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Role of the Cannabinoid Receptor Type 1 (CB1) in Synaptic Plasticity, Memory and Emotionality

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“If a man will begin with certainties, he shall end in doubts; but if he will be content to begin with doubts he shall end in certainties.”

Francis Bacon

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Abbreviations

ACh	acetylcholine
2-AG	sn-2-arachidonylglycerol, an endocannabinoid
AM404	n-(4-hydroxyphenyl)-arachidonoylamide, an AMT inhibitor
AM251	N-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide, a CB1 antagonist
AMPA-R	α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid receptor
AMT	anandamide membrane transporter
BNST	bed nucleus of the stria terminalis
CA1	cornu ammonis field 1
CB1	cannabinoid receptor type 1
CCK	cholecystokinin
Cox-2	cyclooxygenase 2, a 2-AG degrading enzyme
CP55940	(-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol, a CB1 agonist
CS	conditioned stimulus
DAGL	diacylglycerol lipase, the 2-AG synthesizing enzyme
DG	dentate gyrus
DSE	depolarisation-induced suppression of excitation
DSI	depolarisation-induced suppression of inhibition
EEG	electroencephalography
EPSC	excitatory postsynaptic current
FAAH	fatty acid amide hydrolase, an anandamide degrading enzyme
fEPSP	field excitatory postsynaptic potential
GABA	gamma-aminobutyric acid
GR	glucocorticoid receptor
5-HT	5-hydroxytryptamine or serotonin
5-HT3-R	5-hydroxytryptamine receptor type 3
HFS	high-frequency stimulation
HU-210	(6aR)-trans-3-(1,1-dimethylhepthyl)-6a, 7, 10, 10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol, a CB1 agonist
IPSC	inhibitory postsynaptic current
Ko	knockout mouse
LTD	long-term depression
LTD-E	long-term depression of excitation
LTD-I	long-term depression of inhibition
LTP	long-term potentiation
mAChR	metabotropic acetylcholine receptor
MAPK	mitogen-activated protein kinase
MGL	monoglyceride lipase, a 2-AG degrading enzyme
MR	mineralocorticoid receptor
NMDA-R	n-methyl-d-aspartate receptor
PPD	paired-pulse depression
PPF	paired-pulse facilitation

RI	recognition index
SR141716	N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl, also called rimonabant, a CB1 antagonist
TBS	theta-burst stimulation
Δ^9-THC	Δ^9-tetrahydrocannabinol, a CB1 agonist
URB597	cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester, a FAAH inhibitor
US	unconditioned stimulus
VR1	vanilloid receptor 1
WIN55,212-2	R-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinyl)methyl]pyrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate, a CB1 agonist
Wt	wildtype mouse

Abstract

The present work focused on the role of the cannabinoid receptor type 1 (CB1) in synaptic plasticity, memory and emotionality in mice. CB1 is abundantly expressed in the central nervous system and is mainly (if not exclusively) located on GABAergic and glutamatergic nerve cells. CB1 is a G-protein coupled receptor which is essentially inhibiting transmitter release from presynaptic GABAergic or glutamatergic nerve terminals.

To differentiate between the physiological significance of CB1 expressed on glutamatergic and GABAergic nerve terminals, the studies included work with three different CB1-deficient mouse lines: A conventional knock-out mouse line (total-CB1-ko mice) with a deficiency of CB1 in the entire brain and two conditional knock-out mouse lines using the Cre/lox P recombination system, and leading to cell type specific deficiency of CB1 on GABAergic neurons (GABA-CB1-ko mice) or glutamatergic neurons (Glu-CB1-ko mice).

As a common model for alterations in synaptic plasticity and hippocampus-dependent memory, we studied long-term potentiation in the hippocampus at first. The hippocampus is an essential brain structure being involved in spatial and episodic-like memory. We showed that there is an increase of hippocampal LTP *in vivo* at the perforant path-dentate gyrus granule cell synapse in total-CB1-ko mice, but failed to detect any difference in LTP levels for GABA-CB1-ko and Glu-CB1-ko mice. Also, short-term plasticity using a paired-pulse stimulation protocol is unchanged in the three mouse lines. Eventually, augmented theta rhythm that is believed to underlie enhanced cognitive abilities could not be found in total-CB1-ko mice.

Our hypothesis of memory improvement in CB1-deficient mouse lines could not be verified in three tests for memory that are based on a spontaneous preference for novelty: The social recognition test, the object recognition test and the open field habituation test. We consequently tested the mice in two memory tasks that rely on an aversive test situation. In the water maze spatial discrimination task, again no differences could be assessed for acquisition of the task in total-CB1-ko and Glu-CB1-ko mice. Curiously, Glu-CB1-ko mice demonstrate more flexible behaviour in reversal learning indicating that CB1 on glutamatergic neurons may lead to perseverant and persistent behaviour. Eventually, we could show for the first time that there is a differential contribution of CB1 on either GABAergic neurons or glutamatergic neurons in the background contextual fear conditioning task. Here, mice were tested in the shock context and in a different context containing the grid floor as a similar aspect to the shock context, called grid context. GABA-CB1-ko mice reveal increased fearful behaviour specifically in the grid context. This might indicate an increased context generalisation and/or a feature learning strategy in GABA-CB1-ko mice. In contrast, Glu-CB1-ko mice display increased fearful behaviour specifically for the shock context, indicating a conjunctive learning strategy. Total-CB1-ko mice showed an increased fear response in both contexts, representing a mixed phenotype of Glu-CB1-ko and GABA-CB1-ko mice. Another novel finding confirming a large body of evidence is the fact that total-CB1-ko and Glu-CB1-ko mice manifest a deficit of extinction for the conditioned tone, providing first evidence that CB1 on glutamatergic neurons is essential for short-term extinction of auditory-cued fear memory.

Any changes in memory performance might be obscured by altered emotionality in the knockout mouse lines. In classical tests for anxiety such as the elevated plus-maze and the light/dark box, we found a tendency of increased anxiety in total-CB1-ko and Glu-CB1-ko mice and a tendency of a decrease of anxiety in GABA-CB1-ko

mice at most. Strikingly, we were able to show that CB1-ko and Glu-CB1-ko mice, in contrast to GABA-CB1-ko, avoid the open arms of the elevated plus-maze more than wildtype mice on a second exposure to the maze indicating an increased one-trial sensitisation. Furthermore remarkably, CB1-ko and Glu-CB1-ko mice showed increased anxiety-related behaviour whereas GABA-CB1-ko mice revealed an unchanged or anxiolytic phenotype in three different tests of emotionality: The open field test, the novel object exploration test and the novel juvenile exploration test. These tests were carried out under low and high light conditions. Here, as opposed to the elevated plus-maze and the light/dark box, the animals cannot retract from an aversive situation that is bright light in the testing environment which may cause sufficient activation of the endocannabinoid system thus leading to a detectable and profound phenotype in the animals. Interestingly, altered emotionality seems to depend on the averseness of the test situation, as CB1-ko and Glu-CB1-ko animals do not or only mildly differ from their wildtype littermates under lowly aversive conditions but show increased anxiety under highly aversive conditions in the aforementioned tests. This strongly suggests that the endocannabinoid system might dampen states of anxiety in highly aversive and stressful environments. More precisely, CB1 on GABAergic neurons rather leads to an anxiogenic effect, whereas CB1 on glutamatergic neurons prominently leads to an anxiolytic phenotype which we refer to as “the Yin and the Yang effect” of CB1 in emotionality.
Altogether, our study illustrates the value of conditional mouse mutants for which cell-type specific ablation of a gene of interest exist in order to understand the role of CB1 in synaptic plasticity, memory and emotionality. Our findings add another level of complexity to the picture of endocannabinoid action in fear and anxiety, which has to be considered if the endocannabinoid system is going to be exploited as a therapeutic target for the treatment of anxiety disorders.

1. Introduction

1.1 May CB1 be with you!

"*Cytokine network, epilepsy, long-term potentiation, tolerance to morphine, metabolic syndrome, ischemia, neurogenesis, body weight, human breast cancer, seizures, fear extinction, neural cell development, plasticity in the CNS, emotional learning, neuropathic pain, obesity, contextual learning and memory, schizophrenia, addiction, excitotoxic damage, pain suppression, control of motor function, Parkinson's disease*."
Typing in the term "CB1" into the literature search engine *PubMed* leads to a sheer endless list of keywords the cannabinoid receptor type 1 (CB1) is associated with. Especially, CB1 and its ligands, the cannabinoids, have been shown to have a plethora of effects on nerve cells, brain circuits and behaviour and the literature is still expanding.
At least since the psychedelic 1960ies and the hippie era, cannabis products, i.e. leaves and blossoms of the cannabis plant (*Cannabis sativa*), are known for their powerful mind-altering effects. The physiological and psychoactive effects of cannabis intoxication are vast and cannabis products can be used for medical treatment as: Analgesic, anaesthetic, anticonvulsant, tranquilizing, anti-inflammatory, antispasmodic, antiemetic or appetite stimulating agents (Ameri, 1999). The cannabis plant comprises approximately 60 cannabinoid compounds with the primary psychoactive constituent being isolated in the 1960ies as Δ^9-tetrahydrocannabinol (Δ^9-THC) (Mechoulam and Gaoni, 1965), and today we know a large number of naturally occurring or synthetic cannabinoid compounds (Pertwee, 1999).
The effects of cannabinoids could be better understood when, in 1990, the cannabinoid receptor type 1 (CB1) was molecularly cloned from rat brain (Matsuda et al., 1990). This initiated the hunt for an endogenous ligand, that was firstly discovered in 1992 as n-arachidonoylethanolamine, also called anandamide (from the Sanskrit word *ananda*, meaning "bearer of internal bliss and tranquillity") (Devane et al., 1992;Zuardi, 2006). CB1, its endogenous ligands and its synthesising and degrading enzymes constitute what is now referred to as the *endocannabinoid system*. The function of the endocannabinoid system may be very different from the intoxicate effects of exogenously administered cannabinoids, and several functional roles have already been implicated, including the modulation of pain (Calignano et al., 1998), feeding (Di Marzo et al., 2001), drug dependence (Ledent et al., 1999), neuroexcitotoxicity (Monory et al., 2006), depression (Gobbi et al., 2005) and cognition (Terranova et al., 1996).

1.2 Overview of the endocannabinoid system: Neuroanatomy of CB1

To date, two cannabinoid receptors have been cloned that is CB1 and the cannabinoid receptor type 2 (CB2). Whereas CB2 is primarily localised on immune system cells and tissues (Munro et al., 1993), CB1 is exclusively expressed in the central nervous system (Herkenham et al., 1990) and is the subject for investigation of the present study. A simplified illustration of the endocannabinoid system is given in Figure 1. The anatomical localisation of CB1 in the brain has been visualised using *in situ* hybridisation to detect mRNA levels, and autoradiography and immunohistochemistry of the receptor itself. CB1 is virtually omnipresent in the brain and can be found in several regions including neocortex, hippocampus, nucleus accumbens, basal ganglia, hypothalamus, amygdala, and cerebellum (Herkenham et

al., 1990). Within the hippocampus, CB1 is very densely expressed in the molecular layer of the dentate gyrus, and also in the stratum pyramidale and stratum lacunosum moleculare of ammons horn (CA3 and CA1 region) as well as the subiculum (Herkenham et al., 1990).
On a cellular level, CB1 is undetectable on somatic cell membranes and dendrites, but is highly expressed on axons and axon terminals, i.e. the presynaptic compartment, as could be shown by electron microscopical studies (Katona et al., 1999;Katona et al., 2001). Importantly, CB1 can be found on gamma-aminobutyric acid-ergic (GABAergic) neurons, that are mainly interneurons, and glutamatergic neurons (pyramidal cells, granule cells, and mossy cells in the hippocampus and principal neurons elsewhere), which was clearly revealed only very recently (Domenici et al., 2006;Katona et al., 2006;Kawamura et al., 2006;Marsicano et al., 2003;Monory et al., 2006). Remarkably, on excitatory terminals, the level of CB1 is about 10-20 times lower than on inhibitory terminals (Kawamura et al., 2006). Focusing more precisely on the hippocampus, CB1 is expressed at high levels on inhibitory terminals of cholecystokinin(CCK)-positive basket cells (85% of these CB1-positive interneurons contain CCK) (Hajos et al., 2000;Katona et al., 1999;Tsou et al., 1999). CCK-positive GABAergic neurons clasp about 40% of all GABAergic cells in the hippocampus (Marsicano and Lutz, 1999).
CB1 distribution in the amygdala is markedly heterogeneous (Katona et al., 2001;McDonald and Mascagni, 2001). High levels are found in the basolateral complex, nucleus of the lateral olfactory tract, the periamygdaloid cortex, and the amygdalohippocampal areas. In contrast, CB1 is sparsely expressed in the medial, central, and intercalated nuclei. CB1 is primarily expressed on GABAergic CCK-containing axon terminals, but it is likely that glutamatergic neurons carry CB1, too (Domenici et al., 2006), similarly as it is clearly shown for the hippocampus and cerebellum (Kawamura et al., 2006).
It seems to turn out that beside of GABAergic and glutamatergic cells, cholinergic and dopminergic nerve terminals in the hippocampus express CB1 as well to a relatively high percentage (Degroot et al., 2006).

1.3 CB1 and its signal transduction pathways

As member of the G protein-coupled receptor family (GPCR), CB1 is a G-protein coupled receptor of the $G_{i/0}$ type, with a typical seven-transmembrane-spanning structure. It is expressed on the cell surface with its binding domain exposed to the extracellular space. CB1 is much more densely expressed in the brain than any other G-protein-coupled receptor. In several brain regions CB1 is present in densities that are comparable to those of GABA or glutamate receptor channels. Binding of a ligand causes a dissociation of α and βγ G- protein subunits from CB1. Release of the α subunit leads to inhibition of adenylyl cyclase, thereby reducing cAMP levels in the cell and altering the activity of cAMP-dependent protein kinases. The βγ subunit may directly inhibit different ion channels (Freund et al., 2003;Howlett, 2002). CB1 has been reported to modulate the activity of N- and P/Q-type voltage-dependent calcium channels and to enhance the activation of the voltage-dependent A-type potassium channel and the inwardly rectifying potassium channel (Deadwyler et al., 1995;MacKie et al., 1995). A third well characterised messenger system of CB1 is the stimulation of mitogen-activated protein kinase (MAPK) (Bouaboula et al., 1995).

1.4 Endocannabinoids

Beside of the afore mentioned anandamide, a variety of endocannabinoids were identified to date. Endocannabinoids comprise a family of eicosanoid and related unsaturated fatty acid derivatives that can stimulate CB1. Altogether five endocannabinoids have been discovered so far, with anandamide and 2-arachidonylglycerol (2-AG) being the most dominant and best investigated ones (Howlett, 2002). The regional distribution of anandamide and 2-AG correspond quite closely with each other, with the highest concentrations found in the striatum, brainstem, hippocampus, cerebellum and neocortex (Fride, 2005). Noteworthy, anandamide and 2-AG did not match so well with CB1 distribution (Bisogno et al., 1999). One could assume that endocannabinoids may activate additional receptors (as will be discussed in more detail below), but it could also be explained by cellularly inhomogeneous receptor densities on highly specific populations of neurons.
Endocannabinoids are synthesised and released on demand from the soma and dendrites in a non-vesicular manner, and act on cells located near their site of synthesis in a paracrine fashion (Lutz, 2004). Some evidence suggests that endocannabinoids are synthesised constitutively resulting in a constant activation of CB1 (Losonczy et al., 2004). The synthesis and release of endocannabinoids requires two steps: (1) Neuronal depolarisation and activation of voltage-gated Ca^{2+} channels (VGCCs) that elevate intracellular calcium concentration to a micromolar range, and/or (2) activation of the G_q-protein-coupled metabotropic glutamate receptor 1 or 5 (mGluR1 and mGluR5) (Maejima et al., 2001;Ohno-Shosaku et al., 2002a) and/or (3) activation of muscarinic acetylcholine receptors 1 or 3 (mAChR1 and mAChR3) (Kim et al., 2002;Ohno-Shosaku et al., 2003) and the resulting activation of phospholipase Cβ (PLCβ) (Hashimotodani et al., 2005) and (4) there is some evidence that activation of dopamine D_1 receptors can decrease levels of anandamide and 2-AG in the limbic forebrain, and activation of dopamine D_2 receptors can stimulate anandamide efflux in the dorsal striatum (Giuffrida et al., 1999;Patel et al., 2003). Eventually, (5) glucocorticoids can feed back onto hypothalamic neurons via endocannabinoids and thereby block the release of neuropeptides (Di et al., 2003).
The biosynthesis and metabolic pathways of the endocannabinoids, anandamide and 2-AG, have several features in common. For synthesis of anandamide, n-arachidonoyl-phosphatidylethanolamine is cleaved by phospholipase D. In the case of 2-AG, phosphatidylinositol is cleaved to yield 1,2-diacylglycerol, catalysed by phospholipase C. Diacylglycerol in turn is converted into 2-AG, catalysed by diacylglycerol lipase (DAGL). Both steps additively lead to the synthesis of 2-AG (Piomelli, 2003). Anandamide and 2-AG then travel backwards across the synaptic cleft, acting as retrograde messengers at CB1, present on the presynaptic axon terminal (Alger, 2002;Kreitzer and Regehr, 2002). Endocannabinoids are rapidly removed from the extracellular space. Both release and removal seem to be facilitated by a membrane transport process (called anandamide membrane transporter, AMT) that is not molecularly characterised yet. In the presynapse and postsynapse, endocannabinoids are degraded via intracellular enzymatic hydrolysis, being accomplished by fatty acid amide hydrolase (FAAH) for anandamide, and monoglyceride lipase (MGL) and probably cyclooxygenase-2 (COX-2) for 2-AG (Gulyas et al., 2004;Hashimotodani et al., 2007;McKinney and Cravatt, 2005;Slanina and Schweitzer, 2005). A simplified overview of the endocannabinoid system is depicted in Figure 1.

The affinity and activity of endocannabinoids at CB1 can be very different. Anandamide rather behaves like a partial agonist of CB1 in most assays of functional activity, whilst 2-AG appears to be a full agonist, although its affinity to CB1 is lower than that of anandamide (Pertwee, 2005b). Also, anandamide is degraded in the brain primarily at a postsynaptic site but not in presynaptic terminals whereas 2-AG degradation occurs pre- and postsynaptically. Consequently, effects of anandamide on neurons are longer lasting than those of 2-AG, which acts only transiently (Hashimotodani et al., 2007). Therefore, 2-AG and anandamide might function as short-lived and long-lived retrograde messengers in the brain, respectively.

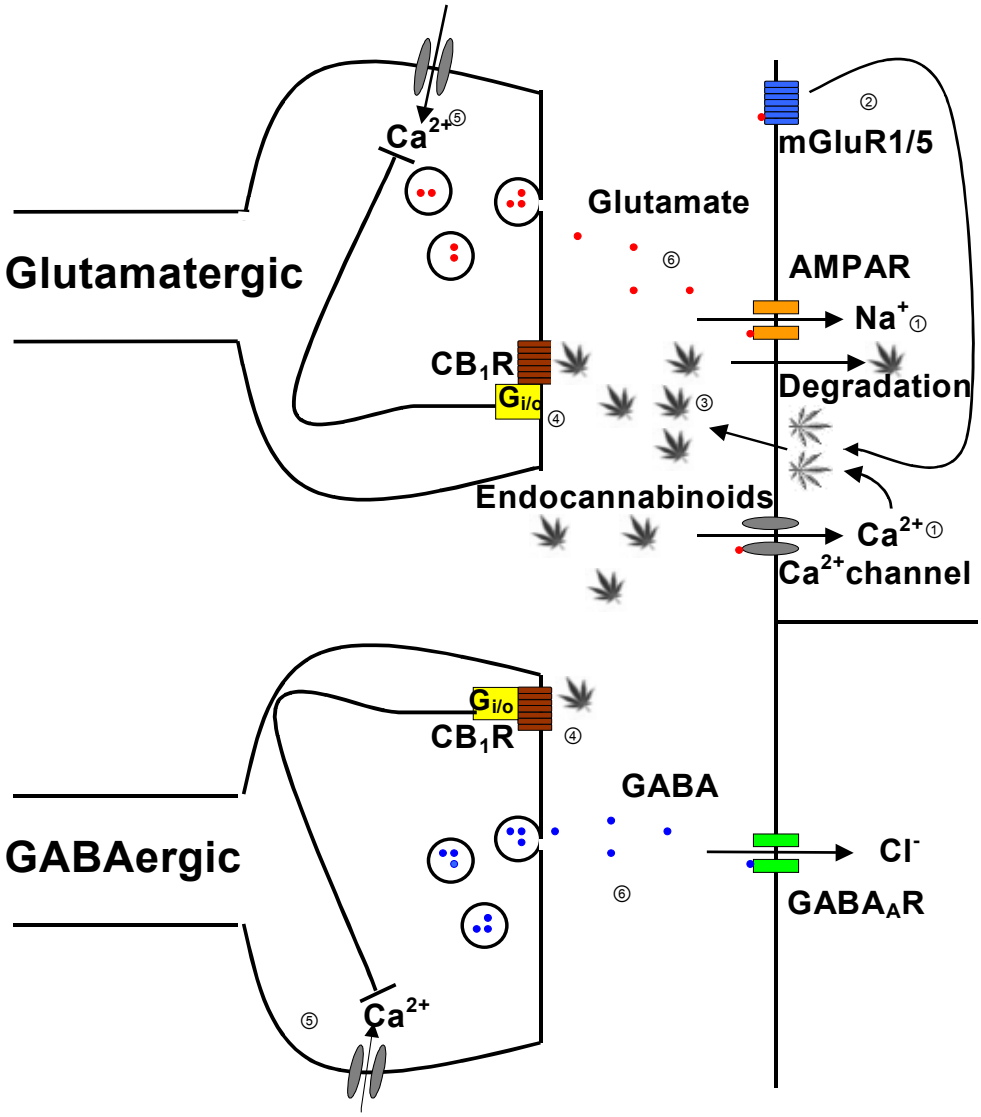


Figure 1: Schematic and simplified overview of the encocannabinoid system. After depolarisation of the postsynaptic membrane and influx of calcium ions (1) or activation of mGluRs (2), endocannabinoids are synthesised from membrane lipids and released from the postsynpase (3). They then travel backwards through the synaptic cleft and bind to CB1 expressed by glutamatergic and GABAergic neurons (4). Activation of CB1 which is a $G_{i/o}$ coupled receptor leads to a block of Ca^{2+} channels amongst other processes (5). Eventually, transmitter release is decreased (6). Endocannabinoids are degraded in the pre- and postsynaptic compartment (not shown).

1.5 Effects of CB1 activation on transmitter release

Activation of CB1 has several effects, with most of them potentially and ultimately resulting in hyperpolarisation of the cell and reduction of release of the transmitters GABA and glutamate at the presynapse:

1) CB1 activation can lead to downregulation of voltage-dependent N-type and P/Q-type calcium channel (VGCC) activity (Mackie et al., 1995), that are known to be required for release of transmitter from hippocampal synapses (Shen and Thayer, 1998),
2) CB1 activation can cause upregulation of activity of A-type and inwardly rectifying potassium channels (Deadwyler et al., 1995;MacKie et al., 1993;Schweitzer, 2000), an effect that is mediated by inhibition of adenylyl cyclase and decreases PKA-dependent phosphorylation of the channel (Mu et al., 2000),
3) CB1 activation inhibits the production of cyclic adenosinmonophosphate (cAMP) by negatively regulating adenylyl cyclase. Thereby, cAMP-dependent protein kinase A (PKA) signalling is downregulated with consequences on possibly reduced LTP and less depolarisation (Childers and Deadwyler, 1996;Hoffman and Lupica, 2000;Twitchell et al., 1997;van Beugen et al., 2006).
4) CB1 activation leads to phosphorylation and thereby activation of p42/p44 mitogen-activated protein kinase (MAPK, also known as extracellular signal-regulated kinase 1 and 2, ERK1 and ERK2), leading to the expression of immediate early gene zif268 and c-fos (Derkinderen et al., 2003). ERK is involved in synaptic plasticity and appears to be important during the early phase of LTP (Adams and Sweatt, 2002;Winder et al., 1999). Zif268 is a transcription factor that is essential for the transition from short- to long-term synaptic plasticity and for the expression of long-term memory (Jones et al., 2001a). The localised increase in endocannabinoid production could facilitate local synaptic efficiency by activating signalling pathways important for a long-term modification.

A direct evidence for the regulation of transmitter release of GABA and glutamate is the decrease of the frequency of evoked quantal synaptic events after application of CB1 agonists, measured electrophysiologically (Auclair et al., 2000;Shen et al., 1996). A decrease of radiolabelled neurotransmitter GABA and glutamate can also be measured chemically in hippocampal slices that were previously incubated with tritium-labelled GABA or glutamate (Katona et al., 1999;Kofalvi et al., 2003). The CB1 agonist WIN55,212-2 inhibited evoked $GABA_A$ receptor mediated inhibitory postsynaptic currents (IPSCs) recorded from CA1 pyramidal cells in the slice preparation (Hajos et al., 2000;Hoffman and Lupica, 2000), an effect that is absent in CB1-knockout (CB1-ko) mice (Hajos et al., 2001). In addition, a reduction of field excitatory postsynaptic potentials (fEPSPs) or excitatory postsynaptic currents (EPSCs) could be shown for different agonists thus suppressing glutamatergic transmission (Ameri et al., 1999;Ameri and Simmet, 2000;Misner and Sullivan, 1999;Shen et al., 1996;Sullivan, 1999) whereas application of the antagonist SR141716 slightly facilitates neuronal excitation (Ameri et al., 1999). Eventually, *in vivo* microdialysis could directly reveal a decrease of glutamate and GABA after application of WIN55,212-2 (Ferraro et al., 2001;Fujiwara and Egashira, 2004). WIN55,212-2 blocks IPSCs with an EC_{50} value 10 times smaller than the one necessary to suppress EPSCs in hippocampal slices (Hajos and Freund, 2002). Thus, axon terminals releasing GABA are much more sensitive to

(endo)cannabinoids than terminals that release glutamate. One could speculate that moderate neuronal activity may preferentially reduce inhibitory input, while stronger activity could suppress both excitatory and inhibitory inputs. This also suggests that two endocannabinoid signalling pathways exist in parallel in the brain with possibly different stages of recruitment. Nevertheless, endocannabinoid-mediated modulation is dependent on postsynaptic activity (Wilson and Nicoll, 2002), although it is suggested that GABAergic transmission in the hippocampus is tonically inhibited by endocannabinoids (Hentges et al., 2005;Neu et al., 2007).
Beside of the main inhibitory and excitatory transmitter, GABA and glutamate, an inhibitory effect of CB1 activation can be also observed for the monoamines noradrenalin, dopamine, and serotonin and for acetylcholine. The exact mechanism for this regulation is unclear and may be secondary to excitation or inhibition of the respective monoaminergic and cholinergic cells. Noradrenalin release can be inhibited in the hippocampus of guinea-pig and human, but not of mouse and rat (Schlicker et al., 1997). Dopamine release can be inhibited in rat caudate-putamen (Cadogan et al., 1997). Serotonin release can be blocked in mouse neocortex (Nakazi et al., 2000). Moreover, cannabinoids inhibit acetylcholine (ACh) release in the rat, mouse and human hippocampus and neocortex but not in the striatum (Gifford and Ashby, Jr., 1996;Kathmann et al., 2001a;Steffens et al., 2003). However, all these data were obtained from *in vitro* preparations and the situation might be different *in vivo*. CB1 antagonist SR141716 increases noradrenalin release in the prefrontal cortex and hypothalamus, dopamine release in the prefrontal cortex and serotonin release in the prefrontal cortex and nucleus accumbens *in vivo* (Tzavara et al., 2001;Tzavara et al., 2003a). Low doses of cannabinoids increase, whereas high doses decrease the release of ACh in prefrontal cortex and hippocampus *in vivo* (Acquas et al., 2000;Gessa et al., 1998).

1.6 Effects of CB1 activation on synaptic plasticity

Already in the 1980ies, it was shown that Δ^9-THC can inhibit high frequency stimulation (HFS)-induced long-term potentiation (LTP) in the CA1 region of the hippocampus (Nowicky et al., 1987). Later on, anandamide was also proven to have a concentration-dependent effect on LTP, although it did not block LTP completely (Terranova et al., 1995). Speaking about synaptic plasticity, we have to distinguish between short-term synaptic plasticity (in the range of seconds) and long-term synaptic plasticity (in the range of hours or days).
(1) Short-term synaptic plasticity: In the hippocampus and in the cerebellum, it could be demonstrated that brief postsynaptic depolarisation of a neuron or a train of postsynaptic action potentials can transiently suppress inhibitory GABAergic synaptic transmission onto that neuron, a process called depolarisation-induced suppression of inhibition (DSI). It could be further on shown that DSI is a retrograde signalling process, i.e. it is induced in the postsynaptic cell and expressed as a presynaptic reduction in GABA release from interneurons. This presynaptic inhibition is caused by postsynaptically released endocannabinoids (Ohno-Shosaku et al., 2001;Wilson and Nicoll, 2001). In a similar manner, endocannabinoids affect glutamatergic synapses which is called depolarisation-induced suppression of excitation (DSE) (Kreitzer and Regehr, 2001;Ohno-Shosaku et al., 2002b). DSE and DSI are responsible for short-term modifications of synaptic plasticity, and are thought to last between 5 and 60 s (Freund et al., 2003). Their resulting inhibition can not account for long-term plasticity of synapses, but may be important in facilitating depolarisation, and therefore induction of LTP during a high-frequency stimulation (Carlson et al., 2002). Indeed,

CB1-dependent long-term DSI potentiation (at least in the range of 15 min) takes place in CA1 of the hippocampus after a tetanic stimulation as has been show recently. This phenomenon could not be described for DSE (Chen et al., 2007).
(2) Long-term synaptic plasticity: A very different mechanism for endocannabinoids has been suggested recently. A normally subthreshold high frequency stimulation could induce LTP if it was delivered during the phase of DSI suggesting that the endocannabinoids released following depolarisation of a pyramidal cell may facilitate the induction of LTP as it would be predicted if they selectively reduced inhibitory GABAergic input (Carlson et al., 2002;Varma et al., 2001).
Another endocannabinoid-mediated modulation of synaptic plasticity is long-term depression (LTD). In the hippocampal CA1 region or in the basolateral amygdala, stimulation protocols that cause long-term potentiation at excitatory synapses onto pyramidal neurons simultaneously produce long-term depression at adjacent inhibitory synapses called long-term depression of inhibition (LTD-I), which is mediated by endocannabinoids (Azad et al., 2004;Chevaleyre and Castillo, 2003;Marsicano et al., 2002). Whereas DSI is triggered by a brief release of endocannabinoids, LTD-I is induced after an activation of CB1 for minutes. Together with activity-dependent DSI potentiation (Chen et al., 2007), LTD-I is a mechanism of localised and persistent reduction of certain GABAergic synapses. Ultimately, by disinhibition, LTD-I can enhance excitatory synaptic transmission. CB1 dependent long-term depression of excitation (LTD-E) was similarly shown for cortical pyramidal neurons projecting onto medium spiny neurons of the striatum (Gerdeman et al., 2002) and also at cortical pyramidal neurons synapsing onto neurons in the nucleus accumbens (Robbe et al., 2002).
What might be the functional significance of these phenomena? For example, if the excitability of the postsynaptic neuron is high, DSI can facilitate LTP of glutamatergic inputs (Carlson et al., 2002). On the other hand, in CB1 knock-out mice that lack DSI (Wilson et al., 2001), LTP is enhanced (Bohme et al., 2000;Reibaud et al., 1999). The behavioural consequence remains unclear as Hampson and colleagues observed that only very high frequency firing rates (>30 Hz) produced cannabinoid release and lead to DSI, which usually do not occur at a behavioural level of spatial exploration but do occur during slow wave sleep and in epilepsy (Hampson et al., 2003).

1.7 Aim of the study

What is the physiological role of CB1, a receptor that is so widely distributed and that simultaneously influences antagonistic (i.e. glutamatergic and GABAergic) components of the central nervous system? There are several keystone publications that lead us to the here presented investigation:

1) Genetical disruption of CB1 leads to an enhancement of LTP in two brain structures among others: The hippocampus and the amygdala (Bohme et al., 2000;Marsicano et al., 2002).
2) It was suggested that blocking of CB1, either pharmacologically or genetically, can have memory enhancing effects (Bohme et al., 2000;Reibaud et al., 1999;Terranova et al., 1996).
3) The release of endocannabinoids and the activation of CB1 modulate fear and anxiety (Bortolato et al., 2006;Kathuria et al., 2003;Marsicano et al., 2002).

As the hippocampus is essential for episodic or spatial memory (Squire, 1992) and the amygdala is required for emotional memory (LeDoux, 2000), both of which richly express CB1, we were interested in the question of which effect the activation of CB1

has on hippocampal synaptic plasticity, hippocampus-dependent learning and anxiety-related behaviour.
There are three ways of tackling the endocannabinoid system and its functions: (1) Administration of exogenous cannabinoids, (2) boosting the effect of endocannabinoids by blocking their degradation or reuptake, (3) pharmacological or genetical disruption of CB1. By the means of the first approach it is questionable if a physiological role of CB1 can be depicted when the whole brain is flooded with an agonist. Moreover, it is nearly impossible to distinguish between GABAergic and glutamatergic effects under *in vivo* conditions. Admittedly, the second approach is promising as it would focus concisely on those synapses that are active during certain behaviour. As already described above, CB1 is primarily localised on GABAergic and glutamatergic nerve terminals, where its primary effect is to decrease transmitter release. As a result of this distribution, CB1 activation might have dramatically different physiological and behavioural consequences, depending on the balance of its effects on GABAergic and glutamatergic transmission within a neural network.
We were in the ideal situation of having three different mouse lines in order to study the endocannabinoid system: (1) A conventional knock-out mouse line (below referred to as total-CB1-ko) and two conditional knock-out mouse lines using the Cre/loxP system, (2) leading to cell type specific deficiency of CB1 in GABAergic neurons (below referred to as GABA-CB1-ko) or (3) in glutamatergic neurons (below referred to as Glu-CB1-ko). It was the overall goal to pin down which population of cells carrying CB1 is mainly involved in synaptic plasticity, learning and memory, and emotionality. We were interested in the differential contribution of CB1 expressed on glutamatergic neurons and GABAergic neurons in synaptic plasticity and memory on the one hand and anxiety on the other hand, as it is summarised in Figure 2.

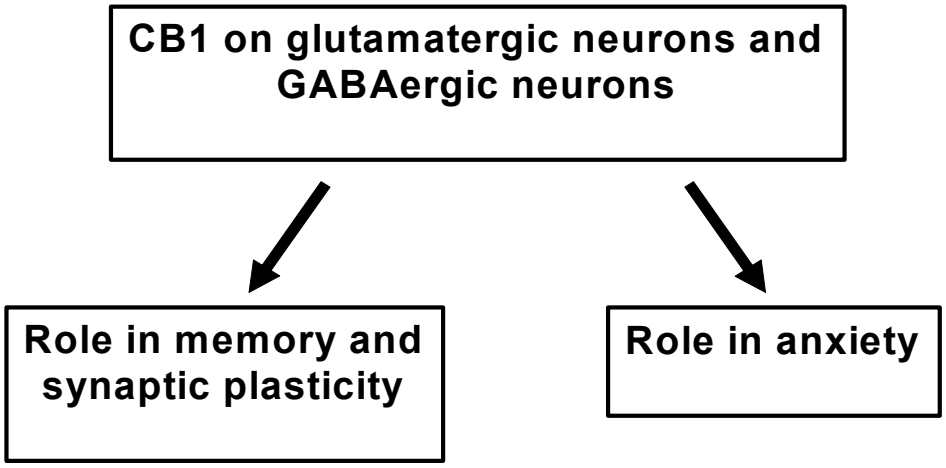


Figure 2: Schematic outline of the experimental work: We hypothesised that CB1 expressed on glutamatergic or GABAergic neurons may differentially affect memory and synaptic plasticity on the one hand and anxiety on the other hand.

We have to consider a couple of difficulties when integrating our data in available concepts. Assessing the function of CB1 will depend on many parameters such as the genetic background of the animals tested, the experimental conditions used, the steady-state level of endocannabinoids and the neurobiological substrate examined. For example, most of the studies dealing with CB1 investigate systemic effects of agonists and antagonists in artificial systems like cell culture or slice preparations which can be very different from the situation in the behaving animal. Knock out

animal models miss CB1 during the whole ontogeny of the animal and at any time of an experiment. Most of the times, it is not the endogenous ligands (e.g. anandamide or 2-AG) used in the studies but newly developed synthetic ligands with different binding kinetics and potencies. These investigations are for sure valuable in allowing us to discover basic mechanisms of CB1 receptor activation and its physiological and behavioural consequences. However, it is likely that the effects of endocannabinoids may be different as they cause their effects at activated synapses in a precise temporal and spatial manner and the reader should bear in mind that physiological mechanisms can be fairly different from experimental models used in the cited and also in our studies. Another challenge is the fact that inbred C57Bl/6 and outbred Swiss CD1 strains of mice have been utilised as genetic background for the generation of CB1-ko mice. These strains of mice have been used to develop four independent CB1-ko mouse lines, with Zimmer et al., Robbe et al. and Marsicano et al. using the C57Bl/6 strain and Ledent et al. using the CD1 strain (Ledent et al., 1999;Marsicano et al., 2002;Robbe et al., 2002;Zimmer et al., 1999). Inconsistencies in published experiments might depend on the respective knockout mouse line that is used in a study (some further information on this issue is given to the reader in chapter 4.4.2 Genetic background of CB1-ko mice). Beside of that, many pharmacological studies were carried out in rats. Altogether, the use of rats or mice as classical animal models is not further indicated in this work.
The presented thesis falls into three parts that we would like to introduce shortly.

1.7.1 An electrophysiological model for synaptic plasticity in the hippocampus: Is LTP altered in CB1-deficient mice?

Both, learning and memory and synaptic plasticity are believed to involve changes in the connection between neurons. The synaptic plasticity and memory hypothesis states that activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation and is both necessary and sufficient for the information storage underlying memory processes (Martin et al., 2000). The most widely studied model for these phenomena is called long-term potentiation (LTP). LTP, firstly described in 1973 by Tim Bliss and Terje Lomo (Bliss and Lomo, 1973), is an abrupt and sustained increase in synaptic efficacy of a specific brain pathway resulting from the application of brief high-frequency electrical bursts. LTP has been shown to occur in many different brain regions of the brain, and it is mostly investigated in the hippocampus (Bliss and Collingridge, 1993). At the same time, the hippocampus is a region of the brain that is necessary for the formation of episodic memories in humans and for spatial learning and memory in rodents (Squire, 1992). The literature on these issues is vast, and only very recently, direct evidence was presented that hippocampus-dependent learning leads to observable LTP at a subset of hippocampal synapses, and suppression of LTP after learning a task abolishes the very same memory of that task (Pastalkova et al., 2006;Whitlock et al., 2006).
We were interested if synaptic transmission and especially synaptic plasticity is altered in our mutant mice. Bohme et al. measured fEPSPs in the CA1 region of hippocampal slice preparations after stimulation of Schaffer collaterals (Bohme et al., 2000). They could show that mice lacking CB1 had stronger LTP than their wild type controls. The mutant mice showed no alterations in the basic excitability after stimulation of the Schaffer collaterals. Also, they did not see any difference for short-term synaptic plasticity like paired-pulse facilitation (which is further described below). Anatomically, the hippocampus forms part of the medial temporal lobe (Amaral and Witter, 1989). The hippocampal formation, as all other cortical areas, consists of two major neuron types: Excitatory principal cells and inhibitory interneurons. The former

include hippocampal pyramidal cells and dentate gyrus granule cells accounting for about 90% of the entire neuronal population, and the remaining 10% of cells are GABAergic interneurons (Freund and Buzsaki, 1996). We focused on the hippocampus as it is one of the areas with the highest density of CB1 receptor (Herkenham et al., 1991), and relatively large amounts of anandamide have been found in the hippocampus of rats and humans (Felder et al., 1996). As DAGL, the enzyme mediating the formation of 2-AG, is being found in the dentate gyrus granule cells (Katona et al., 2006), CB1 mRNA is expressed in the entorhinal cortex which projects to the dentate gyrus molecular layer (Mailleux and Vanderhaeghen, 1992;Marsicano and Lutz, 1999) and cannabinoid receptor is dense in the molecular layer and moderate in the granule cell layer and the hilus of the dentate gyrus (Herkenham et al., 1991), these findings suggest that endocannabinoids are released in the dentate gyrus and CB1 is located on perforant path axonal terminals. The perforant path, coming from the entorhinal cortex, is the major excitatory input pathway to the dentate gyrus of the hippocampus. For the electrophysiological recordings, we hence focused on the perforant path-dentate gyrus granule cell synapse.

We investigated the phenomenon of LTP in anaesthetised mice *in vivo* for several reasons. Firstly, it resembles physiological processes more closely than experiments done in brain slices. The *in vivo* preparation potentially makes enough substrate available for subsequent biochemical analysis after LTP induction. Furthermore, it allows the investigation of extrahippocampal influences on LTP that are lost in the slice preparation. Previous studies of LTP in genetically modified mice have revealed discrepancies between results obtained *in vitro* or *in vivo* (Bordi et al., 1997;Errington et al., 1997;Nosten-Bertrand et al., 1996) in that enhanced LTP was found *in vivo* but not *in vitro*. On the other hand, *in vitro* brain slices show limitations of recording duration due to tissue deterioration (Teyler, 1980). The artificiality of the slice preparation is further revealed in a recent study: Al Hayani and coworkers could show that the CB1 agonist WIN55,212-2 causes a decrease of paired-pulse depression of population-spike recorded in hippocampal slices at 28-30°C, whereas it causes an increase of paired-pulse depression of pop-spike at 35°C (Al Hayani and Davies, 2002) , a quite dramatic difference.

Six electrophysiological characteristics of the dentate gyrus were compared across the knockout mouse lines: (1) The relationship between perforant path stimulation and the resulting granule cell field excitatory postsynaptic potential (fEPSP), i.e. the number of granule cells synchronously depolarising. (2) The relationship between perforant path stimulation and the resulting granule cell population-spike (pop-spike), i.e. a number of granule cells synchronously firing an action potential. (3) Presynaptic facilitation, measured by a paired-pulse protocol of fEPSP. (4) Polysynaptic feedback inhibition of granule cells, measured by a paired-pulse protocol of pop-spike. (5) Induction and maintenance of LTP of fEPSP and pop-spike. (6) Lastly, we assessed theta oscillations in the dentate gyrus after a sensory stimulation. Theta rhythm is a prevalent electroencephalographic (EEG) signal in the hippocampus that oscillates in a frequency range of 3 to 12 Hz. It is commonly regarded as a physiological encoding frequency in memory formation (Buzsaki, 2002). An increase in theta rhythm might be one underlying mechanism for an enhancement in learning and synaptic plasticity (Holscher et al., 1997;Wiebe and Staubli, 2001).

1.7.2 Behavioural paradigms for the measurement of learning and memory: Are cognitive abilities altered in CB1-deficient mice?

Learning and memory can be defined as an experience-dependent generation of enduring internal representations, or lasting modifications in such representations (i.e. learning) and the retentions of these representations, or the capacity to reactivate these representations (i.e. memory) (Dudai, 1989). Because memories in the brain cannot directly be observed, their expression must be inferred from overt behavioural expressions in an indirect manner.

It was shown that local administration of the CB1 agonist CP55940 into the dorsal hippocampus impaired the choice accuracy in a radial arm maze task. This effect was specific to cognition since other pharmacological effects of the agonist were absent, thus highlighting the important role of the hippocampus for cognitive alterations due to CB1 activation (Lichtman et al., 1995). When dealing with learning and memory tasks, one has to bear in mind that a novel environment or situation that is intended to be suitable for gathering information and thus learning, elicits an emotional and cognitive response at the same time. Hence, learning and memory tasks used here are never purely cognitive tasks.

To check for hippocampus-dependent memory performance and to have a potential correlation to our electrophysiological recordings from the hippocampus, we applied several hippocampus–dependent tasks. A set of various memory tasks was chosen that rely on different performance requirements as the outcome of individual tests can vary dramatically and lead to a wrong result (Gerlai, 2002). We chose tasks that do not rely on appetitive motivation as it has been shown that differences in CB1-ko mice from the respective wildtype animals can not be expected in those tasks (Holter et al., 2005). In addition, the endocannabinoid system seems to be a modulator of the brain reward system, as CB1-ko mice have e.g. a reduced sensitivity in the rewarding effects of sucrose (Sanchis-Segura et al., 2004), which is an obvious pitfall when using appetitively motivated learning tasks with food reward. Briefly, we would like to outline our applied tasks.

Water maze spatial discrimination task: In contrast to the classical Morris water maze, two platforms swimming in a pool of water are used in this test that are visible to the animal. The mouse escapes from the pool by using distal spatial cues to locate the unsinkable platform that provides support whereas the other platform sinks if the animal enters it. Whereas the safe platform stays at the same position relative to the spatial cues, the sinkable platform changes the position around the water maze rim. Mice use an allocentric spatial strategy in this test to locate the safe platform which depends on an intact hippocampus. As an exposure to water is aversive to rodents, escape from the water is a positive reinforcement in this test (Arns et al., 1999;Steckler et al., 1999). Compared to the Morris water maze, the water maze used in the present study task is less stressful to the animals as it is considerably less time the mouse is enforced to swim in order to reach the safe platform. In our test, the accuracy, i.e. the choice to swim to the stable or to the floating platform, is used as a measurement for learning performance compared to the latency to swim to the platform in the Morris maze reported by many authors which might include sensory or motor impairments or inappropriate search strategies of the animals.

Object recognition task: This task is based on the spontaneous tendency of rodents to explore a novel object more than a familiar one. It consists of a first trial during which mice are placed in an open field in the presence of an object (such as a piece of plastic or a small glass bottle) and a second trial during which mice are allowed to explore the open field in the presence of a familiar object, that was previously explored, and a novel object. Recognition memory is assessed by

comparing the time spent exploring each object during the second trial (Dodart et al., 1997;Ennaceur and Delacour, 1988). Long-term object recognition memory requires the hippocampus in mice for the completion of the task (Hammond et al., 2004). An advantage of the object recognition test is that it neither needs positive reinforcement (e.g. food reward) nor negative reinforcement (e.g. electric foot shock) nor rule learning (e.g. operant conditioning) but rather exploits the animal's innate preference for novelty.

