation by Southern blot analysis. A: The targeted allele before and after site-specific recombination mediated by flp-recombinase. A single frt site is left after the recombination. B: Southern blot analysis with EcoRV restricted genomic DNA obtained from GAD67^{CRE/+} and GAD67^{+/+} mice hybridised with the 5' probe to confirm the germline transmission of the targeted allele (4.2 kb fragment; 10 kb fragment derives from the wild-type allele). C: SacI restricted genomic DNA from GAD67^{CRE/+;NEO/+} and GAD67^{CRE/+} mice was hybridised with the NEO probe to demonstrate that the NEO cassette was removed. Positions of relevant restriction endonuclease recognition sequences are indicated: BI: BamHI; E: EcoRV; N: NotI; S: SacI; SI: Sal I. frt: Recognition site of flp recombinase.

3.1.4. Expression of CRE recombinase in adult GAD67^{CRE/+;ROSA/+} mice

A reliable, frequently used approach to investigate the functionality and expression pattern of CRE recombinase is to breed transgenic mice expressing CRE recombinase with ROSA26 CRE reporter mice (R26R) (Soriano, 1999). The latter possess the gene encoding for β -galactosidase (lacZ) whose translation is prevented by an upstream floxed NEO cassette. In cells that express CRE recombinase the NEO cassette is excised, the lacZ gene is transcribed and its product can be

visualised either by a X-Gal-staining or immunohistochemically with an antibody against β -galactosidase. Thus $GAD67^{CRE/+}$ mice lacking the NEO cassette, were bred with ROSA26 mice and the offspring were analysed for β -galactosidase expression. Figure 17 shows an overview of β -galactosidase expression in several regions of an adult $GAD67^{CRE/+;ROSA/+}$ mouse brain that was cut coronally and stained with an anti- β -galactosidase antibody. The expression pattern resembles the pattern that was expected for GAD67 expressing cells. The location of the stained cells in the hippocampus and the absence of staining from putative pyramidal cells indicate that labelled cells are interneurons (Fig. 17 B). The same holds true for the cerebral cortex (Fig. 17 A). Strong staining for β -galactosidase is visible in the striatum, the reticular thalamus as well as in the dorsomedial hypothalamic nuclei. The medial septum of the basal forebrain also contains β -galactosidase-positive cells (Fig. 17 C - F). The expression of β -galactosidase in the cerebellum can be detected in Purkinje cells as well as in some cells in the molecular and granular cell layer that are presumably basket/stellate or Golgi cells, respectively. Furthermore some strongly labelled cells are located in the deep cerebellar nuclei (Fig. 17 G). In the olfactory bulb, most cells in the periglomerular and granular cell layer are intensely stained for β -galactosidase. Few labelled cells are found in the external plexiform layer and in the accessory olfactory bulb (Fig. 17 H). These results indicate that CRE recombinase is functional under the control of the GAD67 promoter *in vivo* and can thus be used to ablate floxed genes of interest.

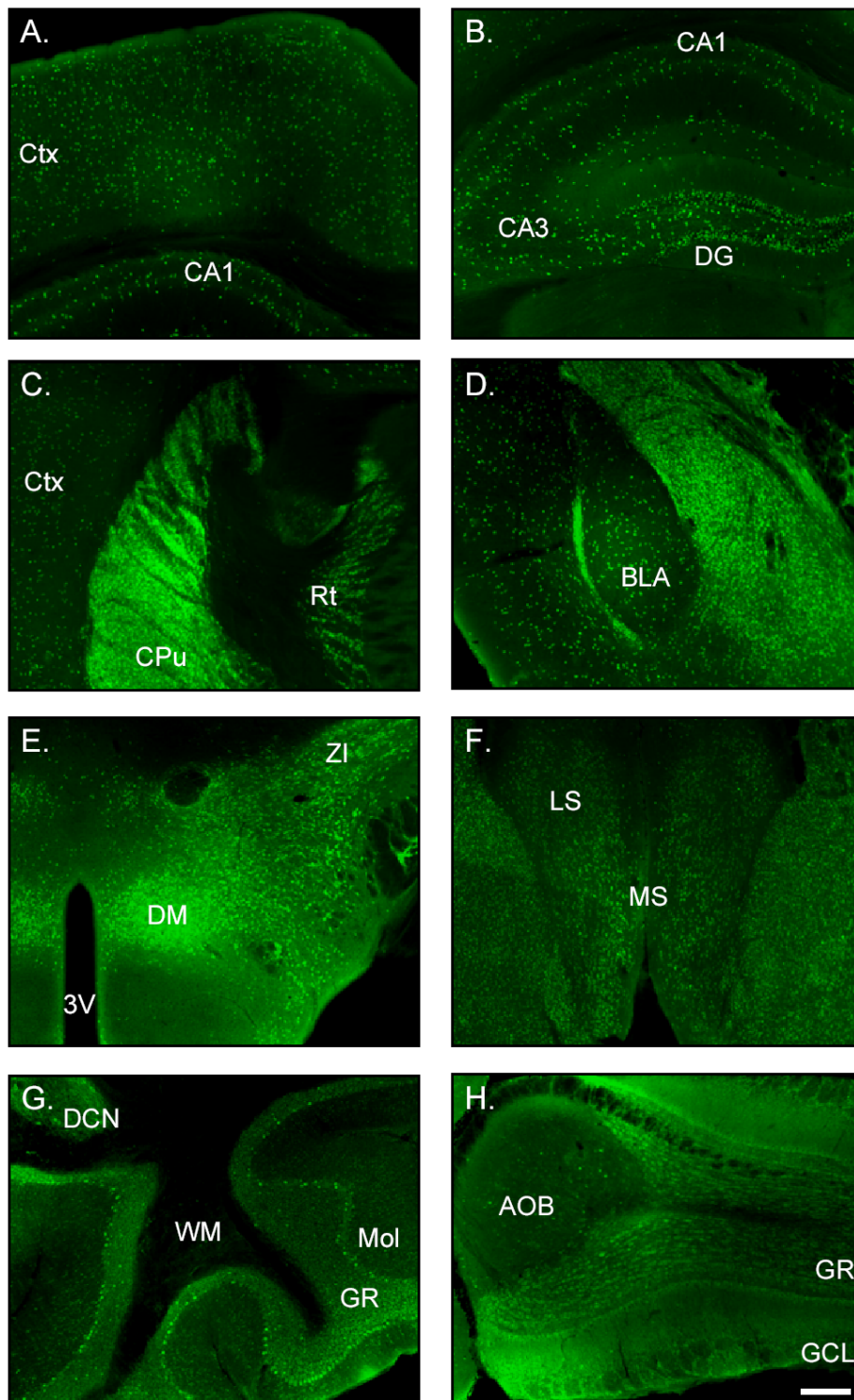


Fig. 17 Distribution of β -galactosidase-expressing cells in the adult brain of a $GAD67^{CRE/+};ROSA^{+/+}$ mouse. A-H: Immunohistochemical experiments were performed with an anti- β -galactosidase antibody (coronal sections) and the resulting expression pattern of β -galactosidase in several brain regions are shown: the cerebral cortex (A), the hippocampus (B), the striatum and the reticular thalamus (C), the basolateral amygdala (D), the hypothalamic region (E), the septal region (F), the cerebellum (G) and the olfactory bulb (H). 3V: Third ventricle; AOB: Accessory olfactory bulb; BLA: Basolateral amygdala; Ctx: Cortex; CPu: Striatum; DCN: Deep cerebellar nuclei; DG: Dentate gyrus; DM: Dorsomedial hypothalamic nucleus; GCL: Glomerular cell layer; GR: Granule cell layer; Rt: Reticular thalamus; LS: Lateral septum; Mol: Molecular cell layer; MS: Medial septum; WM: White matter; ZI: Zona incerta. Scale bar: 250 μ m.

3.1.5. CRE recombinase expressing cells in the brain of $GAD67^{CRE/+;ROSA/+}$ mice

Since the labelling for β -galactosidase only provides information about the location of expression but not whether these cells are GAD67-positive cells, a more thorough analysis had to be performed. Thus double-labelling experiments including a X-Gal-staining and an immunohistochemical staining for GAD67 were performed and evaluated. Two male and two female $GAD67^{CRE/+;ROSA/+}$ mouse brains (adult, at least 8 weeks) were cut coronally (50 μ m) and on every fourth section double labelling experiments were performed. The analysed regions were the hippocampus subdivided in the subregions: CA1, CA3 and dentate gyrus (without granular cell layer), and two cortical areas: motor cortex and somatosensory cortex (Fig. 18). Areas of 46 mm² in size from 10 sections per brain were investigated and the mean value was determined followed by calculating the overall mean of all four brains.

The results are summarised in Tab. 1. In each subregion of the hippocampus around 50% of analysed cells were double-positive for β -galactosidase and GAD67. The remaining 50% (approximately) were positive only for β -galactosidase. A similar result is found in the motor and the somatosensory cortex, about half of all analysed cells were double-labelled whereas the other half was positive for β -galactosidase but not for GAD67. No differences between female and male brains were detected.

Tab. 1 Analysis of β -galactosidase- positive cells in the $GAD67^{CRE/+;ROSA/+}$ mouse.

	β -gal & GAD67 positive cells (%)	β -gal positive cells (%)	n = analysed cells
Hippocampus			
DG	50.2 \pm 6.1	49.5 \pm 5.8	994
CA3	51.8 \pm 6.9	48.1 \pm 6.7	1157
CA1	55.3 \pm 4.6	44.6 \pm 4.6	1028
Cortex			
Motor	47.3 \pm 5.9	52.4 \pm 6.0	1137
Somatosensory	49.5 \pm 7.4	50.6 \pm 6.5	1207

Data are indicated as the mean \pm SEM values and were obtained from four adult $GAD67^{CRE/+;ROSA/+}$ mice. β -gal: β -galactosidase.

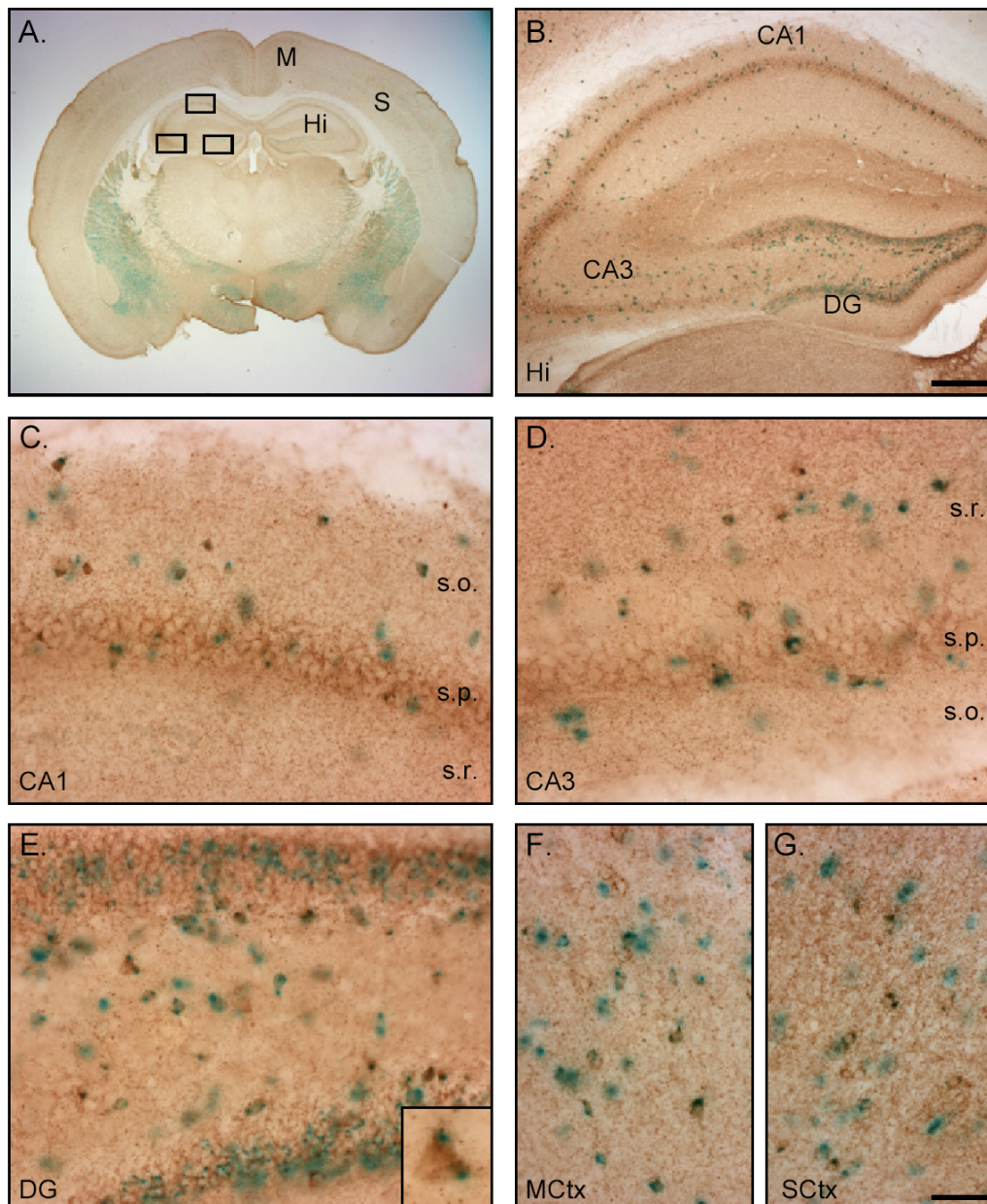


Fig. 18 Analysis of cells expressing β -galactosidase within distinct brain regions of the $GAD67^{CRE/+};ROSA/+$ mouse. Coronal sections were co-stained for β -galactosidase (blue) and GAD67 (brown) using X-Gal- and immunohistochemical staining methods, respectively. A: Overview of a coronal section. B-G: Higher magnifications of the marked areas in A (boxes). C: CA1 region. D: CA3 region. E: Dentate gyrus, the inset shows an example of a double-labelled cell. F: Motor cortex. G: Somatosensory cortex. CPu: Striatum; DG: Dentate gyrus; Ent: Entorhinal cortex; Hi: Hippocampus; M: Motor cortex; S: Somatosensory cortex; s.o.: Stratum oriens; s.p.: Stratum pyramidale; s.r.: Stratum radiatum. Scale bars: 250 μ m (B), 25 μ m (C-G).

To further validate the expression of CRE recombinase in GAD67-positive cells, double-labelling experiments with combinations of an anti- β -galactosidase antibody and antibodies against several interneuronal markers - parvalbumin, calbindin and somatostatin - were performed (Fig. 19). The experiments were performed on

sections obtained from three adult $GAD67^{CRE/+;ROSA/+}$ mice (male and female). Labelled cells of two sections from each mouse were counted and the mean value was calculated. In Tab. 2 the results are listed. Almost all cells in the hippocampus and the cortex were double-positive for β -galactosidase and parvalbumin. The double-labelling experiment with β -galactosidase and calbindin showed as well a nearly complete overlap in the hippocampus. In the motor and somatosensory cortex the percentage of double-positive cells was around 90%. All cells of the hippocampus were co-labelled for β -galactosidase and somatostatin. More than 90 % of all counted cells in the cortical areas exhibited an overlapping signal.

These results convincingly demonstrated that specific gene ablation in all $GAD67$ -expressing cells can be obtained utilizing $GAD67^{CRE/+}$ mice.

Tab. 2 Characterisation of β -galactosidase-expressing cells in the $GAD67^{CRE/+;ROSA/+}$ mouse.

