Genetic Models of the Endocannabinoid System

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Contents

Abstract The endocannabinoid (ECB) system comprises cannabinoid receptors, ECBs and the whole machinery for the synthesis and degradation of ECBs. It has emerged as an important signalling system in the nervous system, controlling numerous physiological processes, including synaptic transmission, learning and memory, reward, feeding, neuroprotection, neuroinflammation, and neural development. This system is also implicated in various diseases of the nervous system, and thus has become a promising therapeutic target. The use of genetically modified mice has contributed crucially to our rapidly expanding knowledge of the ECB system. In this chapter, the existing mouse mutants targeting the ECB system will be discussed in detail. The use of conditional mutants has given an additional dimension to the analysis of the system, and, it is hoped, will finally enable us to understand this widespread and complex system in the context of intricate networks where different brain regions and neurotransmitter systems interact tightly with each other.

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Abbreviations

2-AG	2-Arachidonoyl-glycerol
AAV	Adeno-associated virus
AEA	Arachidonoyl-ethanolamine (anandamide)
CaMK	Calcium/calmoduline-dependent kinase
DAGL	Diacylglycerol lipase
ES	Embryionic stem (cell)
FAAH	Fatty acid amide hydrolase
GABA	Gamma aminobutyric acid
MGL	Monoacyl-glycerol lipase

1 Introduction

Δ^9-Tetrahydrocannabinol (THC), the major pharmacologically active compound of marijuana (Gaoni and Mechoulam 1964), has an impressively wide range of pharmacological effects in mammals including humans. For some time, this multitude of effects was believed to be the consequence of the lipophilic nature of THC molecules, which would change membrane fluidity and thus non-specifically affect neuronal communication (Hillard et al. 1985). However, the availability of radiolabelled synthetic cannabinoid ligands in the 1980s enabled the demonstration of specific cannabinoid binding sites in the brain (Howlett et al. 1988). A few years later, two different cannabinoid receptors were identified (CB_1 and CB_2 receptors); the CB_1 receptor is highly enriched in the central nervous system (CNS) (Matsuda et al. 1990), while the CB_2 receptor is prominently expressed in immune cells (Munro et al. 1993). Radioligand binding (Herkenham et al. 1990), immunolabelling (Tsou et al. 1998; Katona et al. 1999) and in situ hybridisation (Marsicano and Lutz 1999) showed a widespread expression of CB_1 receptors in the adult rodent brain, especially in the cerebellum, basal ganglia, cerebral cortex, hippocampus and amygdala. Electron microscopy showed that the CB_1 receptor is mainly localised on axon terminals (Katona et al. 1999), and electrophysiology demonstrated that the CB_1 receptor is able to modify neurotransmitter release by presynaptic mechanisms (Wilson and Nicoll 2001).

The CB_1 receptor is present at highest density on presynaptic terminals of cholecystokinin-positive GABAergic inhibitory interneurons (Tsou et al. 1998; Katona et al. 1999). Electrophysiological studies and coexpression analysis, however,

have revealed the complexity of the ECB system. CB_1 receptors are also present in many other types of neurons, including glutamatergic (Sullivan 1999; Monory et al. 2006); cholinergic (Degroot et al. 2006), serotonergic (Häring et al. 2007), noradrenergic (Oropeza et al. 2007) and possibly on dopaminergic (Degroot et al. 2006) neurons. This may explain the many diverse effects of *Cannabis* and also suggests an intrinsic complexity of the ECB system in the regulation of neurotransmission. However, several other different factors increase further the complexity of physiology and pharmacology of the ECB system.

First, after the initial belief of mutually exclusive expression of CB_1 receptors in the CNS and CB_2 receptors in the peripheral nervous system, it has become evident that the expression pattern of both receptors is more complex. Namely, the CB_1 receptor was found in several peripheral, non-neuronal tissues (Cota et al. 2003; Bensaid et al. 2003), while the CB_2 receptor was also identified in the brain (Van Sickle et al. 2005; Onaivi et al. 2008). Moreover, several lines of evidence have suggested the existence of yet-to-be identified cannabinoid receptors (Brown 2007). Indeed, the orphan G protein-coupled receptor GPR55 seems to be one of the suspected new cannabinoid receptors (Baker et al. 2006; Lauckner et al. 2008). Furthermore, variants of CB_1 receptor protein were shown to derive from different splice forms in humans (Shire et al. 1995; Ryberg et al. 2005). However, the physiological relevance of these variants has still to be elucidated.

Furthermore, there are different endogenous ligands that can activate cannabinoid receptors. Five ECBs have been identified to date: anandamide (AEA), 2-arachidonoylglycerol (2AG), noladin ether, virodhamine and *N*-arachidonoyl dopamine (NADA) (Di Marzo 2008). They are polyunsaturated fatty acids derived from arachidonic acid. Undoubtedly, AEA and 2AG are the two best characterised ECBs, but it is expected that novel lipids may be identified as ligands of cannabinoid receptors in the near future.

For the different ECBs, different synthesising and degrading enzymes are engaged (Di Marzo 2008). As ECBs are lipids, primarily generated from membrane phospholipid precursors, they are not stored nor released like "classical" neurotransmitters, but are synthesised on-demand after increase of intracellular Ca^{2+} concentration or after stimulation of metabotropic glutamate and acetylcholine receptors. These enzymes are the *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) for AEA, and diacylglycerol lipase (DGL) α and β for 2AG. Both DGL isoforms are postsynaptically localised, but expressed in a different temporal manner, the α isoform being the main isoform postnatally present, while the β isoform is the main isoform prenatally. These synthesising enzymes are presumably anchored to the plasma membrane (DGL) or to internal membrane compartments (NAPE-PLD). DGL and NAPE-PLD are the main but not the only synthetic enzymes of AEA and 2AG. For example, 2AG can also be synthesised by lyso-PLC (Sugiura et al. 1995). For AEA, two alternative pathways have been proposed (Liu et al. 2008).

After receptor binding, ECBs are presumably taken up quickly by the cells and are inactivated. The uptake is possibly mediated by a membrane transporter protein, though it has not yet been identified. Similarly to their synthesis, degradation of

ECBs also follows different pathways (Di Marzo 2008). AEA is degraded by FAAH-1 and FAAH-2, the latter found only in humans (Wei et al. 2006). 2AG is degraded by monoacylglycerol lipase (MGL). In both cases, the end product of the degradation is arachidonic acid. Supporting the concept of retrograde transmission of ECBs, where 2AG appears to be the main player as a retrograde transmitter, MGL is presynaptically localised, while FAAH is postsynaptically associated with membranes of cytoplasmic organelles.

The dynamics of the ECB system activity is determined by a multitude of factors and poses numerous questions: Which biosynthetic pathway is initiated under which circumstances? Which receptor is activated by which ligand? How fast are ECBs taken up by the cell and how fast is the degradation? Such questions have also to be brought into the context of the anatomical complexity of the various components of the ECB system in the nervous system.

To tackle the multifaceted process of ECB signalling, state-of-the-art genetics offers appropriate and powerful tools. Gene inactivation in all cells of the body or only in certain cell types or brain regions should help to decipher physiological and pathophysiological processes in which ECBs participate. Indeed, in the last few years, a growing number of mutant mouse lines have become available in the field of ECB research. In the following paragraphs, we will first outline the background of the transgenic techniques and the available mutants. Then, we discuss the advantages as well as possible pitfalls of genetic approaches; we will address several considerations in the context of behavioural experiments with mutant mice and talk further about possible caveats of pharmacology. Finally, we would also like to point out possible aspects of the use of other advanced genetic models in analysing the ECB system.

2 Genetic Models

2.1 Available Tools

During the last two decades, targeted mutagenesis in mice has become one of the most powerful tools for studying gene function in mammals, as illustrated by the 2007 Nobel Prize in Physiology or Medicine awarded to Mario Capecchi, Martin Evans and Oliver Smithies (Mak 2007). Their discoveries and technological advancements in introducing highly specific modifications in the mouse genome by the use of homologous recombination in embryonic stem cells revolutionised medical research and have led to fundamental discoveries in all fields of mammalian biology, ranging from embryonic development to the generation of animal models for human diseases.