Social recognition: Similar to the object recognition task, this task takes advantage of the innate drive of an animal to investigate nonfamiliar over familiar conspecifis and does not need positive or negative reinforcement (e.g. food reward or electric foot shock, respectively), too. Social recognition can be studied in rodents by placing a juvenile in a cage with an adult. The adult will spontaneously investigate the younger animal, and the duration of this investigation reflects the familiarity between the two animals. A repeated exposure to the same juvenile and a novel juvenile at the same time results in a decrease in investigation time of the familiar juvenile compared to the novel one. Hence, the difference in investigation times between the first and second exposure can be used as an index of social memory. This effect diminishes as the length of interexposure period increases. Social recognition memory is dependent on the hippocampus (Engelmann and Landgraf, 1994;Kogan et al., 2000;Richter et al., 2005).

Contextual fear conditioning: In a Pavlovian or classical conditioning procedure, the presentation of a neutral stimulus, such as a tone, is paired with an aversive stimulus, such as an electric foot shock. By associating it with the shock (i.e. the unconditioned stimulus = US), the previously neutral tone (i.e. the conditioned stimulus = CS) becomes aversive when subsequently given alone and can be used to elicit a number of behaviours associated with fear. The mouse will display a natural defensive response termed freezing that is an immobile posture in which the animal is highly alert (Blanchard and Blanchard, 1969). Rodents can learn simple associations between US and e.g. tone or light as CS (called elemental learning) but they can also learn contextual stimuli, i.e. a set stimuli of the shock chamber itself (configural learning). We applied the background contextual fear conditioning paradigm, in which mice are placed into a distinctive context where they receive an aversive footshock. Animals subsequently display conditioned fear-related behaviours, i.e. freezing, in the presence of the context alone. Learning of the contextual stimuli requires a normally functioning hippocampus and amygdala (Gerlai, 1998;LeDoux, 2003;Paylor et al., 1994). Previous studies indicate that the hippocampus is differentially involved in contextual fear learning depending on whether or not the unconditioned stimulus is signalled by a discrete cue. When the footshock immediately follows presentation of an auditory stimulus (which hence is in the foreground), conditioning to the context is thought to occur in the "background" since the phasic auditory cue is the stimulus that principally becomes associated with the footshock. In the absence of a discrete cue, conditioning to the context occurs in the "foreground" since contextual cues are the only stimuli available to be associated with the footshock. It has been shown that hippocampal lesions specifically disrupt background contextual conditioning (Phillips and LeDoux, 1994).

Open field habituation: Neglecting the component of anxiety and focusing on the exploratory activity, the open field task can be defined as a memory task. As an open field is a novel environment, the animal acquires information of this current environment by means of exploratory activity. Rodents submitted for the first time to an open field display higher spatial exploration (i.e. locomotion and rearing) than in successive exposures. Thus, the decrement in the response to successive

exposures, i.e. a decrease of locomotion, is taken as an index of habituation, a form of non-associative memory (Cerbone and Sadile, 1994). Animals with enhanced cognitive ability habituate more easily to a novel environment. The hippocampus is an essential structure that detects and responds to novel stimuli and is involved in open field habituation memory (Degroot et al., 2005;Vianna et al., 2000;Winograd and Viola, 2004).

1.7.3 Behavioural models for the measurement of anxiety and emotionality: Is emotionality altered in CB1-deficient mice?

Anxiety can be viewed as an appropriate, adaptive response to impending danger that is integral to an organism's preparations to either cope with or avoid a potential environmental threat (Holmes and Cryan, 2006). Generally, if one speaks of emotionality, anxiety or fear, we certainly do not know whether an animal is indeed "anxious" or "fearful". However, by measuring behavioural and physiological responses (e.g. changes in heart rate, blood pressure or hormone level), it is possible to elicit similar anxiety-associated behavioural and physiological responses as can be observed in humans. Ultimately, drugs that are known to reduce anxiety can be studied in the respective behavioural test paradigm and should alter the behaviour under investigation.

Rodents show a strong propensity to explore novel objects and environments. Exploration is a fundamental component of the behavioural repertoire of a rodent and allows the animal to familiarize itself with the surrounding on the search for food, social partner or shelter. The exploratory behaviour is inhibited by a wide variety of aversive stimuli that are thought to induce anxiety. For example, rodents explore less under bright illumination, after receiving an electric shock or in the presence of a predator. A very profound behavioural inhibition is freezing, when an animal remains immobile in a state of readiness for possible flight. Thus, by measuring the exploratory activity, it is possible to quantify an animal's state of anxiety (Belzung and Griebel, 2001). If there is an increase of anxiety-related behaviour, this is called to have an *anxiogenic* effect whereas if there is a decrease of anxiety-related behaviour, this is called to have an *anxiolytic* effect. A number of tests have been developed which assess anxiety by measuring changes in exploratory behaviour or coping behaviour. Most importantly, all these models are sensitive to anxiolytic drugs (such as the "gold standard" benzodiazepine), evidencing their validity. We applied three categories of anxiety tests: On the one hand there are tests where the animal can choose whether to stay in a safe environment or in a putative risky environment (elevated plus maze, light/dark test). On the other hand there are tests where the mouse is enforced to remain in an aversive environment without any choice (open field, social exploration, and object exploration). Thirdly, we included a test in which mice that are more anxious must engage in an active behaviour (defensive marble burying) as opposed to passive behaviours utilized to avoid anxiogenic stimuli (plus maze, light/dark test). Briefly, we would like to introduce the reader to the applied test paradigms.

Elevated plus-maze: The apparatus comprises a plus-shaped maze with four arms radiating out from a central platform. Only two opposing arms are enclosed on three sides by walls. The entire maze is raised of the floor. Rodents will show a preference for the closed areas with walls because of thigmotaxic behaviour. The avoidance of the open arms is assumed to stem from a rodent's aversion to open, exposed spaces without any thigmotaxic cues. The standard measures taken are the number of entries made from the central platform into the open and closed arms and the amount of time being spent there (Lister, 1987).

The elevated plus-maze trial 1/2 protocol allows investigating a memory of the fearful experience: Animals are exposed to the elevated plus-maze on two consecutive days, and prior test experience produces enduring changes in the behavioural response. There is a decrease in exploration of the open arm on day two compared to day one, which is called one-trial sensitization. It seems like rodents acquire some kind of memory of the potentially dangerous areas (Carobrez and Bertoglio, 2005).

Light/dark test: The apparatus consists of two compartments, one painted black and being dark, and a second that is brightly illuminated. As nocturnal rodents like mice and rats find bright light aversive they will avoid the lit compartment. The number of transitions, the distance moved and time spent in the lit compartment is a measure of anxiety (Bourin and Hascoet, 2003;Costall et al., 1989).

Defensive marble burying: Rodents spontaneously sue available bedding material to bury unpleasant sources of discomfort present in their home environment (Archer et al., 1987). Burying behaviour consists in forward-shoving the diggable material over the source of aversion using the snout and forepaws in order to avoid and protect from the localized threat. Marble burying is described as a defensive behaviour reflecting the anxiety state of the animals (Nicolas et al., 2006;Poling et al., 1981). This test is of special interest to us as it consists of an active coping strategy of the animal in contrast to the passive avoidance of aversive surroundings like in the plus-maze or light/dark test.

Social interaction: Social behaviour between animals, such as sniffing, close following, grooming, mounting and aggression are inhibited by anxiety and can be used to infer changes in anxiety. The amount of social interaction is also dependent on aversive stimuli in the environment, such as bright light (de Angelis and File, 1979;File and Seth, 2003).

Novel object exploration: A response to a novel object is thought to be a measure of a conflict between an exploratory drive and a neophobic avoidance of the potentially threatening object. Aversive environmental conditions can enhance the anxiogenic response (Belzung and Le Pape, 1994).

Open field: For this test procedure, the animal is placed in a rectangular arena, and the horizontal (i.e. locomotion) and vertical (i.e. rearing) exploratory activity is measured. However, rodents tend to explore an open field less under bright illumination, providing an aversive stimulus used to elicit anxiety (Hale et al., 2006). Another index of anxiety is thigmotaxis that is the tendency of an animal to stay in close proximity with perimeters within the environment (e.g. walls), supposably because of increasing an animals chance of avoiding detection by potential predators. The time spent in contact with the walls as well as the distance walked along the walls serves as a measure of thigmotaxis (Archer, 1973).

2. Methods

2.1 Animals

2.1.1 Animal housing

Male mice, aged between 3–5 months, were maintained in standard conditions with food (Altromin Standard-Diät 1310, Altromin GmbH, Germany) and tap water *ad libitum* in Makrolon type II cages with sawdust bedding (Altromin Faser Einstreu, Altromin GmbH, Germany), at 22 +/- 2° C room temperature and 55 +/- 5% humidity. Mice were kept in a reversed light-dark cycle with lights off at 9 a.m. and lights on at 9 p.m. if not stated otherwise. Experiments were conducted in the dark phase of the day between 9:30 a.m. to 2 p.m. All mutant mice were bred in house. C57BL/6NCrlBR mice for social interaction and recognition test were purchased from Charles River (Charles River WIGA (Deutschland) GmbH, Sulzfeld, Germany).

The fact that the animals used in this study were singly housed shall be briefly discussed here. Principally, rodents are considered to be social animals, and it is recommended to avoid individual housing (Van Loo et al., 2003). Moreover, the performance in learning and memory tests decreases in singly housed mice (Voikar et al., 2005). On the other hand, mice establish strong dominance hierarchies (Poshivalov, 1980) and social status affects anxiety levels in this species (Ferrari et al., 1998). Singly housed mice show less behavioural variability, probably due to the hierarchical aggressiveness and rivalry found in group housed mice (Voikar et al., 2005).

To avoid confounds from social status, subjects were kept individually in cages. The only exception were mice used for social interaction test that were group housed as this is a requirement for an intact olfactory memory and prevents from the development of aggression.

The Committees on Animal Health and Care of the local government (Regierungspräsidium Oberbayern) approved all experimental procedures.

2.1.2 Generation of CB1 mutant mice

Gene-targeted mice can be created using the phage P1-derived Cre/LoxP recombination system. Marsicano and colleagues generated a line of mice that carries a CB1 gene flanked by lox P sites (the CB1 gene is now called “floxed”). The Cre enzyme, a DNA recombinase derived from P1 bacteriophages, recognizes these lox P sites. When such mice are bred to transgenic strains that express Cre recombinase, floxed genes will be deleted in all tissues in which the Cre recombinase is active. This strategy is now frequently used for the tissue-specific inactivation of genes (Sauer, 1998).

Briefly, a construct containing the entire open reading frame of CB1 gene flanked by two lox P sites was generated and electroporated into mouse embryonic stem cells to obtain the floxed allele. Mice bearing the floxed allele were then crossed with transgenic mice expressing Cre recombinase ubiquitously. Mice carrying a germ-line transmissible deletion of CB1 were backcrossed into C57BL/6N mice (Marsicano et al., 2002). These mice are called total-CB1-ko mice in the presented work.

Using the Cre/Lox P system in which Cre recombinase was under the control of regulatory sequences of the Nex gene (only expressed in glutamatergic neurons of the forebrain), Marsicano and colleagues created conditional knockout mice in which CB1 was deleted in all principal glutamatergic neurons of the forebrain (including the neocortex, amygdala and hippocampus), but was spared in GABAergic interneurons (and cerebellum), henceforth termed as Glu-CB1-ko mice (Monory et al., 2006). A

third line of mice, in which the Cre recombinase was under control of the regulatory sequences of the Dlx5/Dlx6 gene lacks CB1 in forebrain GABAergic neurons, henceforth called GABA-CB1-ko mice (Monory et al., 2006). A brief description of the mouse lines is summarised in Table 1. The anatomical distribution of CB1 mRNA in brains of the mouse lines with an *in situ* hybridisation is depicted in Figure 3.

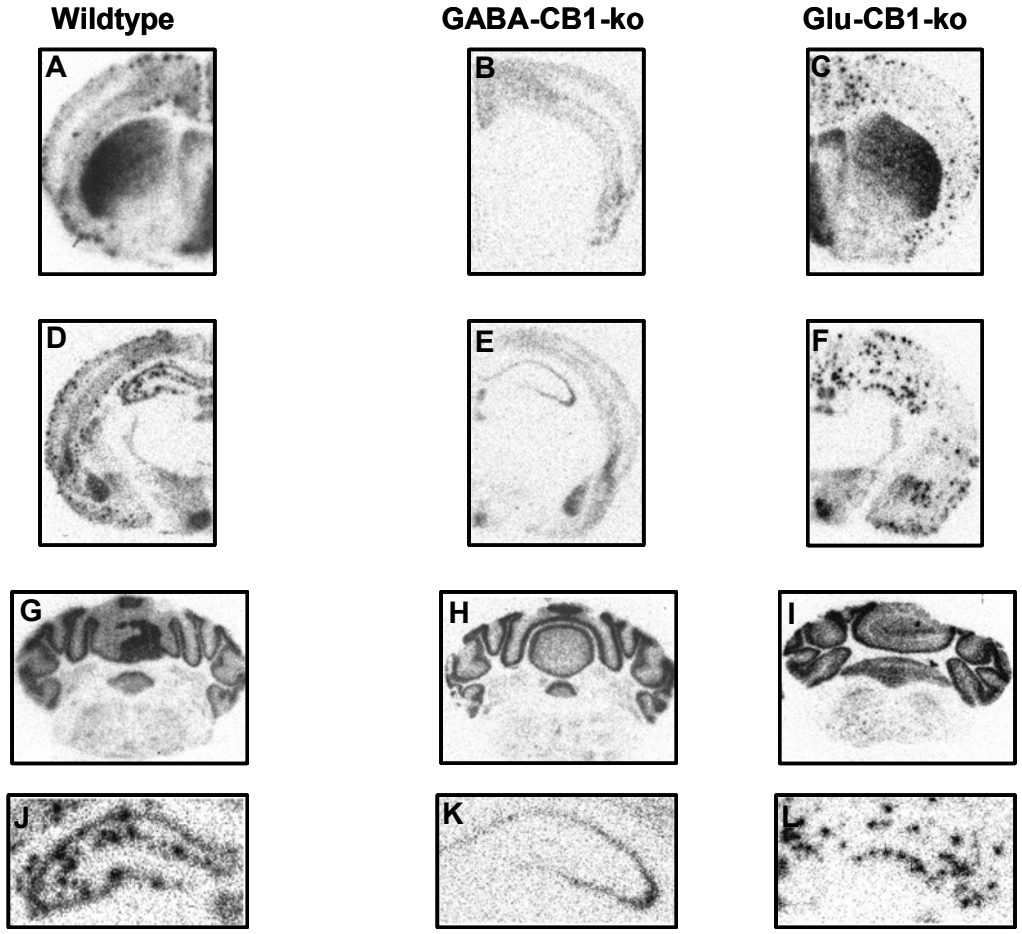


Figure 3: Specific deletion of CB1 in different neuronal subpopulations in conditional CB1 mutant mice as revealed by *in situ* hybridisation. Micrographs showing CB1 mRNA expression of wildtype **(A, D, G, J)**, GABA-CB1-ko **(B, E, H, K)** and Glu-CB1-ko **(C, F, I, L)** mice. Sections were taken from the level of the caudate putamen **(A-C)**, dorsal hippocampus **(D-F)** and cerebellum **(G-I)** and detailed enlargements of the hippocampus are given **(J-L)**. In the mutant lines, deletion of CB1 is mainly restricted to the forebrain. In GABA-CB1-ko mice **(B, E, H, K)**, CB1 mRNA is absent in all GABAergic neurons and is expressed only in non-GABAergic cells (low uniform staining). In Glu-CB1-ko mice **(C, F, I, L)**, CB1 mRNA is absent in the majority of cortical glutamatergic neurons. The intense scattered dots indicate high expression levels of CB1 in GABAergic interneurons. Modified from Monory et al., 2006.

Table 1: Overview of the three knockout mouse lines

Mouse Line	Total-CB1-ko	GABA-CB1-ko	Glu-CB1-ko
Promotor control	Conventional	Dlx5/Dlx6	Nex
Affected cells	All CB1-bearing cells in the brain	All GABAergic neurons in the forebrain i.e. neocortex, hippocampus, amygdala, but present in other brain regions, such as midbrain, and cerebellum	Glutamatergic cells in the forebrain, i.e. neocortex, striatum, hippocampus, amygdala, but present in other brain regions, such as midbrain and cerebellum

All lines were in a mixed genetic background, with a predominant C57BL/6NCrlBR contribution to which they were backcrossed for at least 3-6 generations. Mice used in single experiments were littermates. The breeding pairs for total-CB1-ko and wildtype mice were heterozygous animals. For Glu-CB1-ko and GABA-CB1-ko mice and the respective littermates, the breeding pairs consisted of a mother negative for Cre recombinase (C-) and a father positive for Cre recombinase (C+). The genotype was verified using polymerase chain reaction (PCR) according to protocols previously established (Marsicano et al., 2002;Monory et al., 2006) The experimenter was always blind to the genotype of the mice. We were allowed to use the mutant mouse lines in this study with very kind permission from Dr. Giovanni Marsicano (University of Bordeaux, France) and Prof. Beat Lutz (University of Mainz, Germany).
Electrophysiological recordings in an *in vivo* preparation were carried out with naïve animals.
Behavioural experiments were conducted in four screens with separate cohorts of animals for every screen, as it is depicted in Figure 4:

1. Screen: Separation of animals and individual housing. Object recognition test (30lux). Open field test (0 lux). Contextual fear conditioning (0.7 mA).
2. Screen: Social recognition test (500 lux). Separation of animals and individual housing. Open field test (700 lux).
3. Screen: Social interaction test (0 lux). Separation of animals and individual housing. Object exploration test (500 lux). Elevated plus-maze test. Contextual fear conditioning (1.5 mA).
4. Screen: Separation of animals and individual housing. Light/dark box, elevated-plus maze, marble burying test.

Another cohort of animals was employed for the water maze spatial discrimination task.
Animals were transferred from the vivarium to the laboratory always 1-2 days before the experiment. The different tests were carried out with 2-3 days apart from each other.

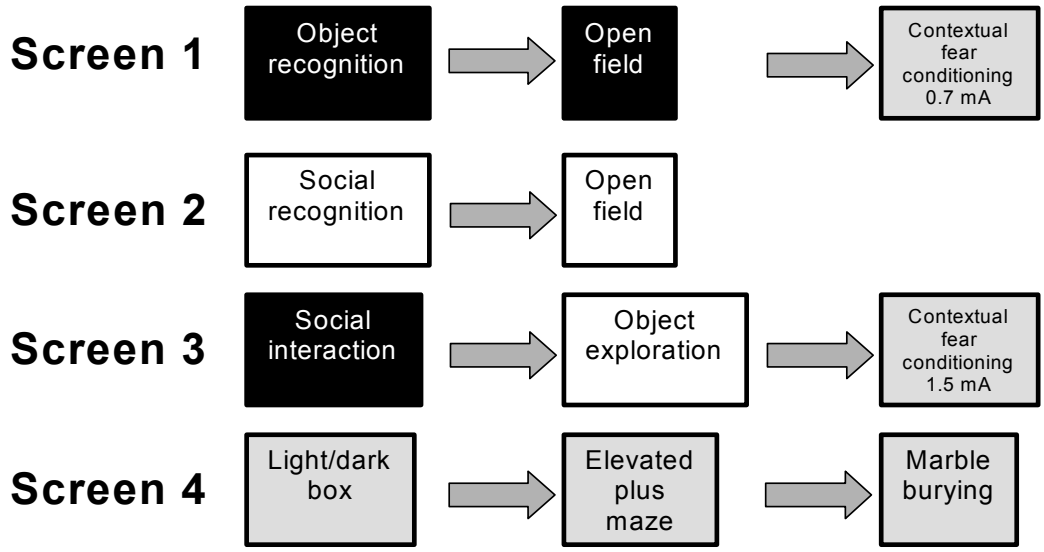


Figure 4: Schedule of the behavioural testing. Four screens were carried out to analyse memory and anxiety in the three mouse lines. Please note that experiments that were more aversive to the animals were always done in the end of a series. Experiments of one screen were conducted within about three weeks. Black boxes highlight lowly aversive testing conditions whereas white boxes highlight highly aversive testing conditions for experiments where we specifically and intentionally changed the environmental conditions. Grey boxes indicate experiments under standard laboratory conditions. The water maze spatial discrimination task was carried out with a separate cohort of animals and is not included in the scheme.

2.2 Electrophysiology

2.2.1 Surgery and field recordings

Experiments were carried out in a Faraday cage constructed in house. Mice were anaesthetised with 1-1.5% isoflurane (Forene, Abbott, Wiesbaden) under control of a vapor (Vapor 19.3, Drägerwerk AG, Lübeck), in 99.9% O_2 and placed in a stereotaxic frame (TSE Systems, Bad Homburg, Germany). Deep anaesthesia was indicated by lack of a pedal reflex and by regular breathing. Temperature was maintained at 37.5°C with a heating blanket and constantly monitored via a rectal probe and a homoeothermic blanket control unit (Harvard Apparatus, Edenbridge, UK). The open eyes of the animals were protected from desiccation with eye ointment (Bepanthen, Roche Basel, Switzerland). Surgical procedure was done under control of a binocular (OPMI pico, Zeiss, Jena). The skin was cut and the bone surface treated with 3% H_2O_2 to make the suturae of the skull better visible. Skull surface was exposed and holes in the skull were drilled with a dental turbine (KaVo EWL Typ 4912, Biberach/Riss, Germany, speed: 30000 rpm). A reference silver wire electrode was positioned on the frontal cortical surface. A borosilicate-glass recording micropipette (GC120T-10, Harvard Apparatus, Edenbridge, UK) was manufactured with an electrode puller (Zeitz-Instrumente, DMZ-Universal Puller, München, Germany) and back-filled with 0.9% NaCl (with an *in vitro* impedance of 0.5-2 MΩ). The pipette was lowered into the cell body layer of the dentate granule cells (stereotactic co-ordinates: 1.8 mm posterior to bregma, 1.6 mm lateral to the midline, 1.4 mm below the cortical surface, according to the mouse brain atlas (Paxinos and Franklin, 2001). The granule cell layer was located by observing the spontaneous multiunit activity (with filter adjustment of 300 Hz – 10 kHz, gain of 100 000) using a differential amplifier (A-M Systems Model 1700, Carlsborg, USA). Coming from the cortical surface, there are

two prominent cell layers that is the pyramidal cell layer of the cornu ammonis CA1 and eventually the granule cell layer of the dentate gyrus. Multiunit activity was monitored with an oscilloscope (Grundig Electronic, MO 53, Nürnberg, Germany) and a loud speaker (RIM Electronics RSV 70 B, Loomis, USA), respectively. When approaching the pyramidal or granule cell layer, this can be seen or heard as an intense spontaneous firing of cells. As soon as the granule cell layer was reached, the recording electrode was left in position and the stimulating electrode was inserted. Now, evoked field excitatory postsynaptic potentials (fEPSP) were amplified through the differential amplifier (at a gain of 100, 0.1-3 kHz bandpass filtered, and digitised at 10 kHz using an analogue-digital converter (Pico Technology, St. Neots, UK). A bipolar stimulating electrode was used that consisted of a two-pin connector (Harwin M22-6140306, Portsmouth, UK) with a 1 cm long stainless steel cannula and a Teflon-isolated tungsten wire (WT-3t, Science Products GmbH, Hofheim, wire tip diameter: 0.03 mm). The tip of the wire protruded from the cannula with 0.4 mm. It was positioned in the angular bundle of the perforant path (stereotactic co-ordinates: 3.5 mm lateral to lambda, 1.0-1.5 mm below the cortical surface). Electrode depth was adjusted in order to maximise the field response to a square wave test stimulation (100-900 µA, 50 µs) that was applied with a stimulator (World Precision Instruments Stimulus Isolator A265, Sarasota, USA). Initially, coming from the cortical surface, the tissue was stimulated maximally with 900 µA. As soon as a fEPSP became visible, current was lowered so that eventually a fEPSP could be observed with about 100 µA. When the position of the stimulation electrode was optimal the recording electrode was slightly shifted ventrally or dorsally to improve the signal. After both stimulating and recording electrodes were properly positioned in the perforant path and the dentate granule cell layer, respectively, the preparation was allowed to stabilise for 30 min prior to baseline recording, with test stimuli injected every 30 s. Data acquisition was done with a personal computer using the LTP program (LTP Program Version 2.4, Bristol, UK). An example of typical waveforms from the dentate gyrus can be seen in Figure 6.

At the end of the experiment, in a subset of mice, small electrolytic lesions were made through the stimulating and recordings electrode with a voltage generator manufactured in-house (DC 1.5 mA for 10 s). 10 min after lesioning the brain, the mice were given an overdose of isoflurane. The brain was removed, deep-frozen and sectioned on a cryostat microtome (Microm Laborgeräte GmbH, HM 500 OM, Waldorf, Germany). The position of the electrode tracks in the dentate hilus and angular bundle of the perforant path could then be verified using sections stained with cresyl violet. An example of correct placement of the electrodes is depicted in Figure 5A and B.

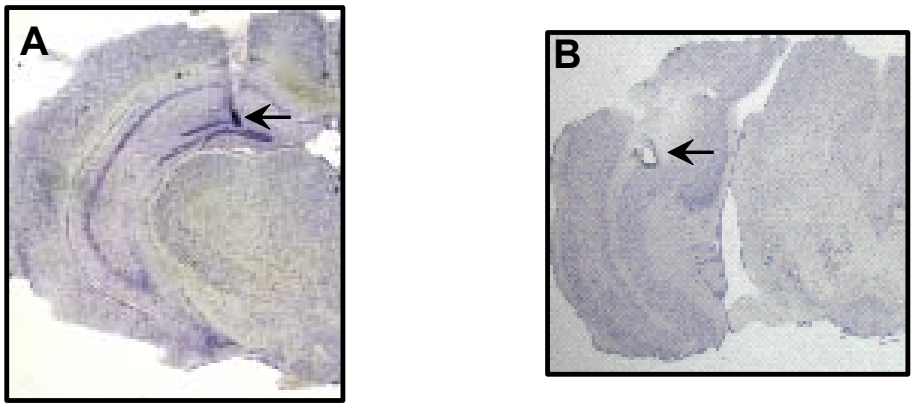


Figure 5: Marking of the stimulating and recording site in the mouse brain. Recording electrode in the upper granule cell layer of the dentate gyrus **(A)** and the stimulating electrode in the perforant path **(B)** of the left hemisphere. Black arrows indicate electrolytic lesions made at the tip of the electrodes, as was typical for the electrophysiological experiments.

2.2.2 Current-response relationships

The relationship between stimulation current and evoked response (i.e. input/output curve, I/O curve) was determined at the beginning of each experiment. Single square-wave pulses (50 μs) were delivered at currents of 100-1000 μA in 100 μA steps at 0.033 Hz with each test current repeated three times. The fEPSP initial slope, measured as the voltage difference between two constant time-points set at the onset of fEPSP and between fEPSP and pop-spike onset (20-80%), and the pop-spike amplitude, measured as the voltage difference between the peak of the initial and late positive components and trough of the pop-spike, were measured for each response, as it is depicted in Figure 6A-C.

2.2.3 Paired-pulse protocols

Pairs of identical stimulation pulses were applied with varying the inter-pulse interval. Two protocols were used:

1. Pre-synaptic stimulation: Stimulation current was set to below the population spike threshold for each animal and inter-pulse intervals (IPIs), i.e. the time between two pulses, were varied from 10-100 ms in 10 ms steps and from 500-2000 ms in 500 ms steps. fEPSP peak heights of each of the pair of responses were compared, and the second peak height expressed as a ratio of the first (Figure 6D). Three determinations were made at each inter-pulse interval and a mean value calculated.
2. Poly-synaptic feedback inhibition: Stimulation currents were set to evoke a population spike with 40-60% amplitude of the maximal response and interpulse intervals were varied from 10-100 ms in 10 ms steps and from 500-2000 in 500 ms steps. Pop-spike amplitudes of each of the pair of responses were compared, and the second pop-spike amplitude expressed as a ratio of the first. Three determinations were made at each inter-pulse interval and a mean value calculated.

The changes are expressed by the paired-pulse ratio (PPR), which is the ratio of the amplitude of the second response divided by that of the first. A ratio bigger than one is called paired-pulse facilitation (PPF) whereas a ratio smaller than one is called paired-pulse depression (PPD).

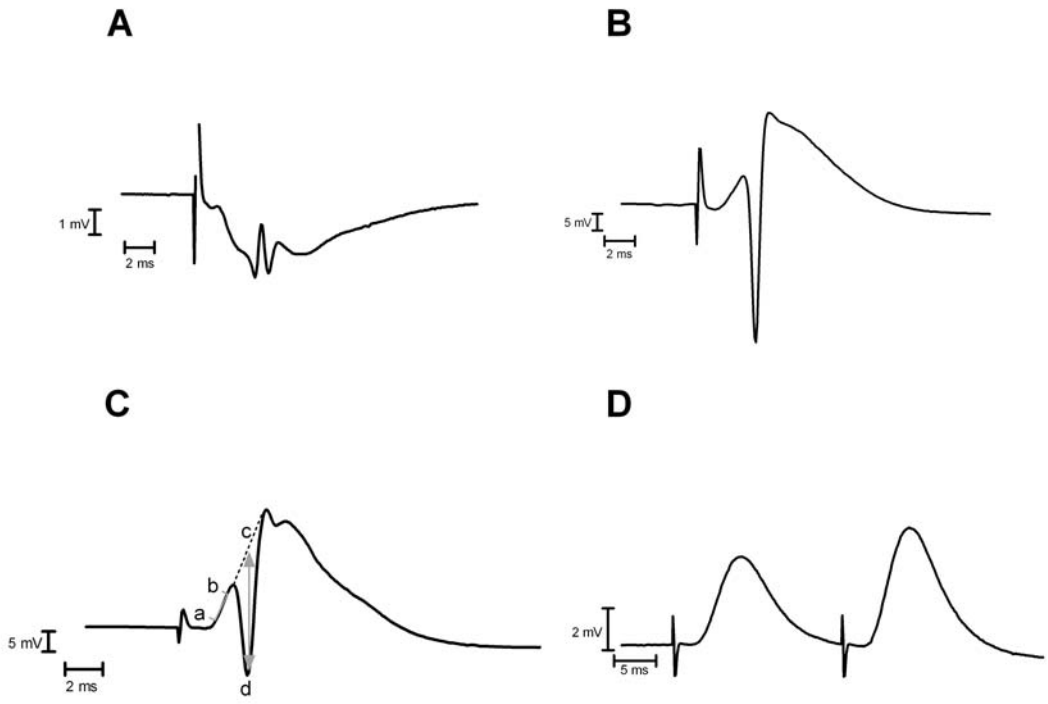


Figure 6: Local field potential traces recorded from the dentate gyrus. Local field potential traces recorded from the molecular layer of the dentate gyrus **(A)** and from the granule cells layer of the dentate gyrus **(B)** evoked by stimulating the perforant path. A negative going fEPSP is overlaid by a positive going pop-spike in the molecular layer in **(A)**, whereas a positive going fEPSP is overlaid by a negative going pop-spike in the granule cell layer in **(B)**. **(C)** Parameters measured of an evoked potential recorded in the granule cell layer: fEPSP slope (difference in point a and b divided by their difference in time) and pop-spike amplitude (difference in amplitude between points c and d). **(D)** Paired-pulse stimulation for fEPSP with an interpulse interval of 20 ms. The peak height of the two fEPSPs was measured for analyses. Calibration is indicated with a horizontal and vertical bar, respectively.

2.2.4 Induction of long-term potentiation (LTP)

Induction and maintenance of LTP was examined following high-frequency stimulation (HFS) of the perforant path. Test stimuli (50 µs), using a current sufficient to produce a population spike of 1-3 mV , were given at 30 s intervals for 30 min in order to establish a steady baseline response. HFS (six trains of six biphasic pulses of 50 µs length at 400 Hz, 200 ms intertrain interval, at a selected current that was at the lowest asymptotic level that evoked maximum pop-spike amplitude as derived from the I/O curve) was then applied to induce LTP. Recording of test responses was then continued, with the same current as before, for a 1 h period post-HFS. The fEPSP slope and the population-spike amplitude were measured for each test response. The baseline fEPSP slope and population-spike amplitude were calculated from the average of responses over a 10 min period prior to the HFS. All fEPSP slope and population-spike amplitude values were then expressed as a percentage of the baseline value and 10 consecutive responses were averaged.

2.2.5 Hippocampal electroencephalography (EEG)

Hippocampal electroencephalographic activity was recorded in the dentate gyrus granule cell layer. Surgery and positioning of the recording electrode was accomplished as described above. For EEG experiments, mice were anaesthetised with urethane (ethyl carbamate) (Sigma-Aldrich Chemie GmbH, München, Germany) with a dose of 1.5 mg/kg intraperitoneally. Deep anaesthesia was indicated by lack of a pedal reflex and by regular breathing. At this plane of anaesthesia no spontaneous theta activity was present in the hippocampal EEG, but could be elicited robustly by sensory stimulation. A frequency of 3-6 Hz was analysed as this is the typical rhythm under urethane anaesthesia that can be eliminated by metabotropic acetylcholine-receptor (mACh-R) antagonists (Kramis et al., 1975). A tail pinch with a forceps for 10 s served as a sensory stimulus. Animals which did not show theta activity in the 10 s epoch after stimulation were discarded. EEG was recorded continuously and the tail pinch was administered for three times with a 10 min interval in each mouse. During this interval theta activity came back to baseline level. EEG activity was filtered at 0.1-500 Hz and amplified with a gain of 500. EEG trace was recorded with a Signal analogue/digital board (Signal micro 1401, Cambridge Electronic Design Limited (CED), Cambridge, UK) and Signal software (Signal Version 2.03, Cambridge Electronic Design Limited (CED), Cambridge, UK) at a rate of 2000 Hz. Hippocampal EEG records were analysed offline with Igor software (Igor Pro, Version 5.0, WaveMetrics Inc., Lake Oswego, USA). For further characterisation, a 30 s-activity epoch before tail pinch and a 500 s-activity epoch after tail pinch were analysed. EEG amplitude and frequency spectra were generated from 10 s activity epochs by Fast Fourier Transform (FFT). FFT analysis provided maximal peak power (μV^2) and was assessed for a 0.1-20 Hz band and subsequently for the theta band (3-6 Hz) over time. Theta activity was expressed as a percentage of the 30 s-baseline activity measured before the tail pinch.

2.3 Behavioural Testing

2.3.1 Water maze spatial discrimination task

A circular swimming pool (80 cm in diameter, 30 cm high, white plastic) was filled to a depth of 20 cm with water (21 +/- 1°C, rendered opaque by addition of a non-toxic dye). At the outside of the maze, eight start boxes (10 x 10 x 26 cm), also filled with water and fitted with sliding doors, were fixed, from which the animal could swim into the centre of the maze when the door was raised. Introduction of start boxes allowed tracking of the animal as soon as it entered the circular open field of the maze. Two identically looking circular platforms with white surface and dark grey rim were used (each 10 cm in diameter, protruding approximately 0.5 cm above the water surface). One platform was stable and provided support (“correct platform”), the other platform was floating and sank when a mouse tried to climb on it (“incorrect platform”). Performance was recorded by the experimenter always standing behind the start box. Illumination in the testing room was about 50 lux on the water surface.

Animals were first habituated to the testing by placing them for 1 min on the stable platform in the middle of the pool. On the following day, subjects were trained to choose between the two platforms over six sessions (acquisition stage, ten trials per session, one session in the morning per day). On each trial, the stable platform remained in the same position (counterbalanced within groups), while the floating platform changed position from trial to trial in a pseudorandom manner (five possible positions). It was ensured that the spatial relationship between the platforms did not consistently reward turns into one direction and the distance between the start

position and each of the two platforms was equal over ten trials. A trial started by placing a mouse in one of six possible start boxes in a pseudorandom sequence and the door was opened. All positions except the start positions in front of and opposite to the correct platform were used. Data recording started when the subject had left the start box. A trial terminated when a mouse climbed onto one of the two platforms or after 30 s. If the mouse climbed onto the stable platform within 30 s, it was allowed to stay there for another 10 s before it was returned to the home cage. If an animal made an incorrect choice (climbing on the incorrect platform), or after 30 s had lapsed, it was gently placed on the correct platform and allowed to stay there for 10 s before it was returned to the home cage. Animals were trained in squads of four. Inter-trial-intervals were approximately 2-4 min and each session lasted approximately 30 min.
On day six, the floating platform was moved to the position of the stable platform and remained in the same position over the trials whereas the stable platform changed the position from trial to trial according to the rules previously applied to the floating platform (reversal stage). This was done to ensure that animals had learnt a spatial location rather than the ability to discriminate the two platforms by other means. Furthermore, hippocampus-dependent reversal learning and memory extinction could be assessed.
The accuracy measure includes percentage of correct choices in the water maze. Choice latency was measured during water maze performance with a handheld stopwatch. A choice was made if an animal touched one of the platforms with its forepaws or its snout.

2.3.2 Social interaction and olfactory recognition

Olfactory recognition was tested during the beginning of the light phase (between 8:00 and 12:00 a.m.). Group-housed experimental subjects were separated by transferring them to new Makrolon Type II cage (27 x 16 x 12 cm) with fresh bedding material 2 h before starting the session. Light intensity in the testing room on the level of the cages was 500 lux.
A social discrimination session consisted of two 4 min exposures of juveniles of the C57BL/6NCrlBR strain (20-30 days old) to the adult mouse in the adult's cage. During the first exposure (sampling trial), a juvenile was exposed to the adult animal, and the duration of the active investigatory behaviour (mainly sniffing and licking of the anogenital region, mouth, ears, trunk and tail of the juvenile and close following of the juvenile) and aggressive behaviour (biting, riding on back) of the adult towards the juvenile were recorded manually by a trained observer with a personal computer. Exploration time of this first encounter was used for the social interaction test. The juvenile was then removed and kept individually in a fresh cage. Short-term and long-term memory was tested after a retention interval of either 1 h or 24 h. The juvenile was re-exposed to the adult (choice trial), but this time with an additional, previously not presented juvenile of the same age and strain. The duration of investigatory and aggressive behaviour of the adult towards each juvenile was measured simultaneously. Significantly longer investigation duration of the new juvenile compared to the familiar juvenile was taken as evidence for an intact recognition memory. A recognition index was calculated for each animal and was expressed by the ratio $(T_{new}) / (T_{old} + T_{new})$ with T being the time spent exploring the juveniles. After each session, the experimental subjects were transferred back to their cage mates. Whereas social recognition was carried out under 500 lux illumination, the social interaction was accomplished under 0 lux or 500 lux illumination.

2.3.3 Novel object exploration and object recognition

The object recognition test was performed in the dark phase of the animals' light/dark cycle. Experimental subjects were habituated to the test arena (a Makrolon Type I cage, 36 x 22 x 14 cm, with sawdust bedding material and transparent walls) on two days for 10 min. Throughout the experiment, no cleaning of the box was allowed in order to saturate it with the animal's own olfactory stimuli. The light intensity for testing was 30 lux at the level of test apparatus. On the third day, the mouse was transferred into the same test cage and after 10 min two objects were placed in a symmetrical position at the short walls of the cage. When placing the objects into the cage, the mouse was put in another Makrolon Type II cage for about 10 s and eventually put back into the test cage. Two identical objects were presented in the first trial (sampling trial). The duration of the sampling session was 10 min. Exploration time of this first encounter was used for the novel object exploration test. The retention interval was either 3 h or 24 h, for which the mouse was left in the test cage (with food and water ad libidum in the latter case) and short-term and long-term memory was tested, respectively. During the second encounter, one familiar object and one new object were introduced to the animal (test trial). The testing session again comprised 10 min. A small CCD camera (Conrad Electronics, München, Germany) was mounted above the Makrolon cage to enable behavioural observation and leaving the animal undisturbed. The time spent exploring each object during sampling and test were recorded manually online with a personal computer and also videotaped. Exploration was defined as follows: Directing the nose towards the object at a distance of no more than 2 cm and/or touching the object with the nose and paws. Sitting on the object was not considered as exploratory behaviour. Between sessions, objects were thoroughly cleaned with soap water to eliminate olfactory cues. We used four different sets of objects that were: (1) A cone made of aluminium (height: 12 cm x diameter: 6 cm), (2) a light bulb on a plastic socket (9 cm x 4 cm), (3) a flask made from yellow plastic filled with sand (12 cm x 4 cm), (4) a black bottle made of glass filled with water (13 cm x 5 cm). We used objects that were rather big (so that the mouse will spend more time exploring the object) and that differed in material and texture (so that the mouse could discriminate it more easily) (Abdul Ennaceur, personal communication). Objects had to be heavy enough that a mouse could not displace them. The order of objects used per subject per session was determined randomly, but it was always combined the cone with the light bulb and the black bottle with the plastic flask, so that novel and familiar objects were counterbalanced. A recognition index was calculated for each animal and was expressed by the ratio $(T_{new}) / (T_{old} + T_{new})$ with T being the time spent exploring the objects. Whereas object recognition was carried out under 30 lux illumination, the novel object exploration was accomplished under 30 lux or 500 lux illumination.

2.3.5 Contextual fear conditioning

Conditioning chambers (ENV-307A, MED Associates, St. Albans, USA) consisted of a house light (0.6 lux, ENV-215M, MED Associates, St. Albans, USA), transparent plastic walls and aluminium walls, and mouse-shock floor consisting of grids (grid harness package: ENV-407, Shocker/Scrambler: ENV 414, MED Associates, St. Albans, USA). The conditioning context, which is further referred to as *shock context*, had a cubic shape. The grid was cleaned with 70% ethanol. The tray underneath the cage was filled with sawdust and sprayed with 70% ethanol to provide an olfactory cue. For memory testing, two different contexts were used. The first test context, further referred as *grid context*, had a shape of a hexagonal prism, with opaque side walls with rough surface. It was illuminated with a stimulus light (12 lux, ENV-221M,

MED Associates, St. Albans, USA) and cleaned with water containing isoamylacetate (1:2000, banana aroma) as a monomolecular odour. The same grid floor of the conditioning context served as a floor, and the tray underneath the cage was filled with sand (Quarzsand, Sakret Trockenbaustoffe Europa GmbH & Co. KG, Berlin, Germany) and sprayed with isoamylacetate as an olfactory cue. The second test context, further referred to as *neutral context*, consisted of a Plexiglas cylinder. It was illuminated with a house light (0.3 lux, ENV215-M, MED Associates, St. Albans, USA) and cleaned with 1% acetic acid. The floor was covered with sawdust bedding that was sprayed with 1% acetic acid.
All contexts were located in soundproof isolation cubicles (ENV-018M, MED Associates, St. Albans, USA) that were additionally isolated with acoustic foam. Tones were generated by audio stimulus generators (ANL-926, MED Associates, St. Albans, USA) and applied by speakers (DTW 110 NG, Visaton, Conrad Electronics, München, Germany) mounted to the ceiling of the isolation cubicle over the respective context chambers. Small CCD cameras (Conrad Electronics, München, Germany) were mounted onto the back plane of the isolation cubicles to enable behavioural observation and recording on videotape. Experiments were controlled by commercial software (MED-PC for Windows v1.17) via interfaces (DIG 715) and the respective control panels (SG 215, all MED Associates, St. Albans, USA). For background contextual fear conditioning, animals were placed into the conditioning chamber. After 3 min, a 20 s tone (9 kHz sine wave, 80 dB) was presented that terminated with a 2 s scrambled electric footshock (0.5, 0.7 or 1.5 mA). It is important to know here that the tested mouse strains did not differ in pain threshold or auditory perception (C.T. Wotjak, personal communication). Animals were returned to their home cages 60 s after shock application. To measure the contextual freezing response, animals were placed into the grid context for 5 min, 24 h after conditioning (day1). Animals were then returned to their home cages. 2 h later, animals were placed into the original conditioning chamber, i.e. the shock context, for 5 min. This protocol was again carried out on day 2 and day 7. To measure the freezing response to the tone without confounding influences of contextual memory, conditioned mice were tested in the neutral context. After 3 min, the conditioning tone was presented (9 kHz, 80 dB) for 5 min. Mice were returned to their home cages 60 s after the end of tone presentation. An overview of the fear conditioning schedule is given in Figure 7.

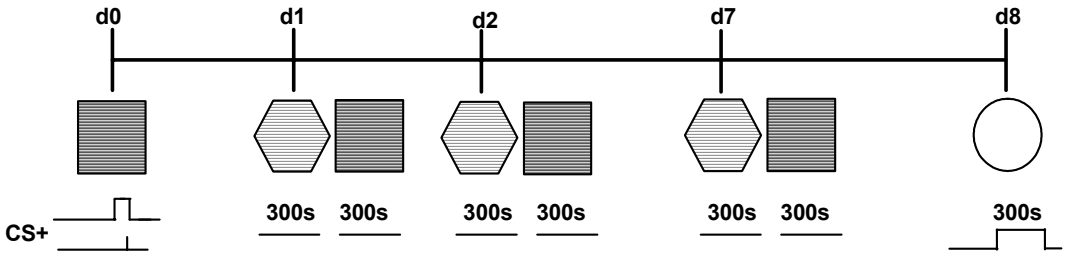


Figure 7: Schedule of the background contextual fear conditioning task. Mice were conditioned with a single tone shock association (either at 0.7 mA or at 1.5 mA) on day 0. On day 1, 2, and 7 following the conditioning procedure, animals were firstly exposed to the grid context and subsequently to the shock context for 5 min, respectively. On day 8, mice were exposed to a novel context where the conditioning tone was presented for 5 min.

The animals' behaviour was videotaped. We focused on freezing as a measure of fear. This response is frequently used in studies with aversive classical conditioning. It is easy to quantify and not induced in naïve mice by a neutral unconditioned stimulus of intermediate intensity. Freezing behaviour is defined as the absence of all movements except for respiration and was quantified off-line by a trained observer and measured second-by-second.

Table 2: Overview of the three different contexts used for fear conditioning

Context	Shock context	Grid context	Neutral context
Shape	Cubicle	Hexagonal Prism	Cylinder
Walls	two aluminium walls and two transparent Plexiglas walls	opaque Plexiglas walls with rough surface	Transparent Plexiglas walls
Floor	Metal grid floor	Metal grid floor	sawdust bedding
Cleaning/odour	70% ethanol	Water containing isoamylacetate (1:2000, banana aroma)	1% acetic acid
Illumination	Lighting of 0.6 lux	Lighting of 12 lux	Lighting of 0.3 lux

2.3.6 Elevated plus-maze test

The elevated plus-maze (EPM) was made of black plastic and consisted of two open arms (30 x 5 cm), and two enclosed arms (30 x 5 x 15 cm). A neutral zone of 5 x 5 cm interconnected all four arms. The open arms had a rim with a height of 0.5 cm. The EPM was located 120 cm above the floor. The light intensity was 300 lux at the end of the open arms and 70 lux at the starting point of the open arms. In the closed arms light intensity was 50 lux and 60 lux on the central platform. A CCD camera (Conrad Electronics, München, Germany) was mounted over the plus-maze in order to observe the mouse' behaviour and leave it undisturbed behind a curtain. At the beginning of the experiment, each mouse was placed on the central platform facing a closed arm. During the 5 min test, the time in percent spent on the open arms (time open arm/ (time open arm + time closed arm) x 100), open arm entries in percent (entries open arm/ (entries open arm + entries closed arm) x 100) and the number of entries into the closed and open arms were scored using the computer software program Plus maze (E. Fricke, München, Germany). Mice were considered to have entered an open or closed arm when all four paws were on the arm. After each test, the plus-maze was cleaned with water containing detergent and dried with tissue.