	β -gal & PV positive cells (%) (n)	β -gal & CB positive cells (%) (n)	β -gal & SOM positive cells (%) (n)
Hippocampus			
DG	96 \pm 5.3 (29)	100 \pm 0 (3)	100 \pm 0 (60)
CA3	100 \pm 0 (46)	95 \pm 6.2 (13)	100 \pm 0 (33)
CA1	100 \pm 0 (43)	92 \pm 11.1 (9)	100 \pm 0 (40)
Cortex			
Motor	99 \pm 1.3 (97)	92 \pm 5.1 (38)	93 \pm 1.4 (111)
Somatosensory	96 \pm 3.1 (70)	88 \pm 8 (43)	92 \pm 3.2 (94)

Data are indicated as the mean \pm SEM values and were obtained from three adult $GAD67^{CRE/+;ROSA/+}$ mice. β -gal: β -galactosidase; CB: Calbindin; PV: Parvalbumin; SOM: Somatostatin.

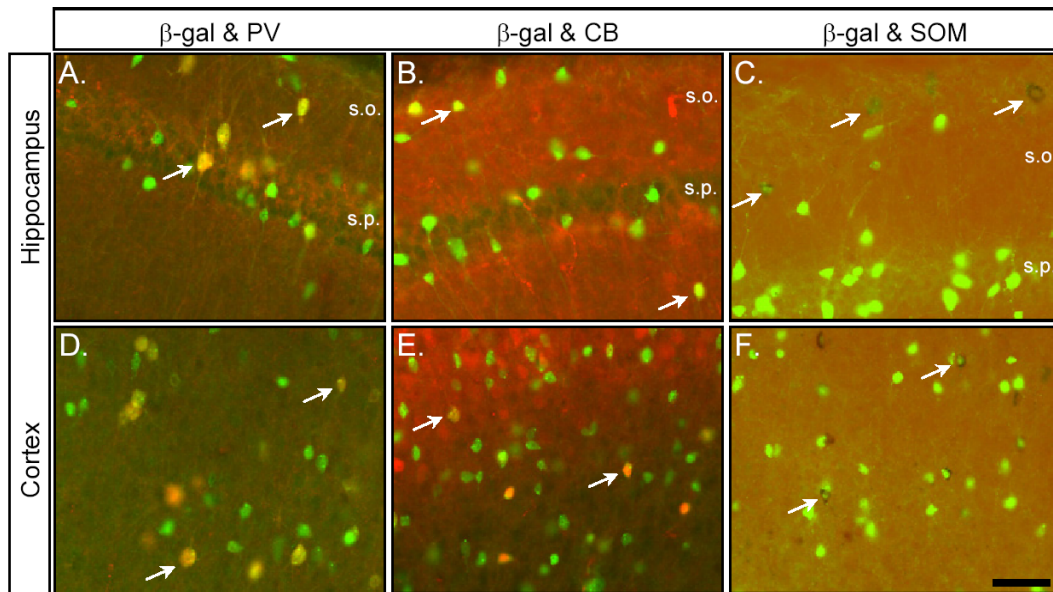


Fig. 19 Interneuronal markers are co-expressed in β -galactosidase-positive cell in the hippocampus and the cortex. A, D: Immunolabelling for β -galactosidase (green) and parvalbumin (red). B, E: Double-immunohistochemical experiments with an anti- β -galactosidase antibody (green) and an anti-calbindin antibody (red). C, F: Co-labelling of β -galactosidase (green) and somatostatin-positive cells (brown, DAB immunohistochemical staining). Cells marked with an arrow serve as examples for double-positive cells (yellow). β -gal: β -galactosidase; CB: Calbindin; SOM: Somatostatin; s.o.: Stratum oriens; s.p.: Stratum pyramidale; PV: Parvalbumin. Scale bar: 25 μ m.

3.1.6. Expression of CRE recombinase in embryonic $GAD67^{CRE/+};ROSA^{+}$ mice

The demonstration of the functionality of CRE recombinase in embryonic mice is a prerequisite to plan experiments that address developmental questions. By analysing embryonic day (E) 16 offspring resulting from the breeding of $GAD67^{CRE/+}$ mice with R26R reporter mice, the functionality of CRE recombinase could be demonstrated by X-Gal staining experiments. In sagittal (whole embryo) as well as in coronal sections (head) the staining pattern of CRE recombinase expression resembled the known expression pattern for $GAD67$. There was staining in the embryonic brain in the olfactory bulb, the cerebral neocortex, the developing hippocampal formation, the thalamic regions, the midbrain and the cerebellum (Fig. 20 A-E). The labelled cells in the cerebral neocortex and the hippocampus were mainly located in the cortical plate and subventricular zone with some scattered cells in the intermediate zone (Fig. 20 D, E). Labelled cells were also detected in the dorsal and ventral horn of the spinal cord (Fig. 20 A, I). In addition, stained cells were observed in non-neuronal tissues, such as in the developing fore- and hindlimbs, the mesenchyme and neuroepithelium of the tailbud, the vibrissae and the kidney (Fig. 20 F-J).

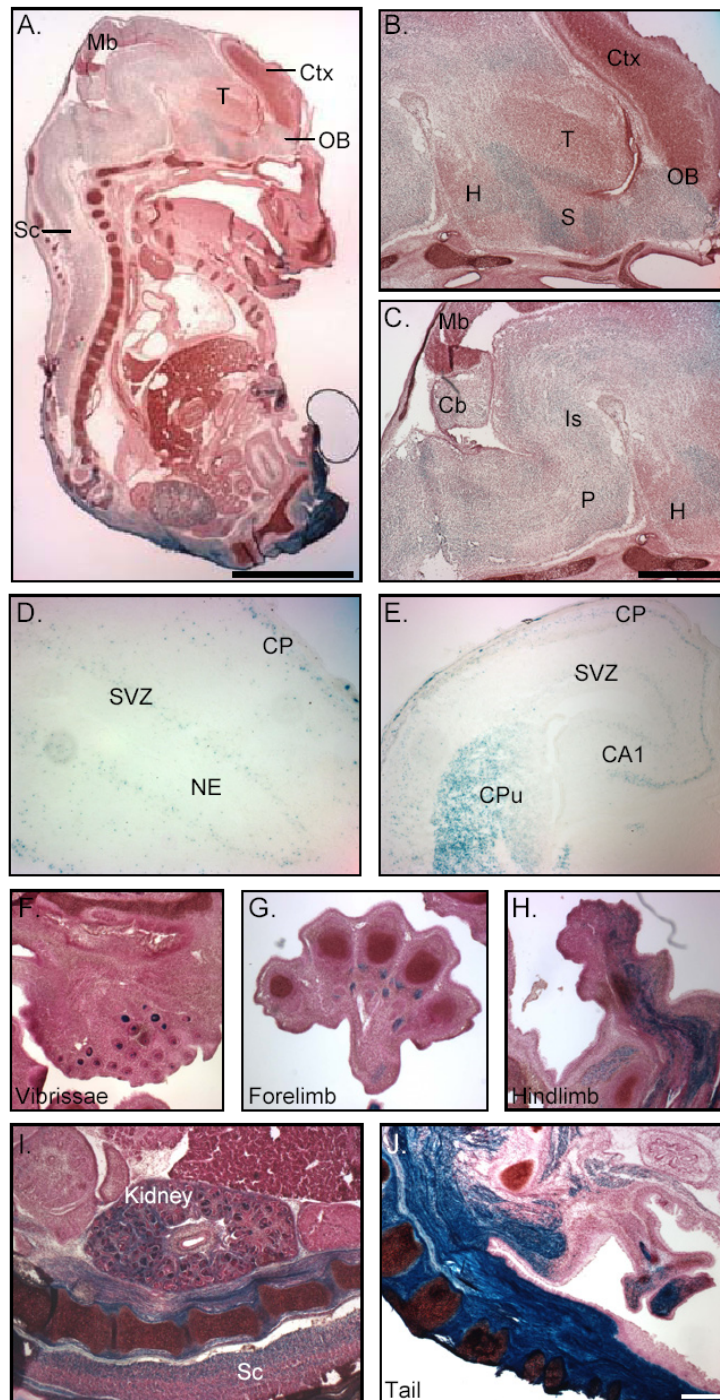


Fig. 20 Expression pattern of β -galactosidase in embryonic $GAD67^{CRE/+};ROSA^{+/+}$ mice. X-Gal staining experiments (blue colour) were performed on sagittal (A-C, F-J) and coronal sections (D, E) of $GAD67^{CRE/+};ROSA^{+/+}$ mice at embryonic day 16. A: Overview of an E16 embryo. B: Higher magnification of the forebrain. C: Higher magnification of the midbrain, the cerebellum and the medulla oblongata. D: Overview of the neocortex with its distinct layers: cortical plate, subventricular zone and neuroepithelium. E: Overview of the developing hippocampus. F: Expression of β -galactosidase in the vibrissae. G: β -galactosidase positive cells in forelimb. H: Stained cells in the hindlimb. I: β -galactosidase labelled cells in the kidney and in the spinal cord. J: Expression of β -galactosidase in the tailbud. The red colour in A-C and F-J derived from counterstaining of the sections with Neutral Red. Cb: Cerebellum; CP: Cortical Plate; CPu: Striatum; Ctx: Cortex; H: Hypothalamus; Is: Isthmus; MB: Midbrain; NE: Neuroepithelium; OB: Olfactory bulb; P: Pons; SVZ: Subventricular zone; Sc: Spinal cord; T: Thalamus. Scale bars: A: 0.25 cm; B-J: 250 μ m.

3.1.7. The GAD67 promoter of GAD67^{CRE/+} mice is not active in gametes

Analysis of GAD67^{CRE/+;ROSA/+} mice included experiments that address whether the promoter driving the CRE recombinase is already active in the gametes of the mouse. If this was the case, cell-type specific gene ablation would not be possible. To this end GAD^{CRE/+;ROSA/+} mice were bred with wild-type C57Bl/6 mice and the offspring were analysed concerning their ability to express β -galactosidase. X-Gal staining was performed on sections of these mice as well as on control sections (derived from GAD67^{CRE/+;ROSA/+}) and staining for β -galactosidase was detected only on the control sections (data not shown). This result indicated that CRE recombinase is not active in gametes and the mice can be used to generate gene knock-outs in GAD67 expressing cells only.

3.1.8. Preliminary results of the behavioural analysis of GAD67^{CRE/+} mice

The behavioural tests were performed to exclude phenotypic deficits of GAD67^{CRE/+} mice due to reduced expression level of GAD67 deriving from the modified allele that is not expressing the GAD67 gene anymore. The tests were performed by Dr. Elke Fuchs (Department of Clinical Neurobiology, University of Heidelberg, Germany) on adult GAD67^{CRE/+} mice and their wild-type littermates as control animals.

The results of the behavioural tests can only be considered as preliminary since the number of tested animal was too low. For behavioural studies (Crawley, 2000) at least 15 animals of the same gender (male) should be tested to reduce the influence of an individual performance of one animal on statistics. GAD67^{CRE/+} mice were indistinguishable from wild-type mice upon visual inspection.

To analyse motor learning ability of GAD67^{CRE/+} mice, the accelerating rotarod test was performed on three consecutive days (= three blocks) with three trials per mouse on each day. GAD67^{CRE/+} mice were able to learn the task but, as mentioned above, to conclusively answer the question more animals have to be tested (Fig. 21 A). No impairment in the explorative behaviour of the GAD67^{CRE/+} mice was observed during the open field test (Fig. 21 B). There were neither significant differences in the squares that GAD67^{CRE/+} and control mice crossed nor in the number of rearings on both days of the testing. Furthermore the novel object recognition test also did not reveal differences in the explorative behaviour of GAD67^{CRE/+} mice (Fig. 21 C).

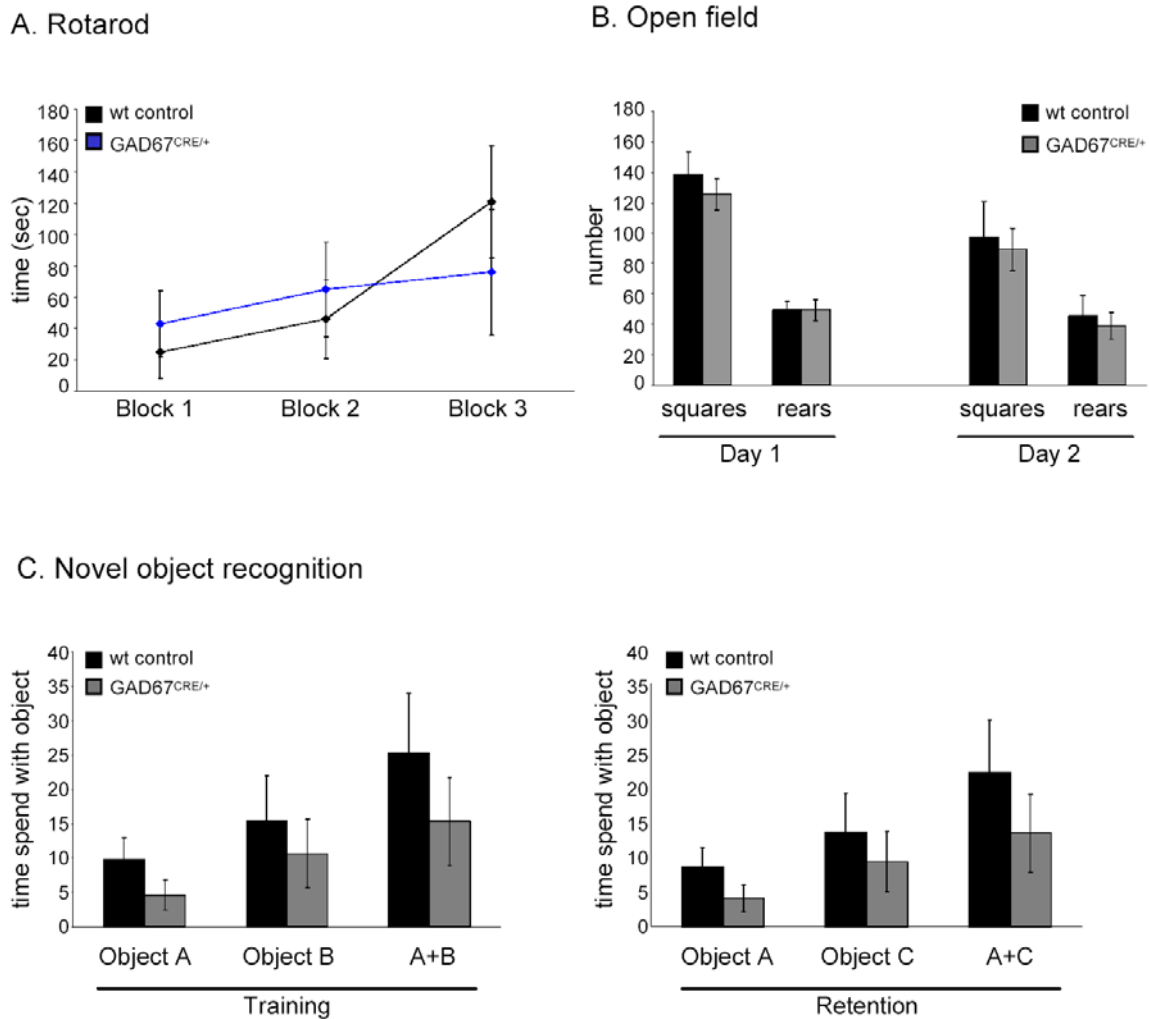


Fig. 21 Preliminary behavioural tests with GAD67^{CRE/+} mice. A: Rotarod test. GAD67^{CRE/+} and wild-type mice performed equally well on three consecutive days (three trials per day) in the accelerated rotarod motor test. (wt n= 6; GAD67^{CRE/+} n= 6). B: Open field test. GAD67^{CRE/+} mice performed similarly compared to control animals. The test was performed twice on two consecutive days. (wt n= 7; GAD67^{CRE/+} n= 6). C: Novel object recognition test. GAD67^{CRE/+} mice showed no differences in recognising a new object compared to the wild-types. The test was performed five minutes after a five minutes training phase. In the training phase the mouse was allowed to explore two different objects (A and B). The bars show the time spent with the objects. During the retention phase object B was exchanged with object C and the time spent with the objects was measured again. (wt n= 6; GAD67^{CRE/+} n= 6). Each column represents the mean \pm SEM.