The first milestone in the development of the gene targeting technology was the establishment of totipotent cell lines (i.e. embryonic stem cells) derived from mouse blastocysts. If kept in appropriate culture conditions, these cells can

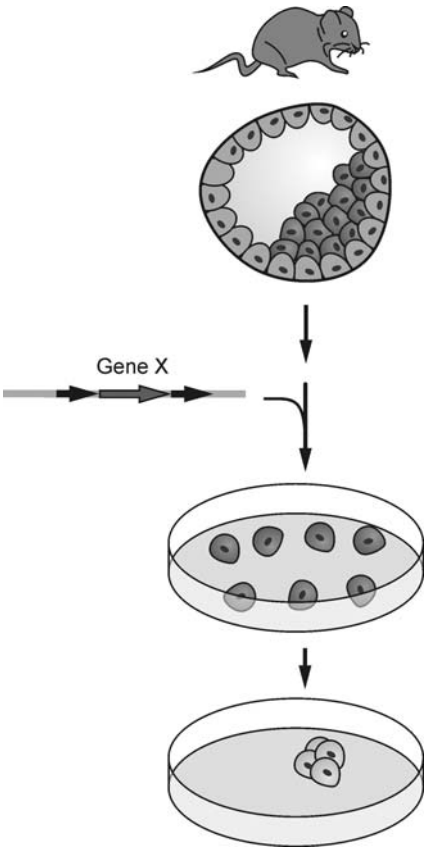

Fig. 1 Genetic manipulation of embryonic stem cells. Embryonic stem (ES) cells are derived from mouse blastocysts and are kept under conditions that allow the maintenance of their totipotency. A gene targeting construct containing selection markers is electroporated into ES cells, plated on culture dish in the presence of a selection medium. Only cells that received the targeting construct by homologous recombination survive selection, forming a colony of cells which contains the genetic modification in a heterozygous state. Here, the targeted Gene X (*grey arrow*) is flanked by two loxP sites (*black arrows*)

contribute to all cell types of the body, including germ cells. The second seminal discovery was the observation by Capecchi and colleagues that mammalian somatic cells possess the enzymatic machinery for mediating homologous recombination between exogenously introduced DNA sequences and homologous sequences in the genome. Both discoveries led to the first gene disruption experiments in mice (Thomas and Capecchi 1987; Doetschman et al. 1987), now currently referred to as gene "knockout" technology (Figs. 1 and 2).

The number of knockout mouse lines has grown almost exponentially after the establishment of this technique, which helped scientists to understand protein functions in the context of the entire animal, avoiding many of the limitations of in vitro models or pharmacological tools. Gene knockout technology has enabled the study of specific gene products in a highly complex context such as the nervous system, leading to seminal discoveries in neurosciences, including learning and memory, cognition and synaptic plasticity. As an example, the study of learning and memory in gene targeted mutant mice has led to insights into key mechanisms

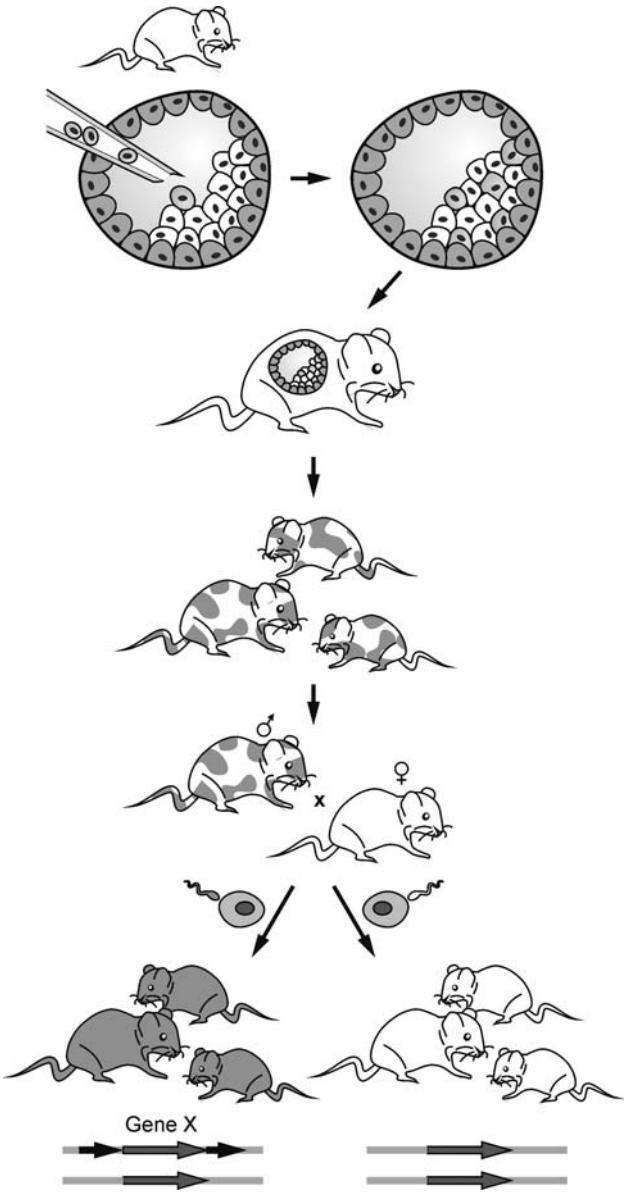

Fig. 2 Generation of mutant mice by gene targeting of embryonic stem cells. Genetically modified ES cells (as male karyotype) are injected into blastocysts, which are then transferred into the uterus of foster mothers. As the blastocysts are obtained from a mouse strain different from that used for the establishment of ES cell lines, the offspring are chimaeras (as mixture of ES cell-derived cells and of inner cell mass from the injected blastocyst). This is later visible by the coat colour. The crossing of male chimaeras with wild-type mice of the appropriate strain will lead to offspring with a distinct coat colour, showing that the injected ES cells contributed fully to the animal via the gametes of the chimaera. Genotyping of these mice will show whether the genetically modified Gene X is present in a heterozygous state. Gene X (*grey*) is flanked by two loxP sites (*black arrows*)

underlying the brain's ability to select, to store and to recall information (Silva et al. 1992; Grant et al. 1992).

Despite the progress enabled by this new technology, some of its intrinsic limitations have become evident. First, complete knockout mice lack the targeted gene product during their entire lifespan, i.e. from embryogenesis to adult life. It has been recognised that many genes with distinct expression and functions during embryogenesis also have specific functions in the adult. If the role during development is essential, the mutation might cause embryonic lethality. Developmental deficits might also induce particular phenotypes in the adult. Thus, such phenotypes may not be easily interpreted at the mechanistic level, as early developmental disturbances may distort or strengthen the phenotypes observed in the adult. Observable defects in the embryo, however, may also be ameliorated due to compensatory mechanisms. Biological processes are highly regulated to maintain cellular homeostasis. The disruption of a gene in the entire organism might trigger the up- or down-regulation of related or unrelated gene products. This, again, might mask or distort the observed phenotype in these mice and hamper the interpretation of the phenotype observed.

In addition, in these mutants, the targeted gene is lost in all cells of the body. Frequently, a single gene product is responsible for distinct functions in different organs or cell types. But even if the biochemical activity of a protein *per se* is similar or is the same in different cell types, the distinct physiological role of the gene product might depend on the context, i.e. the tissue or cell type. In particular in the brain, containing a myriad of different cell types that communicate extensively with each other, it makes an important difference whether a certain protein is present, e.g. in an inhibitory or an excitatory neuron, or in glial cells. Moreover, complex brain functions are a result of co-ordinated interactions of many regions and cell types. Thus, a single gene product might differentially participate in an activated neuronal circuit, depending on the physiological or pathophysiological context; e.g. emotional behaviour will recruit other neuronal circuits and brain areas than reward-related behaviour. However, these distinct functions are unlikely to be uncovered with "conventional" knockout mice.