2.3.7 Light/dark exploration

Testing took place in four boxes (Coulbourn Instruments, Allentown, USA). Each box was divided into two equally sized compartments (L26 x W13 x H38 cm). One compartment was made of clear plastic walls and was illuminated with bright light of 350 lux, an intensity that seems to be sufficiently aversive to reduce exploration in the lit compartment (Costall et al., 1989;Marsch et al., 2007), while the other compartment was made of black plastic, not illuminated and covered by a black roof. An opening, 7.5 x 7.5 cm wide, connected the two parts of the box. Two infrared sensor rings allowed measurement of vertical and horizontal activity, as will be further described below for the open field test.

A session started by placing the animal in the centre of the dark compartment and lasted for 15 min. From the raw data, entries made into, relative distance travelled in

and relative time spent in the lit compartment over the 15 min period were calculated using Tru Scan Software Version 1.1 A (Coulbourn Instruments, Allentown, USA) and by means of customised macros implemented into Microsoft Excel (TruScan).

2.3.8 Defensive marble burying

One day prior to behavioural testing, mice were housed in a Makrolon type II cage on a thick layer (5 cm) of sawdust. On the testing day, 12 glass marbles with a diameter of 1 cm (Simba Toys GmbH, Fürth, Germany) were evenly spaced in the home cage in the presence of the mouse. Illumination in the cage was 0 lux. After 15 min the number of marbles at least two-thirds covered by sawdust was counted.

2.3.4 Open field test

Animals were tested in four open field boxes (L26 x W26 x H38 cm high) (Coulbourn Instruments, Allentown, USA), made of a white floor and clear plastic walls. The arena was subdivided in a centre and margin compartment with the margin clasping an area 4.5 cm away from the walls. The apparatus was equipped with infrared photocell sensors (sensor spacing 1.52 cm) and allowed measurement of vertical and horizontal activity (sampling frequency 4 Hz) and was connected to a personal computer equipped with TruScan Software Version 1.1 A. Each box, including its sensor rings, was surrounded by an additional box (47 x 47 x 38 cm, white plastic), which prevented the animals from seeing each other.
A session started by placing the animal in the centre of the field. Mice were allowed to explore the apparatus for 30 min. The test was carried out at two different light conditions: 0 lux or 700 lux. The mice had to complete two runs using identical light conditions in the open field with a retention interval of 24 h. The total distance travelled, time rested, time spent in the margin, relative distance travelled in the margin and vertical activity (rearing) were measured offline in 3 min time-bins using the Tru Scan Software Version 1.1 A (Coulbourn Instruments, Allentown, USA) and by means of customised macros implemented into Microsoft Excel (TruScan).

2.4 Statistical analysis

Data were analysed by analyses of variance (ANOVA) for repeated measurements and followed by multiple pair-wise comparisons with the Student-Newman-Keul's *post hoc* test separately per line with genotype as an independent and time/day as a dependent variable. Data were Greenhouse-Geisser adjusted if they failed to pass Mauchley's test of sphericity indicating that the groups' variances were significantly different. Two-tailed Student's t-test was used to compare genotypes for data containing two groups. For another subset of experiments, one-sample t-test was used to check for differences from chance level. 2 x 2 contingency tables and two-sided Chi-square test were used to analyse responder versus non-responder characteristics. Data are presented as means +/- standard errors of the mean (S.E.M.). Statistical significance was set at $p \leq 0.05$.
For data illustration, we used GraphPad Prism Version 4.0 software (GraphPad Software Inc., San Diego, USA). For statistical analysis, SPSS 12.0 for Windows (SPSS Inc., Chicago, USA), GraphPad Prism Version 4.0 and Statistica 5.0 for Windows (StatSoft, Tulsa, USA) software were applied.

3. Results

For the matter of clarity and brevity, all statistical parameters of a given experiment are summarised in Tables 3-37 in the Appendix. In the results sections we report only those non-significant and significant differences with the respective level of significance that are essential for the experiments under study. For further parameters, the degrees of freedom, error values and sample sizes the reader is kindly referred to the Tables shown in the Appendix. The respective tables are indicated for each experiment.

3.1 Electrophysiology

3.1.1 Current-response relationships

As an indicator of basal synaptic transmission and excitability, the fEPSP slope and pop-spike amplitude across a range of stimulation currents were compared across the knockout mouse lines. Knockout mice and their respective wildtype littermates did not have significantly different responses for fEPSP slope for all three mouse lines (Figure 8A-C, Table 3). However, for pop-spike amplitude, GABA-CB1-ko mice had significantly larger responses than the wildtype mice over the whole range of stimulus intensities ($p < 0.001$) (Figure 8E, Table 3). Glu-CB1-ko had a strong tendency of increased pop-spike amplitudes ($p = 0.067$) as well (Figure 8F, Table 3). No differences were found for pop-spike responses for total-CB1-ko mice compared to their wildtype littermates (Figure 8D, Table 3).

3.1.2 Paired-pulse facilitation of fEPSP slope

Presynaptic short-term synaptic plasticity was investigated using the paired-pulse protocol for fEPSP. The paired-pulse facilitation profiles (inter-pulse interval: 10-100 ms) were similar between ko and wt mice of all three lines (Figure 8G-I, Table 3). In each case, maximum facilitation of the second response was obtained with a 10-20 ms inter-pulse interval. There were no significant differences in the degree of facilitation between knockout and wildtype mice of the three lines.

3.1.3 Paired-pulse inhibition and facilitation of pop-spike

Inhibitory feedback onto granule cells could be investigated using the paired-pulse protocol of pop-spike. Paired-pulse stimulation at intensities above threshold for evoking a pop-spike of about 50% of the maximal response was conducted with the three knockout lines (Figure 8J-L, Table 3). At shorter inter-pulse intervals (< 40 ms) the pop-spike evoked by the second pulse was inhibited relative to the first pulse. At the shortest interval, i.e. at 10 ms, complete inhibition of the second spike was observed for all three knockout mouse lines. At inter-pulse intervals between 40-100 ms, the second pulse was facilitated relative to the first pulse. Spike facilitation peaked at inter-pulse intervals of 60-80 ms in all mice tested. At inter-pulse intervals ranging from 500-2000 ms, paired-pulse ratio declined to 1, indicating decreasing facilitatory or inhibitory processes occurring for these intervals. For the three intervals, that is 10-30 ms, 40-100 ms and 500-2000 ms, no statistically significant differences could be found between knockout mice and the respective wildtype littermates of the three mouse lines. Only total-CB1-ko mice were close to significance to show less paired-pulse facilitation between 40-100 ms compared to wildtype mice (genotype: $p = 0.058$).

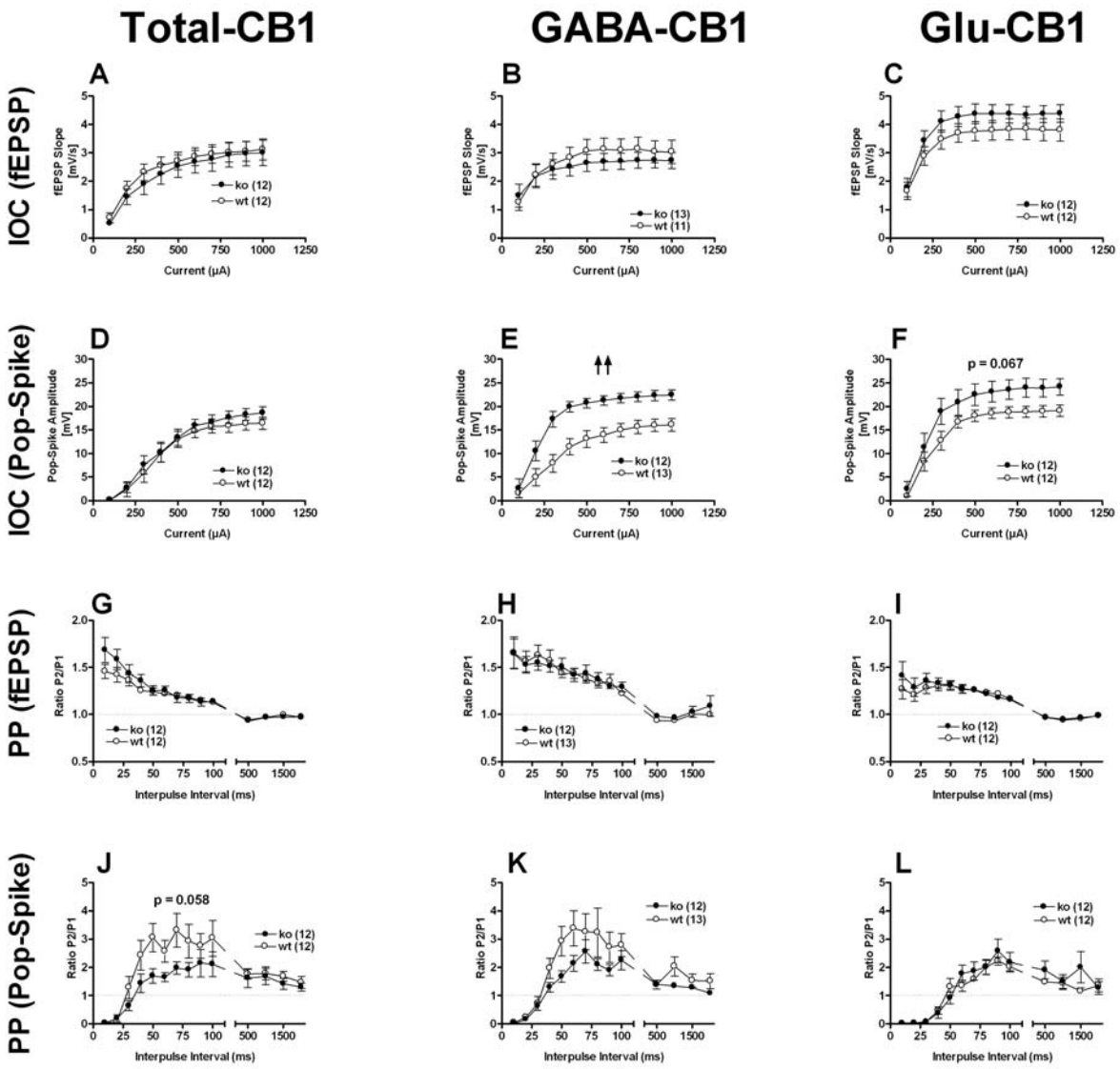


Figure 8: Current-response relationships and short-term synaptic plasticity. Evoked potentials were recorded from the granule cell layer *in vivo* from anaesthetized mice. **(A-F)** Current-response relationship for total-CB1-ko, GABA-CB1-ko and Glu-CB1-ko mice and the respective wildtype littermates. **(A-C)** Slope of the evoked fEPSP over a range of stimulation currents. **(D-F)** Pop-spike amplitude over a range of stimulation currents. Data are shown as mean +/- S.E.M. ↑↑ $p < 0.01$ (main effect of genotype). Sample sizes are indicated for genotypes.
(G-I) Paired-pulse facilitation of fEPSP. The relative change of fEPSP peak height from the first to the second response of each pair is plotted against the inter-pulse interval. Paired-pulse facilitation was observed over an inter-stimulus interval (IPI) range of 10-100 ms. Data are shown as mean +/- S.E.M. Sample sizes are indicated for genotypes. Dotted lines indicate equal peak heights of both pulses.
(J-L) Paired-pulse inhibition and facilitation of pop-spike. The relative change in pop-spike amplitude from the first to the second response of each pair is plotted against the inter-pulse interval. Paired-pulse depression was seen over an inter-stimulus interval range of 10-30 ms and paired-pulse facilitation was observed over an inter-stimulus interval (IPI) range of 40-100 ms. Facilitation recovered to a level of 1 as the IPI increased. Data are shown as the mean +/- S.E.M. Sample sizes are indicated for genotypes. Dotted lines indicate equal pop-spike amplitudes of both pulses.
Please see Table 3 for statistical details.

3.1.4 Long-term potentiation (LTP)

We next investigated long-term synaptic plasticity using the model of LTP. The baseline pop-spike amplitude for each mouse was set in the range of 1-3 mV pop-spike amplitude. We measured baseline responses of fEPSP and pop-spike with a frequency of 0.03 Hz for 30 min. Then, high-frequency stimulation was administered into the perforant path and responses were again measured with the same current used for baseline stimulation. We found no genotype differences for LTP of fEPSP over the period of 1 h after tetanus injection for all three mouse lines when comparing knockout mice with the respective wildtype littermates (Figure 10A-C, Table 3).

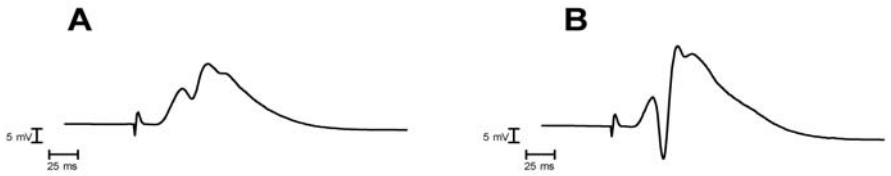


Figure 9: Evoked potential traces of a representative total-CB1-wt mouse. (A) Evoked potential during baseline stimulation. **(B)** Evoked potential 30 min after HFS. Enlargement of the pop-spike can be seen after LTP induction. Calibration is indicated with a horizontal and vertical bar, respectively.

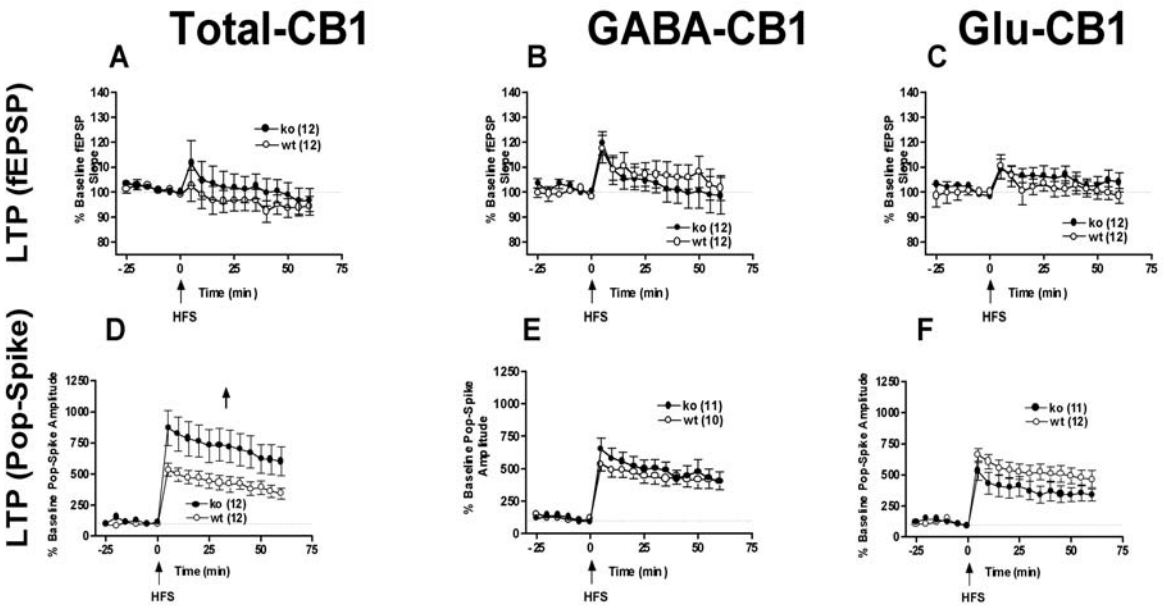


Figure 10: Long-term potentiation (LTP). (A-F) LTP of fEPSP slope and pop-spike amplitude in the granule cell layer of the dentate gyrus following high-frequency stimulation. **(A-C)** Time course of the slope of fEPSP responses evoked by a test pulse, expressed as a percentage of the baseline response (10 min before HFS) that is set to 100% (dotted line). One data-point is the average of ten responses. Data are the mean +/- S.E.M. **(D-F)** Time course of the amplitude of the pop-spike responses evoked by a test pulse, expressed as a percentage of the baseline (10 min before HFS) response that is set to 100% (dotted line). One data-point is the average of ten responses. Data are the mean +/- S.E.M. Sample sizes are indicated for genotypes. Arrows indicate the point of application of HFS. ↑ $p < 0.05$ (main effect of genotype). Please see Table 3 for statistical details.

A robust enhancement of pop-spike amplitudes persisted for at least 60 min in all genotypes of the three mouse lines (Figure 9 and Figure 10 D-F, Table 3). However, we could observe no difference for pop-spike amplitudes for the GABA-CB1-ko and the Glu-CB1-ko mice compared to the wildtype control mice (Figure 10E+F, Table 3). In contrast, total-CB1-ko mice showed significantly enhanced LTP compared to their wildtype littermates over the time course of 1 h after tetanus application ($p < 0.05$) (Figure 10D, Table 3). When comparing the individual levels of LTP of pop-spike in the total-CB1-ko mice, obviously only a subset of mice is responsible for the genotype difference in LTP (Figure 11).

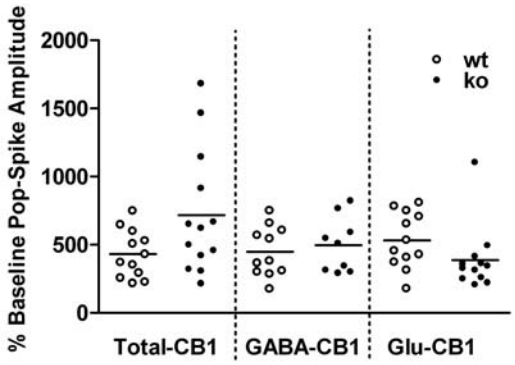


Figure 11: Pop-spike amplitude after LTP induction. Pop-spike amplitude in percent from baseline for the indicated mouse line and genotype for the one point in time 60 min after HFS. Each point represents one animal. Bars indicate the mean.

3.1.5 Hippocampal electroencephalography (EEG) after tail pinch

EEG activity was recorded from the granule cell layer of the dentate gyrus in urethane-anaesthetised total-CB1-ko mice and the respective wildtype controls. EEG activity in the theta range may be an indicator for enhanced cognitive abilities and possibly altered emotionality in animals. Pinching the mouse's tail for 10 s elicited robust theta rhythm that occurred within 1-5 s and lasted over the analysed time-frame of 6 min. Figure 12 shows representative 10 s traces of raw EEG activity before and after tail-pinch in total-CB1-ko mice. Spectral analysis of EEG revealed a prominent theta band that was evident 10 s following tail-pinch (Figure 13A). In order to study the sequence of theta oscillation over time, a baseline was measured for 30 s. Then, a tail pinch of 10 s was administered and EEG was again measured for 360 s. Theta activity was raised to about 115% of baseline level which was significantly different from baseline (interval: $p < 0.001$) (Figure 13B, Table 4). There were no significant differences for the total-CB1-ko mice from the respective wildtype littermates.

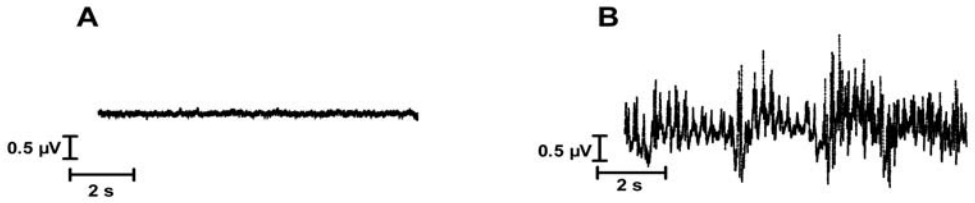


Figure 12: Representative local field potential EEG recordings in the dentate gyrus of a total-CB1-wt mouse. Two 10 s epochs are shown, either 10 s before (baseline) **(A)** or 10 s after tail pinch **(B)**. Calibration is indicated with a horizontal and vertical bar, respectively.

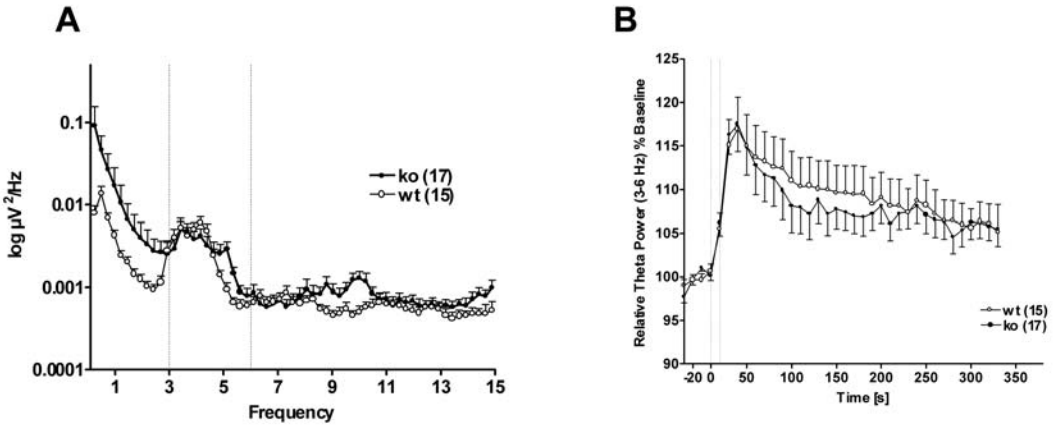


Figure 13: Theta oscillations in the dentate gyrus of anaesthetised mice. (A) Fast Fourier Transform (FFT) analysis of total-CB1-ko animals and the respective wildtype controls. Each FFT analysis is the average of 10 s, recorded 10 s after the tail pinch. Dotted lines indicate the 3-6 Hz range that was defined as theta frequency. Data are the mean +/- S.E.M. Sample sizes are indicated for genotypes. **(B)** Averaged power spectra from all total-CB1-ko mice and the wildtype littermates for a sequence 30 s before and 500 s after the tail-pinch. EEG activity appeared within 10 s following initiation of the tail-pinch and lasted the whole 500 s epoch. When compared with wildtype mice, the averaged 3-6 Hz EEG spectral power band of total-CB1-ko mice was unaltered. Dotted lines indicate the range of the 10 s-tail pinch. Sample sizes are indicated for genotypes. Data are the mean +/- S.E.M. Please see Table 4 for statistical details.

3.2 Learning and Memory

3.2.1 Water maze spatial discrimination task

Allocentric spatial discrimination learning was tested with a two-choice water maze task. Total-CB1-ko and Glu-CB1-ko mice and their respective wildtype littermates were trained to choose between two platforms with one platform being stable and providing support (correct platform) and the other platform floating and sinking when a mouse tried to climb on it (incorrect platform). For total-CB1-ko and Glu-CB1-ko mice and the respective wildtype mice, we found a significant increase in accuracy over days (day: $p < 0.01$ each), and a significant decrease in latency over days (day: $p < 0.001$ each), thus the animals successfully learned the task (Figure 14, Table 5). There was no overall effect between genotypes for the latency to swim to a platform over the days. Furthermore, no genotype difference could be seen in accuracy for the two mouse lines for the acquisition of the task. On day 6, the former stable platform became the sinkable platform and vice versa. For accuracy, reversal training

revealed that Glu-CB1-ko mice showed significantly less tendency to swim to the old location of the correct platform on day 6 and day 7 (genotype: $p < 0.01$) compared to the wildtype mice (Figure 14D, Table 5). No such differences could be found for total-CB1-ko mice and the wildtype controls for reversal training (Figure 14C, Table 5).

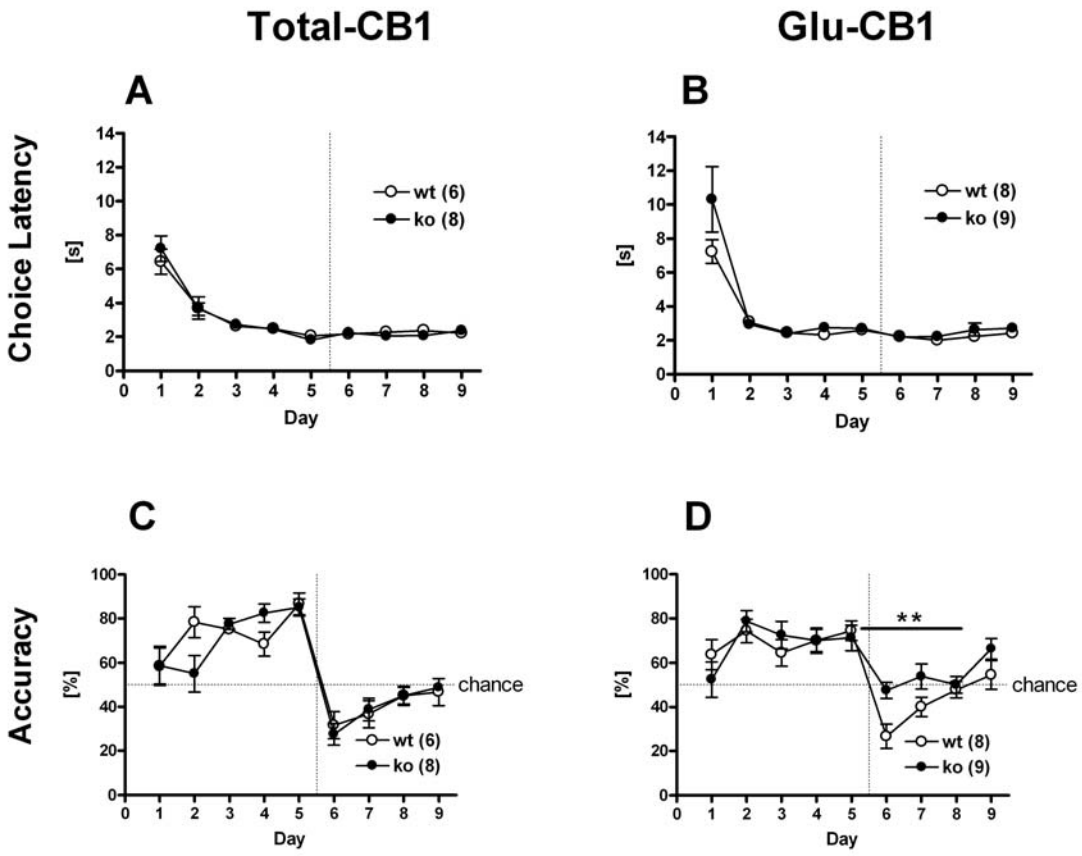


Figure 14: Water maze spatial discrimination task. (A-B) Choice latency to reach one of the platforms in total-CB1-ko mice **(A)** and Glu-CB1-ko mice **(B)** and the respective wildtype mice during the water maze spatial discrimination task. Day 1-5 displays acquisition training which is followed by reversal training on day 6-9 (separated by a vertical dotted line). **(C-D)** Accuracy (percentage correct choices) during water maze spatial discrimination task in total-CB1-ko mice **(C)** and Glu-CB1-ko **(D)** mice and the respective wildtype littermates. Day 1-5 displays acquisition training which is followed by reversal training on day 6-9 (separated by a vertical dotted line). The horizontal dotted lines represent chance performance (50 %). Data are expressed as mean values with error bars denoting S.E.M. ** $p < 0.01$. Sample sizes are indicated for genotypes. Please see Table 5 for statistical details.

3.2.2 Social recognition

We conducted a social recognition task after a short interval (1 h) or a long interval (24 h) to test short-term and long-term memory of mice for a familiar conspecific. As assessed by a one-sample t-test with a column mean different from 0.5 (i.e. chance level), total-CB1-ko ($p < 0.01$), total-CB1-wt ($p < 0.001$), GABA-CB1-ko ($p < 0.05$) and Glu-CB1-wt ($p < 0.001$) mice were able to discriminate between an already encountered familiar conspecific and a new juvenile mouse 1h after the first exposure whereas GABA-CB1-wt and Glu-CB1-ko mice failed to do so (Figure 15A, Table 6). After 24 h, only total-CB1-wt ($p < 0.01$), GABA-CB1-ko ($p < 0.01$) and GABA-CB1-wt ($p < 0.05$) mice were able to discriminate familiar from new juveniles (Figure 15B, Table 6).

Genotype differences were analysed with a two-tailed t-test. For recognition memory after 1 h or 24 h, there were no significant genotype differences except for Glu-CB1-ko mice that showed a decrease in memory performance compared to their wildtype littermates ($p < 0.05$) after an 1 h interval (Figure 15A, Table 7).

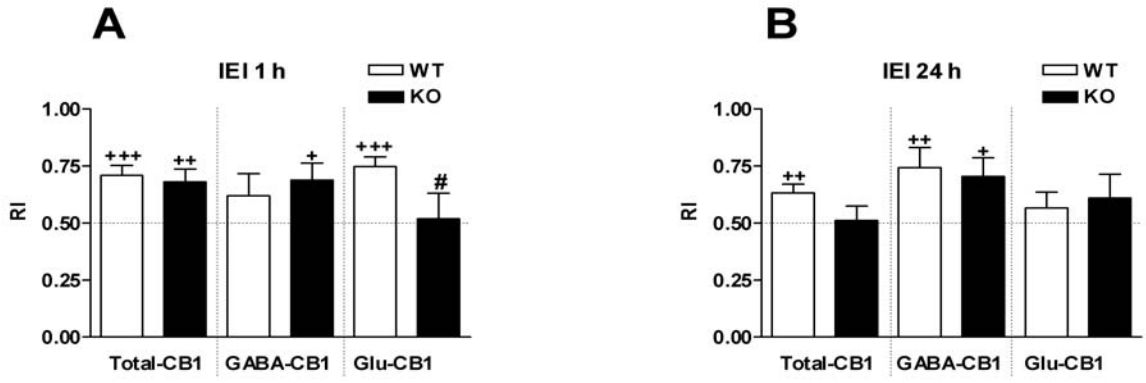


Figure 15: Social recognition memory. Short-term (1h) and long-term (24h) social recognition memory in total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype mice. After a 4 min-sampling trial, social recognition was tested in a 4 min-choice trial 1h or 24h thereafter. A recognition index RI (T_{new}) / (T_{old} + T_{new}) with T being the respective exploration time) was calculated. **(A)** Recognition index after 1 h. **(B)** Recognition index after 24 h. The dotted lines indicate chance level (0.5). Values are means +/- S.E.M. Sample sizes are given in Table 7 (Appendix). + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$, one-sample t-test against chance level. # $p < 0.05$, two-tailed t-test. Please see Table 6+7 for statistical details.

3.2.3 Object recognition

An object recognition task was accomplished to test mice for short-term and long-term memory performance. After having become acquainted with an object, mice were exposed to a novel object and a familiar one 3 h or 24 h thereafter. We applied a one-sample t-test with column mean different from 0.5 (i.e. chance level). After a 3 h intertrial interval, mice of all three lines were able to discriminate the new object except for total-CB1-wt mice which showed a strong tendency to discriminate between the familiar and the new object ($p = 0.08$) (Figure 16A, Table 6). After a 24 h-intertrial interval, in contrast, no genotype of the three lines could discriminate the new object from the old one except for total-CB1-wt mice that showed a strong tendency to discriminate between the familiar and the new object ($p = 0.06$) (Figure 16B, Table 6). Two-tailed t-tests performed separately for the mouse lines failed to reveal significant genotype differences for the three mouse lines neither at the 3 h nor at the 24 h interval (Figure 16, Table 7).

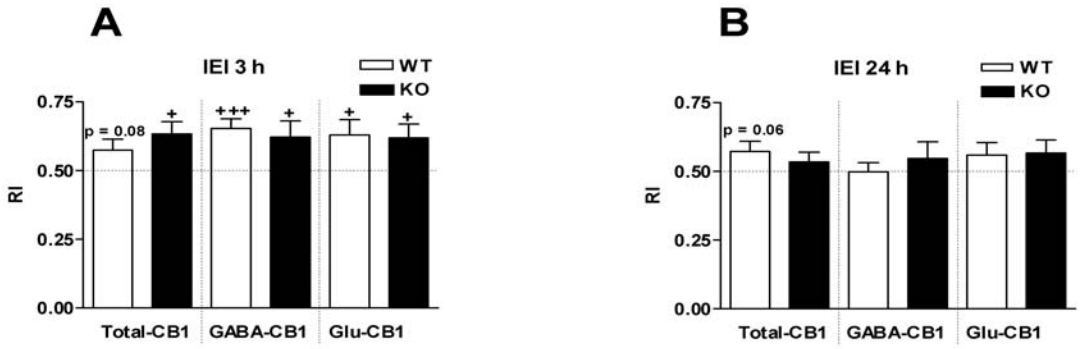


Figure 16: Object recognition memory. Short-term (3h) and long-term (24h) object recognition memory in total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates. After a 10 min-sampling trial, object recognition was tested in a 10 min-choice trial 3h or 24h later. A recognition index RI (T_{new}) / (T_{old} + T_{new}) with T being the respective exploration time) was calculated. **(A)** Recognition index after 3 h. **(B)** Recognition index after 24 h. The dotted lines indicate chance level (0.5). Values are means +/- S.E.M. Sample sizes are given in Table 7 (Appendix). + $p < 0.05$, +++ $p < 0.001$, one-sample t-test against chance level. P-value is indicated if it is close to significance. Please see Table 6+7 for statistical details.

3.2.4 Contextual fear conditioning

In order to understand the ability of mice of the three lines to discriminate between a shock context where the animals experienced an electric footshock and a grid context where they received no shock, we assessed fear conditioning to a context and to a tone in a background contextual fear conditioning task. Mice were exposed to the grid context and to the shock context on days 1, 2 and 7 after conditioning. On day 8, mice were exposed to another neutral context where the tone of the conditioning procedure was presented to the mice. Furthermore, mice were conditioned either with 0.7 mA or with 1.5 mA footshock intensity.

On day 1 after conditioning with 0.7 mA, GABA-CB1-ko mice showed increased levels of freezing in the grid context (genotype: $p < 0.05$) whereas no significant differences could be found for total-CB1-ko and Glu-CB1-ko mice, compared to the respective wildtype controls (Figure 17A, C, E, Table 8). Glu-CB1-ko mice revealed higher levels of freezing in the shock context (genotype: $p < 0.05$) but total-CB1-ko and GABA-CB1-ko mice showed no significant differences here, compared with the wildtype controls, respectively (Figure 17A, C, E, Table 8). On day 2, total-CB1-ko and GABA-CB1-ko mice had increased levels of freezing in the grid context (genotype: $p < 0.05$ and $p < 0.01$, respectively) whereas Glu-CB1-ko mice showed no significant difference, compared to the respective wildtype littermates (Figure 17A, C, E, Table 8). However, Glu-CB1-ko mice showed augmented freezing-levels in the shock context (genotype: $p < 0.05$) and total-CB1-ko mice had a strong tendency to freeze more in the shock context (genotype: $p < 0.07$), but GABA-CB1-ko mice did not show any significant differences, compared to the wildtype littermates (Figure 17A, C, E, Table 8). On day 7, freezing of GABA-CB1-ko mice was elevated in the grid context (genotype: $p < 0.01$) but no significant elevation could be seen in total-CB1-ko and Glu-CB1-ko mice, compared to the wildtype controls, respectively (Figure 17A, C, E, Table 8). Total-CB1-ko mice had increased freezing levels in the shock context (interval x genotype: $p < 0.01$) and Glu-CB1-ko mice had a strong tendency to show increased freezing in the shock context (genotype: $p < 0.06$), but

GABA-CB1-ko mice revealed no significant differences, always compared with the respective wildtype control animals (Figure 17A, C, E, Table 8).
On day 1 after conditioning with 1.5 mA, total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice displayed increased levels of freezing in the grid context (genotype: $p < 0.001$, $p < 0.005$ and $p < 0.05$, respectively), always compared with the respective wildtype animals (Figure 17B, D, F, Table 9). Total-CB1-ko mice revealed higher levels of freezing in the shock context (genotype x interval: $p < 0.05$), whereas no significant differences were found for GABA-CB1-ko mice and Glu-CB1-ko mice, compared with the wildtype littermates (Figure 17B, D, F, Table 9). On day 2, total-CB1-ko mice had increased levels of freezing in the grid context (genotype: $p < 0.001$), whereas GABA-CB1-ko and Glu-CB1-ko mice showed no significant differences, compared with the respective wildtype controls (Figure 17B, D, F, Table 9). GABA-CB1-ko mice had a strong tendency to freeze more in the shock context (genotype: $p < 0.08$), but total-CB1-ko mice and Glu-CB1-ko mice did not display any significant differences, compared to the wildtype control mice (Figure 17B, D, F, Table 9). On day 7, freezing of total-CB1-ko mice was elevated in the grid context (genotype: $p < 0.001$), but no significant difference was found for GABA-CB1-ko and Glu-CB1-ko mice, compared with the wildtype controls, respectively (Figure 17B, D, F, Table 9). Glu-CB1-ko mice had increased freezing in the shock context (genotype: $p < 0.01$), but no significant differences were seen in total-CB1-ko and GABA-CB1-ko mice, compared to the wildtype controls (Figure 17B, D, F, Table 9).
We next tested whether mice of the three lines were able to discriminate between the two contexts. When being conditioned with 0.7 mA, on day 1, total-CB1-ko mice (genotype x context: $p < 0.01$), total-CB1-wt mice (genotype x context: $p < 0.05$), GABA-CB1-ko mice (genotype x context: $p < 0.005$) and GABA-CB1-wt mice (genotype x context: $p < 0.001$) had higher freezing levels in the shock context compared to the grid context, but Glu-CB1-ko and Glu-CB1-wt mice failed to do so (Table 12). On day 2, GABA-CB1-wt mice (genotype x context: $p < 0.005$) and Glu-CB1-ko mice (genotype x context: $p < 0.05$) displayed higher freezing levels in the shock context, but total-CB1-ko, total-CB1-wt, GABA-CB1-ko and Glu-CB1-wt mice failed to do so (Table 12). On day 7, GABA-CB1-wt mice were close to significance to reveal higher levels of freezing (genotype x context: $p < 0.059$) and Glu-CB1-ko mice (genotype x context: $p < 0.01$) indeed revealed higher levels of freezing in the shock context compared to the grid context, whereas total-CB1-ko, total-CB1-wt, GABA-CB1-ko and Glu-CB1-wt mice showed similar levels of freezing in both contexts (Table 12).
On day 1 after conditioning with 1.5 mA, GABA-CB1-wt mice showed more freezing in the shock context (context: $p < 0.01$), whereas total-CB1-ko, total-CB1-wt, GABA-CB1-ko, Glu-CB1-ko and Glu-CB1-wt mice failed to do so (Table 13). On day 2, total-CB1-wt mice (context: $p < 0.05$), GABA-CB1-ko mice (context: $p < 0.01$) and GABA-CB1-wt mice (context: $p < 0.05$) displayed more freezing in the shock context compared to the grid context, but there was no significant difference for total-CB1-ko, Glu-CB1-ko and Glu-CB1-wt mice (Table 13). On day 7, total-CB1-ko mice (context x interval: $p < 0.05$), Glu-CB1-ko mice (context: $p < 0.01$), Glu-CB1-wt mice (context: $p < 0.05$), GABA-CB1-ko mice (context: $p < 0.05$) and GABA-CB1-wt mice (context: $p < 0.05$) had higher levels of freezing in the shock context compared to the grid context, but total-CB1-wt mice showed no significant differences (Table 13) .

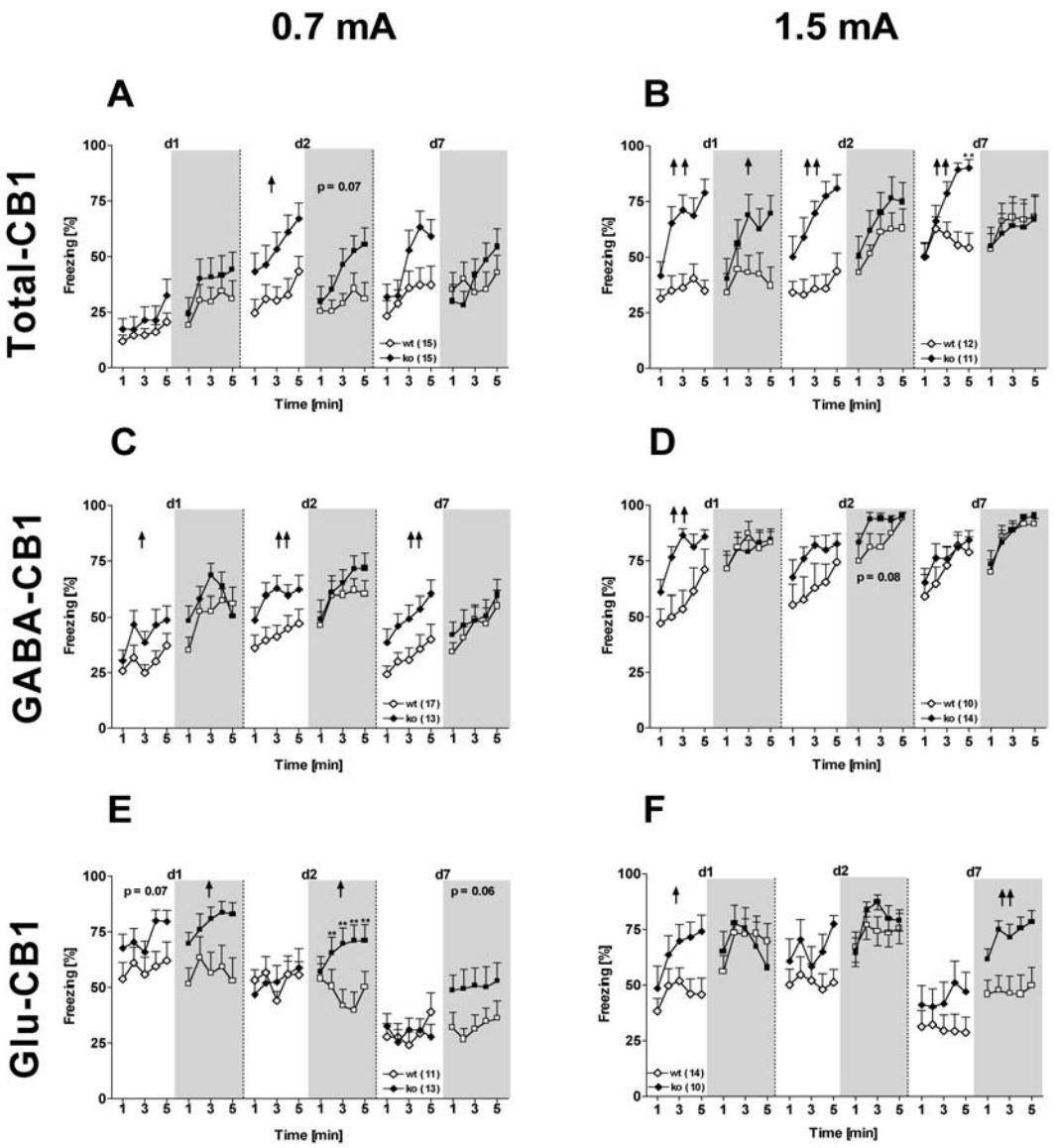


Figure 17: Background contextual fear conditioning. Freezing levels of total-CB1-ko mice **(A-B)**, GABA-CB1-ko mice **(C-D)** and Glu-CB1-ko mice **(E-F)** and the respective wildtype littermates in the contextual fear conditioning paradigm on day 1, 2 and 7 after conditioning. Mice were conditioned with 0.7 mA **(A, C, E)** or with 1.5 mA **(B, D, F)**. Grey insets and square symbols highlight the freezing values in the shock context compared to the grid context with diamond symbols. Data are shown in 1-min-intervals. Values are means +/- S.E.M. Arrows indicate significant main effects of genotype. Asterisks indicate post-hoc comparisons following significant genotype x interval interaction. ↑ $p < 0.05$, ↑↑ $p < 0.01$ and ** $p < 0.01$. Effects close to significance are indicated by the given p value. Sample sizes are indicated for genotypes. Please see Table 8+9 for statistical details.

If the development of the freezing response on the repeated test days was analysed separately for context, at 0.7 mA, total-CB1-ko mice and GABA-CB1-ko mice showed generally more freezing in the grid context than the wildtype control mice (genotype: $p < 0.05$ and $p < 0.01$, respectively) independently of the testing days (genotype x day: $p = 0.3$ and $p = 0.7$, respectively) but there was no significant difference in

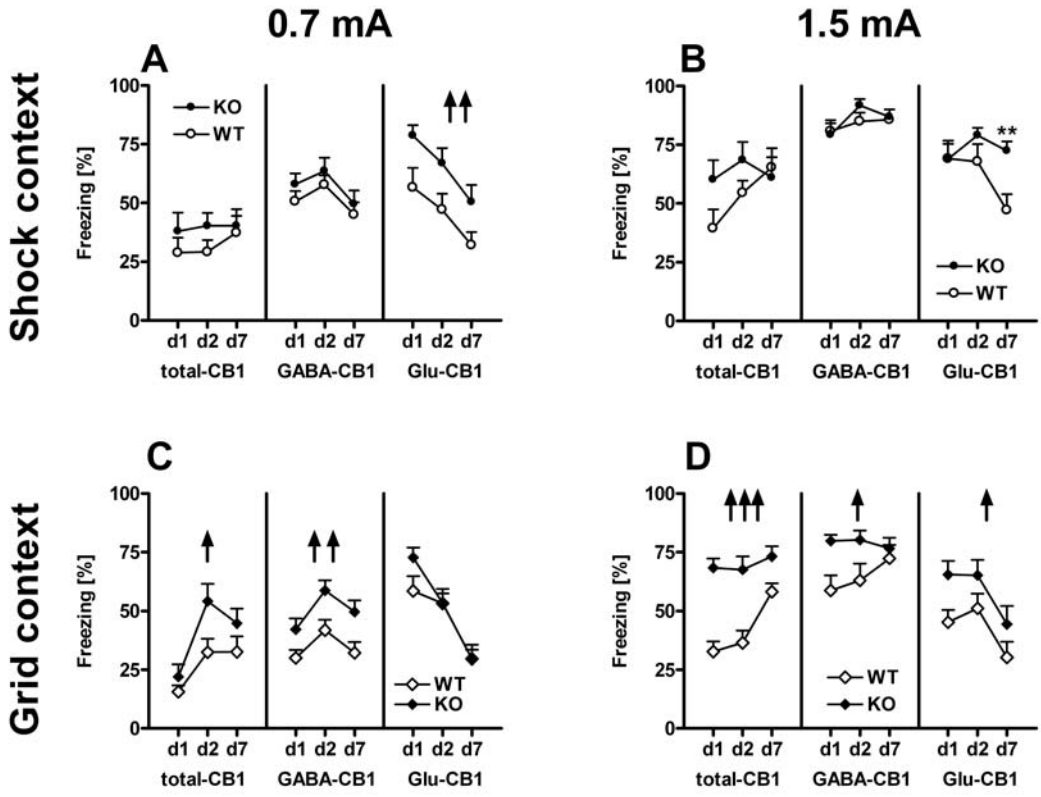


Figure 18: Development of the fear response in the two contexts. Freezing levels of total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates in the shock context **(A, B)** or the grid context **(C, D)** following background contextual fear conditioning with 0.7 mA **(A, C)** or 1.5 mA **(B, D)**, respectively over the three testing days. Values are means +/- S.E.M. Sample sizes are the same as in Fig. 17. Arrows indicate significant main effects of genotype. Asterisks indicate post-hoc comparisons following significant genotype x interval interaction. ↑ $p < 0.05$, ↑↑ $p < 0.01$, ↑↑↑ $p < 0.001$ and ** $p < 0.01$. Please see Table 14+15 for statistical details.