3.1.9. Decreased GAD67 protein expression levels in GAD67^{CRE/+} mice

GAD67^{CRE/+} mice and their wild-type littermates that were used for behavioural tests (3.1.8.) were subsequently used to investigate the GAD67 protein levels in forebrains of GAD67^{CRE/+} mice. Total protein of forebrains from five GAD67^{CRE/+} mice and five wild-type control mice were prepared and were analysed in triplicates by Western blot analysis (Fig. 22). The ECL-stained Western blot films were scanned and the intensity of the detected signals for GAD67 as well as the β -actin as a control, were

determined with the ImageJ program (from the National Institute of Health, Maryland, USA). The quantification was performed by normalising the value for GAD67 to β -actin and calculating the mean value of each triplicate. The reduction of GAD67 protein expression in each pair and the overall mean from all pairs was determined. This value revealed a reduction of GAD67 protein expression in $GAD67^{CRE/+}$ mice to 48.5% (\pm 15 SEM) compared to wild-type mice. In one $GAD67^{CRE/+}$ mouse the GAD67 protein level was reduced to 26% compared to wild-type expression levels accounting for the high SEM.

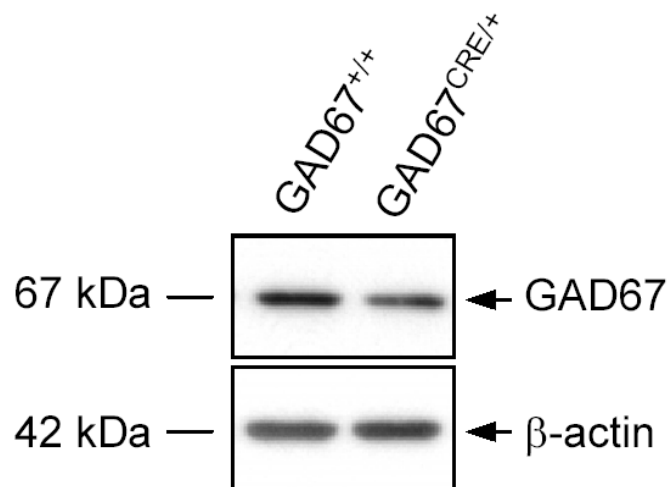


Fig. 22 *Reduced GAD67 protein expression in $GAD67^{CRE/+}$ mice.* The GAD67 protein levels of five $GAD67^{CRE/+}$ mice and five wild-type littermates were quantified by measuring the intensity of the Western blot signal with the ImageJ program from the NIH. The analysis revealed that the GAD67 protein expression in $GAD67^{CRE/+}$ mice is half of the expression levels detected in wild-type mice.

3.2. Generation and analysis of the SOM^{CRE/+} mouse line

3.2.1. Expression pattern of the somatostatin gene in the developing and mature mouse brain

In situ hybridisation (ISH) experiments with radioactive labelled oligonucleotides were performed to investigate the regional distribution of the somatostatin gene during development. Three different oligonucleotides for the somatostatin gene were chosen to compare the expression pattern and thus control the specificity.

ISH experiments were performed on horizontal mouse brain sections at embryonic day (E) 14, postnatal days (P) 1, 9, 15, 25 and in the adult. The three oligonucleotides showed similar expression patterns with different intensities of the ISH signal and corresponded with the results of former publications. Images from the ISH experiment with highest signal intensity are shown in Figure 23.

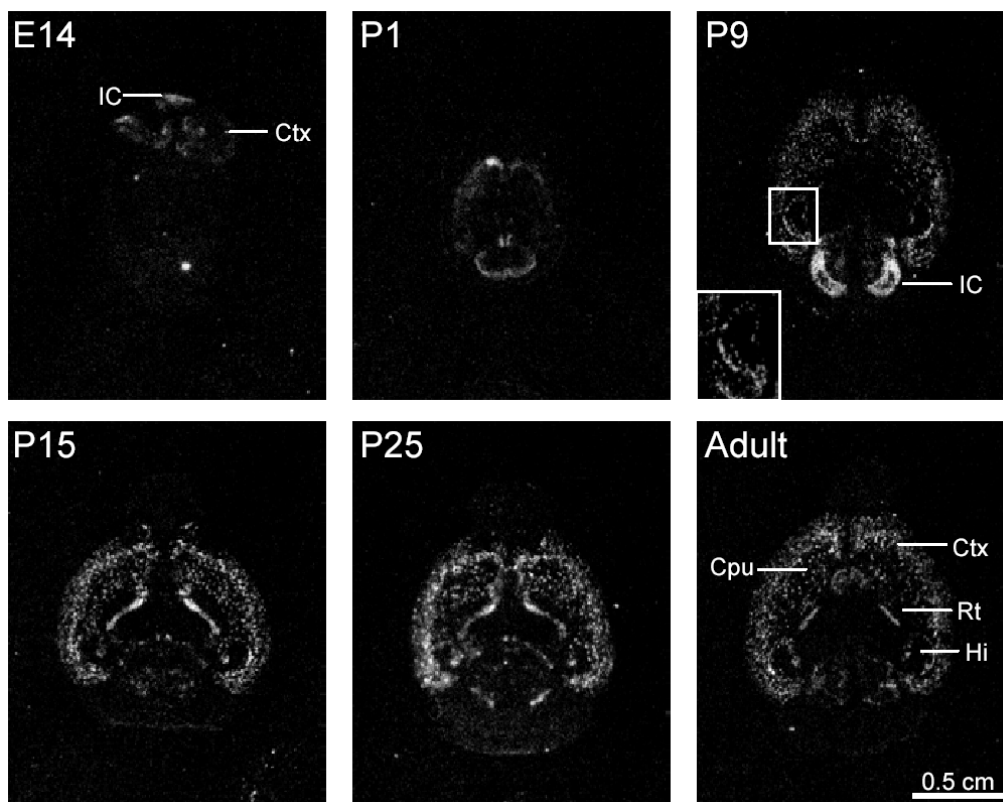


Fig. 23 Expression of somatostatin mRNA in the developing and mature mouse brain. The spatial and temporal distribution of somatostatin mRNA was determined by radioactive *in situ* hybridisation experiments on embryonic (E14; sagittal) and postnatal (P1, P9, P15, P25 and adult; horizontal) mouse brain sections. The inset illustrates a higher magnification of the hippocampus. Hi: Hippocampus; Ctx: Cortex; Cpu: Striatum; IC: Inferior colliculus; Rt: Reticular thalamus. Scale bar: 0.5 cm.

Preprosomatostatin mRNA is expressed already at high levels at E14 and was restricted to the central nervous system. At postnatal stages (P1-adult), mRNA expression was detected throughout all layers of the cerebral cortex, the striatum, the reticular thalamus and the hippocampus with a more intense signal in the CA1 region (Fig. 23, P9, inset). A strong signal was visible in the inferior colliculus already at E14 and was also visible on the section from the P9 mouse brain.

There was hardly any mRNA expression detectable in the olfactory bulb in contrast to the accessory olfactory bulb where some expression was detected (Fig. 23, P15). No expression was found in the cerebellar cortex.

3.2.2. Construction of the targeting vector

The somatostatin gene comprises two exons and the startcodon is located in exon one. The strategy for generating mice expressing CRE recombinase under the promoter of somatostatin was to replace the translational startcodon of somatostatin by the one of CRE recombinase.

A detailed description of the cloning strategy is shown in Fig. 25. A 10 kb fragment of the BAC 220B24 containing the somatostatin locus was subcloned into pBluescript SK+ (pBS) via shotgun-cloning (Fig. 24). Shortly, the BAC DNA and the pBS DNA were digested with BamHI, ligated and transformed into *E.coli* bacteria. The resulting colonies were lifted on a membrane that subsequently was hybridised with a ³²P-labeled 3'probe to identify colonies containing the somatostatin locus. Thus, one positive clone contained the somatostatin locus including 3 kb upstream and 7 kb downstream that were used as recombination arms (RA) for homologous recombination in ES cells (pBS-SOM).

For building the targeting construct, pBS was used as a vector and it consisted of the following components: a CRE recombinase cassette encoding the recombinase of phage P1 (subcloned from pMC-CRE recombinase, (Gu et al., 1993)). Downstream a cassette was introduced containing the neomycin resistance gene (neomycin phosphotransferase, NEO) with its own promoter (derived from the herpes simplex virus) for negative selection in ES cell culture. The NEO cassette was flanked by frt sites. The original NEO cassette was from Cope et al. and was modified by Dr. Irinel Coserea by removing the loxP site and subcloning it to Lit29 (Cope et al., 2004). The targeting construct itself was flanked by a 5' and 3' recombination arm (RA, 280 and 260 bp, respectively) to introduce it into the correct position of the somatostatin gene

(Fig. 25 D). The correct assembly of the targeting construct (pBS-SOM/TG) was confirmed by enzymatic restriction analysis and by sequencing.

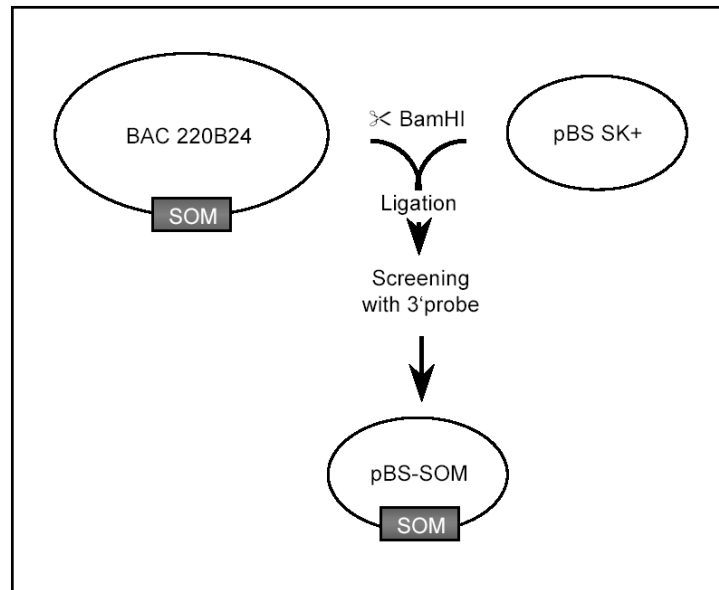


Fig. 24 Subcloning of a fragment of BAC 220B24. A 10 kb fragment containing both exons of the somatostatin gene was subcloned utilising the shotgun cloning method into pBS. Both the BAC and pBS were restricted with BamHI, ligated and transformed into *E.coli*. The resulting colonies were screened with a ^{32}P -labeled 3' probe for clones possessing a BAC fragment including the somatostatin gene.

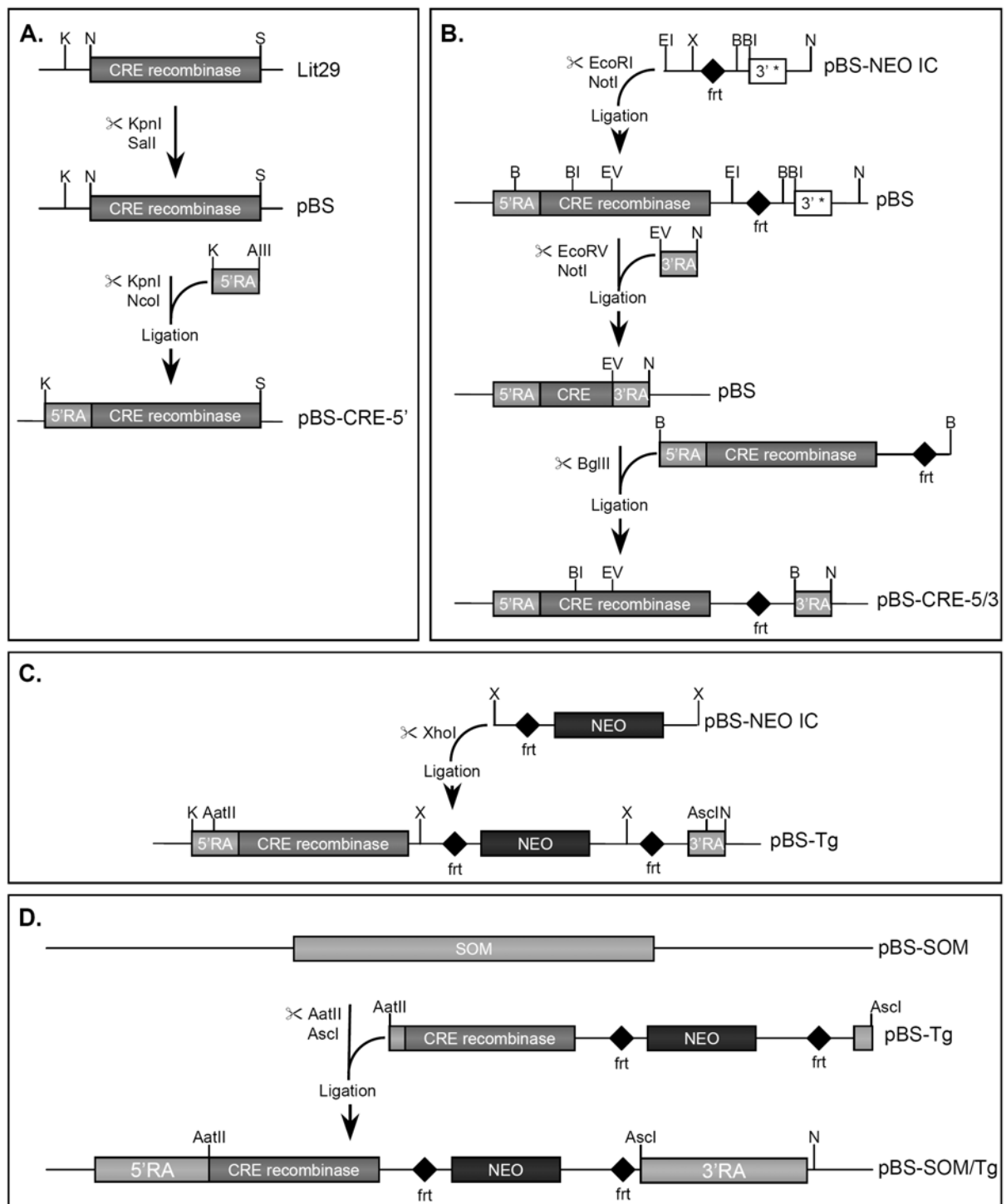


Fig. 25 Strategy for cloning of the targeting construct. A: Subcloning of the CRE recombinase cassette in pBluescript and cloning of the 5'RA resulting in pBS-CRE-5'. B: Adding the 3'RA to pBS-CRE-5' by cloning the RA (3'*) of another construct including one frt site and exchanging the old RA with the new 3'RA (pBS-CRE-5/3). C: Cloning of the NEO-cassette into pBS-CRE-5/3 (pBS-Tg). D: Cloning of the targeting cassette into the startcodon of the somatostatin gene that was included in the vector pBS-SOM obtained from the shotgun-cloning experiment (Fig. 24). Relevant restriction endonuclease recognition sites are indicated: AIII: AflIII; BI: BamHI; B: BglI; EI: EcoRI; EV: EcoRV; K: KpnI; N: NotI; S: Sall; X: XhoI. RA: Recombination arm; frt: Recognition site of flp recombinase. The constructs are not drawn to scale.

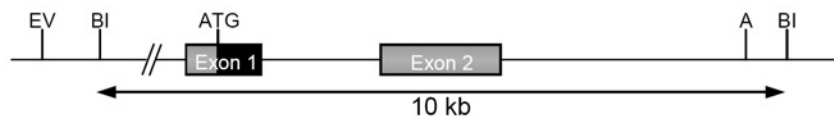
3.2.3. Selection of targeted ES cell clones

The targeting construct pBS-SOM/Tg was linearised with NotI and electroporated into mouse embryonic stem cells derived from the ES cell line R1 (Nagy et al., 1993). ES cell clones were selected based on their resistance to G418 guaranteed by the NEO cassette present in the targeting construct. 960 G418-resistant clones were picked and 360 were further screened for the correct targeting event by Southern blot analysis.