For the functional dissection of the nervous system, it is therefore essential to relate gene function to particular anatomical brain regions and cell types. This is made possible by the technique of conditional mutagenesis. The most widespread technique for generating conditional knockout mice is the Cre/loxP system (Sauer and Henderson 1988) (Fig. 3); however, other systems are also emerging (Feil 2007). Cre recombinase is a P1 bacteriophage-derived enzyme which is able to mediate sequence-specific recombination between a 34-bp-long sequence referred to as loxP (locus of crossover in P1) (Sternberg and Hamilton 1981).

In the conditional mutagenesis approach, two transgenic mouse lines have to be established and crossed (Fig. 4). First, the so-called "floxed" mouse has to be generated, where the gene or region of interest is flanked by two loxP sites. Introducing two loxP sites upstream and downstream in the same orientation induces the deletion of the loxP-enclosed sequence, when the Cre recombinase

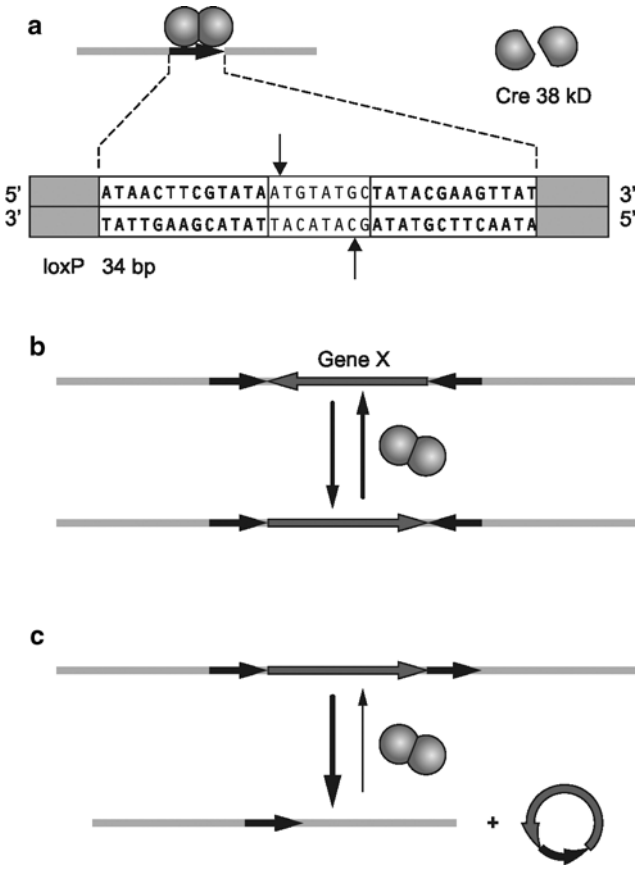

Fig. 3 Principle of the Cre/loxP system. (**a**) One loxP site consists of 34 bp, composed of two 13-bp-long repeats forming a palindrome, which is interrupted by an 8-bp-long non-palindromic sequence. This 8-bp sequence determines the orientation of the loxP site. Cre recombinase binds as a dimer to the loxP site. After binding of one Cre recombinase monomer to each inverted repeat, the DNA strands are cleaved in the spacer region (*vertical arrows*) and exchanged between two loxP sites. (**b**) When loxP sites are oriented "head-to-head", Cre recombinase catalyses an inversion of the sequence "Gene X" between the two loxP sites. (**c**) When the two loxP sites are oriented "head-to-tail", Cre recombinase catalyses the excision of the intervening sequence, leading to a loss of Gene X

protein is present. If appropriately performed, the introduction of these two short loxP sequences should not cause any phenotype. Thus, the "floxed" mice behave like wild-type mice. Second, a mouse line is needed with a cell-type specific expression of Cre recombinase. To this end, specific regulatory sequences should drive the expression of Cre recombinase in the second transgenic mouse line (Fig. 4). In the offspring from crossing of these two mouse lines, the target gene will be deleted in all cells where Cre recombinase is expressed, while the gene

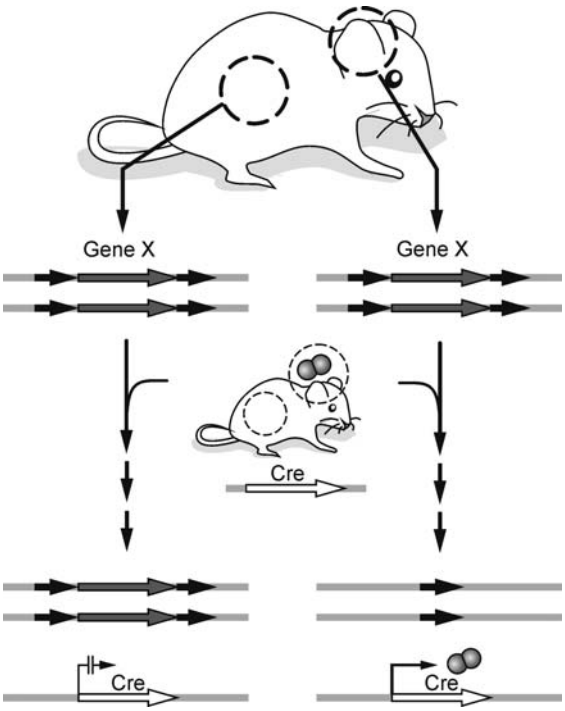

Fig. 4 Principle of the generation of cell- and tissue-specific gene inactivation. Essential sequences of Gene X are flanked by two loxP sites. Mice with the so-called "floxed" allele will be crossed with a transgenic mouse line expressing Cre recombinase in a cell- and/or tissue-specific manner. After an appropriate breeding schedule, mice will be obtained which either lack the expression of Cre recominase not shown or which express Cre recombinase in specific cells, leading to the excision of the loxP-flanked sequences, i.e. Gene X

will keep the wild-type expression pattern in all cells missing Cre recombinase expression. Currently, the "zoo" of Cre recombinase-expressing transgenic mouse lines is continuously growing. A recent review gave an exhaustive list of lines that are useful in the analysis of the nervous system (Gaveriaux-Ruff and Kieffer 2007). In Table 1, transgenic lines are listed that are particularly interesting in the analysis of the ECB system or that have been established only very recently.

In a more advanced approach, not only spatial, but temporal regulation of gene excision is possible. Generating a fusion protein of Cre recombinase with a modified ligand-binding domain of the oestrogen receptor renders the system inducible by tamoxifen (Feil et al. 1996). Cytoplasmic binding of the heat-shock protein Hsp90 to the ligand-binding domain traps the protein to the cytoplasm and inhibits the enzymatic activity of Cre recombinase by conformational inhibition (Fig. 5). When tamoxifen binds to the modified ligand-binding domain of the oestrogen receptor, Hsp90 dissociates, and the fusion protein translocates to the nucleus, where Cre recombinase can excise the "floxed" target sequence. Tamoxifen can be injected into mice intraperitoneally at any chosen time in postnatal (Erdmann et al. 2007), but also in prenatal life (Erdmann et al. 2008). Thus, gene function can be studied after developmental processes are finished. If this fusion protein is expressed under the control of specific regulatory sequences, the gene deletion will be spatially as well as temporally regulated (Feil 2007).