Glu-CB1-ko mice, compared with the wildtype littermates (Figure 18C, Table 14). On the other hand, Glu-CB1-ko mice revealed generally more freezing in the shock context (genotype: $p < 0.01$) which is not the case in total-CB1-ko and GABA-CB1-ko mice, compared to the wildtype mice (Figure 18A, Table 14).
At 1.5 mA, total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice showed generally more freezing in the grid context than the wildtype control mice (genotype: $p < 0.001$, $p < 0.05$ and $p < 0.05$, respectively) (Figure 18D, Table 15). In the shock context, no differences in the levels of freezing could be found in total-CB1-ko and GABA-CB1-ko mice, compared to their wildtype control mice, respectively, whereas there was augmented freezing in Glu-CB1-ko mice compared to the wildtype littermates (day x genotype: $p < 0.01$) (Figure 18B, Table 15).

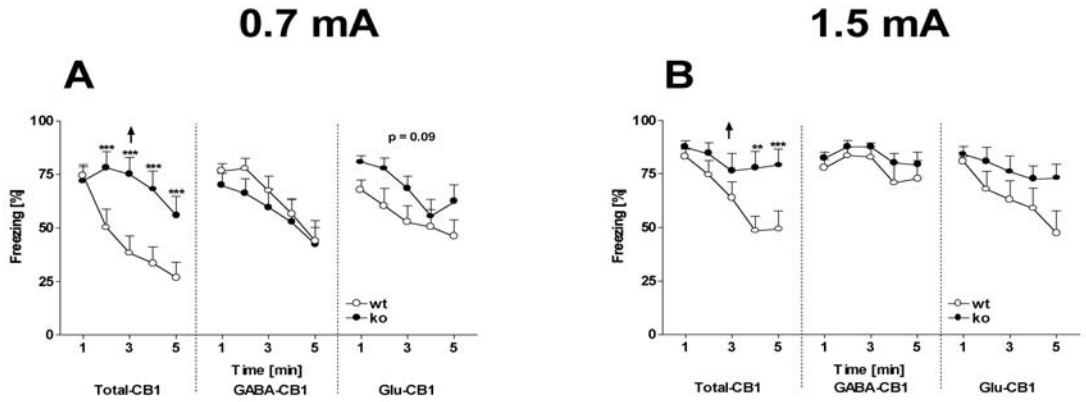


Figure 19: Conditioning response to the auditory cue. Freezing levels of total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates for the auditory cue in the background contextual fear conditioning paradigm on day 8. The same mice as shown in Fig. 15 were conditioned with 0.7 mA **(A)** or with 1.5 mA **(B)**. Data are shown in 1-min-intervals. Values are means +/- S.E.M. Sample sizes are the same as in Fig. 17. Arrows indicate significant main effects of genotype. Asterisks indicate post-hoc comparisons following significant genotype x interval interaction. ↑ $p < 0.05$, ↑↑ $p < 0.01$ and ** $p < 0.01$, *** $p < 0.001$. Effects close to significance are indicated by the given p value. Please see Table 8+9 for statistical details.

Next, we tested whether mice of the three lines displayed different freezing behaviour to the tone used for fear conditioning. The animals were exposed to a neutral context and the conditioning tone was presented for 5 min.
Being conditioned with 0.7 mA, total-CB1-ko (interval x genotype and genotype: $p < 0.001$ and $p < 0.05$, respectively) showed higher freezing levels and Glu-CB1-ko (genotype: $p < 0.092$) mice showed a tendency to display more freezing to the tone, whereas there was no significant difference for GABA-CB1-ko mice, always compared to the wildtype control mice (Figure 19A, Table 8).
Being conditioned with 1.5 mA, total-CB1-ko (Interval x genotype and genotype: $p < 0.05$ each) showed higher freezing levels whereas there was no significant difference for GABA-CB1-ko mice and Glu-CB1-ko mice, always compared to the wildtype control mice (Figure 19B, Table 9). Additionally, we conditioned Glu-CB1 with 0.5 mA (these results are summarised in the Appendix section). Here, Glu-CB1-ko mice revealed higher freezing levels than their wildtype littermates when the tone was presented (Interval x genotype: $p < 0.01$) (Figure 35B, Table 17).
Eventually, we checked whether the mice of the three lines revealed altered freezing behaviour in a new environment, which was the neutral context on day 8 before tone presentation. For 0.7 mA, there was no difference in freezing for total-CB1-ko, GABA-CB1-ko and Glu-CB1-ko mice compared to the wildtype littermates (Figure 20A, Table 22). At 1.5 mA, no difference could be detected for total-CB1-ko and Glu-CB1-ko mice, but GABA-CB1-ko mice showed augmented levels of freezing (genotype: $p < 0.01$), always compared to the wildtype animals (Figure 20B, Table 22).

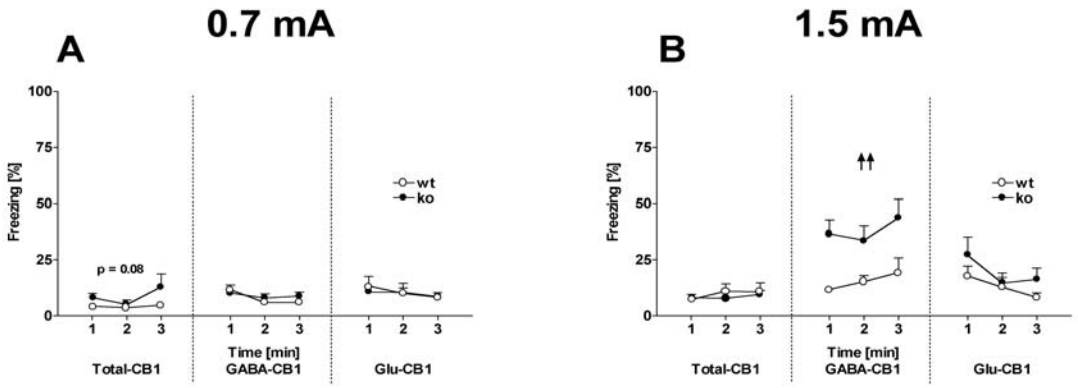


Figure 20: Response of fear in a neutral context. Freezing levels of total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates before the auditory cue in the contextual fear conditioning paradigm on day 8 after conditioning. Mice were conditioned with 0.7 mA **(A)** or with 1.5 mA **(B)**. Data are shown in 1-min-intervals. Values are means +/- S.E.M. Sample sizes are the same as in Fig. 17. Arrows indicate significant main effects of genotype. ↑↑ $p < 0.01$. Please see Table 22 for statistical details.

3.3 Emotionality and Anxiety

3.3.1 Elevated plus-maze

As an indicator of anxiety-related behaviour, the time and frequency with which a mouse explored an unprotected and aversive environment, i.e. the open arms, was assessed with the elevated plus-maze on two consecutive days.

On day one, no significant differences for open-arm entries, open-arm time, closed-arm entries and total-arm entries could be found for the genotypes of the three mouse lines, although total-CB1-ko showed a strong tendency to enter the open arms less often ($p < 0.08$) and to spend less time in the open arms ($p < 0.09$) whereas GABA-CB1-ko had a strong tendency to enter the open arms more frequently ($p < 0.06$), always compared to the respective wildtype controls (Figure 21A+C, Table 23). For total arm entries and closed arm entries, i.e. parameters to control for locomotion, no differences could be observed for any of the three mouse lines (Figure 21B+D).

On day two, no significant differences for open-arm entries, open-arm time, total-arm entries and closed-arm entries could be found for the genotypes of the three mouse lines, although total-CB1-ko showed a tendency to spend less time on the open arms ($p < 0.097$) (Figure 22A-D, Table 23).

Using a chi square test, we next analysed how many mice per genotype explored the open arms at all. On day one, no differences could be found for the genotypes of the three mouse lines (Figure 23A, Table 24). On day two, however, significantly less total-CB1-ko mice ($p < 0.05$) and Glu-CB1-ko mice ($p < 0.01$) explored the open arms compared to the respective wildtype mice (Figure 23B, Table 24).

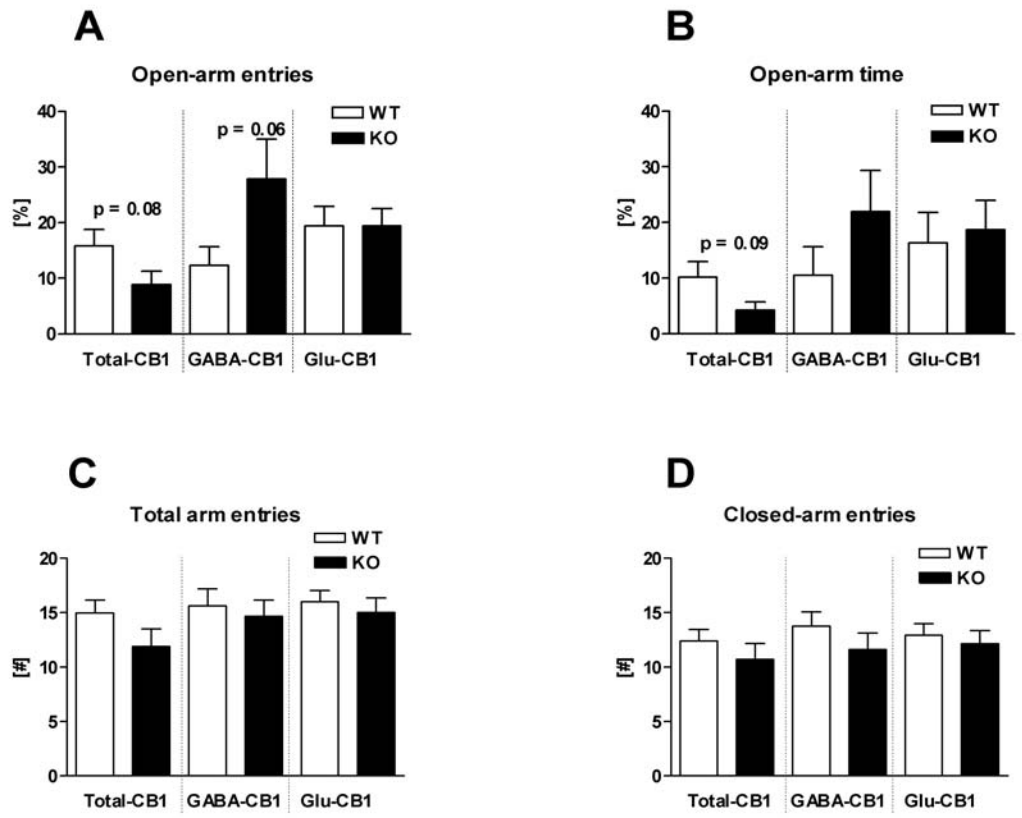


Figure 21: Elevated plus-maze behaviour on day 1. Behaviour on the elevated plus-maze of total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates on day 1. Open-arm entries **(A)**, open-arm time **(B)**, total number of arm entries **(C)** and closed-arm entries **(D)** are shown. Bars represent means +/- S.E.M. Sample sizes are given in Table 23 (Appendix). Effects close to significance are indicated by the given p value. Please see Table 23 for statistical details.

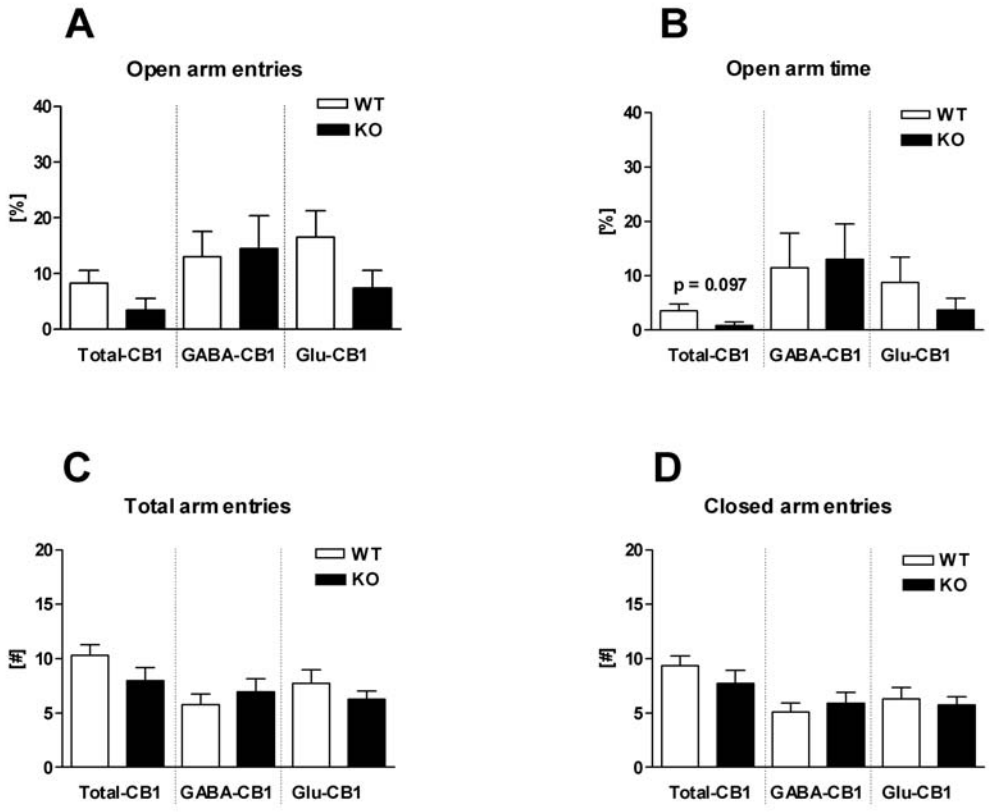


Figure 22: Elevated plus-maze behaviour on day 2. Behaviour on the elevated plus-maze of total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates on day 2. Open-arm entries **(A)**, open-arm time **(B)**, total number of arm entries **(C)** and closed-arm entries **(D)** are shown. Bars represent means +/- S.E.M. Sample sizes are given in Table 23 (Appendix). Effects close to significance are indicated by the given p value. Please see Table 23 for statistical details.

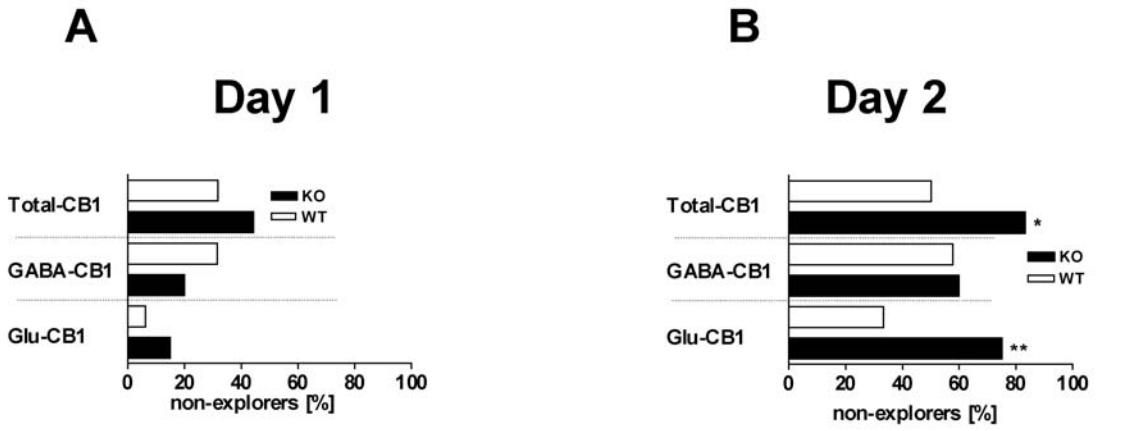


Figure 23: Proportion of non-explorers of the open arms. Relative number of animals of total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates which refrained from open arm exploration at all on day 1 **(A)** and on day 2 **(B)**. Sample sizes are given in Table 24 (Appendix). * $p < 0.05$, ** $p < 0.01$ versus respective wildtype mice (chi-square test). Please see Table 24 for statistical details.

3.3.2 Light/dark exploration

To test the avoidance of animals to explore brightly lit environments, animals were exposed to the light/dark box for 15 min. The time spent in the lit compartment, the relative distance moved in the lit compartment normalised to the total distance moved and the entries into the lit compartment were analysed. No significant genotype differences could be ascertained for either parameter for total-CB1, GABA-CB1 and Glu-CB1 mice (Figure 24A-C, Table 25).

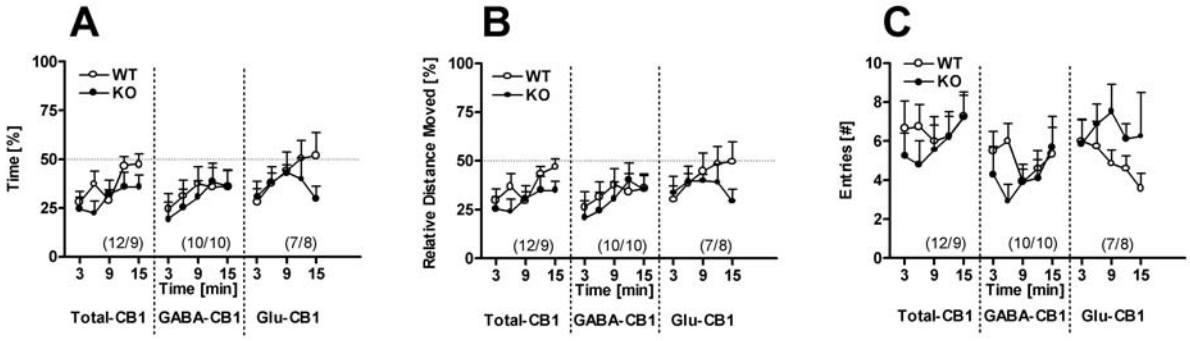


Figure 24: Light/dark box. Light/dark box exploration of total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates. **(A)** Relative time the animals spent in the illuminated compartment **(B)** relative distance travelled in the illuminated part **(C)** entries made into the lit compartment are shown for the entire 15 min exposure in 3-min-intervals. Dotted lines in **(A)** and **(B)** indicate an equal distribution in both compartments (50%). Data are presented as means +/- S.E.M. Sample sizes are indicated for genotypes (WT / KO). Please see Table 25 for statistical details.

3.3.3 Defensive marble burying

Marbles that are distributed in the home cage can be considered as an aversive stimulus to mice. Therefore, the number of marbles that are buried in the home cage of the mice was assessed with the marble burying test during a 15 min interval. No significant differences in the number of buried marbles could be found between the genotypes (Figure 25, Table 27).

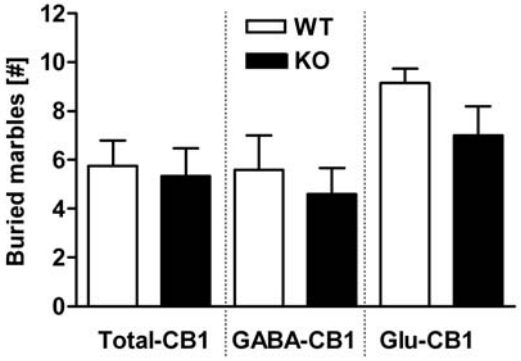


Figure 25: Defensive marble burying test. Number of marbles buried during a 15 min session by total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates. Data are presented as means +/- S.E.M. Sample sizes are given in Table 27 (Appendix). Please see Table 27 for statistical details.

3.3.4 Social interaction

As a marker of anxiety-related behaviour, the time spent by a mouse in social interaction, i.e. sniffing, close following or grooming the partner, was tested in the three mouse lines under lowly or highly aversive lighting conditions, i.e. 0 lux and 500 lux. At an illumination of 0 lux, we found no significant genotype differences for the

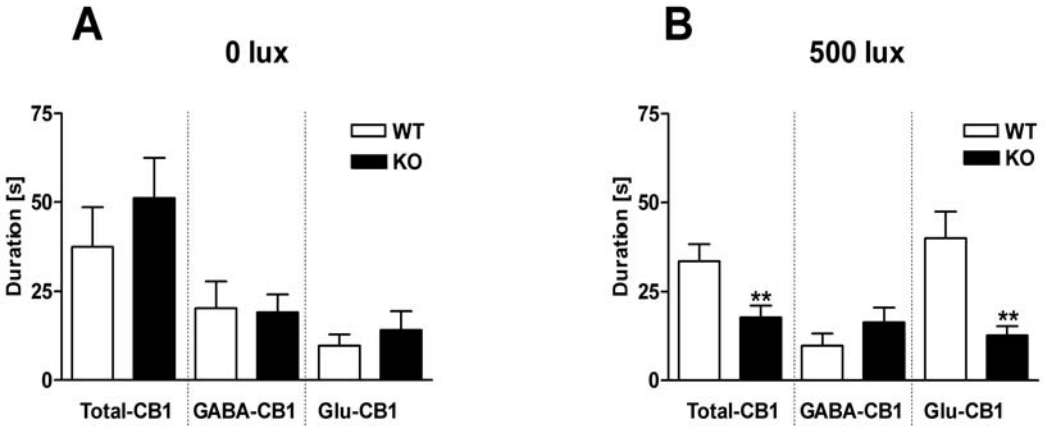


Figure 26: Exploration of a novel juvenile mouse. Exploration of a novel juvenile mouse by total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates at 0 lux (A) or 500 lux (B). Values are means +/- S.E.M. Sample sizes are given in Table 26 (Appendix). ** $p < 0.01$ versus respective wildtype mice (unpaired t-test). Please see Table 26 for statistical details.

three mouse lines (Figure 26A, Table 26). But at an illumination of 500 lux, the time in which total-CB1-ko ($p < 0.01$) and Glu-CB1-ko ($p < 0.01$) mice engaged in social

interaction with a juvenile mouse was significantly lower compared to their wildtype littermates whereas there was no significant difference between GABA-CB1-ko mice and the respective wildtype controls (Figure 26B, Table 26).

3.3.5 Novel object exploration

As a marker of anxiety-related behaviour, the time spent by a mouse in exploring a novel object was tested in the three mouse lines under two lighting conditions that were 30 lux and 500 lux. At an illumination of 30 lux, we found no difference for total-CB1 mice, an increase of exploration for GABA-CB1-ko mice ($p < 0.05$) and a decrease of exploration in Glu-CB1-ko mice ($p < 0.01$) always compared with the respective wildtype control mice (Figure 27A, Table 26). At an illumination of 500 lux, total-CB1-ko ($p < 0.01$) and Glu-CB1-ko ($p < 0.001$) mice showed decreased levels of exploration whereas GABA-CB1-ko mice ($p < 0.05$) showed an increase of exploration of the novel object compared to their wildtype littermates, respectively (Figure 27B, Table 26).

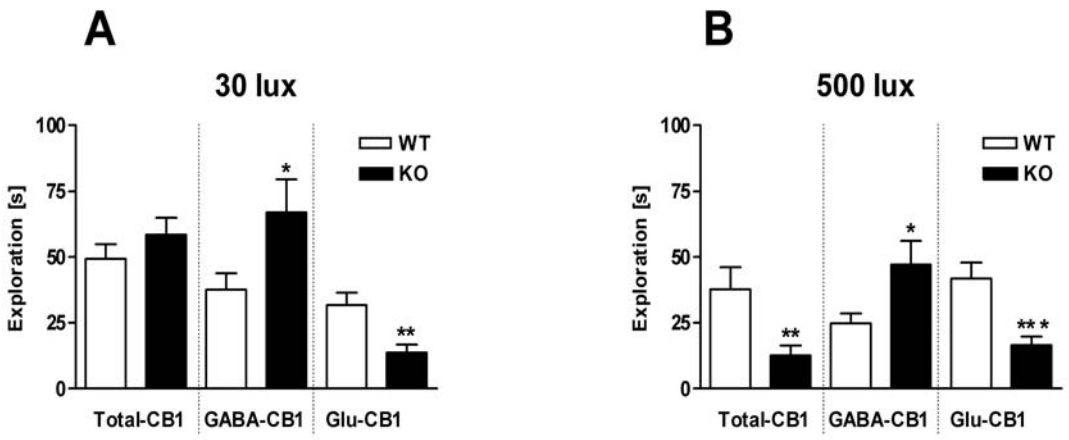


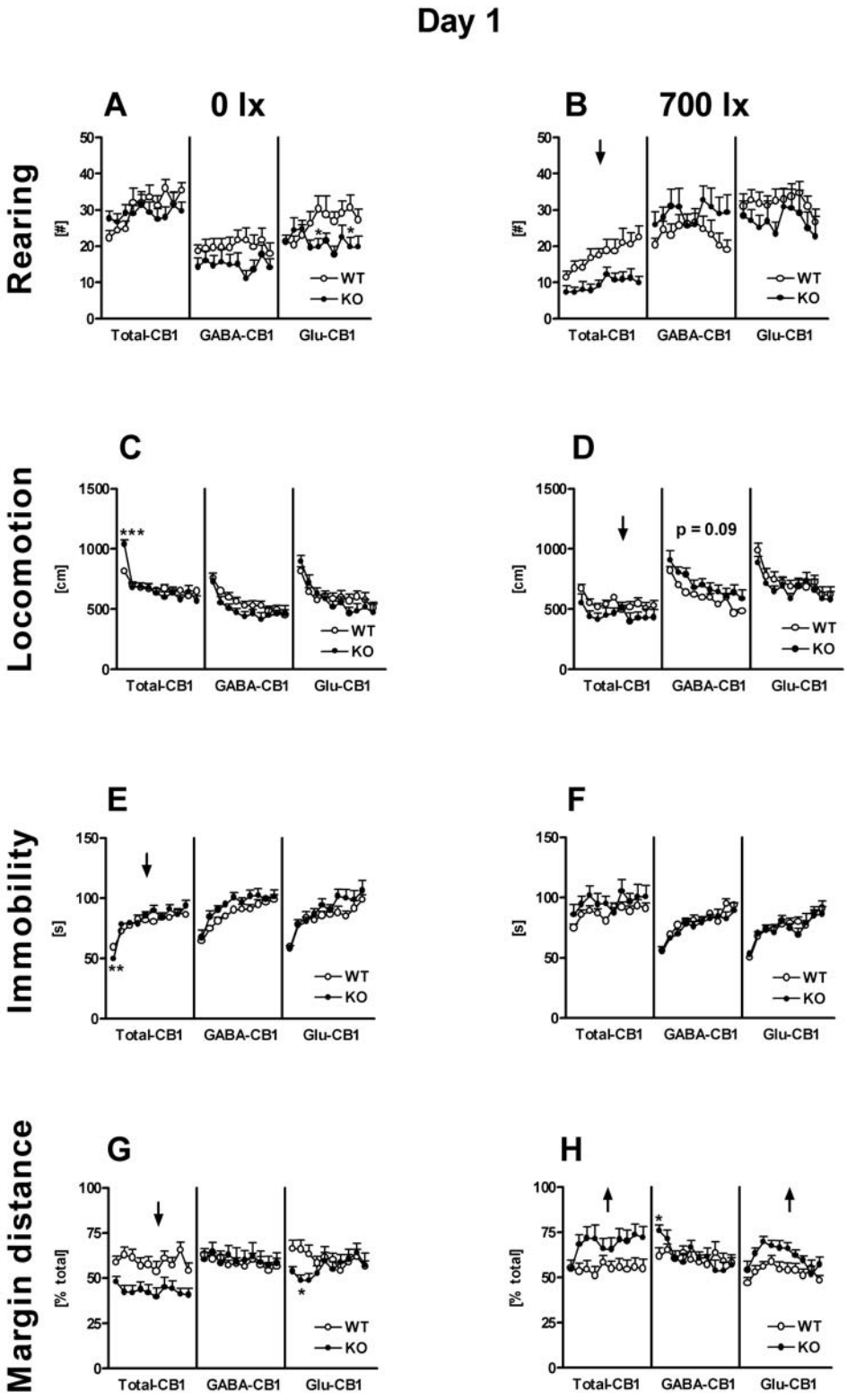
Figure 27: Exploration of a novel object. Exploration of a novel object by total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates at 30 lux **(A)** or 500 lux **(B)**. Values are means +/- S.E.M. Sample sizes are given in Table 26 (Appendix). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus respective wildtype mice (unpaired t-test). Please see Table 26 for statistical details.

3.3.6 Open field test

Mice were exposed to an open field arena for 30 min. The test was conducted under two illuminations, 0 lux and 700 lux, on two consecutive days.

On day 1 at 0 lux, we found a significant difference in the development of rearing over the time of Glu-CB1-ko mice (genotype x interval: $p < 0.05$), indicating that the mutants showed a decrease in the number of rearing towards the end of exposure, and no significant difference for total-CB1-ko and GABA-CB1-ko mice, always compared to the respective wildtype littermates (Figure 28A, Table 28). There was an increase for the total distance moved of total-CB1-ko mice (genotype x interval: $p < 0.001$) whereas no significant difference could be found for GABA-CB1-ko and Glu-CB1-ko mice, always compared to the respective wildtype mice (Figure 28C, Table 28). At the same time, we observed a decrease of immobility of total-CB1-ko mice (genotype x interval: $p < 0.001$) and no significant difference for GABA-CB1-ko and Glu-CB1-ko mice, always compared to the respective wildtype controls (Figure 28E,

Table 28). Distance moved close to margin is significantly decreased in total-CB1-ko mice and in Glu-CB1-ko mice (genotype or genotype x interval: $p < 0.05$ and $p < 0.05$, respectively) but unchanged in the GABA-CB1-ko mice, always compared to the wildtype control mice, respectively (Figure 28G, Table 28).
On day 1 at 700 lux, we found a decrease of rearing of total-CB1-ko mice (genotype: $p < 0.05$) whereas GABA-CB1-ko and Glu-CB1-ko mice showed no significant difference, always compared to the respective wildtype controls (Figure 28B, Table 29). There was a decrease for the total distance moved of total-CB1-ko (genotype: $p < 0.05$) and a tendency of an increase of total distance moved for GABA-CB1-ko mice (genotype: $p < 0.097$) whereas no significant difference could be found for Glu-CB1-ko mice, always compared to the respective wildtype controls (Figure 28D, Table 29). No differences were found for immobility in the three mouse lines (Figure 28F, Table 29). Distance moved close to margin was significantly increased in total-CB1-ko mice, Glu-CB1-ko mice (genotype: $p < 0.05$ each) and in GABA-CB1-ko mice (genotype x interval: $p < 0.05$) compared to the wildtype controls (Figure 28H, Table 29).
On day 2 at 0 lux, we found a decrease of rearing of total-CB1-ko and Glu-CB1-ko mice (genotype x interval and genotype: $p < 0.05$ each), but levels of rearing were not significantly different in GABA-CB1-ko mice, always compared with the respective wildtype control mice (Figure 29A, Table 30). Total distance moved was not significantly different in the three mouse lines, although there was a tendency of increased locomotion in the total-CB1-ko mice (genotype x interval: $p = 0.08$), compared to the wildtype controls (Figure 29C, Table 30). We observed an increase of immobility in Glu-CB1-ko mice (genotype: $p < 0.05$) but no significant differences in total-CB1-ko mice and GABA-CB1-ko mice, always compared with the wildtype mice, respectively (Figure 29E, Table 30). Distance moved close to the margin was significantly decreased in total-CB1-ko mice (genotype: $p < 0.05$), but did not reach significant differences in GABA-CB1-ko and Glu-CB1-ko mice, compared to the wildtype mice, respectively (Figure 29G, Table 30).
On day 2 at 700 lux, we found a decrease of rearing of total-CB1-ko and Glu-CB1-ko mice (genotype: $p < 0.001$ and $p < 0.05$, respectively) but not in GABA-CB1-ko mice, always compared with the respective wildtype mice (Figure 29B, Table 31). There was a decrease for the total distance moved of total-CB1-ko and Glu-CB1-ko mice (genotype: $p < 0.05$ each) but not in GABA-CB1-ko mice, compared with the respective wildtype animals (Figure 29D, Table 31). Immobility was unaltered for the genotypes, although there was a tendency of increased immobility in total-CB1-ko and Glu-CB1-ko mice (genotype: $p = 0.06$ and $p = 0.099$, respectively) always compared to the respective wildtype littermates (Figure 29F, Table 31). Distance moved close to margin was significantly increased in Glu-CB1-ko mice (genotype: $p < 0.05$), whereas no significant difference could be found in total-CB1-ko and GABA-CB1-ko mice, compared with the respective wildtype controls (Fig 29. H, Table 31).


Day 1
A
0 lx
B
700 lx
Rearing
C
D
Locomotion
E
F
Immobility
G
H
Margin distance
[#]
[cm]
[s]
[% total]
WT
KO
Total-CB1
GABA-CB1
Glu-CB1
***
**
*
p = 0.09

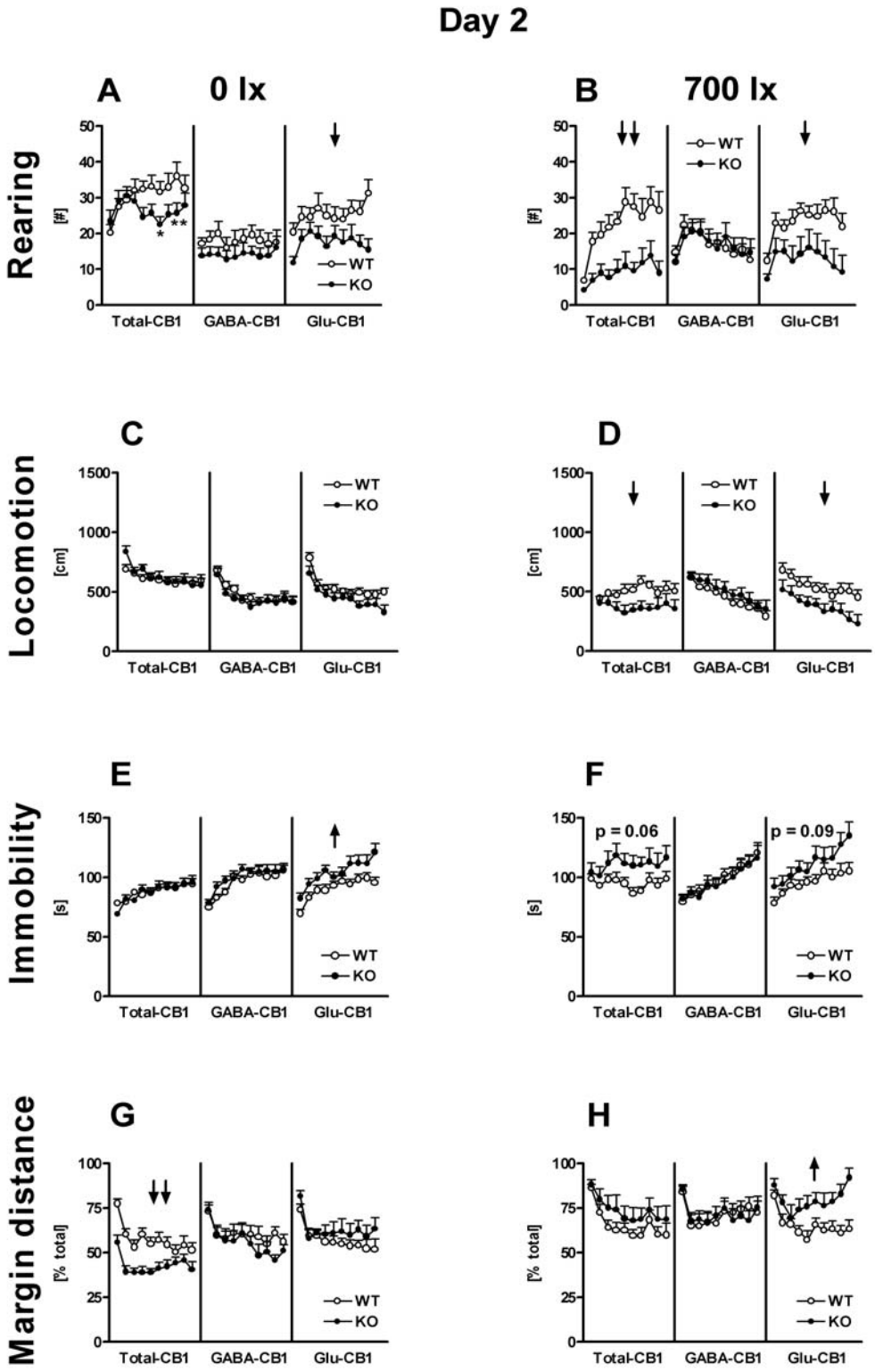

Day 2
A
0 lx
B
700 lx
Rearing
[#]
C
D
Locomotion
[cm]
E
F
Immobility
[s]
p = 0.06
p = 0.09
G
H
Margin distance
[% total]
WT
KO
Total-CB1
GABA-CB1
Glu-CB1

Figure 28: Open field test on day 1. Open field behaviour of total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates on day 1 at 0 lux **(A, C, E, G)** or 700 lux **(B, D, F, H)**. Vertical activity (i.e. rearing) **(A-B)**, total distance moved (i.e. locomotion) **(C-D)**, immobility **(E-F)** and relative distance moved along the margin walls (i.e. thigmotaxis) **(G-H)** are shown for the entire 30 min exposure in 3-min-intervals. Data are presented as means +/- S.E.M. Sample sizes are given in Table 34 and 35. Arrows indicate significant main effects of genotype. Asterisks indicate post-hoc comparisons following significant genotype x interval interaction. ↑ or ↓ $p < 0.05$, ↓↓ $p < 0.01$, and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Effects close to significance are indicated by the given p value. Please see Table 28+29 for statistical details.

Figure 29: Open field test on day 2. Open field behaviour of total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates on day 2 at 0 lux **(A, C, E, G)** or 700 lux **(B, D, F, H)**. Rearing **(A-B)**, locomotion **(C-D)**, immobility **(E-F)** and relative distance moved along the margin walls **(G-H)** over 30 min are shown. Data are presented as means +/- S.E.M. Sample sizes are given in Table 34 and 35. Arrows indicate significant main effects of genotype. Asterisks indicate post-hoc comparisons following significant genotype x interval interaction. ↑ or ↓ $p < 0.05$, ↑↑ $p < 0.01$ and * $p < 0.05$, ** $p < 0.01$. Effects close to significance are indicated by the given p value. Please see Table 30+31 for statistical details.

3.3.7 Open field habituation from day 1 to day 2

We assessed the development of the behavioural performance from day 1 to day 2 by means of ANOVA for repeated measurements for 0 lux. We found a tendency of increased immobility of Glu-CB1-ko (genotype: $p = 0.088$) but no significant difference for total-CB1-ko and GABA-CB1-ko mice compared to the wildtype control mice, respectively (Figure 30E, Table 32). No significant differences could be found for total distance moved in the three mouse lines (Figure 30C, Table 32). Glu-CB1-ko mice revealed a decrease in rearing over the two days (genotype: 0.05), but there was no difference for total-CB1-ko and GABA-CB1-ko mice, compared to the wildtype controls, respectively (Figure 30A, Table 32). However, in no case there was a significant interaction between genotype and day for immobility, total distance moved, and rearing indicating that the degree of habituation did not differ for the three mouse lines.

We then tested with a paired t-test for every genotype whether habituation occurred (Table 34). For rearing, there was a significant decrease in Glu-CB1-ko mice ($p < 0.05$) but not in Glu-CB1-wt mice as being the case in total-CB1-ko, total-CB1-wt, GABA-CB1-ko and GABA-CB1-wt mice. For locomotion, total-CB1-ko mice did not show a decrease whereas total-CB1-wt mice did show a decrease ($p < 0.05$, respectively) as it can be found in Glu-CB1-ko ($p < 0.01$), Glu-CB1-wt ($p < 0.001$), GABA-CB1-ko ($p < 0.05$) and GABA-CB1-wt ($p < 0.001$) mice. For immobility, a significant increase could be observed for total-CB1-wt ($p < 0.01$) mice and GABA-CB1-wt mice ($p < 0.001$) whereas for total-CB1-ko mice ($p < 0.058$) and GABA-CB1-ko mice ($p < 0.056$) the increase was close to significance and both Glu-CB1-ko ($p < 0.01$) and Glu-CB1-wt mice ($p < 0.01$) showed increased levels of immobility always compared to the wildtype controls.

By means of ANOVA for repeated measurements we analysed habituation of the three mouse lines over days for 700 lux. We found a significant decrease of rearing in total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice over the course of the days as compared to the respective wildtype controls (genotype, genotype x day and genotype x day: $p < 0.05$ each), compared to the littermate controls (Figure 30B, Table 33). For total distance moved, a decrease was seen in Glu-CB1-ko mice (genotype x day: $p < 0.05$) and in total-CB1-ko mice (genotype: $p < 0.05$) but not in GABA-CB1-ko mice, compared to the respective wildtype controls (Figure 30D, Table 33). For immobility, a significant increase was found in Glu-CB1-ko mice (genotype x day: $p < 0.05$) whereas total-CB1-ko and GABA-CB1-ko mice showed no significant

differences when being compared with the respective wildtype control animals (Figure 30F, Table 33).
Using a paired t-test, we then tested for every genotype whether habituation occurred (Table 35). At 700 lux, significant decrease in rearing and total distance moved and a significant increase for immobility could be assessed for all genotypes. Only for total-CB1-wt mice we found an increase of rearing under these conditions.
Eventually, we examined habituation within the first open field exposure (Table 36). For total-CB1-ko, total-CB1-wt, GABA-CB1-ko, GABA-CB1-wt, Glu-CB1-ko and Glu-CB1-wt mice we found a decrease of locomotion over time ($p < 0.001$ each) at 0 lux. We could see a corresponding increase of immobility for total-CB1-ko, total-CB1-wt, GABA-CB1-ko, GABA-CB1-wt, Glu-CB1-ko and Glu-CB1-wt mice ($p < 0.001$ each).
At 700 lux, there was a decrease in locomotion only in total-CB1-wt, GABA-CB1-ko, GABA-CB1-wt, Glu-CB1-ko and Glu-CB1-wt mice ($p < 0.001$ each) but not for total-CB1-ko mice (Table 37). In addition, we observed a significant increase of immobility in total-CB1-wt, GABA-CB1-ko, GABA-CB1-wt, Glu-CB1-ko and Glu-CB1-wt mice ($p < 0.001$ for each genotype, respectively) but not for total-CB1-ko mice.

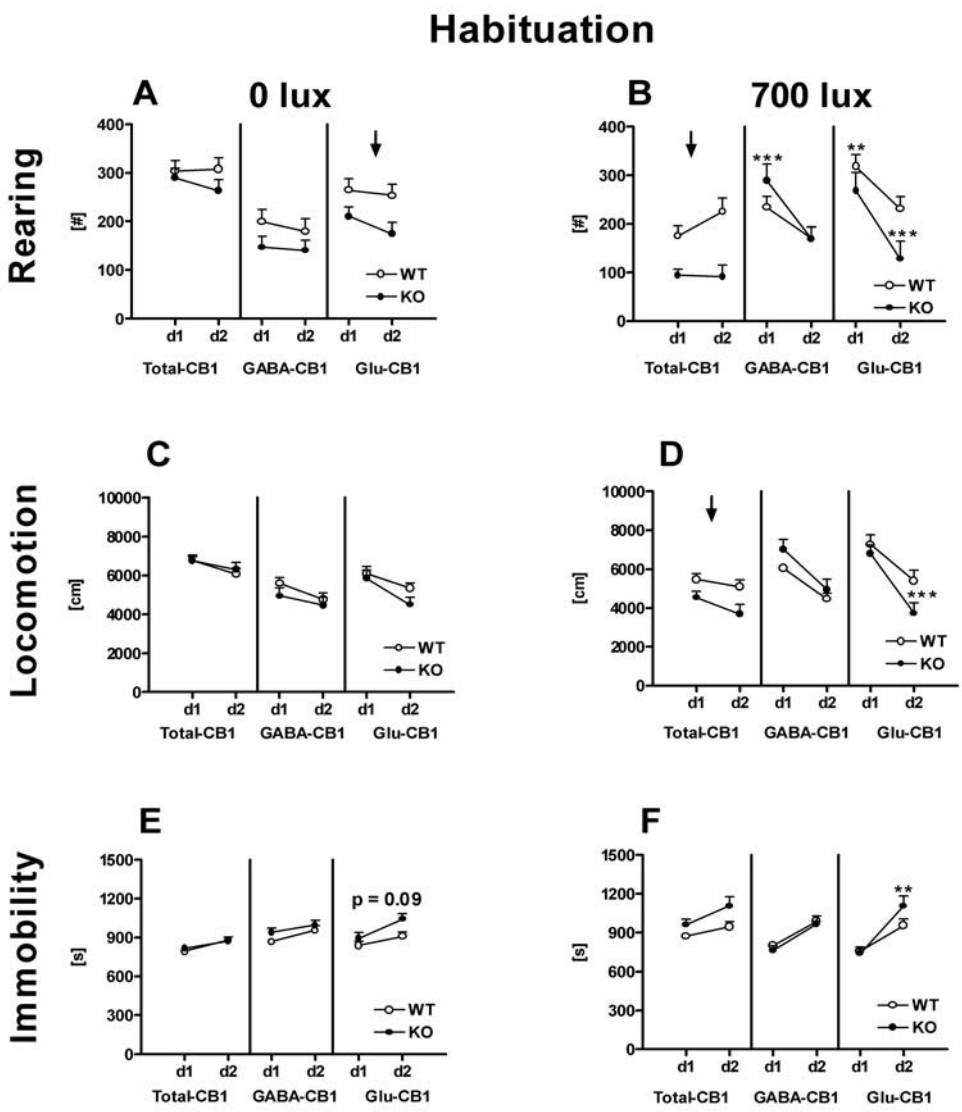


Figure 30: Open field test habituation. Open field habituation of total-CB1-ko mice, GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype littermates form day 1 to day 2 under 0 lux **(A, C, E)** or 700 lux **(B, D, F)**. Rearing **(A-B)**, locomotion **(C-D)** and immobility **(E-F)** are shown. Data are presented as means +/- S.E.M. Sample sizes are given in Table 34 and 35. Arrows indicate significant main effects of genotype. Asterisks indicate post-hoc comparisons following significant genotype x interval interaction. ↓ $p < 0.05$ and ** $p < 0.01$, *** $p < 0.001$. Effects close to significance are indicated by the given p value. Please see Table 32+33 for statistical details.