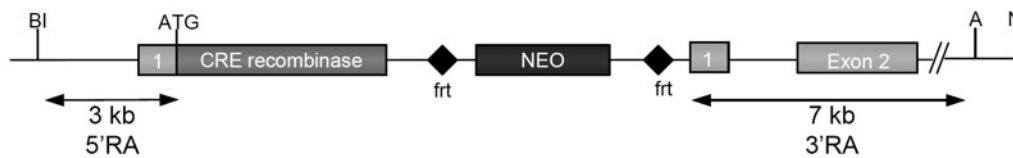
EcoRV restricted genomic DNA obtained from these clones was hybridised with the external 5' probe (Fig. 26 C, position of the probe) demonstrating either the presence of a 10 kb fragment derived from the wild-type allele or both a 10 kb 'wild-type' fragment and a 5.8 kb fragment that is specific for the modified allele. Nine clones showed the expected signal with the 5'probe for a correct targeting event via homologous recombination.

Six of these clones were further analysed by Southern blot analysis with the 3'-, NEO- and CRE probe (Fig. 26 E-G). In three clones the correct targeting was demonstrated by the specific fragment sizes: one fragment larger than 10 kb (wild-type allele) and a 6.9 kb fragment (modified allele) detected with the 3' probe; one 1.2 kb and one 1 kb fragment detected with the NEO and the CRE probe, respectively.

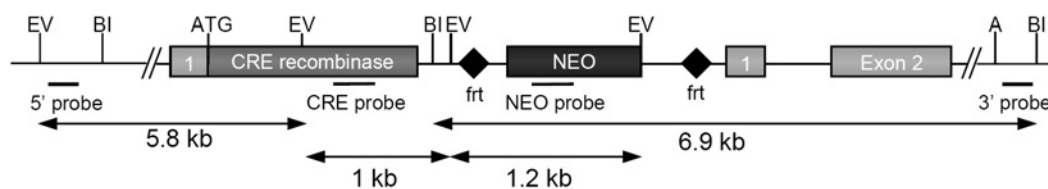
A. Somatostatin locus



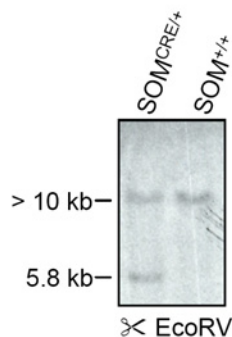
B. Targeting construct



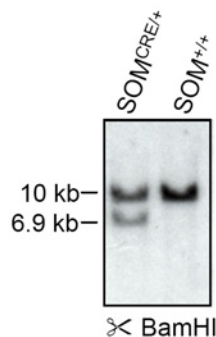
C. Targeted allele



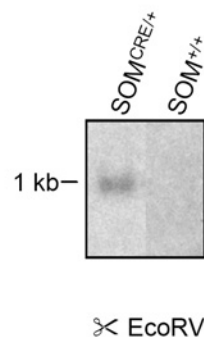
D. 5' probe



E. 3' probe



F. CRE-probe



G. NEO-probe

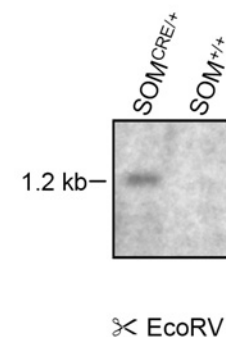


Fig. 26 Generation of $SOM^{CRE/+}$ mice via a knock-in approach. A, B, C: Relevant features of the somatostatin wild-type allele ($SOM^{+/+}$), the targeting construct and the modified ($SOM^{CRE/+}$) allele are shown. Start of the coding region in exon 1 is indicated by a black box, and the black diamonds stand for the two *frt* sites (recognition site of *flp* recombinase.). Positions of relevant restriction endonuclease recognition sequences are indicated: A: *Agel*; B: *BamHI*; E: *EcoRV*; N: *NotI* as well as the locations of the used probes. D: Southern blot analysis of *EcoRV* restricted genomic DNA from $SOM^{CRE/+}$ and $SOM^{+/+}$ ES cells hybridised with the 5' probe demonstrating the presence of a 5.8 kb fragment specific for the modified allele (the 10 kb fragment derives from the wild-type allele). E: *BamHI* restricted genomic DNA obtained from $SOM^{CRE/+}$ and $SOM^{+/+}$ ES cells was analysed by Southern blot experiments with the 5' probe and resulted in a 6.9 kb and a > 10 kb fragment for the modified and the wild-type allele, respectively. F: *EcoRV* restricted genomic DNA obtained from $SOM^{CRE/+}$ and $SOM^{+/+}$ ES cells was hybridised with the CRE probe in a Southern blot experiment showing a 1 kb fragment indicating the presence of the CRE cassette. G: Southern blot analysis of *EcoRV* restricted genomic DNA from $SOM^{CRE/+}$ and $SOM^{+/+}$ ES cells with the NEO probe revealed a 1.2 kb fragment corresponding to the NEO cassette.

3.2.4. Generation of $SOM^{CRE/+}$ mice

One of the three positive ES cell clones was injected into 45 C57Bl/6 mouse blastocysts that were transferred into four foster mothers (the injection was performed by Frank Zimmermann, IBF, University of Heidelberg, Germany). Twelve male and ten female mice were born and the female offspring were eliminated. The chimeric level of the male mice were determined as previously described in chapter 3.1.3.. Out of the twelve male mice, two were high, seven middle and three low chimeric. The two high and three middle chimeric mice were bred with C57Bl/6 mice to determine germline transmission of the altered allele (Fig. 27). The offspring of the breeding was again investigated for the coat colour and all agouti-coloured mice were genotyped by DNA tail analysis (Fig. 28). Mice positive for the modified somatostatin allele were bred with flp-deleter mice (Rodriguez et al., 2000) to remove the NEO cassette. Subsequently $SOM^{CRE/+}$ mice without the NEO cassette were bred with C57Bl/6 mice to propagate the line and to increase the C57Bl/6 genetic background (Fig. 29). 15 litters were analysed taking into consideration the inheritance of the modified allele. From 34 % (± 11.2 SEM) of mice positive for CRE recombinase (n= 115), 15 % were female and 20 % male, indicating that the inheritance of the modified allele follows the Mendelian pattern.

Germline transmission as well as the removal of the NEO cassette was additionally confirmed by Southern blot analysis with the 5'-, 3'- and NEO probe (Fig. 29 C-E).

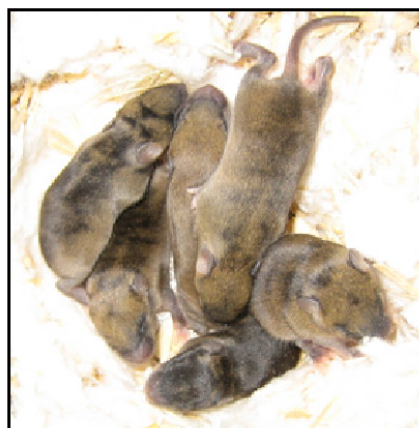


Fig. 27 *Chimeric $SOM^{CRE/+}$ mice.* The coat colour of the male progeny can be used to determine the chimeric level since the coat colour of the mouse strain from which the ES cells were derived (R1, agouti) and the one of the foster mother (C57Bl/6, black) differed. A higher contribution of ES cells to the germline will result in a higher proportion of agouti-coloured coat.

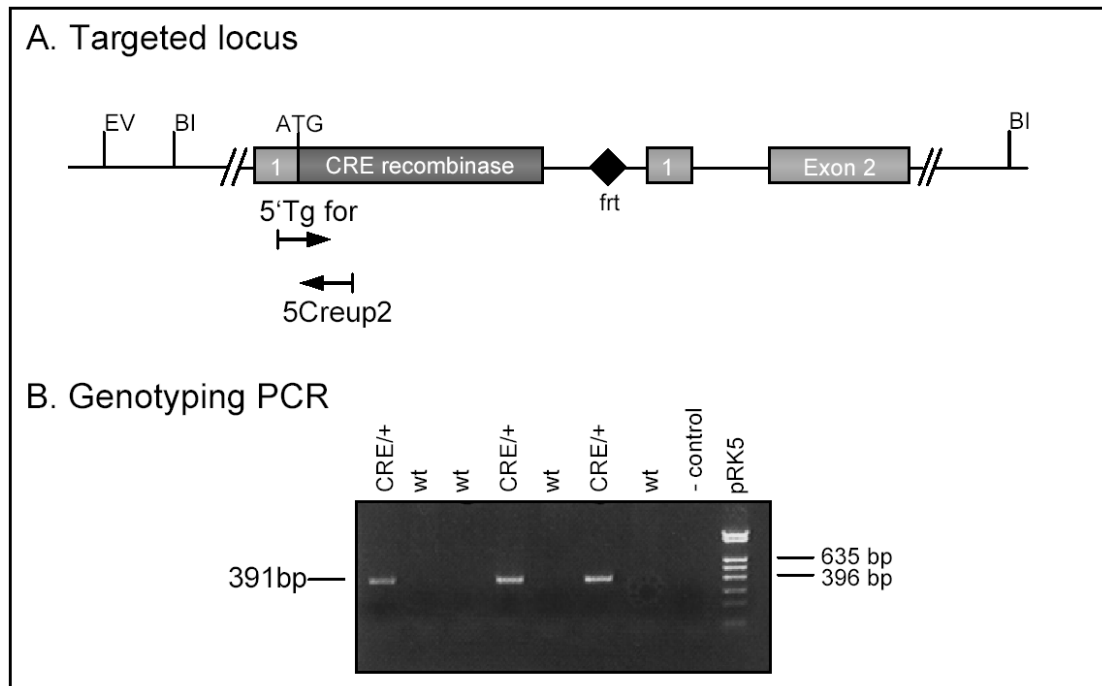


Fig. 28 Genotyping of $SOM^{CRE/+}$ mice by PCR analysis. A: Overview of the targeted SOM locus and position of the oligonucleotides (5'Tg for, 5Creup2) that were used for genotyping by PCR analysis. B: PCR amplification of a fragment (391 bp) that is specific for the targeted SOM allele using genomic tail DNA. Bp: Basepair; frt: Recognition site of flp recombinase; pRK5: DNA size marker; wt: Wild-type control.

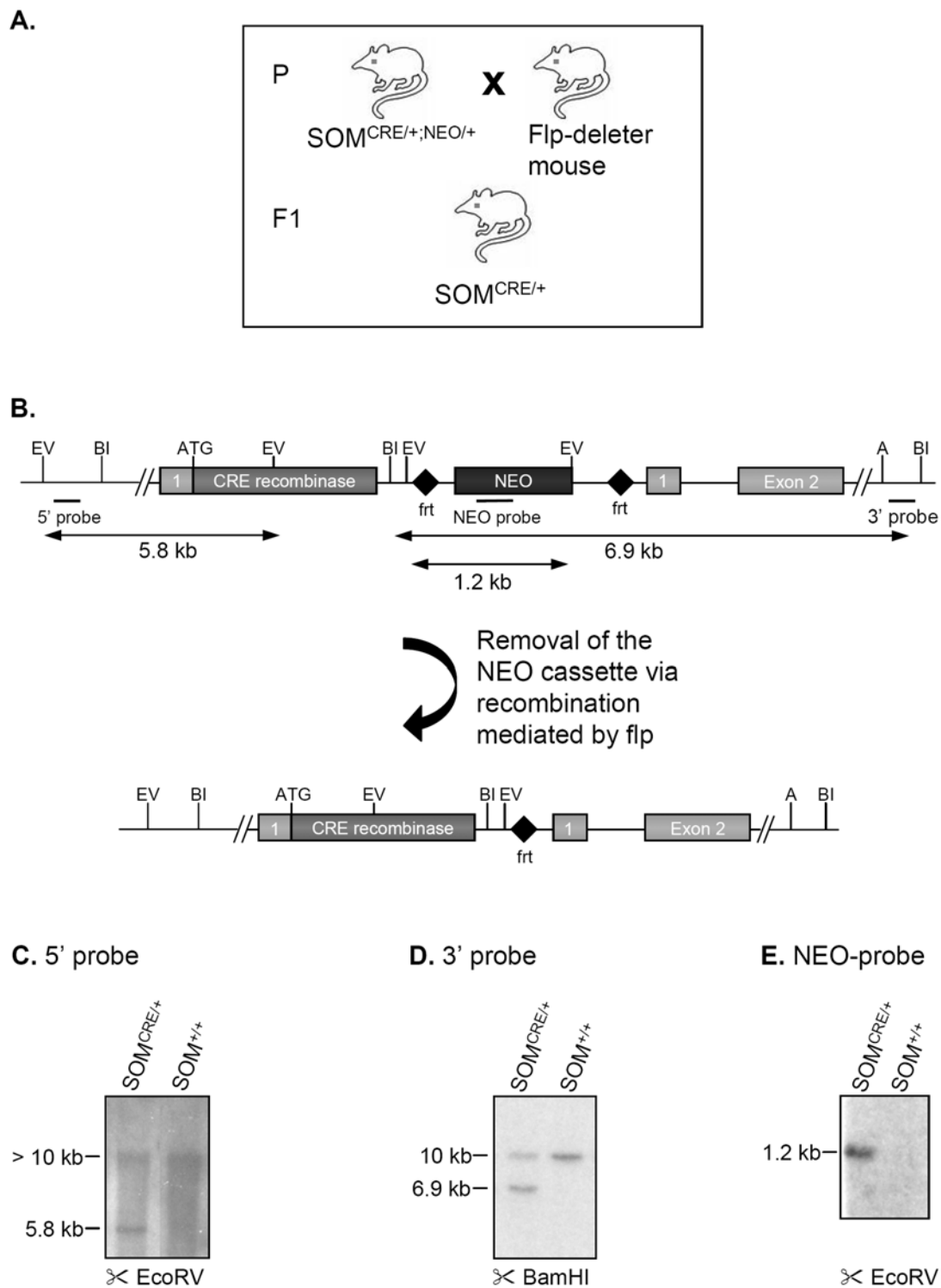


Fig. 29 NEO-cassette removal and validation by Southern blot analysis. A: Breeding scheme for removal of the NEO cassette. $SOM^{CRE/+};NEO/+$ mice were bred with flp-deleter mice (Rodriguez et al., 2000) that is able to promote site-specific recombination of floxed fragments by expressing flp recombinase. B: The modified allele with and without the NEO cassette. C, D: Southern blot analysis of EcoRV (EV) and BamHI (BI) restricted genomic DNA of $SOM^{CRE/+}$ mice with the 5' and 3' probe, respectively, to confirm germline transmission of the modified allele. E: Confirmation of the recombination of the NEO cassette by Southern blot analysis of EcoRV restricted genomic DNA obtained from $SOM^{CRE/+};NEO/+$ and $SOM^{CRE/+}$ mice. frt: Recognition site of flp recombinase.

3.2.5. CRE recombinase expression in adult SOM^{CRE/+;ROSA/+} mouse brains

SOM^{CRE/+} mice without the NEO cassette were bred with mice of the ROSA26 CRE reporter line (Soriano, 1999) to investigate the functionality of CRE recombinase under the somatostatin promoter and to monitor the expression pattern. The ROSA26 mice contain a floxed NEO cassette that keeps a lacZ gene inactive. In cells containing CRE recombinase NEO is excised, the lacZ gene that encodes for β -galactosidase is transcribed and β -galactosidase can be visualised either with an anti- β -galactosidase antibody staining or a X-Gal stain.

In Fig. 30 an overview of β -galactosidase expression in several regions of an adult SOM^{CRE/+;ROSA26/+} mouse brain is shown. This staining indicated that the CRE recombinase is able to mediate site-specific recombination of loxP sites and thus removes floxed genes of interest *in vivo*. The localisation of the stained cells in the hippocampus resembled the known expression pattern of somatostatin: most of the β -galactosidase-positive cells were located in the stratum oriens of the CA3 and CA1 regions and some in the hilus of the dentate gyrus (Fig. 30 B). The cortex showed β -galactosidase staining that was homogeneously distributed in all layers (Fig. 30 A). Only few β -galactosidase expressing cells were located in the striatum (Fig 30 C). The reticular thalamus demonstrated an intense labelling (Fig. 30 D). Strong staining was also visible in the central amygdaloid nuclei in contrast to the lateral and basolateral amygdala (Fig. 30 E). In the lateral hypothalamic area as well as the zona incerta of the thalamus some β -galactosidase positive cells were located (Fig. 30 F). The same applied to the medial and lateral septum (Fig. 30 G).