Table 1 Available mouse mutants targeting components of the endocannabinoid system

Targeted gene	Genetic modification	Reference
CB_1	Null mutant	Ledent et al. (1999)
CB_1	Null mutant	Zimmer et al. (1999)
CB_1	Null mutant	Marsicano et al. (2002)
CB_1	Null mutant	Robbe et al. (2002)
CB_1	Conditional mutant *CB_1R deleted from principal forebrain neurons*	Marsicano et al. (2003)
CB_1	Conditional mutant *CB_1R deleted from forebrain GABAergic neurons*	Monory et al. (2006)
CB_1	Conditional mutant *CB_1R deleted from cortical glutamatergic neurons*	Monory et al. (2006)
CB_1	Conditional mutant *CB_1R deleted from dopamine receptor D1 expressing neurons*	Monory et al. (2007)
CB_1	Conditional mutant *CB_1R deleted from nociceptive dorsal root neurons*	Agarwal et al. (2007)
CB_1	Conditional mutant *CB_1R deleted from all neural precursors*	Maresz et al. (2007)
CB_1	Conditional mutant *CB_1R deleted from T cells*	Maresz et al. (2007)
CB_1	Conditional mutant *CB_1R deleted in hepatocytes*	Jeong et al. (2008)
CB_2	Null mutant	Buckley et al. (2000)
CB_2	Null mutant	Wotherspoon et al. (2005)
GPR55	Null mutant	Staton et al. (2008)
FAAH	Null mutant	Cravatt et al. (2001)
FAAH	NES-FAAH *FAAH expression under the control of the neural-specific enolase promoter*	Cravatt et al. (2004)
NAPE-PLD	Null mutant	Leung et al. (2006)
TRPV1	Null mutant	Caterina et al. (2000)
TRPV1	Null mutant	Davis et al. (2000)

Abbreviation: CB_1R. CB_1 receptor

2.2 Null Mutant Mouse Lines

The first gene targeting addressing the function of the ECB system was performed for CB_1 receptors. There are four different knockout mouse lines available (Ledent et al. 1999; Zimmer et al. 1999; Marsicano et al. 2002; Robbe et al. 2002). In three of these lines, distinct parts of the CB_1 receptor gene were replaced by the 3-phosphoglycerate kinase-neomycin (PGK-Neo) resistance cassette. The following parts were abolished in the CB_1 receptor gene: amino acids 32–448 (Zimmer et al. 1999), the first 233 amino acids of the receptor (Ledent et al. 1999), and part of the 5' intronic region plus the protein encoding region up to the sixth transmembrane domain (Robbe et al. 2002), respectively. Thus, in any of these lines, small parts of the CB_1 receptor protein-encoding sequences remained in the mutated gene locus. Whether or not some small peptide parts of CB_1 receptor are still translated is

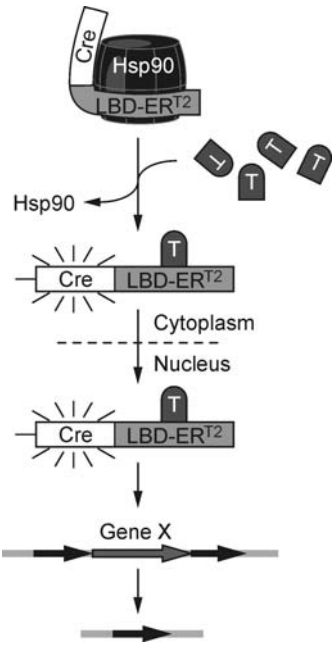

Fig. 5 Principle of ligand-inducible Cre recombinase. Cre recombinase is fused to the modified ligand binding domain of the oestrogen receptor (LBD-ER^{T2}), the latter being unable to bind any endogenous steroid hormones. This fusion protein binds the heat-shock protein 90 (Hsp90), leading to a conformation of the Cre recombinase that prevents enzymatic activity. Application of tamoxifen leads to binding to the LBD-ER^{T2}, and Hsp90 is released from the fusion protein. Next, Cre recombinase reconstitutes its enzymatic activity, translocates into the nucleus, recognises the two loxP sites, and excises Gene X

difficult to evaluate, but functional studies, e.g. ligand binding (Zimmer et al. 1999), electrophysiological experiments (Robbe et al. 2002) and behavioural experiments (Zimmer et al. 1999; Ledent et al. 1999), strongly indicated complete loss of CB_1 receptor function. In the fourth line (Marsicano et al. 2002), two loxP sites were introduced, one into the intron preceding the exon encoding CB_1 receptor protein and the other one into the 3'UTR immediately after the open reading frame. Afterwards, these loxP flanked sequences were removed by Cre recombinase-mediated excision, leading to the complete loss of CB_1 receptor protein-encoding sequences. The PGK-Neo selection cassette, which was flanked by two FRT recombination sites, was also introduced by homologous recombination, but was not removed, and thus it is still present in the 3'UTR of this null mutant line. In addition to the targeting strategies, differences exist regarding the genetic background. Two lines are in the inbred strain C57BL/6J (Zimmer et al. 1999; Robbe et al. 2002), one in C57BL/6N (Marsicano et al. 2002), and one in the outbred strain CD1 (Ledent et al. 1999).

The analysis of these mutant CB_1 receptor mice provided a myriad of extraordinary insights into the numerous functions of the ECB system as mediated by CB_1 receptors in the nervous system. These functions include the roles of CB_1 receptors in drug addiction and reward (Ledent et al. 1999; Racz et al. 2003; Wang et al. 2003), pharmacological effects of THC (Zimmer et al. 1999), cognitive processes (Reibaud et al. 1999; Bilkei-Gorzo et al. 2005), emotional behaviours (Marsicano et al. 2002; Haller et al. 2002), neuroprotection (Marsicano et al. 2003), pain (Ledent et al. 1999; Zimmer et al. 1999), and feeding behaviour (Di Marzo et al. 2001; Cota et al. 2003; Ravinet et al. 2004). Furthermore, CB_1

receptor-deficient mice were important to prove the involvement of ECBs in the regulation of synaptic transmission, such as short-term and long-term suppression of GABA transmission (Wilson et al. 2001; Marsicano et al. 2002). In most of the studies, the phenotypic changes in CB_1 receptor knock-out mice were able to be mimicked by using specific CB_1 receptor antagonists, such as rimonabant and AM251. In recent years, CB_1 receptor mutant mouse lines have become a crucial tool for proving CB_1 receptor functions in peripheral, non-neuronal tissues, such as liver (Osei-Hyiaman et al. 2005), adipocytes (Cota et al. 2003) and skin (Karsak et al. 2007).

The inactivation of the CB_2 receptor also used the strategy of a PGK-Neo replacement vector (Buckley et al. 2000). By homologous recombination, it is reported that a fragment of 341 bp of the exon was deleted. Accordingly, this led to a loss of the C-terminal amino acids from positions 217 to 347, but leaving the splice acceptor of the protein-encoding exon unaffected as well as the sequences coding for the first 216 amino acids of the CB_2 receptor protein. These amino acids potentially code for the N-terminal CB_2 receptor protein, containing the first five transmembrane domains. This CB_2 receptor-deficient mouse line showed no binding activity in the spleen for the radiolabeled non-selective CB_1 receptor/CB_2 receptor agonist CP55940, suggesting that ligand binding to CB_2 receptor is indeed completely lost in this line, but the existence of remaining CB_2 receptor protein-encoding sequences requires further attention.

This mutant line has emerged as an important tool for studying CB_2 receptor functions in non-immune cells. In particular, the functional presence of CB_2 receptor in brain neurons (Van Sickle et al. 2005; Onaivi et al. 2008), liver (Julien et al. 2005) and bone (Ofek et al. 2006) gave new insights into the roles of this receptor.

A second CB_2 receptor-deficient mouse line was also established (Wotherspoon et al. 2005) and is distributed by The Jackson Laboratory (Bar Harbor, USA; for further information see http://www.informatics.jax.org/external/ko/deltagen/614_MolBio.html). Based on the available data sheet, this gene targeting also used a PGK-Neo replacement vector, leading to the deletion of sequences encoding amino acids 26–140, thus deleting the first 3 transmembrane domains. The first 25 amino acids might still be translated. After the inserted PGK-Neo cassette, the rest of the CB_2 receptor coding region remained in the genome. Theoretically, there might be a splicing over the PGK-Neo cassette, but this is rather unlikely. Thus, only minor remaining parts of the CB_2 receptor protein are present in this mouse line, likely not to interfere with cellular processes.