4. Discussion

In the present study, we compared electrophysiological, memory-related and emotional characteristics of three CB1-deficient mouse lines: A conventional knock-out mouse line (total-CB1-ko mice) and two conditional knock-out mouse lines with a cell type specific deficiency of CB1 on GABAergic interneurons (GABA-CB1-ko mice) or glutamatergic neurons (Glu-CB1-ko mice).

4.1 Synaptic transmission and plasticity in CB1-ko mice

4.1.1 Effects of CB1 signalling on basal synaptic transmission

CB1 is located on GABAergic and glutamatergic nerve terminals and can unfold an inhibitory effect on transmitter release (Freund et al., 2003). It is well studied that activation or block of CB1 can influence the synaptic transmission in a cellular pathway. Application of CB1 agonists reduced evoked EPSCs and IPSCs in dentate granule cells (Hajos and Freund, 2002). A reversible depression of both fEPSP and pop-spike can be seen after anandamide application (Terranova et al., 1995), although it had no effect in another study (Lees and Dougalis, 2004). Conversely, the CB1 antagonists SR141716 and AM251 can augment basal excitatory synaptic transmission (Slanina and Schweitzer, 2005). The latter study indicates that endocannabinoids might have a tonic activity to decrease excitatory neurotransmission.

In the present study, comparison of the current-response relationship of the perforant path input to the granule cells of the dentate gyrus revealed no significant differences in shape of the fEPSP increase. For pop-spike amplitude, there was a difference in that GABA-CB1-ko mice showed increased amplitudes over a range of stimulating currents. This difference in the input-output relationship for pop-spike is quite remarkable as it indicates an alteration in basal cellular excitation to fire action potentials of GABA-CB1-ko mice in contrast to total-CB1-ko and Glu-CB1-ko mice but not in depolarisation of the cells as it is reflected in the fEPSP for the three mouse lines. What might be the reason for the specific enhancement of pop-spike amplitude in GABA-CB1-ko mice? One line of evidence points towards altered input properties of the medial septum. The projection of the medial septum to the hippocampus consists of two components: One is cholinergic afferents which contact both pyramidal and inhibitory cells in the hippocampus (Frotscher and Leranth, 1985). The other component is GABAergic neurons that seem to selectively contact interneurons (Freund and Antal, 1988). About 40% of medial septal projection neurons are cholinergic and about 30% are GABAergic (Amaral and Kurz, 1985;Kiss et al., 1990). Cholinergic afferent neurons from the septum increased the amplitude of IPSPs in inhibitory interneurons, with disinhibition in the pyramidal cell (Toth et al., 1997). Disinhibition of granule cells can also be established by septohippocampal GABAergic neurons selectively innervating inhibitory interneurons in the dentate gyrus (Frotscher et al., 1992). Electrical stimulation of the medial septum *in vivo* is reported to increase population spikes but not the fEPSPs generated in the dentate gyrus by the perforant path (Bilkey and Goddard, 1985;Fantie and Goddard, 1982). Application of the acetylcholine esterase inhibitor physostigmine (acetylcholine esterase degrades ACh in the synaptic cleft) raises the levels of ACh and leads to inhibition of fEPSP. Interestingly, the CB1 antagonist AM251 eliminated this inhibitory effect to reduce fEPSP in the hippocampus (Colgin et al., 2003). Furthermore, a recent study of Monory and colleagues seems to indicate a lack of CB1 mRNA in the septum of GABA-CB1-ko mice (Monory et al., 2006). On the whole, as an inhibitory

effect of CB1 is missing on the cholinergic and/or GABAergic projections, disinhibition in the dentate gyrus might be facilitated. This could lead to the increased input-output relationship of pop-spike as we observed it in the GABA-CB1-ko animals. Our finding raises the question why we failed to detect a similar effect in total-CB1-ko mice? The cholinergic input seems to be modulated by CB1 on glutamatergic neurons, too, for which the exact mechanism remains unclear (Tzavara et al., 2003b). Perhaps, glutamatergic CB1 counteracts the disinhibition effect and leaves the I/O curve unchanged in total-CB1-ko animals.
Interestingly, an increase in synaptic strength, measured by input-output relationships, can be observed in the hippocampus of rats exposed to environmental enrichment or after learning a contextual fear conditioning task (Foster et al., 1996;Sacchetti et al., 2001). This phenomenon suggests that basal synaptic transmission can be altered by learning events. Although we did not find improved memory, the anxiolytic phenotype of GABA-CB1-ko mice might correlate with increased excitability in the hippocampus (as it will be discussed in detail below).

4.1.2 Effects of CB1 signalling on short-term synaptic plasticity

We would like to briefly introduce the reader to the experimental phenomenon of short-term synaptic plasticity. When e.g. the perforant path is stimulated with pairs of stimuli, the amplitudes of fEPSP and pop-spike components of the first and second response can vary depending upon the time interval between the stimuli. When the second responses are larger than the first responses, the effect is referred to as paired-pulse facilitation (PPF). When the second responses are smaller than the first responses, the effect is termed paired-pulse depression (PPD).
The analysis of fEPSPs generated by paired stimulation in granule cells has been shown that PPF develops almost instantaneously and that the amplitude of facilitation rapidly decreases with bigger intervals between stimuli. The reported duration of facilitation in granule cells *in vivo* varies tremendously depending upon the experimental conditions and range from about 40 ms to about 200 ms (Lomo, 1971). Here, we could observe facilitation to about 100 ms inter-pulse-interval.
The explanation of this phenomenon is as follows: Two stimuli given in a rapid succession produce two different synaptic responses. When the probability of transmitter release during the first response is high, the second response is relatively smaller than the first, and when the probability of release of the first is low, the second response is relatively larger than the first. PPF is thought to be caused by summation of residual calcium following the first response with even more calcium entering the presynapse on the second stimulation of the cell. This produces a larger second response. Hence, PPF is likely to be the consequence of enhanced transmitter release. Ideally, any process such as presynaptic inhibition that alters the probability of transmitter release should also alter PPF (Zucker, 1989).
This is the first time we report about short-term plasticity phenomena in the dentate gyrus of CB1-ko mice *in vivo*. We could not find a difference of PPF of fEPSP in any of the three mouse lines. In line with our findings, no difference of PPF was detected in slices from hippocampal region CA1 of CB1-ko mice (Bohme et al., 2000). Furthermore, it was shown that activation of CB1 inhibits excitatory synaptic transmission in neonatal, but not adult rat hippocampus (Al Hayani and Davies, 2000). Most studies measuring effects of activation or block of CB1 on transmitter release that found profound effects were done on a single cell level (Hirasawa et al., 2004;Katona et al., 2001;Melis et al., 2004b). Perhaps, differences in transmitter release are too subtle as they could be detected with the here used extracellular field

potential recordings or they simply do not play a role in our adult mice in an *in vivo* preparation.

If stimuli are applied that are of sufficient amplitude to produce a pop-spike, paired-pulse stimulation leads to a diphasic change in granule cell response to the second pulse. At short intervals (10-40 ms) the granule cell responsiveness to the second stimulus is decreased, i.e. PPD occurs. The PPD is due primarily to the activation of interneurons producing GABA-mediated inhibition (Alger and Nicoll, 1982a;Alger and Nicoll, 1982b). The granule cell pop-spike evoked by the first pulse drives polysynaptic GABAergic feedback loops to generate granule cell hyperpolarisation via $GABA_A$-receptors. Subsequent depolarisation of the granule cells arising from the second pulse is shunted by a residual inhibitory postsynaptic potential, reducing the probability of the granule cells reaching firing threshold and thus contributing to the decline of the pop-spike amplitude (Steffensen and Henriksen, 1991;Tuff et al., 1983). Equivalent degrees of PPD evident in knockout and wildtype mice of the three lines suggest that CB1 is not implicated in the IPSPs induced by release of GABA from interneurons and activation of $GABA_A$-receptors on the granule cells.

At intermediate intervals (40-200 ms) granule cell response to the second stimulus is facilitated. An increase in PPF is indicative of a greater number of granule cells firing in response to the second stimulus compared to the first stimulus (Andersen et al., 1971). Three mechanisms have been proposed to account for facilitation at intermediate interpulse intervals: Firstly, within specific time windows, the second pulse is delivered when the presynaptic terminals are still hyperpolarised. This leads to an increase in presynaptic calcium influx into the nerve terminal and augmentation of transmitter release for the second pulse (Fisher et al., 1997;Zucker, 1989). Secondly, PPD at longer IPIs is strongly diminished by activation of presynaptic $GABA_B$-autoreceptors located on interneurons that serve to limit subsequent GABA release (Bordi et al., 1997;Brucato et al., 1992;Canning and Leung, 2000;Steffensen and Henriksen, 1991) which has the effect that firing of granule cells is less restricted and PPF can be accomplished. Thirdly, PPF is also influenced by activation of glutamatergic n-methyl-d-aspartate receptors (NMDA-R), and that their activation comprises a large part of the facilitation response (Joy and Albertson, 1993). These findings suggest that augmentation in glutamatergic and/or reductions in GABAergic mediated neurotransmission promote the facilitation effect.

Activation of the NMDA-R increased the levels of 2-AG in primary cultures of rat neocortical neurons (Stella and Piomelli, 2001). Anandamide can inhibit NMDA-dependent calcium influx in a cortical slice preparation. Interestingly, when the CB1 receptor is blocked, anandamide still produced a stimulatory effect on NMDA-induced calcium responses and NMDA-stimulated currents in the slice, a process obviously independent of CB1 (Hampson et al., 1998). Bearing in mind that FAAH and AMT activity is increased in the hippocampus of CB1-ko mice (Maccarrone et al., 2002) which might lead to reduced anandamide levels in these mice, it might be that anandamide affects NMDA-R activity in total-CB1-ko and wildtype mice. This might explain the tendency of a decreased PPF in total-CB1-ko mice if we assume that less anandamide binds to NMDA-R in these mice compared to the wildtype animals. As further described below, anandamide can also activate the vanilloid receptor 1 (VR1) (Szallasi and Blumberg, 1999). Perfusion of anandamide increases PPD of pop-spike, an effect that was not blocked by the CB1 antagonist AM281, but was blocked by the VR1 antagonist capsazepine (Al Hayani et al., 2001). However, we could not find a significant difference of PPF of pop-spike in all three mouse lines. Perhaps, the inhibitory effect of CB1 is taken over by a similar inhibition of transmitter release by presynaptic $GABA_B$-receptors and thus changes in PPF of pop-spike do not occur.

4.1.3 Effects of CB1 signalling on long-term potentiation (LTP)

It is known for a long time that application of CB1 agonists such as Δ^9-THC, HU-210, WIN55,212-2, anandamide or 2-AG all inhibited LTP of hippocampal fEPSPs *in vitro* (Collins et al., 1995;Misner and Sullivan, 1999;Nowicky et al., 1987;Stella et al., 1997;Terranova et al., 1995). On the other hand, mice devoid of CB1 exhibited enhanced LTP *in vitro* in the hippocampus (Bohme et al., 2000) and in the basolateral amygdala (Marsicano et al., 2002). Whether antagonists of CB1 lead to enhanced LTP is a matter of debate: In some studies, the CB1 antagonist SR141716 failed to enhance LTP in the hippocampus (Terranova et al., 1995) and in the basolateral amygdala (Marsicano et al., 2002) of brain slices. However, other studies could show that the antagonists AM251 and SR141716 clearly increased the level of LTP in hippocampal CA1 region (Slanina et al., 2005) and in the prefrontal cortex (Auclair et al., 2000) in a slice preparation. As all of the pharmacological brain slice studies rely on an acute incubation, one reason for contradictory findings might be the duration of a pharmacological treatment: In a recent study by Hoffman and coworkers, no acute effect of the antagonist AM251 could be assessed but a 7-day treatment led to an increase of LTP levels in CA1 (Hoffman et al., 2007). Undoubtedly, endocannabinoid signalling occurs for a limited time window during or for a few minutes after LTP induction, as there is a lack of effect of antagonists after a certain time interval on LTP (Chevaleyre and Castillo, 2003;Ronesi et al., 2004;Sjostrom et al., 2003). As CB1 is located on GABAergic and glutamatergic synapses, one could expect GABA and/or glutamate to be causally involved in alterations of LTP.

Generally speaking, synaptic plasticity represents a mechanism by which environmental stimuli could alter responsiveness of neurons, such as the storage of information gained through experience. The durability of such changes in synaptic strength is extremely variable, such that synaptic efficacy can fluctuate with time scales ranging from milliseconds to years. LTP is defined as an enduring enhancement of synaptic efficacy, which occurs in a specific synapse following e.g. bursts of high-frequency electrical stimulation, and that can persist for hours to weeks. LTP can be subdivided based on the molecules involved in its induction and expression, as well as the synaptic locus of the change that underlies the alteration in efficacy. Some forms of plasticity are initiated and maintained by purely postsynaptic mechanisms, others by purely presynaptic mechanisms, and still others by mechanisms initiated in the postsynaptic neuron and are then communicated to the presynaptic neuron by retrograde messengers (Bliss and Collingridge, 1993;Tao and Poo, 2001). Retrograde messengers are molecules released from the postsynaptic neuron and alter the presynaptic neurotransmitter release. CB1 is located on the presynaptic terminal and postsynaptically synthesised endocannabinoids indeed function as a retrograde signal and are crucial to an alteration of synaptic efficacy (Alger, 2002;Wilson and Nicoll, 2002). In principal, any modulatory neurotransmitter capable of inhibiting synaptic transmission could disrupt LTP. As stated above, it is shown for many different CB1 agonists that they profoundly block LTP induction and maintenance. This LTP inhibiting action is likely not a unique action of cannabinoid drugs, but given the remarkably widespread presynaptic expression of CB1 throughout the entire brain, it may be a common mechanism by which endocannabinoids regulate different forms of synaptic plasticity. It is of importance for our studies that LTP stimulation paradigms indeed increase the formation of endocannabinoids (at least in the hippocampal slice) and hence might play a role for the processes under investigation (Stella et al., 1997).

In this study, we found for the first time a CB1-dependent LTP enhancement in the dentate gyrus in an *in vivo* preparation. Increased LTP could be observed only in total-CB1-ko mice. This finding was quite unexpected as it was our hypothesis that either GABAergic or glutamatergic neurons lacking CB1 were responsible for enhanced LTP and that there would be also a difference in either GABA-CB1-ko mice or in Glu-CB1-ko mice.

One explanation for our failure to detect any elevations of LTP in Glu-CB1-ko or GABA-CB1-ko mice might be that LTP in these animals was already saturated. We stimulated the perforant path using a theta-burst stimulation (TBS) protocol consisting of six series of six pulses of 400 Hz, with 200 ms inter-series interval. Theta-burst stimulation, that is short bursts of stimuli delivered at an interburst-frequency of 5 Hz, mimic the firing of pyramidal cells of rodents involved in exploratory behaviour and produces reliable, robust and saturated LTP (Staubli and Lynch, 1987). The theta-burst paradigm, but not HFS, also exploits the temporal characteristics of firing of the GABAergic interneurons for effective induction of LTP (Davies et al., 1991). The powerful potentiation enabled through TBS perhaps prevents the observation of more subtle differences in GABA-CB1-ko and Glu-CB1-ko mice compared to the respective wildtype mice. It would be interesting to apply rather moderate LTP-inducing protocols in order to detect differences in GABA-CB1-ko mice or Glu-CB1-ko mice.

Total-CB1-ko mice showed enhanced LTP compared to the respective wildtypes but the statistical significant difference was close. One reason for this finding might be that C57BL/6 mice, which is the background for all three knock-out mouse lines in this study, *per se* exhibit excellent levels of LTP induction and maintenance compared to other mouse strains both *in vitro* and *in vivo* (Jones et al., 2001b;Matsuyama et al., 1997;Nguyen et al., 2000). Hence, we possibly reached a ceiling effect in GABA-CB1-ko and Glu-CB1-ko mice that obscures enhanced LTP in CB1-deficient mice.

It was suggested that LTP has two components: Firstly an increase in the EPSP and secondly an increase in the ability of an equal-sized EPSP to fire an action potential. The latter is called the EPSP-spike (E-S) coupling component of LTP (Bliss and Lomo, 1973). Some studies postulate that the E-S coupling could be mediated by a persistent reduction of inhibitory input and hence a disinhibition of a glutamatergic neuron (Abraham et al., 1987;Chavez-Noriega et al., 1989). Our finding that only the pop-spike is enhanced fits to the results of Chevaleyre and Castillo who could demonstrate that neuronal excitability, measured as E-S coupling, is specifically blocked by CB1 antagonists and seems to be particularly increased following high-frequency stimulation (Chevaleyre and Castillo, 2003). Our TBS-protocol resulted in a pure EPSP-spike (E-S) potentiation: Pop-spike potentiation was not accompanied by any maintained increase in fEPSP slope. This phenomenon was already described by other groups for mouse and rat (Jones et al., 2001b;Veinbergs et al., 1998;Yanagihashi and Ishikawa, 1992). In the mouse, it seems to be much more difficult to induce LTP of fEPSP as also reported by Namgung and colleagues, who found LTP of fEPSP in only 38% and Bliss and Errington et al. who found LTP of fEPSP in 20% of the cases (corresponding to 100% for pop-spike) (Bliss and Errington, 1984;Namgung et al., 1995). We suggest that LTP of dentate granule cells spike generation, reflected in the pop-spike, was caused without potentiating the synaptic input in the present experiment.

CB1 blockade may not generally improve synaptic plasticity but may rather shift the occurrence of LTP. In a patch-clamp study in pyramidal cells of the prefrontal cortex, application of the agonist WIN55212-2 led to a shift of synaptic plasticity in favour of long-term depression (LTD). On the other hand, the antagonist SR141716 favoured

LTP at the expense of LTD. Nevertheless, in both cases, there were still cells that were non-plastic or showed LTP or LTD, respectively (Auclair et al., 2000). Bearing in mind that not all total-CB1-ko mice showed enhanced LTP compared to the wildtype mice in our experiments, LTP does not seem to be increased in an all-or-none fashion but rather in a subgroup of animals being prone to the tetanic stimulation.
The picture gets even more complex as the endocannabinoid anandamide, in contrast to the synthetic cannabinoid WIN55212-2, failed to inhibit theta-burst-LTP (Lees and Dougalis, 2004). Anandamide binds to and activates CB1 but also VR1 (Smart et al., 2000), two receptors with opposite effects on excitation. Furthermore, low concentrations of Δ^9-THC can even enhance the persistence of LTP (Nowicky et al., 1987).
Thus, the physiological role of endocannabinoids with their lipophilic nature and rapid reuptake may be fairly different from the effect of synthetic cannabinoids and remains to be fully explained.
We would like to make detailed suggestions to the reader what might be the underlying reason for LTP enhancement after blockade of CB1 and which transmitter systems might be involved. These suggestions can be found in the Appendix (section 6.1). All in all, it remains to be enigmatic why enhanced LTP can be observed in total-CB1-ko mice but not in Glu-CB1-ko and GABA-CB1-ko mice. Several candidates for transmitters exist of which the contribution cannot be ascertained yet. Future studies in different brain regions (e.g. CA1 of hippocampus, neocortex, and amygdala) might shed light onto altered levels of LTP in Glu-CB1-ko and GABA-CB1-ko mice.

4.1.4 Pitfalls of LTP in anaesthetised mice

We investigated the phenomenon of LTP in anaesthetised mice *in vivo* as it resembles physiological processes more closely than recordings done in brain slice preparations. Admittedly, we have to consider the effects of isoflurane anaesthesia on different transmitter systems in our *in vivo* preparation. Similar to many other anaesthetics, isoflurane potentiates the activity of the $GABA_A$ receptor, but also of 5-HT_3 and kainate receptors, whereas it blocks alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and nicotinic ACh receptors (Rudolph and Antkowiak, 2004). In addition, isoflurane anaesthesia increases the extracellular concentration of dopamine and its metabolites (Adachi et al., 2005). This simply highlights the fact that multiple transmitter systems are altered under isoflurane anaesthesia. Indeed, enhanced LTP in Thy-1-knockout mice could be observed in awake animals but not in an anaesthetised preparation (Errington et al., 1997) clarifying the difference between an awake and an anaesthetised state. Whether this is actually the case and has an effect on our results could be only revealed by measuring LTP in awake, freely behaving animals.

4.1.5 Brain oscillatory activity influenced by CB1 activation

Inhibitory interneurons in the hippocampus contact up to 1000 of pyramidal neurons, and this widespread connectivity is important in synchronising the firing of principal neurons (Freund and Buzsaki, 1996). Interneurons in the hippocampus form fast synapses and are thought to orchestrate synchronous oscillations in the gamma band that is a frequency of 20-80 Hz present in local field potentials (Banks et al., 2000). Gamma oscillations are synchronised over long distances in the brain and are hypothesised to bind together sensory perceptions and to play a role in cognition (Singer, 1999). *In vitro* evidence suggests that exogenously applied cannabinoids can selectively suppress gamma oscillations (Hajos et al., 2000).

Another endogenous oscillation in the brain is theta rhythm that is waves with a 4-12 Hz frequency present in local field potentials. Theta activity was originally described in the hippocampus to characteristically accompany exploratory behaviour (Vanderwolf, 1969) and has been suggested to serve as a temporal reference for coding relevant environmental information represented by place cells (O'Keefe and Recce, 1993).

Theta rhythm might be of interest to us as it is associated with both mnemonic function and cholinergic neurotransmission. The medial septum and the diagonal band of Broca seem to play a prominent role in generating theta rhythm (Smythe et al., 1992;Vertes and Kocsis, 1997). The major septal neurons projecting to the hippocampus use ACh or GABA as neurotransmitters (Freund and Antal, 1988;Frotscher and Leranth, 1985). Blockade of mAChR in the medial septum suppresses theta rhythm, as does $GABA_AR$ activation, and lesioning the respective neurons eliminated theta rhythm in the hippocampus (Givens and Olton, 1990;Yoder and Pang, 2005). Acetylcholine release in the hippocampus is increased during theta activity, and correlates with theta power (Keita et al., 2000). In general, rises in cholinergic transmission are found during novelty-related processes such as attention and learning (Everitt and Robbins, 1997). As learning and memory are powerfully influenced by the cholinergic input of the medial septum, it is widely believed that theta rhythm generated here is possibly a property enabling memory (Wiebe and Staubli, 2001;Winson, 1978) and the underlying neuronal plasticity (Holscher et al., 1997).

Besides of a role in memory processing, the medial septum seems to be essential for anxiety-related behaviour, too, as lesioning the septum disrupts anxiety-like behaviour in several tasks (Bannerman et al., 2004;Deacon et al., 2002;Degroot and Treit, 2003;Miller et al., 1986). Theta rhythm might couple together brain regions like the amygdala and the hippocampus in order to cope with an anxiety-provoking situation (Seidenbecher et al., 2003). Increased magnitude of theta oscillations on the open arms of an elevated plus-maze might indicate an anxiogenic phenotype in mice (Gordon et al., 2005).

Cannabinoids can diminish the power of hippocampal activity in the theta (4-10 Hz), gamma (30-80 Hz) and fast ripple (100-200 Hz) band in the freely behaving rat, a process clearly activated by CB1 (Robbe et al., 2006), and endocannabinoids can interrupt theta rhythm in CA1 of the hippocampus (Reich et al., 2005). Nevertheless, the antagonist SR141716 has no effect on the network activity (Robbe et al., 2006). This suggests that at least a tonic level of endocannabinoids does not seem to be involved in the formation of network oscillations.

Theta stimulation after a sensory stimulus in anaesthetised rodents is an established model to study e.g. cholinergic or GABAergic modulations in the hippocampus (Keita et al., 2000;Kinney et al., 1999;Yoder and Pang, 2005). We hypothesised that increased theta activity might be another factor explaining enhanced memory abilities in total-CB1-ko mice that are found by others. This was especially of interest as increased levels of ACh in CB1-ko mice could be shown *in vitro* (Kathmann et al., 2001) and *in vivo* (Degroot et al., 2006) and cholinergic neurons are considered to regulate the magnitude of hippocampal theta rhythm (Lee et al., 1994;Monmaur et al., 1997). Nevertheless, we found no change of theta activity after a sensory stimulus over time in total-CB1-ko mice. Assuming increased levels of ACh in CB1-ko mice, our finding leads rather to the conclusion that no increased theta activity accompanies a boosted cholinergic system. However, we have to bear in mind that elevated levels of ACh in behaving CB1-ko mice were only seen if the animals were exposed to a stressful environment but not under basal conditions (Degroot et al.,

2006). Therefore, our applied tail pinch that shall resemble a sensory stimulation was possibly not drastic enough to elicit enhanced ACh levels and thereby enhabced theta rhythm in the hippocampus. Also, if we assume that an alteration of theta rhythm is a prerequisite for anxiety-related behaviour, this fits well to our finding that changes of anxiety in total-CB1-ko mice are rather context-specific (as it is further discussed below), and tail-pinch-induced theta rhythm might not be the appropriate model to test this hypothesis. Lastly, we recorded local field potentials from the dentate gyrus which may not be the essential area for memory acquisition and retrieval processes. This may rather be restricted to CA1 of the hippocampus (Hall et al., 2001;Wiebe and Staubli, 1999). EEG recordings in behaving animals and in different areas of the hippocampus could enlighten these issues.
Beside of ACh, serotonin might be involved in alterations of theta rhythm in the hippocampus. Although there is no data of serotonin levels for CB1-ko mice, it could be suggested that levels are rather increased as CB1 activation leads to a decrease of serotonin in the mouse neocortex (Nakazi et al., 2000). Furthermore, CB1 and 5-HT_3 are co-localised in CCK-positive-interneurons of the hippocampus, neocortex and amygdala and receptor activation has opposite effects on GABA release, with 5-HT_3 activation increasing and CB1 activation decreasing GABA release (Hermann et al., 2002;Morales and Backman, 2002). Eventually, a 5-HT_3 receptor antagonist increases the frequency of theta rhythm in the hippocampal CA1 region *in vivo* (Staubli and Xu, 1995). This would rather suggest a decrease of theta rhythm in CB1-ko mice through elevated levels of serotonin.
Altogether, these findings might indicate that even if there is an increased level of serotonin or ACh in total-CB1-ko mice this does not lead to any alteration of hippocampal theta rhythm under our experimental conditions.

4.2. Memory functions in CB1-ko mice

4.2.1 Is there a genuine physiological role of CB1 in memory?

Blockade of CB1 signalling has been found to enhance performance in several memory tasks. SR141716 improved social recognition memory of rats and attenuated recognition memory deficits displayed by aged rats and mice (Terranova et al., 1996). Rats trained in a radial 8-arm maze made fewer errors after treatment with the antagonist SR141716 if the delay between sessions was in the range of hours (Lichtman, 2000) and showed increased performance in the elevated T-maze (Takahashi et al., 2005). Also, chickadees, after an intrahippocampal infusion of SR141716, showed enhanced long-term memory for the location of hidden food (Shiflett et al., 2004). However, there is one study finding no memory alteration in a Morris water maze task after SR141716 application (Da and Takahashi, 2002). Furthermore, SR141716 failed to enhance performance in a variety of operant conditioning paradigms (Brodkin and Moerschbaecher, 1997;Hampson and Deadwyler, 2000;Mallet and Beninger, 1998;Mansbach et al., 1996). This is probably because these tasks are strongly dependent on working or short-term memory where subjects have to retain the information in the range of 10 s and long-term memory processes do not play an essential role here[1]. Finally and conversely, administration

[1] Another difficulty may be that the antagonist SR141716 has properties of an inverse agonist (Mato et al., 2002;Terranova et al., 1996b). That is, some effects of the antagonist appear to be produced in the absence of any ongoing endogenous cannabinoid release, prompting the hypothesis that cannabinoid receptors can exist in a constitutively active state in which they undergo some degree of coupling to their effector mechanisms (Pertwee, 2005a). However, SR141716 is over 7000-fold more potent as a CB1 antagonist than as an inverse agonist and this suggests that it may have limited

of the antagonist AM251 into the rat hippocampus after training disrupted memory of an inhibitory avoidance task in a subsequent session and let the authors suggest that endocannabinoids contribute to facilitate memory consolidation (de Oliveira et al., 2005).

The phenotype of CB1-ko mice, as being reported in the literature, is partly in line with memory improvement after CB1 antagonist application. CB1-ko mice exhibited an enhanced performance in the active avoidance paradigm (Martin et al., 2002) and in an object recognition task (Maccarrone et al., 2002;Reibaud et al., 1999). However, unexpected was the finding that CB1-ko mice did neither show improved performance in a long-term appetitively-motivated operant conditioning task nor in the extinction of this task (Holter et al., 2005). Furthermore, CB1-ko mice have been reported to exhibit similar acquisition rates in the Morris water maze as wild-type littermates (Varvel and Lichtman, 2002). In addition, adult CB1-ko mice even showed a deficit in long-term social memory, skill learning on a rotarod and in an operant conditioning paradigm (Bilkei-Gorzo et al., 2005). Finally, CB1-ko mice revealed increased perseverance during reversal learning in a water maze task and showed a deficit to unlearn the task (Varvel and Lichtman, 2002). CB1-deficient mice exhibited less extinction of fear memory after a tone-footshock conditioning procedure (Marsicano et al., 2002) which is confirmed by administration of CB1 antagonist SR141716 that leads to a decrease of extinction of conditioned fear (Chhatwal et al., 2005;Marsicano et al., 2002;Niyuhire et al., 2007;Pamplona et al., 2006;Suzuki et al., 2004) and in a passive avoidance task (Niyuhire et al., 2007). Thus, the endocannabinoid system may aid extinction of aversive memories. Extinction is believed to involve active suppression of previously learned associations (Lattal and Abel, 2001). If the endocannabinoid system were involved in extinction processing, then disrupting CB1 genetically or pharmacologically may pretend improved memory in some models because it prolongs retention of the respective information.

Given the extent to which the endocannabinoid system appears to modulate short-term and long-term forms of synaptic plasticity and as we could find elevated levels of LTP in total-CB1-ko mice, it should not be surprising that this system may have a tonic activity in mnemonic processes. Curiously, although an improvement of memory was not seen in a first study (Varvel et al., 2006a), FAAH-ko mice, that possess approximately 10-fold elevated brain levels of anandamide, and mice treated with the FAAH inhibitor OL-135 acquired a water maze task faster than the respective controls in a follow-up study (Varvel et al., 2006b). Particularly, this study provides a dramatic example of how manipulations that elevate the endocannabinoid tone can lead to different results compared to exogenously applied agonists. Interestingly, an enriched environmental exposure that is known to improve hippocampus-dependent memory results in 10-fold elevations in hippocampal endocannabinoid content (Wolf and Matzinger, 2003). Perhaps, endocannabinoids are rather essential for cognition and unfold a cognitive enhancing effect.

What would then account for the observation that exogenous cannabinoids like Δ^9-THC can inhibit LTP and learning (Nowicky et al., 1987;Varvel et al., 2005)? The concentration of exogenously administered cannabinoids is likely to be greater at CB1 than the concentration of the highly labile endogenous cannabinoids suggesting

inverse agonist activity *in vivo* (Sim-Selley et al., 2001). Furthermore, SR141716 seems to bind to another receptor, as it was biologically active in mice deficient for CB1 which led to the hypothesis of “CB3” (Hajos et al., 2001). If "CB3” is crucial for effects on learning and memory is a matter of debate, as CB1-ko mice showed no impairment of working memory in a water maze task after application of different CB1 agonists (Varvel and Lichtman, 2002).

that exogenous cannabinoids are likely to do more than simply mimic the function of endocannabinoids. It could be that learning and memory relies on precise spatial and temporal ordering for information storage and recall in a tightly integrated neural circuit, and indeed LTP on a subset of synapses can be elicited by endocannabinoids (Carlson et al., 2002). Global activation of CB1 of all types of neurons and in several brain regions might disrupt this precision and lead to memory impairment.
All things considered, it is fairly controversial whether CB1 is essentially implicated in learning and memory and if so in which aspects of it. To investigate a possible contribution of CB1, we performed a set of learning and memory tasks especially in order to point out the type of receptor system (i.e. CB1 on GABAergic or glutamatergic neurons) that might be needed for such effects.

4.2.2 Water maze

We could not see any differences in acquisition for the total-CB1-ko mice and the Glu-CB1-ko mice indicating that CB1 is dispensable for learning the water maze task. However, we have to be cautious in drawing definite conclusions as Varvel and colleagues failed to find improved acquisition in a first study with FAAH-ko mice but found better acquisition in a second study. This was probably due to a general slower acquisition phase in the second study (that was performed in a bigger water maze tank) that made any differences apparent (Varvel et al., 2006a;Varvel et al., 2006b). These researchers used the latency to locate the platform as a measure of memory. For our experiments, we have to assert that the acquisition of the task occurred very rapidly within two days and there are no differences for the latency to swim to any platform. In the water maze spatial discrimination task, as it was applied by us, accuracy to reach the stable platform is the essential measure for learning abilities, which is dependent on an intact hippocampus (Arns et al., 1999). As the latency to reach the platform is very short, levels of stress due to water exposure are minimised in our test compared to the conventional Morris water maze procedure. This allowed us to isolate true cognitive effects of endocannabinoids from more indirect effects due to stress coping.
Our results confirm water maze studies from different laboratories which indicate no effect of CB1 antagonism once mice have learned the position of the platform, i.e. CB1 activation seems not to be involved in memory retrieval. Moreover, there is no deficit in learning performance, i.e. acquisition, of the Morris water maze in CB1-ko mice (Da and Takahashi, 2002;Varvel et al., 2001;Varvel and Lichtman, 2002). However, knockout mice showed what these authors call “perseverance” in the reversal test. They continued to return to the location where the platform had been previously located and spent more time returning to the position where the platform was formerly located, which interfered with them finding the new platform position (Varvel et al., 2005;Varvel and Lichtman, 2002). The same phenomenon could be observed in chickadees that received an intrahippocampal injection of SR141716. Here, the birds showed better memory of a location with hidden food but this memory was still observable when it extinguished again in the control group (Shiflett et al., 2004). In another study, rats were exposed to unpredictable stress. These animals show decreased levels of 2-AG and CB1 receptor density in the hippocampus and have impaired reversal learning (i.e. increased perseveratory behavior) in the water maze task. Reversal learning worsened by application of the CB1 agonist HU-210 (however, it improved if a low dose was given) (Hill et al., 2005)[2]. These cognitive

[2] We would like to stress here that only the latency to swim to the platfrom was affected, which does not mean that hippocampus-dependent spatial memory was indeed impaired but rather the locomotive

impairments might be a consequence of deficient endocannabinoid signalling. Endocannabinoids may be needed to inhibit a previously learned behaviour such as in extinction or in reversal learning.
We assessed reversal learning by replacing the stable platform with the floating platform in the water maze. The animals were enforced not to swim to the old location of the stable platform anymore in order to find the safe platform. As the stable platform was changing its position from trial to trial, it was difficult for the mouse to efficiently learn to always reach the stable platform. Thus, a chance level of 50 % right choices was defined as a reasonable performance for the reversal training. In our hands, total-CB1-ko mice showed the same rate of reversal learning contradicting the finding of Varvel and colleagues. Glu-CB1-ko mice showed even enhanced abilities for reversal learning. It was suggested that the endocannabinoid system plays a key role in the extinction of memory, i.e. an unlearning that the former stable platform is not safe anymore in our case. Extinction seems to be affected by block of CB1 in aversive tasks (Marsicano et al., 2002), but no effect is apparent in appetitively-motivated tasks (Holter et al., 2005;Niyuhire et al., 2007). What might be the reason that we did not see any effect on reversal learning in the total-CB1-ko mice? Varvel et al. compared a massed extinction protocol, in which the extinction trials were given over days, with a spaced extinction procedure, in which the extinction trials were given over weeks. They pointed out that there were only extinction deficits for the spaced extinction protocol (Varvel et al., 2005). According to this paradigm, our experimental protocol with 10 trials per day and an intertrial-interval of 3 min might resemble a highly massed extinction protocol which might not be suitable to detect any differences in extinction in total-CB1-ko mice. Also, as the latency to reach the platform is very short, our water maze task is perhaps not aversive enough to detect an effect on extinction. Oddly enough, we were able to see facilitated reversal learning in the Glu-CB1-ko mice. Obviously, lack of CB1 on glutamatergic neurons eases the behavioural flexibility to relearn or learn a new platform location. Recently, it was found that administration of the CB1 antagonist AM251 alleviated perseverance in a set-shifting task in rats (Hill et al., 2006a), indicating the possibility that blockade of CB1 can indeed enhance behavioural flexibility under certain circumstances. Behavioural flexibility, or a decrease in perseverance, is most often described in the water maze task. The hippocampus seems to be an essential structure enabling a reversal learning in that damaging the hippocampus or its afferent/efferent fibre bundles leads to a perseverant behaviour (Morris et al., 1986b;Whishaw and Jarrard, 1995). Similarly, the process of extinction, i.e. an ‘unlearning’ of the expression of the original memory (Myers and Davis, 2002), might be enhanced in Glu-CB1-ko mice which is however not supported by our own results from the contextual fear conditioning experiments (please see below). Still, an alteration in glutamatergic transmission in the hippocampus possibly allows Glu-CB1-ko to relearn the new location of the safe platform more rapidly. It remains to be tested whether GABA-CB1-ko mice reveal any alterations in reversal learning and whether CB1 of GABAergic neurons plays a role for behavioural flexibility.

4.2.3 Object and social recognition

There is good evidence that CB1 deletion improves recognition memory. Especially, both young (1 month) and adult (4 months) CB1-ko mice showed better memory in the object recognition task (Maccarrone et al., 2002;Reibaud et al., 1999).

performance and the behavioural coping with cold-water stress of the animals. A more appropriate measure would have been the time spent searching in the target quadrant.

Additionally, administration of the antagonist SR141716 improved memory of young (2-3 months) and old (10-12 months) rats and mice in the social recognition test (Terranova et al., 1996). Having said that, only young CB1-ko mice (6-8 weeks) showed a better performance in the social recognition test in another study, whereas adult (3-5 months) and old (14-17 months) CB1-ko mice showed a memory deficit when compared to their wildtype littermates (Bilkei-Gorzo et al., 2005) which has been interpreted by the authors as accelerated aging in these animals. Altogether, this points towards a nootropic effect of CB1 antagonism at a certain age and that the endocannabinoid system may negatively influence some mnemonic processes.
Admittedly, in the present study we could neither observe improved memory in the object recognition task nor in the social recognition task. There is rather the trend that memory performance is attenuated in total-CB1-ko mice and Glu-CB1-ko mice, as the former fail to show social recognition memory after 24 h and the latter fail to show social recognition memory both after 1 and 24 h. There is no indication for any better memory performance in our three knockout mouse lines. Partly, this might be due to a decrease in exploration time as it is seen in the Glu-CB1-ko mice for the first encounter of a novel object or juvenile (as it will be further discussed in section 4.3.4). A certain amount of exploration might be needed for an effective gathering of information to build up the recognition memory at least for the object recognition as accuracy in olfactory discrimination requires only milliseconds of exploration (Uchida and Mainen, 2003) and even decreased exploration does not lead to impaired social recognition memory (Richter et al., 2005). Hence, instead of memory, we think that rather motivational or emotional properties are altered in total-CB1-ko and Glu-CB1-ko mice.
On the other hand, it was suggested that improved memory in CB1-ko mice might be due to increased ACh efflux in the hippocampus (Chaperon and Thiebot, 1999). Enhanced ACh levels in CB1-ko mice can be only found under stressful conditions (Degroot et al., 2006). At least the object recognition task, which is one of the few tasks for which memory improvement was clearly shown (Maccarrone et al., 2002;Reibaud et al., 1999), was carried out under very lowly stressful conditions in our hands whereas the aversive conditions of the social recognition task (i.e. illumination of 500 lux) were possibly too drastic. Perhaps this did not lead to a sufficient or superior elevation of ACh levels, respectively, in order to enhance memory.

4.2.4 Open field habituation

Habituation, a form of non-associative learning, can be measured by examining exploratory behaviour in a novel environment such as an open field and is reflected by decreased locomotion. It was shown that deletion of the CB1 receptor significantly improved habituation learning in the open field and those authors argue that CB1-ko mice display a prolonged memory retention (Degroot et al., 2005). On the other hand, intrahippocampal infusion of the CB1 antagonist AM251 in the rat after the training session in the open field did not lead to a decrease of locomotion in the second trial (de Oliveira et al., 2005). This suggests that consolidation of open field exploration memory is not influenced by hippocampal endocannabinoids. However, the latter study could show a memory-disrupting effect in an inhibitory avoidance task[3]. These findings point towards the direction that the endocannabinoid system requires some degree of averseness or alertness in order to be recruited.

[3] In this test, the animal receives a foot shock as soon as it steps down from a platform in a training session. In the test session, the latency the animal requires to step down from the platform is measured as an index of memory.

Taking this into consideration, we tested mice in the open field under two different lighting conditions, i.e. 0 lux or 700 lux and accordingly lowly aversive or highly aversive testing conditions. We could show that the level of habituation is dependent on the illumination of the open field. At 700 lux, all genotypes of the three mouse lines revealed habituation indicated by a decrease in locomotion and rearing from day 1 to day 2. In total-CB1-ko mice and in Glu-CB1-ko mice there was a more pronounced decrease in locomotion and rearing compared to their wildtype littermates. At 0 lux, the situation gets a bit more complex in that total-CB1-ko mice showed no decrease in locomotion whereas GABA-CB1-ko mice and Glu-CB1-ko mice did show a decrease in locomotion from day 1 to day 2 and only Glu-CB1-ko mice showed a decrease in rearing. Additionally, we checked for habituation within the first session on day 1. At 0 lux, mice of all three lines were able to habituate within a session which is reflected in a decrease of locomotion. At 700 lux, total-CB1-ko mice failed to habituate whereas GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype animals were able to habituate. However, total-CB1-ko mice started at a lower level of locomotion already. No genotype differences could be revealed for the three mouse lines for within session habituation.
All in all, we can confirm a greater habituation in total-CB1-ko mice and Glu-CB1-ko mice only under highly aversive (i.e. 700 lux) conditions. However, it is questionable whether this truly reflects an enhanced cognitive ability. As we see similar habituation within the first session for the genotypes and a lack of habituation of total-CB1-ko mice from day 1 to day 2 under 0 lux, we rather believe that the observed effects are due to altered levels of anxiety (as being described below in section 4.3.4)[4].

4.2.5 Contextual fear conditioning

It is generally accepted that processing of contextual information in a fear conditioning paradigm may rely on the hippocampus and the amygdala (Desmedt et al., 2003;Kim and Fanselow, 1992;Selden et al., 1991) whereas processing of distinct stimuli (such as a tone) requires the amygdala but not the hippocampus (Phillips and LeDoux, 1994). When rats were treated with the CB1 antagonist AM251 either before the training or before the test session of a background contextual fear conditioning task, freezing was decreased in the AM251-treated rats in the shock context session 48 h later, indicating that expression of contextual fear is impaired by blocking CB1. At the same time, freezing to the tone was increased in AM251-treated rats (Arenos et al., 2006). This finding supports the notion that CB1 does not have the same role in contextual and cue-specific fear conditioning. A study by Mikics and colleagues studied background contextual fear-conditioning in CB1-ko mice (Mikics et al., 2006). They could show that the conditioned fear response was abolished by the disruption of CB1 and was reduced by the CB1 antagonist AM251 in wildtype mice whereas it was increased by the agonist WIN-55,212-2 when administered before the testing session. These findings demonstrate again that blockade of CB1 impairs contextual

[4] The fact that we found contradictory effects of habituation memory compared to Degroot and colleagues may be due to the housing conditions of our mice, too. Individually housed mice display reduced habituation and show an hyperactive phenotype (Voikar et al., 2005). Furthermore, the experimental conditions could explain a lack of habituation: Mice tested during the dark phase of a light-dark cycle (like our mice) are more aroused by the testing environment than those tested during the light phase, as evidenced by increased ambulation during the dark phase (Valentinuzzi et al., 2000).

memory and, quite unexpectedly, activation of CB1 promotes the expression of conditioned fear.[5]

Other studies present a different picture of CB1 function. In a trace eyeblink conditioning task that is dependent on an intact hippocampus and cerebellum, CB1-ko mice revealed the same performance as the wildtype mice. The very same result could be observed after application of the antagonist SR141716 before the training session (Kishimoto and Kano, 2006). Moreover, application of the CB1 agonist WIN55212-2 before a contextual fear conditioning task reduced the freezing levels and thus impaired memory in rats. Here, the antagonist SR151716 had no effect when given alone (Pamplona and Takahashi, 2006). These authors could show that administration of WIN55212-2 had only an effect when given before conditioning, whereas Mikics et al. applied the agonist before the memory test, indicating a differential effect on acquisition and retrieval of aversive memories to environmental cues.

Finally, regarding the extinction process, WIN55212-2, when given before the extinction session, facilitated extinction of fear-potentiated startle, and the anandamide reuptake inhibitor AM404 enhanced extinction, too (Chhatwal et al., 2005), whereas SR147778 or SR141716, both CB1 antagonists, blocked extinction in a cued or contextual fear conditioning task (Chhatwal et al., 2005;Marsicano et al., 2002;Pamplona et al., 2006;Suzuki et al., 2004).

Altogether, some studies suggest that CB1 blockade has no effect on acquisition of contextual fear memory whereas others show attenuated acquisition of contextual fear memory after blockade of CB1. Generally, the process of extinction seems to be blocked by antagonism of CB1 function.

We also used background contextual fear conditioning to examine the response of the three mouse lines to aversive contextual cues. We tested our three mouse lines on three days (day 1, day 2 and day 7 after conditioning) in two different contexts, the shock context and a grid context. By using two contexts, we checked for the specificity of memory for the conditioning environment and discrimination of fear conditioning cues. The two contexts differed in several aspects that were mainly their shape, illumination and the present odour, but shared the grid floor as a common feature (an overview is given in Table 2, page 38). Furthermore, we tested the mice with two shock intensities, with 0.7 mA being a standard and moderate shock intensity whereas 1.5 mA was a highly aversive shock intensity that was expected to promote generalised fear (Shaban et al., 2006).