The cerebellar cortex showed labelling for β -galactosidase of some Purkinje cells as well as of some cells in the granular cell layer - presumably Golgi cells - and the molecular layer (Fig. 30 H, I). In addition a strong staining was also detected in the deep cerebellar nuclei (Fig. 30 I). The olfactory bulb demonstrated only very weak staining of a few cells in the glomerular cell layer (data not shown).

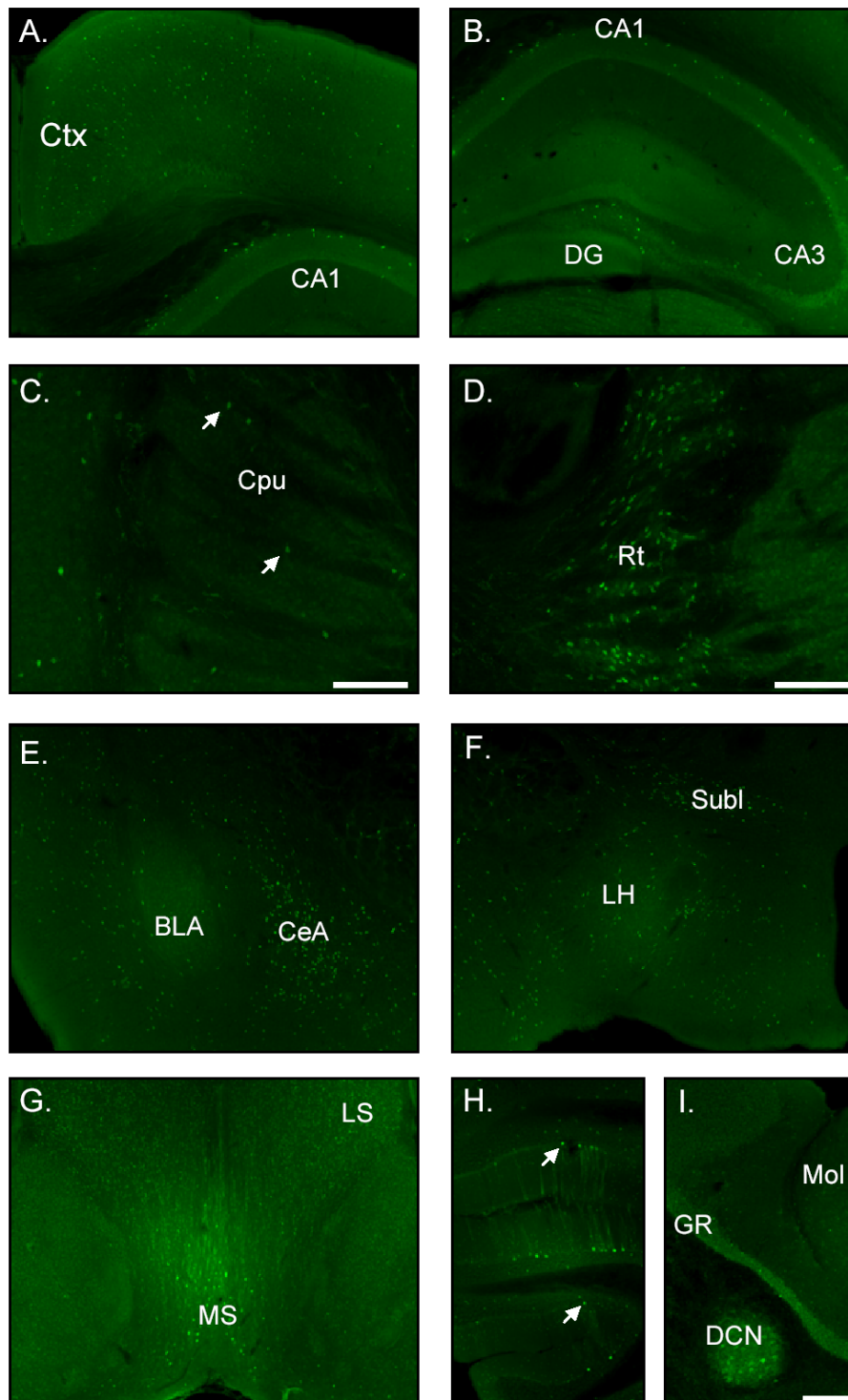


Fig. 30 Localisation of β -galactosidase expression in the $SOM^{CRE/+};ROSA^+$ mouse brain. Immunohistochemistry with an anti- β -galactosidase antibody demonstrated the expression of β -galactosidase in different regions of an adult $SOM^{CRE/+};ROSA^+$ mouse brain (coronal sections). Pictures show overviews of A: Cerebellar cortex. B: Hippocampus. C: Striatum. D: Reticular thalamus. E: Basolateral and central amygdala. F: Hypothalamic region. G: Septal area. H, I: Cerebellum. Arrows in highlight examples of β -galactosidase-expressing cells. BLA: Basolateral amygdala; CeA: Central amygdala; Ctx: Cortex; CPu: Striatum; DCN: Deep cerebellar nuclei; DG: Dentate gyrus; GR: Granule cell layer; Rt: Reticular thalamus; LH: Lateral hypothalamic area; LS: Lateral septum; Mol: Molecular cell layer; MS: Medial septum; Subl: Subincertal Nucleus. Scale bars: 150 μ m (C, D); 250 μ m (A, B, E-I).

3.2.6. Analysis of CRE-expressing cells in the SOM^{CRE/+;ROSA/+} mouse brain

For more detailed analysis three brains of adult (at least 8 weeks old) SOM^{CRE/+;ROSA/+} male mice were cut coronally (50 μ m) in serial sections and on every fourth section a double-labeling experiment was performed consisting of a X-Gal staining followed by a immunohistochemical staining for somatostatin-positive cells. The analysed areas were the hippocampus subdivided in the CA1, CA3 region and the dentate gyrus as well as three cortical regions: motor-, somatosensory- and entorhinal cortex, and the striatum (Fig. 31). For each region cells within an area of 46 mm² were counted and the mean value of all sections from one brain was calculated. The results of all analysed brains were summarised by defining the overall mean value (Tab. 3). In the hippocampus the percentage of cells co-expressing β -galactosidase and somatostatin showed great variance among the different areas of the hippocampus. with between 25% in the CA3 region and around 60% in the CA1 region. The cells that show no co-labelling are either only somatostatin-positive (29 - 67% depending on the hippocampal area) or only β -galactosidase-positive (2 - 17%).

Tab. 3 Analysis of β -galactosidase-positive cells in the SOM^{CRE/+;ROSA/+} mouse.

	β -gal & SOM positive cells (%)	SOM positive cells (%)	β -gal positive cells (%)	n = analysed cells
Hippocampus				
DG	30.4 \pm 5.6	66.9 \pm 4.3	2.9 \pm 1.1	766
CA3	25.1 \pm 3.3	58.1 \pm 2.7	16.8 \pm 4.5	533
CA1	60.2 \pm 4.4	29.6 \pm 4.4	10.0 \pm 4.6	642
Cortex				
Motor	34.4 \pm 7.6	64.0 \pm 6.5	1.6 \pm 0.7	492
Somatosensory	52.7 \pm 1.9	45.9 \pm 2.0	2.2 \pm 0.6	452
Striatum	33.1 \pm 3.6	66.7 \pm 4.1	0.4 \pm 0.5	231

Data are indicated as the mean \pm SEM values and were obtained from three adult SOM^{CRE/+;ROSA/+} mice. β -gal: β -galactosidase; SOM: Somatostatin.

For the evaluated cortical regions the percentages of cells double-labelled for both markers were as well not consistent either. In the motor cortex over 30% of all analysed cells were double-positive and 64% were only positive for somatostatin. In the somatosensory cortex approximately 50% were double-labelled. In the striatum

around 30% showed β -galactosidase and somatostatin staining and more than 60% expressed only somatostatin. The percentages of cells expressing β -galactosidase only is very low (0.4 - 2.2%).

These results allow the conclusion that in $SOM^{CRE/+}$ mice the removal of floxed genes is possible but it does not occur in all somatostatin-expressing cells. The incomplete removal has to be taken into consideration in mice aiming at functional analysis after deletions of specific genes.

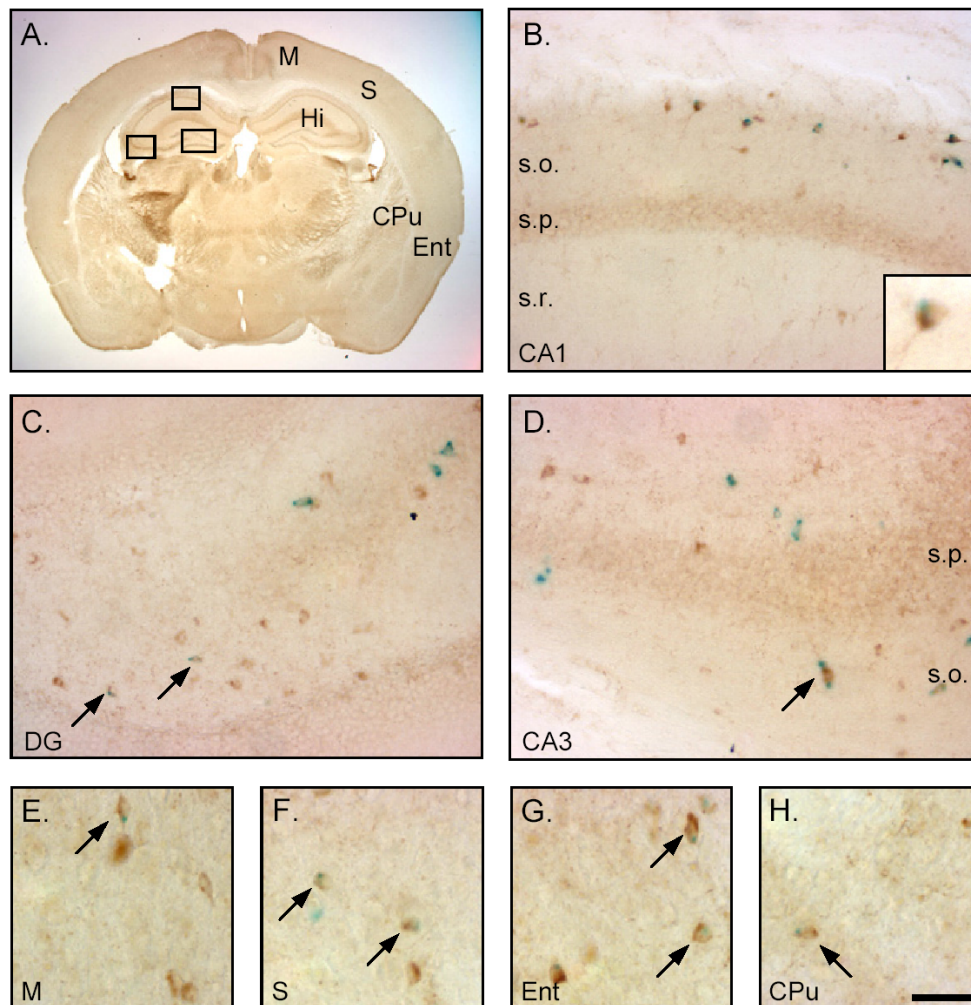


Fig. 31 Analysis of the β -galactosidase-positive cells in different brain areas in $SOM^{CRE/+; ROSA/+}$ mice. Double-labelling experiments including a X-Gal staining (blue) followed by immunolabelling for somatostatin (brown) were performed on coronal sections of adult $SOM^{CRE/+; ROSA/+}$ mice indicating that not all somatostatin-expressing cells also express β -galactosidase. A: Overview of a coronal section illustrates the analysed areas (boxed). B-H: Higher magnifications of the examined areas. B: CA1 region. The inset shows a cell expressing both β -galactosidase and somatostatin. C: Dentate gyrus. D: CA3 region. E: Motor cortex. F: Somatosensory cortex. G: Entorhinal cortex. H: Striatum. Arrows indicate examples for double-labelled cells. CPu: Striatum; DG: Dentate gyrus; Ent: Entorhinal cortex; Hi: Hippocampus; M: Motor cortex; S: Somatosensory cortex; s.o.: Stratum oriens; s.p.: Stratum pyramidale; s.r.: Stratum radiatum. Scale bar: 25 μ m.

3.2.7. CRE recombinase expression in embryonic SOM^{CRE/+;ROSA/+} mice

Since the expression of the endogenous somatostatin gene starts as early as embryonic day 7.5 (Gittes and Rutter, 1992) it was important to show that CRE recombinase in prenatal SOM^{CRE/+} mice is functional and the expression pattern corresponds with the endogenous one. X-Gal staining experiments of SOM^{CRE/+;ROSA/+} mice at embryonic day (E) 15 showed that CRE recombinase is expressed in cells of the neocortex, the hypothalamus, the midbrain, the cerebellum and the pons as well as the medulla oblongata (Fig. 32). In the neocortex labelled cells were mainly located in the cortical plate and a few in the intermediate zone (Fig. 32 C, D). The spinal cord showed labelling in both the dorsal and ventral horn with a more prominent labelling in the latter (Fig. 32 H). Furthermore, staining was detected in the dorsal root ganglia and the trigeminal ganglion (Fig. 32 E, F). Stained cells were additionally detected in non-neuronal tissues, such as the teeth, the midgut and in the kidney as well as the adrenal gland (Fig. 32 G, I, J). In summary, these results indicated that CRE recombinase is already promoting site-specific recombination during early prenatal development.