The orphan G protein-coupled receptor GPR55 (Sawzdargo et al. 1999) has recently been proposed to be a third cannabinoid receptor (Baker et al. 2006; Ryberg et al. 2007), and a null mutant mouse line was recently generated (Staton et al. 2008). A combined LacZ PGK-Neo cassette was used to replace a major part of the GPR55 open reading frame. The deletion removed the sequences coding for amino acids 39–281 of GPR55, representing the second to sixth transmembrane domains. Thus, the mutation retains the first 118 bp of the GPR55 open reading frame encoding 39 amino acids, containing 20 of the 22 amino acids of the first transmembrane domain. In recent experiments, it was shown that GPR55-

deficient mice lack inflammatory mechanical hyperalgesia and neuropathic hypersensitivity (Staton et al. 2008), suggesting GPR55 as a potential target in pain treatments.

Unlike CB_1 and CB_2 receptors (Matsuda et al. 1990; Munro et al. 1993), which are encoded by one exon, the protein-coding sequence of FAAH spans across 15 exons of the gene (Wan et al. 1998). For the FAAH null mutant (Cravatt et al. 2001), standard targeted gene disruption procedures were used and replaced the first exon of the FAAH gene (encoding amino acids 1–65) and 2 kb of upstream sequence with the PGK-Neo cassette. Loss of FAAH protein was evidenced by immunostaining, and by enzymatic activity measurements. The mutants basically lacked the degradation of AEA and oleamide, leading to 15-fold increased levels of AEA in the brain (Cravatt et al. 2001). The second FAAH gene (FAAH-2) is not relevant in this context, as it exists only in humans (Wei et al. 2006). FAAH-deficient mice served as an excellent model in order to understand the physiological consequences of increased AEA levels. They were analysed in several behavioural paradigms, including pain (Cravatt et al. 2001), seizure susceptibility (Clement et al. 2003) spatial memory (Varvel et al. 2007), anxiety (Moreira et al. 2008), emotionality (Naidu et al. 2007), nicotine reward (Merritt et al. 2008), ethanol drinking (Basavarajappa et al. 2006), and in experimental autoimmune encephalitis (Maresz et al. 2007, Webb et al. 2008). In addition, these mice were very useful in elucidating the roles of ECBs in embryonic proliferation of cortical neurons and in radial migration (Mulder et al. 2008), and in proliferation and differentiation of neural progenitors in the adult brain (Aguado et al. 2006). Finally, the analysis of FAAH-deficient mice led to the description of novel AEA degradation pathways (Mulder and Cravatt 2006).

In order to dissociate the peripheral and the central nervous system functions of FAAH, a transgenic mouse line was established in which FAAH is expressed under the control of the neural-specific enolase (NSE) promoter (Cravatt et al. 2004). This transgenic line was crossed with the FAAH knock-out line to in order to obtain $FAAH^{-/-}$ mice that contain the NSE-FAAH transgene. Thus, this approach is a conditional rescue experiment of FAAH, re-expressing FAAH in the central nervous system, but lacking it in all peripheral organs. The analysis of these mice revealed that the anti-inflammatory effects of FAAH deficiency were mediated by peripheral FAAH.

To date, NAPE-PLD is the only ECB-synthesising enzyme which was inactivated by gene targeting (Leung et al. 2006). Using a PGK-Neo replacement strategy, exon 4, which codes for amino acids 98–313, was completely deleted. As NAPE-PLD consists of 396 amino acids, most of the coding region was lost, in fact leading to the loss of the enzyme as detected by Western blot. However, this study revealed that NAPE-PLD is not the only enzyme in the biosynthesis of ECBs. Apparently, a Ca^{2+}-independent PLD activity must exist in NAPE-PLD-deficient mice, accepting substrates including the AEA precursor C20:4 NAPE (Leung et al. 2006). Clearly, this investigation points to serious difficulties in the genetic targeting of the synthesising enzymes, caused by redundant pathways.

The transient receptor potential vanilloid receptor 1 (TRPV1, formerly called VR1) (Caterina et al. 1997) is a heat- and proton-sensitive cation channel implicated mostly in noxious heat sensation. In 1999, however, it was shown that the ECB AEA is also able to specifically activate TRPV1 channels (Zygmunt et al. 1999). Since then, there has been an ever-increasing amount of data showing the interplay between the ECB and the endovanilloid systems (Starowicz et al. 2007). TRPV1 knockout mice became available from two different laboratories (Caterina et al. 2000; Davis et al. 2000). These mice were generated by homologous recombination, replacing part of the TRPV1 sequence with a PGK-Neo selection cassette. The 839 amino acid long TRPV1 protein is encoded by 15 exons. Caterina et al. (2000) replaced the receptor's fifth and sixth transmembrane domains and its pore loop, while Davis et al. (2000) replaced transmembrane domains 2–4. Following the original reports on deficits of pain perception in these mice, not unexpectedly recent investigations discovered also roles of TRPV1 in the CNS, including an involvement in emotional memory and anxiety (Marsch et al. 2007) and synaptic transmission (Gibson et al. 2008).

2.3 Conditional CB_1 Receptor Mutants

Gene targeting using homologous recombination in embryonic stem cells offers unprecedented precision in manipulating single genes and in investigating the in vivo roles of gene products in mice. As discussed above, this has proved to be true in the case of the ECB system, too. However, it has become clear that conventional gene targeting has several limitations. The loss of the gene product throughout development and the lack of spatial and temporal specificity of the generated mutation might give rise to complex, secondary phenotypical alterations. This is clearly a disadvantage in the functional analysis of genes associated with complex brain functions. One current solution of this problem is the generation of conditional mutants where the mutation will be present only in certain cell populations and/or only after a certain time point.

Unfortunately, at present, ECB system-related conditional mutants are only available for the CB_1 receptor gene. Several conditional CB_1 receptor mutant lines have been established in the last few years and have been instrumental in deciphering detailed functions of CB_1 receptors in numerous expression sites in neuroprotection (Marsicano et al. 2003; Monory et al. 2006), synaptic plasticity (Domenici et al. 2006; Azad et al. 2008), stress responses (Steiner et al. 2008b), pain perception (Agarwal et al. 2007), a model of multiple sclerosis (Maresz et al. 2007), in the response to THC (Monory et al. 2007), responses to cocaine (Corbille et al. 2007), in neural development (Berghuis et al. 2007; Mulder et al. 2008), and in hepatic functions of CB_1 receptor (Jeong et al. 2008).

Neuroprotective effects of the ECB system were shown in different models. In particular, in the kainic acid-induced acute seizure model, CB_1 receptor null mutants showed greatly increased seizures compared to their wild-type littermates

(Marsicano et al. 2003). To understand which CB_1 receptor-containing neuronal population is responsible for this function, a series of experiments was carried out with three different CB_1 receptor conditional mutant lines (Monory et al. 2006) (Table 2). Mice lacking CB_1 receptor on GABAergic neurons ($CB_1^{f/f;Dlx5/6\text{-}Cre}$, also

Table 2 Useful Cre recombinase-expressing transgenic lines for the analysis of the nervous system