In general, animals showed high freezing levels in both contexts indicating that the conditioning protocol is appropriate to induce a conditioned fear response. If only considering day 1 (as most of the cited studies do), at 0.7 mA, effects seem to be puzzling: Total-CB1-ko mice did not show any freezing differences in the shock context and the grid context whereas GABA-CB1-ko mice froze more in the grid context and Glu-CB1-ko mice had a significant higher freezing response in the shock context. The picture becomes clearer if we compare the development over days and consider both shock intensities: Total-CB1-ko mice showed increased freezing in both contexts, but especially at 1.5 mA, they outperformed wildtype mice with increased freezing in the grid context. GABA-CB1-ko mice always revealed elevated freezing levels in the grid context whereas Glu-CB1-ko mice nearly always showed

[5] We would like to point out here, that in the study of Mikics and colleagues, each mouse received a pairing with 10 shocks of 3 mA which is an extremely drastic conditioning protocol. It is quite astonishing that the CB1-ko mice show such a poor contextual memory which might be explained with the genetic background of these mice which is the CD1 strain.

enhanced freezing in the shock context. Finally, in the neutral context on day 8, freezing to the tone that was previously presented for conditioning pointed out increased freezing in total-CB1-ko mice and to some extent in Glu-CB1-ko mice.

To begin with, we showed that total-CB1-ko and, as shown for the first time here, Glu-CB1-ko mice froze more to the tone following conditioning with 0.7 mA and 0.5 mA, respectively, which is very consistent with previous reports in which pharmacological blockade of CB1 or genetic deletion of CB1 increased freezing to an auditory cue during a memory testing session (Arenos et al., 2006;Cannich et al., 2004;Kamprath et al., 2006;Marsicano et al., 2002;Niyuhire et al., 2007). We propose that this is due to an impaired habituation (which is a nonassociative learning process), as it is elegantly shown for CB1-ko mice already (Kamprath et al., 2006). We can furthermore specify that it is CB1 located on glutamatergic neurons that has profound effects on short-term extinction.

The finding that total-CB1-ko, GABA-CB1-ko and Glu-CB1-ko mice differently froze in the two types of context may be explained by two possibilities, namely that the balance between reconsolidation and extinction is altered or that the acquisition of contextual information and its representation is modulated.

Firstly, memory testing causes memory reactivation and initiates two dissociable but opposite processes: Reconsolidation, i.e. a stabilisation of the expression of the original memory, and extinction, i.e. a weakening or rather 'unlearning' of the expression of the original memory (Myers and Davis, 2002;Nader, 2003). It remains difficult to differentiate between these two processes, but brief reminders to a conditioning context may lead to reconsolidation, whereas longer reminders may result in memory extinction, which tends to inhibit the expression of the original memory (Suzuki et al., 2004). Total-CB1-ko and especially Glu-CB1-ko mice either show less extinction or these mice possess a stronger tendency for reconsolidation. Application of the CB1 agonist WIN55212-2 into the amygdala after reactivation of the conditioned fear response reduced fear memory when animals were reexposed to the conditioned stimulus, suggesting that reconsolidation is diminished or extinction is facilitated (Lin et al., 2006). On the other hand, the application of AM404, an inhibitor of endocannabinoid reuptake, prior to extinction training enhances extinction in a fear-potentiated startle paradigm and the antagonist SR141716 blocked extinction (Chhatwal et al., 2005). Very convincingly, FAAH-ko mice or mice treated with the FAAH inhibitor OL-135 showed facilitated extinction in the water maze task (Varvel et al., 2006b). Thus, activation of CB1 could facilitate extinction on the one hand and block reconsolidation on the other hand. CB1 activation leads to activation of MAPK among others (Derkinderen et al., 2003). CB1-ko mice have decreased levels of phosphorylated MAPK and calcineurin in the basolateral amygdala, and higher levels of phosphorylated MAPK and calcineurin were observed in the central amygdala after fear conditioning (Cannich et al., 2004). Block of MAPK in the basolateral amygdala reduces extinction in an acoustic startle paradigm (Lu et al., 2001). Altogether, this may indicate that indeed the extinction process is altered in total-CB1-ko and Glu-CB1-ko mice. Most remarkably, calcineurin is specifically expressed in glutamatergic neurons but not in GABAergic interneurons in the hippocampus (Sik et al., 1998). This might be a reason for the specific extinction deficit in total-CB1-ko and Glu-CB1-ko mice in terms of short-term extinction of auditory-cued fear but not GABA-CB1-ko mice taking into consideration the potential role of calcineurin in extinction (Lin et al., 2003a;Lin et al., 2003b). Moreover, the effect on extinction seems to be specific for aversive learning tasks as no effects on extinction could be found in appetitively-motivated memory tasks (Holter et al., 2005;Niyuhire et al., 2007).

Supporting this view, strong impairment of short-term and long-term extinction of auditory-cued fear memory has been reported in CB1-ko mice (Marsicano et al., 2002). The protocol employed resulted in elevated levels of endocannabinoids in the basolateral amygdala (Marsicano et al., 2002) and the dorsal hippocampus (Kamprath et al., 2006) of wildtype mice, regions known to control extinction of aversive memories, which indicates that specifically endocannabinoids might facilitate extinction of aversive memories. In contrast to the tone, we observed no extinction over days for the shock context in total-CB1-ko and GABA-CB1-ko mice and Glu-CB1-ko mice and the respective wildtype animals, with the only exception of Glu-CB1-ko mice showing extinction at a 0.7 mA shock current. This is probably due to the short reexposure of the mice to the shock context (that was 5 min) as only a reexposure to the context for 30 min led to an extinction of the fear response (Suzuki et al., 2004).

After conditioning with 1.5 mA, mice of the three lines revealed a very intense freezing response in the shock and the grid context which can be described as a ceiling effect. We believe that the absence of context discrimination is partly due to a generalised freezing response as it is seen by others (Shaban et al., 2006). This is especially true for GABA-CB1-ko mice that have increased levels of freezing also in the neutral context before tone presentation on day 8.

As a second explanation for the differing freezing behaviour of the three mouse lines, and to see it from a different angle, total-CB1-ko mice showed increased freezing in both contexts whereas Glu-CB1-ko mice generally revealed higher levels of freezing in the shock context. Most curiously, GABA-CB1-ko mice had increased freezing levels in the grid context but not in the shock context as compared to the wildtype littermates. What might lead to the dissociation between the contexts in the mouse lines? Three hypotheses could explain this discrepancy. On the one hand, total-CB1-ko mice and GABA-CB1-ko mice could have generalized the conditioned fear to any novel environment. Here, generalization is a process of judging different sensory stimuli as being similar enough to predict e.g. a footshock (Shepard, 1987) which consequently leads to a loss of memory specificity. Even the unavoidable manipulations inherent in fear conditioning testing (i.e. animal handling, carriage, to be placed in a context, particular time of the day) might induce enough fear to become sufficient to elicit the freezing response. The generalization of the fear response may be linked to the overall behavioral suppression of fear-conditioned mice. This emotional suppression then leads to a loss of discrimination between contexts and can be observed in rats and mice also by other groups (Baldi et al., 2004;Balogh and Wehner, 2003;Fanselow, 1980;Laxmi et al., 2003;Radulovic et al., 1998;Shaban et al., 2006).

A mechanism that more specifically explains the generalization phenomenon is sensitization. Sensitization is a non-associative learning process characterized by a general increase in responsiveness to potentially harmful stimuli after an aversive experience (Groves and Thompson, 1970;Kamprath and Wotjak, 2004). In contrast to contextual fear conditioning, where the freezing response would be tested towards the conditioning context, in sensitization the freezing response would be tested towards an unrelated stimulus, or, as in our case, to an unrelated neutral context. Learning about the association between a tone and a potential footshock, i.e. the associative component, and sensitization to a tone after having received an inescapable footshock, i.e. the non-associative component, occur at the same time and can be basically dissociated (Kamprath and Wotjak, 2004) for auditory cued fear conditioning. Most interestingly, CB1-ko mice are impaired in habituation of the fear response to a tone after sensitization by a footshock (Kamprath et al., 2006). In our

hands, total-CB1-ko and GABA-CB1-ko mice seem to reveal such a sensitized fear as they show increased freezing levels in the grid context compared to the wildtype littermates, beside of per se high freezing levels in the shock context.
If it is indeed enhanced generalization or more specifically increased sensitization that we observe in total-CB1-ko and GABA-CB1-ko mice, then we should expect that they also show enhanced freezing levels in the neutral context on day 8 before tone presentation. Admittedly, this is not the case after conditioning with 0.7 mA, and total-CB1-ko, Glu-CB1-ko and GABA-CB1-ko mice have similar freezing levels compared to their wildtype controls. This rules out the possibility that generalization or sensitization to any new environment explains our findings. However, after conditioning with 1.5 mA, GABA-CB1-ko mice indeed reveal increased freezing in the neutral context possibly indicating increased sensitization under more aversive conditions.

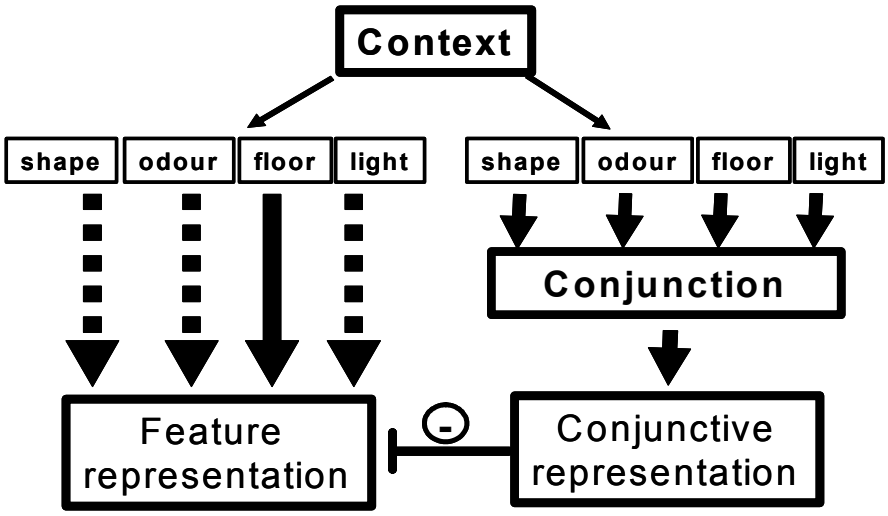


Figure 31: A two-process model of context representation as it is adopted from Rudy et al. (2004). A context consists of a set of cues. Contextual fear conditioning can be supported by associations emphasising an individual feature of the context (as it is the grid floor in our example) and leading to a representation of this feature for the fear memory. On the other hand, conditioning can be supported by associations of contextual cues into a conjunction and a representation of the entire conjunction in the brain is stored as the fear memory. Under normal circumstances, conjunctive representation inhibits feature representation.

A third explanation for our findings is, therefore, that mice could differentially use a subset of the conditioned cues to recall the aversive association. Rudy and co-workers proposed a model that explains how contextual fear memory can be represented in the brain (Rudy et al., 2004) as it is shown in Figure 31. Here, physical elements of a context can be stored as either (1) a set of independent features in association with an event or (2) as features bound into a conjunctive representation encoding the co-occurrence with an event and supporting pattern completion. Rudy and colleagues further put forward the hypothesis that a conjunctive representation relies on the amygdala and the hippocampus whereas for a feature representation only the amygdala is needed. Under normal circumstances, a conjunctive representation inhibits a feature representation. If the hippocampus is blocked or an interaction between the hippocampus and the amygdala is impaired for other reasons, then a feature representation might take over to represent a context. Thus,

the interplay between the hippocampus and the amygdala is important for the kind of context representation (Figure 32).
Both contexts, the shock context and the grid context, contained the grid floor that was used for conditioning of the animals with a footshock. GABA-CB1-ko mice might learn about the contexts by using a feature learning strategy, abstracting a small number of contextual elements that is the grid floor in our experimental condition. Perhaps they then make a stronger association between the grid floor as part of the context and the aversive stimulus during training and thus loose the ability to discriminate between contexts as could be also observed by others (Klemenhagen et al., 2006). If this is true, then GABA-CB1-ko mice favour a feature representation and rely on the grid floor as a cue. One could also argue for a pattern completion strategy, in that they consider the grid floor as a dominant cue that enables them to access the whole context memory. Also, one could bring forward the argument that total-CB1-ko mice and especially GABA-CB1-ko mice reveal declined safety learning, i.e. they cannot distract from the shock memory by combining the new cues (e.g. shape of the grid context, odour in the grid context) with a safe environment (Rogan et al., 2005) and learn that it is harmless. In contrast to that, Glu-CB1-ko mice prefer a conjunctive representation relying more on the context as a whole to learn and remember about the shock context and to specifically freeze more herein.

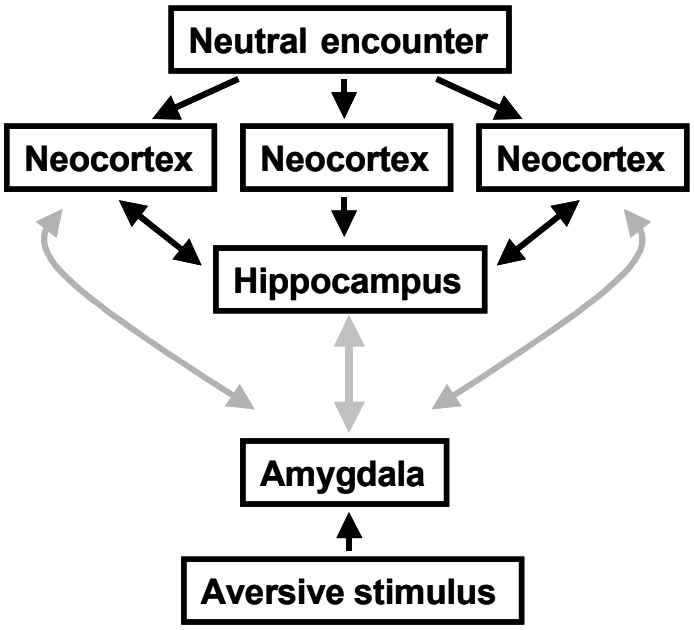


Figure 32: Schematic representation of the brain regions involved in the contextual fear conditioning task. An emotionally neutral encounter is presented in association with an aversive stimulus. Processed information of the encounter is fed from neocortical regions to the hippocampus and the amygdala. The amygdala is receiving direct information on the averseness at the same time. An intense interplay between the hippocampus and the amygdala ensures the conjunction of context information that is represented in the hippocampus with the emotional load of the information that is represented in the amygdala. Neuronal connections of the amygdala are highlighted in grey.

We pointed out earlier already that the medial septum, which uses ACh and GABA as neurotransmitters for its projection to the hippocampus (Freund and Antal, 1988;Frotscher and Leranth, 1985), seems to play a prominent role in generating theta rhythm (Smythe et al., 1992;Vertes and Kocsis, 1997). Elevated levels of ACh specifically promote contextual fear conditioning at the expense of conditioning to a

tone (Calandreau et al., 2006). CB1-ko mice reveal heightened levels of ACh (Degroot et al., 2006;Kathmann et al., 2001b) which might influence the generation and distribution of theta rhythm in the hippocampus. Eventually, enhanced levels of theta oscillations in the hippocampus could be found in mice with increased anxiety-related behaviour on the elevated plus maze (Gordon et al., 2005). More specifically, Seidenbecher and colleagues showed that during normal exploration and during presentation of an unconditioned tone, there was no evidence of theta rhythm in the amygdala, despite strong theta in the hippocampus. However, elicitation of conditioned fear with a context or a tone is associated with theta activity and theta synchronisation in the amygdala and the hippocampus (Seidenbecher et al., 2003). These findings suggest that theta oscillations that are possibly enhanced by ACh can be propagated to downstream targets, from the hippocampus to the amygdala. This might reflect an increase in neuronal communication that promotes the retention of the fear memory in favour of a conjunctive representation. If a synchronisation of the two brain structures is not assured anymore, one could speculate that this would bring forward a feature representation that requires the amygdala only. The fact that we did not find any differences in theta rhythm in the dentate gyrus of anaesthetised total-CB1-ko mice does not contradict this hypothesis as it is specifically the CA1 region but not the dentate gyrus of the hippocampus that is involved in a contextual fear conditioning task (Hall et al., 2001).

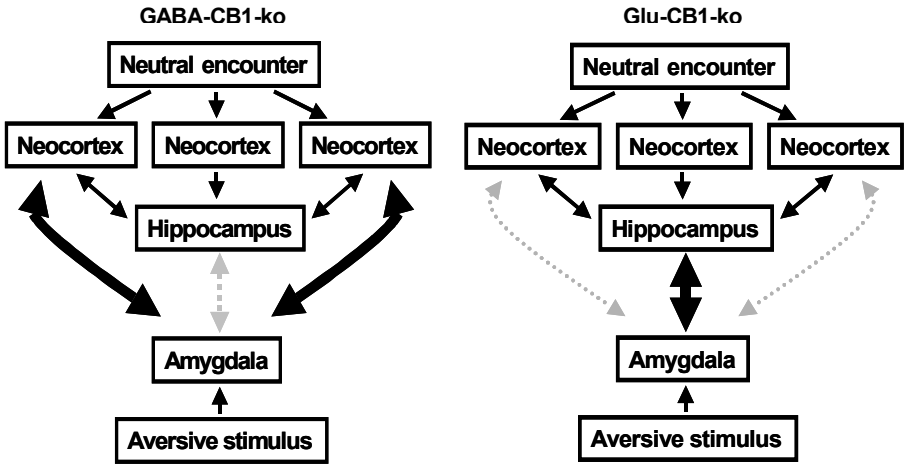


Figure 33: Model of different brain regions involved in the contextual fear conditioning task in GABA-CB1-ko and Glu-CB1-ko mice according to Rudy et al. (2004). A feature learning strategy is accomplished in GABA-CB1-ko mice that rely on a direct input of sensory information coming from the neocortex (left). In Glu-CB1-ko mice, a conjunctive learning strategy is favoured and contextual information is associated in the hippocampus and then fed into the amygdala.

Importantly, GABAergic interneurons play a pivotal role in the generation of theta and gamma rhythm (Bartos et al., 2007;Buzsaki, 2002). It is impossible to state here which CB1-bearing neurons in the brain (GABAergic neurons in the septum, GABAergic neurons in the hippocampus, GABAergic neurons in the amygdala, cholinergic neurons in the septum, or glutamatergic neurons in the hippocampus projecting to the amygdala) are particularly involved in the observed phenotypes. However, it is tempting to speculate that a lack of theta synchronisation or gamma oscillations might be a reason for the amygdala-dependent feature representation in the GABA-CB1-ko mice, and a more strengthened synchronisation of theta rhythm

might underlie the hippocampus/amygdala-dependent conjunctive representation in the Glu-CB1-ko mice (Figure 33).
In conclusion, deletion of CB1 does not play a pivotal role in exploration-based memory tasks like the object recognition test, social recognition test and open field habituation test. However, in aversive learning paradigms, the endocannabinoid system plays a role in reversal learning as could be seen in Glu-CB1-ko mice in the water maze task, and differentially affects learning of a context in the contextual fear conditioning paradigm in all three mouse lines. We would like to make some speculative suggestions referring to transmitter systems and brain structures involved in these processes in the Appendix (section 6.2).

4.3 Modulation of emotionality in CB1-ko mice

4.3.1 Effects of CB1 signalling on emotionality

Blockade of CB1 with an antagonist increased anxiety-like behaviour in rodents (Akinshola et al., 1999;Arevalo et al., 2001;McGregor et al., 1996;Navarro et al., 1997;Patel and Hillard, 2006) whereas other studies could show an anxiolytic effect of antagonist administration (Akinshola et al., 1999;Haller et al., 2002;Rodgers et al., 2003).
Data from mice with a deletion of CB1 were similarly inconsistent. CB1-ko mice showed decreased (Degroot and Nomikos, 2004), unaltered (Aguado et al., 2005;Ledent et al., 1999;Maccarrone et al., 2002;Marsicano et al., 2002) or increased (Martin et al., 2002;Uriguen et al., 2004) anxiety-related behavior in a variety of tests of unconditioned fear such as the open field, shock probe burying, elevated plus-maze, social interaction, and the light/dark test.[6]
On the other hand, CB1 activation with the agonists WIN55212-2 or CP 55940 or potentiation of endocannabinoid signalling with the degradation inhibitor URB532 and URB597 or the endocannabinoid reuptake inhibitor AM404 reduced anxiety-related behavior in rats or mice (Bortolato et al., 2006;Kathuria et al., 2003;Patel and Hillard, 2006). The fact that endocannabinoids affect anxiety-related behaviour is pivotal and suggests an involvement of the endocannabinoid system under normal physiological conditions.
One reason for the apparent discrepancies might be differences in environmental factors. It has been shown that CB1-ko mice only showed an anxiogenic-like phenotype under conditions of high stress: Light in the elevated plus-maze (that was 200 lux compared to 0.5 lux, respectively) and the familiarity of the testing environment (novel cage compared to home cage, respectively) (Haller et al., 2004a). In another study, FAAH-ko mice or mice treated with the FAAH-blocker URB597 revealed no difference to the control mice from the background strain on the elevated plus maze under standard laboratory conditions (i.e. normal overhead light) but showed an anxiolytic phenotype under high-light conditions for the open arms (i.e. overhead lights turned off and two 60-W light bulbs illuminating open arms and casting a shadow on the closed arms) (Naidu et al., 2007). These findings would explain why the same drug or the same knockout animal has different effects or displays different behaviors, respectively, under different conditions because behavioural effects of CB1 blockade depend on the averseness or stressfulness of the testing environment.

[6] Curiously, SR141716A still decreased anxiety-related behavior in CB1-ko mice, suggesting another target of the drug which might be “CB3” (Haller et al., 2002). There was no such an effect with the antagonist AM251, suggesting that this is a truly specific CB1 antagonist (Haller et al., 2004b).

The context dependency is indirectly supported by the “one-trial sensitization” phenomenon described by Rodgers and colleagues in the plus-maze. An anxiolytic effect of the antagonist SR141716 is revealed in mice previously exposed to the plus maze apparatus (Rodgers et al., 2003). Thus, the anxiolytic effect of SR141716 is more pronounced when animals have increased basal levels of anxiety. Another study could show an anxiolytic effect of the CB1 antagonist AM251 in the highly aversive step-down inhibitory avoidance task but no effect in the lowly aversive open field habituation task (de Oliveira et al., 2005;de Oliveira et al., 2006).
We tried to figure out if at all and under which conditions mice of the three lines revealed anxiety-related behaviour. Taken from the studies cited above, our hypothesis was the following: In tests where an animal is exposed to an aversive situation, the endocannabinoid system is activated and eventually leads to an attenuation of the anxiety state. This condition permits the mouse not to remain in a state of anxiety but to access its behavioral repertoire and apply adequate behaviour in the respective situation. Indeed, augmented levels of endocannabinoids can suppress an anxious reaction when rats are exposed to a predator odour (Hill et al., 2006b). Obviously, endocannabinoids are able to dampen activation of stress-related brain circuits, e.g. the HPA axis (Patel et al., 2004;Patel et al., 2005b) and may act as a buffer system in the stress response (Di et al., 2003;Tasker, 2004).

4.3.2 Elevated plus-maze and light/dark box

Grossly, we can divide the applied emotionality tests used in this study into two categories: Choice and no-choice tests of a confrontation with an aversive environment. In the light/dark test and plus-maze test, the animal can choose whether to stay in the safe compartment or whether to explore the unsafe and brightly illuminated or elevated areas of the maze, respectively. Here, no significant differences are found for total-CB1, GABA-CB1 and Glu-CB1 mice. Even so, there was a tendency of total-CB1-ko mice to show an anxiogenic response and in GABA-CB1-ko mice to show an anxiolytic response in the plus-maze test. This makes sense as the elevated plus maze includes two additional anxiety-provoking parameters, height and totally open area (Crawley et al., 1997) as compared to the light/dark test. However, the tendency of a mouse, either wildtype or knockout, to stay in the safe areas and to avoid the unsafe areas will not require the endocannabinoid system and an effect of CB1 deletion cannot be ascertained. Indeed, this is in keeping with pharmacological blockade of CB1, after which no effect on anxiety could be seen in the plus-maze and the light/dark box (Rodgers et al., 2003;Rutkowska et al., 2006).

4.3.3 Defensive marble burying

On the other hand, we employed test situations with a no-choice condition to avoid an aversive surrounding in terms of exposure to bright light or glass marbles, respectively, that were the social interaction test, the novel object exploration test, the open field test and the marble burying test.
We found no differences in the marble burying test as it could be observed by others (Hodgson et al., 2007). Assuming that the marbles constitute an aversive situation this would contradict our hypothesis that mice with an anxiogenic phenotype bury more marbles. It has been argued that marble burying is a better measure of obsessive-compulsive behaviour: Rather than responding to an aversive object, the test is thought to reflect spontaneous digging behaviour (with buried marbles just being a quantification of the digging behaviour) where habituation does not occur and selective serotonin reuptake inhibitors are highly effective (Gyertyan, 1995;Njung'e and Handley, 1991). In the end, the marble burying test might not be an appropriate

test to investigate emotionality in our mouse lines. It has been furthermore suggested that burying is proportional to a subjects' tendency to explore (Londei et al., 1998). As we conducted the test in darkness, the situation might have been not aversive enough, as it could have been observed for the social recognition and object recognition test (please see below). However, as the marbles were spread over the whole cage, there was no opportunity for the animal to avoid contact to the marbles, which might have lead to a severe state of anxiety in both wildtype and knockout animals. We can exclude this possibility as it has been shown that mice did not avoid the marbles when given the opportunity to do so in a two-compartment box (Njung'e and Handley, 1991)[7]. Altogether, marbles do not seem to be as aversive as e.g. brightly lit environments. Ultimately, digging of bedding material, whether being a test for obsessive compulsive disorder or not, is not different in the investigated mouse lines under low light conditions. Future studies are planned to figure out whether this finding holds true for a brightly lit environment, too.

4.3.4 Social interaction, novel object interaction and open field

The case is very different for the social interaction test, the novel object exploration test and the open field test. We conducted these tests under two conditions, bright light or dim light/darkness, that is highly aversive or lowly aversive for rodents, respectively. For example, the startle response in rats was markedly increased under bright light conditions compared to dark conditions, an effect that was disrupted by anxiolytic drugs (Walker and Davis, 1997a). Furthermore, exploration of a novel space, a novel object or a conspecific mouse under bright light conditions was reduced, and this effect could be prevented by administration of a benzodiazepine as an anxiolytic drug (Crawley and Goodwin, 1980;File and Hyde, 1978).

Under low light, we found moderate or no differences for the genotypes. Glu-CB1-ko mice showed less novel object exploration and decreased thigmotaxis in the open field, i.e. an anxiogenic and anxiolytic phenotype, respectively. GABA-CB1-ko, in contrast, showed more novel object exploration compared to the wildtype littermates, i.e. an anxiolytic phenotype. Total-CB1-ko mice had decreased levels of thigmotaxis in the open field task similar to Glu-CB1-ko mice indicating an anxiolytic phenotype.[8] This highlights that under the lowly aversive conditions the endocannabinoid system gets moderately activated as can be observed in some test parameters.

Our results become very clear and unambiguous under the highly aversive condition. Here, we could show that total-CB1-ko and Glu-CB1-ko mice showed a decrease of exploration of a novel object or juvenile and revealed increased thigmotaxis in the open field test emphasizing an anxiogenic phenotype. GABA-CB1-ko mice still have increased exploratory activity in the object exploration test, a tendency of increased locomotion in the open field and a minor increase in thigmotaxis in the open field rather pointing towards an anxiolytic phenotype. We hypothesize that under the highly aversive conditions the endocannabinoid system gets fully activated and unfolds its anxiety-modulating effect. Our findings support the anxiogenic phenotype of CB1-ko mice and the anxiolytic phenotype of FAAH-ko mice that is only seen under high levels of stress associated with the environmental conditions (Haller et al.,

[7] There is another study clearly demonstrating the avoidance of glass marbles in the zone containing the marbles, although only for C57BL/6J mice but not in two other mouse strains (Nicolas et al., 2006).

[8] We would like to emphasise here that a decrease of thigmotaxis is not due to a general augmentation of locomotion as we analysed the relative rate of thigmotaxis by normalizing the distance moved along the walls by the total distance moved. We did not consider the distance moved in the center of the open field as this area is arbitrarily defined and is too small for an appropriate measurement that could consistently reflect an anxiety-like behaviour in our laboratory.

2004b;Naidu et al., 2007). Our findings make sense from a physiological point of view, as corticosterone, that is released during stress, has similar levels in CB1-ko mice and wildtype mice under basal conditions but there is increased levels of corticosterone after a stressful experience in CB1-ko mice (Uriguen et al., 2004;Wade et al., 2006) so that CB1-ko mice are perhaps more prone to stress by that.

We cannot fully exclude the possibility that total-CB1-ko mice and Glu-CB1-ko mice possess a disturbance in their visual system (e.g. a deterioration of the retina) so that they are not able to detect a novel object or a novel juvenile mouse under bright light conditions. This is rather unlikely to our minds: The novel juvenile exploration not only relies on approaching the juvenile mouse by the adult test mouse but also vice versa by approaches of the juvenile mouse towards the adult mouse. Secondly, although we tested total-CB1-ko and Glu-CB1-ko mice in the water maze under moderate illumination (50 lux) we could not detect any differences for acquisition of the task suggesting that the visual acuity of the mice is unaltered.

Behavioural consequences of endocannabinoid system activation become even more pronounced in a second trial in our experiments. Experienced total-CB1-ko mice and Glu-CB1-ko mice now show an anxiogenic phenotype in the plus-maze. Differences are known to exist in the nature of the anxiety response in plus-maze naïve and plus-maze experienced rodents. After an initial apparatus exploration it seems that rodents acquire, consolidate and retrieve some kind of memory related to exploration of potentially dangerous areas of the maze that is the open arms and consequently avoid these areas (Carobrez and Bertoglio, 2005). In particular, test experienced animals do not longer respond to the anxiolytic effect of benzodiazepines (Holmes and Rodgers, 1999). Obviously, in exploration-based tests (e.g. elevated plus-maze, open field) that rely on a conflict of approach and avoidance of a novel environment, learning shifts the balance towards avoidance (as there is no reason to search for food, shelter, escape anymore) (Belzung and Griebel, 2001;Millan, 2003). However, the antagonist SR141716 displayed a significant anxiolytic effect in maze-experienced mice (Rodgers et al., 2003). One explanation of this finding might be that only in the second trial on the plus-maze, the endocannabinoid system is adequately activated and the antagonist can unfold its complete efficacy. Nevertheless, we cannot support the anxiolytic effect of a deletion of CB1 on trial two with our mouse lines but see the opposite effect namely that total-CB1-ko and Glu-CB1-ko mice reveal an anxiogenic phenotype if the number of mice not exploring the open arms at all are considered. However, we have to remark critically here that the mice treated with the antagonist in Rodger's experiment were undrugged in the first trial, whereas our mice were deficient of CB1 in both trials, which may change the outcome of the second trial. Perhaps, Rodger's mice were rather affected in memory retrieval of the first plus-maze trial which resets their emotional response so that it resembles an anxiolytic phenotype. In the same way, total-CB1-ko mice and Glu-CB1-ko display drastically less locomotion and rearing in the open field under bright light conditions and both mice have increased levels of thigmotaxis during a second exposure. We suggest a similar memory of the aversive situation as it is perhaps the case for the plus-maze test.

In conclusion, CB1 on glutamatergic and GABAergic nerve cells has an antagonistic effect on anxiety which we refer to as the "Yin and the Yang effect" of CB1 on anxiety (Figure 34). Total-CB1-ko and Glu-CB1-ko mice show an axiogenic phenotype under highly aversive conditions and at the same time lack CB1 on glutamatergic neurons. We hypothesize that in wildtype animals downregulation of glutamatergic transmission via CB1 can unfold an anxiolytic phenotype. Otherwise, GABA-CB1-ko

mice rather show an anxiolytic phenotype. This mouse line misses CB1 on GABAergic neurons preferentially in the forebrain. Hence, in wildtype mice, downregulation of GABAergic transmission by activation of CB1 might manifest an anxiogenic phenotype and deletion of CB1 on GABAergic neurons consequently leads to the anxiolytic phenotype in the GABA-CB1-ko mice. We would like to make some speculative suggestions referring to transmitter systems and brain regions involved in these processes in the Appendix (section 6.3 and 6.4).

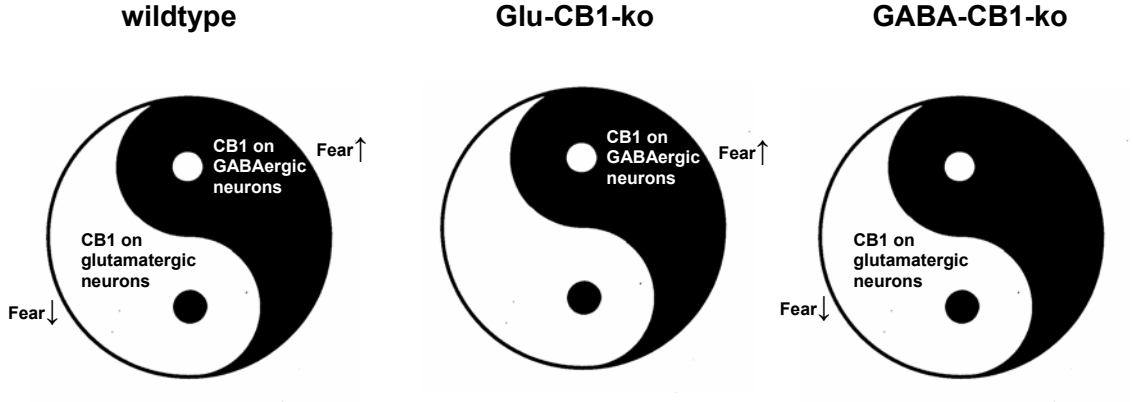


Figure 34: The Yin and Yang of CB1 on emotionality. Activation of CB1 has antagonistic effects on anxiety. Depicted is the distribution of CB1 on either glutamatergic neurons or GABAergic nerve cells. CB1 on glutamatergic cells reveals an anxiolytic effect whereas CB1 on GABAergic neurons appears to be anxiogenic. The converse phenotype can be observed in the Glu-CB1-ko and GABA-CB1-ko mice as CB1 is missing on the respective neuronal populations. Under highly aversive conditions, CB1 on glutamatergic terminals outweighs the effects of CB1 on GABAergic terminals with the consequence that total-CB1-ko mice display an anxiogenic-like phenotype under those circumstances.

All in all, activation of CB1 has a major influence on anxiety, critically depending on the severity of a potential threat and the neuronal subpopulation (i.e. glutamatergic or GABAergic neurons) with respective neuronal circuits participating in certain fearful situations.

4.4 Pitfalls of the CB1-ko animal model

Genetic deletion of CB1 offers several advantages over the pharmacological blockade, most importantly an increased receptor specificity (i.e. no concomitant block/activation of VR1 or occurrence of inverse agonist properties of the CB1 antagonist SR141716 in our case) and a completeness of receptor inactivation. Another advantage is that the lack of receptor and its associated function is not restricted to the moment of the experiment (and then could unfold its effect at an earlier or later moment) but is persistent. Eventually, by using conditional knockout mice, it is possible to target specific neuronal populations or developmental stages.
However, one has to be cautious about the drawbacks of genetically modified mice. Genetic mutations or deletions can lead to molecular or cellular changes that have been interpreted as an attempt of the organism to compensate for the missing or malfunctioning gene product (Nelson and Young, 1998;Pich and Epping-Jordan, 1998;Routtenberg, 2002). Potential drawbacks in CB1-ko mice shall be highlighted in the next two paragraphs.

4.4.1 Compensatory mechanisms in CB1-ko mice

CB1-ko mice develop apparently normally in the absence of the CB1 receptor (Harkany et al., 2007). They are fertile, care for their offspring, and do not show any obvious behavioral abnormalities. However, the elimination of CB1 may still alter the development of the animals, leading to behavioral changes that are not the direct result of acute disruption of endocannabinoid transmission. CB1-ko mice show some gross abnormalities in that they have reduced body weights, tend to have spontaneous seizures in their home cages (G. Marsicano, C.T. Wotjak, personal communication; Zimmer et al., 1999), swam poorly during a first exposure to the water maze pool (Varvel and Lichtman, 2002), have a higher mortality rate, and have a decrease in the number of cells in the hippocampus (Bilkei-Gorzo et al., 2005;Zimmer et al., 1999). On the other hand, young (6-8 weeks old) CB1-ko mice are not impaired in learning and memory test as it has been shown for adult and old CB1-ko mice (Bilkei-Gorzo et al., 2005). Therefore, if there are compensatory changes that affect behaviour, then they set in rather late in ontogeny.

There is some evidence that CB1-ko animals exhibit changes in neurochemical and hormonal states. The study of Maccarrone and colleagues compared 1-month-old and 4-month-old CB1-ko mice with the respective wildtype animals. They could show that there are no changes in endogenous anandamide and 2-AG brain contents as well as in AMT and FAAH activity in the 1-month-old animals. In contrast, the 4 month old CB1-ko animals, that resemble our animals with a range of age of 3-5 months, showed a decrease in hippocampal and striatal anandamide content and an increase in hippocampal and cortical AMT and FAAH activity (Di Marzo et al., 2000;Maccarrone et al., 2002). On the other hand, in a different study, no age-dependent differences in the tissue levels of anandamide and 2-AG or the density of CB1 could be found in 2 month old or 6-12 month old CB1-ko mice (Wang et al., 2003).

In contrast to acute pharmacological blockade with SR141716 or AM251, genetic inactivation of CB1 did not modulate basal steady-state hippocampal ACh efflux (this happened only under stress-inducing conditions) (Degroot et al., 2006;Kathmann et al., 2001b). Furthermore, genetic deletion of CB1 did not result in increased basal corticosterone levels in some studies (Wade et al., 2006;Wenger et al., 2003) whereas application of the antagonist SR141716 or genetic deletion of CB1 clearly increases levels of corticosterone in other studies (Barna et al., 2004;Cota et al., 2006;Wade et al., 2006). Neuropeptide mRNA levels of dynorphin and substance P are increased in the striatum of CB1-ko mice. In addition, locomotion was drastically reduced in CB1-ko mice (level of illumination of the open field in this study is not denoted), but motor coordination on the rotarod was not impaired (Steiner et al., 1999). This led the authors to the conclusion that initiation of movement is affected in CB1-ko mice. However, we could show that the inhibition of locomotion in CB1-ko mice is dependent on the averseness of the test situation and is not seen under low stress conditions. Morover, GABA-CB1-ko mice show a recombination of CB1 in the striatum whereas Glu-CB1-ko mice do not. At the same time, GABA-CB1-ko mice show no differences in locomotion in the open field. Thus, it is rather unlikely that the striatum as a central player of locomotion is the main cause of the observed phenotype.

By and large, these data indicate that brain region-specific adaptive and compensatory changes may occur in endocannabinoid metabolism of CB1-ko mice. However, it is questionable whether they significantly contribute to the phenotypes observed in the present study.

4.4.2 Genetic background of CB1-ko mice

Inbred C57BL/6 substrains and the outbred CD1 strain of mice have been utilised as genetic background for the generation of CB1-ko mice. These strains of mice have been used to develop four independent CB1-ko mouse lines, with Zimmer et al., Robbe et al. and Marsicano et al. using C57BL/6 substrains and Ledent et al. using the CD1 strain (Ledent et al., 1999;Marsicano et al., 2002;Robbe et al., 2002;Zimmer et al., 1999). Particularly, as the Ledent line has been crossed to an outbred CD1 genetic background, individual mutant animals from this strain can be expected to have a more heterogeneous genetic background.

In pharmacological, electrophysiological or behavioral experiments, results can be very different due to different genetic make-up of the used mouse strain. Most importantly for our study, it seems to be very distinct that CD1 mice show declined LTP maintenance and declined non-spatial learning whereas C57Bl/6 mice show excellent learning capabilities and LTP maintenance (Gerlai, 2002). Therefore, it might be much easier to find LTP and memory enhancing effects in mutant CD1 mice, and it is difficult to compare knockout animals of these two strains as a background. To avoid a ceiling effect, it might be more appropriate to use a mouse strain that can improve poor performance and the C57BL/6 mouse strain might not be the best tool for this purpose. Discrepancies between CD1 and C57BL/6 mice are also found in tests of memory (Gerlai, 2002) and anxiety (Bouwknecht and Paylor, 2002) and have to be kept in mind if knockout animals with different background mouse strains are compared. Anyhow, experiments with mutant mice have to be confirmed by acute pharmacological treatment which would, furthermore, allow addressing the question on to the different involvement of CB1 in different phases of the learning process (i.e. acquisition, consolidation, retrieval, extinction). In the present study, however, pharmacological treatment was not indicative as it fails to discriminate between CB1 on GABAergic versus glutamatergic neurons and hence could not help us to answer the questions under study.

5. Conclusion

The present study suggests that CB1 is involved in long-term synaptic plasticity although it could not be ascertained which neuronal population (i.e. glutamatergic or GABAergic nerve cells) accomplished LTP enhancement. The endocannabinoid system does not have a general nootropic effect. CB1 activation seems to specifically affect learning and memory of aversive learning tasks while leaving exploration-based learning tests unaffected. For the first time, a dissociation of contextual fear memory representation could be observed that is affected by cell-type specific CB1 ablation. CB1 activation plays a pivotal role in coping with stressful and potentially threatening situations. A “Yin and Yang principle” of CB1 on emotionality is evident in that CB1 on GABAergic neurons unfolds an anxiogenic effect and CB1 on glutamatergic nerve cells reveals an anxiolytic effect as it is shown for the first time in this study. These effects are most prominent under very stressful and aversive testing conditions. The exact neuroanatomical and neurophysiological processes behind our findings remain to be investigated.

Our findings emphasise the importance of conditional mutant mice to investigate the endocannabinoid system. With pharmacological tools, we would not have been able to distinguish between CB1 on glutamatergic and GABAergic neurons which was only possible with a specific ablation of CB1 on these neuronal populations in conditional mutant mice.

Taken into consideration that a variety of anxiety disorders are characterised by altered processing of trauma-related and stressful contextual fear memories and by impairments in fear extinction (e.g. posttraumatic stress disorder, panic disorder, and social phobia), the data of the present study imply a dysregulation of the endocannabinoid system to play an important role in such pathological states.

6. Appendix

6.1 Factors for LTP enhancement

We would like to introduce the reader to some possible mechanisms by which LTP enhancement could be assured.

6.1.1 Factors for LTP enhancement I: GABA

It is controversial whether the enhancement of LTP is a direct consequence of an unrestrained excitatory transmission between principal neurons, or whether it occurs indirectly as a reduction of inhibitory transmission onto pyramidal or granule cells so that glutamatergic transmission can pass off more efficiently.

Stimulation of hippocampal afferent fibers activates excitatory synapses on principal cells, but also local populations of GABAergic interneurons, which exert potent inhibitory actions on principal cells (Thompson, 1994). Release of endocannabinoids in the hippocampus might be related to reduced inhibition. More precisely, the perforant path-granule cell LTP is normally accompanied by long-term potentiation of a feed-forward inhibitory pathway (Kairiss et al., 1987) and induction of NMDA-R-dependent LTP in the dentate gyrus is facilitated by a reduction of GABAergic IPSCs (Wigstrom and Gustafsson, 1983;Wigstrom and Gustafsson, 1985). LTP of GABAergic interneurons may arise from changes at both excitatory synapses on to interneurons and inhibitory synapses on to pyramidal cells. Activation of CB1 would block GABAergic interneurons that restrain their target glutamatergic pyramidal neurons. Endocannabinoids would then be the natural disinhibitor of local synaptic plasticity in the hippocampus.

In two single-cell recording studies, a high spatial and temporal precision of endocannabinoid action onto GABAergic interneurons could be demonstrated. As stated above, a brief stimulus train, that did not induce LTP under normal conditions, was then delivered during a DSI period, when GABAergic transmission is suppressed after depolarisation of a principal neuron. It now could induce NMDA-R-dependent LTP (Carlson et al., 2002). The same effect holds true for I-LTD in that induction of I-LTD can prime excitatory synapses, so that LTP of excitatory transmission can subsequently be induced by stimuli that were previously subthreshold for LTP induction. The priming effect is spatially very specific for a small dendritic area 10 µm away from the stimulating electrode (Chevaleyre and Castillo, 2004). Accordingly, endocannabinoids can facilitate LTP induction through a heterosynaptic interaction with GABAergic synapses. These studies also suggests that the effect of endocannabinoids to facilitate LTP induction occurs in a highly spatially restricted way as released endocannabinoids travel ≤ 20µm (Wilson and Nicoll, 2001). The results are also remarkable as they do not contradict the many reports that (endo)cannabinoids antagonise LTP, because exogenously applied (endo)cannabinoids globally affect all cells and types of cannabinoid receptors, including those on glutamatergic and GABAergic synapses. Thus, a loss of endocannabinoid control on GABA release, like in CB1 knock-out mice, could rather increase inhibition, which likely counteracts LTP. The fact that this effect can not be supported by our study is perhaps due to the global activation of large hippocampal areas in our experimental *in vivo* preparation.

Remarkably, endocannabinoids may also decrease GABA reuptake and thus augmented GABA levels after HFS could hinder LTP (Al Hayani and Davies, 2002;Maneuf et al., 1996). This suggests, that endocannabinoids released after depolarization may impair the induction of LTP through upregulation of GABAergic transmission.