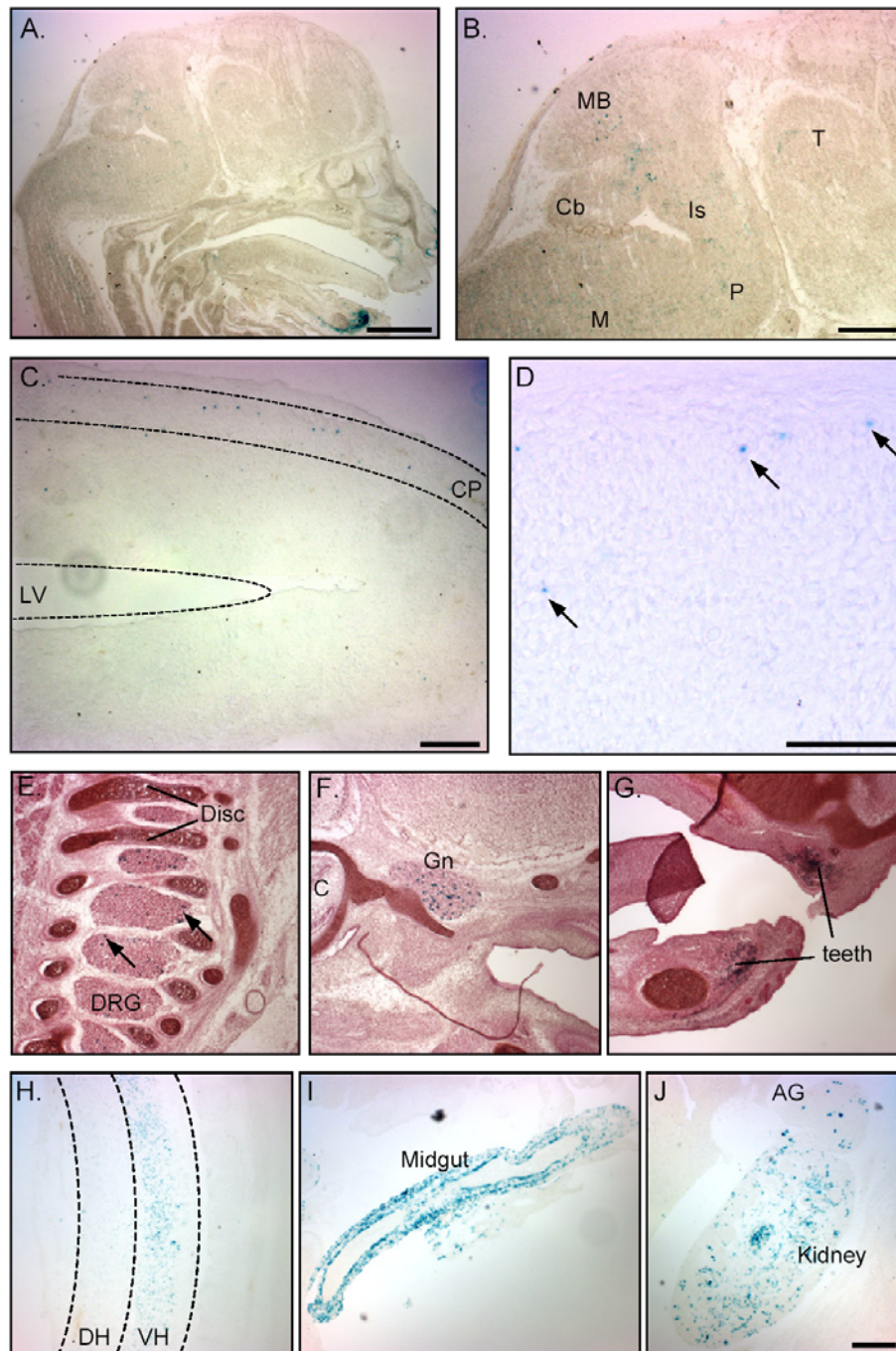


Fig. 32 Localisation of β -galactosidase expression in embryonic $SOM^{CRE/+;ROSA/+}$ mice. X-Gal staining experiments (blue colour) of coronal (C, D) and sagittal (A, B, E - J) sections of $SOM^{CRE/+;ROSA/+}$ mice at embryonic day (E) 15. A: Overview of the central nervous system. B: Higher magnifications of the dorsal brain. C: Overview of the neocortex with labelled cells located mainly in the cortical plate. D: Higher magnification of the cortical plate. Arrows indicate β -galactosidase-positive cells. E: Expression of β -galactosidase in the dorsal root ganglia. F: β -galactosidase labelled cells in the trigeminal ganglion. G: X-Gal staining of the teeth. H: Expression of β -galactosidase in the spinal cord, with a higher number of labelled cells in the ventral horn. I: β -galactosidase stained cells in the midgut. J: Expression of β -galactosidase in cells of the kidney and the adrenal gland. The red colour of the sections in E - G derived from counterstaining with Neutral Red. AG: Adrenal gland; Cb: Cerebellum; C: Cochlea; CP: Cortical plate; DH: Dorsal horn; DRG: Dorsal root ganglion; Gn: Trigeminal ganglion; Is: Isthmus; LV: Lateral ventricle; M: Medulla; MB: Midbrain; P: Pons; T: Thalamus; VH: Ventral horn. Scale bars: A: 1 mm; B: 500 μ m; C: 125 μ m; D: 25 μ m; E - J: 250 μ m.

3.2.8. Misexpression of CRE recombinase in pyramidal cells of the hippocampus

The analysis of the co-expression of β -galactosidase and somatostatin revealed the labelling of additional β -galactosidase-positive cells in the pyramidal cell layer of the hippocampus (Fig. 33 A). These cells were mainly located in the CA1 region. Double-labelling experiments with interneuronal markers such as parvalbumin and GAD67 were performed to gain more information about their identity. None of the markers was expressed in the β -galactosidase-positive cells (Fig. 33). Based on the location, the shape of the soma and the position of the neurites, it can be concluded that these cells are possibly pyramidal cells and that CRE recombinase is misexpressed in these cells.

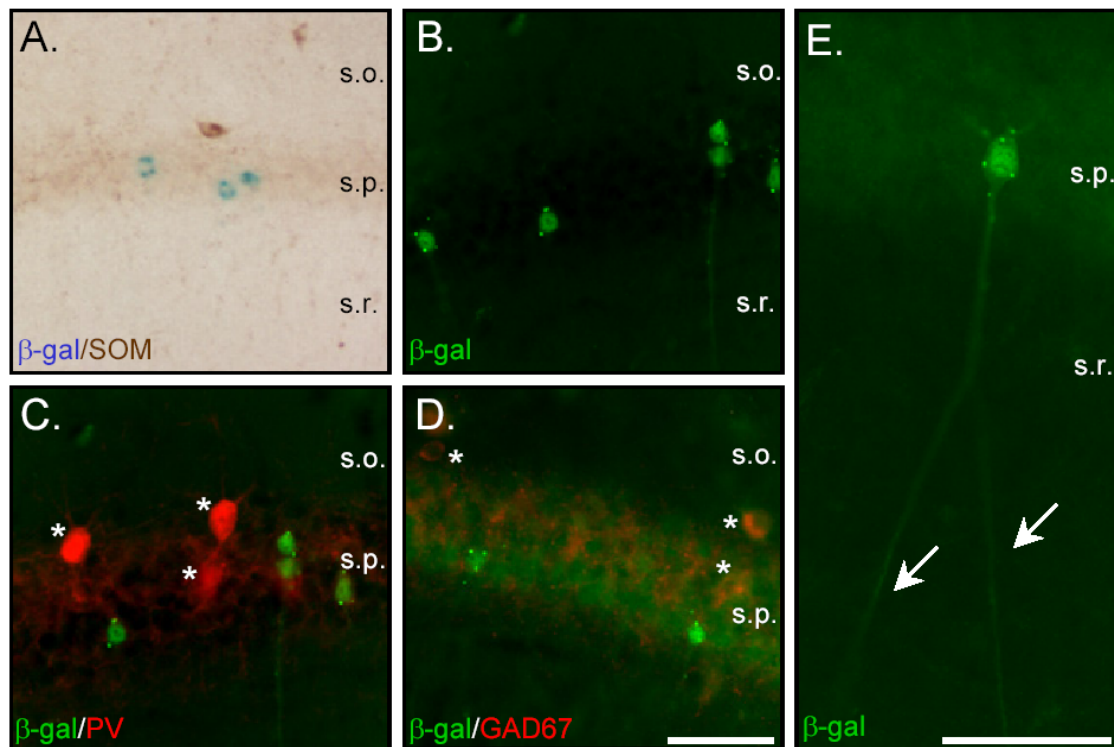


Fig. 33 Cells positive for β -galactosidase in the pyramidal cell layer of the hippocampus do not express interneuronal markers. A: A representative image of a double-labelling for β -galactosidase (blue) and somatostatin (brown) is shown and no co-localisation of both stainings was detected in the pyramidal cell layer. B: Immunohistochemical staining of the pyramidal cell layer with an anti- β -galactosidase antibody (green). C,D: Further double-labelling experiments with antibodies against β -galactosidase (green) and parvalbumin (C; red) or GAD67 (D; red) were performed and they showed no overlapping expression. Parvalbumin- and GAD67-positive cells are pointed out with asterisks. E: High magnification of a β -galactosidase positive cell in the pyramidal cell layer. The arrows mark the neurites of the cell. β -gal: β -galactosidase; SOM: Somatostatin; s.o.: Stratum oriens; s.p.: Stratum pyramidale; s.r.: Stratum radiatum; PV: Parvalbumin. Scale bars: 25 μ m.

3.2.9. The somatostatin promoter in $SOM^{CRE/+}$ mice is not active in gametes

The aim of the $SOM^{CRE/+}$ mouse line is to selectively ablate genes in somatostatin-positive cells hence it had to be investigated whether the somatostatin promoter is already active in the gametes. Expression of CRE recombinase in the gametes would affect additional cell populations and thus impedes the generation of cell-type specific knock-out mice. $SOM^{CRE/+;ROSA/+}$ mice were bred with C57Bl/6 mice and the offspring genotyped positively only for β -galactosidase were further analysed. X-Gal stainings were performed on sections of β -galactosidase-positive ($SOM^{+/+;ROSA/+}$) and control mice ($SOM^{CRE/+;ROSA/+}$). No staining for β -galactosidase was detected on sections of the $SOM^{+/+;ROSA/+}$ mice (Fig. 34). Hence, it is possible to generate cell-type specific knock-outs with $SOM^{CRE/+}$ mice.

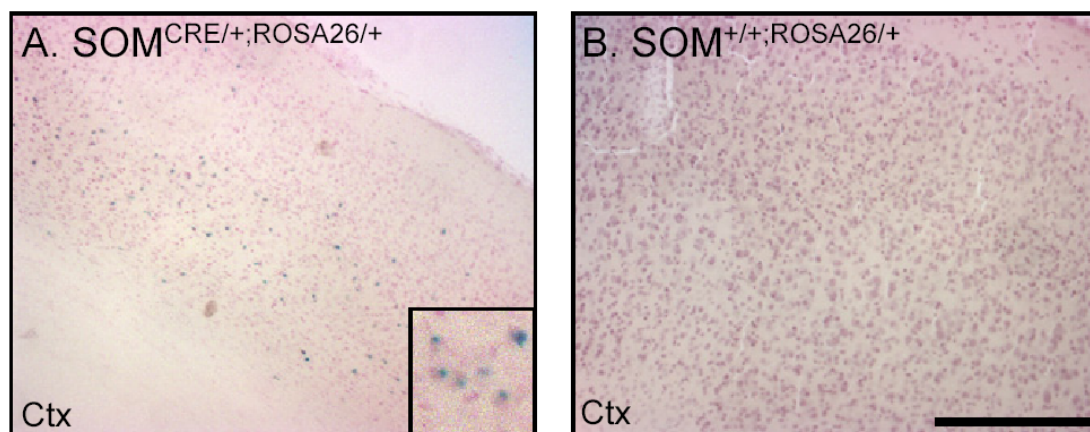


Fig. 34 *The somatostatin promoter is not active in the gametes.* X-Gal stainings were performed on sections of a $SOM^{CRE/+;ROSA/+}$ and a $SOM^{+/+;ROSA/+}$ mouse. A: β -galactosidase-positive cells in the cortex (inset shows higher magnification) of the $SOM^{CRE/+;ROSA/+}$ mouse which serves as a control. B: There was no β -galactosidase-positive cell detected in section from the $SOM^{+/+;ROSA/+}$ mouse. Ctx: Cortex. Scale bar: 250 μ m.

3.3. Ablation of glutamate receptor subunits in somatostatin-expressing interneurons – preliminary observations

$SOM^{CRE/+}$ mice were generated to provide a tool to selectively modify gene expression in somatostatin-positive interneurons. Somatostatin-expressing interneurons in the hippocampus, the so called O-LM cells, are involved in theta oscillatory activity and exhibit feedback inhibition on the dendritic compartment of principal cells. Since not much is known about the functional properties of different glutamate receptors mediating the excitatory input from principal cells onto O-LM

cells, the ablation of specific subunits and the functional consequences are an interesting starting point. Breeding of $SOM^{CRE/+}$ mice with both $NR1^{2lox}$ mice (Shimshek et al., 2006) and $GluR-A^{2lox}$ mice (Zamanillo et al., 1999) were performed to obtain knock-out mice lacking these subunits. Electrophysiological analysis was performed and the preliminary results are described in the following two paragraphs.

3.3.1. Ablation of the NR1 subunit of NMDAR in somatostatin-positive interneurons

$SOM^{CRE/+;NR1^{-/-}}$ mice were generated by breeding $SOM^{CRE/+}$ mice with $NR1^{2lox}$ mice that possess loxP sites flanking exons 11-18 of the NR1 gene (Shimshek et al., 2006). The offspring (F1) were genotyped for both loci and mice positive for CRE recombinase and heterozygous for the floxed NR1 gene were further bred with $NR1^{2lox}$ mice to obtain knock-out mice (F2).

The first pilot electrophysiological experiments were performed on acute hippocampal slices of $SOM^{CRE/+;NR1^{-/-}}$ and control mice ($SOM^{+/+;NR1^{-/-}}$, $SOM^{CRE/+}$ or wild-type) at postnatal day (P) 14. AMPAR and NMDAR currents of O-LM cells of the CA1 region of the hippocampus were measured and their ratio was calculated. Successful ablation of the NR1 subunit would result in smaller or no NMDAR-mediated currents in somatostatin-positive cells. 11 O-LM cells from two knock-out mice were analysed so far and only one cell was found to lack NMDAR-mediated currents.

3.3.2. Ablation of the GluR-A subunit of AMPAR in somatostatin-positive interneurons

The ablation of the GluR-A subunit in somatostatin-expressing cells was attempted by breeding $SOM^{CRE/+}$ mice with $GluR-A^{2lox}$ mice that possess loxP sites flanking exon 11 of the GluR-A subunit (Zamanillo et al., 1999). The offspring of the F1 generation were genotyped for the modified somatostatin and for the GluR-A subunit. Mice that possessed both modified alleles ($SOM^{CRE/+;GluRA^{+/-}}$) were subsequently bred with $GluR-A^{2lox}$ mice and the resulting knock-outs (F2; $SOM^{CRE/+;GluRA^{-/-}}$) were further analysed by electrophysiological recordings.

Measurements on acute hippocampal slices of $SOM^{CRE/+;GluRA^{-/-}}$ mice and control animals ($SOM^{+/+;GluRA^{-/-}}$, $SOM^{GluRA^{-/-}}$ or wild-type) at postnatal day (P) 14 were already performed. AMPAR- and NMDAR-mediated currents of O-LM cells in the CA1 region

of the hippocampus were measured and a reduction or ablation due to the ablation of the GluR-A subunit in $SOM^{CRE/+;GA^{-/-}}$ mice was expected. Analysis of O-LM cells of one knock-out mouse (n = 4 cells) and one control mouse (n = 5 cells) were performed and no change in AMPAR-mediated currents were observed. Evoked AMPAR currents of O-LM cells were measured from one $SOM^{CRE/+;GA^{-/-}}$ mouse at P14 (n = 14 cells) and one $SOM^{CRE/+;GA^{-/-}}$ mouse at P28 (n = 18 cells) but no reduction was detected.

4. Discussion

GABAergic inhibitory interneurons comprise up to 20% of all neurons in the brain and are the counterpart to the larger group of excitatory principal cells (Freund and Buzsaki G, 1996). The processing of all afferent and efferent information in the mammalian brain is based upon reciprocal action of these two major neuronal populations.

Based on morphological and physiological features, interneurons can be divided into several subgroups in contrast to principal cells that are a more homogenous group. But regardless of the criteria that were applied to classify these subgroups a clear delimitation cannot be made. For instance, interneurons with common anatomical features do not necessarily share the same neurochemical or physiological characteristics. However, this great variety enables interneurons to exert a huge influence on the activity of principal cell populations in the mature brain. One major characteristic is the involvement of defined interneuronal subgroups in the generation and maintenance synchronous oscillatory network activity. Oscillations occur at different frequencies and organise the activity of different groups of principal cells in a spatio-temporal manner (Klausberger et al., 2003). Furthermore, different oscillations can be associated with distinct behavioural states. GABAergic interneurons also are a major source of inhibition in the developing brain influencing numerous maturational processes. During embryonic and early postnatal development of the brain the excitatory action of GABA influences cell proliferation and acts as a trophic factor concerning cell migration and neurite growth (Spoerri PE, 1988).

Genetic modifications of defined molecular components, such as receptors, of GABAergic interneurons help to gain more information about the function of interneurons in specific processes such as network activity. An elegant way to perform such modifications is to specifically ablate the desired components in defined interneuronal populations by utilising the CRE/loxP system (Kwan, 2002; Nagy, 2000). This system is based on the expression of the site-specific CRE recombinase under a cell-type specific promoter. The recombinase catalyses homologous recombination at two specific recognition sequences - loxP sites - and thereby deletes the fragment in between. In this study two mouse lines with differential cell-type specific CRE recombinase expression were generated and analysed. In one line the CRE recombinase expression is controlled by the GAD67 promoter

(GAD67^{CRE/+}). This promoter was chosen, because GAD67 is a common feature of GABAergic interneurons and thus the knock-out mice generated by breeding with GAD67^{CRE/+} mice would affect the whole interneuronal population of the brain. In the second mouse line, CRE recombinase is under the control of the somatostatin promoter (SOM^{CRE/+}) that is present in a specific interneuronal subpopulation.