Regulatory elements	Sites of recombination	Reference
AgPR-Cre (Tg)	Neurons expressing agouti-related peptide (hypothalamus)	Gropp et al. (2005)
CaMKIIα-iCre (BAC)	Principal projecting forebrain neurons (cerebral cortex, hippocampus, thalamus, striatum)	Casanova et al. (2001)
CaMKIIα-iCreER^{T2} (BAC)	Principal projecting forebrain neurons (cerebral cortex, hippocampus)	Erdmann et al. (2007)
DAT-iCre (BAC)	Dopaminergic neurons (in areas A8, A9, A10, A11, A12 and A16)	Turiault et al. (2007)
DBH-Cre (PAC)	Noradrenergic neurons (locus coeruleus, sypmpathetic ganglia)	Parlato et al. (2007)
Dlx5/6-Cre (Tg)	Forebrain GABAergic neurons (interneurons in cerebral cortex and hippocampus, and in striatum)	Monory et al. (2006)
Dlx5/6-Cre-IRES-GFP (Tg)	Forebrain GABAergic neurons (interneurons in cerebral cortex and hippocampus, and in striatum)	Stenman et al. (2003)
D1-Cre (YAC)	Dopamine receptor D1-expressing neurons	Lemberger et al. (2007)
GFAP-CreER^{T2} (Tg)	Astrocytes	Hirrlinger et al. (2006)
GLAST-CreER^{T2} (KI)	Astroglia and radial glia	Mori et al. (2006)
Emx-Cre (PAC)	Dosal telencephalon	Iwasato et al. (2004)
K_v3.2-Cre (BAC)	Thalamic projections neurons	Anderson et al. (2005)
Na_v1.8-Cre (BAC)	Nociceptive neurons in dorsal root ganglia	Agarwal et al. (2004)
Nestin-Cre (Tg)	Neuronal and glial cell precursor, neural stem cells	Tronche et al. (1999)
Nestin-CreER^{T2} (Tg)	Neuronal and glial cell precursor, neural stem cells	Imayoshi et al. (2006)
NEX-Cre (KI)	Glutamatergic neurons in cerebral cortex and hippocampus	Goebbels et al. (2006)
Pcp2-Cre (BAC)	Purkinje cells and retinal rod bipolar neurons	Zhang et al. (2004)
Peripherin-Cre (Tg)	Peripheral nervous system	Zhou et al. (2002)
PLP-CreER^{T2} (Tg)	Oligodendrocytes	Leone et al. (2003)
POMC-Cre (BAC)	Arcuate nucleus in hypothalmus, nucleus of the solitary tract	Balthasar et al. (2004)
P0 (Tg)	Myelinating Schwann cells	Akagi et al. (1997)
SF1-Cre (BAC)	Ventromedial hypothalamus	Dhillon et al. (2006)
Sim1-Cre (BAC)	Posterior hypothalamus (line 2)	Balthasar et al. (2005)
Six3-Cre#69 (Tg)	Layer 4 sensory cortex, hypothalamus	Liao and Xu (2008)

Abbreviations: BAC, bacterial artificial chromosome; GFP, green fluorescence protein; IRES, internal ribosomal entry site; KI, knock-in; PAC, phage artificial chromosome; Tg, promoter transgene; YAC, yeast artificial chromosome

named as GABA-$CB_1^{-/-}$) did not differ from wild-types, demonstrating that CB_1 receptor on these neurons does not participate in the protection from kainic acid-induced seizures. However, the mutants that lack CB_1 receptor on principal forebrain neurons and in cortical glutamatergic neurons ($CB_1^{f/f;CaMKII\alpha\text{-}Cre}$, also named as CaMK-$CB_1^{-/-}$; and $CB_1^{f/f;Nex\text{-}Cre}$, also named as Glu-$CB_1^{-/-}$, respectively) had the same phenotype as the complete knockout animals, suggesting that the protective function of the CB_1 receptor is mediated by the cortical glutamatergic neurons.

The neuroprotective function of CB_1 receptors is partly caused by the decreased glutamate release from the glutamatergic axon terminals. This notion was explored by Domenici et al. (2006) using CB_1 receptor conditional mutants. They found that in slices of the basolateral amygdala, the CA1 region of the hippocampus, and the primary somatosensory cortex of wild-type mice, application of a CB_1 receptor agonist reduced evoked excitatory postsynaptic responses. This effect was not seen in mice lacking CB_1 receptors in all principal forebrain neurons (CaMK-$CB_1^{-/-}$). However, CB_1 receptor agonist reduced glutamatergic responses in slices obtained from mice lacking CB_1 receptors exclusively in GABAergic neurons (GABA-$CB_1^{-/-}$), thus excluding the involvement of CB_1 receptor expressed on GABAergic neurons in this effect of the drug. On the other hand, CB_1 receptors on GABAergic neurons play an important role in the modulation of long-term depression (LTD) in the amygdala (Azad et al. 2008). Exogenous CB_1 receptor agonist treatment blocked LTD in the amygdala; an effect that was not present in CB_1 receptor null mutants and in GABA-$CB_1^{-/-}$ mice. These results showed that the CB_1 receptor expressed on either glutamatergic or GABAergic neurons plays a differential role in the control of synaptic transmission and plasticity.

Likewise, in brain development, both aforementioned populations of CB_1 receptor play important roles. Berghuis et al. (2007) revealed that the CB_1 receptor is enriched in axonal growth cones of GABAergic interneurons in the rodent cortex during late gestation and that cannabinoids induce chemorepulsion and collapse of axonal growth cones of these GABAergic interneurons. To strengthen these findings, GABA-$CB_1^{-/-}$ mice were investigated later in development. Indeed, impaired target selection of cortical GABAergic interneurons onto glutamatergic principal neurons in the hippocampus was found. Very recently, the role of ECB signalling in cortical pyramidal cell development was investigated with the help of Glu-$CB_1^{-/-}$ mice (Mulder et al. 2008). In this study, CB_1 receptor null mutants and Glu-$CB_1^{-/-}$ mice were shown to develop axon fasciculation deficits. Accordingly, in wild-type mice, ECB signalling was shown to be operational in subcortical proliferative zones from embryonic day 12 in the mouse telencephalon, controlling the proliferation of pyramidal cell progenitors, and radial migration of postmitotic pyramidal cells. These experiments identified ECBs as axon guidance cues and demonstrate that ECB signalling regulates synaptogenesis and target selection in vivo. Apart from clarifying the ECBs' roles in brain development, these studies may help understanding the damaging effects of marijuana smoking during pregnancy and early postnatal development until puberty.

Marijuana and its main pharmacologically active component THC have a number of pharmacological effects in the adult. This multitude of effects is the

result of complex interactions between different neuronal populations and circuits. To tackle this question, conditional mutants are excellent tools. Testing a series of conditional mutants, each carrying a deletion of the CB_1 receptor in different neuronal populations, for their response to high dose (10 mg/kg) of THC (Monory et al. 2007) has brought cannabinoid research closer to understanding the mechanisms of the pharmacological effect of this drug. Mice lacking CB_1 receptors in GABAergic neurons (GABA-$CB_1^{-/-}$) responded to THC in a similar way to wild-type littermates, whereas deletion of the receptor in all principal forebrain neurons (CaMK-$CB_1^{-/-}$) abolished or strongly reduced the behavioural and autonomic responses to the drug. Deleting CB_1 receptors only from glutamatergic cortical neurons (Glu-$CB_1^{-/-}$) strongly affected locomotor and hypothermic effects of THC, but left THC-induced nociception and catalepsy unaffected, suggesting that these effects are probably mediated by non-cortical projecting neurons. Deletion of CB_1 receptors from the majority of striatal neurons and a subpopulation of cortical glutamatergic neurons (D1-$CB_1^{-/-}$), on the other hand, blocked the cataleptic effect of the drug. However, there are still open questions regarding the in vivo THC pharmacology in mice. For example, cataleptic effects of THC are abolished in D1-$CB_1^{-/-}$, where the CB_1 receptor is missing in dopamine receptor D1-expressing striatal medium spiny neurons and in a small group of layer VI cortical pyramidal cells (Monory et al. 2007). However, cataleptic effects of THC are present in GABA-$CB_1^{-/-}$ where all GABAergic neurons (including medium spiny neurons) lack CB_1 receptors. Similarly, this effect is present in the Glu-$CB_1^{-/-}$, where cortical pyramidal cells (including those in layer VI) lack the CB_1 receptor. However, in CaMK-$CB_1^{-/-}$, where both striatal GABAergic and cortical glutamatergic neurons lack CB_1 receptor expression, THC was not able to induce catalepsy. These apparently puzzling results point to the importance of neuronal circuits, rather than specific neuronal populations, in mediating complex brain functions.

In the above study, THC-induced analgesia was not affected by deleting CB_1 receptors from either GABAergic, cortical glutamatergic or dopaminoceptive neurons. However, THC had no nociceptive effects in CaMK-$CB_1^{-/-}$. This suggests that non-cortical, non-striatal projecting neurons might play an important role in CB_1 receptor-mediated nociception. However, as the recombination pattern of CB_1 receptors in the spinal cord of CaMK-$CB_1^{-/-}$is not known, it is unclear how much of these effects are mediated by spinal versus supraspinal CB_1 receptors. The participation of CB_1 receptors in pain was recently studied (Agarwal et al. 2007). These authors generated a conditional mutant line in which the CB_1 receptor was specifically deleted in nociceptive neurons localised in dorsal root ganglia, while preserving its expression in the CNS. The nociceptor-specific loss of CB_1 receptors substantially reduced the analgesia produced by local and systemic, but not intrathecal, delivery of cannabinoids, suggesting that the peripheral rather than the central terminals of nociceptors are the important site of cannabinergic modulation. However, a smaller albeit important part of nociception is mediated by centrally expressed CB_1 receptors.