Disinhibition does not have to be mediated within the hippocampus but may be mediated from GABAergic septohippocampal afferents that selectively inhibit hippocampal interneurons and thus disinhinbit pyramidal cells (Toth et al., 1997). Remarkably, each hippocampal inhibitory cell contacts 500-1200 pyramidal cells (Buhl et al., 1994), thus the effect might be very striking in disinhibiting a large population of neighbouring pyramidal cells. Disinhibition could eventually lead to a facilitated induction of LTP. As total-CB1-ko and GABA-CB1-ko mice miss CB1 on GABAergic neurons this could mean that disinhibition occurs in these mice and ultimately enhances LTP.
On the other hand, and quite astonishingly, it is not mandatory that LTP of an afferent excitatory input to an interneuron necessarily improves the inhibition in a target cell of the respective interneuron. For example, when LTP is induced in an interneuron, IPSP in a postsynaptic pyramidal neuron may be depressed rather than potentiated, because of presynaptic effects (Stelzer and Shi, 1994). It is known that $GABA_B$ autoreceptors, located on the interneuron terminals that synapse on pyramidal cells and unfold postsynaptic hyperpolarisation via $GABA_A$ receptors, can be activated during LTP induction, depress the release of GABA and lead to a prolongation of EPSPs, thereby creating facile conditions to trigger LTP (Davies et al., 1991;Mott and Lewis, 1991;Pacelli et al., 1989). $GABA_B$ antagonists can block theta-burst-LTP (Davies et al., 1991) or lead to suppressed LTP (Brucato et al., 1996). The effect of autoreceptor-mediated reduction of GABA is more sensitive to LTP induced through theta-burst stimulation that we also applied in our study (Staubli et al., 1999).
In conclusion, increased GABA levels could decrease LTP or paradoxically increase LTP or have simply no net effect at all as the increase of GABA due to the lack of CB1 is decreased to the net level due to $GABA_B$-receptor activation. If these mechanisms play a role in total-CB1-ko and GABA-CB1-ko mice, where CB1 is missing on interneurons, it remains to be elucidated why LTP enhancement can only be observed in total-CB1-ko mice.

6.1.2 Factors for LTP enhancement II: Glutamate

Another obvious mechanism for the action of CB1 on LTP is a modulation of glutamatergic transmission during LTP induction. A straightforward hypothesis would state that the lack of CB1, having an inhibitory effect on glutamatergic transmission, leads to an enhancement of LTP.
Whether endocannabinoids suppress excitatory glutamatergic transmission is a matter of debate. CB1 agonists can decrease EPSPs in hippocampal pyramidal neurons (Al Hayani and Davies, 2000;Ameri and Simmet, 2000;Hajos and Freund, 2002;Misner and Sullivan, 1999) or no inhibition of excitatory synaptic transmission in the hippocampus could be found (Al Hayani and Davies, 2000;Paton et al., 1998;Terranova et al., 1995). Most importantly, suppression of glutamatergic transmission after application of the CB1 agonist WIN55,212-2 could be shown in the amygdala, neocortex, cerebellum and hippocampus in GABA-CB1-ko mice but no such suppression has been detected in mice lacking CB1 on glutamatergic neurons, that are similar to our Glu-CB1-ko mice, or in CB1-ko mice (Domenici et al., 2006;Kawamura et al., 2006). This unequivocally shows that it is indeed CB1 on glutamatergic neurons causing the effect and not a secondary process.
Inhibition of COX-2, that is an endocannabinoid degrading enzyme, which consequently boosts levels of 2-AG, decreases basal excitatory transmission in the hippocampus. Slanina and colleagues could further show that, again by blocking COX-2, hippocampal LTP of fEPSP is greatly restricted. On the other hand, block of CB1 with the antagonists SR161714A or AM251 increased levels of LTP.

Interestingly, only moderate or theta-burst stimulation for LTP induction lead to increased levels of LTP after CB1 block. If strong HFS protocols were applied, no difference could be found anymore (Slanina et al., 2005;Slanina and Schweitzer, 2005).
These data, obtained in the slice preparation, cannot be directly conferred to our *in vivo* preparation. Our LTP-inducing protocol is a moderate theta-burst protocol with 36 stimuli altogether (compared to 216 pulses usually applied for *in vivo* experiments in mouse or rat (Jones et al., 2001a), hence contradicting the finding of Slanina et al., as we did not find any changes in Glu-CB1-ko mice. However, for LTP induction, we used a current with asymptotic pop-spike amplitude compared to baseline current used in the study of Slanina et al. In conclusion, it might be that our protocol for LTP induction is too drastic and differences get blurred between knockout and wildtype animals. The question again arises why we are able to find increased LTP levels only in total-CB1-ko animals but not in Glu-CB1-ko and GABA-CB1 ko mice?
As stated already above, enhanced LTP could not always be mimicked by application of the CB1 antagonist (Marsicano et al., 2002) suggesting some other factor being involved in the increase. Curiously, the antagonist AM251 even inhibited the induction of LTP in a recent study (de Oliveira et al., 2006) whereas only a chronic but not acute administration of the antagonist AM251 increased levels of LTP (Hoffman et al., 2007). Which other transmitter may account for this discrepancy?

6.1.3 Factors for LTP enhancement III: Acetylcholine

If it is not the GABAergic or glutamatergic transmission leading to enhanced LTP then another additional factor must be required for this effect. One possibility is the cholinergic system. ACh has been accorded an important role in supporting learning and memory processes in the hippocampus (Parent and Baxter, 2004). Cholinergic projection neurons that innervate the forebrain (including the hippocampus) arise in the medial septum and the nucleus basalis of Meynert. As mentioned above, the projection of the medial septum to the hippocampus consists of two components: One is cholinergic afferents which contact both pyramidal and inhibitory cells in the hippocampus (Frotscher and Leranth, 1985). The other component are GABAergic neurons that seem to selectively contact interneurons (Freund and Antal, 1988). Interneurons of the hippocampus feed back to the medial septum and innervate cholinergic and non-cholinergic neurons herein (Amaral DG and Witter MP, 1995).
CB1 is present in cholinergic projecting neurons in the medial septum and the nucleus basalis of Meynert (Lu et al., 1999;Nyiri et al., 2005) and FAAH is found in these cells as well (Harkany et al., 2003) indicating that cholinergic neurons might utilize endocannabinoids for the control of efficacy of cholinergic input and are itself under the control of endocannabinoids. The cannabinoid receptor agonists anandamide, WIN55,212-2 and CP55940, decrease acetylcholine release from electrically stimulated hippocampal slices (Gifford et al., 1997a;Gifford et al., 2000;Gifford and Ashby, Jr., 1996), whereas the antagonists SR141716 and AM281 increase the ACh release in mouse and rat suggesting that ACh output is tonically inhibited by endocannabinoids (Freund et al., 2003;Gifford et al., 1997b;Gifford et al., 2000;Kathmann et al., 2001b;Redmer et al., 2003;Steffens et al., 2003). The CB1 antagonist AM251 eliminated the effect of the ACh esterase inhibitor physostigmine to reduce fEPSP in the hippocampus (Colgin et al., 2003). Also, the hippocampus of CB1-ko mice showed enhanced release of ACh in response to electrical stimulation (Kathmann et al., 2001a) although another study found no difference for CB1-ko animals under basal conditions (Degroot et al., 2006;Wade et al., 2006). Eventually, CB1 agonists reduce acetylcholine levels in the neocortex and hippocampus *in vivo*

but only at relatively high doses (mg/kg) whereas lower doses (µg/kg) cause the opposite effect elevating the acetylcholine level (Acquas et al., 2000;Gessa et al., 1998)[9].
Importantly, cholinergic agonists facilitated LTP (Blitzer et al., 1990;Burgard et al., 1993) and endogenous ACh, that activates muscarinic ACh receptors, lowers the threshold for LTP induction (Ovsepian et al., 2004). Interestingly, specific activation of the M1-mAChR leads to enhanced LTP and the same receptor triggers release of endocannabinoids and DSI (Kim et al., 2002;Shinoe et al., 2005). Thus, increased ACh levels may participate in the facilitation of LTP in total-CB1-ko mice. So far, it is unknown whether ACh levels are altered in GABA-CB1-ko and Glu-CB1-ko mice which could consequently contribute to enhance LTP.

6.1.4 Factors for LTP enhancement IV: Cholecystokinin (CCK)

CCK is one of the most abundant neuropeptides in the brain (Fink et al., 1998). CB1 is located on the axon terminals of a specific GABAergic cell population characterised by the expression of CCK. About 80% of CCK-positive interneurons coexpress CCK and CB1. Altogether, about 40% of all hippocampal interneurons express CB1 (Marsicano and Lutz, 1999). Indeed, cannabinoid agonists inhibit CCK release in the hippocampal slice (although CCK release is unchanged in the frontal cortex after agonist application, where CB1 and CCK are coexpressed, too) (Beinfeld and Connolly, 2001). Hence, a reduction of GABAergic transmission by CB1 activation might be paralleled by a reduction of CCK. Another line of evidence showed that deficiency of CCK receptor decreased LTP, and CCK receptor agonists increased LTP in the hippocampus (Balschun and Reymann, 1994;Nomoto et al., 1999;Yasui and Kawasaki, 1995). So far, no study investigated levels of CCK *in vitro* or *in vivo* in CB1-ko animals, however this discloses another possibility of enhanced LTP in total-CB1-ko mice if these mice would indeed reveal enhanced levels in CCK transmission. However, the fact that we did not see any differences in LTP in GABA-CB1-ko mice does not support a major role of CCK in LTP enhancement (provided that CCK levels are actually increased in GABA-CB1-ko mice).

6.1.5 Factors for LTP enhancement V: Vanilloid receptor 1

The transient receptor potential vanilloid subtype 1 channel (TRPV1 or vanilloid receptor 1 VR1) is a calcium-permeable non-selective cation channel that was cloned

[9] The biphasic dose-dependent effect on cholinergic neurotransmission might be explained by a dose-dependent engagement of excitatory or inhibitory pathways of the forebrain. Suppressing, high-dose effects seem to be locally mediated in the hippocampus, whereas low-dose excitatory effects seem to be mediated in the septum (Tzavara et al., 2003b). WIN55,212-2 is much more potent in reducing GABAergic than glutamatergic neurotransmission (Hajos and Freund, 2002). Thus, the higher sensitivity of septal CB1 circuits could be attributed to GABAergic septal neurons in controlling cholinergic neurotransmission. Indeed, blockade of septal GABAergic activity leads to cholinergic stimulation (Moor et al., 1998). Interestingly, a primary reduction of cholinergic activity in the septum can also induce a moderate increase in hippocampal ACh via deactivation of a negative autoregulatory loop (Wu et al., 2000). The inhibitory effect of WIN55,212-2 mediated locally in the hippocampus and observed with higher doses, could result from activation of low-sensitivity CB1 localized on cholinergic terminals or in projections that modulate ACh release. Hence, although ACh levels are increased in CB1-ko mice according to one study (Kathmann et al., 2001b), this does not exclude the possibility of a dysregulated septal-hippocampal network. Altogether, this suggests that there may be an endogenous and tonic CB1-mediated inhibition of ACh release. Given that the septohippocampal pathway is important for learning and memory, it is reasonable to think that excitation of this cholinergic pathway is one of the means by which the lack of CB1 might enhance LTP and memory.

in 1997 (Szallasi and Blumberg, 1999). The channel is activated by both chemical (capsaicin, the red hot chilli pepper ingredient) and physical (noxious heat and low pH) stimuli (Caterina and Julius, 2001). VR1 is expressed in neocortex, hippocampus, amygdala, hypothalamus, thalamus, substantia nigra, cerebellum among others (Mezey et al., 2000). VR1 and CB1 are co-expressed on pyramidal neurons of the hippocampus and in the molecular layer on the dentate gyrus as could be shown by immunohistochemistry (Cristino et al., 2006;Mezey et al., 2000). In contrast to CB1, VR1 is expressed on postsynaptic dendritic spines and cell somata (Toth et al., 2005). The identity of endogenous activators of VR1 remains currently unclear. Endocannabinoids can activate VR1 as could have been shown for anandamide and n-arachidnoyldopamine (NADA) (Al Hayani et al., 2001;Di Marzo et al., 2002a;Zygmunt et al., 1999), thus the term “endovanilloids” may be appropriate for these messengers in the future, too. However, VR1 has opposing effects on cellular excitability in that cation and calcium influx through VR1 channel lead to excitation and to an activation of calcium-dependent kinases. Thus, opposite to actions of CB1, they can increase glutamatergic transmission (Marinelli et al., 2006). There seems to be interplay between VR1 and CB1. For example, although WIN55,212-2 and 2-AG increase the paired-pulse ratio for population spike in CA1 (i.e. less paired-pulse depression), anandamide and also capsaicin have the opposite effect and decrease the paired-pulse ratio (i.e. increased paired-pulse depression), an effect being blocked with the VR1 antagonist capsazepine (Al Hayani et al., 2001). This suggests that these effects are mediated via VR1 and that its activation leads to an increase in inhibitory GABAergic transmission in the hippocampus. Interestingly, in slices prepared from wildtype mice, the CB1 agonist WIN55,212-2 inhibited evoked EPSCs and evoked IPSCs whereas in slices from CB1-ko mice, WIN55,212-2 no longer inhibited IPSCs, but still inhibited EPSCs (Hajos et al., 2001). The inhibition of excitatory transmission was mimicked by the VR1 agonist capsaicin, and was blocked by the VR1 antagonist capsazepine whereas depression of inhibitory currents is not (Hajos and Freund, 2002) indicating that VR1 plays a major role for glutamatergic neurotransmission.

The discrepancy whether CB1 activation reduces glutamatergic transmission could be resolved if one emanates from an interplay with VR1 and it might be that these effects are mediated by VR1 or the so far undiscovered “CB3”. We suggest that the latter possibility is more likely because VR1 forms a non-selective cation channel (Caterina and Julius, 2001) and a lack of VR1 leads to reduction of LTP in the hippocampus (Marsch et al., 2007). On the other hand, neither basal synaptic transmission nor LTP were modulated by the VR1 agonists capsaicin or resiniferatoxin (Lees and Dougalis, 2004). Furthermore, cannabinoid action on hippocampal glutamatergic terminals is clearly mediated via pertussis toxin-sensitive G-proteins (Misner and Sullivan, 1999), hence VR1 which is an ion channel might not be involved. Lastly, a slice study in prefrontal cortex indicated that bath application with cannabinoids facilitated LTD, at the expense of LTP. Conversely, blockade of CB1 with the antagonist SR141716 led to increased levels of LTP (Auclair et al., 2000). It remains to be investigated whether CB1 and VR1 may interact and if endocannabinoids switch to the role of “endovanilloids” in total-CB1-ko mice. However, LTP enhancement by activation of VR1 does not seem to occur in our three mouse lines.

6.1.6 Factors for LTP enhancement VI: Glucocorticoids

Yet another hypothesis for enhanced LTP in total-CB1-ko mice comes from the finding that these mice show alterations in the hypothalamus-pituitary-adrenal (HPA) axis function (Cota et al., 2006;Wade et al., 2006).
Blockade of mineralocorticoid receptors (MRs) suppresses the ability to induce LTP, whereas blockade of glucocorticoid receptors (GRs) leads to an increase LTP in the hippocampus *in vivo* (Avital et al., 2006). It is important to know that MRs are preferentially activated at low corticosterone concentrations. When high corticosterone levels are present, MRs saturate and GRs take over (Reul and de Kloet, 1985). Antagonists of CB1 increased corticosterone levels in mice (Patel et al., 2004;Wade et al., 2006). Furthermore, there is a basal increase of corticosterone and ACTH in CB1-ko mice (Barna et al., 2004;Cota et al., 2006) although another study could not find any difference between CB1-ko mice and the respective wildtype mice (Wade et al., 2006). Furthermore, stressed CB1-ko mice show enhanced levels of ACTH and corticosterone as well (Cota et al., 2006;Wade et al., 2006). Perhaps altered levels of corticosterone in the brain of total-CB1-ko mice lead to permanently increased occupancy of MR in the hippocampus and further on to enhanced LTP. Imagining that the respective occupancy of MR and GR and their opposite effects on LTP is in a fragile balance this could also explain our observation that only some total-CB1-ko mice have increased levels of LTP whereas others show levels similar to wildtype mice. Whether dysregulated occupancy of MR and GR may also take place in Glu-CB1-ko and GABA-CB1-ko mice remains to be investigated.

6.1.7 Factors for LTP enhancement VII: The "cannabinoid receptor type 3 (CB3)"

Although CB1-ko mice lost responsiveness to most cannabinoids, Δ^9-THC still produced nociception in the tail-flick test of analgesia (Zimmer 1999). Anandamide produced the full range of behavioural effects (the so called "mouse tetrad" consisting of antinociception, catalepsy, reduced locomotion and hypothermia) in CB1-ko mice (Di Marzo et al., 2000). However, compounds that potently stimulate VR1 are also very potent and efficacious in the tetrad and can be elicited e.g. with capsaicin (Di Marzo et al., 2002b). Furthermore, anandamide-stimulated GTPγS activity can be elicited in brain membranes from CB1-ko mice (Breivogel et al., 2001). The CB1 agonist WIN55,212-2 reduced both IPSCs and EPSCs in wildtype mice, and most surprisingly the agonist still reduced EPSCs in CB1 knock-out mice, hence the receptor is found on excitatory (pyramidal) axonal terminals. The effect could be blocked by the antagonist SR 141716 and the VR1 antagonist capsazepine in CB1-ko mice (Hajos et al., 2001). Thus, deletion of CB1 clearly altered the action of the cannabinoid agonists on inhibitory transmission, but left its effect on glutamate release unchanged. This might suggest that there is a third cannabinoid receptor "CB3" present on excitatory terminals in the hippocampus that inhibits glutamate release. However, Hoffman et al. could show that the effect of WIN55,212-2 could not be seen in mice of the C57BL/6J strain. As the above mentioned study relies on mice with a CD1 background, the authors suggest that "CB3" is present in hippocampi of CD1 mice but not in C57Bl/6J mice (Hoffman et al., 2005). Intricately, in very recent two studies, no effects of WIN55,212-2 on glutamatergic transmission in CB1-ko mice, neither on a C57Bl/6J background nor on a CD1 background, could be shown at all (Kawamura et al., 2006;Takahashi and Castillo, 2006). The reason for the discrepancy to the study of Hajos et al. and Hoffman et al. remains unknown. Most of the electrophysiological studies support the idea that CB1 is the exclusive cannabinoid receptor responsible for cannabinoid-dependent presynaptic modulation

at inhibitory and excitatory synapses in the brain (Azad et al., 2003;Gerdeman et al., 2002;Melis et al., 2004a;Robbe et al., 2002;Whalley et al., 2004). We believe that "CB3", if it exists at all, does not play a role in our three mouse lines.

6.1.8 Factors for LTP enhancement VIII: Effects of CB1 activation on neuronal growth and neuroneogenesis

One of the cellular mechanisms of learning and memory and synaptic plasticity is the formation of new synapses or the remodelling of existing ones (Martin et al., 2000;Toni et al., 1999). An enhancement of synaptogenesis and increase in the number of cells in a brain area such as the hippocampus could cause enhancement of learning and memory and LTP, too.

Analysis of neuronal progenitor cell proliferation indicate that endocannabinoids promote cell proliferation in a CB1-dependent manner, and this proliferation is impaired in CB1-ko animals (Aguado et al., 2005), although another study showed a clear increase in the number of cells after administration of the CB1 antagonist AM251 *in vivo* (Hill et al., 2006b). Assuming that neurogenesis might contribute to the generation of new functional neurons with the ability to be integrated in hippocampal circuits, this would paradoxically favour a weakening of the hippocampal network in CB1-ko mice. Neurogenesis in the dentate gyrus and the subventricular zone is indeed impaired in CB1-ko mice (Jin et al., 2004), implying that endocannabinoids promote basal levels of neurogenesis *in vivo*. In addition, in 3-5 month old CB1-ko mice, the number of cells is decreased in the CA1 and CA3 region (but not significantly in the dentate gyrus) of the hippocampus (Bilkei-Gorzo et al., 2005). Furthermore, treatment of rats with the CB1 agonist WIN55212-2 over 20 days lead to an increase of dendritic arborisation in CA3, dentate gyrus and subiculum of the hippocampus and an increase in the number of cells could be observed in the dentate gyrus after treatment (Lawston et al., 2000). Finally, chronic treatment of rats with the agonist HU210 promotes neurogenesis *in vitro* and *in vivo*, and interestingly this effect is accompanied with an anxiolytic phenotype in these animals (Jiang et al., 2005). Altogether, one could argue that there might be a decrease in the dendritic arborisation and/or cell number in the hippocampus of CB1-ko mice.

There is some evidence that supports the other view. Using a mouse neuroblastoma cell line as an *in vitro* model to study receptor-mediated neurite remodelling, it could be shown that the CB1 agonist HU-210, causes neurite retraction in a concentration-dependent manner in these cells. CB1 antisense oligonucleotides blocked this effect indicating that it was indeed CB1 dependent (Zhou and Song, 2001). In a primary cell culture model of hippocampal neurons, anandamide as well as synthetic cannabinoids (WIN55212-2, Δ^9-THC) inhibited recruitment of new synapses by activation of CB1, and decreased levels of cAMP and hence proteinkinase A activation within a time frame of 2 h (Kim and Thayer, 2001). Eventually, anandamide inhibited the neuronal differentiation *in vitro* and *in vivo*, whereas the antagonist SR141716 enhanced neurogenesis (Rueda et al., 2002).

In conclusion, beside of direct effects on synaptic transmission, the activation of CB1 potentially up or downregulates the number of functional synapses and cells in certain brain areas. This could especially explain effects found after an acute or chronic pharmacological treatment (Hoffman et al., 2007). Whether this is a genuine role of the endocannabinoid system and whether it is of importance in the here investigated mutant mouse lines for synaptic plasticity remains to be elucidated.

6.2 Transmitters involved in the memory enhancing effects mediated by CB1

It is very speculative to state which neurotransmitter might lead to memory enhancement but we would like to make a few suggestions here.

GABA and glutamate: Most obviously, as CB1 is located on presynapses of GABAergic and glutamatergic neurons, its activation reduces GABA and glutamate transmission release *in vitro* and *in vivo* (Domenici et al., 2006;Katona et al., 1999;Katona et al., 2006;Marsicano et al., 2003;Monory et al., 2006). Pharmacological studies indicate that administration of $GABA_A$-receptor antagonist bicuculline after application of Δ^9-THC specifically alleviates memory impairments in mice (Varvel et al., 2005) and that bicuculline alone can facilitate retention of memory after intrahippocampal administration (Zarrindast et al., 2002). Increase of GABA levels enhances spatial learning in the water maze (O'Connell et al., 2001). There is a vast research line highlighting the involvement of glutamatergic transmission in learning and memory (Riedel et al., 2003). For example, blocking the NMDA receptor causes severe memory deficits in a Morris water maze task (and also decrease in LTP) in rats (Morris et al., 1986a).

Cholecystokinine: Because reduction of GABA by CB1 activation is paralleled by a reduction of CCK (Beinfeld and Connolly, 2001), it is interesting to note that blockade of CCK receptors impairs learning of a 8-arm radial maze task (Harro and Oreland, 1993).

Acetylcholine: ACh can lead to memory enhancement. ACh has been accorded an important role in supporting learning and memory processes by the hippocampus (Parent and Baxter, 2004). Cholinergic projections of the nucleus basalis magnocellularis and of the medial septum to the cerebral cortex and the hippocampus have been regarded as critical for memory (Dutar et al., 1995). A recent study showed that CB1-ko mice exhibited an increased ACh release in the hippocampus (Katmann 2001), although there is evidence that there is no change under basal conditions but increased levels if CB1-ko mice are stressed (Degroot et al., 2006;Wade et al., 2006). Inhibition of ACh activity has been associated with cannabinoid-induced impairment of memory (Braida and Sala, 2000). Furthermore, enhancement of social recognition memory with the CB1 antagonist SR141716 can be reversed by simultaneous administration of the mACh-receptor antagonist scopolamine (Terranova et al., 1996) and coadministration of subthreshold doses of the SR141716 and the ACh-esterase inhibitor donepezil significantly enhanced memory (Wise et al., 2007), suggesting an interaction between the cholinergic and cannabinergic system.

6.3 Transmitters involved in emotional effects mediated by CB1

It is highly speculative to propose which neurotransmitter might lead to modulation of anxiety in our mouse lines but we would like to make a few suggestions here.

GABA and glutamate: Endocannabinoids seem to play a prominent role in modulating anxiety and unfold an anxiolytic effect (Bortolato et al., 2006;Kathuria et al., 2003). Generally, CB1 is predominantly localised on axon terminals of GABAergic and glutamatergic neurons, and the lack of CB1 potentially enhances the levels of GABA and glutamate in the hippocampus, amygdala and other regions of the brain (Freund et al., 2003). Thus, the modulation of either inhibitory or excitatory transmitter systems may be involved in the regulation of emotional behaviour. Especially, activation of the GABAergic system is known to reduce levels of anxiety for a long time (Mohler et al., 2004). It is interesting to know that application of a $GABA_A$-receptor agonist into the medial septum has an anxiolytic effect (Degroot and Treit, 2003). Similarly to GABA, glutamate is an ubiquitous transmitter in the brain

and has profound effects on anxiety (Bergink et al., 2004;Swanson et al., 2005), however this critically depends on the glutamtergic receptor subset and brain region under study. Especially, if GABA levels would be raised in GABA-CB1-ko mice, this could elegantly explain the anxiolytic phenotype in these mice.

Colecystokinine: In addition, endocannabinoids might also influence the release of anxiogenic neuropeptides, such as corticotropin-releasing hormone (CRH) and Cholecystokinine (CCK) (Beinfeld and Connolly, 2001;Weidenfeld et al., 1994). Both CRH and CCK neurotransmission were shown to play a role in anxiety (Chen et al., 2006;de Kloet et al., 2005;Fink et al., 1998;Schulkin et al., 2005). CCK-expressing interneurons co-express CB1 (Marsicano and Lutz, 1999). In addition, the postsynaptic membranes that are contacted by CCK-containing GABAergic interneurons are rich in α_2 subunit of the $GABA_A$ receptor (Nyiri et al., 2001), which was shown to mediate the anxiolytic effect of benzodiazepines (Low et al., 2000). Nevertheless, as we rather observe an anxiolytic phenotype in GABA-CB1-ko mice, it is unlikely that CCK is the main factor that induces states of anxiety.

Serotonin: Another candidate for effects on anxiety is serotonin. CB1 and serotonergic 5-HT_{1B}- and 5-HT_3-receptor are coexpressed on neurons of the hippocampus, striatum and neocortex (Hermann et al., 2002). Also, enhancing levels of anandamide with the FAAH inhibitor URB 597 increases firing activity of serotonergic neurones in the dorsal raphe nucleus (Gobbi et al., 2005). This is all the more interesting as the anxiolytic effect of the anandamide transport inhibitor AM 404 can be antagonised by a $5HT_{1A}$-receptor antagonist suggesting a synergistic role of the $5HT_{1A}$- and the CB1 receptor activation in emotional reactivity (Braida et al., 2007). Hence, if CB1 is missing in the brain and can not support the activation of the serotonergic system, perhaps this could induce the anxiogenic phenotype seen in total-CB1-ko and Glu-CB1-ko mice.

Acetylcholin: Finally, CB1-ko mice have elevated levels of ACh under high stress conditions (Degroot et al., 2006) and activation of postsynaptic muscarinic receptors leads to a facilitated release of endocannabinoids (Fukudome et al., 2004;Ohno-Shosaku et al., 2003) and eventually endogenous levels of ACh or AChR agonists in the hippocampus are anxiolytic in a set of behavioural tests (Degroot et al., 2001;Degroot and Treit, 2003;File et al., 2000;Hess and Blozovski, 1987;Smythe et al., 1998). We can not support an upregulated cholinergic tone as total-CB1-ko conversely show an anxiogenic phenotype but increased and anxiolytic levels of ACh might play a role for GABA-CB1-ko mice.

6.4 Brain regions involved in emotional effects in relation to CB1

What might be the cellular and anatomical substrate of the altered emotional behavior in our mouse lines? Anxiety states and anxiety-related behavior appear to be regulated by a distributed but highly interconnected system of brain structures including the septohippocampal system (McNaughton, 1997), the basolateral amygdala (Campbell and Merchant, 2003), the bed nucleus of the stria terminalis (BNST) (Walker et al., 2003), hypothalamic regions (Singewald et al., 2003), the medial prefrontal cortex and the anterior cingulate cortex (Duncan et al., 1996), as well as hindbrain regions such as the periaqueductal grey, locus coeruleus and dorsal raphe nuclei (Singewald et al., 2003). At the same time, CB1 is richly expressed in most of these brain regions (Herkenham et al., 1990).

The amygdala is a key station in the neural circuitry that processes emotions and mediates stress and fear responses to aversive sensory stimuli. The amygdala consists of the basolateral amygdala that in turn projects to the central nucleus of the amygdala and to the BNST. These structures, in turn, project to a common set of

target areas, mainly hypothalamus and brainstem, that mediate the autonomic and behavioral responses to aversive stimuli (Walker et al., 2003). There is sparse distribution of CB1 in the BNST (Herkenham et al., 1991) and CB1-bearing interneurons are localized in the basolateral complex of the amygdala (Katona et al., 2001;McDonald and Mascagni, 2001). Indeed, the presentation of anxiogenic stimuli increases anandamide and 2-AG concentrations in the amygdala (Marsicano et al., 2002). In addition, stress-induced amygdala activation is potentiated by blockade of CB1 receptors in rodents (Patel et al., 2005a), further implicating CB1 in the processing and integration of emotionally salient information. Endocannabinoids could modify the output of the amygdala in two complementary ways: Firstly, they could depress glutamate release from axon terminals originating in the cortex (Azad et al., 2003;Domenici et al., 2006), which richly innervate the basolateral amygdala. Pyramidal cells in the basolateral amygdala, receiving excitatory sensory inputs, may need to remove an inhibitory control to be able to fire and create associations between emotionally relevant and neutral stimuli, as in the fear conditioning paradigm. Secondly, by reducing GABA release from basolateral interneurons, they might disinhibit GABA cells in the adjacent so-called intercalated nuclei, whose GABAergic neurons generate feedforward inhibition to the central nucleus and consequently decrease the activity of GABAergic neurons in the central nucleus of the amygdala, which constitutes the structure's primary efferent pathway towards autonomic and endocrine centers of the brain (Pare et al., 2004;Royer et al., 1999). Indeed, it has been shown that endocannabinoids induce LTD-I of inhibitory GABAergic neurotransmission in the basolateral amygdala, and this effect is even more pronounced in mice lacking the endocannabinoid-degrading enzyme FAAH (Azad et al., 2004). Also, the central nucleus and the basolateral nucleus of the amygdala project to cholinergic nuclei in the forebrain (Jolkkonen et al., 2002). Bearing in mind that cholinergic neurons of the forebrain express CB1 and FAAH (Harkany et al., 2003;Lu et al., 1999;Nyiri et al., 2005), it is tempting to speculate that cholinergic output to the cortex is modulated by the central amygdala. We could show that CB1 inactivation causes an anxiety-like response in total-CB1-ko and Glu-CB1-ko mice, which might be enabled by an altered processing in the amygdala. On the other hand, the basolateral amygdala also projects to the BNST. The startle reflex can be enhanced by bright light, a factor that we and others found to be important for an anxiogenic response (Crawley, 1981;File and Hyde, 1978;Naidu et al., 2007). Most interestingly, blocking AMPA receptors in the BNST but not the central amygdala abolished light-enhanced startle (Walker and Davis, 1997b). Thus, the BNST might be an essential brain structure involved in our effects on anxiety, too.

6.2 Tables of statistical analysis

Table 3: Statistical analysis of electrophysiological recordings

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
I/O curve, fEPSP slope	Intensity	**F(9, 207) = 51.2, p < 0.001**	**F(1.3, 28.8) = 24.2, p < 0.001**	**F(2.1, 47.8) = 36.8, p < 0.001**
	Intensity x genotype	F(9, 207) = 0.23, p = 0.99	F(1.3, 28.8) = 1.0, p = 0.35	F(2.1, 47.8) = 0.34, p = 0.72
	Genotype	F(1, 23) = 0.21, p = 0.65	F(1, 21) = 0.39, p = 0.54	F(1, 23) = 1.49, p = 0.24
I/O curve, pop-spike amplitude	Intensity	**F(2.7, 63.6) = 83.9, p < 0.001**	**F(2.2, 57.9) = 83.4, p < 0.001**	**F(2.3, 53.2) = 78.2, p < 0.001**
	Intensity x genotype	F(2.7, 83.9) = 0.33, p = 0.78	**F(2.2, 57.9) = 3.8, p < 0.05**	F(2.3, 53.2) = 0.75, p = 0.50
	Genotype	F(1, 24) = 0.42, p = 0.52	**F(1, 26) = 14.7, p < 0.001**	**F(1, 23) = 3.7, p = 0.067**
PPS of fEPSP, 10-100 ms intervals	Interval	**F(2.1, 50.9) = 27.1, p < 0.001**	**F(2.3, 56.8) = 10.1, p < 0.001**	**F(1.2, 28.5) = 4.2, p < 0.05**
	Interval x genotype	F(2.1, 50.9) = 2.0, p = 0.14	F(2.3, 56.8) = 0.54, p = 0.61	F(1.2, 28.5) = 0.96, p = 0.36
	Genotype	F(1, 24) = 0.76, p = 0.39	F(1, 25) = 0.001, p = 0.98	F(1, 23) = 0.144, p = 0.71
PPS of pop-spike, 40-100 ms intervals	Interval	F(3.8, 91.3) = 1.7, p = 0.16	**F(2.8, 72.9) = 4.4, p < 0.05**	**F(3.3, 75.1) = 21.0, p < 0.001**
	Interval x genotype	F(3.8, 91.3) = 0.45, p = 0.76	F(2.8, 72.9) = 1.0, p = 0.38	F(3.3, 75.1) = 1.0, p = 0.41
	Genotype	**F(1, 24) = 4.0, p < 0.058**	F(1, 26) = 0.8, p = 0.37	F(1, 23) = 0.19, p = 0.67
LTP of fEPSP slope, 1 h post tetanus	Time	**F(1.5, 33.3) = 3.9, p < 0.05**	**F(2.5, 41.7) = 10.4, p < 0.001**	**F(2.9, 64.6) = 3.6, p < 0.05**
	Time x genotype	F(1.5, 33.5) = 0.34, p = 0.66	F(2.5, 41.7) = 0.76, p = 0.5	F(2.9, 64.6) = 0.77, p = 0.5
	Genotype	F(1, 22) = 0.63, p = 0.44	F(1, 17) = 0.05, p = 0.82	F(1, 22) = 0.54, p = 0.47
LTP of pop-spike amplitude, 1 h post tetanus	Time	**F(2.3, 53.2) = 16.8, p < 0.001**	**F(2.9, 61.0) = 13.2, p < 0.001**	**F(3.9, 85.8) = 13.3, p < 0.001**
	Time x genotype	F(2.3, 53.2) = 0.83, p = 0.46	F(2.9, 61.0) = 1.3, p = 0.29	F(3.9, 85.8) = 0.47, p = 0.75
	Genotype	**F(1, 23) = 4.2, p < 0.05**	F(1, 21) = 0.56, p = 0.46	F(1, 22) = 2.5, p = 0.13

Table 4: Statistical analysis of hippocampal theta activity

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1
Theta power after tail pinch	Interval	**F(3.07, 92.1) = 9.7, p = 0.001**
	Genotype x Interval	F(3.07, 92.1) = 0.38, p = 0.78
	Genotype	F(1, 30) = 0.13, p = 0.72

Table 5: Statistical analysis of water maze spatial discrimination task
(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	Glu-CB1
Accuracy: acquisition	Day	**F(2.5, 30) = 5.6, p < 0.01**	**F(2.6, 36.8) = 5.2, p < 0.01**
	Genotype x day	**F(2.5, 30) = 2.4, p = 0.097**	F(2.5, 36.8) = 1.5, p = 0.2
	Genotype	F(1, 12) = 0.19, p = 0.67	F(1, 15) = 0.005, p = 0.95
Accuracy: retrieval	Day	**F(3, 36) = 5.9, p < 0.01**	**F(3, 45) = 7.9, p < 0.001**
	Genotype x day	F(3, 36) = 0.19, p = 0.9	F(3, 45) = 1.3, p = 0.3
	Genotype	F(1, 12) = 0.001, p = 1	**F(1, 15) = 11.2, p < 0.01**
Latency: acquisition	Day	**F(1.6, 19.7) = 41.5, p < 0.001**	**F(1.2, 17.8) = 40.2, p < 0.001**
	Genotype x day	F(1.6, 19.7) = 0.42, p = 0.62	F(1.2, 17.8) = 2.4, p = 0.14
	Genotype	F(1, 12) = 0.16, p = 0.7	F(1, 15) = 2.0, p = 0.18
Latency: retrieval	Day	F(3, 36) = 0.64, p = 0.6	F(3, 45) = 1.8, p = 0.15
	Genotype x day	F(3, 36) = 2.1, p = 0.11	F(3, 45) = 0.8, p = 0.5
	Genotype	F(1, 12) = 0.16, p = 0.7	F(1, 15) = 1.1, p = 0.3

Table 6: Statistical analysis of object and social recognition I
(Two-tailed t-test between genotypes)

Test	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Object recognition, 3 h	Genotype	t(29) = 0.94, p = 0.36	t(28) = 0.50, p = 0.62	t(22) = 0.15, p = 0.88
	Sample size	Wt = 14 Ko = 17	Wt = 17 Ko = 13	Wt = 11 Ko = 13
Object recognition, 24 h	Genotype	t(51) = 0.75, p = 0.46	t(28) = 0.73, p = 0.47	t(22) = 0.11, p = 0.92
	Sample size	Wt = 25 Ko = 28	Wt = 17 Ko = 13	Wt = 11 Ko = 13
Social recognition, 1 h	Genotype	t(22) = 0.4, p = 0.69	t(22) = 0.08, p = 0.93	**t(21) = 2.1, p < 0.05**
	Sample size	Wt = 11 Ko = 13	Wt = 12 Ko = 12	Wt = 13 Ko = 10
Social recognition, 24 h	Genotype	t(22) = 1.56, p = 0.13	t(21) = 0.05, p = 0.96	t(21) = 0.35, p = 0.73
	Sample size	Wt = 11 Ko = 13	Wt = 12 Ko = 11	Wt = 13 Ko = 10

Table 7: Statistical analysis of object and social recognition II.

(One-sample t-test with column mean different from 0.5-chance level)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Object recognition, 3 h	wt	**t(13) = 1.87, p = 0.08, n = 14**	**t(16) = 4.5, p < 0.001, n =17**	**t(9) = 2.36, p < 0.04, n = 10**
	ko	**t(16) = 2.9, p < 0.01, n = 17**	**t(12) = 2.1, p < 0.05, n = 13**	**t(13) = 2.3, p < 0.05, n = 14**
Object recognition, 24 h	wt	**t(24) = 1.95, p = 0.06, n = 25**	t(16) = 0.04, p = 0.97, n = 17	t(9) = 1.6, p = 0.14, n = 10
	ko	t(27) = 1.0, p = 0.32, n = 28	t(12) = 0.75, p = 0.47, n = 13	t(13) = 1.2, p = 0.23, n = 14
Social recognition, 1 h	wt	**t(10) = 4.88, p < 0.001, n = 11**	t(11) = 1.63, p = 0.13, n = 12	**t(12) = 5.99, p < 0.001, n = 13**
	ko	**t(12) = 3.18, p < 0.01, n = 13**	**t(11) = 2.12, p < 0.05, n = 12**	t(9) = 0.17, p = 0.87, n = 10
Social recognition, 24 h	wt	**t(10) = 3.50, p < 0.005, n = 11**	**t(11) = 2.75, p < 0.01, n = 12**	t(12) = 0.96, p = 0.35, n = 13
	ko	t(12) = 0.18, p = 0.86, n = 13	**t(10) = 2.52, p < 0.05, n = 11**	t(9) = 2.03, p = 0.33, n = 10

Table 8: Statistical analysis of contextual fear conditioning, 0.7 mA

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Day1	Context	**F(1, 28) = 16.8, p < 0.001**	**F(1, 28) = 31.49, p < 0.001**	F(1, 22) = 0.13, p = 0.72
	Context x genotype	F(1, 28) = 0.15, p = 0.7	F(1, 28) = 0.58, p = 0.45	F(1, 22) = 0.50, p = 0.49
	Interval x genotype	F(2.8, 79) = 0.79, p = 0.5	F(2.6, 73.1) = 0.75, p = 0.5	F(2.6, 57.2) = 2.25, p = 0.30
	Context x interval x genotype	F(3.3, 92.4) = 0.17, p = 0.93	F(2.8, 79.0) = 1.9, p = 0.14	F(2.7, 59.4) = 0.53, p = 0.65
	Genotype	F(1, 28) = 1.1, p = 0.31	**F(1, 28) = 3.5, p = 0.07**	**F(1, 22) = 8.7, p < 0.05**
Day 2	Context	F(1, 27) = 1.48, p = 0.23	**F(1, 28) = 10.0, p < 0.01**	F(1, 22) = 0.81, p = 0.38
	Context x genotype	F(1, 27) = 0.23, p = 0.64	F(1, 28) = 2.7, p = 0.11	**F(1, 22) = 5.48, p < 0.05**
	Interval x genotype	**F(3.3, 89.6) = 2.99, p < 0.05**	F(2.9, 80.0) = 0.29, p = 0.83	**F(3.1, 68.9) = 4.8, p < 0.005**
	Context x interval x genotype	F(3.0, 81.2) = 0.57, p = 0.64	F(3.1, 86.8) = 0.56, p = 0.65	F(2.8, 61.8) = 0.98, p = 0.41
	Genotype	**F(1, 27) = 5.23, p < 0.05**	**F(1, 28) = 3.9, p = 0.058**	F(1, 22) = 1.7, p = 0.2
Day 7	Context	F(1, 25) = 0.001, p = 0.99	F(1, 28) = 1.99, p = 0.17	**F(1, 22) = 5.9, p < 0.05**
	Context x genotype	F(1, 25) = 2.4, p = 0.13	F(1, 28) = 2.1, p = 0.16	**F(1, 22) = 3.7, p = 0.067**
	Interval x genotype	**F(3.1, 76.7) = 6.6, p < 0.001**	F(2.8, 79.7) = 0.14, p = 0.93	F(2.6, 47.1) = 0.17, p = 0.89
	Context x interval x genotype	F(3.5, 87.0) = 0.38, p = 0.99	F(2.7, 87.0) = 0.42, p = 0.72	F(2.6, 57.0) = 0.8, p = 0.5
	Genotype	F(1, 25) = 1.9, p = 0.18	**F(1, 28) = 3.5, p = 0.072**	F(1, 22) = 1.8, p = 0.19
Day 8	Interval	**F(4, 108) = 19.2, p < 0.001**	**F(2.5, 68.7) = 16.6, p < 0.001**	**F(2.6, 56.7) = 8.7, p < 0.001**
	Interval x Genotype	**F(4, 108) = 8.2, p < 0.001**	F(2.5, 68.7) = 0.39, p = 0.72	F(2.6, 56.7) = 0.62, p = 0.58
	Genotype	**F(1, 27) = 6.9, p < 0.05**	F(1, 28) = 0.55, p = 0.47	**F(1,22) = 3.1, p = 0.092**

Table 9: Statistical analysis of contextual fear conditioning, 1.5 mA

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Day1	Context	F(1, 21) = 0.02, p = 0.90	**F(1, 22) = 9.6, p < 0.005**	**F(1, 22) = 11.5, p < 0.01**
	Context x genotype	F(1, 21) = 1.2, p = 0.29	**F(1, 22) = 7.69, p < 0.01**	**F(1, 22) = 6.5, p < 0.05**
	Interval x genotype	**F(2.99, 62.7) = 8.0, p < 0.001**	F(2.7, 60.1) = 0.33, p = 0.78	F(2.7, 58.8) = 0.08, p = 0.96
	Context x interval x genotype	F(2.5, 51.7) = 0.39, p = 0.73	F(2.40, 52.75) = 2.04, p = 0.13	**F(2.2, 49.2) = 4.0, p < 0.05**
	Genotype	**F(1, 21) = 8.36, p < 0.01**	**F(1, 22) = 3.4, p = 0.079**	F(1, 22) = 1.5, p = 0.23
Day 2	Context	**F(1, 21) = 4.2, p < 0.05**	**F(1, 22) = 14.1, p < 0.001**	**F(1, 20) = 10.9, p < 0.01**
	Context x genotype	**F(1, 21) = 4.9, p < 0.05**	F(1, 22) = 0.45, p = 0.5	F(1, 20) = 0.5, p = 0.5
	Interval x genotype	F(2.7, 56.1) = 1.4, p = 0.24	F(2.3, 50.2) = 1.2, p = 0.3	F(2.5, 49.6) = 0.5, p = 0.6
	Context x interval x genotype	F(2.6, 55.3) = 0.74, p = 0.52	F(2.3, 51.1) = 0.05, p = 0.96	F(2.9, 58.0) = 0.94, p = 0.43
	Genotype	**F(1, 21) = 8.3, p < 0.01**	**F(1, 22) = 4.9, p < 0.05**	F(1, 20) = 2.2, p = 0.15
Day 7	Context	F(1, 21) = 0.12, p = 0.73	**F(1, 22) = 9.7, p < 0.005**	**F(1, 21) = 14.8 , p < 0.001**
	Context x genotype	F(1, 21) = 2.1, p = 0.17	F(1, 22) = 0.2, p = 0.6	F(1, 21) = 0.9, p = 0.3
	Interval x genotype	**F(2.3, 48.4) = 3.8, p < 0.05**	F(2.7, 58.5) = 0.2, p = 0.87	F(4, 84) = 1.4, p = 0.2
	Context x interval x genotype	**F(2.8, 58.8) = 3.1, p < 0.05**	F(2.9, 64.6) = 0.6, p = 0.63	F(4, 84) = 0.6, p = 0.67
	Genotype	F(1, 21) = 2.04, p = 0.17	F(1, 22) = 0.55, p = 0.47	**F(1, 21) = 7.2, p < 0.01**
Day 8	Interval	**F(2.9, 61.1) = 9.6, p < 0.001**	**F(2.2, 49.1) = 3.1, p < 0.05**	**F(4, 84) = 5.6, p < 0.001**
	Interval x Genotype	**F(2.9, 61.1) = 3.5, p < 0.05**	F(2.2, 49.1) = 0.14, p = 0.89	F(4, 84) = 0.76, p = 0.56
	Genotype	**F(1, 21) = 5.0, p < 0.05**	F(1, 22) = 1.2, p = 0.28	F(1, 21) = 2.3, p = 0.14

Table 10: Statistical analysis of contextual fear conditioning (0.7 mA) separated for context