Both lines were analysed by breeding the mice with the ROSA26 reporter mice (Soriano, 1999) to investigate the functionality of CRE recombinase as well as to quantitatively analyse its expression pattern in GAD67- and somatostatin-expressing cells by immunohistochemical methods.

Furthermore, cell-type specific knock-out mice were generated: SOM^{CRE/+} mice were bred with GluR-A^{2lox} mice (Zamanillo et al., 1999) that contain a floxed exon11 of the GluR-A subunit of AMPAR as well as with NR1^{2lox} mice (Shimshek et al., 2006) that possess loxP site flanked exons 11-18 of the NR1 subunit of the NMDAR. Finally, some preliminary electrophysiological experiments were performed.

4.1. Generation and analysis of the GAD67^{CRE/+} mouse line

4.1.1. Generation of GAD67^{CRE/+} mice by a knock-in approach

The GAD67^{CRE/+} mouse line was generated by a knock-in approach performed by homologous recombination in mouse embryonic stem cells. The targeting construct (cloned by Dr. Irinel Coserea, Abbott, Ludwigshafen, Germany) was designed to place the CRE recombinase 30 bp upstream of the translational start codon of the GAD67 gene. This position was chosen since another previous knock-in approach indicated that the insertion into that locus did not affect the promoter activity (Fuchs et al., 2001). Based on the expression of a NEO cassette (flanked by frt-sites) that was introduced into the targeting construct downstream of the CRE recombinase cassette, enabled the selection in ES cells. After blastocyst injection of one correctly targeted ES cell clone and germline transmission of the modified GAD67 gene, the NEO cassette was removed by breeding GAD67^{CRE/+;NEO/+} mice with flp-deleter mice (Rodriguez et al., 2000) that express the site-specific recombinase flippase. This step is necessary since the presence of the NEO cassette can influence the expression of the modified gene and thus can lead to a different or false-positive expression pattern of CRE recombinase (Fiering et al., 1995). After the removal of the NEO cassette, GAD67^{CRE/+} mice were used for the analysis as well as for backcrossing with C57Bl/6

wild-type mice to obtain a common genetic background. Since the embryonic stem cells were derived from a different mouse strain (E14.1) up to 10 consecutive breedings with C57Bl/6 mice are required to avoid possible influences concerning different genetic properties of distinct mouse strains (Joyner A.L., 1993) and furthermore to obtain basic standard conditions for future experiments.

4.1.2. Functional analysis of adult GAD67^{CRE/+} mice

A frequently used approach to validate the functionality of CRE recombinase is to breed the CRE recombinase expressing mice with the ROSA26 reporter mice. The ROSA26 line was generated by Soriano *et al.* (Soriano, 1999) and expresses β -galactosidase upon removal of a loxP-site flanked NEO cassette that can be visualised by a X-Gal staining or immunohistochemical experiments. Thus GAD67^{CRE/+} mice were bred with ROSA26 mice and the immunohistochemical analysis revealed a β -galactosidase expression pattern resembling the known expression pattern of GAD67. These results proved that CRE recombinase was functional *in vivo* and was able to ablate floxed genes of interest.

To demonstrate that β -galactosidase was indeed expressed in GAD67 containing cells, double-labelling experiments were performed. X-Gal staining was combined with immunohistochemical staining (DAB method) employing an antibody against GAD67. Quantitative analysis of these experiments showed that in the evaluated brain areas – motor cortex, somatosensory cortex, striatum and hippocampus – all cells expressing the endogenous GAD67 were also labelled for β -galactosidase.

Around 50% of all analysed cells in adult GAD67^{CRE/+;ROSA26/+} mice, independent of the brain area, were only labelled for β -galactosidase. A possible explanation might be the insufficient sensitivity of the GAD67 antibody. Furthermore, the precipitate that was formed during the X-Gal staining procedure to detect β -galactosidase might have prevented by steric hindrance the binding of the anti-GAD67 antibody.

Since the detectability of GAD67-positive cells with the used antibody was not satisfactory double-labelling experiments were performed with an anti- β -galactosidase antibody and antibodies against markers for interneuronal subpopulations: parvalbumin, calbindin and somatostatin. The results demonstrated CRE recombinase expression in almost all neurons of the analysed interneuronal subtypes and the number of cells that were only β -galactosidase-positive was

comparably low. These results also indicated that the GAD67 antibody used before might not label all GAD67-expressing cells.

However, most likely the presence of β -galactosidase positive cells cannot be explained only by the lack of sensitivity of the GAD67 antibody. Early transient expression in a subset of cells that later do not have a GABAergic phenotype may be in part responsible for the presence of β -galactosidase-positive cells that are GAD67-negative.

In $GAD67^{CRE/+;ROSA26/+}$ mice β -galactosidase is expressed and can be visualised after the removal of the stop-cassette by CRE recombinase-mediated homologous recombination. An example for transient expression of GAD67 in precursor cells or early in development is the presence of β -galactosidase in granule cells of the dentate gyrus of the hippocampus (see Fig. 18). These cells are in fact glutamatergic but additionally express GABA and thus GAD67 early during development for fine-tuning of synaptic circuits (Ramirez and Gutierrez, 2001; Sandler R and Smith AD, 1991; Sloviter RS et al., 1996). Another reason accounting for additional β -galactosidase staining in granule cells is that pathological stages in the mature brain, such as seizure activity, can also induce a switch of a glutamatergic to a GABAergic phenotype to compensate the hyperexcitability of principal cells (Gutierrez, 2005).

4.1.3. CRE recombinase is already functional in embryonic $GAD^{CRE/+}$ mice

The X-Gal staining experiments of embryonic $GAD67^{CRE/+;ROSA/+}$ mice (E16) were performed to proof that CRE recombinase recapitulates the temporal and cellular expression of GAD67. This is essential to know if $GAD67^{CRE/+}$ mice are thought to be employed in future experiments involving selective gene ablation in GABAergic interneurons. Depending on the expression profile of the gene of interest, e.g. a developmentally important gene, it is necessary to know when CRE recombinase mediated ablation is performed. Immunohistochemical experiments confirmed the functionality of CRE recombinase in $GAD67^{CRE/+}$ embryos. The CRE recombinase expression in neuronal tissues, such as the brain (Katarova Z et al., 2000) and the spinal cord (Feldblum S. et al., 1995) was in accordance with the GAD67 gene expression pattern. Cells in the neocortex and cells invading the hippocampal formation that were stained for β -galactosidase were mainly distributed in two layers- one in the marginal zone and the cortical plate and one in the intermediate zone and the subventricular zone. This pattern was previously shown by fluorescent dye

labelling (De Carlos et al., 1996; Lavdas et al., 1999) and by the migration pattern analysis in transgenic mice lacking the homeobox genes Distal-less 1 and 2 (DLX-1/2). In these mice the migration of GABAergic interneurons from the subpallium telencephalon to the neocortex was severely affected (Anderson et al., 2001). In GAD67-GFP knock-in mice visualisation of GFP-positive cells aided in the establishment of these migrational routes and in addition revealed the modes of migration of neocortical interneurons (Tanaka et al., 2003; Tamamaki N et al., 2003; Manent et al., 2006).

Furthermore, the CRE expression pattern in non-neuronal tissue, like kidney (Liu Z.H. et al., 1996) and ectodermal structures, such as vibrissae, limb buds or tailbud concurred with the one of the GAD67 gene (Maddox and Condie, 2001). The expression of GAD67 in ectodermal non-neuronal tissues points towards a participation of GAD67 and GABA in their development.

4.1.4. Reduced GAD67 protein levels in GAD67^{CRE/+} mice do not have obvious developmental or behavioural consequences

Manipulations of the GAD67/GABA system have severe consequences that can be seen, for instance, in the loss-of-function mutations of the GAD67 gene. GAD67^{-/-} mice die during the first day after birth (P0.5) due to a cleft palate in combination with malformations of the respiratory control system in these mice (Asada et al., 1997; Condie et al., 1997). The GAD activity is reduced to less than 20% in homozygous GAD67 knock-out mice compared to wild-type mice and additionally the GABA content is reduced to 7 % but no further apparent defects in brain morphogenesis were observed (Asada et al., 1997). Experiments in organotypic cultures of GAD67 knock-out mice confirmed the preceding results by showing a normal development of GABAergic networks and an increase of GABA content due to compensatory mechanisms presumably mediated not only by GAD65 but also by other factors (Ji et al., 1999; Ji and Obata, 1999). One candidate for such a compensating factor is putrescine, a possible precursor for GABA synthesis (De Mello FG et al., 1976; Jakoby and Fredericks, 1959; Seiler N. and Al-Therib M.J., 1974). It was shown that putrescine is present in cells of the developing retina before the appearance of GAD expression and is additionally responsible for the GABA synthesis (Hokoç JN et al., 1990). Furthermore, in recent work of Sequerra *et al.* it was demonstrated that in the SVZ of the rat, in addition to very low levels of GAD

activity, putrescine is a source of GABA synthesis and that it is also produced by neuroblasts (Sequerra et al., 2007). It is assumed that the putrescine pathway is substituted by the GAD pathway as the organism matures (De Mello FG et al., 1976). In addition, it was shown that defects of the cerebral cortex as well as of the hippocampus in neurological disorders, such as schizophrenia, bipolar disorder or autism, are accompanied by an altered inhibitory GABAergic signalling (Akbarian and Huang, 2006). These observations were made on post-mortem brains of subjects with neurological disorders. These studies focused on the prefrontal cortex (Akbarian S et al., 1995) but also other cortical regions and the hippocampus were analysed (Heckers S. et al., 2002). The observed decreased GABA contents in the mentioned disorders are due to reduced GAD67 expression levels but not to a loss of specific interneuronal subpopulations.

Despite of the apparent and expected normal development of GAD67^{CRE/+} mice (e.g. normal body weight and breeding behaviour) possible subtle deficits resulting from the knock-in of CRE recombinase in one GAD67 allele had to be investigated. Therefore, preliminary behavioural tests with a pilot group of knock-in and wild-type mice were performed. The results indicated that the tested GAD67^{CRE/+} mice did not show impairments in their explorative behaviour and furthermore their motor learning ability seemed to be normal. To draw final conclusions concerning the behaviour of GAD^{CRE/+} mice the number of tested individuals must be increased to gain statistical validity. Subsequently to the behavioural analysis, the GAD67 protein levels in these mice were checked by Western blot analysis. As expected from the previously mentioned results of the GAD67 knock-out mice (Asada et al., 1997; Condie et al., 1997), the Western blot analysis revealed a decrease of around 50% concerning the GAD67 protein level in GAD^{CRE/+} mice compared to their wild-type littermates.

4.1.5. Future plans: Ablation of the trkB receptor in all GABAergic interneurons

A factor that is involved in activity-dependent maturation of interneurons is the brain-derived neurotrophic factor (BDNF) (Marty et al., 1997; Thoenen, 1995). BDNF belongs to the family of neurotrophins, together with neurotrophin-3 and neurotrophin-4/5, and is expressed exclusively by principal cells (Altar C.A. et al., 1994). Its receptor is the trkB receptor tyrosine kinase (Klein R. et al., 1989) that is expressed only by interneurons (Zachrisson et al., 1996) and mediates its effects via

the phospholipase C γ - pathway (Widmer H.R. et al., 1993;Huang and Reichardt, 2003).

The ablation of trkB signalling in trkB-null mice (Klein R. et al., 1993;Widmer H.R. et al., 1993) demonstrated reduced migration of interneurons in the developing neocortex and hippocampus and confirmed the role of BDNF in interneuronal migration (Polleux et al., 2002). It was also shown that BDNF is able to promote dendritic growth of neocortical interneurons in organotypic slice cultures (Jin et al., 2003). Furthermore, BDNF regulates synaptogenesis that was demonstrated *in vitro* in cell culture (Rutherford et al., 1997;Vicario-Abejon et al., 1998) as well as *in vivo* in mice overexpressing BDNF postnatally in the visual cortex (Huang et al., 1999).

Developmental functions of the BDNF/trkB signalling and its influence on network activity will be further analysed in mice with ablated trkB receptors in all GABAergic interneurons. To this end GAD67^{CRE/+} mice are currently being bred with trkB^{lox/lox} mice (Minichiello et al., 1999).

In addition, GAD67^{CRE/+;trkB^{-/-}} knock-out mice could also be utilised to focus on the involvement of GABAergic interneurons in specific cognitive disorders, such as schizophrenia. Several studies indicate that interneurons play a central role in the pathogenesis of these diseases and might explain a number of the disease characteristics (Akbarian S et al., 1995). Reduction of expression level of the trkB receptor, for instance, is reflected in reduced GAD67 mRNA expression in GABAergic interneurons that can also be detected in subjects with schizophrenia (Akbarian and Huang, 2006). Furthermore, it was shown that in the trkB knock-out mice parvalbumin-expressing interneurons are specifically affected and exhibit a decrease in parvalbumin and GAD67 expression that is directly correlated to the decrease of trkB gene expression (Hashimoto et al., 2005). A reduction in the activity of parvalbumin-expressing interneurons has been correlated with a reduction in gamma oscillations as well as with impaired working memory as demonstrated in a recent study (Fuchs et al., 2007). These findings show similarities with certain clinical features exhibited by patients with schizophrenia. Patients have an impaired onset of gamma activity in response to a stimulus and presumably, as a consequence of that, show deficits in working memory (Spencer et al., 2003).

Since the above-mentioned studies were performed in mice with incomplete ablation of the trkB receptor, the ablation of the trkB receptor in all GAD67-expressing

interneurons with GAD67^{CRE/+} mice could open up new possibilities to investigate the underlying mechanisms of cognitive disorders.

4.2. Generation and analysis of the SOM^{CRE/+} mouse line

4.2.1. Generation of SOM^{CRE/+} mice via a knock-in approach

The generation of a mice expressing CRE recombinase under the control of an interneuronal subtype-specific promoter was performed by a knock-in approach. SOM^{CRE/+} mice were generated by gene targeting in R1 mouse embryonic stem cells (Nagy et al., 1993). The targeting cassette consisting of CRE recombinase followed by a NEO cassette, flanked by frt-sites, was inserted right after the translational start codon of the somatostatin gene via homologous recombination in mouse ES cells. In contrast to the generation of the GAD67^{CRE/+} mice, where the CRE recombinase was inserted upstream of the ATG (since previous experiments were successful using this approach) (Fuchs et al., 2001), CRE recombinase in the SOM^{CRE/+} mice was inserted after the ATG. This should prevent a negative effect on the promoter activity by the insertion of CRE recombinase.

Following blastocyst injection of a positive ES clone and successful germline transmission of the targeted somatostatin gene, the NEO cassette was removed by breeding the mice with flp-deleter mice (Rodriguez et al., 2000). The NEO cassette that was used for negative selection in ES cell culture can severely influence the expression of the modified gene and lead to an incomplete or to a false-positive expression pattern (Fiering et al., 1995). SOM^{CRE/+} mice without NEO cassette were further backcrossed to the genetic background of the C57Bl/6 strain. This is required since the used ES cells were derived from another mouse strain (R1, (Nagy et al., 1993)) and a common genetic background is needed as a basis for further experiments. SOM^{CRE/+} mice inherited the targeted gene according to the Mendelian ratio, furthermore they did not show any obvious anatomical, behavioural or physiological defects. Therefore one can assume that the missing somatostatin expression of the targeted allele in the heterozygous knock-in mice is compensated by specific mechanisms. This was expected since already the complete knock-out of the somatostatin gene demonstrated no severe deficits in their development (Buckmaster PS et al., 2002; Low et al., 2001). One of the compensating mechanisms for the lack of somatostatin expression in the knock-out mice was shown to be an

upregulation of the somatostatin receptors (Ramirez et al., 2002; Videau et al., 2003). This especially applies for the somatostatin receptor 2 (sst2) that exhibited a significantly increased expression level in somatostatin knock-out mice (Cammalleri et al., 2006). Furthermore, it was concluded that somatostatin is actively participating in the regulation of its receptors, especially sst2 (Csaba et al., 2004). Another compensating mechanism could be mediated by the related neuropeptide cortistatin that shares 11 of 14 aminoacid residues with somatostatin-14 and, additionally, binds *in vitro* with a similar affinity like somatostatin to the five somatostatin receptors (de Lecea et al., 1996). Cammalleri *et al.* could show that the cortistatin expression level was 60% higher in somatostatin knock-out compared to wild-type mice (Cammalleri et al., 2006).