The participation of specific CB_1 receptor populations was also addressed in experimental autoimmune encephalomyelitis (EAE) (Maresz et al. 2007). To this

end, two new CB_1 receptor conditional mouse lines were generated; one lacks CB_1 receptor expression in the entire CNS and another lacks CB_1 receptor in T cells. Additionally, in this study, CB_2 receptor-deficient mice were also included. Results showed that CB_1 receptors in the CNS, but not on T cells, were able to ameliorate disease symptoms, while the presence of CB_2 receptors on T cells was critical in suppressing autoimmune reaction in EAE.

The analysis of these conditional CB_1 receptor mutants also provided new insights into the role of the ECB system in behavioural and endocrine stress responses (Steiner et al. 2008b). Glu-$CB_1^{-/-}$ showed decreased passive stress coping (i.e. decreased immobility) in the forced swim test (FST), while GABA-$CB_1^{-/-}$ and CaMK-$CB_1^{-/-}$ behaved as wild-type littermate controls. Interestingly, FST-induced corticosterone secretion was only increased in CaMK-$CB_1^{-/-}$, but not in Glu-$CB_1^{-/-}$ and GABA-$CB_1^{-/-}$, indicating that behavioural and neuroendocrine acute stress coping in response to FST engage different neuronal subpopulations containing CB_1 receptors. While the CB_1 receptors on GABAergic terminals are not crucially involved in these responses, CB_1 receptors on glutamatergic terminals appear to have differential functions: CB_1 receptors on cortical neurons are accountable for the behavioural responses and on subcortical neurons for the endocrine responses. In addition, these conditional mutants may help in understanding the puzzling effects of the CB_1 receptor antagonist rimonabant in the FST, where antidepressant-like effects were reported (Steiner et al. 2008a).

Recent investigations using conditional CB_1 receptor mutants were also able to show the functional importance of CB_1 receptor in hepatocytes (Jeong et al. 2008, Osei-Hyiaman et al. 2008). Diet- and ethanol-induced steatosis did not occur in the conditional mutants. Indirect calorimetry furthermore showed that CB_1 receptor deletion in hepatocytes leads to increased fat burning, which was further enhanced by systemic rimonabant treatment (Osei-Hyiaman et al. 2008), indicating that organs other than the liver are involved in metabolic processes controlled by CB_1 receptors.

2.4 Complications with Cre Recombinase-Expressing Lines

Conditional mutants are valuable tools in deciphering the exact function of CB_1 receptors and other components of the ECB system at their numerous expression sites. However, even these sophisticated tools are not without limitations.

One such limitation is the unwanted ectopic Cre recombinase expression, causing target gene deletion in cell populations that were not intended to be included. Such recombination may lead to serious problems in the interpretation of the resulting phenotype. It is therefore very important to analyse thoroughly the expression pattern of the targeted gene in the conditional mutants, before a detailed phenotype analysis takes place.

Another possible problem is that the Cre recombinase-expressing transgene might cause some unspecific effects apart from excising the desired target gene. For this reason, it is very important to perform control experiments with mice that express

only Cre recombinase, but do not contain the targeted "floxed" alleles. The phenotype of these mice should be compared to wild-type littermates in order to ensure that Cre recombinase expression alone has no effect on a specific behaviour studied.

Transgenes that engage a cell-type specific expression of Cre recombinase in the adult may also be active in germ cells to some extent under certain circumstances. Subsequently, this causes germ line deletion of the target gene. Offspring resulting from such a gamete will have a complete knockout on the allele that is derived from the affected gamete. Should this mutation not be recognised in time, the mutation could spread and soon the breeding colony would contain increasing numbers of complete knockout mice. Certainly, these mice would still express Cre recombinase in the normal, Mendelian manner. Consequently, genotyping for the presence of Cre recombinase itself would not reveal this problem. It is, therefore, highly important to genotype conditional mutants regularly for the existence of the "floxed" target gene, too.

3 Methodological Considerations on Behavioural Experiments with Mutant Mice

There are several important considerations when planning to carry out behavioural experiments with transgenic mice. These include sample size, genetic background, age and sex of the animals, breeding scheme, and choice of proper controls. The following paragraphs are not exhaustive, but do reflect numerous issues which are often encountered by researchers performing behavioural experiments with transgenic animals. Several recent overview articles have also covered this theme (Crawley 2008; Sousa et al. 2006).

The individual variations in behaviour are considerable, even though congenic lines are used in most of the cases. Therefore, the number of animals per experimental group has to be large enough for the standard deviation not to exceed the level that would conceal between-group differences. In practical terms, this mostly means a sample size of 10–12 (Crawley and Paylor 1997). Very few tests give such uniform data that less than 10 mice are enough for the experiments.

To reduce within-group variability, it is important that the genetic background of the animals is as homogenous as possible. Scores of new mutant mouse lines are generated every year using embryonic stem cell techniques. The genomic composition of these animals depends on the origin of genomic DNA used to generate the targeting construct, the origin of the ES cell line, and the strain of mice for mating the chimaeras and for conducting the backcrossing (Bockamp et al. 2002). The very robust ES cell lines that contribute most effectively to the germ line following blastocyst injections are derived from the 129/Sv mouse strain, while this strain is only seldom used by behavioural scientists. Therefore, before any behavioural experiment can be done, the new transgenic mice have to be backcrossed through a minimum of 5–6 generations into the desired wild-type strain.

The choice of the wild-type strain for backcrossing depends on the type of experiments that are to be carried out, as genetic differences between mouse strains can have a great impact on the observed phenotype. To begin with, certainly, as the goal is genetic uniformity, an inbred strain is called for. However, there are many available inbred strains (and sub-strains), and their characteristics have to be kept in mind for the right choice (Brooks et al. 2004, 2005; Nguyen and Gerlai 2002; Taft et al. 2006). A mouse strain that has difficulties in learning *per se* will not be suitable for studying a gene function in learning and memory. Similarly, a mouse strain that will not drink ethanol cannot be used for alcohol addiction studies.

Another consideration is the fertility and productiveness of the strain in question: if there are only 2–3 pups in one litter, there might be only one or less animal per litter with the desired experimental genotype. This would require the establishment of about 30–35 breeding pairs to secure ten mutant and ten wild-type control mice (considering that normally only males are used for behavioural experiments). Bearing in mind the costs, the animal facility space requirements and the manpower needed, together with the risk of not observing a phenotype in the experiment, this is by and large not a viable plan.

Wild-type strains are available from several companies that have their own colonies. These colonies, though originating from the same founder, might develop some gene mutations over time. Some of these mutations are simply not known, but others are identified. For example, the C57BL/6J line from Harlan carries a null mutation for α-synuclein (Specht and Schoepfer 2001; Wotjak 2003), a gene known to be involved in learning and memory as well as in neurodegenerative diseases such as Parkinson and Alzheimer disease. Therefore, if the mutation could potentially influence the outcome of the experiments (e.g. studies on Parkinson disease in this case) choosing another strain or another supplier is advisable.