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Day 1 neutral context	Interval	**F(3.2, 88.5) = 5.6, p < 0.001**	**F(2.3, 63.6) = 4.5, p < 0.01**	**F(2.6, 57.9) = 2.7, p < 0.05**
	Interval x genotype	F(3.2, 88.5) = 0.8, p = 0.53	F(2.3, 63.6) = 0.7, p = 0.5	F(2.6, 57.9) = 0.7, p = 0.5
	Genotype	F(1, 28) = 1.2, p = 0.29	**F(1, 28) = 4.7, p < 0.05**	F(1, 22) = 3.6, p = 0.07
Day 1 shock context	Interval	**F(2.8, 79.8) = 6.4, p < 0.001**	**F(4, 112) = 5.1, p < 0.001**	F(2.4, 52.6) = 2.0, p = 0.1
	Interval x genotype	F(2.8, 79.8) = 0.3, p = 0.8	F(4, 112) = 1.5, p = 0.2	F(2.4, 52.6) = 1.2, p = 0.3
	Genotype	F(1, 28) = 0.8, p = 0.4	F(1, 28) = 1.2, p = 0.28	**F(1, 22) = 5.9, p < 0.05**
Day 2 neutral context	Interval	**F(4, 112) = 11.7, p < 0.001**	F(2.9, 82.4) = 2.6, p = 0.057	F(2.9, 63.7) = 1.8, p = 0.15
	Interval x genotype	F(4, 112) = 1.1, p = 0.4	F(2.9, 82.4) = 0.4, p = 0.7	F(2.9, 63.7) = 1.1, p = 0.35
	Genotype	**F(1, 28) = 5.5, p < 0.05**	**F(1, 28) = 7.0, p < 0.01**	F(1, 22) = 0.0001, p = 0.9
Day 2 shock context	Interval	**F(2.7, 74.1) = 7.2, p < 0.001**	**F(2.8, 77.2) = 5.3, p < 0.005**	F(2.4, 53.6) = 0.9, p = 0.4
	Interval x genotype	F(2.7, 74.1) = 2.1, p = 0.1	F(2.8, 77.2) = 0.4, p = 0.7	**F(2.4, 53.6) = 5.4, p < 0.005**
	Genotype	F(1, 27) = 3.4, p = 0.075	F(1, 28) = 0.8, p = 0.4	**F(1, 22) = 4.3, p < 0.05**
Day 7 neutral context	Interval	**F(4, 112) = 10.9, p < 0.001**	**F(2.6, 75.5) = 8.5, p < 0.001**	F(2.9, 64.7) = 1.0, p = 0.4
	Interval x genotype	F(4, 112) = 1.9, p = 0.1	F(2.6, 75.5) = 0.2, p = 0.9	F(2.9, 64.7) = 1.8, p = 0.2
	Genotype	F(1, 28) = 1.8, p = 0.2	**F(1, 28) = 6.7, p < 0.01**	F(1, 22) = 0.001, p = 0.99
Day 7 shock context	Interval	**F(2.9, 72.7) = 5.5, p < 0.01**	**F(2.7, 74.7) = 7.8, p < 0.001**	F(2.6, 57.8) = 0.6, p = 0.6
	Interval x genotype	**F(2.9, 72.7) = 3.9, p < 0.01**	F(2.7, 74.7) = 0.4, p = 0.8	F(2.6, 57.8) = 0.2, p = 0.9
	Genotype	F(1, 25) = 0.1, p = 0.7	F(1, 28) = 0.3, p = 0.6	F(1, 22) = 3.8, p = 0.063

Table 11: Statistical analysis of contextual fear conditioning (1.5 mA) separated for context

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Day 1 neutral context	Interval	**F(2.9, 61.1) = 8.3, p < 0.001**	**F(4, 88) = 7.9, p < 0.001**	**F(3.0, 65.3) = 3.6, p < 0.05**
	Interval x genotype	**F(2.9, 61.1) = 4.8, p < 0.005**	F(4, 88) = 1.5, p = 0.2	F(3.0, 65.3) = 1.6, p = 0.19
	Genotype	**F(1, 21) = 16.4, p < 0.001**	**F(1, 22) = 9.5, p < 0.005**	**F(1, 22) = 6.5, p < 0.05**
Day 1 shock context	Interval	**F(2.5, 53.5) = 5.6, p < 0.005**	**F(2.3, 50.9) = 3.0, p < 0.05**	**F(2.1, 46.1) = 3.5, p < 0.05**
	Interval x genotype	**F(2.5, 53.5) = 2.8, p < 0.05**	F(2.3, 50.9) = 0.5, p = 0.6	F(2.1, 46.1) = 1.6, p = 0.2
	Genotype	F(1, 21) = 2.8, p = 0.11	F(1, 22) = 0.02, p = 0.9	F(1, 22) = 0.001, p = 0.98
Day 2 neutral context	Interval	**F(4, 84) = 6.0, p < 0.001**	**F(2.6, 58.2) = 4.1, p < 0.01**	F(2.3, 48.0) = 1.5, p = 0.24
	Interval x genotype	**F(4, 84) = 2.3, p = 0.061**	F(2.6, 58.2) = 0.5, p = 0.67	F(2.3, 48.0) = 0.8, p = 0.45
	Genotype	**F(1, 21) = 16.1, p < 0.001**	F(1, 22) = 2.8, p = 0.11	F(1, 21) = 2.4, p = 0.14
Day 2 shock context	Interval	**F(2.2, 46.5) = 6.9, p < 0.005**	**F(1.6, 36.1) = 5.3, p < 0.01**	**F(2.8, 55.6) = 4.1, p < 0.01**
	Interval x genotype	F(2.2, 46.5) = 0.11, p = 0.91	F(1.6, 36.1) = 1.0, p = 0.36	F(2.8, 55.6) = 0.9, p = 0.45
	Genotype	F(1, 21) = 1.3, p = 0.3	**F(1, 22) = 3.4, p = 0.078**	F(1, 20) = 0.7, p = 0.4
Day 7 neutral context	Interval	**F(2.6, 54.9) = 7.2, p < 0.001**	**F(2.7, 58.6) = 6.5, p < 0.001**	F(2.8, 57.8) = 0.4, p = 0.7
	Interval x genotype	**F(2.6, 54.9) = 5.9, p < 0.005**	F(2.7, 58.6) = 0.5, p = 0.7	F(2.8, 57.8) = 0.97, p = 0.4
	Genotype	**F(1, 21) =13.3 , p < 0.001**	F(1, 22) = 0.5, p = 0.5	F(1, 21) = 1.9, p = 0.18
Day 7 shock context	Interval	**F(2.4, 50.5) = 3.1, p < 0.05**	**F(2.8, 62.1) = 10.5, p < 0.001**	**F(2.8, 59.0) = 2.3, p = 0.09**
	Interval x genotype	F(2.4, 50.5) = 0.2, p = 0.9	F(2.8, 62.1) = 0.27, p = 0.8	F(2.8, 59.0) = 1.2, p = 0.3
	Genotype	F(1, 21) = 0.04, p = 0.8	F(1, 22) = 0.13, p = 0.7	**F(1, 21) = 8.8, p = 0.01**

Table 12: Statistical analysis of contextual fear conditioning (0.7 mA) in terms of context comparison separately per genotype

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Day 1	ko: context	**F(1, 14) = 9.3, p < 0.01**	**F(1, 12) = 11.6, p < 0.005**	F(1, 12) = 0.7, p = 0.4
	ko: context x interval	F(4, 56) = 1.5, p = 0.2	**F(4, 48) = 3.6, p < 0.01**	F(1.8, 21.3) = 1.0, p = 0.4
	wt: context	**F(1, 14) = 7.5, p < 0.05**	**F(1, 16) = 21.7, p < 0.001**	F(1, 10) = 0.05, p = 0.8
	wt: context x interval	F(4, 56) = 1.2, p = 0.3	**F(2.4, 38.3) = 2.1, p = 0.09**	F(2.1, 20.8) = 0.8, p = 0.5
Day 2	ko: context	F(1, 13) = 0.9, p = 0.4	F(1, 12) = 1.2, p = 0.3	**F(1, 12) = 5.7, p < 0.05**
	ko: context x interval	F(4, 52) = 0.2, p = 0.9	F(4, 48) = 0.7, p = 0.6	F(4, 48) = 0.2, p = 0.9
	wt: context	F(1, 14) = 0.7, p = 0.4	**F(1, 16) = 12.0, p < 0.005**	F(1, 10) = 1.0, p = 0.3
	wt: context x interval	F(4, 56) = 1.3, p = 0.3	F(4, 64) = 0.4, p = 0.7	F(2.1, 20.9) = 1.2, p = 0.3
Day 7	ko: context	F(1, 13) = 1.5, p = 0.2	F(1, 12) = 0.001, p = 0.99	**F(1, 12) = 8.5, p < 0.01**
	ko: context x interval	F(4, 52) = 0.6, p = 0.6	F(4, 48) = 0.2, p = 0.93	F(2.3, 27.2) = 0.3, p = 0.8
	wt: context	F(1, 12) = 1.0, p = 0.3	**F(1, 16) = 4.1, p = 0.059**	F(1, 10) = 0.2, p = 0.7
	wt: context x interval	F(4, 48) = 1.3, p = 0.3	F(2.3, 36.7) = 0.4, p = 0.7	F(2.4, 24.0) = 0.7, p = 0.5

Table 13: Statistical analysis of contextual fear conditioning (1.5 mA) in terms of context comparison separately per genotype

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Day 1	ko: context	F(1, 10) = 0.8, p = 0.4	F(1, 13) = 0.1, p = 0.7	F(1, 9) = 0.24, p = 0.64
	ko: context x interval	F(1.9, 19.3) = 0.3, p = 0.7	F(2.1, 27.9) = 1.7, p = 0.2	**F(1.8, 16.1) = 5.2, p < 0.05**
	wt: context	F(1, 11) = 0.4, p = 0.5	**F(1, 9) = 9.1, p < 0.01**	**F(1, 13) = 26.7, p < 0.001**
	wt: context x interval	F(2.4, 26.5) = 0.3, p = 0.8	F(2.0, 18.3) = 1.8, p = 0.2	F(2.2, 28.8) = 0.53, p = 0.61
Day 2	ko: context	F(1, 10) = 0.02, p = 0.9	**F(1, 13) = 8.5, p < 0.01**	**F(1, 9) = 4.6, p = 0.06**
	ko: context x interval	F(3.0, 30.4) = 0.3, p = 0.9	F(2.6, 34.3) = 0.3, p = 0.8	**F(4, 36) = 2.7, p = 0.05**
	wt: context	**F(1, 11) = 6.7, p < 0.05**	**F(1, 9) = 5.7, p < 0.05**	**F(1, 11) = 6.9, p = 0.05**
	wt: context x interval	F(2.1, 23.3) = 1.1, p = 0.4	F(1.8, 16.4) = 0.09, p = 0.9	F(4, 44) = 0.06, p = 0.99
Day 7	ko: context	F(1, 10) = 1.9, p = 0.2	**F(1, 13) = 4.6, p < 0.05**	**F(1, 9) = 9.8, p = 0.01**
	ko: context x interval	**F(1.8, 17.7) = 3.6, p < 0.05**	F(4, 52) = 0.2, p = 0.9	F(4, 36) = 1.2, p = 0.3
	wt: context	F(1, 11) = 0.5, p = 0.5	**F(1, 9) = 4.9, p < 0.05**	**F(1, 12) = 5.0, p = 0.05**
	wt: context x interval	F(4, 44) = 0.5, p = 0.8	F(4, 36) = 0.8, p = 0.5	F(4, 48) = 0.3, p = 0.87

Table 14: Statistical analysis of the development of contextual fear conditioning over days (0.7 mA) separately per context

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Neutral context	Day	**F(2, 56) = 15.6, p < 0.001**	**F(2, 56) = 8.3, p < 0.001**	**F(2, 44) = 42.7, p < 0.001**
	Day x genotype	F(2, 56) = 1.4, p = 0.3	F(2, 56) = 0.3, p = 0.7	F(2, 44) = 2.1, p = 0.1
	Genotype	**F(1, 28) = 4.7, p < 0.05**	**F(1, 28) = 10.7, p < 0.01**	F(1, 22) = 0.7, p = 0.4
Shock context	Day	F(2, 50) = 0.9, p = 0.4	**F(2, 56) = 4.4, p < 0.05**	**F(2, 44) = 10.7, p < 0.001**
	Day x genotype	F(2, 50) = 0.7, p = 0.5	F(2, 56) = 0.05, p = 0.95	F(2, 44) = 0.05, p = 0.9
	Genotype	F(1, 25) = 1.5, p = 0.2	F(1, 28) = 1.5, p = 0.2	**F(1, 22) = 9.5, p < 0.01**

Table 15: Statistical analysis of the development of contextual fear conditioning over days (1.5 mA) separately per context

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Neutral context	Day	**F(2, 42) = 8.4, p < 0.001**	F(2, 44) = 0.9, p = 0.4	**F(2, 42) = 10.3, p < 0.001**
	Day x genotype	F(2, 42) = 1.4, p = 0.3	F(2, 44) = 1.5, p = 0.2	F(2, 42) = 0.24, p = 0.79
	Genotype	**F(1, 21) = 29.5, p < 0.001**	**F(1, 22) = 6.7, p < 0.05**	**F(1, 21) = 5.0, p < 0.05**
Shock context	Day	F(1.5, 32.3) = 1.9, p = 0.2	**F(2, 44) = 2.7, p = 0.079**	**F(2, 40) = 7.5, p < 0.005**
	Day x genotype	F(1.5, 32.3) = 1.1, p = 0.3	F(2, 44) = 1.0, p = 0.4	**F(2, 40) = 4.9, p < 0.01**
	Genotype	F(1, 21) = 1.8, p = 0.2	F(1, 22) = 0.8, p = 0.4	F(1, 20) = 2.5, p = 0.13

Table 16: Statistical analysis of contextual fear conditioning over days in terms of context discrimination

(3-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
0.7 mA	Context x genotype	F(1, 25) = 1.1, p = 0.3	**F(1, 28) = 3.9, p < 0.05**	**F(1, 22) = 7.8, p < 0.01**
	Day x genotype	F(2, 50) = 1.1, p = 0.4	F(2, 56) = 0.04, p = 0.96	F(2, 44) = 1.0, p = 0.4
	Context x day x genotype	F(2, 50) = 1.0, p = 0.4	F(2, 56) = 0.4, p = 0.7	F(2, 44) = 0.4, p = 0.7
	Genotype	**F(1, 25) = 3.3, p = 0.081**	**F(1, 28) = 6.6, p < 0.05**	**F(1, 22) = 5.0, p < 0.05**
1.5 mA	Context x genotype	**F(1, 21) = 6.6, p < 0.05**	**F(1, 22) = 4.2, p < 0.05**	F(1, 20) = 0.3, p = 0.58
	Day x genotype	F(2, 42) = 1.8, p = 0.2	F(2, 44) = 1.0, p = 0.4	F(2, 40) = 1.1, p = 0.34
	Context x day x genotype	F(2, 42) = 0.3, p = 0.7	F(2, 44) = 1.7, p = 0.2	**F(2, 40) = 4.6, p < 0.05**
	Genotype	**F(1, 21) = 13.1, p < 0.005**	**F(1, 22) = 5.5, p < 0.05**	**F(1, 20) = 4.7, p < 0.05**

Table 17: Statistical analysis of contextual fear conditioning, 0.5 mA
(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	Glu-CB1
Day1	Context	F(1, 19) = 1.97, p = 0.18
	Context x genotype	F(1, 19) = 0.51, p = 0.49
	Interval x genotype	F(2.37, 45.0) = 0.14, p = 0.9
	Context x interval x genotype	F(2.80, 53.1) = 0.41, p = 0.73
	Genotype	F(1, 19) = 1.37, p = 0.26
Day 2	Context	F(1, 19) = 0.12, p = 0.7
	Context x genotype	F(1, 19) = 0.56, p = 0.46
	Interval x genotype	F(2.9, 55.2) = 1.0, p = 0.42
	Context x interval x genotype	**F(3.1, 59.1) = 3.9, p < 0.01**
	Genotype	F(1, 19) = 0.84, p = 0.37
Day 7	Context	**F(1, 19) = 3.5, p = 0.076**
	Context x genotype	F(1, 19) = 0.16, p = 0.7
	Interval x genotype	F(2.1, 40.7) = 1.8, p = 0.5
	Context x interval x genotype	F(2.7, 50.8) = 0.29, p = 0.8
	Genotype	F(1, 19) = 2.6, p = 0.12
Day 8	Interval	**F(3.4, 65.1) = 13.0, p < 0.001**
	Interval x Genotype	**F(3.4, 65.1) = 6.2, p < 0.001**
	Genotype	F(1, 19) = 0.16, p = 0.69

Table 18: Statistical analysis of contextual fear conditioning (0.5 mA) separated for context

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	Glu-CB1
Day 1 neutral context	Interval	**F(2.4, 46.4) = 2.7, p = 0.065**
	Interval x genotype	F(2.4, 46.4) = 0.29, p = 0.8
	Genotype	F(1, 19) = 0.3, p = 0.6
Day 1 shock context	Interval	F(2.9, 55.5) = 0.4, p = 0.73
	Interval x genotype	F(2.9, 55.5) = 0.26, p = 0.9
	Genotype	F(1, 19) = 2.0, p = 0.18
Day 2 neutral context	Interval	F(4, 76) = 1.7, p = 0.15
	Interval x genotype	**F(4, 76) = 2.3, p = 0.071**
	Genotype	F(1, 19) = 0.12, p = 0.7
Day 2 shock context	Interval	**F(2.9, 55.9) = 2.4, p = 0.08**
	Interval x genotype	**F(2.9, 55.9) = 2.9, p < 0.05**
	Genotype	F(1, 19) = 1.5, p = 0.23
Day 7 neutral context	Interval	F(2.9, 64.7) = 1.0, p = 0.4
	Interval x genotype	F(2.9, 64.7) = 1.8, p = 0.16
	Genotype	F(1, 22) = 0.001, p = 0.9
Day 7 shock context	Interval	F(2.7, 51.0) = 1.7, p = 0.2
	Interval x genotype	F(2.7, 51.0) = 0.8, p = 0.5
	Genotype	**F(1, 19) = 3.0, p = 0.097**

Table 19: Statistical analysis of contextual fear conditioning (0.5 mA) in terms of context comparison separately per genotype

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	Glu-CB1
Day 1	ko: context	F(1, 11) = 0.5, p = 0.5
	ko: context x interval	F(2.5, 27.7) = 0.7, p = 0.5
	wt: context	F(1, 8) = 1.2, p = 0.3
	wt: context x interval	F(2.7, 21.5) = 1.9, p = 0.2
Day 2	ko: context	F(1, 11) = 0.7, p = 0.4
	ko: context x interval	**F(4, 44) = 2.8, p < 0.05**
	wt: context	F(1, 8) = 0.08, p = 0.8
	wt: context x interval	**F(2.1, 16.9) = 3.4, p < 0.05**
Day 7	ko: context	F(1, 11) = 1.0, p = 0.4
	ko: context x interval	F(2.5, 27.6) = 0.4, p = 0.7
	wt: context	**F(1, 8) = 4.2, p = 0.075**
	wt: context x interval	**F(4, 32) = 2.4, p = 0.07**

Table 20: Statistical analysis of the development of contextual fear conditioning over days (0.5 mA) separately per context

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	Glu-CB1
Neutral context	Day	**F(2, 38) = 5.0, p < 0.01**
	Day x genotype	F(2, 38) = 0.08, p = 0.9
	Genotype	F(1, 19) = 0.8, p = 0.4
Shock context	Day	**F(1.3, 25.3) = 7.6, p < 0.01**
	Day x genotype	F(1.3, 25.3) = 0.3, p = 0.6
	Genotype	F(1, 19) = 3.0, p = 0.1

Table 21: Statistical analysis of contextual fear conditioning over days (0.5 mA) in terms of context discrimination

(3-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	Glu-CB1
0.5 mA	Context x genotype	F(1, 19) = 1.1, p = 0.3
	Day x genotype	F(2, 38) = 0.2, p = 0.8
	Context x day x genotype	F(2, 38) = 0.09, p = 0.9
	Genotype	F(1, 19) = 2.3, p = 0.2

Table 22: Statistical analysis of baseline freezing in novel context before tone on day 8

(2-way ANOVA for repeated measurements

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
0.7 mA	Interval	F(1.2, 31.1) = 2.1, p = 0.15	**F(2, 56) = 4.6, p < 0.01**	F(2, 44) = 1.8, p = 0.18
	Interval x genotype	F(1.2, 31.1) = 1.2, p = 0.28	F(2, 56) = 1.2, p = 0.3	F(2, 44) = 0.36, p = 0.7
	Genotype	**F(1, 26) = 3.4, p = 0.076**	F(1, 28) = 0.53, p = 0.47	F(1, 22) = 0.03, p = 0.87
1.5 mA	Interval	F(1.3, 26.9) = 0.8, p = 0.4	F(1.4, 29.8) = 1.6, p = 0.2	**F(2, 42) = 7.6, p < 0.01**
	Interval x genotype	F(1.3, 26.9) = 0.54, p = 0.51	F(1.4, 29.8) = 0.3, p = 0.65	F(2, 42) = 1.1, p = 0.34
	Genotype	F(1, 21) = 0.15, p = 0.7	**F(1, 22) = 9.3, p < 0.01**	F(1, 21) = 0.8, p = 0.38

Table 23: Statistical analysis of elevated plus-maze, day 1 and 2

(unpaired t-test, two-sided)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Open arm entries, day 1	Genotype	**t(39) = 1.78, p = 0.08**	**t(37) = 1.94, p = 0.06**	t(34) = 0.008, p = 0.99
	Sample size	Wt = 23 Ko = 18	Wt = 19 Ko = 20	Wt = 16 Ko = 20
Open arm time, day 1	Genotype	**t(39) = 1.74, p = 0.09**	t(37) = 1.25, p = 0.22	t(34) = 0.30, p = 0.76
	Sample size	Wt = 23 Ko = 18	Wt = 19 Ko = 20	Wt = 16 Ko = 20
Total arm entries, day 1	Genotype	t(39) = 1.56, p = 0.13	t(37) = 0.44, p = 0.66	t(34) = 0.56, p = 0.58
	Sample size	Wt = 23 Ko = 18	Wt = 19 Ko = 20	Wt = 16 Ko = 20
Open arm entries, day 2	Genotype	t(39) = 1.5, p = 0.14	t(37) = 0.20, p = 0.84	t(33) = 1.66, p = 0.11
	Sample size	Wt = 23 Ko = 18	Wt =19 Ko = 20	Wt = 15 Ko = 20
Open arm time, day 2	Genotype	**t(39) = 1.7, p = 0.097**	t(37) = 0.16, p = 0.87	t(33) = 1.09, p = 0.28
	Sample size	Wt = 23 Ko = 18	Wt = 19 Ko = 20	Wt = 15 Ko = 20
Total arm entries, day 2	Genotype	t(39) = 1.5, p = 0.13	t(37) = 0.75, p = 0.46	t(33) = 1.07, p = 0.29
	Sample size	Wt = 23 Ko = 18	Wt = 19 Ko = 20	Wt = 15 Ko = 20

Table 24: Statistical analysis of elevated plus-maze in terms of animals which refrained from exploring the open arms at all

(chi-square test, two-sided)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Non-explorer, day 1	Genotype	t(1) = 0.67, p = 0.41	t(1) = 0.69, p = 0.41	t(1) = 0.69, p = 0.41
	Sample size	Wt = 22 Ko = 18	Wt = 19 Ko = 20	Wt = 16 Ko = 20
Non-explorer, day 2	Genotype	**t(1) = 4.8, p < 0.05**	t(1) = 0.02, p = 0.89	**t(1) = 6.08, p < 0.01**
	Sample size	Wt = 22 Ko = 18	Wt = 19 Ko = 20	Wt = 15 Ko = 20

Table 25: Statistical analysis of dark/light box-test
(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Time in lit compartment	Interval	**F(2.6, 49.4) = 3.4, p < 0.05**	**F(2.36, 42.45) = 3.3, p < 0.05**	F(2.4, 31.2) = 1.53, p = 0.23
	Genotype x interval	F(2.6, 49.4) = 0.9, p = 0.44	F(2.36, 42.5) = 0.38, p = 0.72	F(2.4, 31.2) = 1.07, p = 0.37
	Genotype	F(1, 19) = 2.2, p = 0.15	F(1, 18) = 0.08, p = 0.78	F(1, 13) = 0.65, p = 0.44
Total distance moved in lit compartment	Interval	**F(2.5, 47.6) = 2.66, p = 0.068**	**F(2.3, 42.4) = 3.2, p < 0.05**	F(2.2, 28.9) = 0.97, p = 0.4
	Genotype x interval	F(2.5, 47.6) = 0.68, p = 0.55	F(2.3, 42.4) = 0.73, p = 0.51	F(2.2, 28.9) = 1.02 p = 0.38
	Genotype	F(1, 19) = 2.36, p = 0.14	F(1, 18) = 0.08, p = 0.78	F(1, 13) = 0.7, p = 0.41
Entries into lit compartment	Interval	F(3.1, 59.0) = 0.96, p = 0.42	F(2.1, 38.0) = 0.87, p = 0.43	F(4, 52) = 0.76, p = 0.55
	Genotype x interval	F(3.1, 59.0) = 0.32, p = 0.82	F(2.1, 38.0) = 1.1, p = 0.34	F(4, 52) = 0.75, p = 0.56
	Genotype	F(1, 19) = 0.84, p = 0.37	F(1, 18) = 0.67, p = 0.42	F(1, 13) = 1.5, p = 0.25

Table 26: Statistical analysis of novel object and novel juvenile exploration
(unpaired t-test, two-sided)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Novel object exploration, 30 lux	Genotype	t(51) = 1.04, p = 0.30	**t(28) = 2.25, p < 0.05**	**t(22) = 3.10, p < 0.01**
	Sample size	Wt = 25 Ko = 28	Wt = 17 Ko = 13	Wt = 10 Ko = 14
Novel object exploration, 500 lux	Genotype	**t(22) = 3.5, p < 0.005**	**t(40) = 2.22, p < 0.05**	**t(19) = 3.7, p < 0.001**
	Sample size	Wt = 12 Ko = 12	Wt = 22 Ko = 20	Wt = 10 Ko = 11
Novel juvenile exploration, 0 lux	Genotype	t(22) = 0.85, p = 0.40	t(22) = 0.54, p = 0.59	t(14) = 0.52, p = 0.61
	Sample size	Wt = 12 Ko = 12	Wt = 12 Ko = 12	Wt = 9 Ko = 7
Novel juvenile exploration, 500 lux	Genotype	**t(22) = 2.8, p < 0.01**	t(21) = 1.1, p = 0.28	**t(21) = 3.17, p < 0.01**
	Sample size	Wt = 11 Ko = 13	Wt = 11 Ko = 12	Wt = 13 Ko = 10

Table 27: Statistical analysis of marble burying
(unpaired t-test, two-sided)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Buried marbles	Genotype	t(19) = 0.27, p = 0.79	t(18) = 0.57, p = 0.58	t(13) = 1.53, p = 0.15
	Sample size	Wt = 12 Ko = 9	Wt = 10 Ko = 10	Wt = 7 Ko = 8

Table 28: Statistical analysis of open field, day 1, 0 lux

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Rearing	Interval	**F(5.9, 164.7) = 4.5, p < 0.001**	F(9, 6.4) = 1.1, p = 3.5	F(5.0, 110.6) = 1.4, p = 0.35
	Genotype x interval	**F(5.9, 164.7) = 4.5, p < 0.01**	F(9, 6.4) = 1.2, p = 3.1	**F(5.0, 110.6) = 4.2, p < 0.01**
	Genotype	F(1, 28) = 0.24, p = 0.629	F(1, 28) = 2.13, p = 0.16	**F(1, 22) = 3.1, p = 0.09**
Total distance moved	Interval	**F(9, 252) = 25.4, p < 0.001**	**F(4.8, 135.5) = 26.8, p < 0.001**	**F(4.6, 101.6) = 19.0, p < 0.001**
	Genotype x interval	**F(9, 252) = 5.8, p < 0.001**	F(4.8, 135.5) = 1.3, p = 0.27	**F(4.6, 101.6) = 2.3, p < 0.05**
	Genotype	F(1, 28) = 0.001, p < 0.97	F(1, 28) = 1.7, p = 0.21	F(1, 22) = 0.17, p = 0.68
Immobility	Interval	**F(9, 252) = 40.4, p < 0.001**	**F(5.6, 157.6) = 32.2, p < 0.001**	**F(4.8, 105.2) = 24.8, p < 0.001**
	Genotype x interval	**F(9, 252) = 3.3, p < 0.001**	F(5.6, 157.6) = 0.89, p = 0.51	F(4.8, 105.2) = 2.2, p = 0.13
	Genotype	F(1, 28) = 0.48, p = 0.49	F(1, 28) = 2.7, p = 0.11	F(1, 22) = 0.73, p = 0.4
Margin Distance	Interval	F(9, 252) = 1.61, p = 0.15	F(9, 252) = 1.1, p = 0.38	F(5.0, 108.6) = 0.9, p = 0.5
	Genotype x interval	F(9, 2.52) = 1.2, p = 0.32	F(9, 252) = 0.4, p = 0.91	**F(5.0, 108.6) = 2.4, p < 0.05**
	Genotype	**F(1, 28) = 11.5, p < 0.05**	F(1, 28) = 0.11, p = 0.75	F(1, 22) = 1.3, p = 0.3

Table 29: Statistical analysis of open field, day 1, 700 lux

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Rearing	Interval	**F(3.6, 73.9) = 4.3, p < 0.001**	F(9, 198) = 1.1, p = 0.42	F(9, 180) = 1.7, p = 0.1
	Genotype x interval	F(3.6, 73.9) = 0.42, p = 0.46	F(9, 198) = 1.9, p = 0.3	F(9, 180) = 0.34, p = 0.96
	Genotype	**F(1, 22) = 3.31 p < 0.05**	F(1, 22) = 0.68, p = 0.2	F(1, 20) = 0.56, p = 0.26
Total distance moved	Interval	**F(2.7, 59.9) = 2.6, p < 0.05**	**F(4.8, 104.4) = 18.9, p < 0.001**	**F(4.7, 94.5) = 15.9, p < 0.001**
	Genotype x interval	F(2.7, 59.9) = 0.6, p = 0.63	F(4.8, 104.4) = 0.97, p = 0.44	F(4.7, 94.5) = 1.1, p = 0.35
	Genotype	**F(1, 22) = 4.5, p < 0.05**	**F(1, 22) = 3.0, p = 0.097**	F(1, 20) = 0.45, p = 0.51
Immobility	Interval	F(2.7, 59.8) = 2.0, p = 0.13	**F(9, 198) = 27.9, p < 0.001**	**F(4.5, 89.9) = 20.6, p < 0.001**
	Genotype x interval	F(2.7, 59.8) = 0.49, p = 0.67	F(9, 198) = 1.2, p = 0.32	F(4.5, 89.9) = 1.0, p = 0.41
	Genotype	F(1, 22) = 2.7, p = 0.11	F(1, 22) = 0.7, p = 0.4	F(1, 20) = 0.15, p = 0.71
Margin Distance	Interval	F(4.5, 99.6) = 1.2, p = 0.3	**F(5.0, 110.5) = 3.9, p < 0.001**	**F(9, 180) = 4.7, p < 0.001**
	Genotype x interval	F(4.5, 99.6) = 1.6, p = 0.18	**F(5.0, 110.5) = 2.8, p < 0.05**	F(9, 180) = 1.3, p = 0.2
	Genotype	**F(1, 22) = 4.9, p < 0.05**	F(1, 22) = 0.04, p = 0.84	**F(1, 20) = 5.8, p < 0.05**

Table 30: Statistical analysis of open field, day 2, 0 lux

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Rearing	Interval	**F(5.1, 136.4) = 3.9, p < 0.05**	F(5.2, 145.8) = 0.6, p = 0.64	**F(4.8, 104.7) = 2.3, p < 0.05**
	Genotype x interval	**F(5.1, 136.4) = 3.4, p < 0.01**	F(5.2, 145.8) = 0.3, p = 0.9	F(4.8, 104.7) = 1.4, p < 0.2
	Genotype	F(1, 27) = 1.7, p = 0.2	F(1, 28) = 1.2, p = 0.29	**F(1, 22) = 5.5, p = 0.05**
Total distance moved	Interval	**F(5.3, 143.4) = 10.3, p < 0.001**	**F(5.8, 161.7) = 21.6, p < 0.001**	**F(4.9, 107.0) = 20.8, p < 0.001**
	Genotype x interval	**F(5.3, 143.4) = 2.0, p = 0.078**	F(5.8, 161.7) = 1.0, p = 0.42	F(4.9, 107.0) = 1.2, p = 0.3
	Genotype	F(1, 27) = 0.21, p = 0.65	F(1, 28) = 0.34, p = 0.57	F(1, 22) = 2.9, p = 0.1
Immobility	Interval	**F(5.0, 134.8) = 12.2, p < 0.001**	**F(9, 252) = 22.7, p < 0.001**	**F(4.5, 99.9) = 15.4, p < 0.001**
	Genotype x interval	F(5.0, 134.8) = 1.2, p = 0.33	F(9, 252) = 1.2, p = 0.3	F(4.5, 99.9) = 1.3, p = 0.28
	Genotype	F(1, 27) = 0.03, p = 0.86	F(1, 28) = 0.59, p = 0.45	**F(1, 22) = 5.4, p < 0.05**
Margin Distance	Interval	**F(9, 252) = 10.9, p = 0.001**	**F(5.9, 166.4) = 7.0, p = 0.001**	**F(4.7, 104.4) = 6.9, p = 0.001**
	Genotype x interval	**F(9, 252) = 2.1, p < 0.05**	F(5.9, 166.4) = 1.2, p = 0.31	F(4.7, 104.4) = 0.58, p = 0.71
	Genotype	**F(1, 28) = 14.9, p < 0.01**	F(1, 28) = 0.70, p = 0.41	F(1, 22) = 1.2, p = 0.29

Table 31: Statistical analysis of open field, day 2, 700 lux

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Rearing	Interval	**F(2.9, 63.7) = 7.3, p < 0.001**	**F(4.8, 106.3) = 4.0, p < 0.05**	**F(4.8, 95.9) = 5.0, p < 0.001**
	Genotype x Interval	F(2.9, 63.7) = 1.9, p = 0.14	F(4.8, 106.3) = 0.6, p = 0.7	F(4.8, 95.9) = 1.2, p = 0.3
	Genotype	**F(1, 22) = 13.2, p < 0.001**	F(1, 22) = 0.001, p = 0.98	**F(1, 20) = 5.9, p < 0.05**
Total distance moved	Interval	F(3.9, 86.3) = 0.64, p = 0.64	**F(9, 198) = 21.2, p < 0.001**	**F(4.2, 84.5) = 11.8, p < 0.001**
	Genotype x Interval	F(3.9, 86.3) = 1.1, p = 0.34	F(9, 198) = 0.39, p = 0.86	F(4.2, 84.5) = 0.74, p = 0.58
	Genotype	**F(1, 22) = 5.3, p < 0.05**	F(1, 22) = 6.0, p = 0.45	**F(1, 20) = 4.4, p < 0.05**
Immobility	Interval	F(3.8, 82.8) = 1.2, p = 0.33	**F(4.3, 94.9) = 19.2, p < 0.001**	**F(4.0, 79.7) = 14.4, p < 0.001**
	Genotype x Interval	F(3.8, 82.8) = 0.64, p = 0.63	F(4.3, 94.9) = 0.28, p = 0.89	F(4.0, 79.7) = 1.6, p = 0.17
	Genotype	**F(1, 22) = 3.8, p = 0.063**	F(1, 22) = 0.08, p = 0.79	**F(1, 20) = 3.0, p = 0.099**
Margin Distance	Interval	**F(4.9, 107.8) = 9.0, p < 0.001**	**F(5.2, 114.7) = 5.1, p < 0.001**	**F(9, 180) = 5.5, p < 0.001**
	Genotype x Interval	F(4.9, 107.8) = 0.3, p = 0.9	F(5.2, 114.7) = 0.7, p = 0.6	**F(9, 180) = 2.5, p < 0.05**
	Genotype	F(1, 22) = 1.4, p = 0.25	F(1, 22) = 0.001, p = 0.99	**F(1, 20) = 7.6, p < 0.05**

Table 32: Statistical analysis of open field habituation, 0 lux

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Rearing	Day	F(1, 27) = 0.34, p = 0.56	F(1, 28) = 1.6, p = 0.22	**F(1, 22) = 3.2, p = 0.089**
	Day x genotype	F(1, 27) = 2.6, p = 0.12	F(1, 28) = 0.37, p = 0.6	F(1, 22) = 0.99, p = 0.33
	Genotype	F(1, 27) = 0.73, p = 0.4	F(1, 28) = 1.8, p = 0.19	**F(1, 22) = 5.2, p = 0.05**
Total distance moved	Day	**F(1, 27) = 5.7, p < 0.05**	**F(1, 28) = 20.3, p < 0.001**	**F(1, 22) = 23.3, p < 0.001**
	Day x genotype	F(1, 27) = 0.15, p = 0.7	F(1, 28) = 1.4, p = 0.24	F(1, 22) = 2.0, p = 0.18
	Genotype	F(1, 27) = 0.14, p = 0.7	F(1, 28) = 0.9, p = 0.34	F(1, 22) = 1.2, p = 0.28
Immobility	Day	**F(1, 27) = 14.1, p < 0.001**	**F(1, 28) = 22.8, p < 0.001**	**F(1, 22) = 23.3, p < 0.001**
	Day x genotype	F(1, 27) = 0.43, p = 0.52	F(1, 28) = 0.95, p = 0.34	F(1, 22) = 3.1, p = 0.19
	Genotype	F(1, 27) = 0.013, p = 0.99	F(1, 28) = 1.5, p = 0.23	**F(1, 22) = 3.1, p < 0.088**
Margin Distance	Day	F(1, 28) = 0.19, p = 0.66	F(1, 28) = 0.075, p = 0.79	F(1, 22) = 1.6, p = 0.22
	Day x genotype	F(1, 28) = 0.37, p = 0.86	F(1, 28) = 2.5, p = 0.13	**F(1, 22) = 6.4, p < 0.05**
	Genotype	**F(1, 28) = 14.1, p < 0.001**	F(1, 28) = 0.07, p = 0.8	F(1, 22) = 0.26, p = 0.61

Table 33: Statistical analysis of open field habituation, 700 lux

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Rearing	Day	**F(1, 22) = 44.8, p < 0.001**	**F(1, 22) = 75.6, p < 0.001**	**F(1, 20) = 86.2, p < 0.001**
	Day x genotype	F(1, 22) = 2.91, p = 0.1	**F(1, 22) = 7.0, p < 0.05**	**F(1, 20) = 4.8, p < 0.05**
	Genotype	**F(1, 22) = 11.2, p < 0.05**	F(1, 22) = 5.3, p = 0.5	**F(1, 20) = 3.5, p = 0.075**
Total distance moved	Day	**F(1, 22) = 154.0, p < 0.001**	**F(1, 22) = 103.7, p < 0.001**	**F(1, 20) = 104.3, p < 0.001**
	Day x genotype	F(1, 22) = 2.8, p = 0.11	F(1, 22) = 1.8, p = 0.2	**F(1, 20) = 6.2, p < 0.05**
	Genotype	**F(1, 22) = 4.7, p < 0.05**	F(1, 22) = 1.6, p = 0.22	F(1, 20) = 2.3, p = 0.15
Immobility	Day	**F(1, 22) = 321.0, p < 0.001**	**F(1, 22) = 64.9, p < 0.001**	**F(1, 20) = 78.2, p < 0.001**
	Day x genotype	F(1, 22) = 1.6, p = 0.22	F(1, 22) = 0.13, p = 0.72	**F(1, 20) = 7.1, p < 0.05**
	Genotype	F(1, 22) = 2.8, p = 0.12	F(1, 22) = 0.3, p = 0.61	F(1, 20) = 1.2, p = 0.3
Margin Distance	Day	**F(1, 22) = 12.4, p < 0.05**	**F(1, 22) = 18.4, p < 0.001**	**F(1, 20) = 0.001, p < 0.001**
	Day x genotype	F(1, 22) = 0.88, p = 0.36	F(1, 22) = 0.29, p = 0.60	F(1, 20) = 0.87, p = 0.36
	Genotype	F(1, 22) = 2.2, p = 0.15	F(1, 22) = 0.002, p = 0.96	**F(1, 20) = 7.0, p < 0.05**

Table 34: Statistical analysis of open field habituation for individual genotypes at 0 lux

(paired t-test)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Rearing	wt	t(12) = 1, p = 0.34, n = 13	t(17) = 1.19, p = 0.25, n = 18	t(9) = 0.55, p = 0.60, n = 10
	ko	t(15) = 1.34, p = 0.19, n = 16	t(11) = 0.99, p = 0.35, n = 12	**t(13) = 2.25, p < 0.05, n = 14**
Total distance moved	wt	**t(12) = 2.64, p < 0.05, n = 13**	**t(17) = 4.47, p < 0.001, n = 18**	**t(9) = 3.24, p < 0.01, n = 10**
	ko	t(15) = 0.13, p = 0.23, n = 16	**t(11) = 2.16, p < 0.05, n = 12**	**t(13) = 3.8, p < 0.01, n = 13**
Immobility	wt	**t(12) = 3.65, p < 0.01, n = 13**	**t(17) = 5.1, p < 0.001, n = 18**	**t(9) = 3.72, p < 0.005, n = 10**
	ko	**t(15) = 2.05, p = 0.058, n = 16**	**t(11) = 2.13, p = 0.056, n = 12**	**t(13) = 3.78, p < 0.01, n = 14**

Table 35: Statistical analysis of open field habituation for individual genotypes at 700 lux

(paired t-test)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Rearing	wt	**t(11) = 6.37, p < 0.001, n = 12**	**t(11) = 5.34, p < 0.001, n = 12**	**t(12) = 5.53, p < 0.001, n = 13**
	ko	**t(11) = 3.31, p < 0.01, n = 12**	**t(11) = 6.67, p < 0.001, n = 12**	**t(8) = 7.50, p < 0.001, n = 9**
Total distance moved	wt	**t(11) = 16.12, p < 0.001, n = 12**	**t(11) = 8.0, p < 0.001, n = 12**	**t(12) = 5.60, p < 0.001, n = 13**
	ko	**t(11) = 5.97, p < 0.001, n = 12**	**t(11) = 7.05, p < 0.001, n = 12**	**t(8) = 9.5, p < 0.001, n = 9**
Immobility	wt	**t(11) = 22.72, p < 0.001, n = 12**	**t(11) = 5.66, p < 0.001, n = 12**	**t(12) = 5.11, p < 0.001, n = 13**
	ko	**t(11) = 10.31, p < 0.001, n = 12**	**t(11) = 5.78, p <0.001 , n = 12**	**t(8) = 6.95, p < 0.001, n = 9**

Table 36: Statistical analysis of open field habituation within the first session for individual genotypes at 0 lux

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Total distance moved	wt: interval	**F(4.2, 54.9) = 5.2, p < 0.001**	**F(9, 153) = 21.8, p < 0.001**	**F(3.5, 34.7) = 6.1, p < 0.01**
	ko: interval	**F(4.9, 73.1) = 24.2, p < 0.001**	**F(3.3, 36.4) = 8.9, p < 0.001**	**F(9, 108) = 15.1, p < 0.001**
Immobility	wt: interval	**F(9, 117) = 16.3, p < 0.001**	**F(9, 153) = 23.6, p < 0.001**	**F(4.2, 41.5) = 12.8, p < 0.001**
	ko: interval	**F(9, 135) = 27.2, p < 0.001**	**F(9, 99) = 11.6, p < 0.001**	**F(9, 108) = 15.8, p < 0.001**

Table 37: Statistical analysis of open field habituation within the first session for individual genotypes at 700 lux

(2-way ANOVA for repeated measurements)

Parameter	Statistical Parameter	total-CB1	GABA-CB1	Glu-CB1
Total distance moved	Wt: interval	**F(3.9, 43.3) = 4.1, p < 0.001**	**F(9, 90) = 16.7, p < 0.001**	**F(3.9, 46.3) = 12.9, p < 0.001**
	Ko: interval	F(2.3, 24.9) = 0.9, p = 0.5	**F(9, 108) = 7.6, p < 0.001**	**F(9, 72) = 5.5, P < 0.001**
Immobility	Wt: interval	**F(9, 99) = 4.4, p < 0.001**	**F(9, 90) = 18.1, p < 0.001**	**F(3.1, 37.3) = 17.0, p < 0.001**
	Ko: interval	F(2.3, 25.7) = 0.8, p = 0.6	**F(9, 108) = 12.4, p < 0.001**	**F(9, 72) = 6.7, p < 0.001**

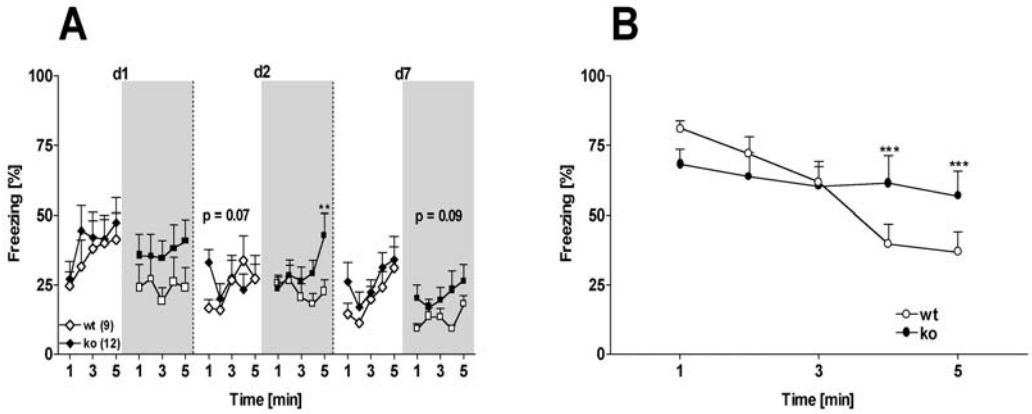


Figure 35: Background contextual fear conditioning of Glu-CB1-ko mice at 0.5 mA. Freezing levels Glu-CB1-ko mice and the respective wildtype littermates in the contextual fear conditioning paradigm on day 1, 2 and 7 after conditioning **(A)**. Mice were conditioned with 0.5 mA. Grey insets and square symbols highlight the freezing values in the shock context compared to the grid context with diamond symbols. Data are shown in 1-min-intervals. Freezing levels of Glu-CB1-ko mice and the respective wildtype littermates for the auditory cue in the background contextual fear conditioning paradigm on day 8 **(B)**. Values are means +/- S.E.M. Asterisks indicate post-hoc comparisons following significant genotype x interval interaction. ↑ $p < 0.05$, ↑↑ $p < 0.01$ and ** $p < 0.01$. Effects close to significance are indicated by the given p value. Sample sizes are indicated for genotypes. Please see Table 17+18 for statistical details.

7. References

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2. Conference Abstracts

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W. Jacob, R. Marsch, G. Marsicano, B. Lutz, C.T. Wotjak (2005): Altered short-term and long-term plasticity in the dentate gyrus of mice lacking the cannabinoid CB1 receptor. *24th Symposium of the Working Committee of Neuropsychopharmacology and Pharmacopsychiatry (AGNP), Munich*

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