Possible changes in the protein levels of somatostatin in SOM^{CRE/+} mice compared to wild-type littermates could not be determined since antibodies against somatostatin for its detection in Western blot analysis are not commercially available.

4.2.2. Analysis of the functionality of CRE recombinase in adult SOM^{CRE/+} mouse brains

Crossing of SOM^{CRE/+} mice with ROSA26 reporter mice (Soriano, 1999) was used on the one hand to proof the functionality of CRE recombinase *in vivo*, and on the other hand to analyse its expression pattern and the correspondence with that of the endogenous somatostatin gene. ROSA26 mice contain a floxed NEO cassette in the ROSA26 locus whose presence suppresses the transcription of a β -galactosidase gene. Upon CRE recombinase mediated ablation of the floxed NEO cassette β -galactosidase is expressed and can be visualised with distinct immunohistochemical staining methods.

The immunohistochemical staining experiments for β -galactosidase proofed the *in vivo* functionality of CRE recombinase and the expression pattern indicated that it is expressed in somatostatin-positive interneurons. But the quantitative analysis that was performed by double-labelling experiments for β -galactosidase and the endogenous somatostatin revealed that CRE recombinase is not expressed in all somatostatin-positive cells. The percentage of double-positive cells lies between 25 and 60% depending on the analysed brain region. The highest amount of double-labelled cells can be found in the CA1 area of the hippocampus (60.2%) followed by the striatum (52.7%). Since this mouse line was generated by a knock-in approach

that should guarantee a targeted allele in every somatostatin-expressing cell, the reason for this incomplete CRE recombinase expression is not clear. One explanation could be related to the complex promoter region of the somatostatin gene. It consists of a proximal cAMP response element (CRE) (Montminy et al., 1986), a tripartite regulatory domain consisting of an upstream enhancer (SMS-UE) and two neighbouring tissue-specific enhancers (TSEI and II), as well as silencer elements (PS1, PS2) (Vallejo et al., 1992a; Vallejo et al., 1992b; Vallejo et al., 1995). Dynamic and complex interactions between these promoter elements and its factors, such as transcriptional activator and repressor proteins, allows cell-specific expression of somatostatin that is expressed in neuronal as well as non-neuronal tissues of different developmental origin. CRE, for instance, positively regulates the basal expression level of somatostatin in neurons (Schwartz and Vallejo, 1998). Whereas the SMS-UE region represents a D-cell-specific enhancer and is thus responsible for somatostatin expression in pancreatic cells and is furthermore negatively regulated during development by at least one of the two proximal silencer (PS) elements (Vallejo et al., 1992a; Vallejo et al., 1992b). The multi-layered organisation of the somatostatin gene expression makes it also more sensitive towards genetic manipulations. The introduction of CRE recombinase by homologous recombination might have caused minor structural changes in the sequence of the promoter region and thereby affected the responsible elements for somatostatin expression in neurons so that in some neurons CRE recombinase cannot be transcribed. Alternatively, the expression level of CRE recombinase in these cells is so low due to modifications of enhancer or repressor elements that either site-specific recombination cannot be performed or be detected by immunohistochemical methods.

The missing CRE recombinase expression has also an advantage. Future planned experiments with SOM^{CRE/+} mice are mainly focused on the hippocampus and especially the CA1 region. This region shows the highest amount of co-expression and therefore has the highest probability of specifically modified cells. Furthermore, the cell-type specific ablation of genes is always present in the whole brain. The resulting influences derived from other brain regions than the analysed one have normally to be taken into account while evaluating an experiment. But these influences will be diminished in the SOM^{CRE/+} mice due to the CRE expression pattern.

The number of cells that are only positive for β -galactosidase is highest in the CA3 and CA1 region of the hippocampus (10-16%) and rather low in the other areas (up to 2.9%; see also Tab. 3). These percentages coincide with the development of somatostatin-containing neurons in the hippocampus as well as the cortical regions. This developmental process includes transient expression of the somatostatin gene. Therefore a number of only β -galactosidase-positive cells are detected in adult $SOM^{CRE/+;ROSA/+}$ mouse brains since CRE recombinase is expressed as soon as its controlling promoter is active and then CRE recombinase is able to mediate site-specific recombination throughout life. It was shown by many studies in rat (Burgunder J.-M., 1994; Naus C.C.G. et al., 1988a; Naus C.C.G. et al., 1988b; Shiosaka S et al., 1982) and mouse (Bendotti C. et al., 1990; Forloni G. et al., 1990) that somatostatin is highly expressed during the first two to three postnatal weeks and afterwards decreases to the lower adult expression level. During the development of the hippocampus high somatostatin expression can be detected in the whole stratum oriens and the hilus, that is in accordance with the number of cells only expressing β -galactosidase and not somatostatin in the CA1 and CA3 region of the hippocampus of $SOM^{CRE/+;ROSA/+}$ mice. This is also valid for the β -galactosidase-positive cells in other regions since a decrease of somatostatin expression during development was also shown for other brain regions, such as the cerebral cortex. The only difference is that the decline of expression in these areas is not as striking as in the hippocampus. Another example for a brain area where transient somatostatin expression occurs and was detected during analysis of the $SOM^{CRE/+;ROSA/+}$ mice is the lateral septum (Bendotti C. et al., 1990; Shiosaka S et al., 1982).

The temporary distinct expression profile of the somatostatin gene might also explain the few β -galactosidase expressing cells in the pyramidal cell layer of the hippocampus that are not of GABAergic origin. Possibly, the somatostatin promoter is active in a small subset of principal cells during development. The fact that CRE recombinase is active in few pyramidal cells has to be kept in mind since CRE recombinase is able to promote site-specific recombination of floxed genes of interest also in these cells. This misexpression might have an influence on results in experiments performed with cell-type specific knock-out mice generated with the $SOM^{CRE/+}$ line.

4.2.3. CRE recombinase is already functional in embryonic SOM^{CRE/+} mice

The analysis of CRE recombinase expression in embryonic SOM^{CRE/+} mice was performed by breeding SOM^{CRE/+} mice with mice of the ROSA26 reporter line (Soriano, 1999). X-Gal staining experiments of SOM^{CRE/+;ROSA/+} mouse embryos at embryonic day (E) 15 showed that CRE recombinase is already expressed in neuronal as well as non-neuronal tissues. This corresponds with previous studies on the developmental expression of somatostatin by *in situ* hybridisation and immunohistochemical experiments (Bendotti C. et al., 1990; Burgunder J.-M., 1994; Shiosaka S et al., 1982; Soriano, 1999). The expression of β -galactosidase in the central nervous system of E15 SOM^{CRE/+;ROSA26/+} mice is rather low, since an increase of somatostatin expression and thus the promoter activity, primarily appears after birth during the first three weeks of postnatal life (Bendotti C. et al., 1990; Forloni G. et al., 1990). The detected 'expression gradient', with a higher β -galactosidase expression level in the thalamic region and a lower one in the neocortex was described by J.-M. Burgunder in the rat (at a comparable age) (Burgunder J.-M., 1994). The β -galactosidase-labelling of somatostatin-positive cells in the spinal cord with a more dominant staining of the ventral horn corresponds with previous studies in the prenatal rat (Senba E et al., 1982). It indicates that somatostatin is involved in the development of the spinal cord since the expression in the ventral horn decreases remarkably after birth and the moderate levels in the dorsal horn remain also in the adult. CRE recombinase activity in the developing cerebellum, as well as in the dorsal root ganglia and the trigeminal ganglion is consistent with somatostatin expression in these tissues found by other research groups (Naus, 1990; Ichikawa et al., 2003; Baxter and Smith, 1998).

The expression of CRE recombinase in non-neuronal tissue can also be confirmed by previous observations, such as expression in the guts (Gittes and Rutter, 1992), the kidney (Bates et al., 2004) and the adrenal gland (Maurer and Reubi, 1986). Taken together these results demonstrate that cell-type specific ablation of genes of interest by CRE recombinase mediated site-specific recombination can already be performed in the developing nervous system of the SOM^{CRE/+} mouse. Hence also developmental questions pertaining to the functional role of somatostatin-expressing interneurons can be approached.

4.3. Ablation of AMPAR and NMDAR subunits in somatostatin-positive interneurons: Preliminary results

Interneurons exhibit a great influence on network activity by shaping the output of principal cells via inhibition. Interneurons are recruited by the activation of fast ionotropic glutamate receptors, of the AMPA- and NMDA-type.

The use of genetically modified mice with glutamate receptor subunit ablations was helpful in gaining more insight into their function. The global knock-out of the NR1 subunit, for instance, first revealed the importance of the NMDAR in prenatal development since the mutant mice died within the first 8-15 hours after birth (Li et al., 1994). The life span was dependent on the level of the transgene expression and thus it could be shown that NMDAR play a crucial role in formation of sensory-related neural patterns (Iwasato et al., 1997; Li et al., 1994). Cell-type specific ablation of the NR1 subunit in the CA1 region, furthermore, showed impaired hippocampal space representation due to the lack of NMDAR-mediated synaptic plasticity (McHugh et al., 1996; Tsien et al., 1996).

Complete ablation of the GluR-A subunit leads to a reduction of the amount of AMPAR in the CA1 region of the hippocampus associated with a loss of long-term potentiation at CA3 to CA1 synapses (Zamanillo et al., 1999). In another study, the GluR-A subunit was ablated selectively in fast-spiking parvalbumin-expressing interneurons and revealed reduced excitation of parvalbumin-positive interneurons and as a consequence disrupted hippocampal gamma oscillations *in vitro* (Fuchs et al., 2007). On a behavioural level the animals showed impairment of hippocampus-dependent working memory and of short-term recognition memory. Parvalbumin-expressing interneurons comprise a large number of hippocampal interneurons and it was obvious to analyse their role in hippocampal network activity and the behavioural consequences. In contrast, not much is known about the functional properties of hippocampal somatostatin-expressing interneurons, especially O-LM cells that are thought to be involved in the generation of theta oscillation by exhibiting feedback inhibition on principal cells (Lacaille et al., 1987). Hence, it would be interesting to investigate the consequences of changed excitatory input on O-LM cells by genetic manipulations. Therefore ongoing work includes the cell-type specific ablation of the NR1 subunit of NMDR as well as the GluR-A subunit of AMPAR in somatostatin-positive cells using SOM^{CRE/+} mice. First preliminary electrophysiological results will now be shortly discussed.

4.3.1. Ablation of the NR1 subunit of the NMDAR in somatostatin-positive cells

Breedings of SOM^{CRE/+} mice with NR1^{2lox} mice (Shimshek et al., 2006) are currently in progress and one SOM^{CRE/+;NR1^{-/-}} and two control mice (age: P14) were analysed by electrophysiological experiments so far. Out of four measured cells, one showed a reduced NMDAR-mediated current. This preliminary result, of course, has no statistical validity but it indicates that the ablation of the NR1 subunit in O-LM cells mediated by CRE recombinase site-specific recombination is functional. Additionally, it has to be taken into account that not all somatostatin-expressing in the stratum oriens of the CA1 region, where the measurements were performed, are also expressing CRE recombinase. However, further experiments will clarify whether this cell-type specific knock-out approach can help to gain more insight into the role of NMDAR in O-LM cells and theta oscillatory activity in the hippocampus.

4.3.2. Ablation of the GluR-A subunit in interneurons expressing somatostatin

Mice with cell-type specific ablation of the GluR-A subunit in somatostatin-positive interneurons were generated by breeding SOM^{CRE/+} with GluRA^{2lox} mice (Zamanillo et al., 1999). Until now one SOM^{CRE/+;GluRA^{-/-}} and one control mouse (P14) were subjected to electrophysiological analysis. No reduction of AMPAR-mediated currents were detected. To exclude that CRE recombinase mediated site-specific recombination of the floxed GluR-A subunit takes longer than two weeks, electrophysiological measurements were performed on P28 SOM^{CRE/+;GluRA^{-/-}} animals. Again no altered AMPAR currents were measured. Since the functionality of the ablation of the GluR-A subunit was demonstrated in previous studies (Fuchs et al., 2007; Zamanillo et al., 1999), the lack of alterations in the AMPAR currents cannot be explained by problems regarding recombination in the GluRA^{2lox} mice. In addition, the analysis of SOM^{CRE/+} mice showed that the CRE recombinase was able to promote site-specific recombination already at embryonic stages. Hence, one has to consider other reasons and compensatory mechanism as a possibility for the lack of effect regarding the measured AMPAR currents. Thus it is conceivable that GluR-A expression in O-LM cells is normally low and does not contribute to AMPAR mediated currents. Since not much is known about expression of AMPAR subunits in O-LM cells further analysis have to be performed to establish AMPAR subunit composition in this cell population.

5. Abbreviations

%	Percentage
α	Alpha
β	Beta
γ	Gamma
$[\alpha^{35}\text{S}]\text{dATP}$	Adenosine triphosphate labeled with ^{35}S in gamma position
$\alpha^{32}\text{P}\text{-dCTP}$	Deoxycytosin triphosphate labeled with ^{32}P in alpha position
μ	Micro
A	Ampere
AMPA	L- α -amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid
AMPA	AMPA receptor
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BDNF	Brain-derived neurotrophic factor
bp	Base pairs
BSA	Bovine serum albumin
C	Celcius
CA1 - 3	Cornus ammonis fields 1 - 3
cAMP	Cyclic adenosine monophosphate
CRE	cAMP-responsive element
CRE	CRE recombinase
Da	Dalton
DAB	Diaminobenzidine
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
DTT	Dithiothreitol
E	Embryonic day
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylene diaminetetraacetic acid
EPSC	Excitatory postsynaptic potential
ES	Embryonic stem cells
et al.	et alii

Fig.	Figure
Flp	Flp recombinase
G418	Geneticin
GABA	γ -Aminobutyric acid
GAD	Glutamic acid decarboxylase
GluR	Glutamate receptor
h	hour
HEPES	N-(2-Hydroxyethyl)piperazine-N'-ethanesulfonic acid
Hz	Hertz
IgG	Immunoglobuline G
ISH	<i>In situ</i> hybridisation
kB	Kilobase
kDa	Kilodalton
lacZ	β -galactosidase
LIF	Leukaemia Inhibitory Factor
loxP	Locus of crossing over (for) phage P
LTP	Long-term potentiation
m	Milli
M	Mol
mGluR	Metabotropic glutamate receptor
min	Minute
mRNA	messenger RNA
n	Number
NEO	Neomycin resistance gene
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NR1	NMDAR subunit 1
P	Postnatal day
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PKC	Protein kinase C
RNA	Ribonucleid acid
RT	Room temperature

s	Second
SDS	Sodium dodecylsulfate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SEM	Standard error of the mean
STDEV	Standard deviation
t	Time
Taq	Therminus aquaticus
TE	TRIS/EDTA buffer
TENS	Tris-EDTA-sodium chloride-SDS buffer
TRIS	Tris(hydroxymethyl)aminomethan
TRIS-HCl	Tris(hydroxymethyl)aminomethan-hydrochloride
U	Unit
V	Voltage
V	Volts
W	Watt
wt	wildtype
X-Gal	5-brom-4-chlor-3-indolyl-b-D-galactopyranoside

6. References

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