Choosing the proper controls for a behavioural experiment is vital. In this regard, there is often a conflict between good scientific practise and available resources. However, the importance of littermate controls cannot be emphasised enough. Why is it so important? As mentioned above, the genetic background of a transgenic mouse line is never 100% identical to a chosen wild-type strain. Therefore, by comparing your mutants to a certain wild-type strain, the effects of several variables are assessed – most of whose identity one does not even know. Importantly, complex behaviours (especially anxiety traits) often depend on early life experiences. Animals that grew up in different environments (e.g. a supplier company's vs. a laboratory's animal facility) will certainly behave differently in a test. Yet this observed phenotype might not have anything to do with the mutation the transgenic animal carries. Importantly, early experiences also include the effects of maternal behaviour. A genetic mutation might influence maternal care in many different ways – starting from extent of fertility through proper milk composition to enough nursing provided. Thus, even breeding mutants and wild-types separately could introduce variables that are not the direct consequences of the mutated gene in the observed individual but in the mother. The best way to exclude these confounding variables is to set up a breeding scheme where the generated mutant and control mice will be littermates. When breeding knockouts, this means heterozygous

mating. The disadvantage of the method is that only 25% of the offspring is knockout, 25% is wild-type littermate and the rest, 50%, is "unwanted" heterozygous. This setup also requires more storage space in the animal facility, more manpower and higher costs for genotyping. Still, in the long run, this method pays back, as it gives clearer results with less room for misinterpretation. Whatever the decision, however, one rule has to be kept in mind: when it comes to publication, the mating scheme and the origin of the control group must be unambiguously traceable for the reader.

On the other hand, with the advent of more sophisticated genetic tools, double transgenic mice, such as conditional mutants, are becoming more widespread. However, setting up a breeding scheme with double heterozygous parents would result in 1 in 16 double transgenic offspring. This is clearly unrealistic in most laboratories. In this case, scientists have to weigh up which genotypes are the most important experimentally and design a breeding scheme where the relevant mutant and its control counterpart are littermates. Taking conditional knockouts as an example, a good solution is breeding mice that are transgenic or wild-type for the Cre recombinase allele, respectively, and all have the "floxed" allele in a homozygous manner.

The other considerations such as gender and age of the experimental animals do not differ in experiments with transgenic or wild-type mice. Often, relatively young adult males are used. These choices have mostly practical reasons, and though they are justifiable, it is important to keep in mind that they will to some extent limit the possible interpretations of the results. Regarding the analysis of the ECB system, it has to be kept in mind that changes in the importance of the system may occur depending on age (Wang et al. 2003; Bilkei-Gorzo et al. 2005).

Behavioural experiments are addressing complex issues, and thus the phenotypes observed are the results of complex – and frequently redundant – mechanisms. When studying knockout animals, redundancy (i.e. compensatory mechanisms) is especially an aspect to keep in mind. Therefore, the magnitude of difference between the mutant group and the controls is critical. Mutations that cause strong phenotypes in basal level conditions are mostly seriously debilitating, and thus are not easily used to tackle the subtle regulatory processes of complex behaviours. On the other hand, subtle and hidden phenotypes are obviously more difficult to deal with. The trick is to challenge the system in a way that will reveal differences between the experimental groups - a requirement easy to demand but often difficult to fulfil.

4 Caveats in Genetics and Pharmacology

To study a biological system in vivo, pharmacology and genetics are two obvious approaches to choose from. However, it is important to regard them as complementary rather than competing methods, as both approaches have some weaknesses and advantages. Therefore, using these two approaches in parallel and confirming the data obtained with the other method strengthens conclusions greatly.

What are then the main limitations of pharmacology? Pharmacology is always "dirty". There is not one drug that has 100% specificity to a given binding site.

Therefore, there will always be some other site influenced as well as that studied, potentially confounding the obtained observations. This site can be another subtype of the studied protein, but it can even be a completely different class of molecule. As an example from the ECB system, AEA is very promiscuous, as it binds, e.g., to CB_1 receptors, CB_2 receptors, and TRPV1. This may also be relevant in cases where a genetic deletion causes altered AEA levels, e.g. in CB_1 receptor-deficient mice (Di Marzo et al. 2000) or in FAAH-deficient mice (Cravatt et al. 2001). Another example is the specificity of the CB_1 receptor antagonist/inverse agonist rimonabant, for which non-CB_1 receptor/non-CB_2 receptor sites were proposed (Jin et al. 2004; Haller et al. 2004).

Another point is whether the receptor in question is constitutively active in vivo. If so, instead of simply blocking the receptor with an antagonist resulting in an effect opposite to agonist stimulation, agonist treatment will prevent in this case the action of tonically released endogenous ligands on the receptor. A similar effect is seen when inverse agonists are used for pharmacological intervention. These molecules stabilise the receptor in a third conformational state different from the agonist-activated or the antagonist-inactivated ones. Importantly, several cannabinoid antagonists, most prominently rimonabant, are considered as inverse agonists. Even though site-directed mutagenesis studies could show which amino acids might be responsible for this effect in the amino acid chain of CB_1 receptor (McAllister et al. 2003; Hurst et al. 2002), in an in vivo situation there is still an open question as to how much the effects of rimonabant are due to its inverse agonist effect and how much to its blocking the effects of tonically released ECBs (Hentges et al. 2005).

This kind of problem is less prevalent in a genetically modified model. If a certain protein is genetically inactivated, the observed phenotype should be a specific consequence of its absence. Shouldn't it? However, there are some important aspects to consider here, too. First, there might be some compensatory mechanisms that try to counterbalance the loss of an important protein – this mostly manifests in the up-regulation of related gene(s) in the same family. For example, this is nicely shown for the leucin-zipper transcription factors CREB/CREM (Mantamadiotis et al. 2002). For cannabinoid receptors, such an up-regulation has not yet been reported. Another important aspect is the developmental effects of genes. Inactivation of genes that participate in developmental processes might leave the animal with malformed organs, aberrant wiring, changed hormonal secretion or altered motor ability, to name but a few. If these effects are not known or hidden, the resulting phenotype might be interpreted as the effect of the lack of acute activation of the investigated gene product. This can be tested by pharmacological treatment of wild-type animals with an antagonist. If the phenotype is the result of an acute lack of the gene product, it will be reproducible by pharmacological blockade of the protein. For example, this is illustrated by the involvement of CB_1 receptors in extinction of aversive memories (Marsicano et al. 2002) and protection against kainic acid-induced seizures (Marsicano et al. 2003).

On the other hand, possible compensatory processes or developmental effects in knockout mice may explain discrepant results between genetic and pharmacological

invalidation. It was reported that CB_1 receptor-deficient mice have different phenotypes as compared to pharmacologically treated wild-type mice, as illustrated by the fact that CB_1 receptor-deficient mice show a depression-like behaviour (Steiner et al. 2008c), while the CB_1 receptor antagonist rimonabant induces antidepressant-like behaviour as monitored in the FST (Steiner et al. 2008a). Another example is that genetic inactivation of CB_1 receptors and pharmacological treatment with rimonabant resulted in differential effects on "non-associative" memory and forebrain monoamine concentrations in mice (Thiemann et al. 2007).

A possible solution to circumvent developmental effects in genetic approaches is gene inactivation with a spatiotemporal specificity. Gene inactivation in the adult stage should ensure that phenotypic alterations are not caused by developmental deficits, although compensatory processes in the adult brain cannot be excluded. In this respect, even conditional mutants might not be fully devoid of developmental effects (Berghuis et al. 2007; Mulder et al. 2008).

5 Perspectives

The combination of genetic and pharmacological experiments has given an impressive number of novel insights into the physiological and pathophysiological roles of the ECB system. This regulatory system, however, contains a high intrinsic complexity, which requires special attention in the design of genetic experiments. (a) As ECB signalling is active throughout neural development, genetic inactivation should occur in the adult stage in case of investigations on adult processes. (b) Particularly in the nervous system, ECB signalling acts in a sophisticated spatiotemporal manner. Thus, spatial and temporal specificity in inactivation experiments are highly important in order to allow firm conclusions from these genetic experiments. (c) As the synthesising and degradation machinery of ECBs appears to be redundant, the genetic analysis of these components will be very difficult and remains a special challenge. (d) The application of adeno-associated virus and other viral systems appears to be very useful in order to inactivate "floxed" genes in specific brain regions (Monory et al. 2006), to introduce RNA silencing constructs (Xia et al. 2004), or to overexpress genes in a spatiotemporal manner (Klugmann et al. 2005). It is hoped that the combination of the techniques discussed will give novel insights into this fascinating neuroregulatory system.

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